

Blood tests may predict early primary myelofibrosis in patients presenting with essential thrombocythemia

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According to World Health Organization (WHO)-defined criteria, patients presenting clinically as essential thrombocythemia (ET) may show early primary myelofibrosis (PMF) with accompanying thrombocythemia [1]. Previous clinicopathological studies revealed that laboratory parameters like gender-matched hemoglobin (Hb), white blood cell (WBC) count, and particularly lactate dehydrogenase (LDH) values are significantly different in PMF [2]. By strictly applying the WHO criteria, our investigation was aimed to study sensitivity and specificity of these features in an exploratory cohort of 536 patients and to validate the results on an independently recruited series of 321 strictly corresponding patients. The discriminatory power of these parameters (Hb, WBC, and LDH) was tested by plotting their receiver operating characteristic curves. The best performance was found for LDH (areas under the curve, AUC = 0.7059). WBC and Hb had superimposable curves, with AUC of 0.6279 and 0.6257, respectively. A diagnostic algorithm was generated by applying these parameters in a stepwise fashion. Nearly half of the patients could be correctly allocated to WHO-defined ET or early PMF in both cohorts investigated. It is important to note that this result does not substitute bone marrow morphology with hematological parameters, however, in clinical practice may alert physicians to get more suspicious of early PMF in a patient presumably presenting with ET.

Following current criteria established by the World Health Organization (WHO), patients presenting with a clinical picture of essential thrombocythemia (ET) can actually have an early primary myelofibrosis (PMF) [3]. Incidences may vary depending on the accuracy of applied diagnostic guidelines [4–6] and was recently reported in about 18% of cases of so-called ET [7]. Laboratory tests which are significantly different in early PMF as compared with histologically confirmed ET (WHO-ET) include decreased Hb, increased white blood cell (WBC) and lactate dehydrogenase (LDH) values. Although bone marrow biopsy represents the gold standard for differentiating WHO-ET from early PMF [8], in clinical practice it might be very useful to know whether one or more of these baseline laboratory parameters may help to differentiate these two entities. To tackle this issue, we evaluated sensitivity (SE) and specificity (SP) of blood cells counts and LDH for the diagnosis of early PMF versus WHO-ET in an exploratory cohort of 536 histologically diagnosed patients aimed to propose an algorithm for the practical clinical use of these tests. This algorithm was then applied to an independently recruited validation cohort of 321 patients presenting with similar diagnosis of early PMF versus WHO-ET. The starting point of this study was an international database of 1,071 patients with ET either confirmed by WHO criteria (891 cases) or revised to early PMF (180 cases) as detailed elsewhere [7]. From this database, 536 patients (50%) who had complete laboratory data measured at diagnosis were extracted and constituted the training set of our study.

Clinical and laboratory characteristics of the 536 selected patients were not different from that of the initial population. To recognize early PMF, the salient parameters SE and SP have been evaluated by receiver operating characteristic (ROC) curves (Fig. 1). The worst performance was registered for platelets (PLT) count: its areas under the curve (AUC) was only 0.5628, not significantly different from the reference value of 0.50 ($P = 0.154$). Thresholds of Hb, WBC and LDH were searched to achieve at least 90% of SE or SP. Table I shows that Hb < 12 g/dL for women or < 13 g/dL for men, or WBC >= 13 × 10⁹/L had higher SP (92 and 91%,

respectively). High SP of these two tests is associated with few false positives (WHO-ET cases, incorrectly identified as early PMF), thus it is highly related to the presence of early PMF. Concerning high levels of LDH, we could not find an upper threshold with a SP >90% for diagnosis of early PMF and including at least 5% of patients. On the contrary, LDH < 200 mU/mL and WBC < 7 × 10⁹/L had good SE (91 and 94%, respectively). High SE produces few false negatives (early PMF cases not recognized and incorrectly classified as ET), thus it is highly related to the confirm of WHO-ET diagnosis.

To optimize the use of this data in daily clinical practice, a step-by-step algorithm was proposed. By applying the criterium “anemia,” SP was 92% indicating that 46/536 (9%) of patients presenting anemia were correctly classified in 92% of cases as early PMF. In the 490 remaining patients, we applied two different WBC cut-offs: 97 (18%) and 43 (8%) patients were classified as WHO-ET or early PMF. Finally, in the remaining patients, LDH value (<200 mU/mL) classified as WHO-ET in another 13% of patients. Thus, following these steps, nearly half of patients (48%) could be classified as WHO-ET (by SE) or early PMF (by SP), assuming at each step a margin of error of about 10% (Fig. 2). For the remaining 50% of patients, laboratory results did not allow to suspect or exclude the presence of early PMF. To validate these results, the performance of the same thresholds of laboratory parameters was tested in 321 patients classified as WHO-ET or early PMF (Cologne cohort). SP of anemia was 84%, WBC below 7 × 10⁹/L or above 13 × 10⁹/L had 91 and 81% of SE and SP, respectively. LDH values < 200 revealed a SE of 85%. By applying the above reported flow chart, the percentage of patients classified as WHO-ET or early PMF was almost similar (46%), even though the margin of error at each step was higher, likely due to smaller number of patients.

In conclusion, while patients presenting clinically with ET can now be discriminated as true ET or early PMF by adopting the WHO 2008 criteria that require bone marrow histology, considering Hb, WBC, and LDH, this differentiation can be recognized in about 50% of patients with a good approximation. On the other hand, for a definitive proof, bone marrow histology remains still an integral part for final diagnosis. For this reason, it should be emphasized that results of our study do not substitute bone marrow biopsy with laboratory parameters to distinguish true ET from early PMF. Instead, it is to provide clinicians with laboratory parameters that should increase suspicion of early PMF in a patient with a working clinical diagnosis of ET.

Methods

Laboratory parameters considered in this study were those already found significantly different between early PMF and WHO-ET: Hb (g/dL), WBC (×10⁹/L), PLT (×10⁹/L), and LDH (mU/mL). The discriminatory ability of these parameters in correctly classifying patients in the early PMF or WHO-ET groups was initially tested by plotting their ROC curves and comparing the relative AUC with the value of 0.50 (which stands for the completely useless application of the test) [9,10]. Three parameters with statistically significant discriminatory power were chosen (Hb, WBC, and LDH) and opportune thresholds searched to guarantee at least 90% of SE or SP [11]. Finally, a diagnostic algorithm was designed. The validation set of this analysis was constituted by 321 patients with WHO-ET ($n = 62$) or early PMF ($n = 259$) diagnosed by the same pathologist who confirmed the training set cohort and collected in the Institute for Pathology, University of Cologne, Germany.

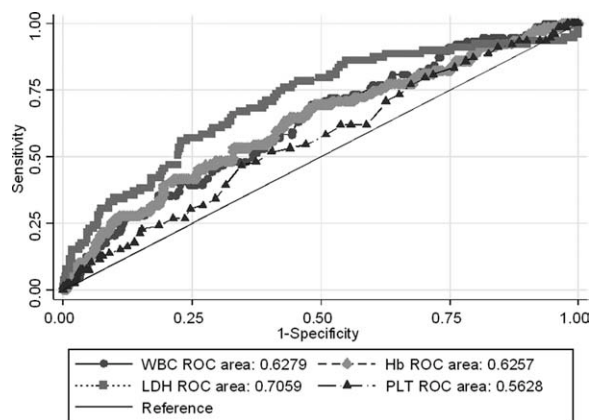


Figure 1. ROC curves of Hb, WBC, PLT, and LDH for the diagnosis of early/ prefibrotic PMF.

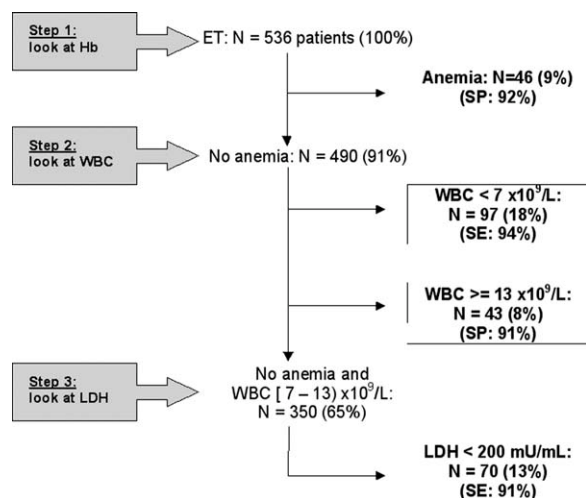


Figure 2. Algorithm of sensitivity (SE) and specificity (SP) to detect or exclude early PMF versus WHO-ET.

TABLE I. Sensitivity and Specificity for Hb, WBC, and LDH Thresholds

Parameters' thresholds	N	Cases	Specificity% - / true-	Sensitivity% + / true+
1 Hb < 13 g/dL for men or Hb < 12 g/dL for women	536	46	422/457 (92%)	11/79 (14%)
2 WBC >= 13 x 10 ⁹ /L	536	56	417/457 (91%)	16/79 (20%)
3 WBC < 7 x 10 ⁹ /L	536	104	99/457 (22%)	74/79 (94%)
4 LDH < 200 mU/mL	536	428	101/457 (22%)	72/79 (91%)

SE and SP for the same parameters and thresholds as well as the final diagnostic algorithm were applied to this set of patients to demonstrate the reproducibility of the results.

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TP53 mutations and polymorphisms in primary myelofibrosis

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A total of 107 patients with chronic-phase primary myelofibrosis (PMF) were screened for TP53 mutations, which were detected in 4 (4%) cases: (i) E204E; GAG>GAA (silent exon 6); (ii) G245D; GGC>GAC (exon 7); (iii) R175H; CGC>CAC (exon 5); and (iv) six base insert (GGCGAG) after bp13767 (exon 6). Three (75%) of the four TP53-mutated cases also carried JAK2V617F whereas none were positive for MPL or IDH mutations. Two of the four TP53 mutated cases were also screened for TET2, ASXL1, DNMT3A, and EZH2 mutations and were negative. There was no significant difference in presenting features or

survival between TP53 mutated and unmutated cases. TP53 exon 4 single nucleotide polymorphism (SNPs) data for codon 72 were available on 104 patients and included 56% with homozygous Arg72Arg, 33% with heterozygous Pro72Arg, and 11% with homozygous Pro72Pro. There were no significant differences among the three codon 72 genotypes in terms of presenting characteristics or survival.

PMF is biologically more aggressive [1–3] than other myeloproliferative neoplasms (MPN) and displays higher prevalence of cytogenetic abnormalities [4–7], immune dysregulation [8,9], and somatic mutations [10], including

JAK2, *MPL*, *TET2*, *ASXL1*, *CBL*, *IDH1*, *IDH2*, *IKZF1*, *LNK*, *EZH2*, *DNMT3A*, *CUX1*, and *SF3B1* mutations [10–13]. These mutations are believed to represent late genetic events derived from an ancestral abnormal clone whose genetic make up remains elusive. The fact that many of these mutations are infrequent and lack disease specificity further undermines their pathogenetic contribution to disease initiation [10]. On the other hand, the absence of mutual exclusivity and the higher prevalence of some MPN-associated mutations, such as *IDH* [14], *LNK* [15], *IKZF1* [16], and *TP53* [17] mutations, in blast-phase, as opposed to chronic-phase, MPN suggests possible pathogenetic contribution to leukemic transformation. Consistent with this contention, we recently showed that the presence of mutant *IDH* signified an increased risk of leukemic transformation in PMF [18] whereas others have demonstrated the same for *EZH2* mutations [19]. In the current study, we examined the possibility of a similar phenomenon with *TP53* mutations in chronic phase PMF and also examined the prognostic effect of *TP53* codon 72 SNPs.

The current study was approved by the Mayo Clinic institutional review board. Study eligibility criteria included availability of bone marrow histology and cytogenetic information at time of referral to the Mayo Clinic. The diagnoses of PMF and leukemic transformation were according to the World Health Organization criteria [20]. Patients with blast-phase disease at the time of their referral to the Mayo Clinic were excluded from the study because one of the objectives of the study was to assess mutation impact on leukemic transformation. Unfavorable karyotype designation and Dynamic International Prognostic Scoring System-plus (DIPSS-plus) risk categorization were as previously described [21,22]. All study patients were fully characterized for karyotype, *JAK2*, *MPL*, and *IDH* mutational status and DIPSS-plus risk category.

MPL, *JAK2*, and *IDH* mutation analyses were performed according to previously published methods [14,23–27]. DNA from bone marrow or peripheral blood was extracted using conventional methods and amplified with one set of primers covering *TP53* exons 4 through 9 (primer sequences available by request). The amplification products were then sequenced with eight primers (primer sequences available by request) using the Big Dye Terminator sequencing kit v1.1 (Applied Biosystems, Foster City, CA) and the ABI 3130xl Genetic Analyzer. Sequences were examined for mutations by the Sequencher software (Gene Codes, Ann Arbor, MI). Any mutation found was confirmed with a second polymerase chain reaction (PCR) and sequencing reaction. A mutation was considered to be any sequence other than genomic reference sequence NC_000017.10 or a well documented polymorphism such as the rs1042522 in exon 4. Mutations were compared with the IARC p53 Database (<http://www.p53.iarc.fr/>) and/or the *TP53* Mutation Database (<http://p53.free.fr/index.html>) to determine whether the mutation had been reported before.

All statistical analyses considered clinical and laboratory parameters obtained at time of first referral to the Mayo Clinic, which in most instances coincided with time of bone marrow biopsy at the Mayo Clinic and study sample collection. Differences in the distribution of continuous variables between categories were analyzed by either Mann–Whitney (for comparison of two groups) or Kruskal–Wallis (comparison of three or more groups) test. Patient groups with nominal variables were compared by chi-square test. Overall survival was calculated from the date of first referral to date of death (uncensored) or last contact (censored). Survival curves were prepared by the Kaplan–Meier method and compared by the log-rank test. *P* values less than 0.05 were considered significant. The Stat View (SAS Institute, Cary, NC) statistical package was used for all calculations.

A total of 107 patients with chronic-phase PMF were studied. Median age at time of referral was 64 years (range 32–81) and 71% were males. DIPSS-plus risk assignment was low in 13%, intermediate-1 in 15%, intermediate-2 in 38%, and high in 34%. Karyotype was normal in 70 (65%) patients, favorable in 26 (24%), and unfavorable in 11 (10%). *JAK2*, *MPL*, and *IDH* mutations were detected in 62 (58%), 7 (7%), and 6 (6%) cases, respectively. To date, 74% of patients had died and 15% had documented leukemic transformation.

TP53 mutations were discovered in 4 (4%) cases: (i) E204E; GAG>GAA (silent exon 6); (ii) G245D; GGC>GAC (exon 7); (iii) R175H; CGC>CAC (exon 5); and (iv) six base insert (GGCGAG) after bp13767 (exon 6). Three (75%) of the four *TP53*-mutated cases carried *JAK2V617F* whereas none were positive for *MPL* or *IDH* mutations. The differences in *JAK2*, *MPL*, and *IDH* mutational frequencies between *TP53* mutated and unmutated cases

were not significant; corresponding *P* values were 0.50, 0.59, and 0.62. Two of the four *TP53* mutated cases were also screened for *TET2*, *ASXL1*, *DNMT3A*, and *EZH2* mutations and were negative. Cytogenetic findings were also similar (*P* = 0.38) between *TP53* mutated (three normal and one unfavorable karyotype) and unmutated (67 normal, 28 favorable, and 10 unfavorable karyotype) cases.

The four *TP53* mutated cases were of ages 52, 53, 75, and 81 years and three were males. Two of the four patients were red cell transfusion dependent and hemoglobin levels were 11 and 12.4 g/dL. Leukocyte/platelet counts were 2/88, 6/210, 16/230, 37/70 × 10⁹/L. DIPSS-plus risk distributions were high in two patients, one intermediate-2, and one intermediate-1. Time of referral to Mayo Clinic was at 2, 6, 20, and 49 months from initial diagnosis and follow-up time from time of referral was 3, 61, 8, and 32 months, respectively. Treatment was conventional and none of the four *TP53* mutated patients underwent allogeneic stem cell transplant. None of the four patients with *TP53* mutation incurred leukemic transformation and two of the four patients were dead at time of this writing. There was no significant difference in presenting features or survival between *TP53* mutated and unmutated cases; the results were the same even when the one patient with silent mutation was excluded from the analysis. *TP53* exon 4 SNPs data for codon 72 were available on 104 patients and included 58 (56%) patients with homozygous Arg72Arg, 34 (33%) patients with heterozygous Pro72Arg, and 12 (11%) patients with homozygous Pro72Pro. Allele frequencies were 0.7 for G (corresponding to arginine) and 0.3 for C (corresponding to proline), which is similar to the general population (0.65 and 0.35, respectively). There were no significant differences among the three codon 72 genotypes in terms of presenting characteristics or survival (data not shown).

The current study reveals a low frequency occurrence of *TP53* mutations in chronic phase PMF. A higher number of informative cases are needed to accurately define their phenotypic or prognostic effects. On the other hand, *TP53* polymorphisms at codon 72 do not appear to influence phenotype or prognosis in chronic-phase PMF.

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Relative efficacy of steroid therapy in immune thrombocytopenia mediated by anti-platelet GPIIb/IIIa versus GPIb α antibodies

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Immune thrombocytopenia (ITP) is characterized by platelet clearance mediated primarily by autoantibodies against the platelet GPIIb/IIIa and/or GPIb α . Steroid therapy is a first-line treatment for ITP. However, some patients are refractory to this therapy and currently no method can predict which patients will respond. To evaluate whether steroids are equally efficacious in treating patients with ITP caused by anti-GPIIb/IIIa versus anti-GPIb α antibodies, we performed a retrospective study on 176 newly diagnosed patients with acute ITP who had severe bleeding symptoms and were admitted as resident patients to the hospital. The patients were treated first with intravenous administration of high-dose dexamethasone (DXM), followed by oral administration of prednisone. Response to therapy was observed in a majority of patients with antibodies specific for GPIIb/IIIa (31/43) or without detectable antibodies against either GPIIb/IIIa or GPIb α (36/45). In contrast, the steroid response was significantly lower in patients with anti-GPIb α antibodies (9/34) or with antibodies against both GPIb α and GPIIb/IIIa (16/54). The preliminary findings of this study suggest that in future prospective clinical trials including corticosteroids, the anti-GPIb α , and -GPIIb/IIIa status should be assessed in order to test its potential relevance in deciding future treatments.

ITP is a relatively common autoimmune bleeding disorder which is caused primarily by autoantibody-mediated platelet destruction in the reticuloendothelial system [1–3]. The major targeted platelet autoantigens have been localized on the glycoprotein (GP) IIb/IIIa and GPIb α complexes [4,5]. Approximately, 70–80% of ITP patients have autoantibodies against GPIIb/IIIa, and 20–40% of patients have antibodies against either GPIb α or both GPIIb/IIIa and GPIb α [1,5]. Our previous studies in a murine model of ITP demonstrated that thrombocytopenia induced by most anti-GPIb α antibodies was refractory to intravenous IgG (IVIg) therapy while thrombocytopenia mediated by anti-GPIIb/IIIa antibodies was responsive to IVIg [6]. These data are consistent with subsequent retrospective studies in human ITP patients by other independent research groups [7,8] How-

ever, it is still largely unknown whether the pathogenesis and therapeutic responses to other therapies are different in patients with ITP caused by autoimmune responses against these two distinct platelet antigens.

Steroids have been used to treat ITP for decades and are the most commonly used treatment for this disorder worldwide [9–16]. Although a majority of patients benefit from traditional steroid therapy, some are refractory to this treatment [11,12] as well as more recent steroid treatment protocols which utilize repeated pulses of high-dose DXM [13]. The mechanisms by which steroid therapy alleviates ITP are not well understood, but it is generally accepted that immunosuppression may be central to the efficacy of this therapy. Since there are few studies reporting the autoantibody specificity of ITP patients receiving steroids [14], it is unclear whether steroid therapy has equal efficacy in patients with ITP caused by either anti-GPIIb/IIIa or anti-GPIb α autoantibodies.

In the present study, we recruited 176 acute ITP patients with severe bleeding symptoms who were admitted as resident patients to the hospital. We found that 24.4% (43/176) of patients had antibodies against GPIIb/IIIa, 19.3% (34/176) had antibodies against GPIb α , 30.7% (54/176) had antibodies against both GPIIb/IIIa and GPIb α (double positive), and 25.6% (45/176) had no detectable antibody to either of these two antigens (double negative) (Table I). No significant difference was observed between the ratio of female and male patients with single positive anti-GPIb α (female: 25/122 = 20.5%, male: 9/54 = 16.7%), single positive anti-GPIIb/IIIa (female: 27/122 = 22.1%, male: 16/54 = 29.6%), double positive (female: 37/122 = 30.3%, male: 17/54 = 31.5%), or double negative (female: 33/122 = 27.1%, male: 12/54 = 22.2%) ($P > 0.05$). There was also no significant difference observed between age and antibody specificities ($F = 1.623$, $P > 0.05$).

