Phase1/-2 study of pomalidomide in myelofibrosis

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We conducted a dose-escalation study to see if higher doses of Pomalidomide (previously shown to be safe and effective for myelofibrosis-associated anemia at 0.5 mg/day [with prednisone] or 2.0 mg/day) increased anemia responses. 3.0 mg/d given for 21 of 28 consecutive days was the maximum-tolerated dose (MTD), with myelosuppression being dose limiting. Nonresponders at the MTD had their dose decreased and the therapy interval increased to daily. Seven of 19 subjects had an anemia-response and two had a spleen response. Most responses occurred after dose-reduction to 0.5 mg/d, suggesting higher doses are associated with increasing myelosuppression without increasing (or possibly decreasing) efficacy.

Anemia and/or thrombocytopenia are common in persons with myelofibrosis (MF) including de novo myeloproliferative neoplasm primary myelofibrosis (PMF) and myelofibrosis after progression from a pre-existing MPN (essential thrombocythemia and/or polycythemia vera myelofibrosis). MF-associated anemia is frequently multifactorial: ineffective erythropoiesis and spleen sequestration of RBC are important. Anemia not only contributes to the substantial symptoms of fatigue associated with MF [1], but is also strongly correlated with shorter survival [2,3]. There is no FDA-approved therapy for anemia in MF, androgens [4], and corticosteroids are occasionally effective. Pharmacologic doses of erythropoietin improves anemia in some subjects with low erythropoietin levels [5] but may adversely affect likelihood of progression to acute leukemia [6].

Immune-modulatory drugs (IMiDs®) are active in MF. Conventional doses of thalidomide (50–100 mg/d) improves anemia and thrombocytopenia but is associated with sedation and neuropathy [7]. Higher doses of thalidomide increased toxicity but not efficacy. Combining low-dose thalidomide with a prednisone results in responses in >50% of subjects with anemia and thrombocytopenia but neuropathy is likely to develop in most patients over time with therapy [8]. Lenalidomide also improves anemia and thrombocytopenia, in persons with MF but is associated with bone marrow suppression (50% grade 3 or 4 neutropenia and/or thrombocytopenia) and gastrointestinal toxicity [9]. Studies of lenalidomide with corticosteroids show no added benefit [10,11].

Pomalidomide (POM) is an IMID significantly more potent than thalidomide, but without the limiting toxicities of neuropathy or sedation [12]. We previously performed a large randomized adaptive phase II trial of (0.5 mg or 2.0 mg/d with or without prednisone) in which (1) significant and durable responses for anemia were observed, (2) no other MF-features (splenomegaly, fibrosis) were improved even at the highest dose, (3) there was no definitive additive benefit of steroids to POM, and (4) there were no dose limiting toxicities (DLT) observed [13]. We performed a subsequent dose-escalation trial to determine whether higher doses of POM are effective and well tolerated.

Methods

The trial was a classic 3 × 3 design that included subjects with MF with hemoglobin less than 8.0 g/dL or requiring RBC-transfusions and/or symptomatic splenomegaly, neutrophils (≥ 1 × 10^9/L), platelets (≥ 50 × 10^9/L), and adequate renal and liver function. POM was started at dose of 2.5 mg/day for 21 of 28 day. Dose increased in cohorts by 0.5 mg/day were done if no subject had a dose-limiting toxicity (DLT) (≥ grade-4 hematological toxicity, ≥ grade-3 febrile neutropenia or ≥grade-3 nonhematological toxicity) in cycle-1. Subsequent cohorts were enrolled until the maximum tolerated dose (MTD) was determined (dose level before that resulting in DLT in >1 of 6 subjects). In the phase 2 portion, subjects were treated at the MTD. Nonresponders after three cycles received 0.5 mg/day for 28 day [13].

Nineteen subjects were enrolled between June 2008 and March 2009. Patient-related variables are indicated in Table I. Most of the subjects were intermediate-2 or high risk by the International Prognostic Scoring System for MF [2]. Median duration of disease at enrollment was 24 months (range, 1–173 months). Most were RBC-transfusion-dependent and had symptomatic splenomegaly.

Three subjects enrolled into the 2.5 mg/day, 3.0 mg/day, and 3.5 mg/day cohorts. The dose-limiting toxicity (DLT) was bone marrow suppression, which occurred in two of three subjects in the 3.5 mg/day cohort. These latter DLTs were the only DLTs seen on the trial. Three additional subjects received 3.0 mg/day confirming this as the MTD. Seven additional subjects were enrolled at the MTD of 3.0 mg/day. Because we saw no efficacy at 3.0 mg/day (21 of 28 days), the dose was decreased to 0.5 mg/day after three cycles in eight subjects. Seven subjects responded (Table II) using the International Working Group for Myelofibrosis Research and Treatment criteria for Clinical Improvement (IWG-MRT CI [14]). All responders had an IWG-MRT CI for anemia (six previously RBC-transfusion-dependent). Two of these anemia responders also had a decrease in splenomegaly. The eligibility criteria excluded the possibility of an IWG-MRT CI for thrombocytopenia. However, all five subjects with prestudy platelets of 50–100 × 10^9/L had an increase in their platelet number, with three of these achieving normal platelet levels. Responses occurred after a median of 4 months (range, 2–9 months) and only after reduction to 0.5 mg/day in five responders including the two subjects with decreased spleen size. These responses occurred after 1 and 2 cycles after dose reduction, respectively (1–3 months). Responses were durable; five responders continue on-study.

POM is well tolerated. Dose-related bone marrow suppression was the main toxicity with substantial reductions in granulocytes and platelets at doses ≥2.5 mg/day (Table II). Nonhematological toxicity was uncommon at any dose with grade 3 fatigue at 3.0 mg/day in one subject. Two subjects with baseline leukocytosis had a substantial increase in leukocytes (baseline

TABLE I. Baseline Clinical and Laboratory Features of 19 Patients with Myelofibrosis Enrolled in a Dose Escalation Study of Pomalidomide

	N = 19
Demographic Information	
Age (median years and range)	67 (43–83) years
Males (%)	8 (42%)
MF subtype	PMF = 14 (74%) Post ET MF = 2 (11%) Post PV MF = 3 (15%)
Disease duration at enrollment	Median 24 months (range, 1–173 months)
MF IPSS (IWG-MRT) [2]	Low = 2 (11%) Intermediate 1 = 2 (11%) Intermediate 2 = 8 (41%) High = 7 (37%)
Disease features at enrollment	
Baseline laboratory studies	
Hemoglobin (g/dL)	Median 9.0 (range −4.4–10.6)
Leukocytes (×10^9/L)	Median 5.4 (range −2.2–204.1)
Absolute neutrophil count (×10^9/L)	Median 4.42 (range −1.1–136.7)
Platelet (×10^9/L)	Median 170 (range −52–582)
Erythrocyte transfusion dependent	*N* = 11 (58%)
Palpable Splenomegaly	*N* = 16 (84%)
Median cm below left costal margin	12.5 cm (range 1–26cm)
*JAK2*V617F-positive (%)	*N* = 16 (84%)

PMF, Primary Myelofibrosis; Post ET MF, Post Essential Thrombocythemia Myelofibrosis; Post PV MF, Post Polycythemia Vera Myelofibrosis; MF IPSS, Myelofibrosis International Prognostic Scoring System.

TABLE II. Efficacy and Toxicity of Single Agent Pomalidomide in a Dose Seeking Study of 19 Myelofibrosis Patients

	POM reduced to 0.5 mg daily ($N = 8$)	POM at Doses ≥ 2.5 mg days 1–21 ($N = 11$)	Total patients ($N = 19$)
Responses observed			
Anemia responses (all eligible for response)			
IWG-MRT CI[14]	5 (63%)	2 (18%)[a]	7 (37%)
Spleen responses (16 eligible for response; 7 in 0.5 mg group)			
IWG-MRT CI[14]	2/7 (29%)	2/9 (22%)[a]	2 (11%)
Toxicity			
Hematological			
Neutropenia (G 3/4)	0	8 (73%)	8 (73%)
Platelets (G 3/4)	0	3 (27%)	3 (27%)
Non Hematological			
Fatigue (Gr 3)	0	1 (9%)	1 (5.3%)
Discontinuation			
Reasons for Stopping			
Lack of Response	0	5 (46%)	5 (26%)
Progression	2 (25%)	4 (36%)	6 (32%)
Toxicity	0	1 (9%)	1 (5%)

IWG-MRT CI (International Working Group for Myelofibrosis Research and Treatment – Clinical Improvement) [14]. Anemia CI (Erythrocyte transfusion independence or hemoglobin increase >2 g/dL for ≥2 months). Splenomegaly CI (≥50% reduction of palpable component for ≥2 months). G 3/4 (Grade 3 or 4 by CTC 3.0 criteria).

[a]Both at 3.0 mg daily.

of 31.4 and 204.1 $\times$ 10^9/L to a peak of 183 and 417 $\times$ 10^9/L, respectively). This latter correlation between IMiD therapy, MF, and a "myeloproliferative reaction" is previously reported [15]. A total of 117 cycles been given (median, 6/subject; range, 2–13). Seven patients remain on POM (all but two at 0.5 mg/day). Of the 12 subjects who withdrew (10 at ≥2.5 mg/day), the reasons for discontinuation were no response ($N = 3$), withdrawal of consent ($N = 2$), progression ($N = 6$), or unrelated worsening comorbidities ($N = 1$).

Low-dose POM is effective in persons with MF and anemia and, less commonly splenomegaly. Its use is neither accompanied by the neuropathy or sedation common with thalidomide nor the bone marrow suppression common with lenalidomide9. Also, low-dose POM reverses MF-associated thrombocytopenia in some subjects but more data are needed, especially in subjects with baseline platelets <50 $\times$ 10^9/L who were excluded from this study. High-dose POM not only failed to produce more response but was also associated with fewer responses than lower doses possibly because of bone marrow suppression. A prior study combined short-term prednisone with low-dose POM [13]. However, the prednisone seems unnecessary as the response rate in the 0.5 mg/day cohort in this study is similar to that of low-dose POM and prednisone in the prior study.

