ENERCA clinical recommendations for disease management and prevention of complications of sickle cell disease in children

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Universal neonatal screening is performed in the United States, England, the Netherlands, and several cities in Belgium, with selective screening targeted on "high-risk" population in France (globally, one quarter of all the babies born in France are screened). Newborns diagnosed with a major sickle cell syndrome (SCD) should be referred to a designated pediatric sickle cell centre, and the parents are informed that their child has SCD; this may be in the sickle cell centre by an expert physician or in the community by an experienced nurse counsellor. The pediatric sickle cell centre should organize the care of the baby [1].

Prevention of infections

A randomized study published in 1986 showed that prophylaxis with penicillin twice a day in SCD children younger than 3 years was associated with an 84% reduction in the incidence of infection, compared with placebo therapy [2]. Penicillin is therefore recommended twice daily starting at 2 months of age, but further research is needed to determine the age at which penicillin prophylaxis can be stopped safely [3]. Typically, in Europe, prophylaxis is extended into adulthood, although the benefits of this are not demonstrated.

Given the risk of poor adherence to daily prophylaxis and the development of penicillin resistant *Streptococcus pneumoniae* strains, pneumococcal immunization as well as prophylactic penicillin is recommended [4]. The recommended immunization schedule for previously unvaccinated children with SCD consists of three doses of conjugated vaccine 6 to 8 weeks apart, followed by a booster dose 1 year later, then by a polysaccharide vaccine after age 2 years, with additional doses every 3–5 years.

Prevention of strokes

Adams et al. [5] demonstrated in 1992 that it was possible to identify early the children at high risk of developing an overt stroke using a transcranial Doppler scanning, showing that 40% of children with an increased blood flow velocity in the internal carotid or middle cerebral artery had an overt stroke within 3 years of scanning. Six years later, Adams et al. [6] demonstrated that a first stroke could be prevented by monthly transfusions in children with abnormal transcranial Doppler (TCD) findings. Finally, he randomized discontinuation of transfusion in children undergoing chronic transfusion for an abnormal transcranial Doppler, during which time the transcranial Doppler ultrasonography became normal. Stopping the transfusions was followed by a high rate of stroke or reversion to abnormal velocities of cerebral blood flow [7]. These well-designed studies led to the recommendation that transcranial Doppler ultrasonography should be performed annually in children aged 2–16 years with SCD and that regular blood transfusions should be strongly considered in those with abnormal results [8].

Education and psychological support

Patients and families should be educated about the factors that increase the risk of vaso-occlusive episodes, such as exposure to cold, fever, dehydration, stress, and tobacco. They should be taught to manage mild pain with rest, hydration, simple analgesia (paracetamol and ibuprofen), and weak opioids (such as codeine or propoxyphene) and to recognize signs that require an immediate visit to the emergency room, such as pallor, asthenia, high fever, and respiratory distress.

SCD is a chronic, painful, and distressing disease. Most parents are very upset when the diagnosis is given and some of them experience thereafter repeated life-threatening complications in their children. Some parents and children experience post-traumatic stress disorders [9]. Children may be unable to attend school regularly, making it difficult to gain good qualifications. Furthermore, silent brain infarcts may be responsible for learning difficulties. Specific individual and family psychological interventions can potentially play an important part in disrupting the vicious circle of pain and fear of pain in SCD children. Early detection of school difficulties may help to identify the need for extra school support. Adolescents and their families should be informed about delayed sexual development and growth and reassured that this proceeds appropriately with achievement of normal adult height in most cases. Finally, transition from pediatric to adult clinics must be carefully presented and prepared. Indeed, a recent review of the causes of mortality in children and adolescents with SCD has shown that the adolescents who move from pediatric to adult care units are at high risk of early death [10].

Yearly checkup

Adult patients with SCD may suffer from significant damage to one or more organs, which in some instances can be detected also in older children and adolescents and treated early. This justifies the organization of yearly checkups to detect any early development of organ deficiency [11] (Table I).

Preoperative preparation

The complications of SCD often require surgical procedures such as adenotonsillectomy, cholecystectomy, hip replacement, and splenectomy. However, patients with the disease are at high risk of perioperative complications, chiefly acute chest syndrome (ACS), and pain. Transfusion or exchange transfusion is therefore recommended for major operations, including neurosurgery, cardiovascular surgery, and operations requiring prolonged anesthesia. The role of preoperative transfusion in children undergoing minor or moderate risk procedures is less certain and should be individually assessed based on the nature of the surgery and the history complications in the child.

Treatment of Disease Complications

Pain, infections, worsening of anaemia, ACS, and vasculopathy (contributing to stroke, priapism, and leg ulcers) may complicate the course of SCD. The various complications may occur together and be difficult to disentangle, a painful crisis frequently causing fever for instance.

Pain

Pain is the hallmark of SCD. The frequency and severity of painful episodes vary widely both across patients and over time in each patient. Effective treatment of acute pain is one of the most common and challenging problems in the management of SCD.

TABLE I. Recommended Exams to Be Performed Annually

	0 – 1 Year	2 years	3 - 5 years	6 - 9 years	10 – 15 years	16 -18 years
Physical examination	■	■	■	■	■	■
Transcutaneous O_2 saturation	■	■	■	■	■	■
Laboratory tests[a]	■	■	■	■	■	■
Assessment of adherence to treatments and appointments	■	■	■	■	■	■
Transcranial Doppler		■	■	■	■	
Liver/Gallbladder Ultrasound			■	■	■	■
Academic Performance			■	■	■	■
Pulmonary function tests			■	■	■	■
Hip X-Ray				■	■	■
Electrocardiography				■	■	■
Ophtalmologic evaluation				[b]	■	■

[a]Blood tests: complete blood count, liver profile, electrolytes, BUN, creatinine, ferritin if transfused, calcium metabolism including vitamin D and PTH, parvovirus B19 serology until positive; urine test: μalbumin.
[b]Since the age of 6 years if Hb SC disease.

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Dactylitis is a common early manifestation of SCD that may occur before 6 months of age. The humerus, femur, and vertebrae are often involved in older children, presenting with acute tenderness, swelling, often fever, and mimicking sometimes so closely osteomyelitis that the differentiating between the two conditions is extremely difficult. Repeated clinical examination, blood counts, blood cultures and C-reactive protein measurements, coupled with ultrasonography, and sometimes MRI are used to help the diagnosis.

Acute abdominal pain is frequent in young children, most often related to constipation, but specific complications of SCD such as acute splenic sequestration must be ruled out by palpation of the spleen and blood count, and cholelithiasis by abdominal ultrasound; the possibility of conditions not related to SCD should also be considered, including acute appendicitis.

Acute vaso-occlusive episodes are defined as new onset of pain that lasts at least 4 hr for which there is no explanation other than vaso-occlusion. This may sometimes be managed at home with simple analgesia but will often require therapy with parenteral opioids or ketorolac in a medical setting.

There is general agreement among experts that paracetamol and adequate hydration are appropriate. The value of nonsteroidal anti-inflammatory drugs is less certain. Opioids are often needed and may be effective orally in some children. For very severe pain, intravenous administration is often necessary, using either a continuous infusion or patient/nurse-controlled analgesia [12]. Regardless of the route of administration, the dosage should be titrated to achieve pain relief, particularly as the analgesic effect varies widely across patients (Table II).

TABLE II. Management of Acute Pain

Hospitalize the child when pain is not relieved by codeine, or in case of fever, pallor, chest pain, respiratory signs
Assess pain intensity
Always look for a cause (e.g., infection)
Treatment: choose the analgesic, dosage, and route of administration
Reassess pain intensity and adjust the treatment
Be empathetic, reassuring, and supportive
Provide distraction (e.g., television, school work, games station)
Start incentive spirometry if back, chest or abdominal pain are present
Monitor fluid balance
Encourage drinking but parenteral fluids may be necessary if intake inadequate
Prescribe laxatives, antipruritics, and antiemetics if on regular opioids
Examine the patient often to ensure that pain relief is adequate and to check for evidence of complications such as acute chest syndrome or anemia

Acute chest syndrome

ACS is defined as an acute illness characterized by fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray. Many possible causes have been identified, including bacterial and/or viral infection, fat embolism, intravascular sickling of red cells in the lung, and hypoventilation related to pain and/or to opioids. More recently, an association between asthma and ACS has been found [13], suggesting that asthma should be systematically looked for and treated in SCD children.

Antibiotics are given routinely, and the high rate of atypical microorganisms requires combination of a macrolide with a broad spectrum antibiotic depending on sensitivities of locally prevalent bacteria. Incentive spirometry can prevent atelectasis and infiltrates associated with ACS in children and young adults admitted with chest or back pain above the diaphragm [14]. Transfusion or exchange transfusion produced improvements in several uncontrolled studies and should be considered at an early stage in deteriorating children. Bronchodilators are often used, particularly if bronchospasm appears to be present. Hydroxyurea is recommended in subjects having had at least two episodes of ACS [8].

Infections

Due to routine immunization against *S. pneumoniae* and *Haemophilus influenzae* Type b, septicaemia and bacterial meningitis are becoming rare in developed countries.

Pneumonias remain extremely frequent. Osteomyelitis is mostly related to *Salmonella* species in patients with SCD. Except in the minority of cases where the diagnosis is confirmed by the positive culture of a joint or subperiostal abscess aspirate, in most cases, the diagnosis is made presumptively in a child with bone pain, swelling, fever, and hyperleucocytosis, all signs which may be also present in bone infarction. Radioisotope and bone scans are unhelpful, and the value of MRI is debated. Ultrasonography of the painful zone may evidence a subperiostal abscess or a contiguous effusion which may be aspirated. Blood cultures may be helpful if positive but negative results do not exclude the diagnosis.

Given that bone infarcts are much more frequent than infections, it can be recommended in children without obvious signs of sepsis to wait some hours for the effects of treatment of vaso-occlusion before introducing intravenous antibiotics, but not to wait more than 1 day to begin antibiotics to avoid osteoarticular sequel. Antibiotic treatment must cover *Salmonella* and *Staphylococcus* species.

Acute anemia

Acute exacerbation of anemia is defined as an acute lowering of the hemoglobin level from baseline by at least 2 g/dL. The most common causes of acute anemia are acute splenic sequestration, transient red cell aplasia, and increased hemolysis in patients with severe infection or vaso-occlusion.

Acute splenic sequestration is defined as rapid intrasplenic trapping of cellular elements of the blood, which causes a precipitous fall in hemoglobin level and is often associated with a relative or absolute thrombocytopenia ($<150 \times 10^9$/L) and hypovolemia. It occurs mostly before age 6 years in homozygous SS patients but may occur later in SC patients. The diagnosis is made on a fall in hemoglobin level of at least 2 g/dL in the presence of an acutely enlarged spleen, typically associated with reticulocytosis. Life is threatened both by acute anemia and hypovolemia. Transfusion is urgent but must be cautious because the red cells sequestered in the spleen are remobilized after transfusion, and hemoglobin levels increase more than expected given the amount of blood transfused. About one half of children who have had a first splenic sequestration have a recurrence, and there is no consensus about the management; either splenectomy or conservative management may be appropriate [15].

Transient red cell aplasia is defined as a transient total or partial suppression of erythropoiesis characterized by a decrease in the hemoglobin level and reticulocytopenia (absolute reticulocyte count $<50 \times 10^9$/L). The most common cause is parvovirus B19 infection. Parvovirus B19 is very infectious, and carers should be warned that other children in the family with SCD may also develop profound anemia and should be seen in hospital if there is concern.

Episodic transfusion is required in these settings. Its goal is to restore hemoglobin to its baseline level and not above, to avoid increasing excessive blood viscosity.

Stroke

Cerebrovascular accident is defined as an acute neurological syndrome secondary to occlusion of an artery or hemorrhage, with resultant brain ischemia, neurological signs, and symptoms.

Experts agree that exchange transfusion should be performed when a stroke occurs. More than half of patients with a first stroke have another. Long-term observational studies showed that monthly blood transfusions decreased the risk of recurrent stroke, although transient neurological events were not completely stopped. Stroke is considered an indication for bone marrow transplantation in children and adolescents who have human leucocyte antigen (HLA)-matched siblings [8].