After patients were subjected to steroid therapy, we found that a majority of patients with antibodies against GPIIb/IIIa (31/43 = 72.1%) were sensitive to the treatment (Table II). Their response rate was 2–3 times higher than the response rate from patients with antibodies against GPIb α (9/34 = 26.5%) ($\chi^2 = 15.832$, $P < 0.01$) or those patients double positive for both

TABLE I. Profile of ITP Patients and Antibody Status

Number of patients	176
Sex (female/male)	122/54
Median age (years)	40 (13–91)
Median number of platelet ($\times 10^9/L$)	6 (1–34)
Bleeding score	14 (4–23)
Antiplatelet antibodies	
GPIIb/IIIa(+),GPIb α (-) (female/male)	27/16
GPIIb/IIIa(-),GPIb α (+) (female/male)	25/9
GPIIb/IIIa(+),GPIb α (+) (female/male)	37/17
GPIIb/IIIa(-),GPIb α (-) (female/male)	33/12

GPIb α and GPIIbIIIa antibodies (16/54 = 29.6%) ($\chi^2 = 17.282$, $P < 0.01$). No significant difference in steroid responsiveness was observed between patients with anti-GPIb α antibodies and those with double positive antibodies ($\chi^2 = 0.102$, $P > 0.05$). Furthermore, no significant difference was observed between patients with anti-GPIIbIIIa antibodies and those with no detectable antibodies (36/45 = 80%) ($\chi^2 = 0.757$, $P > 0.05$). There was also no significant difference observed between patient's age and their responsiveness to steroid therapy ($t = 0.071$, $P > 0.05$).

These data suggest that anti-GPIb α , but not anti-GPIIbIIIa, antibodies may dictate the efficacy of steroid therapy. Patients with anti-GPIb α antibodies, regardless of anti-GPIIbIIIa antibody status, had significantly poorer steroid response rates (25/88 = 28.4%) than patients without detectable anti-GPIb α antibodies (including double negative patients and patients with only anti-GPIIbIIIa, 67/88 = 76.1%, $\chi^2 = 40.17$, $P < 0.01$). Therefore, detection of anti-GPIb α antibodies may have the potential to identify patients with a less favorable response to steroid therapy.

It is interesting that the results from the present study are consistent with our previous report showing that anti-GPIb α -mediated-ITP is less responsive to IVIG treatment in a murine model [6] and subsequent retrospective studies in human patients by other groups [7,8]. It is currently unknown whether steroids and IVIG target similar pathways that efficiently control the pathogenesis and immune response of ITP induced by GPIIbIIIa, but not GPIb α , autoantigens. Our findings that patients negative for both antibody specificities are sensitive to steroid therapy are interesting and worthwhile for further investigation. It is unclear whether some of these patients have autoantibodies that recognize the physiological structure of GPIIbIIIa, but not EDTA-treated samples utilized during current monoclonal antibody immobilization of platelet antigen assay (MAIPA) procedures, since GPIIbIIIa (but less likely GPIb α) needs divalent cations to maintain its proper structure and function on the platelet surface [17]. It is also unclear whether steroids may target the alternative mechanism of platelet destruction by cytotoxic T cells in these antibody double negative patients [18–21], since T cells may be sensitive to steroid treatment [22,23].

To the best of our knowledge, the results from this study are the first to distinguish between the therapeutic effects of steroids in ITP patients with autoantibodies against the two major platelet autoantigens GPIIbIIIa and GPIb α . It is currently mechanistically unknown why anti-GPIb α -mediated ITP is less responsive to steroid and IVIG therapies. However, data from others [24] and our group [25] suggest that anti-GPIb α antibodies may induce platelet activation, aggregation, and apoptosis, which may lead to Fc-independent platelet clearance [6,26,27] in the reticuloendothelial system. Further study of the autoimmune response to GPIb α and comparison of its differences with anti-GPIIbIIIa responses may provide insight into the development of new therapies for treating these patients who are refractory to steroid and IVIG therapies.

Methods

Patients and treatment protocol. ITP patients were recruited between 2005 and 2010. Patients who had severe bleeding symptoms (bleeding scores ranging from 4 to 23) [28] were recruited as resident patients for the therapy in the hospital. A total of 176 newly diagnosed ITP patients, who had no previous ITP therapy [16] but had data available regarding treatment outcomes and anti-platelet antibody status, were analyzed in the present study. The patient profiles are listed in Table I. The therapeutic procedures and the definition of response were predetermined before the study started. The patients were first treated with high-dose DXM (20–40 mg/day \times 3–5 days) intravenously, followed by oral administration of prednisone (1 mg/kg/day) for maintenance [15]. The dose of prednisone was gradually decreased and maintained for 28 days. The patients were considered responders if no

TABLE II. ITP Patients Associated With Specific Antibodies and Response to Steroid Therapy

	GPIb α (+)		GPIb α (-)		Total
	GPIIbIIIa(-)	GPIIbIIIa(+)	GPIIbIIIa(-)	GPIIbIIIa(+)	
R	9 (26.5%)	16 (29.6%)	36 (80%)	31 (72.1%)	92
NR	25	38	9	12	84
Total	34	54	45	43	176

R = response; NR = no response

obvious hemorrhage was detected, and their platelet counts were raised to $\geq 30 \times 10^9/L$ and increased to double their original pre-treatment platelet count [16]. Patients who failed to reach these criteria during DXM and prednisone treatments had to be treated with alternative therapies, and were considered non-responders in the present study. Patients' plasma samples were collected prior to therapy and were aliquoted and stored in $-80^\circ C$. The samples were simultaneously tested blindly for antibody status after therapy was completed.

Preparation of platelet lysate. Whole blood was taken from healthy type-O donors into ethylenediaminetetraacetic acid (EDTA)-Na and centrifuged at 754g for 10 min [19,29,30]. Platelet-rich plasma was transferred to 10-mL tubes and centrifuged at 3,361g for 5 min; supernatant was decanted and platelets were washed with phosphate buffered saline (PBS)/EDTA three times. One hundred microliters platelet suspensions ($200 \times 10^9/L$) were incubated with test sample (50 μL plasma) at $37^\circ C$ for 60 min and then washed with PBS/EDTA three times to remove unbound plasma proteins, including possible non-pathogenic autoantibodies against the GPIIbIIIa cytoplasmic tail. Platelets were lysed with 130 μL lysis buffer (1%NP40, 150 mM NaCl, 50 mM Tris-HCL pH 8.0, 2 mM EDTA, 0.1 mM phenylmethanesulfonylfluoride (PMSF)) at $4^\circ C$ for 30 min, centrifuged at 14,000 rpm at $4^\circ C$ for 20 min, and 100 μL from each supernatant was collected and diluted 1:2 for further testing.

Detection of anti-GPIIbIIIa and anti-GPIb α autoantibodies. Anti-GPIIbIIIa and/or anti-GPIb α were detected with a MAIPA [8,19,21,30]. Briefly, goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MO) was coated (3 $\mu g/100 \mu L$ well) into microplates at $4^\circ C$ overnight. After being washed with TBST buffer (10 mM Tris-HCL pH 7.4, 150 mM NaCl, 0.5 mM CaCl₂, 0.5% NP40, and 0.05% Tween 20) and blocked with 10% BSA, 40 μL (1 $\mu g/mL$) of mouse anti-CD61 (DakoCytomation, Canada Inc, Mississauga, ON) or anti-CD42b (Beckman Coulter, Canada, Inc, Mississauga, ON) were added to each well and incubated for 60 min at $37^\circ C$. Microplates were then washed and 100 μL of prepared platelet lysate (pre-incubated with patient plasma) was added and incubated for 90 min at $4^\circ C$. Alkaline phosphatase-conjugated goat anti-human IgG and *p*-nitrophenyl phosphate (Sigma-Aldrich) was used to develop the color. For each experiment, 30–40 normal sera samples randomly selected from 100 healthy blood donors were used as negative controls, and were tested simultaneously with 40–50 patient samples and 1 positive control in a single ELISA plate to decrease possible variations with MAIPA. An optical density (OD) value >3 standard deviations (SD) from the mean of the healthy donors' sera values was considered as positive. MAIPA was repeated at least three times for each sample and the results were confirmed blindly and independently from the patient therapy.

Statistical analysis. Differences in response between the groups were assessed by χ^2 test, ANOVA, and student *t* test as indicated.

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Practical approach for characterization of glucose 6-phosphate dehydrogenase (G6PD) deficiency in countries with population ethnically heterogeneous: Description of seven new G6PD mutants

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We present a rapid strategy based on Restriction Fragment Length Polymorphism (RFLP) analysis to characterize the more frequent glucose 6-phosphate dehydrogenase (G6PD) variants observed in a population with high gene flow. During a study involving more than 600 patients, we observed mainly G6PD A⁻ (c.202G>A, c.376A>G; p.Val68Met, p.Asn126Asp), G6PD Mediterranean (Med) (c.563C>T, p.Ser188Phe), and

G6PD Betica (c.376A>G, 542A>T; p.126Asn>Asp, 181Asp>Val) with addition of a few rare ones. A number of 10 abnormalities amounted to 92% of all the molecular defects. In addition, seven new mutations were found: three presented with acute hemolytic anemia following oxidative stress [G6PD Nice (c.1380G>C, p.Glu460Asp), G6PD Roubaix (c.811G>C, p.Val271Leu), and G6PD Toledo (c.496C>T, p.Arg166Cys)],

three with different degrees of chronic hemolytic anemia [G6PD Lille (c.821A>T, p.Glu274Val), G6PD Villeurbanne (c.1000_1002delACC, p.Thr334del), and G6PD Amiens (c.1367A>T, p.Asp456Val)] and one found fortuitously G6PD Montpellier (c.1132G>A, p.Gly378Ser).

G6PD deficiency is the most frequent red blood cell (RBC) abnormality worldwide and is essentially divided into three classes [1]. Class I variants are characterized by a severe deficiency with chronic hemolytic anemia. Class II and III variants have substantial remaining enzyme activity and only present hemolytic crises under oxidant stress. [2]. Until last century, the Class II–III G6PD variants were restricted to some populations with a distribution specific to each region [3,4]. Thus, in some countries, only very few molecular defects are encountered, while in industrialized countries, with high immigration flow, the simultaneous presence of several variants is observed. The conventional approach is a time consuming and expensive sequencing of the gene. Therefore, working in a reference laboratory where more than 100 samples are investigated yearly, we propose a simplified procedure for molecular diagnosis of this defect.

During the last decade, we investigated more than 600 cases presenting a G6PD deficiency for which we could obtain an informed consent. We started this study by analyzing the entire sequence of G6PD gene. Rapidly we realized that more than 75% of abnormalities corresponded to G6PD A⁻ and G6PD Med and that a total of 10 variants covered 92% of all the defects [5]. Therefore, we developed the herein described rapid strategy based on RFLP analysis that could easily be adapted to other countries according to the specificity of their population.

First, we look for the presence of G6PD A⁻ and Med by restriction enzymes as previously described [5]. In the remaining 25% of subjects, according to their ethnic origin, the eight other frequent abnormalities are searched for by RFLP using restriction enzymes specific for each mutation. Only the very rare remaining cases required a complete DNA analysis.

In total, we found G6PD A⁻ (62.7%), Med (11.8%), Betica (4.9%), Santa Maria (3.1%), Viangchan (3.1%), Seattle (1.8%), Canton (1.3%), Chatham (1.3%), Valladolid (1.1%), and Coimbra (0.5%). In addition, we observed some rare variants already described in the literature [6] such as G6PD Nankang in a Antillean woman, Chinese-5, in two south East Asian males, and G6PD Genova in a German woman. We observed also three cases of compound heterozygosity (G6PD Med/G6PD Seattle, G6PD Betica/G6PD Santa Maria, and G6PD A⁻/G6PD Santa Maria), with residual enzyme activity similar to that of hemizygotes.

Seven new molecular defects were observed, corresponding DNA sequences are presented as Supporting Information.

G6PD Nice (p.Glu460Asp) was found in a 35-year-old man of Italian origin admitted for a hemolytic crisis following fava bean ingestion. G6PD assay revealed 5%–10% residual enzyme activity. Family study showed that his sister and niece, who never had hemolytic crisis, presented 50% residual G6PD activity. Hematologic parameters of the niece were normal. The molecular study of G6PD locus in his sister revealed an undescribed G6PD mutation: c.1380G>C in exon 12. This residue is the one which follows that mutated in G6PD Canton. Glu 460 and Lys 293, located toward the exterior of the molecule are distant by 5 Å and forms an ionic bond (Fig. 1). In G6PD Nice, the replacement of Glu for Asp increases this distance to more than 6 Å, reducing the interaction between these two sites.

G6PD Roubaix (Val271Leu) was found in a newborn girl presenting a Noonan syndrome and a prolonged neonatal icterus. G6PD assay revealed a deficiency, which was later on characterized at molecular level by direct sequencing. In exon 8, nucleotide 811 was replaced by a cytosine. Her mother and her sister, with normal enzymatic assay were heterozygous for the same mutation. When the proband was 2-year old, her RBC indices were normal. This mutation disturbs a hydrophobic pocket localized not very far from the interaction area between the two subunits of the active dimer (Fig. 1).

G6PD Toledo (p.Arg166Cys) was found in an 11-year-old boy presenting with an acute hemolytic crisis following a viral infection. He presented a neonatal jaundice. G6PD enzyme activity was not detectable. Molecular study revealed a mutation (c.496C>T) in exon 6. No genetic abnormality was found in his mother indicating that it is a neomutation. Eight cysteines are present in the normal enzyme, and it is likely that Cys13 may form a disulfide bridge with Cys446. The introduction of a cysteine near to this region could disturb the local structure.

G6PD Lille (p.Glu274Val) was found in a 23-year-old French Caucasian man. He presented, at birth, a severe jaundice requiring exchange blood transfusion. He needed further several blood transfusions until 7-year-old.

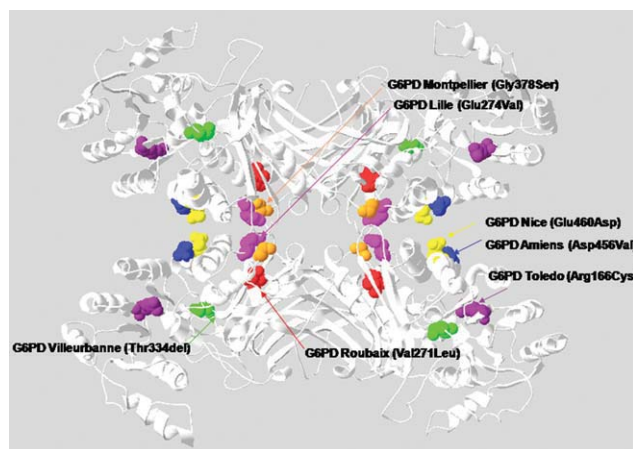


Figure 1. Localization of the structural abnormalities in the 3D model of a G6PD tetramer. Structural modifications of G6PD Lille, Amiens, Montpellier, Roubaix, and Nice are localized near to the area involved in tetramerization. It is likely that Cys13 may form a disulfide bridge with Cys446. In G6PD Toledo, the introduction of an additional cysteine near to Cys446 could disturb the local structure presence of this region. G6PD Villeurbanne resulted from a loss of one of the two consecutive threonines at 333 and 334 may play a role in interaction of subunits forming the tetramer [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

He was seen again when 15-year-old for a mucocutaneous jaundice, a hyperreticulocytosis (16%). At the age of 22 years, a moderate splenomegaly and hemosiderosis were observed. The biological and hematological parameters indicated a compensated hemolysis associated to a macrocytosis (haptoglobin < 0.3 mg/l and ferritin 411 µg/l with 75% of saturation of transferrin). G6PD enzyme activity was 2 IU/g Hb. Direct DNA sequencing revealed a mutation in exon 8 of G6PD gene (c.821A>T). According to the clinical presentation, this abnormality leads to a typical G6PD Class I variant. The 3D model shows that the modified amino acid is located in the neighborhood of 275Lys, which is a key residue involved in dimerization and tetramerization of the protein (Fig. 1) [7,8]. The same residue is affected in G6PD Cleveland (c.820G>A, p.Glu274Lys), which is another Class I variant.

G6PD Villeurbanne (p.Thr334del) was found in a boy presenting a chronic hemolytic anemia and several acute hemolytic crisis following viral infections. G6PD activity is reduced (0.8 IU/g Hb). Direct sequencing of DNA revealed an in frame deletion of three nucleotides, in exon 9, resulting in the loss of one of two consecutive threonines. No molecular abnormality was found in his mother, presenting a normal G6PD activity, showing that G6PD Villeurbanne resulted from a neomutation.

G6PD Amiens (p.Asp456Val) was found in a 3-year-old male with persistent scleral icterus, who had a severe neonatal jaundice. The investigations revealed a G6PD deficiency with 1% residual enzymatic activity. At the age of 6 years, his cell blood count (CBC) and biological parameters showed a chronic hemolytic anemia. When the mother was again pregnant, the G6PD deficiency was characterized. Direct sequencing of the gene revealed a yet undescribed substitution of A>T at nucleotide 1367, in exon 12 in child and her mother. This abnormality modifies the same residue as reported for G6PD Figueira da Foz (c.1366G>A, p.Asp456His) associated with a chronic hemolytic anemia [9]. In addition, the structural modification is close to that observed in G6PD Nice or Canton and may therefore affect association between subunits.

G6PD Montpellier (p.Gly378Ser) was found in a 14-year-old boy of Iberian origin. During a hospitalization indicated for an abdominal trauma, a hyperbilirubinemia was fortuitously found. Hematological parameters were normal. The sonographic examination revealed the presence of gallstones. The patient was found to be homozygous for the (TA)⁷ allele in the promoter region of the gene for bilirubin UDP-glucuronosyltransferase-1. In addition, a complete RBC enzyme study was performed, which revealed a simultaneous presence of two enzyme defects. The first one, a pyruvate kinase (PK) deficiency with a 50% decreased activity and the second one a G6PD deficiency affecting 50% of the enzyme activity. The molecular study of PK gene revealed a common missense mutation in exon 11 (c.1456C>T) [10]. The study of G6PD gene showed a new mutation in exon 10 (c.1132G>A). This substitution affecting an amino-acid located between two β sheets

could alter steric hindrance of Lys275 and may thus slightly affect association into dimers or tetramers.

Human active G6PD is an equilibrium between homodimers and tetramers, while the monomer is unstable. Following its 3D structure determination, it was observed that most of the Class I variants resulted from changes of residues encoded by exon 10. These residues are located in a region that binds a molecule of NADP⁺ involved in the structure of the protein, thus stabilizing the contact area between subunits within the active dimer [7,8]. A few other Type I variants alter directly the catalytic center or some key regions, well conserved during evolution, which are involved in the stability of the protein. Conversely, the Class II–III variants are found distributed all over the molecule and result in local structural changes that may affect more or less the stability or the activity of the enzyme. Among the seven new variants that we report, G6PD Montpellier is the only one encoded by a mutation in exon 10, but its amino acid modification, from a glycine to a serine, is not expected to disturb significantly the spatial structure. None of the new Class I variants here reported concerns a residue encoded by exon 10: G6PD Villeurbanne is a single residue deletion that may induce quite large local changes, G6PD Lille is near a key residue (Lys275) involved in dimerization and tetramerization of the protein, and G6PD Amiens concerns a position, whose modifications have already been described to lead to Class I variants [11]. As shown in Fig. 1, four of the new variants here reported are localized near to the contact areas between dimers and tetramers. It could therefore be hypothesized that keeping the equilibrium between dimers and tetramers is a way to prevent formation of unstable monomers. Any mutation that will impair formation of tetramers will increase the amounts of dimers and could thus displace the equilibrium favoring its dissociation. Further biochemical and biophysical studies are certainly required to validate this mechanism. This study contributes to demonstrate the heterogeneity of molecular mechanism leading to G6PD deficiency.

Methods

Six hundred and four individuals with suspicion of G6PD deficiency were characterized at the molecular level. An informed consent was obtained from each patient, according to the French regulation. The majority of them originated from African and Mediterranean countries. The remaining was from Asiatic origin. In addition, some patients were sent to our laboratory for more thorough investigation because of neonatal jaundice, favism, family studies, or systematic controls before some treatments.

Blood sampling was done using ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were shipped to the laboratory at 4°C for CBC and DNA study. Enzyme activity study was measured on ACD samples. Hematological parameters were determined by routine methods. G6PD assay was done spectrophotometrically according to Beutler et al., 1968 using a 20XTi auto-analyzer (Thermo Electron, Victoria, Australia).

In a first step, we looked for G6PD A⁻ and Med by NlaIII, FokI, and MboII restriction enzymes as previously described [5]. For the remaining cases, we look for G6PD Betica, Valladolid, and Santa Maria in individuals from North African and Mediterranean origin and G6PD Viangchan and Canton for those of Asian origin. Restriction enzymes used in this approach are listed in Table I. DNA fragments carrying these mutations were amplified using the specific primers. The list of primers is given in Supporting Information.

The amplified fragments were restricted according to the manufacturer's recommendation with 20 IU of each of the corresponding enzyme. Ten microliters of the digestion products were size fractionated by electrophoresis through polyacrylamide gels (6%), stained with ethidium bromide (0.25 µg/ml), and analyzed by ultraviolet transillumination. The resulting patterns are shown in Fig. 1 of supporting materials. The creation or abolition of restriction sites is looked for. Variants detected because of abolition of a restriction site require direct sequencing for confirmation of the abnormality.

Variants not detected by this approach were characterized by direct sequencing. Conditions for amplification reactions were as follows: 500 ng DNA were amplified on a GeneAmp[®] PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA) with 1.25 units AmpliTaq[®] DNA Polymerase (Roche, New Jersey, NJ) and 10 pmol of each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂. An initial denaturation step at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 10 sec, annealing at 61°C for 30 sec and extension at 72°C for 30 sec. A final extension at 72°C for 7 min completed the cycling reaction.