There is substantial need for a safe and effective therapy for MF-associated anemia and thrombocytopenia. Although many new therapies, like JAK2-inhibitors, are being tested in MF, these drugs do not correct anemia or thrombocytopenia; they may worsen these conditions [16]. Further study of low dose POM alone or combined with drugs with complimentary activities in MF seems warranted.

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Coexistence of primary AL amyloidosis and POEMS syndrome: Efficacy of melphalan-dexamethasone and role of biochemical markers in monitoring the diseases course

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Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes (POEMS) syndrome and primary (AL) amyloidosis are two rare plasma cell disorders with heterogeneous, deceptive clinical features and severe prognosis. With the exception of autologous bone marrow transplantation for selected patients, the best therapeutic approaches are far from being settled and organ-response rates do not exceed 50% in AL amyloidosis and are fairly better in POEMS syndrome associated with localized osteosclerotic myeloma. Moreover, clinical evidence of therapy efficacy is delayed so that biological markers play a crucial role in monitoring diseases course and assessing therapy efficacy. We report on a 57-year-old woman with the unique coexistence of these two plasma cell disorders in whom the close monitoring of serum vascular endothelial growth factor (VEGF) and free light chains (FLC) helped to evaluate the response to melphalan and dexamethasone therapy.

Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes (POEMS) syndrome is a plasma cell disease with multiorgan involvement and increased serum levels of vascular endothelial growth factor (VEGF). Primary amyloidosis (AL) is a plasma cell disorder, where amyloid fibrils are formed by the N-terminal portion of a monoclonal immunoglobulin light chain.

In both AL amyloidosis and POEMS syndrome, therapy is aimed at the eradication of the plasma cell clone, although in POEMS syndrome the best therapy has still to be defined [1,2]. Considering the slow clinical response to therapy and the lack of prognostic factors, clinicians need to rely on biochemical markers to assess therapy efficacy. We report on a patient with the unusual coexistence of the two plasma cell disorders in whom the close monitoring of serum VEGF [3] and serum free light chains (FLC) [4] helped to evaluate the response to therapy.

Since 2001, a 57-year-old woman complained of progressive difficulties of gait and painful distal paresthesias, secondary to a sensory-motor polyneuropathy. The electrodiagnostic findings were suggestive of chronic inflammatory demyelinating polyradiculoneuropathy (CIDP). Cerebrospinal fluid analysis showed increased protein levels with no cells. Sural nerve biopsy showed a severe axonal neuropathy. Congo-red staining and electron microscopy failed to reveal deposits of amyloid fibrils. Direct immunofluorescence of cryostatic sections did not show deposition of IgG, IgA, IgM, κ- or λ-chains, and C3d. Serum VEGF was increased (>3,000 pg/mL, normal range: <600 pg/mL). Associated systemic symptoms and signs [hyperprolactinemia (36.6 μg/L, normal range: 5–15 μg/L), hypothyroidism (TSH: 6.2 U/L, normal range: 0.2–4.0 U/L), hepatosplenomegaly, new onset hirsutism, erythrodermia, acrocyanosis] prompted a diagnosis of POEMS syndrome. At the diagnostic work up, thrombocytosis (749×10^9/L), erythrocytosis (Hb: 175 g/L; Ht: 53%), and neutrophilia (neutrophils: 9.5×10^9/L) were detected. Capillary electrophoresis revealed one monoclonal component; immunofixation electrophoresis (IFE) on agarose gel also showed a second monoclonal component. The first one, anodic, was IgG/λ-type (9 g/L) and the second one was light chain (λ) only. Lambda FLC concentration and κ/λ FLC ratio were 85.1 mg/L and 0.19 (range: 5.7–26.3 mg/L and 0.26–1.65, respectively). Albuminuria (1.2 g/24 hr), but no Bence-Jones protein were found by IFE. Alkaline phosphatase (ALP) was normal but progressively increased shortly after the diagnosis and peaked at 249 U/L (normal range: 53–141 U/L) 5 months later. Restrictive cardiomyopathy was found by echocardiography; an endomyocardial biopsy showed Congo red-positive deposits. NT-proBNP serum level were 4,200 ng/L (normal range: <900 ng/L). Bone marrow biopsy showed 10% infiltration (interstitial and nodular) of monoclonal (λ) plasma cells. Pelvis magnetic resonance (MR) showed a small round area (13 mm diameter) on the neck of left femur; that area displayed low FDG-avidity on PET-CT scan and might have been a small osteosclerotic myeloma. No other focal bony lesions were evident on X-ray, bone and PET scans, spine and pelvis MR. A diagnosis of amyloidosis (cardiac and hepatic and likely, renal) and POEMS syndrome was made.

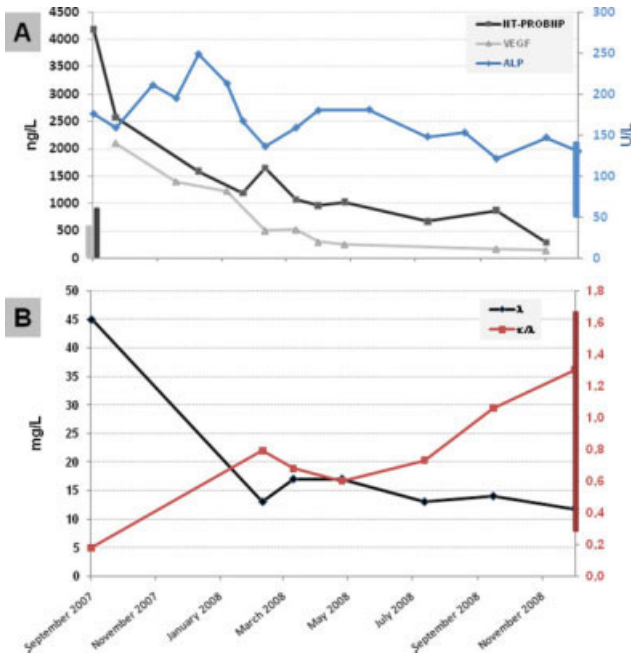

Figure 1. Serum levels of the biochemical markers (Panel A: NT-proBNP, VEGF, ALP; Panel B: lambda free light chain and kappa/lambda ratio) over the time. Thick vertical bars represent normal ranges. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Because of the involvement of the heart, stem cell transplantation was not considered. Chemotherapy with melphalan plus dexamethasone was started in September 2007, and 3 months later, serum λ FLC as well as VEGF and NT-proBNP started decreasing. ALP significantly decreased to 180 U/L and kept steadily dropping over the following months (Fig. 1A). Clinically, a significant improvement was evident only 1 year later, when the patient showed improvement of neurological symptoms and performance and regression of skin abnormalities along with normalization of ALP and reduction of albuminuria. Eventually, the λ FLC levels and κ/λ FLC ratio (Fig. 1B) as well as serum VEGF and NT-proBNP levels returned to normal limits. The monoclonal λ light chain was no more evident by serum IFE.

AL amyloidosis and POEMS syndrome may share many clinical features, i.e., neuropathy, hepatopathy, splenomegaly, hypothyroidism, and proteinuria. In this patient, the clinical and electrodiagnostic characteristics of polyneuropathy were suggestive of CIDP, more consistent with POEMS syndrome than with amyloid neuropathy. Very high serum levels of VEGF confirmed the diagnosis of POEMS syndrome. In fact, serum VEGF levels are increased in multiple myeloma patients to a much lesser extent than in POEMS syndrome [5,6] and are not increased at

all in monoclonal gammopathies of undetermined significance [6,7], immune-mediated neuropathies [8], and in AL (primary) amyloidosis (data not shown).

This is the second reported case of simultaneous coexistence of primary AL amyloidosis and POEMS syndrome. In the case described by Patier et al. [9], however, no biochemical markers were investigated and response to therapy was not reported.

The pathogenesis of POEMS syndrome remains unclear, short of the major role played by the VEGF whose serum levels are abnormally high, useful in the differential diagnosis [10] and necessary to monitor the disease course. VEGF is secreted by the plasma cell clone and deemed to account for most features of the disease [7,11]. In primary AL amyloidosis, the plasma cell clone produces the monoclonal immunoglobulin that aggregates as extra cellular insoluble amyloid fibrils, leading to organ damage and dysfunction. Thus, plasma cell clones, even small, can produce two distinct and severe diseases [12].

The organ response rates to different therapeutic regimens other than stem cell transplantation are variable in POEMS syndrome [2] and do not exceed 50% in amyloidosis [13]. Considering that alternative therapeutic approaches are nowadays available (e.g., bortezomib, lenalidomide, and thalidomide), it is crucial to assess as soon as possible the response to therapy to shift, if necessary, to different treatments and avoid unnecessary toxicity and disease progression. In our patient, both serum VEGF and FLC levels reliably mirrored the response to therapy, and thus remarking the crucial role of a timely serological monitoring for a proper treatment and follow-up.

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Deferasirox treatment may be associated with reversible renal Fanconi syndrome

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Two cases of reversible renal Fanconi syndrome associated with deferasirox treatment are reported. One of these recurred upon rechallenge. Deferasirox (Exjade®) is a new once daily oral iron chelator for the treatment of chronic iron overload due to blood transfusions. Deferasirox became available in the USA in 2005 and is licensed in over 70 countries worldwide [1]. Preclinical studies in animals indicated that the kidney was a potential target organ of toxicity [2]. However, in phase II and III clinical trials, deferasirox was found to be generally well tolerated [3–5]. Cases of fatal, acute, irreversible renal failure have been described in a post marketing report on deferasirox [6], but details of the incidence rate or cause of these adverse events have not yet been reported, raising concerns regarding the toxicity of the drug. Renal Fanconi syndrome is a rare disorder in which the proximal tubular function of the kidney is impaired, resulting in decreased reabsorption of electrolytes and nutrients into the bloodstream. The solutes involved include glucose, amino acids, uric acid, phosphate, and bicarbonate. Both patients described below, having transfusion related iron overload, developed renal tubular dysfunction.

