Paroxysmal nocturnal hemoglobinuria testing in patients with myelodysplastic syndrome in clinical practice—frequency and indications

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ABSTRACT

Background Myelodysplastic syndrome (mds) is characterized by peripheral blood cytopenias, with most patients developing significant anemia and dependence on red blood cell (rbc) transfusion. In paroxysmal nocturnal hemoglobinuria (pnh), mutations in the PIGA gene lead to lack of cell-surface glycosylphosphatidylinositol, allowing complement-mediated lysis to occur. Paroxysmal nocturnal hemoglobinuria results in direct antiglobulin test–negative hemolysis and cytopenias, and up to 50% of patients with mds test positive for pnh cells. We wanted to determine whether pnh is considered to be a contributor to anemia in mds.

Methods Patients with a diagnosis of mds confirmed by bone-marrow biopsy since 2009 were reviewed. High-resolution pnh testing by flow cytometry examined flaer (fluorescein-labeled proaerolysin) binding and expression of CD14, CD15, CD24, CD45, CD59, CD64, and CD235 on neutrophils, monocytes, and rbc.

Results In 152 patients with mds diagnosed in 2009 or later, the mds diagnosis included subtypes associated with pnh positivity (refractory anemia, n = 7, and hypoplastic mds, n = 4). Of 11 patients who underwent pnh testing, 1 was positive (9.0%). Reasons for pnh testing were anemia (n = 3), new mds diagnosis (n = 2), hypoplastic mds (n = 2), decreased haptoglobin (n = 1), increased rbc transfusion requirement (n = 1), and unexplained iron deficiency (n = 1).

Conclusions Testing for pnh was infrequent in mds patients, and the criteria for testing were heterogeneous. Clinical indicators prompted pnh testing in 6 of 11 patients. Given that effective treatment is now available for pnh and that patients with pnh-positive mds can respond to immunosuppressive therapy, pnh testing in mds should be considered. Prospective analyses to clarify the clinical significance of pnh positivity in mds are warranted.

Key Words Paroxysmal nocturnal hemoglobinuria, PNH, myelodysplastic syndrome, mds

INTRODUCTION

Myelodysplastic syndrome (mds) encompasses a group of bone-marrow disorders characterized by ineffective hematopoiesis leading to peripheral blood cytopenias1. Most patients with mds develop significant anemia and dependence on red blood cell (rbc) transfusions, which are associated with inferior survival2–4.

In paroxysmal nocturnal hemoglobinuria (pnh), mutations in the PIGA gene lead to a lack of the glycosylphosphatidylinositol (gpi) anchor on the cell surface, allowing complement-mediated lysis to occur. The pnh phenotype includes hemolysis that is direct antiglobulin test (dat)–negative and hemoglobinuria, resulting in iron deficiency, renal insufficienty, thrombosis, fatigue, and abdominal pain5,6. Positivity for pnh has been reported in varying proportions of patients with mds, from a low of 1.1% to a high of 8% overall7,8 and from 17.6% to 53.3% in the refractory anemia (ra) subtype9,10. The median percentage of pnh-positive cells in mds has been reported to range from a low of 0.18% to 22% in ra11,12.

The presence of pnh-positive cells in mds could potentially confound the reason for rbc transfusion dependence by contributing to hemolysis. For example, in one
study including 585 MDS patients, 5.7% with PNH cells, the combined incidence of signs including hemolytic anemia, unspecified hemolysis, and unspecified iron deficiency was 33.1%[1]. Moreover, patients with PNH-positive MDS might have a better response to immunosuppressive therapy. For example, in a study of 164 patients with MDS, 21 of 119 (17.6%) were positive for PNH, and of those 21, 77.8% had a response to cyclosporine; 0% of the patients without PNH cells had such a response[6,8]. In that analysis, no patient with detectable PNH cells progressed to acute myeloid leukemia, but 6.2% of patients without PNH cells experienced such progression[6].

Testing for PNH in MDS is recommended for patients with the RA subtype; in MDS with evidence of hemolysis [lactate dehydrogenase (LDH) above the upper limit of normal, or haptoglobin below the lower limit of normal, or elevated reticulocyte count] with or without anemia; and MDS classified as low or intermediate-1 by the International Prognostic Scoring System (IPSS), with hypoplastic bone marrow and serum erythropoietin (EPO) measuring 500 mIU/mL or more[4–18]. Other conditions associated with PNH include aplastic anemia.