Nucleotide sequencing of the PCR products was performed with the forward and reverse primers using the BigDye[®] Terminator v3.1 Sequencing

TABLE I. Characteristics of the most frequent G6PD variants observed in our laboratory by RFLP method

Restriction enzyme	G6PD variant	Most frequent ethnical origin
NlaIII/FokI	A ⁻ (202G>A/376A>G)	Sub-Saharan Africa
MboII	Med (563C>T)	Mediterranean and Middle East countries
Acil	Valladolid (c.406C>T)	Iberian
	Coimbra (592C>T)	Iberian
	Chatham (1003G>A)	Middle East
Hpy188I	Santa Maria (542A>T)	Iberian
	Seattle (844 G>C)	Mediterranean countries
NciI	Betica (986T>C)	West Africa
Hyp188III	Viangchan (871G>A)	South East Asia
SmlI	Canton (1376G>T)	Chinese

Kit (Applied BioSystems) and analyzed on an automated sequencer (ABI PRISM[®] 3100 Genetic Analyzer; Applied BioSystems).

The crystallographic model of G6PD was studied using a 3D protein modeling software, Swiss pdb Viewer (<http://www.expasy.ch/spdbv/text/tutorial.htm>) and the pdb file 1qk1 [7,8] with special attention to the affected residues.

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Additional Supporting Information may be found in the online version of this article.

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Treatment of molecular relapse in patients with acute myeloid leukemia using clofarabine monotherapy

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Few studies have examined the treatment of molecular relapse in patients with acute myeloid leukemia (AML) using different treatment regimens. We describe for the first time in the literature experiences with administration of clofarabine monotherapy in the treatment of eight patients with AML with molecular relapse of the disease.

A substantial proportion of patients with AML who initially respond to treatment will relapse on the current options available. In patients with AML with detectable molecular markers (i.e., fusion genes or mutated genes), quantitative real-time polymerase chain reaction (RQ-PCR) provides a sensitive monitoring technique for measuring minimal residual disease (MRD) as well as the early detection of relapse prior to an overt hematological relapse [1]. Several studies have already proven the benefit of early intervention at the stage of molecular relapse in patients with acute promyelocytic leukemia (APL) [2]. However, only limited data related to early intervention in patients with non-APL AML have been reported to-date [3–5], particularly in regards to the beneficial effect of this approach.

Clofarabine, a novel nucleoside analog, has demonstrated efficacy with a good toxicity profile in primary therapy of elderly patients with AML as well as in the salvage treatment of relapsed/refractory AML patients with or without additional allogeneic stem cell transplantation [6,7]. However, so far, this drug has not been used in the early treatment of molecular relapse. Therefore, the aim of this study was to evaluate the efficacy and feasibility of using clofarabine monotherapy for the treatment of molecular relapse in patients with non-APL AML.

All patients with AML treated at our institution who were monitored for MRD and who had a molecular relapse between April 2009 and August 2010 were included in this study. All patients signed an informed consent form for participation in the study, and the study protocol was approved by the IRB of the University Hospital Brno, Brno, Czech Republic.

Peripheral blood (PB) and bone marrow (BM) samples were used to monitor MRD during all phases of initial therapy of AML. After the end of this initial treatment, samples were obtained every 2–3 months for the first two years or more frequently in unstable cases. Moreover, any new reappearance of the molecular marker was confirmed by additional sampling within 2 weeks. After clofarabine therapy, samples for MRD evaluation (PB and BM) were obtained after each cycle (if clofarabine was administered repeatedly), before and after an allogeneic hematopoietic stem cell transplantation (HSCT) (if performed after clofarabine treatment), and every 2–3 months thereafter.

Quantitative reverse-transcription polymerase chain reaction (RQ RT-PCR) and real-time PCR (RQ-PCR) were used to measure fusion transcripts (*RUNX1/RUNX1T1*, *CBFB/MYH11*, and the fusion transcript of the *MLL* gene) and the mutated *NPM1* gene, respectively, in order to monitor MRD as previously described [3,8]. The sensitivity of RQ-PCR assays range from 1:10,000 to 1:10,000,000. All samples were analyzed in duplicate.

Molecular relapse was defined as the reappearance of the molecular marker in PB or BM samples, or a 10-fold increase if detected repeatedly, when the simultaneously assessed BM morphology, immunophenotype, and cytogenetics remained normal [3]. After clofarabine therapy, complete molecular remission (CMoR) was defined as the reduction of the particular molecular marker to a value of 0 (i.e., undetected level) in all monitored compartments. Partial molecular remission (PMoR) was defined as a one order of magnitude reduction of the molecular marker level in the monitored compartment together with complete cytogenetic and hematological remission.

The clofarabine regimen for the treatment of molecular relapse consisted of one cycle of a 40 mg/m² intravenous infusion of clofarabine for 5 days. Any additional therapy for patients who exhibited a response differed by patient and is shown in Table I. If a second cycle of clofarabine therapy was administered, the dosage was identical to the first cycle. All patients received prophylaxis treatment with posaconazole and co-trimoxazole. The

Common Terminology Criteria for Adverse Events (CTCAE), version 4.03 (National Cancer Institute, Bethesda, MD), were used for the classification of adverse events.

During the study period, eight patients with AML exhibited a molecular relapse and were treated with clofarabine monotherapy. Table I shows a summary of the baseline patient characteristics. The median age of patients at the time of molecular relapse was 51 years. Primary therapy of AML consisted of induction 3+7 in all patients, followed by post-remission therapy using conventional chemotherapy in five patients (62.5%), autologous BM transplantation in one patient (12.5%), and allogeneic HSCT in two patients (25%). One patient (no. 7) was treated with clofarabine after relapsing from a previously treated molecular relapse that had occurred after an allogeneic HSCT. Seven patients (87.5%) fulfilled criteria for the reappearance of the molecular marker, and one patient (12.5%) had persistent detection of the marker and fulfilled the criterion of a 10-fold increase. The median time from the end of the last treatment to molecular relapse was 5.7 months (range 2.4–11.8 months).

The efficacy of clofarabine for reinduction as well as additional post-remission treatment is shown in Table I. After one cycle of clofarabine reinduction, all patients had a sustainable complete hematological remission. A molecular response was achieved in 7 of 8 patients (87.5%), 6 patients (75%) achieved CMoR, and 1 patient (12.5%) achieved a PMoR. In one case, a progressive increase in the molecular marker occurred and the patient relapsed hematologically within one month despite clofarabine therapy. Post-remission therapy in patients achieving a CMoR or PMoR included an allogeneic HSCT in three patients (Table I).

During the follow-up period, a new molecular relapse occurred at a median of 151 days (range 42–169 days) in 4 of 7 patients (57%) who exhibited a treatment response to clofarabine. Three of the four patients who did not receive a transplant after the initial treatment for molecular relapse with clofarabine developed second molecular relapse. In contrast, only 1 of 3 patients who underwent an allogeneic HSCT after clofarabine treatment for molecular relapse had a recurrence of the disease during the follow-up period. Moreover, the patient that relapsed after receiving an allogeneic HSCT only achieved a PMoR with clofarabine, and therefore received the transplant when MRD was still detectable (patient SM in Table I).

The 6-month overall survival (OS) rate for the evaluated group of AML patients was 100%, and the 6-month event-free survival (EFS) as well as disease-free survival (DFS) was 75% (95% CI: 50.3–100%), respectively.

Table I shows the associated individual toxicities with the clofarabine regimen used in this study. All patients experienced hematological toxicity (Table II). Also similar frequency and length of myelosuppression were reported in previously published phase 2 study [9], these patients were treated for manifested disease whereas in our study they received therapy only for relapse at a molecular level, being otherwise without any clinical manifestation of the AML. Recently published studies showed efficacy of clofarabine in the treatment of newly diagnosed AML with reduction of myelosuppression when lower doses were used [6,10]. Thus, because worsening of quality of life caused by cytopenia-related complications is an important issue in patients treated for molecular relapse of AML, in future trials the dose of clofarabine might be further reduced possibly with maintenance of its efficacy. Non-hematological toxicity was substantially less frequent. Infection occurred in four patients (50%), but these events were uncomplicated febrile neutropenia without clinically or microbiologically documented infection. In one patient, palmar-plantar erythrodysesthesia syndrome (grade 2) and elevated liver enzymes (grade 3) occurred after clofarabine administration, but these were reversible non-hematological toxicities.

TABLE 1. Baseline Characteristics, Efficacy of Treatment of Molecular Relapse and Associated Toxicities, and Follow-Up of Patients Involved in the Study

Initials	Sex	Age at relapse	Molecular marker	Initial therapy of AML			CLOFARABINE reinduction and post-reinduction treatment				CLOFARABINE toxicity			Follow-up							
				Primary therapy	Effect of primary/previous therapy	First/repeated molecular relapse	Time to relapse from the end of previous therapy (months)	Magnitude of molecular marker at the time of relapse—peripheral blood ^a	Magnitude of molecular marker at the time of relapse—bone marrow ^a	Effect of reinduction therapy with CLO ^b	Postremission therapy	Type of postremission therapy	Neutrophils < 1.0 × 10 ⁹ /l (days)	Lymphocytes < 1.0 × 10 ⁹ /l (days)	Thrombocytes < 50 × 10 ⁹ /l (days)	Non-hematological toxicity	Type of toxicity	Further molecular relapse	Time to further molecular relapse (months)	Death in follow up period	Cause of death
1	F	30	RUNX1/ RUNX1T1	I, C	CMoR	first	6.8	0.17%	0.13%	CMoR	yes	1 × CLO & allo HSCT	25	33	7	yes	febrile neutropenia	no	NA	yes	TRM
2	M	53	CBFB/ MYH11	I, C	CMoR	first	2.4	13.36%	10.43%	PMoR	yes	allo HSCT	21	33	11	yes	febrile neutropenia, hepatopathy, palmar-plantar erythema	yes	5.6	no	NA
3	F	47	NPM1 mutation	I, auto BMT	CMoR	first	5.0	467.8	1568	progression	NA	NA	70	NA	68	no	NA	NA	no	NA	NA
4	F	54	CBFB/ MYH11	I, C	CMoR	first	5.0	5.14%	6.80%	CMoR	no	NA	19	38	12	no	NA	yes	4.5	no	NA
5	F	23	CBFB/ MYH11	I, C	CMoR	first	11.8	0.20%	0.23%	CMoR	yes	allo HSCT	3	15	8	no	NA	no	NA	yes	TRM
6	M	48	NPM1 mutation	I, allo HSCT	CMoR	first	2.8	1640	45635	CMoR	yes	DLI	18	24	23	no	NA	no	NA	no	NA
7	M	56	MLL-ELL fusion gene	I, allo HSCT	PMoR	repeated	7.8	17.89%	NA	CMoR	yes	1 × CLO & interferon	24	47	27	yes	febrile neutropenia	yes	1.4	yes	progression
8	M	66	NPM1 mutation	I, C	CMoR	first	6.4	3517	32928	CMoR	no	NA	37	41	66	yes	febrile neutropenia	yes	5.6	no	NA

F, female; M, male; I, induction 3+7; C, consolidation [usually high dose cytosin arabinoside]; CMoR, complete molecular remission; PMoR, partial molecular remission; CLO, clofarabine; DLI, donor lymphocyte infusion; allo HSCT, allogeneic hematopoietic stem cell transplantation; TRM, transplant related mortality; NA, not applicable.

^aMutant NPM1 normalized copy number for NPM1 mutation; % of fusion gene/abl for RUNX1/RUNX1T1, CBFB/MYH11 and MLL-ELL.

^bBone marrow assessment was used for evaluation of CLO therapy effect.

TABLE II. Hematological Toxicity of Clofarabine Regimen in the Treatment of Molecular Relapse

	Neutropenia	Lymphopenia	Thrombocytopenia
% of patients with toxicity	100%	100%	100%
CTCAE grade \geq 3			
No. of days with toxicity	22 (3–70)	31 (7–46)	18 (7–68)
CTCAE grade \geq 3—median (range)			
Lowest detected count ($\times 10^9/l$)—median (range)	0.03 (0.01–0.21)	0.03 (0.01–0.18)	7.5 (2–45)

CTCAE, The Common Terminology Criteria for Adverse Events, version 4.03; National Cancer Institute, Bethesda, MD.

The data presented in this study can be primarily compared to a study published by our group several years ago on a cohort of AML patients with preemptive therapy for molecular relapse [3]. In this historical cohort, we treated 21 patients with molecular relapse and obtained a 62% response rate; however, only half of the responding patients achieved a CMoR (32%) [3]. The rate of complete molecular response was similar among the regimens used for treatment—conventional chemotherapy “5+2” – 25% ($n = 8$), gemtuzumab ozogomycin—29% ($n = 7$), and immunomodulation after allogeneic HSCT—43% ($n = 7$). Moreover, Doubek et al. showed that of the 71% of patients successfully treated for relapse in their study, a new molecular relapse occurred during the course of follow-up monitoring with a median progression-free duration of 119 days. In consideration of these data, the present study that used clofarabine as a monotherapy achieved a higher rate of complete molecular response compared to previously used strategies at our institution (75% vs. 32%, respectively) [5]. Very recently, a small cohort of AML patients with NPM1 mutation that were treated for molecular relapse using several courses of azacytidine was published. At least temporal molecular response was observed in 7 out of 10 (70%) treated patients. However data about the depth of this response were not provided and thus cannot be compared to our cohort [5]. Rubnitz et al. showed in a childhood AML study that the persistence of significant MRD positivity after induction is an adverse prognostic factor for both EFS and OS [11]. Therefore, if this approach is used for the treatment of molecular relapse, then clofarabine seems to be superior in this situation compared to a “5+2” type chemotherapy or gemtuzumab ozogomycin, at least in patients without a history of allogeneic HSCT.

Regardless of the therapy used for molecular relapse treatment, our present results as well as our historical data [3] clearly showed that without allogeneic HSCT, the further reappearance of the disease after treatment of molecular relapse is highly probable, especially in patients that do not achieve a complete molecular response. In consideration of these findings, clofarabine monotherapy, which has the highest frequency of complete molecular responses and a toxicity profile similar to a conventional “5+2”

chemotherapy or gemtuzumab ozogomycin, could represent an ideal bridge for AML patients with molecular relapse to cover the period of time until an allogeneic HSCT. Therefore, we believe that a prospective trial addressing the role of clofarabine in the treatment of molecular relapse in AML is warranted.

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The potential role of pre-transplant HBcIgG seropositivity as predictor of clinically relevant cytomegalovirus infection in patients with lymphoma undergoing autologous hematopoietic stem cell transplantation: A study from the Rome Transplant Network

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Despite the increased use of intensive immunosuppressive chemotherapies in patients with lymphoma observed in the last decade, current data on cytomegalovirus (CMV) infection following auto-

gous stem cell transplantation (Auto-SCT) are very limited. To address this peculiar aspect, a retrospective study on a cohort of 128 adult patients consecutively transplanted for lymphoma in three Hematology

Institutions was performed with the aim to determine the incidence of and the risk factors for CMV symptomatic infection and/or end-organ disease. Sixteen patients (12.5%) required specific antiviral therapy and 4/16 died (25%); transplant-related mortality (TRM) was significantly influenced by CMV infection ($P = 0.005$). In univariate analysis, a pre-transplant HBcIgG seropositivity, HBV infection according to clinical-virological definitions, a pre-transplant Rituximab treatment, a diagnosis of B-cell non-Hodgkin lymphoma, and age at transplant were significantly associated with the risk of developing a clinically relevant CMV infection. In multivariate analysis, only a pre-transplant HBcIgG seropositivity ($P = 0.008$) proved to be an independent predictor of a clinically relevant CMV infection. These results suggest that a pre-transplant HBcIgG seropositivity could be considered as an independent predictor factor of clinically relevant CMV infection after Auto-SCT.

The large use of new purine analogs and immunotherapeutic drugs for the treatment of hematological malignancies has provided new interest in CMV infection in clinical settings different from the allogeneic transplantation. In particular, the potential impact of these drugs on the risk of clinically relevant CMV infection in patients undergoing Auto-SCT raises important questions [1]. Recently, the European Conference on Infections in Leukemia (ECIL) published a list of recommendations on CMV infection management in patients with hematological malignancies [2]. Briefly, routine monitoring of CMV was considered unnecessary in patients undergoing Auto-SCT because of the low likelihood of progression from infection to disease, with the exception of patients receiving CD34-selected grafts and prior treatment with fludarabine, cladribine, or alemtuzumab. However, in an era of intensive immunosuppressive chemo-immunotherapies in patients with lymphoma, current data on CMV infection and disease following Auto-SCT are, as yet, very limited [3–6]. Therefore, to address this peculiar aspect, we performed a retrospective study on a cohort of 128 consecutive adult patients who received non-selected peripheral blood Auto-SCT for malignant lymphoma with the aim to provide insights on the incidence of and risk factors for clinically relevant CMV infection.

Baseline patient characteristics are summarized in Table I. One hundred twenty-three patients (96%) were conditioned with chemotherapy alone and 5 (4%) with ^{90}Y -Ibritumomab Tiuxetan-containing regimens. Antiviral prophylaxis was based on acyclovir 800 mg/day (77 patients) and valacyclovir 1 g/day (51 patients) given orally for six months after Auto-SCT. Lamivudine 100 mg/day orally was administered in case of HBcIgG seropositivity. Overall, 16/128 patients (12.5%) experienced a clinically relevant CMV infection requiring specific treatment; no case of primary infection in seronegative patients at transplant was documented. Out of 16 patients requiring treatment, 12 were diagnosed to have a CMV symptomatic infection whereas the remaining 4 had an end-organ disease. Clinical, laboratory features, treatment, and outcome of these infectious episodes are reported in Table II. The mortality rate was 25% (4/16); in particular 3/12 for patients with CMV symptomatic infection and 1/4 for patients with end-organ disease. Causes of death were CMV interstitial pneumonia with concomitant bone marrow failure ($n = 1$), subsequent co-infections (Gram negative sepsis, $n = 2$) during antiviral therapy, and multi-organ failure (MOF) at the end of the antiviral treatment ($n = 1$). The occurrence of a clinically relevant CMV infection after Auto-SCT was significantly associated with the risk of TRM [hazard ratio (95% confidence intervals): 0.136 (0.034–0.544), $P = 0.005$]. Two different diagnostic strategies for diagnosis of CMV infection were adopted (see Methods section). Among the 80 patients in which was adopted a clinically driven PCR diagnostic strategy, the CMV infection incidence was 13.7% (11/80), with 10 diagnoses of symptomatic infection or end-organ disease [12.5% (10/80)] and 1 patient with positive DNAemia and fever but not responding to the diagnostic criteria of symptomatic infection. The CMV end-organ disease incidence and attributable mortality were 3.7% (3/80) and 1.2% (1/80), respectively. Two patients suffered from CMV interstitial pneumonia (one of them died) and one suffered from CMV enteritis. The CMV symptomatic infection incidence and concomitant mortality were 8.7% (7/80) and 3.7% (3/80), respectively. Among the 48 patients in which was adopted a surveillance PCR diagnostic strategy, the CMV DNAemia incidence was 39.6% (19/48). The incidence of CMV symptomatic infection and end-organ disease was 10.4% (5/48) and 2.1% (1/48) (CMV interstitial pneumonia), respectively. None of these patients died. There are no statistically significant differences in terms of diagnostic timing (median days to Auto-SCT) of clinically relevant reactivation between two different groups (30 days in clinically driven group vs. 25

days in surveillance group, $P = 0.29$). In univariate analysis, a pre-transplant HBcIgG seropositivity ($P = 0.008$), HBV DNA positivity ($P = 0.027$), HBV infection according to clinical-virological definitions (True occult Hepatitis B infection (OBI), False OBI, ABI, IBI, see Table I, $P = 0.006$, a pre-transplant Rituximab therapy ($P = 0.049$), and a diagnosis of B-cell non-Hodgkin lymphoma ($P = 0.049$) were significantly associated with the risk of developing a clinically relevant CMV infection requiring specific antiviral therapy. As for Rituximab and age at transplant as risk factors for CMV infections, we observed that patients who experienced a clinically relevant CMV infection received a higher number of Rituximab administrations (median number of administration: 7.5 vs. 4, $P = 0.001$) and presented an higher median age at transplant (median age: 54 vs. 45, $P = 0.022$). All others factors analyzed (number of chemotherapy lines before Auto-SCT, disease status at Auto-SCT, hematologic parameters at Auto-SCT, number of CD34+ infused, prior fludarabine administration) are resulted not associated to the risk of CMV infection. Since HBcIgG, HBV DNA, and HBV infection status according to virological/clinical definitions had reciprocal competitive effect, in multivariate logistic regression analysis the final model included only HBcIgG. The results of multivariate analysis revealed that only a pre-transplant HBcIgG seropositivity was an independent significant risk factor ($P = 0.008$) for the appearance of a CMV symptomatic infection or end-organ disease requiring specific antiviral therapy (Table III).