The first case an 18-year-old male patient with pure red cell aplasia (Diamond-Blackfan anemia) has been dependent on blood transfusions every 3–4 weeks since infancy. He had been treated with subcutaneous deferoxamine for iron overload with low compliance. Deferiprone was stopped due to neutropenia. His serum ferritin level remained around 1,500 to 2,000 ng/mL.

At the age of 15 years, deferasirox was started at a dose of 20 mg/kg/day. Serum creatinine levels increased from 0.6 to 1.07 mg/dL. No other side effects were noted initially. Compliance with the treatment was good and ferritin levels declined to 1,200 ng/dL. No additional drugs were taken during this time. Six months after starting treatment, serum phosphorus concentration declined from 3.5 mg/dL to 1.1 mg/dL. Mild hypokalemia and hypouricemia were also noted. At this time, urinalysis revealed glycosuria, uricosuria, phosphaturia, kaliuria, and generalized aminoaciduria. Tubular reabsorption of phosphate (TRP) was 58% (normal $>$ 85%). There was no evidence of metabolic acidosis. Laboratory tests are summarized in Table I. In light of acceptable ferritin levels, treatment with deferasirox was stopped with improvement in all electrolyte disturbances and resolution of phosphaturia and other abnormal urinary losses. However, 6 months after discontinuing treatment, ferritin levels increased to 1,728 ng/mL. After discussing with the patient and his parents the possible link between the renal abnormalities and deferasirox, they opted to restart deferasirox treatment, having considered the alternatives of subcutaneous deferoxamine or no treatment. Deferasirox was restarted at a low dose of 5 mg/kg/day with a gradual increase to 20 mg/kg over 19 months. Mild hypophosphatemia reappeared with phosphaturia within a few weeks, while on 10 mg/kg/day of deferasirox. This was well controlled with oral phosphorus supplements. Mild hypouricemia and low-molecular-weight proteinuria (increased urinary β_2 microglobulin) were also noted, although glycosuria and other abnormalities did not recur.

TABLE I. Laboratory Results of Both Cases, Before Onset of Fanconi Syndrome, During and After Stopping Treatment with Deferasirox

		Pre-deferasirox	Fanconi syndrome	After drug cessation	Rechallenge	Normal values
S Phosphorus (mg/dL)	Case 1	3.5	*1.1*	4.0	*1.9*	2.5–4.5
	Case 2	4.8	*1.2*	4.4		
S Potassium (mEq/L)	Case 1	3.7	*3.2*	3.9	*3.2*	3.6–5.0
	Case 2	4.2	*3.3*	4.3		
S Uric acid (mg/dL)	Case 1	2.8	*1.5*	3.0	*2.2*	3.5–7.2
	Case 2	4.0	*1.4*	3.6		
S HCO3 (mEq/L)	Case 1		23.0	26.7	22.2	22–26
	Case 2		23.9	25.4		
S Creatinine (mg/dL)	Case 1	0.6	1.07	0.8	0.92	0.5–1.0
	Case 2	0.45	0.56	0.46		0.3–0.7
S Ferritin (ng/mL)	Case 1	*2,070*	*1,200*	*1,728*	*1,445*	24–336
	Case 2	*1,146*	313	*701*		
TRP (%)	Case 1		*59.2*	87	*66.5*	>85
	Case 2	92.0	*63.7*	96.1		
U Glucose (mg/dL)	Case 1	Negative	*250*	Negative	Negative	Negative
	Case 2	Negative	*250*	Negative		
U Total prot/ creatinine	Case 1		0.22	0.05	0.1	<0.20
	Case 2	0.23	*0.54*	0.23		
U Uric acid excretion[a]	Case 1		*0.62*	0.33	0.52	<0.57
	Case 2	0.51	*1.01*	0.42		
U β2 Microglobulin (mg/L)	Case 1			<0.21	*1.56*	<0.21
	Case 2		*2.4*	<0.21		
U Amino acids	Case 1		*Positive*	Negative		Negative
	Case 2		*Positive*	Negative		

Case 1 results after treatment was restarted are also shown. Abnormal results are italicised.

S, Serum; U, Urine; TRP, Tubular reabsorption of phosphate.

[a]U Uric acid excretion = U uric acid/U creatinine × S creatinine.

Throughout treatment with deferasirox, serum creatinine remained normal at 0.8 to 1 mg/dL (Table I). He continued treatment with increasing doses of deferasirox with no further deterioration of renal tubular function.

The second case an 11-year-old female patient was diagnosed with β-thalassemia major complicated by iron overload due to chronic blood transfusions. At the age of 4 years, she underwent unrelated bone marrow transplantation which was acutely rejected, and at the age of 9 years, she underwent splenectomy. She was treated with deferoxamine for iron overload. Because of poor compliance, blood ferritin levels rose to levels of around 2,500 ng/mL.

Treatment with deferasirox was started at the age of 8 years, at a dose of 20 mg/kg/day with very good response. No additional drugs were taken at this point. Treatment was stopped after 17 months when ferritin levels decreased to 500–600 ng/mL but was restarted after 5 months due to a rise in ferritin levels to 1,146 ng/mL at 30 mg/kg/day. Six months later, hypophosphatemia of 1.2 mg/dL was found on routine blood tests. Serum creatinine and other electrolytes were normal; however, phosphaturia (TRP—63.7%), glycosuria, mild low-molecular-weight proteinuria, and generalized aminoaciduria were noted on urine tests (Table I). In view of these findings, and normal serum ferritin concentration at that time (313 ng/mL), treatment with deferasirox was again stopped and short term supplemental phosphorus was given. There was rapid resolution of hypophosphatemia and within 2 weeks urinary phosphate excretion normalized with resolution of glycosuria.

In light of the two cases described, we performed systematic screening for renal tubular abnormalities in nine additional children and young adults attending the pediatric hematology clinic, with transfusion-dependent anemia and iron overload, defined as serum ferritin above 1,000 ng/mL before chelation therapy. The group included patients not treated with chelation therapy ($n = 2$), and those treated with deferiprone ($n = 2$) or deferasirox ($n = 5$). Serum electrolytes and creatinine, urinalysis, and urine biochemistry analysis were obtained at each clinic visit. None of these patients exhibited any signs of tubular dysfunction, such as hypophosphatemia, phosphaturia, hypokalemia, hypouricemia, glycosuria, or low-molecular-weight proteinuria.

We describe two cases of renal Fanconi syndrome following treatment with deferasirox. In both cases, serum phosphorus concentrations were normal before initiation of treatment and normalized rapidly after discontinuation of treatment. Rechallenge with deferasirox in one patient resulted in recurrence of the renal tubular disorder, though in a milder form. Because of the mild and reversible nature of the findings, renal biopsy was not performed. However, the similarity of both cases, with prompt resolution of findings upon discontinuation of therapy, and recurrence with treatment reinstatement in one patient, strongly suggest a causative effect of deferasirox.

The first pathological description of deferasirox-related acute kidney injury in humans was recently described in a 62-year-old patient with myelodysplastic syndrome, who developed a progressive decline in renal function after starting deferasirox [7]. A kidney biopsy showed acute interstitial nephritis with increased eosinophils, suggesting a drug hypersensitivity reaction as the cause of renal injury.

An additional report of a 78-year-old diabetic patient with sideroblastic anemia presenting with Fanconi syndrome and acute renal failure on deferasirox treatment was described [8]. However, because of the advanced age of that patient and his additional problems predisposing for kidney disease, the association between deferasirox treatment and Fanconi syndrome in that case was less obvious.

Fanconi syndrome may be hereditary or may be caused by exposure to heavy metals or other chemical agents, kidney transplantation, multiple myeloma, or amyloidosis. The most common cause of Fanconi syndrome in children is cystinosis. Fanconi syndrome is characterized by a generalized defect in proximal tubular function, leading to impaired tubular reabsorption and resulting aminoaciduria, glycosuria with normal serum glucose, low-molecular-weight proteinuria, and wasting of bicarbonate, potassium, uric acid, and phosphate. This may result in hypophosphatemia, hypokalemia, hypouricemia, and acidosis. Chronic urinary solute losses may cause bone disease and growth impairment in children.

Drugs that are known to be associated with Fanconi syndrome include outdated tetracyclines, aminoglycosides, chemotherapeutic agents (cisplatin and ifosfamide), valproic acid, and anti retroviral agents [9]. The mechanism of proximal tubular damage is not completely understood in most of them, although it seems that a variety of mechanisms are involved.

Interestingly, it has been shown that proximal tubular dysfunction exists in children with untreated β-thalassemia major, even in the absence of clinical findings, possibly as a result of oxidative stress due to tissue deposits of iron [10]. It has even been suggested that in patients with β-thalassemia minor, proximal renal tubular dysfunction is a common finding [11]. This does not seem to be the cause in our cases as the Fanconi syndrome was of sudden onset and was reversible upon drug cessation and reappeared upon drug rechallenge. Our screened group did not show any signs of renal tubular dysfunction, and moreover, one of the two reported cases did not have thalassemia.

In the phase III trial, in which 296 patients with β-thalassemia major received deferasirox for 1 year, the most common adverse events included rash, gastrointestinal disturbances, and mild nonprogressive increases in serum creatinine [5]. The mechanism of the changes in creatinine levels is not yet understood [1], but it has been suggested that excessive rapid iron removal may modify renal hemodynamics [12].

In our cases, the iron deposits were depleted by chelation therapy with deferasirox, also suggesting an involvement of an over chelating mechanism. The average ferritin blood level of the patients treated with deferasirox in our screening group was 3,102 ng/mL in the last year, much higher than the ferritin levels of the two cases. This theory is supported by preclinical studies conducted with deferasirox in rats and marmosets, where high doses of deferasirox were found to be associated with vacuolization of the renal proximal tubular epithelium, particularly in animals that did not have iron overload. The tubular damage seen in these animals was attributed to decreased iron content in the renal tubular cells following the pharmacological effect of deferasirox [2].