Eculizumab is the first specific treatment for PNH; it was approved by Health Canada in 2009. It binds to complement protein C5, preventing formation of the membrane attack complex, preventing complement-mediated hemolysis, decreasing anemia and the requirement for RBC transfusions, preventing thrombosis, and improving renal function, quality of life, and overall survival (OS) 18–22,23. The 5-year OS for PNH patients in the pre-eculizumab era was 65%; currently, the OS at 66 months with eculizumab is 97.6%. However, the significance of PNH positivity in MDS is uncertain. In particular, the prevalence of GPI-negative clone sizes greater than 10% in MDS (with the exception of 4 patients with RA in one study) has not, to our knowledge, been reported—which is important, given that that population was included in pivotal clinical trials of anti-complement therapy in PNH patients without MDS[12,24,25]. Given the improvement in outcomes observed with specific treatment for PNH without MDS, and given that, compared with patients having PNH-negative MDS, patients with MDS in which GPI-negative populations are detected appear to benefit more from immunosuppressive therapy, we wanted to determine whether PNH as a contributor to anemia is considered in MDS patients.

METHODS

Patients with MDS seen at St. Paul’s Hospital in Vancouver, British Columbia, were identified from the hematology department clinical database and reviewed. Patients were included if they had undergone a bone marrow aspiration and biopsy that confirmed the MDS diagnosis after the date of Health Canada approval of eculizumab (2009).

Clinical data extracted by chart review included baseline clinical and laboratory characteristics, clinical course, treatment, and outcomes. Potential indicators of hemolysis—including increased LDH, bilirubin, and reticulocyte count; decreased haptoglobin; and DAT results—were recorded (Figure 1). Episodes of unexplained thrombosis, abdominal pain, hemoglobinuria, and unexplained iron insufficiency (serum ferritin < 100 ng/mL) were also noted. Because we wanted to focus on PNH cells as a possible contributor to anemia, we considered the presence of significant anemia at any point in the patient’s disease course (approaching a level requiring clinical intervention: hemoglobin < 100 g/L) and the presence of any indicator of hemolysis (LDH, bilirubin, or reticulocyte count above the upper limit of normal, or haptoglobin below the upper limit of normal) as reasons for PNH testing.

Transfusion requirements were documented. Transfusion dependence was defined as use of transfusions meeting the International Working Group 2006 criteria[36]. Reasons for PNH testing were noted. High-resolution PNH testing by flow cytometry looked for FLAER (fluorescein-labeled proaerolysin) binding and expression of CD14, CD15, CD24, CD45, CD59, CD64, and CD235 on neutrophils, monocytes, and RBCs. High-resolution multicolor flow cytometry used an antibody panel consisting of 3 samples as follows:

- **White blood cell sample:** CD14-APC-H7-A, CD15-V450, CD24-PE, CD45-V500, CD64-APC-A, and FLAER, yielding an analytic sensitivity of at least 0.1% and ranging from 0.01% up to 0.1%, depending on the number of events acquired (for severely pancytopenic patients, the sensitivity of the white blood cell assay could be lower)
- **RBC sample:** CD59-PE and CD235-FTTC, yielding an analytic sensitivity of at least 0.01%
- **Unstained RBC control tube**

The samples were analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) and were interpreted using the FACSDiva 6.1 software (BD Biosciences), following an standardized institutional operating protocol. The study was approved by the University of British Columbia Research Ethics Board.

RESULTS

The MDS database included 395 patients who had been diagnosed since 1981, and 152 of them had a diagnosis confirmed by bone marrow aspiration and biopsy in 2009.
or later. Median age at MDS diagnosis was 73.5 years (range: 38–91 years), and 66% were men. Table I shows the baseline characteristics of the group. The RA and hypoplastic MDS subtypes were found in 7 (5%) and 4 patients (3%) respectively.