CMV infection can be a major problem following Auto-SCT in adult patients with malignant lymphoma. In fact, 12.5% (16/128) of our patients underwent specific antiviral treatment for a clinically relevant CMV infection and 25% of them (4/16) died. The impact of CMV symptomatic infection or end-organ disease on TRM was noteworthy; however, it is difficult to attribute all fatalities directly to CMV. After Auto-SCT, about 40% of seropositive patients develop a CMV infection, diagnosed through routine monitoring of DNAemia [7,8]. If we consider only the 48 patients with weekly performed surveillance PCR, the incidence of CMV infection was 39.6%, according to literature. On the contrary, considering the 80 patients with clinically driven diagnosis, the CMV infection incidence, end-organ disease incidence, and attributable mortality were 13.7%, 3.7%, and 1.2%, respectively, and also in this case are well comparable with those reported in literature in patients tested for CMV on clinical suspicion of infection [9,10]. Han [11] reported a

TABLE I. Patient Characteristics at Transplant ($n = 128$)

Age (years) median (range)	47 (17–71)
Sex M/F	83/45
Diagnosis	
B-cell non Hodgkin lymphoma	81 (63.3%)
Hodgkin lymphoma	36 (28.1%)
T/NK-cell non Hodgkin lymphoma	11 (8.6%)
CMV IgG ^a +	117 (92.9%)
HBsAg+	4 (3.1%)
HBcIgG+	17 (13.3%)
HBV DNA+	6 (4.7%)
HBV according to virological/clinical definitions ^b	
True OBI	10
False OBI	3
ABI	3
IBI	1
HCVAb ^a +	2 (1.6%)
Disease status	
Complete response	92 (71.8%)
Partial response	29 (22.7%)
Stable/progressive disease	7 (5.5%)
Prior chemotherapy lines	
1	32 (25%)
2	76 (59.4%)
≥3	20 (15.6%)
Prior Rituximab treatment	81 (63.3%)
Rituximab administrations number median (range)	7 (2–21)
Prior Fludarabine treatment	7 (5.5%)
Conditioning	
Chemotherapy alone (BEAM or BEAM-like regimens)	123 (96.1%)
Chemotherapy with ^{90}Y -Ibritumomab Tiuxetan	5 (3.9%)
CD34+ infused cells $\times 10^6/\text{kg}$ median (range)	6.14 (2.5–14.5)

^aDatum is missing in two patients.

^bTrue OBI (Occult Hepatitis B Infection) HBcIgG+/HBsAg–/DNA–; False OBI (Occult Hepatitis B Infection): HBcIgG+/HBsAg–/DNA+; ABI (Active Hepatitis B Infection): HBcIgG+/HBsAg+/DNA+; IBI (Inactive Hepatitis B Infection): HBcIgG+/HBsAg+/DNA–. All patients were negative for protective antibodies against HBsAg.

TABLE II. Clinical and Laboratory Features, Treatment, and Outcome of CMV Infection Episodes Requiring Specific Antiviral Treatment (16/128)

	Symptomatic infection, n = 12	End-organ disease, n = 4
Clinical and laboratory features		
Fever (temperature >38°C persistent at least 60 min)	12	4
Signs of bone marrow suppression (lack of platelet recovery at day +30 or drop in neutrophils and/or platelet count after recovery)	12	3
Inversion of neutrophils/lymphocytes ratio	4	1
Alteration of hepatic cytolysis enzymes	2	2
Interstitial pneumonia (chest X-ray, computed tomography scan, bronchoalveolar lavage)	0	3
Enteritis (endoscopy, biopsy)	0	1
DNAemia positivity	12	4
Pre-transplant HBcIgG seropositivity	5	1
Treatment ^a		
Foscarnet sodium	7	1
Ganciclovir	3	3
Valganciclovir	2	0
Non specific immunoglobulins	1	4
Outcome		
Survived, median follow-up 467 days (range 100–1560)	9	3
Died (30, 62, 89, 248 days from transplant)	3	1

^a**Foscarnet sodium dosage:** 60 mg/kg twice daily for 14 days, then 60 mg/kg/day for subsequent 5 days weekly for 2 weeks); **Ganciclovir dosage:** 5 mg/kg twice daily for 14 days, then 5 mg/kg/day for subsequent 5 days weekly for 2 weeks; **Valganciclovir dosage:** 900 mg twice daily for 14 days, then 900 mg/day for subsequent 5 days weekly for 2 weeks.

Median number of viral copies at first PCR detection was of 290 copies/ml for patients tested at Laboratory of Molecular Virology of IFO (LOD: 88 copies/ml) and of 1,790 copies/ml for patients tested at Laboratory of Molecular Virology of PTV (LOD: 556 copies/ml).

TABLE III. Multivariate Analysis of Risk Factors for the Occurrence of CMV Symptomatic Infection or End-Organ Disease

Variables		Univariate analysis		Multivariate analysis	
		Occurrence of symptomatic infection or end-organ disease	P value	Odds ratio (95% CI)	P value
HBcIgG	–	10/102 (9.8%)	0.008	0.148 (0.036–0.606)	0.008
	+	6/15 (40%)			
Diagnosis of B-cell non-Hodgkin lymphoma	No	2/40 (5%)	0.049	1.007 (0.028–35.880)	0.997
	Yes	14/77 (18.2%)			
Pre-transplant Rituximab administration	No	2/40 (5%)	0.049	1.562 (0.035–68.823)	0.817
	Yes	14/77 (18.2%)			
Rituximab administrations, n	Median (range)		0.001	1.238 (0.996–1.539)	0.055
Age at transplant, years	Median (range)		0.022	1.028 (0.971–1.089)	0.323

Multivariate analysis was performed only for pre-transplant CMV-IgG seropositive patients (n = 117); CI: confidence intervals.

“not regularly monitored” CMV antigenemia incidence of 12.9% among lymphoma patients undergoing Auto-SCT and demonstrated that Auto-SCT “per se” did not increase the risk of infection, since non-Auto-SCT lymphoma patients had the same antigenemia incidence (12.7%). Finally, in most prospective and retrospective studies performed in the second half of the nineties, CMV end-organ disease ranged from 1.5% to 6.8% after Auto-SCT [7,9,12,13]. Therefore, our CMV end-organ disease incidence (4/128, 3.1%) is comparable with that of the literature. As for ECIL warnings, these are applicable only to a small part of lymphoma patients undergoing Auto-SCT (i.e., those pre-treated with fludarabine and those receiving CD34-selected grafts) [2]. To our knowledge, this is the first report in which the pre-transplant HBcIgG seropositivity is recognized as independent risk factors for CMV symptomatic infection or end-organ disease after Auto-SCT. HBcIgG is the sole serological surrogate-marker associated with the condition of occult carrier and its seropositivity permits to identify, apart from other serological markers, all HBV carriers. Recently, it has been demonstrated that CMV infection is common in chronic HBV and HCV patients, who can be regarded as patients at high risk for CMV disease [14]. In the study of Bayram [14], CMV infection was diagnosed in 23/44 of chronic HBV patients by detection of CMV DNA in liver biopsy samples, and the presence of CMV DNA was significantly different between chronic HBV patients and control group. Moreover, Lian reported a superinfection rate of 10.3% for HBV and CMV in chronic Hepatitis B cases and an unpromising prognosis with a high mortality rate in those patients [15]. Therefore, the value of the serological status for HBV “core” as independent risk factor for CMV infection observed in our study is not surprising. Virus–virus interactions have been demonstrated capable of modifying the pathogenesis of infections, both in humans and transgenic mice, through mechanisms of lower or greater cross-permissiveness mediated by the immune system [16,17]. In particular, it seems that

many viruses can induce profound modifications in their host and that mechanism of virus–virus interactions is common and crucial to understanding the pathogenesis of viral infections. In a recent review, DaPalma describes three main mechanisms of virus–virus interactions: (1) direct interactions of viral gene or gene products; (2) indirect interactions that results from alterations in the host environment, and (3) immunological interactions, based on adaptive immune system [18]; it is possible to hypothesize that hepatitis B virus is capable of favoring a CMV co-infection through direct interaction of viral molecules, but also through an indirect action on cell-mediated immune system. The role of Rituximab as a risk factor for CMV symptomatic infection or end-organ disease after Auto-SCT remains controversial, since our multivariate analysis did not confirm the predictive value registered in univariate. However, data from the latter suggest that the risk of developing clinically relevant CMV infection after Auto-SCT proportionally increases according to Rituximab administrations.

In conclusion, from our study in lymphoma adult patients undergoing Auto-SCT, two issues may be addressed: (1) the incidence of CMV infection and disease in lymphoma patients undergoing Auto-SCT is about 12%; clinicians must be aware of this potentially life-threatening complication in order to initiate timely and proper treatment, (2) our data suggest that a pre-transplant HBcIgG seropositivity could be considered as independent predictor of clinically relevant CMV infection. However, further studies on a large number of patients are necessary to clarify the role of a pre-transplant HBcIgG seropositivity as a potential risk factor for CMV infection after Auto-SCT.

Methods and Study Design

Patients. From January 2004 to June 2010, a total of 144 adult patients with a diagnosis of malignant lymphoma consecutively underwent Auto-SCT in three different Hematology Institutions, participating in the Rome Trans-

plant Network (RTN). These Institutions were: the Hematology Unit of Regina Elena National Cancer Institute (IRE), the Stem Cell Transplant Unit of Tor Vergata University Hospital (PTV), and the Hematology Unit of Campus Bio-Medico University Hospital (CBM). The clinical features and outcomes were retrospectively obtained by peer review of the medical records. Out of these 144 patients, 128 were eligible for the analysis; 16 patients were excluded because of insufficient available data.

Quantification of CMV DNA. Quantification of CMV DNA was performed in two different laboratories of molecular virology. Patients of PTV and CBM were studied by the Laboratory of Molecular Virology of Tor Vergata University Hospital. Automated nucleic acid sample preparation systems QIAmp DNA Mini Kit[®] (Qiagen, Dusseldorf, Germany) was used for CMV DNA extraction from plasma and bronchoalveolar lavage fluid and DNA amplification for detection and quantification was carried out by commercially available real-time PCR assays according to the manufacturer's instructions of Q-CMV Real Time System Complete Kit[®] (Cepheid, Nanogen Advanced Diagnostics, Sunnyvale, USA, limit of detection 556 copies/ml); Real-time PCR platform was ABI Prism 7300[®] Sequence Detection System (PE Applied Biosystems, Foster City, USA). All patients weekly monitored were studied in this laboratory. Patients of IRE were studied by the Laboratory of Molecular Virology of San Gallicano Dermatological Institute. Automated nucleic acid sample preparation systems NucliSENS easyMAG[®] (BioMerieux, Durham, USA) was used for CMV DNA extraction from plasma and bronchoalveolar lavage fluid and DNA amplification for detection and quantification was carried out by commercially available real-time PCR assays according to the manufacturer's instructions of Affigene[®] CMV Trender diagnostic assay (Cepheid AB, Bromma, Sweden, limit of detection 88 copies/ml); Real-time PCR platform was Mx3000P[®] System (Stratagene, La Jolla, USA).

Criteria for diagnosis of CMV symptomatic infection and end-organ disease. CMV DNAemia, CMV symptomatic infection, and CMV end-organ disease were defined according to published recommendations [2,19–21]. In particular, CMV DNAemia was defined as the detection of DNA in samples of plasma by quantitative PCR. CMV symptomatic infection was defined as a documented CMV DNAemia in presence of fever (temperature >38°C) and overt clinical signs of bone marrow suppression (lack of platelet recovery at day +30 or drop in neutrophils and/or platelet count after recovery) in absence of concomitant documented co-infections at clinical examination, imaging, and repeated cultures from blood and urine. CMV end-organ disease (CMV pneumonia and CMV gastrointestinal disease) was defined by the presence of a combination of clinical, imaging's, and histopathological/molecular features consistent with CMV infection

CMV infection diagnostic strategies. Two different strategies for CMV infection diagnosis were adopted: a clinically driven diagnostic strategy (80 patients, 51 at IRE and 29 at PTV), in which the CMV was investigated on clinical suspicion of infection, and a surveillance strategy (48 patients, 38 at PTV and 10 at CBM), in which plasma PCR was routinely performed weekly in all patients regardless of clinical suspicion for at least 4 weeks after transplantation. In this second cohort of patients, specific antiviral therapy started only in presence of a proven diagnosis of clinically relevant CMV infection and no pre-emptive therapy was initiated at the evidence of clinically asymptomatic CMV DNAemia.

Mobilization procedures and criteria of treatment response. Mobilization, collection, and cryopreservation procedures were previously described [22]. Evaluation of treatment response was performed according to Cheson criteria [23,24].

Statistical analysis. Data were analyzed by Statistical Package of Social Sciences software (SPSS, version 13.0, Chicago, USA). Univariate analysis was performed in order to identify risk factors for clinically relevant CMV infection requiring specific treatment by using chi-square test (Fisher or Pearson) and analysis of variance. Two-sided *P*-values below 0.05 were considered to be statistically significant for the multivariate analysis. In case of two or more significant variables with reciprocal competitive effect, only the variable statistically more significant or clinically more relevant was included in the final model. Binary logistic regression model was used to analyze associations between significant baseline characteristics and the occurrence of CMV infection. Cox regression was used to analyze the influence of the occurrence of a clinically relevant CMV infection after

Auto-SCT and TRM. Enter and remove limits were 0.05 and 0.1, respectively.

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Fetal hemoglobin in sickle cell anemia: Molecular characterization of the unusually high fetal hemoglobin phenotype in African Americans

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Fetal hemoglobin (HbF) is a major modifier of disease severity in sickle cell anemia (SCA). Three major HbF quantitative trait loci (QTL) are known: the *Xmn I* site upstream of $\alpha\text{-}\gamma$ -globin gene (*HBG2*) on chromosome 11p15, *BCL11A* on chromosome 2p16, and *HBS1L-MYB* intergenic polymorphism (HMIP) on chromosome 6q23. However, the roles of these QTLs in patients with SCA with uncharacteristically high HbF are not known. We studied 20 African American patients with SCA with markedly elevated HbF (mean 17.2%). They had significantly higher minor allele frequencies (MAF) in two HbF QTLs, *BCL11A*, and HMIP, compared with those with low HbF. A 3-bp (TAC) deletion in complete linkage disequilibrium (LD) with the minor allele of rs9399137 in HMIP was also present significantly more often in these patients. To further explore other genetic loci that might be responsible for this high HbF, we sequenced a 14.1 kb DNA fragment between the $\alpha\text{-}\gamma$ -(*HBG1*) and δ -globin genes (*HBD*). Thirty-eight SNPs were found. Four SNPs had significantly higher major allele frequencies in the unusually high HbF group. In silico analyses of these four polymorphisms predicted alteration in transcription factor binding sites in 3.

HbF inhibits deoxy-HbS polymerization. Patients with elevated HbF have fewer vaso-occlusive complications and prolonged survival [1]. Three major HbF QTL are known. The C>T polymorphism (rs7482144) at nucleotide –158 upstream of *HBG2* is associated with increased HbF in some patients with SCA [2]. Polymorphisms in intron 2 of *BCL11A* represented by rs766432 was associated with HbF in healthy Northern Europeans [3], African Americans with SCA [4,5], Chinese with β -thalassemia trait, and Thai's with HbE- β thalassemia [5]. *BCL11A* polymorphisms correlate highly with HbF levels in SCA, accounting for 7–12% of the HbF variance [6]. The HMIP polymorphisms are distributed in three LD blocks [7]. HMIP block 2 represented by rs9399137 is most significantly associated with HbF expression and might function as a distal regulatory element [8,9].

We studied a selected group of 20 African American patients with SCA with exceptionally high HbF (mean 17.2%), which differed by more than four times the standard deviation of 30 other patients with low HbF (mean 5.0%; Table I). All study subjects' *HBB* underwent nucleotide sequencing to ascertain that they were HbS homozygotes. Multiplex ligation-dependent probe amplification was carried out to ensure that they did not harbor hereditary persistence of HbF (HPFH) 1, HPFH 2, Black ($\delta\beta$)⁰- and Black ($\alpha\text{-}\gamma\delta\beta$)⁰-thalassemia deletions [10]. Furthermore, their *HBG2* and *HBG1* promoters were also sequenced to be certain that they did not have promoter HPFH single nucleotide mutations [11,12]. They were unlikely to be on hydroxyurea based on their MCV being less than 100 fL.

In addition, we conducted a subset analyses in 56 patients with unusually high HbF (mean 20.7%), which differed by more than 11 times the standard deviation of 489 patients with low HbF (mean 3.1%; Table II). These patients were selected from 1,086 subjects from the Cooperative Study of Sickle Cell Disease (CSSCD), who were previously investigated in a genome-wide association study (GWAS) of HbF [13].

The MAF of rs7482144, also known as the *Xmn I* site, on chromosome 11p15 in the unusually high HbF group (10%) is not significantly different from that in the low HbF group (8%) as shown in Table III. The SNP rs5006884, a missense mutation (CTC>TTC or Leu172Phe) in *OR51B6* (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=5006884) on chromosome 11p15 was reported to be associated with HbF in SCA in a GWAS [13]. In this study, the MAF of rs5006884 in the unusually high HbF group (2%) is actually less than that in the low HbF group (10%), even though the difference is not statistically significant (Table III). It should be recognized that the small sample size in this study does not afford sufficient power to discern possible MAF differences if present at these two SNPs between these two groups of patients.

The SNP rs7482144 did not reach genome-wide significance in previous GWAS of HbF in the full CSSCD cohort [13]. In this study with subsets from the CSSCD cohort, rs7482144 has a significantly higher MAF in the unusually high HbF group (30%) compared to the low HbF group (10%), $P = 0.002$. The MAF of rs5006884 in *OR51B6* in the high HbF group (22%) is significantly higher than that in the low HbF group (10%), $P = 0.00055$. This SNP reached genome-wide significance in the GWAS of the full CSSCD cohort; however, the mean HbF level in homozygotes for this variant was only 10.6%. The present subset analysis shows that for patients with SCA with unusually high HbF the frequency of this mutation is comparable with the general population (Table III).

To examine the *BCL11A* QTL, three SNPs, rs766432, rs4671393, and rs11886868 were chosen for genotyping [6]. These three SNPs are in strong LD. Only data on rs766432 that is most highly correlated with HbF in patients with SCA are presented. The MAF in the unusually high HbF group (45%) is significantly higher than that in the low HbF group (25%), $P = 0.05$ (Table III). The MAF in the subgroups from CSSCD were similar: 47% in the high and 20% in the low HbF groups, $P = 6.399 \times 10^{-10}$. The allele C of this SNP was associated with increased levels of HbF [13] and the mean HbF levels in subjects homozygous for the C allele was 8.26%. The present subset analysis in the CSSCD subgroups shows that in patients with unusually high HbF the frequency of this mutation is almost twice that of the full cohort.

TABLE I. Hematologic Results of High and Low HbF Study Groups

	N	Age	Male/female	Hb (g/dL)	MCV (fL)	HbF (%)
High HbF group	20	16.3 ± 8.3 (6–30)	8/12	9.0 ± 1.3 (5.7–11.7)	87.9 ± 9.0 (77–99)	17.2 ± 4.8 (11–28.9)
Low HbF group	30	19.3 ± 9.8 (5–49)	10/20	8.6 ± 1.4 (5.2–11.4)	81.4 ± 11.1 (65–100)	5.0 ± 2.5 (0.5–8.8)

Values are shown as mean ± SD; Values shown between parentheses represent range of values.

The QTL in HMIP is best represented by rs9399137 [9]. The MAF of rs9399137 among patients with SCA of African descent without European admixture was reported to be 1–2% [14,15]. A GWAS on over 800 African American patients with SCA did not detect genome-wide significance of association of this QTL with HbF [13]. The functional motif for this QTL is most likely a 3-bp (TAC) deletion which is in complete LD with the minor allele of rs9399137 [9]. The frequency of this 3-bp deletion is 23% in non-African HapMap populations but only 5% in Africans [9].

In this study, the MAF of rs9399137 in the African American patients with SCA with unusually high HbF is 18%, significantly higher than that with low HbF (3%), $P = 0.02$ (Table III). Furthermore, the 3-bp deletion as reported by Farrell et al [9] was found for the first time in these African American patients with SCA and it is in complete LD with the minor allele of rs9399137. Among the subset of CSCCD patients, the MAF of rs9399137 in the unusually high HbF group (9%) is also significantly higher than that in the low HbF group (3%), $P = 0.006$ (Table III). These results raise the possibility that some African American patients with SCA with markedly elevated HbF might have inherited the minor allele of chromosome 6q23 QTL due to European genetic admixture.

The minor T allele of rs7775698 tags either an ancestral T nucleotide found mostly in African populations, or a 3-bp deletion often found in European and Chinese populations [9]. In this, the MAF of rs7775698 in the high HbF group (40%) is higher than that in the low HbF group (21%). However, the difference is not statistically significant ($P = 0.07$). Among the patients with CSCCD, the MAF of rs7775698 in the high HbF group is 16% compared with 19% in the low HbF group (Table III).

We found a 2-bp (CC) deletion plus an (A) insertion 19 bp downstream of rs9399137. This deletion/insertion was present in 33% of chromosome 6 in both high and low HbF groups. It is unlikely that it plays a significant functional role in modulating HbF expression. Its relatively high frequency in both groups makes it a probable haplotype marker in African American patients with SCA.

Two of the three known HbF major QTLs are present in the unusually high HbF patient study group at a frequency significantly higher than those with low HbF. Their cumulative effect was estimated by assigning to each minor allele at each locus a score of 1, and adding the scores to generate the number of minor allele present (NOMAP) [16]. There was an average of two minor alleles in the unusually high HbF group compared with one in the low HbF group ($P = 0.001$) as shown in Fig. 1.

To explore other possible genetic loci that may modulate HbF expression, we undertook nucleotide sequencing of a 14.1 kb DNA fragment between *HBG1* and *HBD* in patients with 15 high and 15 low HbF. This DNA fragment was chosen because *BCL11A* binds to this intergenic region and it also encompasses the Corfu deletion that in homozygotes is characterized by markedly elevated HbF [17,18].