Specific Therapies for Severe Disease

Approximately 10% of SCD children have severe complications, either because they have repeated painful episodes or ACSs or because they have evidence of cerebral vasculopathy [16]. Three types of intensification can be proposed to these children: hydroxyurea, chronic transfusion, or hematopoietic stem cell transplant when they have an HLA-identical sibling.

Hydroxyurea

Hydroxyurea was used in children with SCD affected by severe forms of the disease for more than 15 years. The rationale for using it arose from the findings that hydroxyurea increases fetal hemoglobin, which interrupts the elongation of the polymer of deoxyHbS. Secondarily, it was observed that the clinical benefit felt by the patients precedes the reactivation of HbF synthesis, suggesting that other mechanisms of action are involved, including decreased leucocyte numbers and activation, decreased of adhesiveness of blood cells to endothelial cells, and increased in NO production. The clinical efficacy of hydroxyurea in children was demonstrated by a Belgian controlled trial in children with severe SCD [17]. There are now many reports about the use of hydroxyurea in SCD children affected with severe forms of the disease [18], and we are waiting for the results of a controlled trial (the BABY HUG study), the drug being used in this last setting in children aged 9–17 months at entry in the hope of preventing the onset of the complications [19].

Long-term studies on hydroxyurea use in children confirm a sustained efficacy in young patients [20–22].

Indications

Globally, hydroxyurea is now recommended in children with SCD to prevent recurrence of acute pain and ACSs. Many authors treat also children with chronic severe anemia (baseline hemoglobin level <6 or 7 g/dL according to authors). There are more controversies about the use of hydroxyurea as an alternative to chronic transfusion to prevent cerebrovascular events. A 26-site study randomizing transfusion and chelation against hydroxyurea with venesection in children having already had a stroke (the SWiTCH trial) has been recently stopped by the NIH because they noted that no strokes occurred in the 66 participants who received the standard therapy of blood transfusion and 7 strokes occurred in the group of 67 children who received hydroxyurea and venesection (stroke prevention study in children with sickle cell anemia, iron overload stopped early.) [23] (indications for hydroxyurea treatment are given in Table III).

In the United States, the Food and Drug Administration has approved hydroxyurea use only in adult SCA patients, and the children have to be enrolled in protocols, while European regulatory authorities have approved a coated breakable 1,000-mg tablet for adults and children and 100-mg pills for children. Starting doses are generally around 15 gm/kg/day and may be escalated by 5 mg/kg/day until there is evidence of clinical benefit or the maximum tolerated dose is reached.

Side effects and follow-up

Tolerance. The short- and mid-term tolerances of hydroxyurea in children are good [22]. The real questions concern the long-term complications of the drug. Hydroxyurea has been shown to exacerbate the alterations of semen parameters observed in SCD adults [24], and there are uncertainties as to the long-term consequences on fertility of boys treated with hydroxyurea early and for several years. Storage of frozen sperm can be offered to mature boys and adults, although it is rarely accepted.

Transient myelosuppression may occur and usually resolve after decreasing the dosage or temporary interruption of the drug.

Complete blood count must be performed before starting the treatment, 2 weeks after its beginning, at 2–4-week intervals during the initial phase, and then every 8 weeks. These results should be monitored by a medical professional. Nail hyperpigmentation is common. The possibility that hydroxyurea, which was shown to delay splenic infarct, lengthens the period at risk for acute splenic sequestration is debated [22]. An appropriate strategy is to carefully monitor spleen size and blood tests at each evaluation particularly for children with prior splenomegaly or past history of splenic sequestration before starting hydroxyurea treatment.

Risk of malignancies. The other issue related to the use of this cytostatic drug concerns the risk of malignancies. There is no evidence that hemoglobinopathies are associated with an increased risk of malignancy. To date, several malignancies have been reported in patients with SCD receiving hydroxyurea [25,26] but implication of this drug in the pathogenesis of these malignancies is not possible. Recommendations for hydroxyurea treatment monitoring are indicated on Table III.

Chronic Transfusion

Chronic transfusion was for many years the only therapy for patients with a severe form of SCD. More recently, hydroxyurea was proposed as an alternative in several conditions.

Indications

The most frequent indication for chronic transfusion in children is the prevention of cerebrovascular events, either of a first stroke (efficacy proven in the controlled STOP trial [6]) or of a recurrence. Chronic transfusion may be also used in children with recurrent splenic sequestrations aged less than 5 years to delay the time of splenectomy. It is also used in the minority of children having been treated with hydroxyurea because of recurrent pain or ACSs and who fail to respond to this drug.

Top-up versus exchange transfusion

Chronic transfusion may be performed through simple or exchange transfusion. The advantages of the latter include the avoidance of an excessive increase in hematocrit and to reduce the amount of transfused iron. Exchange transfusion can be performed manually or using a cell separator (erythrocytapheresis).

TABLE III. Indications and Requirements for Specific Therapies

Hydroxyurea, indications
Established
Recurrent episodes of severe acute pain ≥ 3 year
≥2 episodes of acute chest syndrome
Postulated
Correction of severe anemia
Stroke prevention
Prevention of organ dysfunction
Hydroxyurea, monitoring
Full blood count and Hb F level each 2 weeks after initiation and after each dose increase; when stable every 8 weeks
Monitor spleen size, particularly if splenomegaly is present or there is an history of splenic sequestration
Propose storage of frozen sperm
Red cell transfusion
Prevention of alloimmunization and iron overload
Phenotypic matching for full Rh, Kell antigens, and more extensively if alloimmunization is known or suspected
Iron chelation should be considered for patients who have received at least 20 top-up transfusion episodes or with a serum ferritin level consistently >1,000 μg/L

Target Hb S values

The target percentage of hemoglobin S in patients receiving regular blood transfusions varies across studies from 30 to 50% and the optimal target remains to be determined.

Side effects

Complications of long-term transfusion programs include alloimmunization, paucity of venous access, leading in many cases to the use of subcutaneous central venous access devices, and iron overload. Transfusion-transmitted infections are now very rare in industrialized countries with established blood transfusion services.

Alloimmunization. Red cell alloimmunization is especially frequent in transfused SCD patients because of the discrepancies between the blood donor and recipient populations in many countries [27]. Extensive blood phenotyping must therefore be done before any transfusion, except in emergencies when delay is likely to cause death or complications. Blood products have to be phenotyped and matched for at least the Rh and Kell antigens and more extensively if alloimmunization is known or suspected. An aspect of alloimmunization causing complications in SCD is a delayed hemolytic transfusion reaction syndrome; typically, 1–2 weeks after a transfusion, a dramatic fall in total hemoglobin level caused by the destruction of both donor and recipient red cells. A negative direct antiglobulin test and reticulocytopenia are often present. It is recommended to avoid additional transfusion if possible. Anecdotally, corticosteroids and intravenous immunoglobulin have been successfully used.

Iron overload. Currently, iron-related organ damages seem less severe in SCD than in thalassemia [28], but this may be related to the fact that fewer children with SCD have undergone long-duration transfusional programs. Recently, a once-daily oral chelator, deferasirox was demonstrated to have similar efficacy to deferoxamine in reducing iron burden in children with SCD, with an acceptable tolerability. Recommendations for minimizing chronic transfusion complications are indicated in Table III.

Hematopoietic Stem Cell Transplantation

Transplantation of hematopoietic stem cells from HLA-identical siblings is the only curative therapy for SCD. Stem cell source may be either bone marrow or cord blood. In a series of 87 patients transplanted between 1988 and 2004, the overall and event-free survival rates were, respectively, 93.1% and 86.1% [29]. Ovarian tissue can be cryopreserved but it is currently unclear how useful this approach is in allowing future pregnancies [30]. Both the immediate risk of death and the long-term uncertainties about fertility must lead health care providers to discuss the indications for transplantation with parents, very carefully.

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Conflict of interest: Nothing to report.
The European network for rare and congenital anaemia (http://www.enerca.org) is cofunded by the European Commission through its Public Health and Consumer Protection Directorate. One of the goals of this network is to promote harmonization of procedures for diagnosis, treatment, and follow-up of patients with rare anemias. Through this network, clinical recommendations for pediatric sickle cell syndromes are proposed.
Published online 30 August 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21865

References

1. Consensus Conference. Newborn screening for sickle cell disease and other hemoglobinopathies. JAMA 1987;258:1205–1209.
2. Gaston MH, Verter JI, Woods G, et al. Prophylaxis with oral penicillin in children with sickle cell anemia. N Engl J Med 1986;314:1593–1599.
3. Hirst C, Owusu-Ofori S. Prophylactic antibiotics for preventing pneumococcal infection in children with sickle cell disease. Cochrane Database Syst Rev 2002;3:CD003427.
4. Davies EG, Riddington C, Lottenberg R, Dower N. Pneumococcal vaccines for sickle cell disease. Cochrane Database Syst Rev 2004;1:CD003885.
5. Adams RJ, McKie V, Nichols F, et al. The use of transcranial ultrasonography to predict stroke in sickle cell disease. N Engl J Med 1992;326:605–610.
6. Adams RJ, McKie V, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial doppler ultrasonography. N Engl J Med 1998;339:5–11.
7. Adams RJ, Brambilla D. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. N Engl J Med 2005;353:2769–2778.
8. National Institutes of Health.The Management of Sickle Cell Disease, 4th ed. NIH publication No 02–2117. Available at: www.nhlbi.nih.gov/health/prof/blood/sickle/index.htm, 2002.
9. Hofmann M, de Montalembert M, Beauquier-Maccotta B, et al. Posttraumatic stress disorder in children affected by sickle-cell disease and their parents. Am J Hematol 2007;82:171–172.
10. Quinn CT, Rogers ZR, McCavit TL, et al. Improved survival of children and adolescents with sickle cell disease. Blood 2010;115:3447–3352.
11. Haute Autorité de Santé. Prise en charge de la drépanocytose chez l'enfant et l'adolescent [Clinical practice guidelines in French]. Available at: www.has-sante.fr/portail/display.jsp?id=c_272479, 2005.
12. Rees DC, Olujohungbe AD, Parker NE, et al. Guidelines for the management of the acute painful crisis in sickle cell disease. Br J Haematol 2003;120:744–752.
13. Boyd JH, Macklin EA, Strunk RC, DeBaun MR. Asthma is associated with acute chest syndrome and pain in children with sickle cell disease. Blood 2006;108:2923–2927.
14. Bellet PS, Kalinyak KA, Shukla R, et al. Incentive spirometry to prevent acute pulmonary complications in sickle cell diseases. N Engl J Med 1995;333:699–703.
15. Owusu-Ofori S, Hirst C. Splenectomy versus conservative management for acute sequestration crises in people with sickle cell disease. Cochrane Database Syst Rev 2002;4:CD003425; DOI: 10.1002/14651858.
16. Ballas SK, Lieff S, Benjamin LJ, et al. Definitions of the phenotypic manifestations of sickle cell disease. Am J Hematol 2010;85:6–13.
17. Ferster A, Vermylen C, Cornu G, et al. Hydroxyurea for treatment of severe sickle cell anemia: A pediatric clinical trial. Blood 1996;88:1960–1964.
18. Kinney TR, Helms RW, O'Branski EE, et al. Safety of hydroxyurea in children with sickle cell anemia: Results of the HUG-KIDS study, a phasel/II trial. Blood 1999;94:1550–1554.
19. Thompson BW, Scott TM, Rogers ZR, et al. The pediatric hydroxyurea phase III clinical trial (BAY HUG): Challenges of study design. Pediatr Blood Cancer 2010;54:250–255.
20. Gulbis B, Haberman D, Dufour D, et al. Hydroxyurea for sickle cell disease in children and for prevention of cerebrovascular events. The Belgian experience. Blood 2005;105:2685–2690.
21. Zimmerman SA, Schultz WH, Davis JS, et al. Sustained long-term efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. Blood 2004;103:2039–2045.
22. De Montalembert M, Brousse V, Elie C, et al. Long-term hydroxyurea treatment in children with sickle cell disease: Tolerance and clinical outcomes. Haematologica 2006;91:125–128.
23. Available at: http://www.nih.gov/news/health/jun2010/nhlbi-03.htm, 2010.
24. Berthaut I, Guignedoux G, Kirsh-Noir F, et al. Influence of sickle cell disease and treatment with hydroxyurea on sperm parameters and fertility of human males. Haematologica 2008;93:988–993.
25. Ferster A, Sariban E, Meulemann N. Malignancies in sickle cell disease patients treated with hydroxyurea. Br J Haematol 2003;123:368–369.
26. Schultz WH, Ware RE. Malignancy in patients with sickle cell disease. Am J Hematol 2003;74:249–253.
27. Vichinsky EP, Earles A, Johnson RA, et al. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. N Engl J Med 1990;322:1617–1621.
28. Wood JC. Cardiac iron across different transfusion-dependent diseases. Blood Rev 2008;22(Suppl 2):S14–S21.
29. Bernaudin F, Socie G, Kuentz M, et al. Long-term results of related myeloablative stem-cell transplantation to cure sickle cell disease. Blood 2007;110:2749–2756.
30. Brachet C, Heinrichs C, Tenoutasse S, et al. Children with sickle cell disease: Growth and gonadal function after hematopoietic cell transplantation. J Pediatr Hematol Oncol 2007;29:445–450.