In the cohort overall, the IPSS score\(^a\) was low in 53 patients, intermediate-1 in 59 patients, intermediate-2 in 32 patients, and high in 8 patients. Hemoglobin was less than 100 g/L in 86 patients at MDS diagnosis and in 103 patients at any point during follow-up. Serum EPO was 500 mIU/mL or more in 4 patients. Measurements of LDH\(^b\), bilirubin\(^c\), and reticulocyte count\(^d\) were obtained (and found to be elevated) in 96, 109, and 142 patients respectively, and haptoglobin was low in 2 of 7 patients. In 6 patients, LDH and bilirubin were both elevated; in 23, only LDH was elevated; and in 5, only bilirubin was elevated. A DAT was recorded for 13 patients, and the result was negative in 9. Serum ferritin was assessed in 116 patients and was less than 100 ng/mL in 14. Table I and Figure 2 summarize the hemolysis investigations.

Treatment for MDS was supportive care only in 53 patients (34%); 81 patients (53%) received specific MDS treatment as documented in Table I. Of 77 patients (51%) with a RBC transfusion requirement at MDS diagnosis, the median requirement was 4 units (range: 1–8 units) per 8 weeks. In 38 patients (25%) with a RBC transfusion requirement exceeding 4 units per 8 weeks, the apparent reasons for no PNH testing were receipt of cytoreductive therapy (n = 21), advanced age (n = 4), lung cancer diagnosis limiting life expectancy (n = 2), hematopoietic stem-cell transplantation (n = 1), DAT positivity (n = 1), acute leukemia progression with patient declining chemotherapy (n = 1), and gastrointestinal bleeding (n = 1). None of the 7 remaining patients underwent PNH testing (although haptoglobin was undetectable in 1). No patients had hemoglobinuria or thrombosis within 12 months of MDS diagnosis or at any point during follow-up.

Of 11 patients who were tested for PNH, 1 was positive. Reasons for PNH testing included anemia (n = 3 (1 with abdominal symptoms and unexplained cytopenias pre-MDS diagnosis, who had undergone new-diagnostic bone marrow biopsies)), new MDS diagnosis (n = 2), hypoplastic MDS (n = 2), decreased haptoglobin (n = 1), increased RBC transfusion requirement (n = 1), and unexplained iron deficiency (n = 1).

Table III shows the characteristics of patients undergoing PNH testing. Median age for those 11 patients was 60.3 years (range: 39–78 years), with 54.5% being men. The MDS diagnoses were RA or hypoplastic MDS in only 2 patients. The IPSS score was low or intermediate-1 in 8 patients (73%), but intermediate-2 in 3 patients (27%). Median hemoglobin was 102 g/L (range: 86–118 g/L) in the group overall and less than 100 g/L in 5 patients. The RBC transfusion requirement per week was 0 units for 6 patients and more than 4 units for 5 patients. The only patient with a positive PNH test result was a 51-year-old man with a diagnosis of refractory cytopenia with unilineage dysplasia, an IPSS score of intermediate-1, and hemoglobin 96 g/L before transfusion dependence. The reason for PNH testing in this patient was a new MDS diagnosis with serum EPO exceeding 500 mIU/mL. Positivity for PNH was reported as 0.08% for granulocytes, 0.16% for monocytes, 0.03% for lymphocytes, 0.02% for platelets, 0.003% for reticulocytes, and 0.002% for megakaryocytes.

### Table I: Baseline clinical and laboratory characteristics of 152 patients with myelodysplastic syndrome (MDS) diagnosed since 2009

| Characteristic | Value [n (%)] |
|---------------|--------------|
| Age at MDS diagnosis | |
| <65 Years | 32 (21) |
| ≥65 Years | 120 (79) |
| Sex | |
| Men | 99 (65) |
| Women | 53 (35) |
| Specific MDS diagnosis | |
| Refractory anemia | 7 (5) |
| RARS\(^a\) | 17 (11) |
| RCMD with/without RS\(^b\) | 40 (26) |
| RAEB | 23 (15) |
| Hypoplastic\(^c\) | 4 (3) |
| Other\(^d\) | 61 (40) |
| IPSS risk group | |
| Low | 53 (35) |
| Intermediate-1 | 59 (39) |
| Intermediate-2 | 32 (21) |
| High | 8 (5) |
| Hemoglobin | |
| < Lower limit of normal | 139 (92) |
| <100 g/L | 86 (57) |
| Serum EPO | |
| <500 mIU/mL | 32 (21) |
| ≥500 mIU/mL | 4 (3) |
| RBCs transfused (per 8 weeks) | |
| 0 Units | 73 (49) |
| 1–2 Units | 11 (7) |
| 3–4 Units | 21 (14) |
| >4 Units | 38 (26) |
| Unclear | 7 (5) |
| Specific MDS treatment | |
| Supportive care only | 53 (34) |
| ESA | 39 (25) |
| Lenalidomide | 4 (3) |
| Azacytidine | 35 (23) |
| HSCT | 3 (2) |