Thirty eight SNPs were found in both the high and low HbF groups (Supporting Information Table I). SNP rs10128558 as described by Galarnau et al [19] was present in our study groups and found to be in complete LD with *Xmn I* polymorphism. Its MAF in the high HbF group (10%) is not different from the low HbF group (8%). Based on in silico analysis by TFSEARCH (threshold

TABLE II. Hematologic Results of High and Low HbF CSCCD Groups

	N	Age	Hb (g/dL)	MCV (fL)	HbF (%)
High HbF group	56	15.2 ± 10.9	9.6 ± 1.5	89.7 ± 8.9	20.7 ± 8.2
Low HbF group	489	19.1 ± 10.8	8.1 ± 1.1	89.2 ± 7.3	3.1 ± 1.5

Values are shown as mean ± SD; Values shown between parentheses represent range of values.

TABLE III. HbF QTL Minor Allele Frequencies in High and Low HbF Groups

Chromosome	SNP	Minor allele	YRI	CEU	Gene	Study groups (N = 50)		CSCCD groups (N = 590)	
						MAF	P	MAF	P
2	rs766432	C	0.25	0.12	<i>BCL11A</i>	0.45/0.25	0.05	0.47/0.2	6.399E-10
6	rs9399137	C	0.042	0.25	<i>HBS1L-MYB</i>	0.18/0.03	0.02	0.09/0.03	0.006
6	rs7775698	T	0.32	0.22	<i>HBS1L-MYB</i>	0.40/0.21	0.07	0.16/0.19	0.52
11	rs5006884	T	0.25	0.22	<i>OR51B5; OR51B6</i>	0.02/0.1	0.2	0.22/0.1	0.00055
11	rs7482144	T			<i>HBG2</i>	0.1/0.08	1.0	0.30/0.1	0.002

YRI, minor allele frequency of subjects from Ibadan, Nigeria based on the HapMap data. CEU, minor allele frequency of northern and Western European subjects, based on the HapMap data. MAF, minor allele frequency (see Fig. 1).

score 85), 22 of the 38 SNPs are associated with alterations in transcription factor binding including erythroid-specific transcription factors. In addition, four SNPs have significantly higher major allele frequency in the high compared to the low HbF groups ($P < 0.05$). Three of these four SNPs result in alteration in transcription factor binding sites (Supporting Information Table I).

This study based on a small cohort of carefully selected African American patients with SCA with unusually high HbF revealed that the MAF for rs766432 (*BCL11A*), rs9399137 and 3-bp deletion both within HMIP are much higher than that found in patients with low HbF. These findings also raise the possibility that some African American patients with markedly elevated HbF might have inherited the minor allele of chromosome 6q23 QTL due to European genetic admixture. Validation of these findings in a larger patient cohort and further functional investigations into these and other polymorphisms are warranted.

Materials and Methods

Study Groups. Blood samples referred to the Hemoglobin Diagnostic Reference Laboratory for DNA-based diagnostics at the Boston Medical Center were selected for this study. The samples were collected between 2003 and 2008. Patients younger than 5-year-old, the time at which HbF levels stabilized [13] and patients with MCV greater than 100 fL were excluded. Nucleotide sequencing of the *HBG* and promoters of *HBG2* and *HBG1* was done after PCR amplification. The presence of *HBB* deletions was excluded by multiplex ligation dependent probe amplification [20]. This study was approved by the Boston University School of Medicine Institutional Review Board.

CSCCD subsets. We used data from the CSCCD. From 1,086 cases, who underwent GWAS [13], we selected 56 patients with unusually high HbF and 489 patients with low HbF for subset analyses.

QTLs. The *Xmn I* polymorphism (rs7482144) was genotyped by polymerase chain reaction (PCR) of the *HBG2* promoter, followed by restriction enzyme digestion analysis.

Genotyping of SNPs in *BCL11A* was done by a TaqMan SNP genotyping assay (Applied BioSystems, Foster City, CA) according to the manufacturer's instruction. Predesigned probes were ordered for genotyping analyses: rs766432 (C_1025980_10), rs11886868 (C_11363852_10), rs4671393 (C_25926414_10). Amplification was done with 5 µl of 2X TaqMan Universal PCR master mix, 0.5 µl of 40X primer and TaqMan probe dye mix, and between 10–50 ng of DNA. Cycling conditions consisted of 10 min at 95°C, followed by 40 cycles 15 sec at 92°C, 1 min at 60°C. Allelic discrimination is performed on Applied BioSystem RT-PCR system.

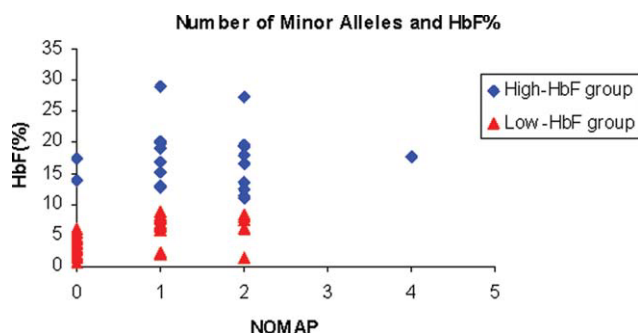


Figure 1. Correlation between NOMAP (Number of HbF QTL Minor Allele Present) and HbF levels in the unusually high and low HbF groups. The presence of either one minor allele, T in rs7482144 (*Xmn I*), C in rs766432 (*BCL11A*), or 3-bp deletion in HMIP is counted as 1.

Genotyping of SNPs in HMIP, rs9399137, rs7775698, and 3-bp (TAC) deletion, and SNP rs5006884 in *OR51B6* was done by PCR, followed by nucleotide sequencing using the BigDye terminator cycle sequencing kit from Applied BioSystems.

HBG1-HBD nucleotide sequencing. Short (500–600 bp) and overlapping fragments of DNA covering the 14.1 kb region were amplified by PCR. All PCR reactions were performed in a total volume of 20 μ l. Master mix concentrations, and cycling conditions were optimized based on the region being amplified. Usually, each reaction contained 100–250 ng of DNA, 1X PCR buffer (Applied BioSystems), 2 mM MgCl₂, 200 μ M dNTP, 1 ng of each primer, and 0.5U AmpliTaq polymerase. Cycling conditions consisted of 5 min at 94°C, followed by 30 cycles of 40 sec at 94°C, 40 sec at 55°C, and 3 min at 72°C and a 7-min elongation step at 72°C.

Statistical Analysis. Association between HbF and the minor alleles of each QTL was statistically analyzed by the Fisher exact test performed in R (www.r-project.org). Comparison of hematological parameters and correlation between NOMAP and HbF in both groups was examined using paired *T*-test. An overall significance level of 0.05 was set for all statistical analyses.

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Spontaneous graft versus host disease occurring in a patient with multiple myeloma after autologous stem cell transplant

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Graft versus host disease (GVHD) is a common complication of allogeneic transplant. Acute GVHD primarily affects the skin, liver, and GI tract generally within the first 100 days after transplant. GVHD following an allogeneic transplant occurs as a result of donor T-cell recognition of host alloantigens. In contrast, patients undergoing ASCT are not subjected to the genetic disparity that occurs with allogeneic transplant, and in principal, should not develop this proinflammatory response.

A clinical syndrome, however, has been described in patients following autologous transplant that shares the same features as GVHD occurring in recipients post-allogeneic transplant [1–3]. Previously reported cases have described skin, liver, and GI tract manifestations consistent with what is seen in allogeneic GVHD. Biopsies of the skin and GI tract mucosa have demonstrated similar histological features

as well. Interestingly, the majority of reported cases seem to occur in patients with multiple myeloma undergoing consolidative ASCT. Historically, however, these patients have been described as having a relatively benign course with mild skin rash, nausea, vomiting, and/or diarrhea that is responsive to immunosuppression.

In this article, we present a case of fatal, spontaneous GVHD in a patient with multiple myeloma following ASCT.

A 55-year-old woman with free kappa light chain multiple myeloma received induction therapy with bortezomib and decadron. She achieved a complete response after six cycles of therapy. The only complication secondary to induction therapy was a bortezomib-related neuropathy that was managed with Lyrica[®]. Prior to transplant she received mobilization with cyclophosphamide (3 g/m²) followed by a 10 day course of G-CSF. Five days after stem cell collection, she received high dose melphalan (100 mg/

m² on day-2 and day-1) followed by stem cell rescue on day 0. Her immediate post-transplant course was complicated by persistent, low grade fevers and mild diarrhea occurring during and after engraftment. Blood cultures, urine cultures, stool cultures, and chest X-ray were unremarkable. The fevers were attributed to engraftment and the patient was discharged home off all antimicrobials. Steroids were not given for the presumed engraftment as, at the time of discharge, she appeared to be clinically improving.

On day +24 post-transplant, the patient presented with fever, nausea, anorexia, and a diffuse, erythematous, maculopapular rash. Her complete blood count was normal. Blood, urine, throat, and stool cultures were negative as was the chest X-ray. Serologic testing for HSV, EBV, adenovirus, influenza, RSV, Rubella, and HIV were unrevealing. The rash progressed, becoming intensely erythematous with desquamation. Concurrently, an eosinophilia developed coupled with elevated transaminases, the ALT peaking at 425 units/L. Serologies for hepatitis A, hepatitis B, and hepatitis C were negative as were anti-smooth muscle antibodies and antimitochondrial antibodies. CT scans of the chest, abdomen, and pelvis were unremarkable.

A skin biopsy was performed and revealed intraepidermal pustules and acute inflammation. Studies for HSV, CMV, and fungus were negative. Given the concern for a spontaneous GVHD process, the patient was started on solumedrol (1 mg/kg) every 12 hr. Within 48 hr, the rash began to improve and fevers subsided. The patient was ultimately discharged from the hospital on a slow prednisone taper, a 20% dose reduction per week.

Following discharge, with each attempt to taper the steroids, her rash worsened requiring an increase in the steroids. In addition, on day +50, the patient developed persistent diarrhea. The average stool output ranged 1 to 2 liters daily. Though *C. difficile* infection was documented, the diarrhea did not improve after a course of flagyl followed by a course of vancomycin. The steroids were not increased after the onset of diarrhea as we believed that the diarrhea was solely due to infection. Notably, 1 week after completion of vancomycin the diarrhea persisted and repeat *C. difficile* testing was negative.

Given our patient's persistent and worsening rash, a second skin biopsy was performed on day +61 post-transplant. The biopsy revealed marked spongiosis with vacuolization along the basal layer of the epidermis with necrotic keratinocytes and a perivascular infiltrate of lymphocytes within the superficial dermis, compatible with acute GVHD. The prednisone was subsequently increased to a total dose of 1 mg/kg/day following the biopsy results.

On approximately day +75 post-transplant, the patient presented with continued diarrhea and a marked worsening of her rash with generalized erythroderma, bullae formation, ulceration, and desquamation. Physical exam was notable for facial and lip swelling with severe mucositis. Laboratory evaluation revealed a new pancytopenia, showing a white blood cell count of 1,000 k/mm³, hemoglobin 8 gm/dL, and platelets 51,000 k/mm³. The patient was admitted to the hospital with presumptive diagnosis of severe autologous GVHD and started on treatment with solumedrol (2 mg/kg) IV daily in combination with tacrolimus. She was placed on broad spectrum antifungal, antiviral, and antibiotic prophylaxis. At this time, we performed chimerism analysis of the patient's buccal mucosa and leukocyte DNA. The DNA from both cell lines was identical, ruling out the possibility that our patient had received another patient's stem cells. In addition, we thoroughly reviewed blood bank records and documentation to ensure that all blood products had been irradiated, ruling out transfusion-associated GVHD as a cause for her symptoms.

Despite aggressive measures, the patient clinically deteriorated. She developed polymicrobial sepsis and ultimately required intubation and vasopressor support. Her condition worsened despite maximum support. She expired 104 days after the transplant with autologous GVHD as the cause of death.

This case illustrates the development of severe acute GVHD in a patient following ASCT as consolidative treatment for multiple myeloma. Despite the dismal outcome described in our case, it is of interest to note that the induction of GVHD following autologous transplant was once thought to be beneficial [1,4,5]. Several studies have investigated cyclosporine as a means to induce GVHD to increase the antitumor effect [4,5]. Such reported cases of GVHD described mild skin rashes that readily responded to steroids. The mechanism for the development of GVHD in these instances is thought to be mediated by the increased expression proinflammatory mediators perforin, IFN- γ , and TNF- α coupled with decreased expression of the proapoptotic protein, fas ligand [6,7].

There have also been several reported cases of spontaneous autologous GVHD that have developed in the absence of calcineurin inhibitor exposure [2,3,8–11]. The majority of these cases, such as the series described by Holmberg et al., have described patients presenting with a mild rash and gastrointestinal symptoms that have rapidly responded to steroids and other immunosuppressants. The case presented here differs in that our patient developed a severe and ultimately fatal form of GVHD. Importantly, our case is not the only instance of severe GVHD occurring after ASCT for multiple myeloma. In a recently published case series by Drobyski et al., five similar cases were described [2]. Four of the five patients ultimately expired as a result of complications directly attributed to steroid-refractory autologous GVHD.

In the Drobyski study, the cases of 386 patients undergoing ASCT were reviewed. Of these 386 patients, 26 underwent a second ASCT. Five patients developed a clinical syndrome with severe skin rash and diarrhea. All five patients had multiple myeloma. Three of the five patients developed GVHD after their second transplant. Researchers subsequently evaluated the stem cell products in an attempt to elucidate a mechanism for the development of the GVHD. Notably, there was no significant difference in the number of CD34+ cells/kg infused in the patients who developed GVHD as compared to those patients who did not develop GVHD. In addition, there was no difference in the composition of CD4+/CD8+ cells, memory B cells, regulatory T-cells, or the production of cytotoxic granules between the two groups. While there was no absolute difference in the composition of the T-cell populations within these groups, the authors postulate that the function of the T-regulatory cells is altered in those developing autologous GVHD. This alteration in T-cell function may be mediated by high dose melphalan, and hence, the effects are amplified by repeat exposure to melphalan during the second transplant.

Though alteration of regulatory T-cell function by high dose melphalan may partially explain the development of autologous GVHD, it is interesting that our case, as well as the series reported by Drobyski et al., occurred uniquely in patients with multiple myeloma and not other hematologic malignancies. This suggests that alteration of immune function may occur as a result of the disease process itself, or, may be secondary to the therapies used in its treatment. Notably, our patient, as well as two patients in the Drobyski study, received bortezomib as part of the induction regimen. One mechanism by which bortezomib produces an antimyeloma effect is through the induction of apoptosis [12]. Previously, apoptosis has been linked to the development of autoimmunity [13]. Deficiencies in the clearance of apoptotic cells may lead to the presentation of auto-antigens to cytotoxic T-cells. This process likely results in failure of self-tolerance and development of GVHD, especially in patients who have received high dose chemotherapy which alters the function of regulatory T-cells. The severity of the syndrome may then be dependent upon unique host factors such as a deficiency in the clearance of apoptotic cells or fas/fas ligand mutations which will prevent the apoptosis of auto-reactive lymphocytes.

Regardless of the mechanism responsible for its development, GVHD following an autologous stem cell transplant appears to be a real, albeit rare phenomenon. The overall incidence in the Drobyski series was 2%. Another larger study by Holmberg et al. demonstrated an incidence rate as high as 13%, though this series described primarily non-fatal cases of autologous GVHD [8].

Nevertheless, patients referred for ASCT should be cautioned regarding the potential development of this devastating syndrome, particularly in those patients referred for consolidative treatment for multiple myeloma. In the appropriate clinical setting, GVHD following an autologous transplant should be a diagnostic consideration when evaluating rash or gastrointestinal symptoms in the post-transplant period. Most importantly, this syndrome requires prompt and aggressive immunosuppressive therapy. Fatalities, such as that described here, may potentially be prevented if intensive immunosuppressive therapy is implemented without delay.

Temple Bone Marrow Transplant Program

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Transfusional iron overload in children with sickle cell anemia on chronic transfusion therapy for secondary stroke prevention

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Chronic transfusion reduces the risk of recurrent stroke in children with sickle cell anemia (SCA) but leads to iron loading. Management of transfusional iron overload in SCA has been reported as suboptimal [1], but studies characterizing monitoring and treatment practices for iron overload in children with SCA, particularly in recent years with the expansion of chelator options, are lacking. We investigated the degree of iron loading and treatment practices of 161 children with SCA receiving transfusions for a history of stroke who participated in the Stroke with Transfusions Changing to Hydroxyurea (SWITCH) trial. Data obtained during screening, including past and entry liver iron concentration (LIC) measurements, ferritin values, and chelation were analyzed. The mean age at enrollment was 12.9 ± 4 years and the mean duration of transfusion was 7 ± 3.8 years. Baseline LIC (median 12.94 mg/g dw) and serum ferritin (median 3,164 ng/mL) were elevated. Chelation therapy was initiated after a mean of 2.6 years of transfusions. At study entry, 137 were receiving chelation, most of whom (90%) were receiving deferasirox. This study underscores the need for better monitoring of iron burden with timely treatment adjustments in chronically transfused children with SCA.

Chronic transfusion therapy greatly reduces the risk of recurrent stroke in children with sickle cell anemia (SCA) [2]. A major concern with this therapy is the development of transfusional iron overload, which can cause endocrinopathies, liver inflammation and fibrosis, and cardiac toxicity [3–5], although some of these complications appear to occur less commonly in SCA compared with thalassemia patients [6,7]. The use of modified transfusion programs that allow higher pretransfusion hemoglobin S levels [8] and automated exchange transfusions may limit iron loading in SCA [9,10]. Chelation therapy also is an effective means of reducing iron burden, and the clinical availability of an oral chelator, deferasirox (DFX), since late 2005, may improve treatment adherence compared with the parenterally administered deferoxamine (DFO). Despite these effective means of controlling iron burden in children with SCA, many children receiving transfusions have elevated iron stores.

The SWITCH trial was a Phase 3 multicenter, randomized trial to compare transfusion therapy and iron chelation to hydroxyurea and phlebotomy for secondary stroke prevention and control of iron burden [11]. Children with SCA receiving red cell transfusions for a history of overt stroke with trans-

sional iron overload were screened to determine trial eligibility. In this report, we characterize the transfusional iron burden and treatment practices in this group of children.

Patient characteristics of the 161 children screened for the study are shown in Table I. The mean serum ferritin level and LIC were elevated at study entry. Of the 151 children who underwent screening liver biopsy, 64 (42.4%) subjects had LIC ≥ 15 mg/g dw and 99 (65.6%) had serum ferritin levels ≥ 2,500 ng/mL, levels associated with a high risk of iron-related complications in other transfused populations [12–14]. Seventeen of 151 (11.3%) children who underwent screening liver biopsy had well controlled iron levels with LIC < 5 mg/g dw so were ineligible for randomization. Their mean LIC was 2.85 mg/g dw (median, 2.781; range, 0.8–4.893) and mean ferritin was 1,102 ng/mL (median, 850; range, 219 – 3,315).

No children had a history of iron-related heart disease but 15 of 79 (19.0%) with past liver biopsies had a history of hepatic fibrosis. Baseline ALT levels positively correlated with both ferritin ($r = 0.27$, $P = 0.0007$) and LIC ($r = 0.26$, $P = 0.0012$). The presence of endocrinopathies was not assessed in this study, but no children were receiving oral hypoglycemics, insulin, or thyroid hormone replacement at the time of study entry.

Transfusion duration weakly correlated with both serum ferritin levels and LIC (Fig. 1); other factors such as method of transfusion and use of chelation affect this relationship. Serum ferritin and LIC also were positively correlated, although the correlation was not precise (Fig. 1), underscoring that other factors including inflammation affect serum ferritin levels. Prior reports have shown conflicting results regarding the correlation of ferritin and LIC in transfused SCA patients [15–17]. A recent report showed ferritin levels < 1,500 ng/mL correlated with an LIC < 10 mg/g dw, while serum ferritin levels above 3,000 ng/mL usually predicted LIC > 10 mg/g dw [18]. This pattern generally held true in our patient population, although disparities occurred in a subset of children: 3 had ferritin < 1,500 ng/mL with LIC > 10 mg/g dw, while 14 had ferritin > 3,000 ng/mL with LIC < 10 mg/g dw. Thus, serial assessment of the serum ferritin level is helpful and, together with monitoring of transfusional intake, may be utilized to monitor iron overload, but periodic LIC measurements are advisable, particularly if MRI is available.

TABLE I. Patient Characteristics (N = 161)

Characteristic	Mean (SD)	Median (range)	N (%)
Demographics			
Age (y)	12.9 (3.97)	13.2 (5–19)	
Sex (% male)			51.6
Age at stroke (y) (N = 158)	5.8 (2.77)	5.5 (1.1–14.8)	
Transfusion history			
Transfusion duration (y)	7.0 (3.75)	6.5 (1.3–15.5)	
Transfusion type (N = 160)			
Simple			102 (63.4)
Partial exchange			40 (24.8)
Erythrocytapheresis			18 (11.2)
Current use of chelation			
Receiving chelation at study entry (N = 160)			137 (85.6)
Current use of DFO monotherapy (N = 137)			12 (8.8)
Current use of DFX monotherapy (N = 137)			123 (89.8)
Current use of DFO and DFX (N = 137)			2 (1.5)
Baseline laboratory (N = 151)			
Ferritin (ng/mL)	3423 (2028)	3164 (219–12419)	
Liver iron concentration (mg/g dw liver)	15.46 (10.09)	12.94 (0.8–61.3)	
Pretransfusion hemoglobin (gm/dL)	9.3 (0.86)	9.3 (7.0–11.9)	
Pretransfusion Hb S%	28.8 (10.4)	29.2 (4.2–60.5)	
Aspartate aminotransferase (U/L)	59.1 (31.9)	51.5 (15–249)	
Alanine amino transferase (U/L)	55.6 (31.4)	44.5 (26–239)	
Creatinine (mg/dL)	0.4 (0.14)	0.4 (0–1)	

RBC, red blood cell; DFO, deferoxamine; DFX, deferasirox; y, years. If the number of patients analyzed for a respective characteristic differs from the total N of 161, a corresponding N value is provided in parentheses after the characteristic.