In conclusion, we report two cases of proximal tubular dysfunction compatible with Fanconi syndrome in association with deferasirox treatment, resolution after discontinuation of the drug and reappearance upon rechallenge. Routine monitoring of serum electrolytes and phosphorus as well as urinalysis and urine electrolytes could detect renal tubular dysfunction before the onset of symptoms or complications. The apparently reversible and nonprogressive nature of this condition suggests that the benefits of deferasirox therapy may still outweigh potential risks. We suggest that gradual rechallenge

of deferasirox with careful monitoring could be considered in those patients with Fanconi syndrome on deferasirox treatment.

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von Willebrand disease type 2N: Uncovering a congenital bleeding disorder in a patient with hepatitis C, cirrhosis, and coagulopathy

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von Willebrand disease type 2N (VWD 2N) is a rare autosomal recessive bleeding disorder caused by mutations in von Willebrand factor that impair its ability to bind to factor VIII (FVIII). Patients with VWD 2N present with abnormal bleeding, low factor VIII levels, and often a prolonged activated partial thromboplastin time. Although both are associated with low factor VIII levels, distinguishing VWD 2N from hemophilia A is clinically important because the prophylactic and therapeutic approach to bleeding is distinctly different. We present an instructive case of a woman with abnormal bleeding in the setting of progressive liver disease who had been previously diagnosed with "hemophilia." Her clinical history and laboratory testing were neither consistent with bleeding solely due to progressive liver dysfunction nor consistent with hemophilia. We outline the diagnostic evaluation of VWD 2N in the context of this case presentation and discuss the rationale for the use of purified VWF/FVIII concentrates for surgical prophylaxis and treatment of bleeding in VWD 2N patients.

A 52-year-old female with chronic hepatitis C virus (HCV) infection and cirrhosis was evaluated for coagulopathy. She and her father carried a diagnosis of "hemophilia." Two siblings and four children had no bleeding history. She described abnormal bleeding after tonsillectomy and vaginal deliveries, once requiring blood transfusion postpartum. She now reported increasing frequency of easy bruising and gum bleeding in the setting of decreasing platelet counts, presumably due to progressive cirrhosis. Physical examination revealed mild splenomegaly but was otherwise unrevealing. She had moderate pancytopenia (Table I). Bone marrow biopsy revealed multilineage dysplasia without an increase in blasts. The activated partial thromboplastin time (PTT) was elevated out of proportion to a mild elevation in prothrombin time. von Willebrand factor (VWF) antigen and ristocetin cofactor activity were in the normal range. A PTT mixing study was consistent with factor deficiency. Levels of factors II, V, VII, IX, X, XI, and XII were mildly reduced (25–65% normal), consistent with impaired hepatic synthesis. Her factor VIII level was severely reduced at 3% of normal.

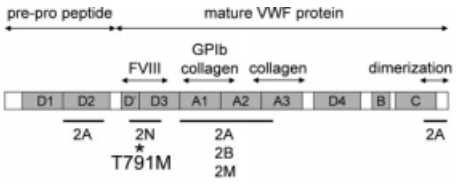
In liver disease, most coagulation factor levels are reduced because of hepatocyte dysfunction, whereas endothelial-derived factor VIII (FVIII) levels are usually preserved or increased [1]. Possible explanations for her very low FVIII were that she was a hemophilia A carrier with extreme X chromosome lyonization, or that she had von Willebrand disease type 2N (VWD 2N). VWD 2N is characterized by mutations in the FVIII binding site on VWF causing reduced binding of FVIII to VWF, and therefore, rapid clearance of FVIII from the circulation [2]. Low FVIII can also be caused by a variety of preanalytical variables, including clotting in the specimen, precipitation of FVIII during specimen transportation as whole blood, or multiple freeze–thaws of the plasma specimen. Based on our clinical suspicions, special diagnostic tests were performed. A binding assay between our patient's VWF and recombinant FVIII was less than the limit of detection (<10% binding, reference ≥70%), indicating VWD 2N. DNA sequence analysis revealed homozygous C->T substitution at VWF base 2372, causing a threonine to methionine mutation at amino acid 791. This mutation lies in the FVIII binding domain of VWF, and when present in a homozygous state predicts decreased FVIII binding to VWF and therefore a low FVIII level, confirming the diagnosis of VWD 2N. More than 20 VWD 2N mutations have been described, three of

TABLE I. Laboratory Values

Test	Result	Reference	Units
WBC	1.3 (L)	4.5–11.0	K/mm^3
Hgb	9.3 (L)	12–16	g/dl
Hct	28.6 (L)	36–46	%
Platelet count	33 (L)	150–400	K/mm^3
PT	17.1 (H)	10.3–13.2	sec
INR	1.6	NA	
PTT	59.3 (H)	21–33	sec
Fibrinogen	135 (L)	150–400	mg/dl
VWF:Ag	171	70–190	%
VWF:RCo	103	70–190	%
FII	25 (L)	60–140	%
FV	48 (L)	60–140	%
FVII	29 (L)	60–140	%
FVIII	3 (L)	50–200	%
FIX	33 (L)	60–140	%
FX	65	60–140	%
FXI	32 (L)	60–140	%
FXII	52 (L)	60–140	%

WBC, white blood cell count; Hgb, hemoglobin; Hct, hematocrit; PT, prothrombin time; INR, international normalized ratio; PTT, activated partial thromboplastin time; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; FII-FXII, factor II–factor XII level.

A



B

VWD subtype	VWF:Ag	VWF:RCo	Ratio RCo:Ag	FVIII:C	VWF multimers	RIPA
1	low	low	normal	low-normal	normal	normal or low
2A	low-normal	low	low	low-normal	loss of HMW	low
2B	low-normal	low	low	low-normal	loss of HMW	high
2M	low-normal	low	low	low-normal	normal or shift toward HMW	low
2N	normal	normal	normal	low	normal	normal
3	very low	very low	N/A	very low	absent	absent

Figure 1. (A) Schematic of the VWF protein, some of its important functional domains, and the locations of mutations corresponding to VWD types 2A, 2B, 2M, and 2N. FVIII, GPIb (glycoprotein Ib), and collagen label the interaction domains between VWF and the named proteins. *T791M shows the location of the homozygous VWF mutation found in our patient. (B) Clinical laboratory characteristics of the VWD subtypes. VWF:Ag, von Willebrand factor antigen level; VWF:RCo, ristocetin cofactor activity; FVIII:C, factor VIII procoagulant activity; RIPA, ristocetin-induced platelet aggregation; HMW, high molecular weight; N/A, not applicable.

which cause the majority of cases (R854Q, R816W, and T791M) [3]. A VWD mutation database is maintained at the University of Sheffield, accessible at http://www.vwf.group.shef.ac.uk/index.html.

VWD is a congenital bleeding disorder associated with qualitative or quantitative defects in VWF (see Fig. 1) [4]. In VWD type 1, patients have partial quantitative deficiency of VWF. VWD type 2 includes qualitative defects in VWF that alter its interaction with platelets (2A, 2B, and 2M) or with FVIII (2N). Type 3 is a recessive, near complete deficiency of VWF. Recognizing VWD 2N can be challenging, because the clinical and laboratory phenotype mimics hemophilia A, but making this distinction is necessary for guiding therapy as discussed later [5]. Family history may be helpful in making a diagnosis of VWD 2N. Hemophilia A is inherited as an X-linked recessive trait; males are affected, females are carriers, and there is no father-to-son transmission. VWD 2N is inherited as an autosomal recessive trait, and therefore both males and females can be affected. The phenotype of VWD 2N can also be present in patients who are compound heterozygous for VWD type 1 on one allele and VWD 2N on the other allele.

DDAVP (desmopressin) increases levels of VWF and FVIII and is used to treat bleeding in many patients with VWD [6]. However, DDAVP is usually not effective in VWD 2N patients even though it initially raises VWF and FVIII levels, because the patient's endogenous mutated VWF cannot protect FVIII from rapid clearance. Similarly, if incorrectly diagnosed with hemophilia A, VWD 2N patients will not adequately respond to exogenous FVIII alone because it will have a short half-life in the circulation. Therefore, for our patient, we recommended treatment with a purified VWF/FVIII concentrate (e.g., Humate-P or Alphanate) before surgery or with trauma [7]. This patient presented a diagnostic challenge: she had a progressive bleeding disorder in the setting of thrombocytopenia, cirrhosis, and HCV infection, but had clinical and laboratory findings that were not simply explained by coagulopathy of liver disease. Thus, in patients with abnormal bleeding (including those with progressive liver disease) who have a disproportionately prolonged PTT and a markedly low FVIII, we suggest that one must consider the diagnosis of VWD 2N, which requires distinctly different prophylactic and therapeutic approaches to control bleeding.

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Resolution of cerebral artery stenosis in a child with sickle cell anemia treated with hydroxyurea

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Stroke is a major cause of morbidity and mortality in individuals with sickle cell anemia (SCA) [1]. Transcranial Doppler (TCD) ultrasound can identify patients at increased risk of stroke [2,3], and chronic transfusion therapy decreases stroke risk by 92% in the highest risk group, defined by "abnormal" [time average mean of the maximum velocity (TAMV) $\geq$ 200 cm/sec] TCD flow velocities [4]. However, chronic transfusions can lead to serious complications, and the optimal management strategy for patients with cerebral artery stenosis whose TCD flow velocities are "conditional" (TAMV = 170–199 cm/sec) is unknown. Hydroxyurea effectively prevents several complications of SCA [5], but there are no controlled studies demonstrating its effectiveness in cerebral artery stenosis or primary stroke prevention in SCA. We report the case of a 4-year-old child with SCA found to have left middle cerebral artery stenosis on evaluation for unilateral "conditional" TCD flow velocities. Hydroxyurea therapy was followed by normalization of TCD flow velocities and resolution of vascular stenosis. This case adds to the literature suggesting that hydroxyurea can successfully reverse cerebral artery stenosis in patients with SCA, further supporting the need for controlled clinical trials of hydroxyurea for cerebral vasculopathy and primary stroke prevention in SCA.