C-MYC rearrangement may induce an aggressive phenotype in anaplastic lymphoma kinase positive anaplastic large cell lymphoma: Identification of a novel fusion gene *ALO17/C-MYC*

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Anaplastic lymphoma kinase (ALK) positive anaplastic large cell lymphoma (ALCL) is usually associated with a favorable prognosis. We describe an 11-year-old girl patient with ALK positive ALCL bearing t(2;5)(p23;q35) and t(8;17)(q24;q25) translocations who had an aggressive clinical course despite various combinations of intensive chemotherapy. Southern blot analysis identified *C-MYC* rearrangement. Immunohistochemistry and Northern and Western blot analyses revealed c-myc overexpression. A new fusion between *ALO17* (ALK lymphoma oligomerization partner on chromosome 17) and *C-MYC* was identified by the 5′-rapid amplification of cDNA ends. This new fusion may have possibly provoked the poor prognosis in this patient with ALK positive ALCL, and *C-MYC* rearrangement may indicate poor prognosis in ALCL.

Anaplastic large cell lymphoma (ALCL) is a type of non-Hodgkin's lymphoma characterized by CD30/Ki-1 expression [1]. Its clinical features include a predominance of systemic symptoms and a usually high frequency of extranodal involvement of the lung, skin, bone, and soft tissue. Hemophagocytosis is observed in some patients with ALCL [2–5]. The underlying etiology is not fully elucidated, however, there are a few reports describing the relationship of *perforin* germline mutations [6,7] and cytokine production by tumor cells [8–10].

Based on immunologic studies, ALCLs are derived from a T cell or null phenotype [11] and half of these tumors express anaplastic lymphoma kinase (ALK) [12]. In 80% of these cases, ALK expression results from t(2;5)(p23;q35), and this chromosomal translocation induces the generation of the chimeric protein nucleophosmin (NPM)-ALK [13]. Compared with ALK negative ALCL, ALK positive ALCL demonstrates a significantly favorable prognosis [11]. Other markers, such as survivin and CD56, are known as predictors of poor prognosis [12,14].

Recently, Monaco et al. [15] reported a unique ALK positive ALCL case with t(3;8)(q26.2;q24) translocation and *C-MYC* rearrangement who showed an aggressive and dismal clinical course. This case suggests that *C-MYC* rearrangement in addition to ALK activation may be associated with an aggressive progression of the disease. In this report, we describe an ALK positive ALCL case showing an aggressive clinical course with hemophagocytosis whose ALCL cells had t(8;17)(q24;q25) and *C-MYC* rearrangement.

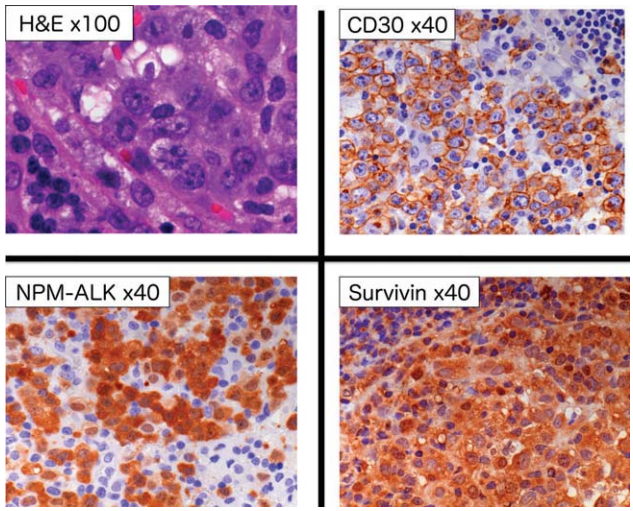

Figure 1. Histology of the lymph node biopsy showing ALCL. Hematoxylin and eosin stain shows proliferation of large lymphoid cells with large irregular-shaped nuclei and conspicuous nucleoli. The tumor cells show strong positive reactivity for CD30 in the cytoplasm and NPM-ALK both in the cytoplasm and nucleus. These cells show uniform reactivity for survivin in an exclusively cytoplasmic distribution.

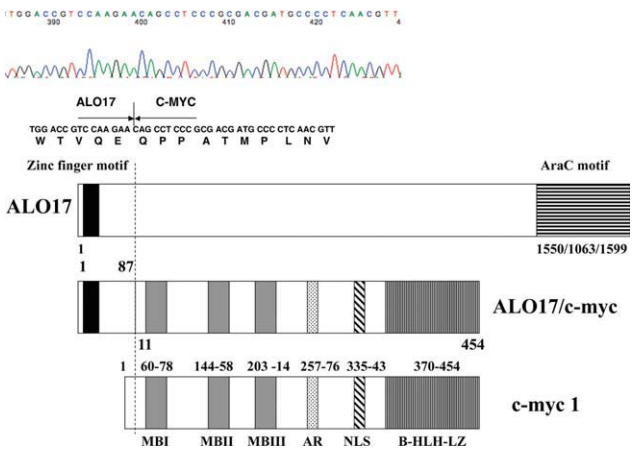

Figure 2. Direct sequencing of RT-PCR products revealed that the ALO17/c-myc fusion transcript was in frame fusion. The ALO17 protein consists of 1,550/1,063/1,599 amino acids as a consequence of alternative splicing. They each have a Zinc finger motif at the N terminal region. The two isoforms with 1,550 and 1,599 amino acids have a weak similarity to the bacterial AraC motif that is supposed to have transcriptional activating function at the C terminus. The c-myc N-terminal domain contains Myc Box I (MBI), Myc Box II (MBII), and Myc Box III (MBIII), and an acidic region (AR). The c-myc C-terminal domain contains a primary nuclear localization signal (NLS) and the basic helix-loop-helix leucine zipper domains (B-HLH-LZ). The novel ALO17/c-myc fusion protein consists of 531 amino acids containing 87 amino acids from the N-terminal part of ALO17 and 444 amino acids from the C-terminal part of c-myc.

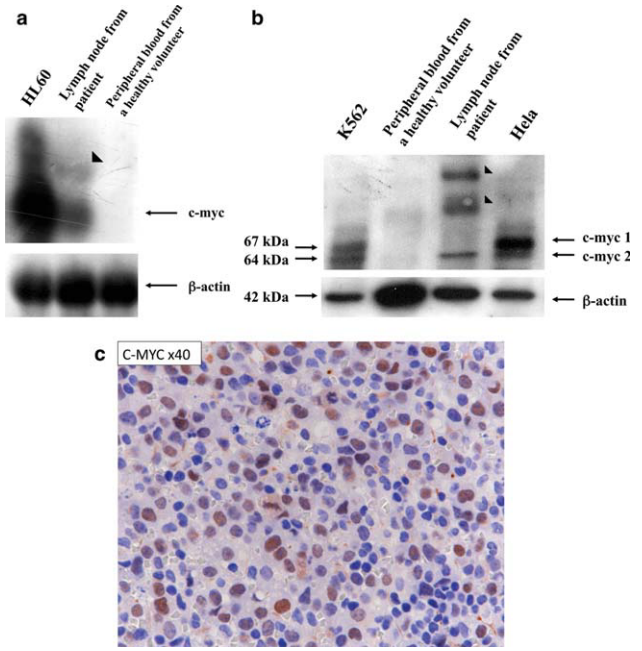

Figure 3. (A) Northern blot analysis of the lymph node from the patient for c-myc. C-myc mRNA of the patient's lymph node and HL60 cell line was greatly overexpressed, as compared with the negative control. The arrowhead indicates the possible fusion transcript of ALO17/c-myc. The β-actin gene was used as an internal control. (B) Western blot analysis of the lymph node from the patient using c-myc antibody. Immunoreactive bands of c-myc 1 and/or c-myc 2 were detected in the K562, Hela, and lymph node from the patient, but not in peripheral blood from a healthy volunteer as a negative control. Arrowheads indicate possible protein of ALO17/c-myc. The β-actin was used as an internal control. (C) The tumor cells show strong positive reactivity for c-myc in the nucleus.

These two ALK positive ALCL cases suggest that *C-MYC* rearrangement may induce poor prognosis. In addition, we were able to identify *ALO17* as the fusion partner of *C-MYC*. The *ALO17* gene, which is also known as *KIAA1618*, has been identified as a fusion partner of ALK in an ALCL case with t(2;17)(p23;q25) [16]. The *ALO17* gene may have an important role in oncogenesis and tumor progression of ALCL.

An 11-year-old girl presented with a 1-week history of right axillary pain and fever in March 2007. Because these symptoms failed to respond to antibiotics, peripheral blood and bone marrow examination were performed. The blood count was as follows: Hb 6.7g/dl, Plts 16 $\times$ 10^9/l, and WBC 2.3 $\times$ 10^9/l. The bone marrow aspirate was hypocellular, revealing hemophagocytosis without existence of any blasts. She was diagnosed with hemophagocytic lymphohistiocytosis (HLH). Treatment including prednisolone and gamma globulin failed to induce remission. The patient was referred to our hospital at the end of March 2007. Treatment including etoposide, prednisolone, and cyclosporine A led to a complete response. In May 2007, the patient showed recurrence with fever, cervical lymphadenopathy, thymic mass, and skin rash. Biopsy of the cervical lymph node was performed. Proliferation of large lymphoid cells with large irregular-shaped nuclei and conspicuous nucleoli was seen (Fig. 1). Immunohistochemically, these lymphoid cells were positive for CD30, T-cell intracytoplasmic antigen (TIA-1), granzyme B, NPM/ALK, and survivin (Fig. 1). They were negative for CD45RO, CD2, CD3e, CD4, CD5, CD7, CD8, CD20, CD79a, Myeloperoxidase, CD13, CD15, CD56, CD68, CMV, EBNA2, and EBV-LMP1 (data not shown). A total of 17 metaphase cells in tumor tissue were examined by G-banding method. Among them, nine cells showed that the modal chromosome number was 46 and a representative karyotype was 46, XX, t(2;5)(p23;q35), t(8;17)(q24;q25) (Supplemental Fig. 1). NPM/ALK chimeric transcript was detected in tumor tissue by reverse transcription (RT)-polymerase chain reaction (PCR). Sequencing revealed the same in-frame junction as previously described (data not shown) [17]. After establishing diagnosis of ALCL, the patient received chemotherapy according to the ALCL99 protocol, which is a multicentric international prospective study for childhood ALCL, involving most European groups as well as Japan. Induction regimen with a combination of dexamethasone (10 mg/m^2, days 1-5), methotrexate (3 g/m^2, day 1), ifosfamide (800 mg/m^2, days 1-5), cytarabine (300 mg/m^2, days 4 and 5), and etoposide (300 mg/m^2, days 4 and 5) led to a response, however, fever and lymphadenopathy recurred after hematological recovery. Reinduction therapy with etoposide (150 mg/m^2, days 1-5), cytarabine (200 mg/m^2, days 6-12), and mitoxantron (5 mg/m^2, days 6-10) also led to a response, however, the disease again progressed after hematological recovery. Stem cell transplantation with high-dose chemotherapy was recommended, however, consent could not be obtained from her guardian. The patient's clinical status continued to deteriorate, and she died of the disease in October 2007.