Only 79 of 161 (49.1%) had undergone liver biopsy in the past. Transfusion duration was longer in those who underwent liver biopsy (8.2 vs. 5.7 years, $P < 0.0001$). This liver biopsy rate is strikingly similar to a rate of 46.5% in transfused pediatric patients with SCA in a prior report, which was significantly lower than the rate of 69.6% in transfused children with thalassemia [1]. The mean LIC at the time of past biopsy was 6.8 mg/g dw ($n = 73$, range, 1.15–44.5) and had risen to 15.4 mg/g dw at study entry (mean, 2.6 years). The mean study entry LIC of children without prior liver biopsy was similar at 15.5 mg/g dw. Magnetic resonance imaging (MRI) techniques for estimation of liver iron loading were not available at most sites at the time of SWITCH study enrollment. The increasing availability of R2 and other MRI techniques to measure LIC should allow more timely and frequent assessment of LIC in this patient population.

One hundred forty-one (87.6%) children had received chelation therapy in the past. The mean transfusion duration prior to chelation initiation was 2.6 years (median, 2.0; range, 0–12.6), which is substantially lower than the duration of 4.1 years for sickle cell patients and 5.5 years for thalassemia patients in a prior report [1]. Treatment guidelines generally recommend initiating chelation after 10–20 transfusions have been administered [19], or after one to two years of transfusions, similar to the time of initiation of chelation in our overall population. Twenty of 161 (12.4%) children had never received chelation therapy before study entry; these children were younger (9.6 ± 2.8 vs. 13.4 ± 3.9 years, $P < 0.001$), had a shorter transfusion duration (3.2 ± 1.9 vs. 7.5 ± 3.7 years, $P < 0.001$) and had a trend towards both lower serum ferritin (2,622 vs. 3,525 ng/mL, $P = 0.084$) and LIC (11.6 vs. 15.95 mg/g dw, $P = 0.096$) than children who received chelation.

Most (105) subjects had received DFO in the past, but by study entry, only 14 of the 105 (13.3%) continued to take DFO (mean dose 33.9 mg/kg/day), including two children who were taking both DFO and DFX. Ninety-nine (94.3%) children switched from DFO to DFX monotherapy (mean dose 25 mg/kg/day) before study entry after a mean of 4.7 years (median, 4.25; range, < 1 month to 12.33 years) of DFO therapy. The predominant use of DFX is not unexpected given that it is an oral agent and easier to administer than parenteral DFO. There was no significant difference in either ferritin levels ($P = 0.6913$) or LIC ($P = 0.7811$) between the DFO and DFX groups at study entry. However, an inverse relationship between duration of DFX, but

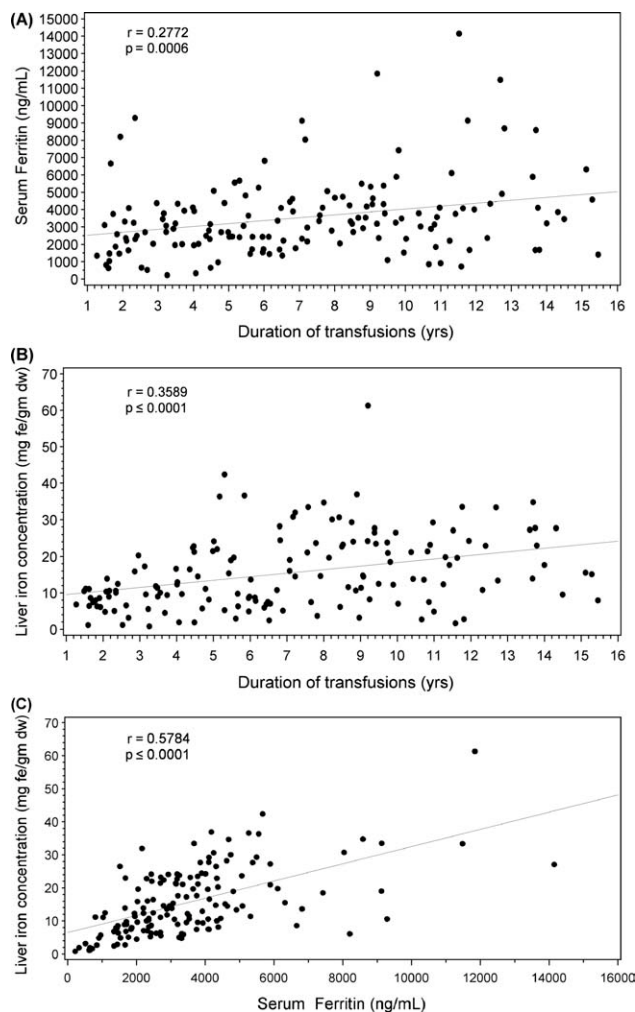


Figure 1. The correlation between the duration of transfusion and (A) baseline study serum ferritin level and (B) baseline liver iron concentration and the correlation between liver iron concentration obtained by liver biopsy and serum ferritin (C) are shown. r = Pearson's correlation coefficient. Please see the accompanying Supporting Information for a list of participating centers.

not DFO, treatment and both serum ferritin level ($r = -0.21$, $P = 0.0018$) and LIC ($r = -0.21$, $P = 0.0137$) was seen. This beneficial effect on reducing iron load was evident despite a short overall duration of DFX use, and could be related to superior adherence to treatment with this drug (data not shown). Further follow-up is required to see if this effect will persist, or perhaps be augmented over time.

The type of transfusion also had an effect on LIC: median LIC was 7.87 mg/g dw (range, 3.17–31.94, $N = 18$), 11.71 mg/g dw (0.8–61.3, $N = 96$), and 18.94 mg/g dw (range, 1.89–42.39, $N = 37$) among children receiving erythrocytapheresis, simple, and partial exchange transfusions, respectively, $P = 0.0417$; duration of transfusion therapy was similar between these groups (7.4, 6.6, and 7.7 years, respectively, $P = 0.24$). The finding of the lowest iron burden in children receiving erythrocytapheresis is not surprising, given that this method is associated with a reduction in net red cell loading [9,10]. Overall treatment costs also are lowered if chelation therapy can be avoided [20], further supporting the early use of erythrocytapheresis for long-term transfusion therapy in SCA when feasible. The finding of the highest iron loading in the subjects receiving partial exchange transfusion is unexpected. It is possible that these subjects were switched to partial exchange in response to high iron burden, and their iron burden will improve with continued treatment, which could not be assessed in this study. In a prior report, with long-term manual partial exchange to maintain hemoglobin S levels below 35–50%, six of seven subjects maintained ferritin levels below 1,000 ng/mL and did not require chelation; costs also were lowered

compared with automated erythrocytapheresis [21]. Thus, manual partial exchange may be beneficial, particularly where chelation is not readily available or the treatment costs are prohibitive.

A number of limitations of this study exist. First, study inclusion criteria required evidence of iron loading and thus excluded patients with well-controlled iron burden. Nonetheless, we found that iron burden was much higher than required for study entry. Second, baseline data collection was retrospective and some subjects may have had prior monitoring that was not reported in the study. Finally, the mean duration of DFX use was short (1.58 years), limiting the ability to assess the impact of this chelator on iron burden.

In summary, the majority of the children in this multicenter cohort had sub-optimal control of iron burden at study entry. Although iron overload was required for study inclusion, the LIC criterion for entry was only 5 mg per g dw while most of the participants had LIC well above this value. Most children initiated chelation in a timely fashion; thus, the high iron burden might be explained by other factors such as poor adherence to therapy or inadequate chelator dosing, which deserves further study. Health care providers should be aware of the importance of monitoring iron burden with timely initiation of treatment and ongoing adjustments to transfusion methods and/or chelation regimens in response to these measurements.

Methods

The primary aim of SWITCH, a multicenter intervention trial, was to compare alternative therapy (hydroxyurea and phlebotomy) with standard therapy (transfusions and chelation) for the prevention of secondary stroke and reduction of transfusional iron overload in pediatric subjects with SCA and previous stroke. The SWITCH study design is described in detail elsewhere [11]. Briefly, children ages 5 to < 19 years old, were eligible to be screened for participation in the randomized portion of the SWITCH study if they had SCA (type SS, S- β^0 -thalassemia, or S-OArab), a history of overt stroke, had received chronic transfusion therapy (simple, partial exchange, or erythrocytapheresis) for at least 18 months with an average pretransfusion hemoglobin (Hb) S level of $\leq 45\%$ during the prior six months, and had evidence of iron overload defined as a LIC of at least 5 mg/g dry weight (dw) liver or serum ferritin of at least 500 ng/mL. At study entry, medical history was taken including data on stroke and other medical complications such as heart and liver disease. Data on transfusion duration and method were collected. Transfusion methods were characterized as simple, partial exchange (manual removal of a determined amount of blood followed by packed red cell transfusion), and erythrocytapheresis (automated red cell exchange). Additional data collection included chelation history (chelator type, dosage and duration of use), and current and prior medications used. Baseline laboratory studies included complete blood count with white blood cell differential and reticulocyte count obtained locally and hemoglobin electrophoresis with quantification of Hb A, S and F levels, chemistry panel, and serum ferritin measured at a central laboratory (Georgia Health Sciences University, Augusta, GA). Liver biopsy was performed and analyzed for LIC by inductively coupled plasma-mass spectrometry at Mayo Medical Laboratories, Rochester, MN.

Statistical Analyses. Descriptive statistics (mean, standard deviation, median, range, and frequency) were performed for demographic variables, transfusion history, chelation history, and baseline laboratory values. LIC values were log-transformed due to a skewed distribution of values. Pearson product-moment correlations were implemented for continuous variable relationships and for significance testing. Independent sample *t*-tests were computed for comparisons of categorical predictors (chelation type, splenomegaly, transfusion type) with continuous outcomes (ferritin, LIC, and hemoglobin S levels). *T*-tests were also used to examine the relationship of baseline chelator type (DFO versus DFX) and both LIC and serum ferritin levels. All *P*-values should be considered descriptive.

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Complications of implantable venous access devices in patients with sickle cell disease

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Implantable venous access devices (VADs) are used in sickle cell disease (SCD) for patients with poor venous access to facilitate chronic blood transfusions and manage acute complications. We attempted to define the frequency of bloodstream infections (BSI) and thrombosis in adults and children with SCD and VADs. We performed a single-institution, retrospective review of VAD-associated infection and thrombosis in patients with SCD. Thirty-two patients (median age 20 years, range, 1–59) had 86 VADs placed (median, 2.7 VADs per patient, range, 1–7) with a total of 41,292 catheter days (median, 1,376 days; range, 323–3,999). Mean catheter lifespan in adults (691 days \pm 123) was not significantly higher than children (614 days \pm 154). A total of 66 VAD-associated BSI (1.59 infections per 1,000 catheter days) occurred in 17 of 32 (53%) patients. Children with VADs had fewer BSI (3 of 10; 30%) than adults (14 of 22; 64%, $P = 0.08$). 24 catheter-associated thromboses (0.49 thromboses per 1,000 catheter days) occurred in 10 of 32 (41%) of patients. Children also had fewer VAD-associated thrombosis (1 of 10; 10%) than adults (9 of 22; 40%, $P = 0.08$). In conclusion, the use of VADs in SCD was linked to a significant rate of infection and thrombosis.

Sickle cell disease (SCD) is one of the most commonly inherited diseases worldwide. Patients with SCD have acute and chronic complications including recurrent or chronic pain, acute chest syndrome, splenic sequestration, priapism, and stroke. Venous access for blood drawing and infusions often become difficult in patients with SCD. Central venous access devices (VADs) may be placed to facilitate laboratory assessments, chronic transfusion therapy or acute interventions such as intravenous fluids, pain medication, and antibiotics. Central VADs are a valuable instrument for long-term intravenous treatment and access; however, they are associated with complications such as infection and thrombosis.

With some degree of baseline thrombophilia and functional asplenia, patients with SCD are predisposed to thrombotic and infectious complications. Although there is difficulty in identifying a cause-and-effect relationship, numerous studies have documented serum abnormalities of cytokines, coagulation markers and surface markers, leading to an overall hypercoagulable state in patients with SCD [1]. In addition, functional asplenia predisposes to infectious complications, with a recent review finding 28% of patients with SCD who were hospitalized had at least one positive blood culture. Although fevers were not recorded, their review also found that 80% of these bloodstream infections were catheter related [2].

There is wide variability in the reported rates of VAD-associated complications [3] with rates of infection ranging from 0 to 5.5 per 1,000 VAD days, and rates of thrombosis from 0 to 0.99 per 1,000 VAD days (see Table 1). An initial report evaluated five adult patients with SCD and concluded that these patients had a greater incidence of VAD-associated BSI as well as an increased rate of premature removal of catheters when compared to cancer patients with VADs [4]. More recently, Jeng et al. observed a higher incidence of VAD-associated infection in 19 patients with SCD aged 1.5–30 years than patients with cancer in their institution. They also observed a significantly higher incidence of infection in adult patients with SCD (7.0 per 1,000 VAD days) than pediatric patients with SCD (4.5 per 1,000 VAD days), $P = 0.05$ [5]. However, a larger review found that the rates of VAD associated complications or premature removal for 25 patients with SCD aged 8 months to 23 years did not differ from those reported for patients with cancer, HIV infection, or cystic fibrosis [6].

The populations in these studies differed in terms of the VAD used, as some patients had external Broviac lines and others had implantable ports. Raj et al. evaluated the use of a single type of implantable venous access device (Cathlink[®] 20 port) in patients with SCD requiring long-term erythrocytapheresis. A total of 15 patients aged 7–20 years were followed for a mean of 44 months. A lower incidence of thrombosis (0.16 per 1000 VAD

days), and no episodes of catheter-related infections were reported, which was thought to be the result of the VAD's polymer material in addition to the practice of allowing only specialty trained nurses to access the devices [7].

The variability in the use of VADs and the reported rates of complications in patients with SCD make it difficult for clinicians to develop evidence-based guidelines. Therefore, we attempted to define the frequency of bloodstream infections and thrombosis in patients with SCD and VADs at our institution as well as summarize past results to guide future clinical recommendations for their use.

A total of 32 subjects were eligible for inclusion with a median age of 20 years (range, 1–59); 10 pediatric and 22 adult patients. There were 86 VADs placed (44 dual lumen ports, 27 single lumen ports, 1 Vortex[®] port, 10 Broviacs, 3 PICCs, and 1 permcath) with a median of 2.7 VAD per patient (range, 1–7). There were a total of 41,292 catheter days with a median of 1,376 days (range, 323–3,999). The mean catheter lifespan in adults (691 \pm 123 days) was not significantly different than pediatric patients (614 \pm 154 days; $P = 0.58$).

There were 66 distinct catheter-related infections with a median of one infection per patient (range, 0–9). The median number of days prior to a documented bloodstream infection was 492 days (range, 25–1,877). There were 1.59 catheter-associated infections per 1,000 catheter days in all patients. Three out of 10 (30%) individual pediatric patients had infections compared to 14 of 22 (64%) adult patients ($P = 0.08$). Rates of catheter-associated infections between pediatric and adult patients were not significant (1.07 per 1,000 days vs. 2.03, $P = 0.23$). Infection rates were not significantly different among catheter types, including dual lumen ports (21/44; 48%); single lumen ports (11/27; 41%); Broviac's (4/10; 40%); and PICCs (2/3; 67%). No infections were noted with both permcath and Vortex[®] port placement. 81% of patients were taking hydroxyurea, although there was no significant difference in the rates of catheter-associated infections between patients taking or not taking hydroxyurea (44% vs. 56%, respectively = 0.45).

Our review was unable to clearly determine the initial indication for placement for the majority of patients requiring VADs; however, four patients were clearly noted to have issues with peripheral phlebotomy for standard blood draws and two patients were receiving chronic transfusions with oral chelation therapy. We also were unable to identify the number of times catheters were accessed prior to positive blood cultures. Five of 10 pediatric patients were on penicillin prophylaxis as age appropriate pneumococcal prophylaxis, and discontinued its use at five years of age.

Review of radiographic records revealed 24 separate episodes of catheter-associated thrombosis in 10 of 32 patients (41%), with a mean of 0.75 thrombotic events per patient (95% CI, 0.31–1.19). No patients had a history of thrombosis prior to placement of VAD. The sites of thrombosis included the following: upper extremity (11); pulmonary embolism (8); superior vena cava (4); and left atrial thrombus (1). Fewer pediatric patients had VAD-related thromboses (1/10; 10%) compared with adults (9/22; 40%), although this was not statistically significant ($P = 0.08$). However, the overall rates of thrombosis was significantly different between adult and pediatric patients (0.75 per 1,000 catheter days vs. 0.09, $P = 0.001$).

Thrombosis was more frequent in patients with ports, with 10 episodes in dual lumen (23%) and 9 episodes in single lumen (33%). Only one broviac associated thrombosis was noted (10%) and the one Vortex[®] port had an associated thrombosis presenting with superior vena cava syndrome (100%). No thrombosis was found associated with use of either PICC or Permcath.

Our review involved an analysis of a larger number of patients ($n = 32$) and longer total catheter days (41,292) than in prior studies and may more accurately reflect VAD-associated complication rates. Similar to Jeng et al., we found an increase in VAD-associated infections or thrombosis in adults

TABLE I. Comparison of Venous Access Device Complications From the Literature

	Patient demographics	Number of patients with SCD	Number of VAD days evaluated	Mean number of VAD days	Rate of Infection (per 1000 days)	Rate of Thrombosis (per 1000 days)
Current study	Adult and Pediatric	32	41,292	672	1.59	0.49
Phillips et al. (1988)	Adult	8	3,440	430	0.46	0.46
Abdul-Rauf et al. (1995)	Pediatric	25	17,444	562	0.86	0.29
McReady et al. (1996)	Adult	5	1,200	240	4.00	NA
Jeng et al. (2002)	Adult and Pediatric	19	12,120	295	5.50	0.99
Raj et al. (2005)	Pediatric	15	19,230	1,068	0.00	0.16
Fratino et al (2006)	Pediatric Cancer patients	0	107,102	256	0.87	0.09
Lee et al (2006)	Adult cancer patients	0	59,931	88	NA	0.03
Maki et al (2005)	Various Medical conditions	0	*	*	1.6 (0.1 for ports)	NA

NA, not assessed; *Review of 200 prospective studies.

when compared with pediatric patients, although nonsignificant. We postulate that age related changes and incidence of additional comorbidities may lead to an increased risk for VAD-associated complications. In addition, other studies have shown evidence of increased thrombin generation and potential "hypercoagulability" with increasing age [9,10].

Compared with other study populations our patients had a higher rate of catheter-associated BSI (see Table I). For example, Maki et al recently performed a large systematic review of 200 published prospective studies of adults with VADs placed for various medical diagnoses. They found long term VADs (cuffed and tunneled) had 1.6 infections per 1,000 catheter days. Central venous ports, however, had 0.1 infections per 1,000 catheter days [8]. Fratino et al. evaluated pediatric oncology patients with >100,000 days of observation and revealed a lower rate of infection (0.87) and thrombosis (0.09) compared with our data involving patients with SCD [9]. This is in contrast to our results with SCD in which the majority of patients (83%) had ports as their VAD and patients with single lumen and dual lumen ports had high rates of infection (41 and 48%, respectively).

Thrombosis rates in adult cancer patients with VADs are reported similar to our study. In a prospective study of adult cancer patients, 19 of 444 patients (4.3%) had VAD-associated thrombosis (0.3 per 1,000 catheter days). Baseline risk factors included having a previous central venous catheter and more than one attempt at insertion, both suggesting that vessel wall injury adds to the predisposition of thrombosis [10]. We did not find information on number of attempts required during VAD placement, however, our review did find that 6 of 10 patients (60%) had a VAD-associated thrombosis following insertion of their second VAD.

Limitations of our study include the retrospective design and lack of an institutional comparative group. Although we did not compare our sickle cell cohort at Duke University to other cohorts with VADs at our institution, we found no patients in our study had deep vein thrombosis (DVT) or pulmonary embolism (PE) prior to placement of VAD. We also compared our rates of complications to previously published data in various patient populations. We found patients with SCD to have higher rates of VAD-associated infection and thrombosis than oncology patients [9,10], despite periods of neutropenia and potential thrombophilia. Our comparison is limited by the fixed periods of time for which oncology patients require central venous access and the variation in access practices and chemotherapy infusions.

Further efforts are needed to determine potential interventions such as thromboprophylaxis to decrease these complications. In addition, care providers need a prospective database to guide further recommendations. A database which includes the reason for VAD placement, the number of times VADs are accessed, and laboratory results such as hematocrit. This will provide additional information for potential interventions to decrease the high complication rates currently found.