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\(^a\) RARS with thrombocytosis in 2 patients.
\(^b\) RCMD with RS in 7 patients.
\(^c\) <20% cellularity.
\(^d\) Chronic myelomonocytic leukemia (n=24); MDS unspecified (n=13); acute myeloid leukemia with 20%–30% blasts (n=7); therapy-related MDS (n=5), del5q (n=4), MDS/myeloproliferative neoplasm overlap syndrome (n=4), refractory cytopenia with unilineage dysplasia (n=4).

RAEB = refractory anemia with ringed sideroblasts; RS = ringed sideroblasts; RARS = refractory anemia with excess blasts; IPSS = International Prognostic Scoring System; EPO = erythropoietin; RBCs = red blood cells; ESA = erythropoiesis stimulating agent; HSCT = hematopoietic stem-cell transplantation.
TABLE II Summary of hemolysis investigations leading to testing for paroxysmal nocturnal hemoglobinuria (PNH) in 152 patients with myelodysplastic syndrome (MDS) diagnosed since 2009

| Patient group | All pts | Proportion of patients (%) |
|---------------|---------|---------------------------|
|               | Overall | Hemoglobin <100 g/L at any point | LDH or BILI (or both) >ULN | Tested for PNH |
| All           | 152     |                           |                          |                 |
| With hemoglobin <100 g/L at any point | 103 | 66.8                      |                          |                 |
| With LDH or BILI (or both) >ULN | 34 | 22.4 | 33.0 |             |
| Tested for PNH | All | 11 | 7.2 | 10.7 |          |
| With increased LDH or BILI | 5 | 3.3 | 4.9 | 14.7 |             |
| Positive for PNH | 1 | 0.7 | 1.0 | 2.9 | 9.0 |

a Whether or not direct antiglobulin test–negative, decreased haptoglobin or increased reticulocyte count, refractory anemia or hypoplastic MDS subtype, or unexplained iron deficiency.

b With or without an increased LDH or BILI (see Figure 2).
Pts = patients; LDH = lactate dehydrogenase; BILI = bilirubin; ULN = upper limit of normal.

FIGURE 2 Reasons for paroxysmal nocturnal hemoglobinuria (PNH) testing in patients with myelodysplastic syndrome diagnosed since 2009.

DISCUSSION AND CONCLUSIONS

This retrospective single-centre review found that, in routine clinical practice, testing for PNH in MDS patients was infrequent. Despite potential indicators of hemolysis in some patients, only 11 patients (7%) diagnosed with MDS in 2009 onward underwent PNH testing. For example, of 29 patients with elevated LDH, only 3 (10%) underwent PNH testing, with none of the 3 being positive. Similarly, of 7 patients in whom haptoglobin was measured, a decreased level was found in 2, but only 1 underwent PNH testing, with a negative result. Clinical rather than laboratory indicators prompted PNH testing in 6 of 11 patients. In 1 patient who presented with ISS intermediate-1 MDS unclassified, complicated by anemia and abdominal symptoms (abdominal computed tomography imaging was negative for thrombosis), a PNH test performed in the absence of indicators of hemolysis was negative. The 1 patient who was found to have a PNH clone had diagnosis of refractory cytopenia with unilineage dysplasia without a hypocellular marrow and, despite normal LDH and bilirubin levels, underwent testing because of a new MDS diagnosis with a transfusion requirement of 4 units per 8 weeks and serum EPO exceeding 500 mIU/mL.

Of 11 patients who underwent PNH testing, 8 had ISS low or intermediate-1 MDS; however, 3 patients had intermediate-2 disease. In those 3 patients, the reasons for testing were a hypoplastic bone marrow in RA with excess blasts−2, a RBC transfusion requirement of 5 units per 8 weeks with elevated LDH in RA, and anemia from chronic myelomonocytic leukemia in a patient 39 years of age. All 3 patients were negative for PNH cells. Because of findings in some studies that patients of all MDS subtypes and risk groups might have PNH cells, PNH testing could be considered in all MDS patients with evidence of hemolysis, with or without elevated serum EPO, thrombosis, or hemoglobinuria 15–17.