Southern blot analysis of the lymph node revealed that the *C-MYC* gene was rearranged (Supplemental Fig. 2). To clone the end of the putative novel c-myc-fusion cDNA, rapid amplification of cDNA ends-PCR (RACE-

PCR) was performed using poly(A)+ RNA from a frozen lymphoma specimen. Using c-myc specific 3′-primer, the ~1300-bp DNA fragment was obtained. DNA sequence analysis revealed an identification of the fusion between *ALO17* and *C-MYC*. The breakpoint in the new fusion transcript was at its 5′ end after nucleotide 261 of ALO17 and 5′ end after nucleotide 30 of c-myc 1 (Fig. 2). The c-myc mRNA was definitely overexpressed in the patient's lymph node tissue and the HL60 cell line, but not in the patient's peripheral blood cells (Fig. 3A). Western blot analysis showed the ALO17/c-myc protein in size beyond c-myc 1 was definitely overexpressed in lymph node tissue from the patient (Fig. 3B). Immunohistochemically, c-myc overexpression was also detected in tumor specimen (Fig. 3C).

Discussion

We experienced an ALK positive ALCL case whose ALCL cells have a representative karyotype 46, XX, t(2;5)(p23;q35), t(8;17)(q24;q25) and *C-MYC* rearrangement, showing an aggressive clinical course. This case had complications with HLH before diagnosis of ALCL. The association of malignancy, especially ALCL, is well known in secondary HLH. The underlying etiology is less elucidated, however there are a few reports describing the relationship of *perforin* germline mutations [6,7] and cytokine production by tumor cells [8–10]. We performed direct sequencing for the *perforin* gene but could not find any mutation (data not shown). In addition, we analyzed 17 cytokines and chemokines in the patient's sera at diagnosis and after chemotherapy. Among them, a total of 10 cytokines and chemokines (IL6, IL7, IL8, IL10, IL12, IL13, G-CSF, TNFα, IFNγ, and MCP1) were significantly elevated (data not shown). We suspected that the cytokines and chemokines were produced by systemic ALK positive cells and accessory cells as described previously [8–10], and that this hypercytokinemia induced HLH in this patient.

In the 4th edition of the 2008 World Health Organization classification, ALK positive and ALK negative ALCLs are distinguished as separate entities [18]. ALK positive ALCL is characterized by a younger age distribution, lower serum LDH levels, less frequent extranodal involvement, and better prognosis [11]. Recently, Monaco et al. described a pediatric ALK positive ALCL case with t(3;8)(q26.2;q24) translocation and *C-MYC* rearrangement [15]. They suggest the presence of *C-MYC* rearrangement in addition to ALK activation may be associated with an aggressive disease course. The Myc family proteins are potent oncogenes that can activate and repress a very large number of cellular target genes. The transactivation domain located in the amino terminal region has an important role for cell transforming activity [19–23]. The c-myc protein has two variants of 454 and 439 amino acids called as c-myc 1 and c-myc 2, respectively. The breakpoint of c-myc 1 fusion protein in our patient is in its immediate upstream that is known to contain sequences involved in the regulation of c-myc expression (Fig. 2). The translocation in our case may have disrupted the regulation of c-myc expression and induced overexpression.

Other prognostic markers, such as survivin and CD56, have been reported [12,14]. Both survivin and CD56 expressions are known to indicate poor prognosis. The immunohistochemical study in our case was positive for survivin (Fig. 1) and negative for CD56. The case reported by Monaco et al. shows the positivity for CD56, however, the reaction to survivin was not performed. Both cases phenotypically have similarity showing null and cytotoxic phenotype, although the presence of cytotoxic phenotype was not related to clinical prognosis [24]. Survivin, a member of the inhibition-of-apoptosis family, blocks apoptotic signals by inhibiting the activation of downstream effectors of apoptosis, caspase-3, and caspase-7, in cells exposed to apoptotic stimuli [25]. C-myc transcriptionally up-regulates survivin by engaging its response element in the survivin core promoter region in breast cancer [26]. There is no report describing the relationship between survivin and c-myc in ALCL, however, in our case, survivin overexpression may be relevant to c-myc overexpression.

Additionally, we succeeded in identifying the *ALO17* gene as the fusion partner of *C-MYC*. The *ALO17* gene, which is also known as *KIAA1618*, has already been identified as a fusion partner of ALK in an ALCL case with t(2;17)(p23;q25) [16]. So far, the mechanism of the *ALO17* gene has yet to be elucidated. As a consequence of alternative splicing, three different sequences are known. According to these results, the ALO17 protein has three variants of 1,550/1,063/1,599 amino acids. Among them, two isoforms of 1,550 and 1,599 amino acids have a weak similarity to the bacterial AraC motif at the C terminus, however, there is no other significant sequence similarity in known proteins. It is possible that the ALO17 has an oligomerization domain in the N terminus, because all ALK fusion partners have these domains to induce oncogenic properties. They are expressed in hematopoietic cells, especially in T lymphocytes and natural killer cells (http://genome.ucsc.edu). The *ALO17* gene may have an important role in oncogenesis and tumor progression of ALCL.

In conclusion, we experienced a case of ALK positive ALCL with an aggressive clinical course, and identified a novel fusion partner *ALO17* of *C-MYC* originating from t(8;17)(q24;q24) translocation. This case suggests poor prognosis associated ALK positive ALCL with *C-MYC* rearrangement. Recently, Andréasson et al. [27] identified molecular targets associated with transformed diffuse large B-cell lymphoma from follicular lymphoma using gene expression analyses. Further genetic studies including DNA copy number and gene expression alterations are necessary to elucidate the molecular mechanism in which *C-MYC* rearrangement induces aggressive phenotypes in ALK positive ALCL.

Methods

Morphologic and immunohistochemical findings. Paraffin-embedded sections of lymph node were stained with hematoxylin and eosin. The sections were also investigated with antibodies directed against: NPM/ALK (Nichirei, Tokyo, Japan), CD30 (DakoCytomation, Carpinteria, CA), CD3e (DakoCytomation), CD5 (DakoCytomation), CD20 (DakoCytomation), CD45RO (DakoCytomation), CD68 (DakoCytomation), CD79a (DakoCytomation), granzyme B (DakoCytomation), MPO (DakoCytomation), CMV (DakoCytomation), EBNA2 (DakoCytomation), EBV-LMP1 (DakoCytomation), CD2 (Novocastra, Newcastle upon Tyne, UK), CD4 (Novocastra), CD7 (Novocastra), CD8 (Novocastra), CD13 (Novocastra), CD56 (Novocastra), TIA-1 (Immunotech, Marseille, France), CD15 (Becton Dickinson & Co., Mountain View, CA), survivin (R&D Systems, Minneapolis, MN), and c-myc (Cell Signaling Technologies, Beverly, MA).

Detection of chimeric transcript NPM/ALK by RT-PCR. Total RNA was extracted from the frozen tissue sample using a TRIZOL Reagent (Invitrogen, Carlsbad, CA). RT and PCR amplification was carried out according to the manufacture's instructions (Takara, Kyoto, Japan). The sense and antisense primers for the fusion gene were 5′-TCCCTTGGGGGC TTTGAAATAACACC-3′ and 5′-CGAGGTGCGGAGCTTGCTCAGC-3′, respectively. Thermal cycling was performed with an initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed in 2% agarose gels in the presence of 0.5 μg/ml of ethidium bromide and visualized by UV irradiation.

Perforin *gene mutation analysis.* Genomic DNA was prepared from peripheral blood obtained from the patient using Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). The primers used were as follows: Exon2-F 5′-CATGGCTTCCCAGAGCCCAAG-3′, Exon2-R 5′-AGCAGC CTCCAAGTTTGATTGG-3′, Exon3-F 5′-CAGCTGAGGTCTCTCTCTTC-3′, and Exon3-R 5′-CCTTTCCAAGCTCACTGTTC-3′. PCR amplification was performed according to the manufacture's protocol (Toyobo, Osaka, Japan). Thermal cycling was performed with an initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 98°C for 10 seconds, and extension at 68°C for 1 min. PCR products were directly sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing was performed with an ABI model 3130XL (Applied Biosystems).

*Analysis of cytokines and chemokines in sera.*Serum cytokine and chemokine analyses were performed on blood collected from the patient at the time of diagnosis and after 2nd chemotherapy. Seventeen cytokines and chemokines (IL1β, IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12, IL13, IL17, G-CSF, GM-CSF, TNFα, IFNγ, MCP1, and MIP1β) were assayed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). The assay was performed according to the manufacture's instructions. Sera obtained from volunteer healthy donors were used as controls.

G-banding chromosome analysis. Karyotype analysis was performed using a cervical lymph node sample. The tumor tissue was minced finely into small pieces with scissors and then digested with 0.08% collagenase type II (Worthington Biochemical Co., Lakewood, NJ) for 3 hours at 37°C. The suspension was then mixed with Hank's balanced salt solution by shaking, and passed through a mesh screen. After washing, the cells were incubated in RPMI 1640 supplemented with 20% fetal calf serum in an atmosphere of 5% CO_2. For a cytogenetic study, chromosomes were prepared by standard techniques

and analyzed by trypsin-Giemsa banding as previously described [28,29]. Karyotypes were determined according to the International system for Cytogenetic Nomenclature.

C-MYC *gene rearrangement*. Ten micrograms of DNA isolated from a cervical lymph node digested with restriction enzyme HindIII (New England Biolabs, Beverly, MA) was electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane as previously described [28]. The membrane was hybridized with a ^{32}P-labeled 660-bp DNA fragment corresponding to the second exon of the *C-MYC* gene. Peripheral mononuclear cells of a healthy volunteer were used as a control.

Rapid amplification of cDNA ends. The poly (A)+ RNA was isolated from total RNA using a mRNA Purification Kit (GE Healthcare Bio-Sciences Inc., Piscataway, NJ). Double-stranded cDNA from poly (A)+ RNA was synthesized by the Marathon cDNA amplification kit (CLONTECH Laboratories Inc., Palo Alto, CA). RACE was also performed using the Marathon cDNA amplification kit [30]. A 3′-specific c-myc primer (5′-CAGTGGGCTGTGAG GAGGTTTGCTG-3′) for 5′ RACE-PCR and a 5′-specific c-myc primer (5′-CTGGATTTTTTTCGGGTAGTG-3′) for 3′ RACE-PCR were used. Purified PCR products were cloned into TA vector (Invitrogen) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit.

Northern blot analysis. Twenty micrograms of total RNA was electrophoresed through a 1.5% formaldehyde-agarose gel and transferred to a nylon membrane as previously described [28]. The membrane was hybridized with the same probe for Southern blot analysis. As an internal control, the β-actin gene was also used. The human promyelocytic leukemia cell line, HL60, which overexpresses c-myc mRNA, was used as a positive control.

Western blot analysis. Frozen tissues of lymph node from the patient were lysed in RIPA lysis buffer (Thermo Scientific, Rockford, IL) supplemented with protease inhibitors (Thermo Scientific) by homogenizing at 4°C. Also, the K562 and Hela cells which are known to overexpress c-myc protein, and peripheral mononuclear cells from a healthy volunteer were lysed by sonication. Samples were loaded onto 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene fluoride membranes (Millipore, Watford, United Kingdom). After blocking, membranes were probed with primary c-myc (Cell Signaling Technologies) or β-actin (Sigma, St. Louis, MO) antibodies overnight at 4°C. After washing, the membranes were incubated with appropriate secondary antibody horseradish peroxidase conjugate (Cell Signaling Technology) for 1 hour at room temperature. After washing, the membrane was developed with Amersham ECL reagent (Amersham, Arlington Heights, IL).

Acknowledgments

We thank Ms. Megumi Obara for her technical assistance and Dr. Kaede Yanagita for her useful comments on this manuscript.