Overall, we report that use of VADs in SCD was linked to a significant rate of infection and thrombosis. Most concerning was the finding of a high proportion of catheter-related thrombosis in adults with SCD, which places patients at risk for post-thrombotic syndrome and adds the burden of anticoagulation to patient management. The total catheter days reviewed in our study is much greater than in similar reviews and may allow a more accurate reflection of complications related to VADs in patients with SCD.

Patients and families considering VAD placement should be specifically counseled on the potential lifespan of VADs and the increased risk of bloodstream infections and thrombosis in patients with SCD. A prospective study involving patients with SCD is needed to evaluate potential interventions to decrease these risks.

Methods

We performed a retrospective analysis of patients with SCD (type SS, SC, or $S\beta^0$ -thalassemia) and VADs at Duke University. After obtaining approval by the institutional review board with waiver of consent, records were reviewed from January 1, 1998 to December 1, 2009. We identified patients through Duke Enterprise Data Unified Content Explorer (D.E.D.U.C.E.), an electronic database of clinical information collected by patient care [11]. Procedural codes for central line placement, flush, and removal were used for confirmation of VAD use, and patients were only included if they had at least 12 months of follow up following VAD insertion. Patients were excluded if they were younger than 1 year old, had care primarily at another institution, or had incomplete medical records. Patients with temporary insertion of venous catheters (placement for less than 24 hr) were also not included in the analysis. The following VADs were included: single and dual lumen ports, Vortex ports[®], broviacs, peripheral inserted central catheters (PICC) and permcaths. Pediatric patients were defined as all patients less than 18-year-old. Adult and pediatric sickle cell providers were subsequently queried, however no additional patients were identified.

Of the greater than 800 patients with SCD followed at our Comprehensive Sickle Cell Center, 44 eligible patients were identified through our retrospective review. Eleven were excluded due to inadequate records from an outside hospital or insufficient follow-up, and 1 was excluded due to a confounding diagnosis of cystic fibrosis.

All blood cultures drawn during the lifespan of a VAD were reviewed to determine the number of catheter-associated bloodstream infections. Catheter-associated blood stream infections were defined as bacterial or fungal growth within 72 hr in cultures drawn through the VAD. Blood cultures were considered contaminant if only one of two sets of cultures yielded *Viridans streptococci*, *Coagulase-negative staphylococci*, *Bacillus* spp, *Corynebacterium* spp, or *Propionibacterium* spp. Similar to Jeng et al., we considered a positive culture as a distinct infection if there was a negative blood culture between two positive cultures, a two-week interval between positive blood cultures, or both [5].

We also reviewed all radiographic records to determine the number of catheter-related thromboses. Thrombosis required confirmation by venogram, duplex ultrasonography, or computed tomography (CT) and was regarded as catheter-related if diagnosed as deep vein thrombosis, superior vena cava syndrome, or pulmonary embolism without lower extremity thrombosis. Minor episodes of occlusion or decreased flow successfully treated by thrombolytics were not recorded.

Complication rates were calculated per 1,000 catheter days. Comparisons were made between pediatric and adult patients using a Student's *t*-test for continuous variables, and a Fisher's exact test for categorical variables. For all comparisons, $P < 0.05$ was considered significant and analysis was performed using Graphpad Prism 5 software.

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Inhibition of cell-mediated immunity by the histone deacetylase inhibitor vorinostat: Implications for therapy of cutaneous T-cell lymphoma

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Several histone deacetylase inhibitors (HDACi), including vorinostat, have been approved for the therapy of cutaneous T-cell lymphoma (CTCL). Emerging data suggest that HDACi may exert immune suppressive effects which would be disadvantageous for therapy of CTCL. We describe a patient with Sezary syndrome who was monitored for drug-induced immunosuppression while undergoing treatment with vorinostat. Analysis of the patient's natural killer cell function before and after initiation of treatment confirmed inhibition of this important cell-mediated immune function. In addition, the *in vitro* effects of vorinostat on the immunity of healthy volunteers confirmed that this class of drug can profoundly suppress multiple arms of the cellular immune response. These findings raise concerns of increased susceptibility to infection in this high-risk population.

Histone deacetylase inhibitors (HDACi) represent a novel class of anti-cancer drugs, with two members of the group, vorinostat and romidepsin, approved for the therapy of cutaneous T-cell lymphoma (CTCL). HDACi modulate chromatin structure, and have been shown to promote growth arrest, differentiation, and apoptosis of tumor cells [1]. Specifically, vorinostat has been shown to cause selective apoptosis of malignant T cells by increasing the expression of pro-apoptotic factors p21^{WAF1}, bax, and caspase-3, and decreasing the expression of anti-apoptotic factors such as Stat-6 in CTCL cell lines and patients' peripheral blood mononuclear cells (PBMC) [2].

There is emerging evidence for the potent immunosuppressive properties of HDACi. Studies have shown that HDACi can have therapeutic benefit in autoimmune disease models [3–5] perhaps owing to the enhancement of regulatory T-cell functions [6]. Moreover, we have reported a patient with CTCL with refractory bullous pemphigoid, who experienced rapid resolution of this autoimmune disorder following initiation of therapy with vorinostat [7]. Furthermore, *in vitro* studies have shown anti-inflammatory properties of HDACi via suppression of cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β [8]. As attempts at preservation of cellular immunity are critical in the management of CTCL, we examined the effects of vorinostat on cellular immunity in a patient undergoing therapy with this agent and in healthy volunteers.

A 65-year old female with a past history of erythroderma, lymphadenopathy, and circulating atypical cells was diagnosed with Sezary syndrome in 2001. She experienced a complete clinical response following three years of therapy with photopheresis, interferon gamma, bexarotene, and psoralen

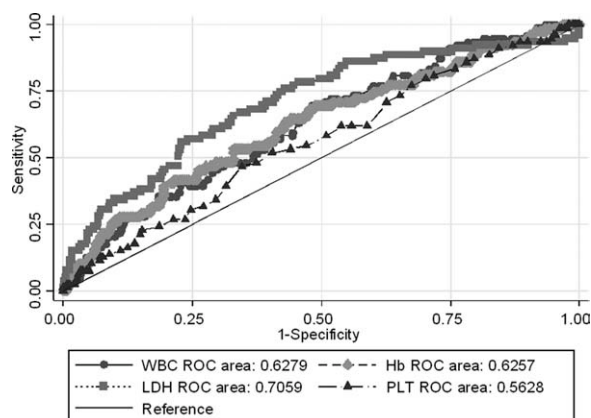


Figure 1. Suppression of NK cell cytotoxicity by vorinostat *in vivo*. PBMC from the patient presented in the case report were collected prior to initiation of systemic CTCL therapy (baseline) and while undergoing treatment with vorinostat (vorinostat) were cultured in medium alone or treated with 10 μ g/mL of a synthetic TLR 7/8 agonist 3M-007 for 48 hr, followed by a 4 hr Cr⁵¹ release assay using K562 cells as targets. Data represent mean of triplicate of percentage of specific lysis per single NK cell at E:T ratio 50:1. Percentage NK cells/total PBMC defined by flow cytometric analysis.

and ultraviolet A radiation (PUVA). She was in remission from 2004 until 2007 when she relapsed with recurrent skin lesion and blood involvement. Her disease progressed despite multimodality therapy. In February 2011, she began vorinostat 400 mg/day with marked improvement in skin erythema within one month. Blood was obtained from the patient for study 30 days following initiation of vorinostat.

As interaction between natural killer (NK) cells and antigen presenting cells (APCs) is critical in the maintenance of antitumor immunity [9], we examined the effects of vorinostat on these cellular functions. NK cell cytotoxicity from our patient's blood specimen obtained 4 months prior to the initiation of vorinostat therapy was compared to a specimen obtained 30 days after initiation of vorinostat therapy. After culturing cells for 48 hr, NK cytolytic activity was assessed as reported [10]. To assess NK cell responsiveness, the patient's cells were stimulated with 10 μ g/mL of a known APC activator Toll-like receptor (TLR) 7/8 agonist, known to indirectly activate NK

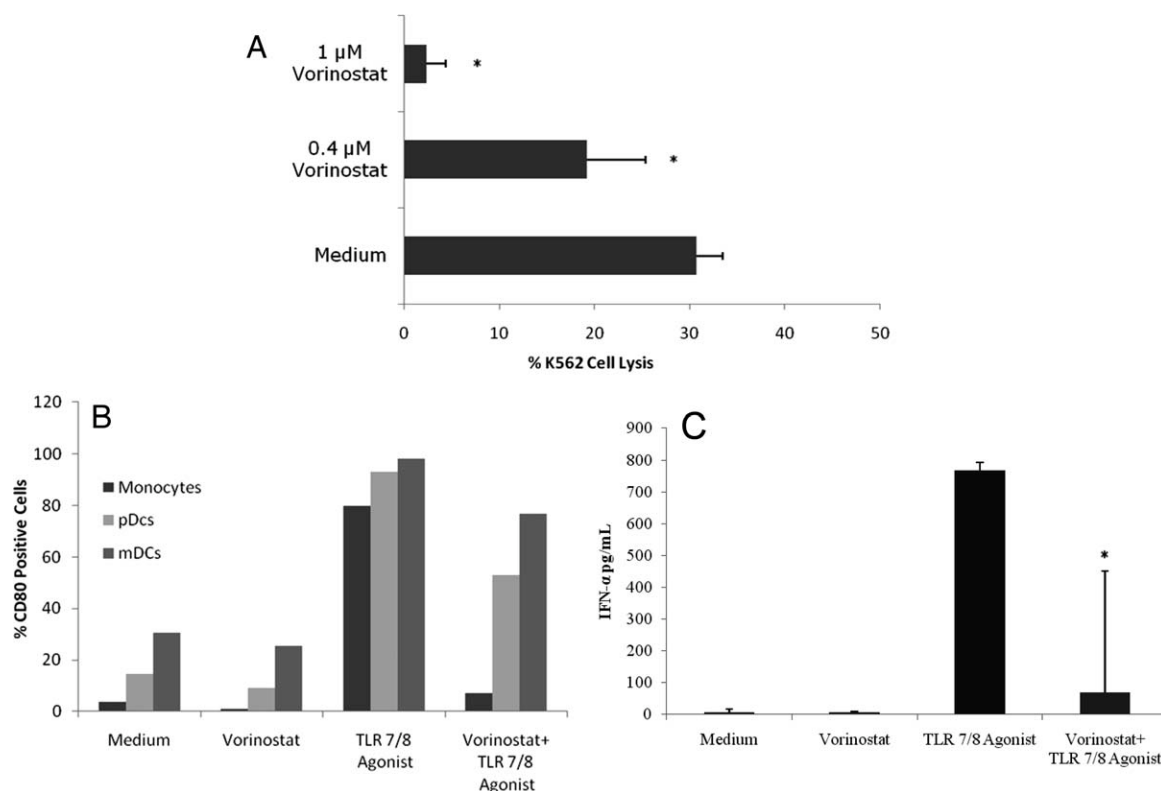


Figure 2. Vorinostat inhibits NK cell cytotoxicity, APC activation, and IFN- α production in vitro. **A:** Highly purified NK cells from healthy volunteers ($n = 3$) were cultured in medium alone or treated with 0.4 μ M and 1 μ M vorinostat for 48 hr, followed by a 4 hr Cr^{51} release assay using K562 cells as targets. Data represent mean \pm SD of tested individuals and are presented as a percentage of specific lysis at E:T ratio 2:1. * $P < 0.05$ when compared to medium. **B:** PBMC from healthy volunteers were cultured in 24-well plates at a density of 2×10^6 /mL/well in media alone, with 1 μ M vorinostat, or 10 μ g/mL synthetic TLR 7/8 agonist 3M-007 for 48 hr, or primed with 1 μ M vorinostat for 4 hr followed by activation with TLR 7/8 agonist for further 48 hr. Cells were then harvested, and analyzed for the expression of CD80. Monocytes were identified by anti-CD64 and anti-CD14 staining. Plasmacytoid dendritic cells (pDCs) were defined as lineage-negative cells (Lin 1-FITC cocktail) that co-express HLADR and CD123. Myeloid dendritic cells (mDCs) were defined as lineage-negative cells co-expressing HLADR and CD11c. The figure illustrated one of three representative experiments. **C:** PBMC from healthy volunteers were cultured in 24-well plates at a density of 2×10^6 /mL/well in media alone, with 1 μ M vorinostat, or 10 μ g/mL synthetic TLR 7/8 agonist 3M-007 for 48 hr, or primed with 1 μ M vorinostat for 4 hr followed by activation with TLR 7/8 agonist for further 48 hr. Cell free supernatants were then collected, and IFN- α levels were measured in cell-free supernatants by ELISA. Data represent mean \pm SD of tested individuals. * $P < 0.05$ compared to the TLR 7/8 agonist sample.

cells. While stimulation with TLR 7/8 agonist resulted in a significant increase in NK activity from 5.4% to 17.6% K562 lysis in the baseline sample, responsiveness upon stimulation was impaired in the sample obtained during vorinostat therapy, increasing only from 3.2% to 6.1% K562 lysis (Fig. 1). These results indicate that compared to baseline, vorinostat treatment resulted in significant blunting of functional NK activity.

Subsequent in vitro studies of vorinostat on the immunity of healthy volunteers revealed that this drug can profoundly suppress multiple arms of the cellular immune response. Purified CD56 $^+$ NK cells isolated from blood from three healthy donors were cultured in medium with 1 μ M or 0.4 μ M vorinostat, doses yielding serum concentrations in the range of those achieved with a standard vorinostat regimen of 400 mg daily. Assessment of NK cytotoxicity after 48 hr incubation with vorinostat, showed that vorinostat profoundly suppressed NK activity in a dose-dependent manner (Fig. 2A). Thus, functional NK activity of healthy volunteers was virtually eliminated following a short exposure to therapeutic levels of vorinostat.

In addition to depressed NK cell function, other important cellular immune functions appear to be inhibited by vorinostat. Activation of antigen presenting cells measured by the expression of the inducible co-stimulatory molecule CD80 [11] on monocytes, plasmacytoid, and myeloid dendritic cells (DCs) collected from healthy volunteers was markedly inhibited by vorinostat. While stimulation with TLR 7/8 agonist (10 μ g/mL for 48 hr) led to significant upregulation of CD80, and, thus activation, across all three cell lineages, this activation was suppressed by vorinostat (Fig. 2B). Furthermore, we observed that interferon- α (IFN- α) production following stimulation of healthy volunteer APCs with a TLR 7/8 agonist was greatly inhibited by 1 μ M vorinostat (Fig. 2C). TLR 7/8 stimulation led to significant (766.6 pg/mL) IFN- α production

by pDCs, which was markedly inhibited (68.4 pg/mL) by vorinostat. As NK cells require interaction with dendritic cells and monocytes for optimal activation, impairment of APC activation in turn diminishes NK functionality.

In summary, while HDACi vorinostat has been shown to have a significant response rate and a high rate of pruritus relief in heavily pretreated, refractory CTCL patients, our findings suggest that vorinostat also potentially suppresses multiple arms of the immune system which may contribute to disease progression and lead to greater susceptibility of these patients to opportunistic infections [12]. Our findings highlight the complexity of the effects of vorinostat and the need to balance its anti-tumor effects and immunosuppressive capabilities. Further studies are necessary to determine whether the present findings of immune suppression are representative of the HDACi class as a whole, or solely of vorinostat.

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The diagnostic value of biopsy of small peripheral lymph nodes in patients with suspected lymphoma

Beverly W. Baron¹ and Joseph M. Baron²

In a patient with suspected lymphoma, it is considered desirable to confirm the diagnosis by excisional biopsy of enlarged lymph nodes. However, sometimes the ideal nodes are positioned internally, requiring a deep invasive procedure for access, or the patient may have underlying medical conditions that make it risky to perform such an invasive procedure. Under a protocol approved by our institution's review board (IRB), we reviewed five patients in whom superficial lymph nodes were biopsied which were smaller than usually considered optimal for diagnosis (≤ 2 cm). In each of these cases, the biopsy yielded diagnostic information upon which treatment could be based, sparing the patient a deep invasive procedure. We suggest that in situations in which large internal lymph nodes are not easily accessible and/or the patient's clinical situation precludes more invasive procedures, including deep core needle biopsy of a large mass, it is worthwhile to consider the removal of smaller, superficial lymph nodes with minimal risk which may suffice for diagnosis.

Selection of which lymph node or mass to biopsy is an important step in the diagnosis of suspected lymphoproliferative disorders. Despite the availability of modern imaging techniques, including positive emission tomography scanning for staging, the initial encounter with the patient usually still involves careful assessment of lymph node-bearing areas for palpable nodes. Conventional wisdom advises excising the largest node [1,2] so that adequate material is available for the various studies needed for modern hematopathologic diagnosis [3]. However, common sense dictates that superficial nodes be biopsied when feasible. Preservation of architectural features of the involved node may provide a more definitive diagnosis than fine-needle aspiration.

Patient 1

A 20-year-old man with a history of infectious mononucleosis 15 months previously presented with several palpable right cervical and supraclavicular nodes which were matted, soft, and nontender. He had no B symptoms. In the aggregate, the involved areas measured 2–3 cm. Surgical excision of five irregular soft, tan fragments of lymph node tissue was performed. None of the fragments had a dimension > 1 cm. A diagnosis of Hodgkin lymphoma, mixed cellularity, was made (Fig. 1, Panel 1). Subsequent workup was negative, except for a single focus of disease in a left axillary node (0.5 cm on physical examination) and an enlarged right femoral node. The patient was treated with total nodal radiation therapy. Evaluation 4 years later revealed no recurrence of disease. The patient did not return for follow-up.

Patient 2

A 74-year-old man with a history of chronic atrial fibrillation (on warfarin) presented with weight loss, abdominal pain, distention, and emesis. Physical examination revealed a single right inguinal lymph node about 2 cm in diam-

eter. It was slightly mobile, firm, and nontender. CT scan of the abdomen and pelvis revealed mesenteric, retroperitoneal, and retrocrural lymphadenopathy. Because of his overall condition, it was elected to remove the inguinal lymph node for diagnostic testing. It measured $2.0 \times 1.5 \times 1.2$ cm. The pathologic diagnosis based on histology (Fig. 1, Panel 2A), positive BCL2 status (Fig. 1, Panel 2B), and flow cytometric analysis (not shown) was follicular lymphoma, Grade 3 of 3. Bone marrow was also involved. Subsequent treatment was combination chemotherapy (cyclophosphamide, hydroxydaunorubicin, and prednisone). Vincristine was omitted because of the patient's intestinal symptoms. The patient was lost to follow-up.

Patient 3

A 31-year-old man presented with fever, weight loss, hepatosplenomegaly, abnormal liver function tests, and severe pancytopenia. An extensive workup, including liver and bone marrow biopsies, was not definitive. The liver biopsy showed a mild predominantly lymphocytic infiltrate in the portal tract and within the sinusoids. A T cell lymphoproliferative process was suspected in the bone marrow, but there was insufficient material for confirmatory study by flow cytometry. A left inguinal lymph node (1.5 cm in greatest dimension) was then removed (Fig. 1, Panel 3). A diagnosis of peripheral T cell lymphoma NOS was made by histologic evaluation and immunohistochemical staining (Fig. 1, Panel 3B). There were no other lymph nodes identified on physical examination or body CT imaging. Despite a single cycle of CHOP chemotherapy (cyclophosphamide, hydroxydaunorubicin, vincristine, prednisone), multiple antibiotics, and antifungal therapy for *Aspergillus fumigatus* identified in respiratory secretions, the patient expired in the hospital within the week post lymph node biopsy.

Patient 4

A 58-year-old man who was asymptomatic presented with a small lymph node in the left supraclavicular region. Physical examination was otherwise unremarkable. A sister had lymphoma. Excisional biopsy of fibroadipose tissue (three pieces averaging $0.6 \times 0.5 \times 0.3$ cm) was performed. A diagnosis of follicular lymphoma, Grade 1–2 of 3 was made based on histology (Fig. 1, Panel 4A) and positive BCL2 staining (Fig. 1, Panel 4B). CT imaging and gallium scan were negative for other lymphadenopathy. Bone marrow was not involved by lymphoma. Radiation therapy was given to upper mantle and neck regions. Lymphoma recurred 2.5 years later in the left iliac region. The subsequent clinical course was characterized by progression of the lymphoma despite combination chemotherapy.