We found no patients with thrombosis or hemoglobinuria in this series. However, thrombosis is frequent in PNH, with an incidence ranging from 5% in patients with a PNH clone size of less than 10%, to 15% in patients with a clone.
size of 50% or greater, and with an incidence of 8% or 16% for patients with serum LDH below or 1.5 times the upper limit of normal respectively. Before the eculizumab era, thrombotic events were responsible for approximately half the deaths in pNH. However, given that we did not observe thrombotic events in our MDS patients, unrecognized pNH as a contributing factor to that particular morbidity might be less than feared. We did not rigorously collect data about renal insufficiency or pulmonary hypertension; however, although we cannot rule out renal insufficiency on the basis of unrecognized subtle hemoglobinuria, other contributors to renal insufficiency in the age group at risk for MDS are common, and those data are likely best collected prospectively. On the other hand, renal insufficiency secondary to hemolysis could account for mortality in a substantial minority of pNH patients. Similarly, pulmonary hypertension and right ventricular failure can present in a subclinical manner in pNH. Given the implications for morbidity and mortality, monitoring for those conditions should be undertaken in MDS patients.

The frequency of pNH clones in MDS has been reported to be up to 8% in all MDS and 53.3% in RA. Elevated LDH was seen in 42% of MDS patients with a pNH clone, although clinical hemolysis was associated with a clone size greater than 50%. In contrast, the pNH clone size in series of MDS patients is reported to range from a low of 0.02% to a high of 2.41%. In keeping with the results of whole-exome sequencing showing that mutations in PIGA can occur as primary or secondary mutational events, pNH clones were found in MDS patients with RA, RA with ringed sideroblasts, refractory cytopenia with multilineage dysplasia with or without ringed sideroblasts, MDS unclassified, del(5q), MDS with RA with excess blasts, del(5q)/MDS, and RA with excess blasts. Cells of all lineages can lack GPI, suggesting that the development of hematopoietic stem cells and lymphocytes might both be deregulated in pNH.

The clinical significance of pNH positivity in MDS is not yet fully understood. Some data in small numbers of patients suggest that pNH clones in MDS arise in committed progenitor cells rather than in hematopoietic stem cells, limiting the lifespan of affected cells. Complement-mediated hemolysis could exacerbate anemia and increase the rbc transfusion requirement in MDS. Clone size can fluctuate in pNH without MDS, and fluctuation is probably also the case in MDS patients with pNH cells. However, the evolution of pNH clone size over time in MDS has been addressed only minimally. In one study of 25 patients with 0.11%–1% positivity, including those with MDS, 10 showed an increase in clone size over time (time points not specified). Although such increases have yet to be demonstrated in MDS, Clinical Cytometry guidelines recommend annual monitoring in patients with a stable pNH clone size and more frequent monitoring in patients with clones that fluctuate. Another unanswered question in MDS is whether pNH clones can be gained or lost during the MDS course.

There is some evidence that patients having MDS with pNH positivity might achieve a better response to immunosuppressive therapy. For example, Ishikawa et al. used cyclosporine A in the treatment of 20 patients with MDS, 17 of them with RA, and found that short duration of MDS, RA subtype, and the presence of pNH cells were associated with a platelet response. In addition, in another study of patients with MDS, 77.8% of patients with a positive test for pNH cells but 0% of those who were pNH-negative responded to cyclosporine. Patients with fewer than 10% pNH cells had LDH levels close to the upper limit of normal, making