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Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports and Technology of Japan

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Conflict of interest: Nothing to report.
Published online 21 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21887

References

1. Stein H, Mason DY, Gerdes J, et al. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: Evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 1985;66:848–858.
2. Invernizzi R, Paulli M, Rosso R, et al. Reactive hemophagocytosis in Ki-1 positive large cell lymphoma: A case study. Haematologica 1989;74:155–159.
3. Wong KF, Chan JK, Ng CS, et al. Anaplastic large cell Ki-1 lymphoma involving bone marrow: marrow findings and association with reactive hemophagocytosis. Am J Hematol 1991;37:112–119.
4. Bhagwati NS, Oiseth SJ, Abebe LS, Wiernik PH. Intravascular lymphoma associated with hemophagocytic syndrome: a rare but aggressive clinical entity. Ann Hematol 2004;83:247–250.
5. Gudgin E, Rashbass J, Pulford KJ, Erber WN. Primary and isolated anaplastic large cell lymphoma of the bone marrow. Leuk Lymphoma 2005;46:461–463.
6. Clementi R, Locatelli F, Dupre L, et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. Blood 2005;105:4424–4428.
7. Cannella S, Santoro A, Bruno G, et al. Germline mutations of the perforin gene are a frequent occurrence in childhood anaplatic large cell lymphoma. Cancer 2007;109:2566–2571.
8. Al-Hashmi I, Decoteau J, Gruss HJ, et al. Establishment of a cytokine-producing anaplastic large-cell lymphoma cell line containing the t(2;5) translocation: potential role of cytokines in clinical manifestations. Leuk Lymphoma 2001;40: 599–611.
9. Siebert S, Amos N, Williams BD, Lawson TM. Cytokine production by hepatic anaplastic large-cell lymphoma presenting as a rheumatic syndrome. Semin Arthritis Rheum 2007;37:63–67.
10. Shimada A, Kato M, Tamura K, et al. Hemophagocytic lymphohistiocytosis associated with uncontrolled inflammatory cytokinemia and chemokinemia was caused by systemic anaplastic large cell lymphoma: A case report and review of the literature. J Pediatr Hematol Oncol 2008;30:785–787.
11. Falini B, Pileri S, Zinzani PL, et al. ALK+ lymphoma: Clinico-pathological findings and outcome. Blood 1999;93:2697–2706.
12. Schlette EJ, Medeiros J, Goy A, et al. Survivin expression predicts poorer prognosis in anaplastic large-cell lymphoma. J Clin Oncol 2004;22:1682–1688.
13. Drexler HG, Gignac SM, von Wasielewski R, et al. Pathobiology of NPM-ALK and variant fusion genes in anaplastic large cell lymphoma and other lymphomas. Leukemia 2000;14:1533–1559.
14. Suzuki R, Kagami Y, Takeuchi K, et al. Prognostic significance of CD56 expression of ALK-positive and ALK-negative anaplastic large-cell lymphoma of T/null cell phenotype. Blood 2000;96:2993–3000.
15. Monaco S, Tsao L, Murty VV, et al. Pediatric ALK+ anaplastic large cell lymphoma with t(3;8)(q26.2;q24) translocation and c-myc rearrangement terminating in a leukemic phase. Am J Hematol 2007;82:59–64.
16. Cools J, Wlodarska I, Somers R, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofiblastic tumor. Genes Chromosomes Cancer 2002;34: 354–362.
17. Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994; 263:1281–1284.
18. Jaffe ES. The 2008 WHO classification of lymphomas: Implications for clinical practice and translational research. Hematology Am Soc Hematol Educ Program 2009:523–531.
19. Stone J, de Lange T, Ramsay G, et al. Definition of regions in human c-myc that are involved n transformation and nuclear localization. Mol Cell Biol 1987;7:1697–1709.
20. Evan GI, Wyllie AH, Gilbert CS, et al. Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992;69:119–128.
21. Freytag SO, Dang CV, Lee WMF. Definition of the activities and properties of c-myc required to inhibit cell differentiation. Cell Growth Diff 1990;1:339–343.
22. Herbst A, Hemann MT, Tworkowski KA, et al. A conserved element in Myc that negatively regulates its proapoptotic activity. EMBO Rep 2005;6:177–183.
23. Cowling VH, Chandriani S, Whitfield ML, Cole MD. A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation and G2 arrest. Mol Cell Biol 2006;26:4226–4239.
24. Dukers DF, ten Berge RL, Oudejans JJ, et al. A cytotoxic phenotype does not predict clinical outcome in anaplastic large cell lymphomas. J Clin Pathol 1999;52:129–136.
25. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–921.
26. Cosgrave N, Hill AD, Young LS. Growth factor-dependent regulation of survivin by c-myc in human breast cancer. J Mol Endocrinol 2006;37:377–390.
27. Andréasson U, Dictor M, Jerkeman M, et al. Identification of molecular targets associated with transformed diffuse large B cell lymphoma using highly purified tumor cells. Am J Hematol 2009;84:803–808.
28. Moritake H, Sugimoto T, Asada Y, et al. Newly established clear cell sarcoma (malignant melanoma of soft parts) cell line expressing melanoma-associated Melan-A antigen and overexpressing C-MYC oncogene. Cancer Genet Cytogenet 2002;135:48–56.
29. Seabright M. A rapid banding technique for human chromosomes. Lancet 1971;2:971–972.
30. Chenchik A, Moqadam F, Siebert PA.A new method for full-length cDNA cloning by PCR. In:Krieg PA, editors.Laboratory Guide to RNA: Isolation, Analysis, and Synthesis. New York:Wiley-Liss; 1996. pp 273–321.

Prolonged ^{18}FDG-PET negative complete remission in a heavily pretreated, elderly patient with diffuse large B cell lymphoma treated with lenalidomide, low dose dexamethasone, and colony stimulating factor (Rd-G)

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Diffuse large B-cell lymphoma (DLBCL) is a common lymphoid malignancy among adults in the developed world and accounts for about a third of all patients newly diagnosed with non-Hodgkin lymphoma each year [1]. The prognosis of patients with DLBCL has improved over the past 10 years since the advent of chemoimmunotherapy regimens such as R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) [2,3]. However, a significant number of patients still experience disease relapse or progression after first or second line therapy, and ~40% of patients will die within 5 years [4]. In particular, elderly patients and those ineligible for high-dose chemotherapy due to comorbidities require effective salvage treatment options with favorable toxicity profile. Several novel therapeutic approaches have been proposed for these patients including monoclonal antibodies, radioimmunotherapy, proteasome inhibitors, mTOR inhibitors, and the immunomodulatory drugs such as thalidomide and lenalidomide.

Here we report a case of an elderly female patient with relapsed DLBCL who achieved a sustained complete remission during salvage therapy with a combination of lenalidomide plus low dose dexamethasone and prophylactic G-CSF.

A 71-year-old woman was diagnosed with DLBCL (Unclassified/type 3) [5]. Based on a lymph node biopsy in 2006, the patient was Epstein-Barr virus negative, epithelial membrane antigen negative, B-cell lymphoma (Bcl)-2$^+$, CD20$^+$, CD10$^-$/Bcl-6$^+$, MUM1/IRF4$^+$, CD30$^{-/+}$, CD138$^-$, CD15$^-$, and Ki-67 was expressed in 50% of cells. Further investigations established that the lymphoma was in the Ann Arbor Stage III disease classification. Examinations using a ^{18}FDG-PET/CT scan revealed multiple metabolically active lymphadenopathy in the abdomen and thorax. No lymphoma symptoms (night sweats, weight loss, fever), or bone marrow infiltration was seen and the patient was classified as having an International Prognostic Index (IPI) score of 3 (high-intermediate risk) based on her high serum lactate dehydrogenase levels, age, and stage III disease [6]. Her past medical history was remarkable for hypertension which was managed pharmacologically.

The patient received six cycles of R-CHOP administered every 21-day and achieved a complete response after completion of the treatment course, which was confirmed by a ^{18}FDG-PET/CT scan being negative for pathologic lesions. Subsequently, she experienced disease relapse within 8 months as shown by a diffuse^{18}FDG-PET/CT scan positivity and lymph node biopsy. According to recent study who emphasize the role of immune status of the host at relapse in the final outcome, the Absolute lymphocyte count at relapse (ALC-R) was $>1.0 \times 10^9$ /L [7]. As per routine clinical practice in our institute, the patient was treated with eight cycles of a weekly, third-generation, combination chemotherapy VNCOP-B (cyclophosphamide, mitoxantrone, vincristine, etoposide, bleomycin, and prednisolone) plus rituximab for four cycles. After the treatment the patient obtained a ^{18}FDG-PET/CT scan negativity confirming the second complete remission.

After 10 months of remission, the patient had a second relapse with a ^{18}FDG-PET/CT scan showing diffuse positive lesions (Fig. 1).

A rebiopsy of a lymph node confirmed the histopathologic diagnosis of DLBCL (Unclassified/type 3). Again, no lymphoma symptoms or bone marrow infiltration was present. The IPI score now was 2 (low-intermediate risk) based on the patient's age and Ann Arbour stage III and ALC-R was again $>1.0 \times 10^9$ /L The patient was now considered unsuitable for

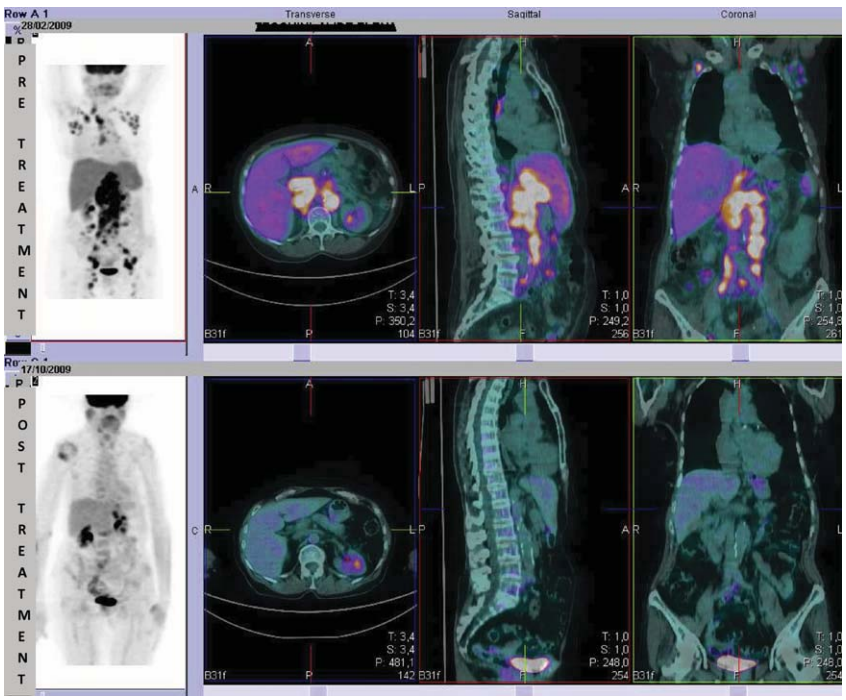

Figure 1. ^{18}FDG-PET/CT scan at baseline (above) and after four cycles of combination treatment with lenalidomide plus low dose dexamethasone (below). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

further polychemotherapy and alternative treatment strategies were explored. As daily oral lenalidomide (25 mg) has demonstrated efficacy in patients with relapsed or refractory DLBCL [8], we started a treatment regimen of lenalidomide 25 mg once-daily on days 1–21 of every 28-day cycle.

A previous study in patients with multiple myeloma demonstrated that lenalidomide plus dexamethasone was more effective than either agent alone [9]. Moreover, a randomized trial in patients with multiple myeloma found that low doses of pulsed dexamethasone was better tolerated and less toxic when used in combination with lenalidomide therapy [10]. Based on these data, we added low doses of dexamethasone (20 mg weekly for 4 weeks) to single agent lenalidomide.