Patient 5

A 68-year-old man with a history of prostate cancer treated with radiation therapy underwent excisional biopsy of a left posterior cervical lymph node ($1.2 \times 0.8 \times 0.5$ cm) and a left supraclavicular lymph node ($1.5 \times 0.4 \times 0.5$ cm). Pathologic diagnosis (Fig. 1, Panel 5) of follicular lymphoma, Grade

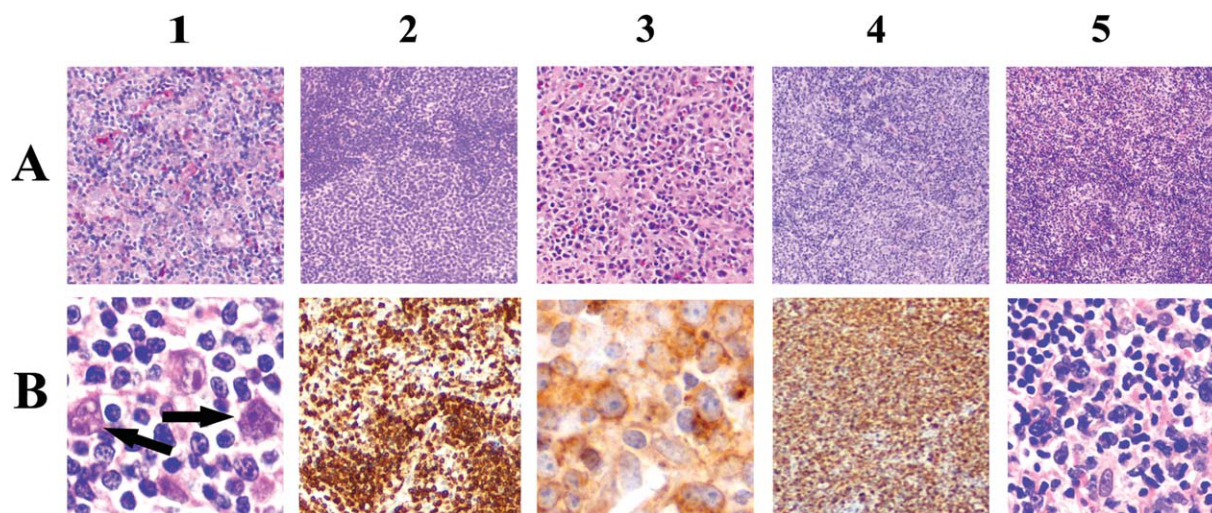


Figure 1. Formalin or B-5-fixed, paraffin-embedded sections of the lymph node biopsies from the five patients described. Panel A: Hematoxylin/eosin-stained sections show effacement of lymph node architecture in each case. Panel B: Patient 1—Hodgkin lymphoma, mixed cellularity: lymph node architecture is effaced by small lymphocytes, histiocytes, and Reed Sternberg cells (arrows). Patient 2—B cell lymphoma, follicular, Grade 3/3: enlarged, back-to-back follicles (Panel A) filled with a proliferation of BCL2-positive neoplastic cells (Panel B); flow immunophenotyping (not shown) indicated the neoplastic cells marked as clonal B cells (IgM, IgD, lambda). Patient 3—peripheral T cell lymphoma, NOS: large neoplastic cells mark with anti-human CD3 (Panel B), indicating their T cell origin. Patient 4—follicular lymphoma, Grade 1–2/3: low power (Panel A) reveals a vaguely nodular architecture; the atypical follicular center cells are strongly BCL2 positive (Panel B). Patient 5—B cell lymphoma, follicular, Grade 2/3: partial effacement of lymph node by back-to-back follicles (Panel A) containing large, atypical cells (Panel B), which by flow cytometry (not shown), demonstrated monoclonality for Kappa light chains. Magnifications: A1, A2, A3 $\times 70$; A4, A5 $\times 40$; B1, B3 $\times 420$; B2, B4 $\times 70$, B5 $\times 300$.

2 of 3 was made. Radiation therapy was administered. Multiple sclerotic bone metastases and rising prostate-specific antigen were noted 2.5 years later. These findings improved with hormonal therapy. Five years after the initial biopsy, extensive paraceliac adenopathy was documented by CT and gallium scans. Ultrasound-guided needle biopsy revealed malignant lymphoma, large cell type. There was subsequent disease progression despite systemic chemotherapy.

We have presented five patients in whom biopsy of small lymph nodes revealed the diagnosis and prepared the way for selection of appropriate treatment of their lymphoproliferative disorder. In Patient 1, the only readily approachable site of disease was in the right cervical/supraclavicular region. A cosmetically acceptable procedure was readily accomplished and diagnostic information was found in small pieces of lymphoid tissue. In Patient 2, there was considerable internal lymphadenopathy evident on body scanning, but the patient's general condition and anticoagulated status made it preferable to remove an inguinal node despite its small size rather than performing a more invasive biopsy procedure. In Patient 3, prior invasive procedures had not yielded a firm diagnosis in a desperately ill patient. A single palpable inguinal node was accessible and provided the diagnostic findings. In Patients 4 and 5, small neck nodes were the only detected foci of malignant lymphoma.

Of interest, in Patients 2 and 3, the diagnostic lymph node came from the inguinal area—a region often ignored for biopsy because of the frequency of benign inflammatory findings in adults [2,4]. In patients in whom a lymphoproliferative disorder is suspected, especially if there is evident retroperitoneal adenopathy, the diagnostic yield of inguinal node biopsy may be increased. Thorns et al. [5] found that the morphology of follicular lymphomas in inguinal lymph node biopsies did not differ from lymphoma biopsies taken from other sites.

Further, it has been shown that, at the time of pathologic examination, the size of a superficial lymph node removed surgically does not always correlate with diagnostic yield [4]. Small, nonpalpable lymph nodes in Hodgkin lymphoma have been found to contain focal involvement when examined microscopically [6]. Similarly, in Patient 4, only focal involvement of the lymph node was found. In other tumors, such as breast cancer and cutaneous melanoma, it has been found that foci of cancer may be detected microscopically in small, nonpalpable sentinel nodes [7]. Excisional biopsy of a lymph node, even if small, is preferable to fine-needle aspiration in cases such as ours, where study of overall tissue architecture is usually essential for diagnosis. Our cases illustrate that, even with limited amounts of tissue, an accurate diagnosis can be made by using modern methodologies, including flow cytometry. Further, modern molecular techniques, such as PCR, require very little sample.

Onciu et al. [8] compared morphology and immunophenotyping in 64 consecutive cases of non-Hodgkin lymphoma (NHL) in which aspiration or biopsy of two sites was performed within 30 days. Some of their cases included peripheral lymph node samples (often by fine needle aspiration) as compared with other anatomic sites, e.g., bone marrow. They found 11 cases (17%) with discordant morphologic features and 14 cases (22%) with discordant antigen expression. All 11 patients with discordant features between the two anatomic sites had B cell chronic lymphocytic leukemia (CLL) with transformation to large B cell lymphoma (Richter transformation). Of the 14 cases with discordant antigen expression (involving one or two antigens at different anatomic sites), 10 had concordant morphology, and only four had histologic discordance (B cell CLL involving one site and large B cell lymphoma involving a second site, i.e., Richter syndrome). Although these authors found that the immunophenotype of NHL cells in most patients is stable from site to site, it is well known that variations in histology may occur in different sites in patients with NHL, since this phenomenon occurs in 20 to 30% of patients who undergo multiple biopsies for staging [9]. The issue of variations in histology in different sites is not unique to small lymph node biopsies and needs to be kept in mind in evaluating any patient with lymphoma.

The patients we present illustrate the need, still, for meticulous physical examination to identify small lymph nodes and appreciate their features—e.g., texture and mobility—which may help in the decision to biopsy. It is also important to use modern imaging techniques to aid in the identification of superficial potentially diagnostic lymph nodes which may not have been detected at the bedside. In addition, radiographic features of lymph nodes, e.g., fatty centers, may be helpful in determining the likelihood of benignity versus the chances of obtaining a diagnostic biopsy.

Methods

Under an IRB-approved protocol, we reviewed the clinical findings, clinical course, and biopsy evaluations from five patients who underwent excisional biopsy of small (≤ 2 cm) superficial lymph nodes for diagnostic evaluation of possible lymphoma. The surgical pathology and flow cytometry reports were re-evaluated by a senior, experienced hematopathologist, including hematoxylin and eosin-stained frozen, formalin, and B5-fixed paraffin-embedded tissues, and immunohistochemistry. Diagnostic terminology is based on the most recent WHO classification [3].

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Safety of plasma-derived protein C for treating disseminated intravascular coagulation in adult patients with active cancer

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Cancer-related disseminated intravascular coagulation (DIC) is a life-threatening condition for which no effective treatment is currently available. Protein C (PC), a modulator of coagulation as well as the inflammatory system, has been successfully tested (in its activated recombinant form [a-rPC]) in sepsis-related coagulopathy, but with an increased risk for major bleeding. Plasma-derived PC (pd-PC) is more suitable than a-rPC in patients at high risk from bleeding due to its self-limiting process. We carried out a single-arm study evaluating the role of pd-PC in adult cancer patients with overt DIC. Over a period of 3 years, we treated 19 patients with overt DIC and a PC plasma concentration <50%; all received PC concentrate (Ceprotin[®], Baxter) for 72 hr in adjusted doses to restore normal PC values (70–120%). Coagulation, haematological tests, and the DIC score were recorded after 12, 24, 48 hr, 7 and 10 days, while clinical outcomes (bleeding, thrombosis and mortality) were recorded up to 28 days. Within 48 hr of starting pd-PC therapy, laboratory tests as well as the DIC score improved in all patients. At 28-days follow-up, no bleeding or thrombosis was observed. This is the first study to investigate the use of pd-PC for treatment of cancer-related overt DIC.

Disseminated intravascular coagulation (DIC) is a syndrome characterized by the systemic activation of blood coagulation, thrombosis of the small and medium size vessels, and multiorgan dysfunction leading to consumption of coagulant factors and platelets; bleeding is usually the overt clinical picture while thrombosis produces organ damage [1]. There are many causes of DIC, such as sepsis, cancer, or pregnancy [1,2]. Its management, still unsatisfactory due to the lack of an effective therapy, is mainly based on the treatment of the underlying condition along with the administration of fresh-frozen plasma (FFP) and replacement therapy (such as anti-thrombin [AT]) [3,4]. Recently, the correlation between sepsis and coagulation abnormalities has been intensively investigated [5–7]; in septic patients with DIC, substitution therapy with activated recombinant protein C (ar-PC) has been proven to be effective, but with an increase in bleeding [8,9]. The protein C pathway is a modulator of the coagulation system, and PC itself plays an integral role in the host response to infection, immune system, and cancer cells [1,2]. The extent of PC deficiency assessed at the time of diagnosis has been found to correlate with increased morbidity and mortality [6,7].

Less is known regarding the correlation between active cancer and coagulation consumption leading to DIC in patients without signs or symptom of sepsis [10–13]. As the presenting condition, DIC occurs in 2–5% of patients suffering from solid neoplasm [10]. Many factors can contribute to coagulopathy in tumors. Cancer cells express different procoagulant molecules as well as fibrinolytic proteins [10–13], and chemotherapy may enhance the risk of thrombosis due to its damaging effect on the endothelium [11]. Some clinical differences exist between the coagulopathy that occurs in sepsis in

comparison to the one occurring in cancer. In general, a less severe picture occurs in cancer patients where chronic activation of coagulation can proceed with few clinical events. This process usually leads to exhaustion of platelets and coagulation factors, thus making bleeding (e.g., at the site of the tumour) the first clinical symptom of DIC, while in other cases, cancer-related coagulopathy occurs in the form of thrombotic microangiopathies [10–13]. In cancer-related DIC, conventional treatment is related to high mortality also due to the difficulty in properly treating the underlying neoplasm [10]. In this situation, pd-PC seems a suitable treatment, due to its ability to modulate coagulopathy, inflammation and cancer activity, and its self-limiting process in activating anticoagulation [1,2]. However, there is little information in the literature regarding the appropriateness of such an approach within cancer populations.

For this purpose, we conducted a single arm study evaluating the role of pd-PC in cancer patients with overt DIC showing no signs or symptoms of sepsis.

From February 2006 to June 2009, 49 consecutive adult cancer patients with overt DIC were considered; 30 patients were excluded because of: normal PC levels at the baseline ($n = 14$), signs/symptoms of sepsis ($n = 8$), reluctance to give consent ($n = 3$), acute promyelocytic leukemia (APL) ($n = 2$), and initial treatment with substitutive AT ($n = 3$). Thus, 19 patients were enrolled. At study entry, all had active cancer and plasma values of PC activity lower than 50%. Patient characteristics are reported in Table I. Six patients received fresh frozen plasma and one (4.5%) received red blood concentrates; seven patients had clinical manifestations of muco-cutaneous and/or genitourinary bleeding. Twelve patients had solid cancer (six gastrointestinal, two pulmonary, four genitourinary) and six had a haematological cancer (three lymphoproliferative disorders, two myeloma, and one acute myeloid leukaemia). Most had an advanced/metastatic neoplasm (Table I), and six had recently undergone previous surgery (within <1 month).

Baseline plasma PC activity was $31.4\% \pm 8.1\%$ (range 19–48%). Our therapeutic approach increased plasma PC activity in all patients. Plasma PC activity normalized after 48 hr and remained stable over the following days. Average bolus dose and daily total amount are reported in Table I. At baseline, most patients showed abnormal PT, aPTT, platelets and D-dimer levels, and had high DIC scores. During the study period, there was a progressive and significant increase in platelets, fibrinogen, and AT, and a progressive and significant decrease in D-dimer, PT/INR, aPTT, and DIC scores (Table II). No adverse reactions or events were observed during PC replacement therapy. Baseline PC levels were lower in nonsurvivors than in survivors (23.4% vs. 31.6%, respectively), although the difference was not statistically significant ($P = 0.08$); there was no difference between PC

TABLE I. Patient Characteristics

Characteristics	All patients	Survivors ^a	Nonsurvivors
Number of patients, female/male	19	15 (78.9%)	4 (21.1%)
Female/male sex	11/8	8/7	2/2
Median age (years)	68 (43–82)	68 (43–82)	68 (52–78)
Weight (kg) (mean ± SD)	72 ± 21.4	77.3 ± 25.84	62.9 ± 5.72
Clinical presentation			
Solid tumors, n (%)	14 (73.6)	11	3
Advanced/metastatic, n/N (%)	11 (78.5)	8	3
Haematological neoplasm ^b , n (%)	6 (31.5)	4	2
Advanced/metastatic, n/N (%)	4 (66.6)	1	3
PC consumption			
PC dose (initial bolus) U.I.	4,310 ± 822	4,134 ± 915	4,325 ± 761
PC dose (daily total amount) U.I.	4,210 ± 1,050	4,252 ± 1,210	4,263 ± 862
Interval between diagnosis and PC administration (hr)	21.5 ± 8.8	21.8 ± 11.7	17.3 ± 5.32

^aAt 28 weeks follow-up.

^bExcept for acute promyelocytic leukaemia (APL).

TABLE II. Changes in Laboratory Findings During the Study Period (mean ± SD)

	Baseline	24 hr	48 hr	72 hr	Day 7	Day 14
PC (%)	27.3 ± 7.1	71 ± 15.6*	85.9 ± 12.5*	91.2 ± 11.6*	92.2 ± 13.4*	99.1 ± 13.5*
WBC ($\times 10^9/L$)	8.2 ± 3.1	7.8 ± 2.2	6.5 ± 1.9	6.7 ± 1.5	7.3 ± 1.5	8.1 ± 0.6
Platelets ($\times 10^9/L$)	49.3 ± 20.4	51.2 ± 19.4	71.2 ± 33.4	91.7 ± 41.1*	113.4 ± 65.1*	154.8 ± 109.2*
D-dimer ($\mu g L^{-1}$)	2.133 ± 1.643	2.366 ± 1.561	1.230 ± 1.045*	800.2 ± 686*	350 ± 225*	541 ± 246*
Fibrinogen ($g L^{-1}$)	2.1 ± 1.4	2.8 ± 1.1	3.6 ± 1.5	4.4 ± 1.4*	4.5 ± 1.2*	4.2 ± 1.3*
INR [20]	1.61 ± 0.12	1.48 ± 0.17	1.44 ± 0.15	1.32 ± 0.11	1.26 ± 0.11*	1.19 ± 0.13*
aPTT (s)	40.1 ± 13.4	40.8 ± 7.6	35.4 ± 6.1	33.4 ± 6.1	32.9 ± 7.5	31.2 ± 3.6
AT (%)	54.2 ± 12.2	61.6 ± 23.3	73.4 ± 21.4	77.7 ± 22.2*	80.6 ± 16.5*	87.1 ± 18.5*
DIC score	5.86 ± 1.12	5.38 ± 1.42	4.26 ± 0.96	3.16 ± 0.98	2.97 ± 0.87*	2.21 ± 1.43*

* $P < 0.05$ versus baseline.

levels in patients who died and those who survived. On average, the total amount of infused PC concentrate was similar in survivors and nonsurvivors.

No difference was found among all other laboratory tests among patients who died in comparison to survivors. In both groups, baseline AT levels were similar, mean D-dimer concentrations decreased significantly over the following days. At baseline, the mean DIC score was 6.26 ± 1.12 ; 14 patients had overt DIC, (DIC score = 5). Regarding the baseline DIC score, we found a significant difference ($P < 0.05$) between survivors (mean DIC score of 6.2) and patients who died (mean DIC score of 8.5). The DIC score decreased significantly after 72 hr in all patients. At the 7-day follow-up, only two patients had laboratory and clinical characteristics of an overt DIC status. No patients developed signs or symptoms of bleeding or thrombosis. Five patients (26.3%) died within 28 days of DIC diagnosis.

The use of PC has been shown to be effective for reducing mortality in adult patients with severe sepsis (in its activated form) and in children with concomitant PC deficiency (plasma-derived zymogen) [8,9,14–16]. Regarding pd-PC, this form acts only when it is converted to activated PC and requires the presence of the thrombin–thrombomodulin complexes. The physiological self-limitation of this process may reduce the risk of bleeding in respect to ar-PC and, to our knowledge, no adverse events on the use of pd-PC have been reported so far [8,9,14–16]. This evidence supports the use of pd-PC zymogen in acquired PC deficiency in septic patients [14] and in adults with purpura fulminans [16].

While nonovert chronic DIC is quite common in cancer patients (and usually does not require any specific therapeutic approach), there are no reliable data regarding the incidence and management of overt DIC. In fact, this complication, although rare, is extremely severe and difficult to treat properly [10,13,17]. Based on the notion that depression of the PC system may contribute significantly to the beginning of DIC in cancer patients, supplementation of PC may be of benefit [1,2,10,12]. However, the role of restoring defective physiological anticoagulant in patients with cancer has not been evaluated and extrapolation from other settings of patients is difficult; this is considering that patients with severe thrombocytopenia (as commonly occurs during cancer treatment) were excluded from the available trials for the use of ar-PC [9]. Therefore, the role of PC (either plasma-derived or activated) for treating cancer-related coagulopathy is unclear.

We are the first to evaluate PC concentrates for managing cancer patients with overt DIC. The observed results of our series are limited to the effectiveness of the dosage and the lack of side effects; our regimen of pd-PC therapy was effective in restoring physiological plasma PC activity, to normalize coagulation laboratory tests and to decrease the DIC score. No adverse events were recorded.

Our study has some limitations. First, we had to conduct a single arm study due to the rarity of the disease; in the absence of a control group, it is not possible to comment on the efficacy of treatment. To reinforce our findings, we compared the mortality rate in the present investigation with historic control values from our hospital. The latter comprised a population of 24 cancer patients with DIC, similar to the present series in respect to median age, tumor type and burden, and severity of coagulopathy, all of whom received substitutive AT and FFP for a median period of 72 hr. Mortality at the 28-days follow-up was around 70% in comparison to 26% reported in the present investigation. Although direct comparisons are not possible, this difference would appear to be clinically relevant. Second, cancer patients differ in terms of prognosis, cancer spread, organ involvement, and staging. We are aware that our population may not be fully comparable between survivors and nonsurvivors; however, only patients with advanced cancer (solid or haematological) were included in the analysis to reduce the risk of considering patients with different degrees of severity. We excluded patients suffering from APL since the associated-coagulopathy is well controlled by *trans-retinoic-acid* treatment [18].

In conclusion, we report for the first time the use of pd-PC concentrates for managing advanced cancer patients with overt DIC. Our results demonstrate the feasibility of pd-PC therapy for normalizing laboratory values and ameliorating the DIC score without bleeding or thrombosis during the study period.

Materials and Methods

Eligible patients were those with active cancers (solid or haematological except than APL) [18], overt DIC score and PC plasma levels below 50%. Patients were excluded in the case of laboratory or clinical findings suggesting multiorgan failure (MOF) and/or severe sepsis. In these cases, patients were admitted to the local ICU and managed accordingly. Treatment was performed at the University Hospital of Palermo, Italy. A diagnosis of DIC was established according to the Scientific Subcommittee on Disseminated

Intravascular Coagulation of the International Society of Haemostasis and Thrombosis [19]. An evolving DIC score was given to each patient: a DIC score = 5 was compatible with overt-DIC, while a score < 5 was suggestive (not affirmative) for nonovert DIC. Overt DIC was defined as the alteration of laboratory parameters according to current international guidelines resulting in a DIC score ≥ 5 with or without bleeding or thrombosis [18,19]. Enrolled patients were treated with pd-PC concentrate (*Ceprotin*[®], Baxter) with the aim of obtaining 100% of the plasma PC activity. The infusion was given for 72 hr at a starting dose of 50 UI kg⁻¹ daily⁻¹, adjusted to maintain a plasma PC activity between 70 and 120%. Laboratory measurements of PC activity were performed every 24 hr with a chromogenic method (amidolytic assay protein C assay, Chromogenix, Milan, Italy; range 70–140%). Fresh-frozen plasma, AT concentrates, and red packed cells or platelet transfusions were given when appropriate. White blood cells, platelets, D-dimer, fibrinogen, PT, aPTT, AT, and DIC score (20) were evaluated before PC infusion (baseline), after 24, 48, 72 hr, and on the 7th and 10th day of treatment. Mortality was evaluated within 28 days.

Parameters evaluated during the study period were analyzed by repeated measures ANOVA and post-hoc comparison (LSD test). A $P < 0.01$ was considered statistically significant. The study design was a single-arm study and approved by the Internal Review Board. Protein C concentrate (*Ceprotin*[®], Baxter) was administered according to the ethical principles of the Declaration of Helsinki for medical research involving human subjects.

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