TABLE I. Results of Hematologic and Radiologic Tests

	Age (years)								
	2.9	3.8	4.0[a]	4.3	5.6	6.0	6.3	7.5	8.0
White blood cell count (K per ml)	22.1	20.7		9.8	6.7	8.1	5.5	8.8	8.8
Hemoglobin (g/dl)	6.8	7.7		7.6	8.1	8.1	8.2	8.8	9.3
Hematocrit (%)	20.0	21.9		21.0	23.2	23.2	23.6	24.1	27.4
Platelet count (K per ml)	420	416		289	168	146	236	291	684
Mean corpuscular volume (fl)	98	97		119	113	120	126	119	120
Absolute neutrophil count (K per ml)	12.8	6.4		2.8	2.2	2.7	2.3	1.5	2.9
Reticulocyte count (%)	15.1	14.3		9.7	5.5	8.3	8.0	8.6	5.8
Lactate dehydrogenase (units/l)		658		635		631		335	437
Bilirubin, Total (mg/dl)		4.3		3.6		1.6		1.2	2.2
Bilirubin, Direct (mg/dl)		0.2		0.2		0.3		0.3	0.3
Hemoglobin F (%)[b]				28.4	34.0	36.8		23.1	16.4
TAMV at left MCA (cm/sec)	158	181[c]		171	177	179	145	132	140
MRA of left MCA			Stenotic			Stenotic			Normal

TAMV, time average mean of the maximum velocity; MRA, magnetic resonance angiography.
[a]Hydroxyurea therapy initiated.
[b]Hemoglobin F not measured between infancy and initiation of hydroxyurea.
[c]TAMV at left MCA repeated 3 weeks later and confirmed to be elevated at 186 cm/sec.

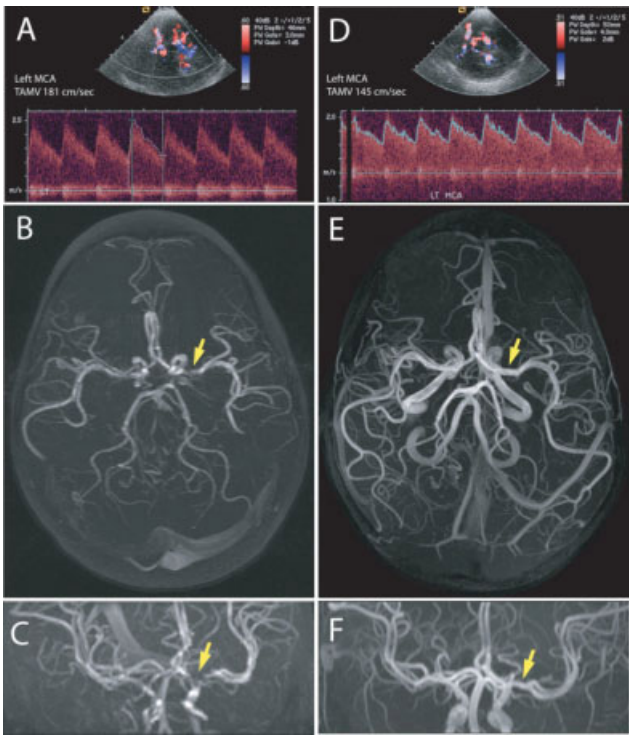

Figure 1. Imaging transcranial Doppler ultrasound (TCD) at age 3.8 years (A) shows normal arterial waveforms but elevated TAMV measuring 181 cm/sec. 3D time-of-flight (TOF) maximum intensity projections (MIPs) in the axial (B) and coronal (C) planes of an MR angiogram at age 4 years demonstrate focal severe stenosis in the distal left M1 vessel (arrows). Following treatment with hydroxyurea, follow-up TCD at age 6.9 years demonstrated TAMV in the normal range at 145 cm/sec (D). Repeat 3D TOF MIPs in the axial (E) and coronal (F) planes of an MR angiogram performed at 8 years of age showed resolution of short segment stenosis of the left middle cerebral artery (arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

A female of Afro-Caribbean descent was diagnosed with homozygous SCA (hemoglobin SS) by in utero genetic testing, which was confirmed by postnatal hemoglobin electrophoresis. Her initial course was complicated by dactylitis at 9 months of age, followed by individual episodes of acute chest syndrome, splenic sequestration, reticulocytopenic anemia, and a febrile urinary tract infection in the first 3 years of life. She additionally had frequent emergency department visits and occasional hospitalizations for pain. The patient's baseline laboratory tests and imaging results are shown in Table I.

At 3.8 years of age, routine imaging TCD identified new elevation in flow velocities in the left middle cerebral artery, with a TAMV of 181 cm/sec, which was confirmed on repeat imaging TCD 3 weeks later (Table I, Fig. 1A). Magnetic resonance imaging and magnetic resonance angiography (MRI/MRA) were then performed, and focal MRA abnormalities consistent with stenosis of the M1 segment of the left middle cerebral artery were identified (Fig. 1B,C). Hydroxyurea therapy was initiated at the age of 4 years, and a maximal tolerated dose of 25 mg/kg was reached. Hydroxyurea therapy was followed by an increase in fetal hemoglobin levels to a peak of 36.8%, which was associated with reductions in reticulocyte count, lactate dehydrogenase, and bilirubin levels, suggesting a reduced rate of hemolysis (Table I). Follow-up imaging TCD studies demonstrated eventual normalization of the TAMV in the left middle cerebral artery (Fig. 1D, Table I). Follow-up MRI/MRA demonstrated stable stenosis of the left middle cerebral artery 2 years after the initiation of hydroxyurea, followed by normalization of the vascular abnormality on repeat MRI/MRA after 4 years of hydroxyurea (Fig. 1E,F, Table I). Hydroxyurea therapy was complicated by the development of hypersplenism at the age of 5.5 years, which was successfully treated by laparoscopic splenectomy. The initial maximal tolerated dose of hydroxyurea was 25 mg/kg, but dose-limiting neutropenia required a gradual reduction in the hydroxyurea dose to 17 mg/kg by 8 years of age. There were no other adverse effects attributable to hydroxyurea.

Direct vasoocclusion by sickled erythrocytes plays a central role in the pathogenesis of SCA, but increasing evidence suggests that a subset of complications, including stroke and pulmonary hypertension, may be primarily due to vasculopathy induced by chronic intravascular hemolysis (reviewed in [6,7]). Intravascular hemolysis releases free plasma hemoglobin, which generates oxygen free radicals and scavenges nitric oxide, a critical regulator of vasodilator tone. Nitric oxide depletion and free radical-mediated endothelial damage promote intimal and smooth muscle proliferation and dysregulation of vasomotor tone, which are thought to play central roles in the pathogenesis of stroke, pulmonary hypertension, and priapism. Hydroxyurea is thought to be effective primarily because it promotes a shift from sickle to fetal hemoglobin synthesis, thus inhibiting sickle hemoglobin polymerization and hemolysis, although the metabolism of hydroxyurea also generates nitric oxide [5]. The effects of hydroxyurea therapy on fetal hemoglobin synthesis and hemolysis rate are expected within weeks of initiation of therapy, but the normalization of our patient's TCD and MRI/MRA abnormalities required more than 2 years of hydroxyurea therapy. This suggests that remodeling of an abnormal arterial wall was required prior to the resolution of arterial stenosis and the normalization of blood flow through this vessel.

The detection of elevated blood flow velocities through the large intracranial vessels by TCD ultrasound reliably identifies individuals with SCA who are at increased risk of stroke. The risk of stroke was found to be 40% when TCD demonstrates a TAMV of $\geq$200 cm/sec ("abnormal"), whereas a TAMV of 170–199 cm/sec ("conditional") was associated with a 7% risk of stroke, over

a mean follow-up period of 64 months [3]. TAMV data in these trials were obtained using nonimaging TCD ultrasound, but our TCD measurements were performed using the more widely available imaging TCD ultrasound, which can identify the major intracranial arteries more effectively than TCD [8]. Although one report identified no significant differences between imaging versus nonimaging TAMV measurements at the distal internal carotid, middle cerebral, and anterior cerebral arteries [9], a number of others suggest that TAMV measurements by imaging TCD are higher than those obtained via the nonimaging TCD technique used in the original trials [8,10–12]. Based on these reports, it has been suggested that the TAMV thresholds for "conditional" and "abnormal" TCDs be lowered by 10% when these are obtained via imaging TCD technique [13]. Although our patient's imaging TAMV measurements were borderline elevated using these adjusted thresholds, this patient's family elected to proceed with hydroxyurea therapy after considering the potential risks versus benefits of chronic transfusions and hydroxyurea.

Monthly transfusions reduce the risk of primary stroke in individuals with "abnormal" TAMV by 92% [4]. However, chronic transfusions are associated with serious complications, including alloimmunization and iron overload [14], and evidence suggests that the benefit of transfusions wanes rapidly once these are discontinued [15]. Furthermore, more recent evidence suggests that chronic transfusion therapy may have limited efficacy in patients with cerebrovascular lesions identified on MRI [16,17]. A current alternative to chronic transfusions is allogeneic bone marrow transplantation (BMT). However, BMT is a high-risk procedure for which not all individuals with SCA are candidates. Whether hydroxyurea is an effective alternative to chronic transfusions or BMT for primary stroke prevention in patients with SCA is currently unknown.

Recent studies have shown that the initiation of hydroxyurea reduces TCD flow velocities in patients with sickle cell disease [18–21], although most patients in these studies had normal pretreatment TCD flow velocities. Furthermore, multiple reports suggest that hydroxyurea may reduce the incidence of secondary stroke [18,21–24], although prudence has been urged in the interpretation of these results until the appropriate randomized trials are performed [25]. The efficacy of hydroxyurea in secondary stroke prevention for individuals with SCA and iron overload is currently being evaluated in a randomized fashion as part of the SWITCH clinical trial (http://clinicaltrials.gov/ct2/show/NCT00122980).