During the first cycle of lenalidomide plus low dose dexamethasone therapy the patient experienced significant neutropenia. Therefore, subcutaneous granulocyte colony-stimulating factor (G-CSF) was administered on days 22–26 of each 28-day cycle. Aspirin (100 mg/day) was given as prophylaxis against deep-vein thrombosis and weekly co-trimoxazole (every Saturday and Sunday) was given as prophylaxis against *Pneumocystis jirovecii* infections. Patient compliance was good and no tumor lysis syndrome or tumor flare reactions were seen. There was a 1-week delay in treatment as a result of neutropenia, but after the introduction of G-CSF adverse events were grade 2 fatigue and grade 1 neutropenia showing that the treatment had low toxicity. After four cycles of treatment with lenalidomide and low dose dexamethasone a ^{18}FDG-PET/CT scan was completely negative for lesions (Fig. 1); after six cycles of treatment (about 6 months) this result was confirmed. Now, about 10 months since documented complete response, the patient remains in remission. Based on tolerance and the convenient oral administration of the drugs, the patient is currently on maintenance treatment with lenalidomide at a lower daily dose (15 mg) plus weekly dexamethasone (10 mg) and prophylactic agents against infection and thrombosis. DLBCL is incurable for patients who experience relapse after first or second line therapy, and are unsuitable for stem cell transplantation because of comorbidities or advanced age. Salvage therapy with lenalidomide has been evaluated in this aggressive non-Hodgkin lymphoma, with an overall response rate ranging from 20 to 35%, and with a low percentage of complete responses [8]. The mechanism of action of immunomodulatory drugs and, in particular, lenalidomide is currently being explored. However, it has been shown that lenalidomide induces enhanced-activity of Th1 immunity and natural killer cell mediated cytotoxicity [11,12]. In vitro models have demonstrated that lenalidomide exerts antiproliferative activity through inhibition the Akt pathway and the upregulation of the *p21* tumor suppressor gene, which leads to cell cycle arrest in the G_1 phase [13–15]. Moreover, lenalidomide has been shown to inhibit T-regulatory cell function and exert antiangiogenic effects on the tumor microenvironment [16,17]. Recently it was reported to promote immune synapse restoration through its effects on malignant B cells and the T-cell microenvironment [18]. It has been shown that the addition of dexamethasone, with its induction of apoptosis via caspase-9, enhances the pro-apoptotic effect of lenalidomide especially as lenalidomide induces apoptosis via caspase-8 [19]. Furthermore, combination lenalidomide pulsed dexamethasone may prevent tumor flare reactions, improve patient compliance, and preserve the antiproliferative effect of lenalidomide, without impairing its immunomodulatory action [20].

The quick response shown by our patient was in agreement with results obtained in a previous study of lenalidomide monotherapy in DLBCL patients in terms of the time to response (about 4 months in our study) [8]. However, the negative for lesions ^{18}FDG-PET/Tc scan, showed that the complete response in this elderly, heavily pretreated patient was of particular interest considering the good compliance with the treatment schedule. Moreover, we can hypothesize that there was synergistic activity between lenalidomide, dexamethasone, and the G-CSF agent via the influence of the G-CSF agent in the production of interleukin (IL)-12 p40 and, in particular, reducing IL-12 p40 and IL-12 (p40/p70) levels, with IL-12 p40 being a strong antagonist of IL-12 p70 immunological activity [21]. This effect might have enhanced lenalidomide antitumor activity by stimulating antitumor immune responses.

To verify these hypothesis, we are planning a prospective, translational study with lenalidomide and low dose dexamethasone, combined with prophylactic G-CSF, in a subset of elderly, heavily pretreated patients.

Acknowledgments

We thank Celgene Italy for providing Revlimid® for compassionate use in this patient.

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Conflict of interest: Nothing to report.

Published online 7 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/ajh.21869

References

1. Anderson JR, Armitage JO, Weisenburger DD. Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project. Ann Oncol 1998;9:717–720.
2. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235–242.
3. Sehn LH, Donaldson J, Chhanabhai M, et al. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. J Clin Oncol 2005;23:5027–5033.
4. Feugier P, Van Hoof A, Sebban C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol 2005;23:4117–4126.
5. Zinzani PL, Dirnhofer S, Sabattini E, Alinari L. Identification of outcome predictors in diffuse large B-cell lymphoma. Immunohistochemical profiling of homogeneously treated de novo tumors with nodal presentation on tissue micro-arrays. Haematologica. 2005;90:341–347.
6. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med 1993; 329:987–994.
7. Porrata LF, Kay R, Habermann TM, et al. Absolute lymphocyte count at time of first relapse predicts survival in patients with diffuse large B-cell lymphoma. Am J Hematol 2009;84:93–97.
8. Wiernik PH, Lossos IS, Tuscano JM, et al. Lenalidomide monotherapy in relapsed or refractory aggressive non-Hodgkin's lymphoma. J Clin Oncol 2008;26:4952–4957.
9. Dimopoulos M, Spencer A, Attal M, et al. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. N Engl J Med 2007;357:2123–2132.
10. Rajkumar SV, Jacobus S, Callander NS, et al. Lenalidomide plus high-dose dexamethasone versus lenalidomide plus low-dose dexamethasone as initial therapy for newly diagnosed multiple myeloma: an open-label randomised controlled trial. Lancet Oncol 2010;11:29–37.
11. Bartlett JB, Dredge K, Dalgleish AG. The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat Rev Cancer 2004;4:314–322.
12. Chang DH, Liu N, Klimek V, et al. Enhancement of ligand-dependent activation of human natural killer T cells by lenalidomide: therapeutic implications. Blood 2006;108:618–621.
13. Gandhi AK, Kang J, Naziruddin S, et al. Lenalidomide inhibits proliferation of Namalwa CSN.70 cells and interferes with Gab1 phosphorylation and adaptor protein complex assembly. Leuk Res 2006;30:849–858.
14. Hideshima T, Chauhan D, Shima Y, et al. Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood 2000;96:2943–2950.
15. Verhelle D, Corral LG, Wong K, et al. Lenalidomide and CC-4047 inhibit the proliferation of malignant B cells while expanding normal CD34+ progenitor cells. Cancer Res 2007;67:746–755.
16. Galustian C, Klaschka DC, Meyer B, et al. Lenalidomide (Revlimid®, CC-5013) and Actimid™ (CC-4047) inhibit the function and expansion of T regulatory (Treg) cells in vitro: Implications for anti-tumor activity in vivo [abstract]. Proc Am Assoc Cancer Res 2006;47:754a [abstract 4882].
17. Lentzsch S, LeBlanc R, Podar K, et al. Immunomodulatory analogs of thalidomide inhibit growth of Hs Sultan cells and angiogenesis in vivo. Leukemia 2003;17:41–44.
18. Witzig TE, Wiernik PH, Moore T, et al. Lenalidomide oral monotherapy produces durable responses in relapsed or refractory indolent non-Hodgkin's lymphoma. J Clin Oncol 2009;27:5404–5409.
19. Richardson PG, Blood E, Mitsiades CS, et al. A randomized phase 2 study of lenalidomide therapy for patients with relapsed or relapsed and refractory multiple myeloma. Blood 2006;108:3458–3464.
20. Gandhi AK, Kang J, Capone L, et al. Dexamethasone synergizes with lenalidomide to inhibit multiple myeloma tumor growth, but reduces lenalidomide-induced immunomodulation of T and NK cell function. Curr Cancer Drug Targets 2010;10:155–167.
21. Toubai T, Tanaka J, Ota S, et al. Effect of granulocyte colony-stimulating factor on IL- 12 p40 production during chemotherapy for B-cell lineage non-Hodgkin's lymphoma patients. Eur J Haematol 2006;77:403–409.

Multiple myeloma and pregnancy

Agustin Avilés[1]* and Natividad Neri[2]

Pregnancy has been reported in patients with hematological malignancies, such as acute leukemia, Hodgkin and malignant lymphoma and chronic myelocytic leukemia. Only 12 cases of pregnancy occurring in patients with multiple myeloma (MM) have been reported. The present report describes 6 additional cases of this rare association that received chemotherapy during pregnancy, including in the first trimester. The newborns were 3 male and 3 female with weight >2500 g and without evidence of fetal malformations. Longer follow-up (>3 years) did not give evidence of late complications in the children. Three mothers received stem cell transplantation.

Multiple myeloma (MM) is a malignant disease characterized by an abnormal proliferation of plasma cells, with a mean age at time of diagnosis of 62 years (1,2) . It is a disease affecting elderly people, more often men between 50 to 70 years. Thus the presence of pregnancy during MM is very rare; until now, only 13 cases of this association have been reported (2–16). Most of these were reported as single cases, without longer follow-up of mother and newborn; thus, adequate treatment has not been defined and the effect of pregnancy on the disease process has remained unknown until now.

Between 1992 and 2006, we diagnosed and treated 6 cases of MM associated with pregnancy, In this report we describe the specific treatment given during pregnancy, and the longer follow-up results and compare our cases with the patients reported in the literature.

In five cases MM was diagnosed before pregnancy, and because all were symptomatic of myeloma, we began treatment with different therapeutic schedules according to the time of diagnosis. Interferon is not considered useful in the treatment of MM, but when case 3 was diagnosed, this drug was common in therapeutic decisions. The combination of interferon and all trans retinoic acid is not common in MM either, but between 1998 to 2005 this combination was the first line of treatment in our Institution with good results (17). One case was diagnosed concomitant with the pregnancy, but she had an aggressive disease with severe anemia, renal insufficiency, fracture of the femur and vertebral collapse, thus the decision was to start treatment. Taking into consideration that all patients had bone lesions in different body parts, a cesarean was performed in all patients for delivery. Chemotherapy was stopped 3 to 4 weeks before the delivery to avoid presence of hematological toxicity in the newborn.

During pregnancy, an expert gynecologist followed the mothers and they received nutrimental support.

All newborns were carefully examined for any congenital abnormalities, and laboratory tests were performed: complete blood and platelets counts , careful review of blood smears, serum chemistry, determinations of immunoglobulin (and if they showed any abnormalities: we performed protein electrophoresis), and cytogenetic studies (18,19). The children were reviewed every three months for the first two years, every 6 months for the following 5 years, and every year until the last follow-up (December 2008) or when they were >21 years old. If they presented clinical indications, additional studies were performed. Laboratory tests were performed at each medical evaluation. At 3, 6 and 12 years psychological and neurological evaluation were performed. Academic progress was monitored by an medical assistant and the corresponding teachers.

The study was begun in 1988 (HO-88-010) to evaluate cancer and pregnancy, including long term follow-up. Parents gave their informed consent to participate in the study and also the studies in the children when corresponding children were <7 years of age and assenting to participate in the analysis.

Table I shows the main clinical characteristics of pregnancy and MM, including the present 6 cases. Different schedules of chemotherapy were administered according to the time of diagnosis. Cases 3, 4 and 6 after chemotherapy achieve complete remission and were allocated to received autologous stem cell transplant (ASCT), 6 to 9 months after delivery. All are alive and completing responding 7, 7 and 2 years after pregnancy. Case 4 died early with active MM. Cases 1 and 2 were treated with different chemotherapeutic combinations, case 1 achieved complete remission, and case 2 achieved partial remission, but they showed progression disease and died 4 and 6 years after pregnancy, respectively.

At delivery, all newborns showed an Apgare score >9, weight was normal, according to the specific week of pregnancy; and in all cases, no congenital abnormalities were observed, and all laboratory tests were negative. During the follow-up the physical, neurological and psychological development of newborns were considered normal. All were alive, without any evidence of any cancer. The placenta was examined in cases 2–6, in all cases without evidence of infiltration.

Pregnancy in the presence of MM occurs rarely, only 15 cases have been reported in the world literature. Haster reported two additional cases diagnosed after delivery (15). In reviewing the literature we found that 7 cases were diagnosed during second and third trimester of pregnancy and 4 during the first trimester. In most cases the use of chemotherapy was considered dangerous to the fetus and they did not receive any specific therapy. Two patients received urethane and interferon during the first trimester; but this was stopped when pregnancy was confirmed. One mother received radiotherapy 1.5 G to the lumbar area and urethane 2 g/day during the first 3 months of pregnancy. Two mothers received cyclophosphamide during the second and third trimester. In the present cases, five were diagnosed during the first and second trimester, when treatment for MM as already underway, because the pregnancy had not been suspected. Taking into consideration that chemotherapy was given during early pregnancy we considered the possibility of fetal damage, but careful evaluation did not show any evidence of fetal damage, Thus, according to the parents wishes we decided to continue the same treatment, (moreover three patients were candidates to SCT). (Table I).