### TABLE III Characteristics of 11 patients with myelodysplastic syndrome (MDS) who underwent testing for paroxysmal nocturnal hemoglobinuria (PNH)

| Pt ID | Age at MDS diagnosis (years) | Sex | MDS type | IPSS score | Hemoglobin (g/L) | Transfusion requirement (RBC units per 8 weeks) | PNH testing | Reason | Result |
|-------|-----------------------------|-----|----------|------------|-----------------|-----------------------------------------------|------------|--------|--------|
| 1     | 51                          | Male | RCU D    | Intermediate-1 | 96              | 4                                             | New MDS    |         | Positive |
| 2     | 55                          | Female | Therapy-related MDS | Low       | 118             | 0                                             | Iron deficiency anemia |         | Negative |
| 3     | 72                          | Male | RAEB-2   | Intermediate-2 | 102             | 0                                             | Hypocellular bone marrow biopsy |         | Negative |
| 4     | 55                          | Female | Therapy-related MDS | Intermediate-1 | 98              | 0                                             | High lactate dehydrogenase, low haptoglobin |         | Negative |
| 5     | 67                          | Male | MDS unspecified | Intermediate-1 | 98              | 4                                             | Pre-MDS diagnosis |         | Negative |
| 6     | 59                          | Female | Hypocellular | Intermediate-1 | 113             | 0                                             | Hypocellular |         | Negative |
| 7     | 44                          | Male | RCMD     | Intermediate-1 | 111             | 8                                             | High transfusion requirement |         | Negative |
| 8     | 78                          | Male | RCMD-RS  | Low         | 117             | 0                                             | New MDS |         | Negative |
| 9     | 71                          | Female | Refractory anemia | Intermediate-2 | 97              | 5                                             | High transfusion requirement, high lactate dehydrogenase |         | Negative |
| 10    | 72                          | Male | MDS unspecified | Intermediate-1 | 110             | 8                                             | Low hemoglobin, abdominal symptoms |         | Negative |
| 11    | 39                          | Female | CMML     | Intermediate-2 | 86              | 0                                             | Low hemoglobin |         | Negative |

a Gastrointestinal bleeding suspected.

Dx = diagnosis; IPSS = International Prognostic Scoring System; RBC = red blood cell; RCU D = refractory cytopenia with unilineage dysplasia; RAEB = refractory anemia with excess blasts; RAEB-2 = refractory anemia with excess blasts; RCMD = refractory cytopenia with multilineage dysplasia; RS = ringed sideroblasts; CMML = chronic myelomonocytic leukemia.
clinical hemolysis unlikely; however, that clone size did not preclude a significant incidence of PNH symptoms.\(^2^7\).

Given that an effective treatment for PNH is otherwise available, testing for PNH should be considered in MDS, and such testing is supported by recommendations from the National Comprehensive Cancer Network’s MDS practice guideline, the International PNH Interest Group, and the International Clinical Cytometry Society.\(^6^,15–18,31\). However, access to specific PNH treatment can be a process. For example, reimbursement for eculizumab in British Columbia is adjudicated by a committee of health care professionals who make a recommendation to BC Pharmacare. Criteria for funding include a diagnosis of PNH confirmed by flow cytometry of FcεR binding, with a granulocyte clone greater than 10% and serum LDH more than 1.5 times the upper limit of normal, accompanied by significant clinical sequelae attributable to PNH, and excluding patients having conditions with a poor prognosis or likely to compromise response to therapy. Immunosuppressive therapy is, however, more readily available and should be considered in MDS patients with PNH cells. In one study, most MDS patients with PNH positivity were also positive for HLA-DR15, which is also predictive of a response to immunosuppressive therapy. However, the contribution of PNH positivity to symptoms and morbidity in MDS is currently unclear and is sufficiently concerning to justify prospective analyses addressing those points.

Limitations of the present study include its retrospective nature and the inclusion of only 7 patients (5%) with RA, the MDS subtype in which PNH cells are most prevalent. Only a few patients with MDS were tested for PNH despite evidence of hemolysis in some, and the criteria for testing were not uniform. Ideally, criteria for PNH testing in MDS would be settled by data from prospective analyses. Until such data are available, and although only 1 of 11 patients tested was positive for PNH cells, given that a specific effective treatment is available and that patients with PNH-positive MDS might have a better response to immunosuppressive therapy, testing should be considered more frequently in clinical practice.

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**CONFLICT OF INTEREST DISCLOSURES**

We have read and understood Current Oncology’s policy on disclosing conflicts of interest, and we declare the following interests: HAL has received speaker fees from Novartis Corporation and also fees as an advisory board member for AbbVie, Alexion, Celgene, and Novartis Corporations; HAL’s institution receives funding from AbbVie Corporation for a trial in which she is a co-investigator. The remaining authors have no conflicts to disclose.

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