Despite the evidence that hydroxyurea can improve TCD flow velocities, MRI findings and TCD flow velocities are not always concordant [26,27], and determining whether hydroxyurea can also reverse vascular stenosis and reduce the risk of stroke in these patients remains an important question. Partial improvement in vascular stenosis has previously been reported in one child with sickle cell disease and moyamoya treated with hydroxyurea to maximal tolerated dose [28], whereas a second report describes the improvement of vascular stenosis in three patients with sickle cell disease treated with hydroxyurea [21]. The case reported here adds to the limited literature that hydroxyurea can successfully reverse cerebral artery stenosis and improve elevated TCD velocities in SCA, further supporting the need for a multicenter controlled trial of hydroxyurea for the treatment of cerebral vasculopathy and primary stroke prevention in SCA.

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Identification of immunophenotypic signatures by clustering analysis in pediatric patients with philadelphia chromosome-positive acute lymphoblastic leukemia

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Detection of Philadelphia chromosome t(9;22) (Ph) in children with precursor-B-ALL (pB-ALL) is an adverse prognostic factor, thus leading to a high-risk protocol for treatment. RT-PCR is the gold-standard for the detection of this abnormality. Specific gene and protein expression signatures have recently been identified for genetic subclasses in childhood and adult ALL using gene profiling and flow cytometric analyses, respectively. Our aim is the characterization of Ph+ pB-ALL for a fast and cheap screening approach in routine immunophenotyping applied at diagnosis. Forty-one children with Ph+ and 99 Ph- newly diagnosed pB-ALL (AIEOP cohort) were analyzed. The expression level of 16 marker proteins was monitored by five color flow cytometry (FC) and quantified in terms of Geometric Mean Fluorescence (GMF). Computational analyses were applied to the patient cohort: we identified a Cluster A, including the majority of Ph+ patients (35/41), associated with upregulation of CD52, TdT, CD45, CD34, HLA-DR, CD33, and downregulation of CD38, CD24, CD58, CD22, CD19; a Cluster B+C gathers most of the Ph− patients (86/99) showing the opposite tendency for listed markers. The immunophenotypic method identifies Ph+ cases with a comprehensive accuracy of 86% providing a rapid and effectual screening method for the identification of Ph+ pB-ALL.

The Philadelphia (Ph) chromosome is a shortened chromosome 22 resulting from the reciprocal translocation of the long arm of the chromosome 9 with the long arm of chromosome 22, t(9;22)(q34;q11). At molecular level, this translocation causes parts of the *BCR* gene from chromosome 22 to be placed in juxtaposition with downstream tyrosine kinase domains of the *ABL* gene from chromosome 9, coding for a chimeric oncoprotein with tyrosine kinase activity. The translocation is present in a heterogeneous group of leukemias: it is detectable in more than 95% of chronic myelogenous leukemia (CML) and 15–20% adult B-lineage acute lymphoblastic leukemia (B-ALL). In children, the Ph chromosome has been reported to occur in 1–5% ALL and in some acute myeloid leukemia (AML) cases [1].

The t(9;22) can be detected by fluorescence in situ hybridization (FISH) and reverse transcription-polymerase chain reaction (RT-PCR) [2,3,4] that are time consuming and require specialized facilities.

Philadelphia chromosome-positive (Ph+) ALL generally carries a dismal prognosis; treatment results can be enhanced by introducing specific inhibitors of the *BCR/ABL* tyrosine kinase such as STI-571 (imatinib mesylate) [5,6]. Therefore, newly diagnosed ALL patients need to be screened for the presence of the t(9;22) translocation for prognostic stratification and treatment decision making.

Recent studies identified specific gene signatures for genetic subclasses performing microarray analyses on childhood and adult ALL cases [7,8,9]. Advances in identification of leukemia subtypes have been recently achieved using FC technique: several leukemia classes could be distinguished on the basis of peculiar antigen expression profiles [10].

The aim of this study is the evaluation of FC in routine immunophenotyping applied at diagnosis as simple and objective approach with adequate accuracy to characterize the phenotype of pediatric Ph+ pB-ALL.

The marker protein expression levels in leukemic cells, from patients diagnosed with pB-ALL bearing the *BCR/ABL* translocation, have been compared with pB-ALL samples not involving this genetic aberration.

Based on the availability of material, the complete antibody panel was not performed on some patients. For analysis purpose, we restricted the number of monitored antigens according to the presence of the data for each patient; only one fluorochrome has been used for each MoAb to avoid possible bias related to utilization of different MoAb combinations or different fluorochromes for the same antibody [11]. The following markers were used for cluster analysis: CD10-PE, CD38-PECy5, CD24-Pecy5, CD22-PECy5, CD45-ECD, HLA-DR-PECy5, CD19-PECy7, CD33-PECy5, CD56-PE, TdT-FITC, CD52-FITC, CD58-FITC, CD34-PE, CD66c-FITC, CD11a-PE, CD44-PE.

Unsupervised cluster analysis was performed on the entire cohort of 140 patients. Two-dimensional cluster analysis (see Fig. 1) for GMF values separated patients into two main groups (A and B+C): a smaller and homogeneous branch (A) is separated from a larger and heterogeneous branch (B+C), which further divides into two sub-branches (B and C). Cluster A included the majority of pB-ALL Ph+ (35/41 as true positive samples (Ph+), 85.37%) and 13/99 (13.13%) pB-ALL Ph-. Cluster B+C gathered 86/99 (true negative cases (Ph-) 86.87%) pB-ALL Ph− and 6/41 (14.63%) pB-ALL Ph+ (Cluster B: 4 Ph+ and 35 Ph−; Cluster C: 2 Ph+ and 51 Ph−).

The accuracy measure, a comprehensive value reflecting the proportion of correctly predicted Ph+ and Ph− patients, indicates that our method identified a total of 121/140 (86.43%) cases that showed concordant results with molecular analysis (Table I).

Supervised analysis was performed on GMF data to find differentially expressed antigens with statistical relevance between Ph+ and Ph− clusters. Six and five antigens, respectively, were found to be statistically relevant (q value = 0) in discriminating Ph+ and Ph− subgroups using SAM algorithm as class comparison approach. The over-expression of CD52, TdT, CD45, CD34, HLA-DR, and CD33 antigens characterizes Ph+ group, whereas the same markers are down-regulated in Ph− group. A higher expression of CD38, CD24, CD58, CD22, and CD19 antigens depicts the Ph− profile, whereas Ph+ group shows low expression values for the same markers (see Fig. 2).

We examined possible differences in presenting features and response to treatment (day +8) between outliers (6 Ph+ enclosed in the cluster B+C, and 13 Ph− enclosed in the cluster A) and the vast majority of patients in each cluster.

Both CD10+ and CD10− cases have been included in the analysis: patients with CD10− phenotype are interspersed throughout the clusters; patients belonging to Ph+ cluster mainly express CD10. DNA index data, available for 130/140 patients, showed no apparent relation between ploidy and phenotype of blast cells (data not shown). In conclusion, no significant associations were recorded in this cohort between outlier patients and biological (expression of CD10, breakpoint on chromosome 22, DNA index) or clinical (response to steroid therapy) features. Moreover, an adequate long-term follow-up would be required to explain different outcomes for outlier patients.

The malignant clone carries specific oncogene rearrangements in many cases of pB-ALL defining both biological and clinical subentities [12].

The presence of Philadelphia chromosome has been associated with poor response to chemotherapy. Patients carrying the t(9;22) translocation are assigned to a higher risk group and receive intensified chemotherapeutic regimens that can include specific tyrosine kinase inhibitors. These findings emphasize the importance to early identify patients carrying this genetic rearrangement.

The detection of genetic rearrangements in ALL has been improved by the introduction of PCR, especially, for cases negative for cytogenetics [13,14]. Recently, a flow cytometric immunobead assay has been proposed for the detection of *BCR/ABL* fusion proteins [15].

Phenotypic aberrations have been shown to be associated with specific cytogenetic abnormalities such as t(1;19) [16–18], t(12;21) [18–20] and 11q23 [17,21,22].

Most of cytogenetic, molecular, and immunologic subgroups in acute leukemia has been successfully characterized by gene expression profiles [7,9,23]. Kern et al. [10] showed that protein expression (by FC) is highly correlated to mRNA abundance (by microarray) in AML and ALL. More recently, Coustan-Smith et al. identified a distinct, previously unrecognised biological subtype of childhood leukemia, ETP-ALL (Early T-cell precursors), with a characteristic

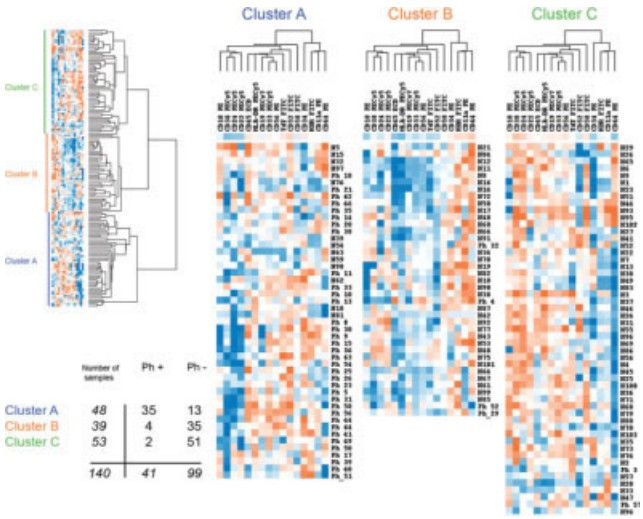

Figure 1. Unsupervised Hierarchical Clustering for 140 pB-ALL patients using 16 surface antigens. Each column represents an antigen; each row is a patient. Antigen expression levels (GMF) are log-transformed, median centered, and color depicted (orange: values higher than the median level; blue: values lower than the median, white: values comparable to the median).

gene expression profile and distinct cell-surface features, associated with high risk of remission induction failure or relapse. The early recognition of ETP-ALL by gene expression and immunophenotypic criteria is essential for the development of an effective clinical management strategy [24].