The newborns reported in the present paper and literature review were normal, no congenital abnormalities were observed and five children were noted alive 2 to 6 years after birth, but 7 children were not mentioned in any follow-up. One child showed an elevation of gamma-globulin, but it disappeared at 6 months; further studies were not mentioned. Although children were exposed to chemotherapy in utero, including in two cases all trans retinoic acid, all were normal with and remain no evidence of any physical or psychological alteration, with normal physical development and scholar degree appropriate to their ages. Based in this small number of cases, it appears that the use of chemotherapy regimens that were employed as cytoreductive therapy before ASCT did not affect the fetal development and survival.

Methods

Records for the Oncology Hospital were reviewed, and women with diagnosis of MM and pregnancy were considered in the present study.

Diagnosis of MM was established according to the criteria at time of diagnosis; all patients have >20% plasmoblasts bone marrow identified with immunoperoxidase technique, presence of abnormal immunoglobulin peak, determination of light-chains in blood and urine, lytic lesions and evidence of organ-damage secondary to neoplasm (hypercalcemia, anemia, renal insufficiency or bone lesions), and determination of beta 2 microglobulin. Retrospectively, all patients will be considered at high-risk and symptomatic of MM according to the most recent criteria. From 1988, all women with a diagnosis of cancer in our hospital haven an immunological test to determine if they were pregnant before they began any specific treatment. In our population, three patients have intrauterine devices, two patients have oral contraception and one woman preferred condom use. Also, all women of fertile age signed a statement that they and their partner continue with the contraceptive measures during the treatment of neoplasm.

TABLE I. Multiple Myeloma and Pregnancy

Ref	Age (years)	Trimester at diagnosis	Treatment during pregnancy (trimester of pregnancy)	Delivery (week/weight)	Current status Mother/Newborn	
3	40	2°	Cy, 800 mg (total dose) (1°)	(38/3000)	NA	alive, 3 months
4	35	1°	RT, 1.5 G, lumbar spine Urethane, 2 g/day (1°)	(38/3100)	dead, 3 months	NA
5	42	1°	Urethane, 2 g/day, 1 month (1°)	(38/NA) (1°)	dead, 6 months	alive, 6 months
6	38	3°	None	(35/NA)	dead (?)	NA
7	21	2°	Cy, 50 mg/day, until delivery	(39/2523)	alive 3 months	alive, 3 months
8	32	3°	None	(38/3000)	alive 2 months	alive 2 months
9	33	2°	None	(36/NA)	alive 2 months	alive 2 months
10	41	1°	IFN, 3.0 MU, three times a week for 2 months (1°)	(38/NA)	alive (?)	NA
11	34	1°	None	(38/3000)	alive (?)	alive (?)
12	41	3°	None	(32/2270)	alive 2 months	NA
13	32	3°	None	(36/2210)	NA	NA
14	39	3°	None	(32/NA)	alive 1 month	NA
15	32	1°	None	(NA/NA)	die (NA)	NA
Present Series						
	32	1°	CMOP (6) MP (2)	(36/2900)	dead (6[a])	alive 19[a]
	37	2°	CMOP-D (3) MP (3)	(38/3100)	dead (4[a])	alive 15[a]
	24	1°	CMOP-I (3) MP (5)	(33/2850)	alive 10[a]	alive 10[a]
	35	1°	DAI (6), MP (5)	(34/2500)	alive (7[a])	alive 10[a]
	39	2°	DAI (6)	(38/2750)	alive (5[a])	alive 5[a]
	32	3°	CMOP (6)	(39/3050)	alive (4[a])	alive 4[a]

Cy, cyclophosphamide; IFN, interferon alfa 2B; RT, radiotherapy; CMOP, cyclophosphamide, melphalan, vincristine, prednisone; MP, melphalan, prednisone; CMOP-D, CMOP+doxorubicine; CMOP-I, CMOP+interferon; DAI, dexamethasone; all trans-retinoic acid and interferon (number of cycles administered); NA, not noted.
[a]Number of years after delivery.

Acknowledgments

To Allison McPhee MD for reviewing the manuscript for language.

Contribution for Authirship

Both authors made substantial contributions to the concept design of study and to the acquisition and analysis of data. The article was critically reviewed for important intellectual content. Final approval version was given for both authors.

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Conflict of interest: The authors disclose any conflict of interest.
The work did not receive any external financial support.
Published online 16 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21876

References

1. Kyle RA, Rajkumar SV. Criteria for diagnosis, staging, risk and response assessment of multiple myeloma. Leukemia 2009;23:3–9.
2. Bladé J, Kyle RA. Multiple myeloma in young patients. Leuk Lymphoma 1998; 30:493–501.
3. Giordano C. Mieloma multiplo e gravidez (premeira observacao da casuistica mundial). Matern Infanc (San Paulo) 1965;24:158–164.
4. Kusuva LA, Schwartz SO. Multiple myeloma and normal pregnancy. Blood 1966;28:102–111.
5. Rosner F, Soong BC, Krim M, Miller SP. Normal pregnancy in a patient with multiple myeloma. Obstet Gynecol 1968;31:811–820.
6. Talerman AM, Serjeant GR, Milner PF. Normal pregnancy in a patient with multiple myeloma and sickle cell anemia. West Indian Med J 1971;20:97–100.
7. Lergier JE, Jimenez E, Maldonado N, Veay F. Normal pregnancy in multiple myeloma treated with cyclophosphamide. Cancer 1974;34:1018–1022.
8. Malee MP. Multiple myeloma in pregnancy. Obstet Gynecol 1990;75:513–515.
9. Caudle MR, Dodd S, Solomon A. Multiple myeloma and pregnancy. Obstet Gynecol 1990;75:516–518.
10. Sakata H, Karamitsos J, Kundara B, Disai PJ. Case report of interferon alpha therapy for multiple myeloma during pregnancy. Am J Obstet Gynecol 1995;172:217–219.
11. Maglione AC, DiGiorgi U, Petrozelli F, Longo MP. Multiple myeloma diagnosed during early pregnancy. Eur J Obstet Gynecol 2003;111:214–215.
12. Fortham CL, Ponce BA, Mankin HJ. Multiple myeloma with an pathological fracture during pregnancy. J Bone Joint Surg 2004;86A:1284–1287.
13. Zun KH, Choi HM. Multiple myeloma presenting as vertebral compression during pregnancy. Int J Gynaecol Oncol 2008;101:89–90.
14. Quinn J, Rabin N, Rodriguez-Justo M, Kwee YBC. Multiple myeloma presenting with spinal cord compresión during pregnancy. Ann Hematol 2009;88:181–182.
15. Harster GA, Krause JR. Multiple myeloma in two young postpartum women. Arch Pathol Lab Med 1987;111:38–46.
16. Lee JC, Francis RS, Smith S, Lee R, Bingham C. Renal failure complicating myeloma in pregnancy. Nephrol Dial Transplant 2007;22:3652–3655.
17. Avilés A, Nambo MJ, Neri N. Biological modifiers as cytoreductive therapy before stem cell transplant in previously untreated patients with multiple myeloma. Ann Oncol 2005;16:219–221.
18. Avilés A, Neri N. Hematological malignancies and pregnancy. Clin Lymph 2001;2:175–177.
19. Avilés A. Hematological malignancies and pregnancy. Rev Recent Clin Trials 2009;4:131–139.

Pain management in children and adolescents with sickle cell disease

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In a cohort of 2,194 children with sickle cell disease (SCD) treated in community-based services, we explored the types of medications used to treat vaso-occlusive (VOC) pain episodes, and the relative effectiveness of nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and adjunctive antidepressants or anticonvulsant medications on reducing acute VOC pain visits over time. Pharmacologic treatments for VOC pain consisted mainly of NSAIDs and weak opioids. Significantly more patients with more than 3 inpatient or ER VOC pain visits during their first year of SCD treatment were prescribed stronger opioids, SSRIs, SNRI/heterocyclics, and anticonvulsants. Prescription of both stronger opioids and SSRI antidepressants or anticonvulsants was significantly associated with lower cumulative rates of acute VOC pain visits over time. Using an observational study design and existing clinical data, these findings are intended to illustrate the potential clinical advantages of combining adjunctive antidepressants or anticonvulsants with primary pain medications for relief of acute VOC pain over time.

TABLE I. Descriptive Analyses of 2,194 SCD Cohort

Independent variable	SCD Cohort
Ethnicity	
African American	1305 (59.5%)
Non African American[a]	889 (40.5%)
Gender	
Female	1041 (47.4%)
Male	1153 (52.6%)
Mean age at SCD diagnosis as documented in Medicaid	5.7 (SD = 5.3)
Years in Medicaid	7.2 (SD = 3.1)
Percent receiving care at specialty clinic	13.4 (SD = 14.9)
SCD VOC pain treatments	
Mean blood transfusions per year	1.6 (SD = 1.9)
Prescribed hydroxyurea: Yes	209 (9.5%)
Adenotonsillectomy performed: Yes	256 (11.7%)
Prescribed NSAID: Yes	740 (33.7%)
Prescribed weak opioid: Yes	1477 (67.3%)
Prescribed stronger opioid: Yes	494 (22.5%)
Prescribed SSRI antidepressant: Yes	122 (12.0%)
Prescribed SNRI/heterocyclic antidepressant: Yes	111 (10.9%)
Prescribed anticonvulsant: Yes	74 (3.4%)

[a]Hispanic, Middle Eastern, Asian Indian, or mixed.

TABLE II. Vaso-Occlusive Pain Severity During First Year of SCD Treatment

Medication Prescribed[a]	VOC Pain First Year Severity–Low (*N* = 1972; 89.9%)	VOC Pain First Year Severity–High (*N* = 222; 10.1%)	*P*-value
NSAIDs	572 (29.0%)	168 (75.7%)	<0.0001
Weak Opioids	1258 (63.8%)	219 (98.7%)	<0.0001
Stronger Opioids	342 (17.3%)	152 (68.5%)	<0.0001
SSRIs	93 (4.7%)	29 (13.1%)	<0.0001
SNRI/heterocylics	91 (4.6%)	20 (9.0%)	0.005
Anticonvulsants	48 (2.4%)	26 (11.7%)	<0.0001

[a]Patients could be prescribed more than 1 medication.
Low = 0–3 pain visits; High = >3 pain visits and Pain Treatments Received.

In children with SCD, erythrocytes become deoxygenated, dehydrated, and crescent-shaped, and tend to aggregate or stick to blood vessel walls, blocking blood flow in bones and organs, and causing recurring, VOC pain episodes [1]. With recurring acute VOC pain episodes, severe SCD represents a chronic pain condition [2]. For effective long-term management, pharmacological interventions for acute VOC pain [3] could be combined with adjunctive interventions for chronic pain [4].

The chronology of pain in children with SCD includes eight phases [3]. The first three phases involve specific pain sites (e.g., dactylitis), prodromal signs of VOC pain, and an early, mild form of VOC pain. A mild oral analgesic may be given (e.g., acetaminophen), and NSAIDs may be effective in reducing bone and joint pain. Phase 4 is characterized by pain accelerating to a moderate level, interfering with daily activities, and requiring stronger oral analgesics (e.g., weak opioids [codeine, hydrocodone]). In Phase 5, VOC pain increases to a severe level and plateaus for an extended time period, requiring hospitalization and intravenous (IV) opioids (e.g., morphine, oxycodone). The acute phase of VOC pain episodes may last 3–5 days. The VOC pain starts to decrease in Phase 6 and in Phase 7 decreases rapidly, as treatment with of IV analgesics is gradually decreased and the use of sustained-release oral opioids is initiated. In Phase 8, VOC pain is resolved or decreased to a "tolerable level," treated at home, using mild to moderate strength analgesics. Some patients may require "breakthrough" oral opiates (e.g., morphine elixir).