Gene expression studies already described differentially expressed genes identifying *BCR/ABL*1 fusion transcript [25]. It would be expected that *BCR/ABL* translocation could be associated with unique pattern of surface antigen expression among pB-ALL.

There is poor information about surface antigen expression in pB-ALL Ph+, especially in children, due to the low incidence in this age group. Previous studies showed that adult pB-ALLs carrying *BCR/ABL* gene rearrangements display unique phenotypic features [26,27].

Our results partially confirm previously reported findings in a smaller cohort of adult pB-ALL Ph+ patients, characterized by bright CD10 and CD34, low CD38 and reactivity for myeloid antigens [27]. We improved the immunophenotypic profiling on pediatric pB-ALL Ph+ samples using a larger group of antigens, some previously unexplored, and five color multiparametric FC.

Yeoh et al. [7] showed a poor gene expression signature defining Ph+ ALL samples with respect to other genetically distinct subclasses, which could correlate with the clinical variability in Ph+ ALL. Chiaretti et al. [25] characterized *BCR/ABL* adult samples using gene expression profiling with an overall classification error rate of 15%, which reflects the accuracy measure (86.43%) obtained by immunophenotyping in this study. More recently, a new classifier identified a high-risk subtype *BCR/ABL* positive-like with a gene-expression pattern and prognosis similar to Ph+ALL [28].

Gene expression analysis confirmed the high expression of *ABL* gene and other protein kinases in *BCR/ABL*+ ALL cells. Treatment results drastically changed by the introduction of a specific inhibitor of the *BCR/ABL* tyrosine kinase (imatinib). New *BCR/ABL* kinase inhibitors with higher efficacy in vitro are presently being evaluated in phase I/II clinical trials. Since few years, targeted immunotherapies have become a new domain of investigation for the treatment of hematological tumors. Our analysis pointed out phenotypic markers representing potential cellular targets for ALL Ph+ patients, such as CD33 and CD52. Several clinical trials have already been carried out in acute leukemia with Gemtuzumab Ozogamicin (GO), a humanized anti-CD33 antibody conjugated with calichemicin-g1 and Alemtuzumab (CAMPATH-1), a humanized monoclonal antibody against the CD52 antigen [29]. GO has been recently used in combination with chemotherapy as a salvage regimen in an adult patient with CD33+ ALL Ph+ [30]. CD52, an excellent target for complement-mediated lysis and antibody-dependent cellular cytotoxicity, has been found to be strongly expressed in Ph+ ALL in this study. This observation may be of clinical importance indicating a potential role for humanized monoclonal antibodies in the treatment of selected Ph+ ALL patients.

TABLE I. The Accuracy Measure for the Immunophenotypic Clustering Method

	Ph+ cluster	%
True positive	35/41	85.37
True negative	86/99	86.87
False positive	13/41	–
False negative	6/99	–
Concordant	121/140	86.43
Discordant	19/140	13.57
Sensitivity	0.85	85.37
Specificity	0.87	86.87
Positive predictive value	0.73	72.92
Negative predictive value	0.93	93.48
Positive diagnostic likelihood Ratios	6.50	–
Negative diagnostic likelihood Ratios	0.17	–
Accuracy	0.86	86.43

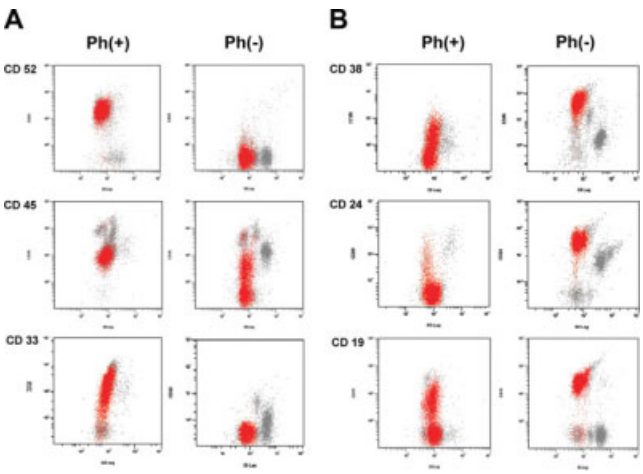

Figure 2. Most representative antigens for Ph+ and Ph− cluster groups analyzed by flow cytometry.

We propose a minimal MoAbs panel to be routinely applied in ALL diagnosis, including the markers identified in this study. Our everyday immunophenotypic analysis clearly identifies a cell-marker profile for a valuable and fast screening of Ph+ pB-ALL cases, and moreover provide information for possible intensification therapy with MoAbs directed to CD52 or to CD33 antigens in poor responders Ph+ pB-ALL.

Methods

Patients. A retrospective analysis has been performed on 140 patients affected by untreated pB-ALL, including 41 Ph+ patients (diagnosed between March 2000 and January 2007), and a control group of 99 Ph− patients (consecutively diagnosed between January and April 2006). For immunophenotypic and molecular analyses, bone marrow samples were centralized to the AIEOP (Associazione Italiana di EmatoOncologia Pediatrica) Reference Laboratory, Padua University.

Among the 41 Ph+ patients, 13 were females and 28 were males with median age at diagnosis 9 years (range 1–16 years). The control group included 46 females and 53 males, with median age at diagnosis 5 years (range 1–16 years). All patients had an unequivocal diagnosis of *de novo* pB-ALL based on morphological, cytochemical, and immunophenotypic criteria [11]. The definitive diagnosis of pB-ALL Ph+ was based on molecular analysis.

All patients have been treated according to the ongoing AIEOP ALL protocols. The study and treatment protocol were approved by the institutional ethical committees, and carried out after an informed consent.

Immunophenotypic studies. Immunophenotypic studies have been performed at diagnosis on erythrocyte-lysed whole bone marrow (BM) samples by FC, using a direct immunofluorescence technique with five color combinations of monoclonal antibodies (MoAbs).

Bone marrow samples were processed within 24 hr after collection, and analyses were performed as previously described [11].

Briefly, in each analysis, 0.5×10^6 cells in a final volume of 100 μl were incubated for 20 min at dark and at room temperature with the appropriate combinations of MoAbs directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem PE-Texas red (ECD), tandem PE-cyanine 5 (PECy5), and tandem PE-cyanine 7 (PECy7). Samples were subsequently lysed using 3 ml of NH4Cl, then washed in phosphate-buffered saline (PBS) and resuspended in 0.5 ml of PBS. Intracellular staining (TdT and CyIg) was performed by two step fixation and permeabilization procedure using a commercial kit (Valter Occhiena–Caltag Laboratories–Fix&PermTM, San Francisco, CA) according to manufacturer's instructions. A prior washing step was performed for light chains assessment.

The five color protocol is routinely employed for diagnosis of B-lineage acute leukemias at the AIEOP Reference Laboratory for Immunophenotypic Studies, Padua University.

The MoAbs used are the following: CD4-FITC, CD34-FITC, CD34-PE, CD2-FITC, CD8-PE, Igλ-light chain-PE, CD38-PE-Cy5, CD25-PE-Cy5, CD34-PE-Cy5 (Becton Dickinson Biosciences, San Jose, CA); CD66c-FITC, CD11a-PE (Becton Dickinson Biosciences Pharmingen, San Jose, CA); CD45-ECD, CD3-ECD, CD3-PE-Cy7, CD34-ECD, CD14-ECD, CD16-FITC, Igk-light chain-FITC, 7.1-PE, CD10-FITC, CD10-PE, CD33-PE-Cy5, CD24-PE-Cy5, CD20-PE-Cy5, CD19-PE, CD19-PE-Cy7, CD20-PE-Cy7, CD56-PE, CD135-PE, CD58-FITC, Tdt-FITC, HLA-DR-PE-Cy5, CD22-PE-Cy5 (Beckman Coulter-Immunotech, Miami, FL); CD7-FITC, CD7-PE-Cy5, CD15-PE, CD65-FITC, CD52-FITC, CD44-PE, (Valter Occhiena–Caltag Laboratories, San Francisco, CA); IgG/Smig-FITC, cyIg/IgM-PE (Dako); CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell acquisition was performed using a flow cytometer FC500, equipped with a 488 nm argon laser, and the software EXPO 32 (both from Beckmann Coulter, Miami, FL). The immunophenotypic diagnoses were performed collecting 15,000 events for each sample.

Instrument setup was routinely optimized by analyzing Calibrite™ beads (BD) and normal peripheral blood T lymphocytes stained with the anti-CD4-FITC/CD8-PE/CD45-ECD/CD7-Pe-Cy5/CD3-Pe-Cy7 five-color combination, as previously reported [11].

Erythrocyte lysis efficiency was monitored by an extra staining combination of SYTO16 (Molecular Probes, Leiden, The Netherlands), a live-cell-permeant nucleated-cell dye together with 7AAD (Beckman Coulter-Immunotech, Miami, FL).

Leukemic cells were identified using an immunological gate based on CD19 expression associated with a physical parameter (Side Scatter or SSC) and CD45 whose expression is low or negative in a large percentage of pB-ALL [11,19]. Once the blast population was selected, the intensity of each antigen expression was analyzed in association with SSC using a logarithmic scale. Fluorescence signals were quantified using GMF values for further comparison of antigen expression.

Reverse-transcription polymerase chain reaction (RT-PCR). Mononuclear cells (MNC) were isolated from diagnostic BM specimens using Ficoll-Hypaque (Pharmacia-LKB, Uppsala, Sweden) density gradient centrifugation, and cryopreserved in 4 mol/l guanidium isothiocyanate. RNA was extracted from BM-MNC at diagnosis by phenol-chloroform standard procedure. cDNA was synthesized from 1 μg total RNA in 20 μl total volume by using random hexamers. RT-PCR for ETV6-RUNX1, BCR-ABL1, and MLL-AFF1(AF4) and TCF3-PBX1 fusion genes was performed by single-round RT-PCR, as previously described, with a sensitivity of around 10^{-3} [3]. The expression of the housekeeping *ABL* gene was assessed to determine the quality of cDNA and the RT efficacy. PCR reaction products were analyzed by electrophoresis (2% agarose gels stained with ethidium bromide).

Statistical analysis. Antigen expression values were calculated for the 16 markers using GMF measures. Hierarchical clustering was performed on the entire cohort of 140 patients to group samples and antigens according to the similarity of their antigen expression profiles. Data have been log transformed and median centered for unsupervised analysis; Spearman rank correlation and centroid linkage were used to compute cluster distances.

We calculated the accuracy of the clustering approach in discriminating Ph+ samples according to unsupervised analysis results. Specificity, sensitivity, and predictive values have been retrieved for the Ph+ cluster group.

Supervised analysis has been applied to the previously identified groups using class comparison statistics. SAM algorithm has been performed on antigen expression values to identify significant differentially expressed markers between Ph+ and Ph− groups.

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The value of monitoring minimal residual disease in the patients with donor lymphocyte infusion as intervention of relapsed/refractory acute lymphoblastic leukemia after allogeneic hematopoietic stem cell transplantation

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Treatment of acute lymphoblastic leukemia (ALL) has achieved significant progress, however the long-term disease free survival (DFS) of adult ALL patients still does not exceed 20%38% [1–3]. Allogeneic HSCT (allo-HSCT) has been proved to be the most effective treatment for a variety of hematologic malignancies, but the benefits were limited [4]. Although donor lymphocyte infusion (DLI) could restore complete remission (CR) in many patients after transplantation, patients received prophylactic DLI with the risk of fatal graft versus host disease (GVHD). Monitoring minimal residual disease (MRD) allowed for the early detection of trance amount of tumor cells in ALL patients[5·6]. Whereas, the value of monitoring MRD in relapsed/refractory ALL patients with DLI as intervention after allo-HSCT remains to be evaluated. In this investigation, we reported monitoring MRD in 47 cases of relapsed/refractory ALL after allo-HSCT for 43 months in median and observed that among them 19 patients with a high risk for disease relapse after allo-HSCT were treated with DLI under the guidance of monitoring MRD. Notably, 6 among the 19 cases had no leukemic progression after DLI, suggesting that MRD monitoring has a value in the treatment of relapsed/refractory ALL patients using DLI as intervention after allo-HSCT.

This was a single center retrospective study that included 47 cases of relapsed/refractory ALL (24 males and 23 females) treated between 2001 and 2007. The median age of the patients was 27 years old (range 8–56 years), except for one patient was 8 years old in CR_4, and the rest of the patients were from 17 to 56 years. The study was performed according to institutional guidelines. Patients were included if they fulfilled at least one of the following criteria defining refractory ALL: (1) primary induction failure after two or more cycles of chemotherapy (16 cases), (2) early relapse (<6 months) after a CR when they still received treatments (14 cases), (3) refractory to salvage combination chemotherapy containing high-dose Ara-C or high-dose methotrexate (6 cases), (4) beyond first CR (CR_2 in 8 cases, CR_3 in 2 cases and CR_4 in 1 case). Among these 47 cases, 19 were transplanted from HLA identical sibling donor (6/6 or 10/10 matched); 18 from HLA-identical (6/6 or 10/10) or mismatched (5/6 or 9/10) unrelated donor; 10 from haplo-identical donor of mother.

From November 2001 to February 2007, a conditioning regimen called "modified TBI+CY" for allo-HSCT has been introduced, with informed consent of the individuals involved. After February 2007, patients received a "modified BU+CY" conditioning regimen. MRD was monitored with flow cytometry using appropriate markers every month till one year post transplant, and MRD monitoring was continued every three months. MRD$\leq 9 \times 10^{-4}$ was considered as a low risk for disease relapse, and MRD $\geq 9 \times 10^{-3,}$ or increasing MRD was considered as a high risk for disease relapse. If MRD was between these two levels it would be redetected in a month. Once monitoring MRD shows a patient with a high risk for disease relapse, the patient was treated with DLI. The initial dose for DLI was 5×10^6 CD3+ cells/kg. Four to six weeks after that, DLI was repeated with an increased dose as 1×10^7 CD3+ cells /kg when the patients were free from GVHD. If it was necessary, an escalating dose of 5×10^7 CD3+ cells /kg or 1×10^8 CD3+ cells /kg would be used in every 1 month. For those patients with immunosuppression, the treatment should be stopped. DLI would be recommended after 4 weeks off immunosuppression in the absence of GVHD. For those patients did not receive immunosuppression therapy, DLI would be used immediately.

After transplant, the median follow up was 43 months (range, 10–77 months). Among the 47 cases of relapsed/refractory ALL, 24 patients were alive and 23 of them were free of leukemia. The estimated cumulative incidence of death of leukemia at 5 years after transplantation was 33.67%. The Kaplan-Meier estimate of DFS at 5 years after transplantation was 46.55%. By monitoring MRD with flow cytometry, 19 patients were eligible (whose MRD were $\geq 9 \times 10^{-3}$) to receive DLI after allo-HSCT. There was one exception that the patient who developed hematologic relapse with MRD level $\leq 9 \times 10^{-3}$. Median time from transplantation to the first DLI was 125 days (range, 37–570 days). Among these cases, one patient received 1 transfusion, 5 received 2, 11 received 3, and 2 received 4 transfusions in escalating doses, containing 5×10^6, 1×10^7, 5×10^7 and 1×10^8 CD3+ cells/kg at DLI 1, 2, 3, and 4, respectively. Some patients stopped receiving DLI, the reason for discontinuing DLI was development of GVHD or hematologic relapse.

The results showed that the amounts of MRD were decreased in 14 patients after DLI, and notably, 6 patients (31.58%) had no disease progression and were alive in continuous CR after DLI, whereas leukemic relapse following DLI was observed in 13 of these 19 patients (Table I), and twelve of them died of leukemia progression. Another patient achieved a stable CR after re-induction with imatinib and was in hematologic remission with persisting MRD. GVHD was the main complication of DLI, including 7 cases of acute GVHD and 5 cases of chronic GVHD. One patient with acute GVHD grade III after DLI finally had leukemia progression.

DLI has shown to improve leukemia patient survival through exerting the graft versus leukemia effect [7,8]. In this study, 6 out of 19 ALL patients were survival whereas other 13 patients were relapse, eventually. It is still an open question whether all patients with ALL should receive prophylactic DLI in the absence of GVHD post allo-HSCT. Although some doctors doubt the beneficial outcome of DLI, some scholars suggested that DLI should be recommended for those ALL patients who had poor-risk cytogenetic abnormalities [9]. The treatment of relapsed/refractory ALL is still a big challenging, as a median survival time is 4 to 6 months only [1–3]. In the light of these poor results with conventional therapy, we introduced intensive MRD monitoring and used the personal data to guide DLI in individual eligible patient after allo-HSCT, and achieved an improved ALL therapy with an obvious decrease in leukemic death rate in 6/19 (31.58%) relapsed/refractory ALL patients after DLI therapy. By the way, if these 6 ALL patients did not receive MRD monitoring-directed DLI, they would have high likely

TABLE I. Results of MRD Monitoring in Patients Received DLI

Patient	MRD results before DLI	MRD results after DLI	Leukemia progression	Suvival
1	9.2×10^{-2}	2.7×10^{-2}	Yes	No
2	7.3×10^{-1}	4.5×10^{-2}	Yes	No
3	3.4×10^{-2}	7.7×10^{-2}	Yes	No
4	5.1×10^{-2}	3.0×10^{-3}	No	Yes
5	5.0×10^{-2}	6.4×10^{-2}	Yes	No
6[a]	7.8×10^{-2}	3.9×10^{-4}	Yes	Yes[a]
7	1.6×10^{-2}	5.5×10^{-3}	No	Yes
8	9.7×10^{-3}	1.9×10^{-2}	Yes	No
9	2.2×10^{-2}	3.3×10^{-3}	Yes	No
10	1.9×10^{-2}	4.6×10^{-4}	No	Yes
11	4.4×10^{-2}	2.0×10^{-4}	No	Yes
12[b]	4.8×10^{-3}	2.9×10^{-3}	Yes	No[b]
13	8.7×10^{-2}	8.6×10^{-3}	Yes	No
14	2.3×10^{-2}	9.6×10^{-2}	Yes	No
15	6.4×10^{-2}	3.8×10^{-3}	No	Yes
16	9.5×10^{-2}	4.3×10^{-3}	Yes	No
17	9.0×10^{-2}	7.9×10^{-3}	No	Yes
18	1.8×10^{-2}	2.6×10^{-2}	Yes	No
19	1.6×10^{-2}	7.7×10^{-3}	Yes	No

[a] The patient had leukemia progression after DLI and were alive with the treatment of imatinib.

[b] Exception, who developed hematologic relapse while the MRD result was still $\leq 9 \times 10^{-3}$.

relapsed and died. Using MRD monitoring and analysis system, we can continuously monitor the amounts of MRD in the patients and the dynamic MRD monitoring data is very useful to guide making treatment decision. To a certain degree, DLI could prevent leukemic progression if it was performed timely. Thus, DLI together with MRD monitoring translated into better DFS in certain ALL patients.

Since most adult ALL patients will relapse after first CR and it has an incubation time from CR to relapse and MRD is a well-known causal of ALL reoccurrence, the intensive MRD monitoring post allo-HSCT allowed to reveal the initiation of clinical relapse and to take measure for intervention timely, and to design new strategy and therapeutic regimen accordingly. It is worth to mention here that MRD monitoring may occasionally fail because the antigens of leukemia cells have changed. Nevertheless, MRD monitoring has been demonstrated in this study that it can provide valuable data to guide ALL treatment and increase the survival rate of the patients. In conclusion, our study suggested that intensive MRD monitoring-directed DLI might represent a step toward optimizing the treatment of relapsed/refractory ALL after allo-HSCT.

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