Hydroxyurea (HU) has been shown to reduce episodes of VOC pain in observational and randomized controlled trials [5–10], and regular blood transfusions are used for prevention of recurrent pain in patients who have not responded to HU [2,4]. Pharmacotherapies for chronic pain include agents from multiple drug classes (simple analgesics, NSAIDs, opioids, anticonvulsants, and antidepressants) [4]. The tricyclic antidepressants [11–13] as well as the serotonin norepinephrine reuptake inhibitors or heterocyclic (SNRI/ heterocyclic) antidepressants venlafaxine, bupropion, and duloxetine [14–17] are efficacious in the management of chronic pain. The SNRI/ heterocyclics produce fewer side effects in children and adolescents and, when used as adjunctive analgesics, they effectively improve patient overall quality of life [11]. When used solely for pain management, the selective serotonin reuptake inhibitors (SSRIs) have either been less robust (i.e., paroxetine, citalopram) or lacked any efficacy at all (i.e., fluoxetine) [11,18]. Two anticonvulsants, gabapentin and pregabalin, are backed by the strongest evidence as analgesics for multiple types of pain [18] but others may be used. No systematic studies of the combined effects of these classes of agents on VOC pain were found in the literature.

TABLE III. Effectiveness of Pain Treatments in Reducing VOC Pain Visits Over Time

Predictor	Estimated Coefficient	95% Confidence Interval	*P*-value
Adenotonsillectomy	−0.21	(−0.42, −0.01)	<0.0001
Transfusions per year	0.17	(0.07, 0.26)	0.0003
Percent receiving specialty care	0.01	(0.009, 0.02)	<0.0001
Amount NSAIDs prescribed per year	0.01	(0.004, 0.02)	0.007
Amount weak opioids prescribed per year	0.08	(0.07, 0.10)	<0.0001
Prescribed stronger opioids and SSRIs	−0.02	(−0.04, −0.01)	0.0009
Prescribed stronger opioids and anticonvulsants	−0.0002	(−0.0003, −0.0001)	<0.0001

This pediatric SCD cohort was 60% African American, 53% male, entered into the Medicaid data set at 5–6 years of age, and remained in the data set for about 7 years. Most were prescribed NSAIDs or weak opioids (Table I). The majority of patients treated with NSAIDs were taking ibuprofen, motrin, and naproxen. The weak opioids prescribed were codeine/acetominophen and hydrocodone. The stronger opioids prescribed were morphine and oxycodone. Those prescribed SSRIs were taking citalopram, escitalopram, paroxetine, fluoxetine, or sertraline. Prescribed SNRI/heterocyclic agents were mainly venlafaxine, mirtazapine, bupropion, or duloxetine. The primary anticonvulsants prescribed were carbamazepine, valproic acid, gabapentin, or phenytoin, with fewer patients receiving pregabalin (Table I). Ten percent of the pediatric SCD cohort had more than three acute VOC pain visits during their first year of SCD treatment, and significantly more of these were prescribed stronger opioids, SSRIs, SNRI/heterocyclics, and anticonvulsants (Table II). As shown in Table III, only those children prescribed both stronger opioids and SSRI antidepressants or anticonvulsants had significantly lower cumulative rates of acute VOC pain visits over time, controlling for other interventions, which could impact VOC pain.

Most of these psychotropic medications have infrequent but potentially important hematologic side effects or may interact with the anticoagulants used in medically ill patients [19]. The SSRIs citalopram, paroxetine, fluoxetine, and sertraline as well as some SNRIs may inhibit platelet function and are associated with an increased risk of bleeding complications (e.g., gastrointestinal bleeding, ecchymoses, epistaxis, hematomas, hemorrhage) or bruising, especially with the concomitant use of aspirin or NSAIDs [19]. Citalopram is associated with leukocytosis and leukopenia, whereas sertraline is associated with thrombocytopenia; both are associated with the development of anemia [19]. The SNRI/heterocyclic agents, venlafaxine, mirtazapine, and bupropion, are associated with leukopenia. Furthermore, venlafaxine is associated with the development of anemia and leukocytosis, and mirtazapine is associated with the development of anemia, eosinophilia, agranulocytosis, pancytopenia, and thrombocytopenia, whereas duloxetine is only associated with bruising and bleeding [19]. The anticonvulsants, carbamazepine, and phenytoin, carry an increased risk of agranulocytosis, leukopenia, and thrombocytopenia, carbamazepine is also associated with the development of anemia, eosinophilia, and leukocytosis, whereas valproic acid is associated with pure red cell aplasia (as is carbamazepine) and thrombocytopenia. Gabapentin is associated only with leukopenia and neutropenia [19].

While this SCD cohort represents a large, heterogeneous group of children and adolescents, and the long-term observational study provides information regarding important clinical interventions and their impact on pediatric VOC pain, the data were not gathered using a prospective, controlled design and structured clinical research interviews were not employed to confirm any of the assigned medical conditions. Previous studies have shown that although Medicaid databases provide much less detailed information on individuals and care than primary data collection, physician diagnoses and

utilization data correspond to clinical medical record reviews in the majority of the cases [20,21]. Furthermore, these results report associations and, as a result, directions of causality cannot be inferred. Pediatric SCD patients who dropped out of treatment or were periodically ineligible for Medicaid coverage are not represented in this data set and their outcomes may differ from those patients who remained in Medicaid over time.

Practitioners will need to evaluate the individual benefit-risk ratio of combining analgesics with antidepressants or anticonvulsants in SCD children and adolescents with severe pain, rather than solely increasing the opioid dose [22], and realize that none of these psychotropic agents is FDA-approved for pain management in children and adolescents. Controlled trials regarding the adjunctive use of psychotropics for VOC pain relief are needed to determine which agent combinations are safe, effective, improve quality of life, and reduce the personal and payer burden associated with multiple acute VOC pain episodes per year [23–25].

Materials and Methods

Medical and pharmacy claims for the calendar years January 1, 1998 through December 31, 2006 were used to identify a cohort of child and adolescent patients (ages 17 and under) enrolled in and eligible for Medicaid for a minimum of 9 months in each calendar year included in this analysis, who had a service encounter with a diagnosis of SCD (thalassemia and SC trait were not included). The selection date was the date of the first encounter in which the SCD diagnosis was documented in the Medicaid system. No information was available on their treatments before this start date. This procedure yielded a total $N = 2194$ pediatric SCD cases during the 11-year period. Medicaid medical claims were used to identify a service encounter, date of service, and the International Classification of Diseases, 9th Clinical Modification diagnosis codes related to that visit. Pharmacy claims identified the medication dispensed, and the date the prescription was filled. This study was approved by the University of South Carolina Institutional Review Board as exempt from human subject research guidelines (45 Code of Federal Regulations part 46).

Primary or secondary diagnosis codes for VOC inpatient or ER pain visits and for blood transfusions, an adenotonsillectomy, and the percent of services received from an SCD specialty clinic were obtained from the Visits file. Prescriptions of HU, NSAIDs, weak and stronger opioids, anticonvulsants, and antidepressants were coded from the Pharmacy file. The analgesics examined were prescribed acetaminophen, NSAIDS (naproxen, ibuprofen, motrin, etc.), weak opioids (codeine or hydrocodone, with or without acetaminophen), or stronger opioids (morphine or oxycodone, with or without acetaminophen). Antidepressants were categorized as SSRIs for citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline, or SNRIs/heterocyclics for bupropion, duloxetine, maprotiline, mirtazapine, nefazodone, trazodone, or venlafaxine. Anticonvulsants were coded for gabapentin, pregabalin, carbamazepine, valproic acid, and phenytoin. The SSRI and SNRI/heterocyclic antidepressants were prescribed for diagnosed depression, and the anticonvulsants were prescribed for seizures, not for the treatment of VOC pain.

To examine the relative impact of pain medication categories over time on frequency of acute pain episodes, a negative binomial regression model (for non-normally distributed count data; PROC GENMOD facility in SAS version 9.1; SAS Institute, Cary, NC) was employed to calculate a ratio of the log rate of VOC acute pain visits per total years in the Medicaid data set with prescribed NSAIDs, opioids, antidepressants, or anticonvulsants as the independent variables. Individual risk factors (age, gender, race), receipt of HU or an adenotonsillectomy (dichotomously coded as yes/no), which might be associated with hypoxic episodes correlating with the onset of pain crises [26,27], mean number of blood transfusions per year, and the percentage of SCD services received from a specialty clinic for SCD were used as control variables to explain differences in total pain episodes. Interaction terms between the strong opioids and antidepressants or anticonvulsants were used to examine the combined effect of these medications. Resulting model estimates, 95% confidence intervals, and *P*-values are reported for statistical significance.

Acknowledgment

Data analysis was supported by the University of South Carolina School of Medicine Departments of Neuropsychiatry, Pediatrics, and Internal Medicine.

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Conflict of interest: The views expressed do not necessarily represent those of the funding agency or official findings of the South Carolina, Department of Health and Human Services (Medicaid).

Published online 16 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/ajh.21873

References

1. Brawley OW, Cornelius LJ, Edwards LR, et al. National Institutes of Health Consensus Development Conference Statement: Hydroxyurea treatment for sickle cell disease. Ann Intern Med 2008;148:932–938.
2. Howard J, Thomas VJ, Rawle HM. Pain management and quality of life in sickle cell disease. Expert Rev Pharmacoecon Outcomes Res 2009;9:347–352.
3. DeBaun MR, Vichinsky E. Hemoglobinopathies. In Kliegman RM, Behrman RE, Jenson HB, Stanton B, editos. Nelson Textbook of Pediatrics, 18th ed. Philadelphia, PA:WB Saunders Elsevier; 2007. Available at http://www.mdconsult.com/das/book/body/143898456-9/853444084/1608/1067.html#4-u1.0-B978-1-4160-2450-7..50464-3--cesec19_8724. Accessed June 17, 2009.
4. Chambliss CR, Heggen J, Copelan DN, Pettignano R. The assessment and management of chronic pain in children. Paediatr Drugs 2002;4:737–746.
5. Ferster A, Vermylen C, Cornu G, et al. Hydroxyurea for treatment of severe sickle cell anemia: A pediatric clinical trial. Blood 1996;88:1960–1964.
6. Kinney TR, Helms RW, O'Branski EE, et al. Safety of hydroxyurea in children with sickle cell anemia: Results of the HUG-KIDS study, a phase I/II trial. Pediatric Hydroxyurea Group. Blood 1999;94:1550–1554.
7. Strouse JJ, Lanzkron S, Beach MC, et al. Hydroxyurea for sickle cell disease: A systematic review for efficacy and toxicity in children. Pediatrics 2008;122:1332–1342.
8. Ferster A, Tahriri P, Vermylen C, et al. Five years of experience with hydroxyurea in children and young adults with sickle cell disease. Blood 2001;97:3628–3632.
9. Hankins JS, Ware RE, Rogers ZR, et al. Long-term hydroxyurea therapy for infants with sickle cell anemia: The HUSOFT extension study. Blood 2005;106:2269–2275.
10. Hoppe C, Vichinsky E, Quirolo K, et al. Use of hydroxyurea in children ages 2 to 5 years with sickle cell disease. J Pediatr Hematol Oncol 2000;22:330–334.
11. Jackson KC, St. Onge EL. Antidepressant pharmacotherapy: Considerations for the pain clinician. Pain Pract 2003;3:135–143.
12. Sharp J, Keefe B. Psychiatry in chronic pain: A review and update. Curr Psychiatr Rep 2005;7:213–219.
13. Gallagher RM. Management of neuropathic pain: Translating mechanistic advances and evidence-based research into clinical practice. Clin J Pain 2006;22:S2–S8.
14. Wolfe GI, Trivedi JR. Painful peripheral neuropathy and its nonsurgical treatment. Muscle Nerve 2004;30:3–19.
15. Sullivan MD, Robinson JP. Antidepressant and anticonvulsant medication for chronic pain. Phys Med Rehabil Clin N Am 2006;17:381–400.
16. Jann MW, Slade JH. Antidepressant agents for the treatment of chronic pain and depression. Pharmacotherapy 2007;27:1571–1587.
17. Chong MS, Brandner B. Neuropathic agents and pain: New strategies. Biomed Pharmacother 2006;60:318–322.
18. Kroenke K, Krebs EE, Bair MJ. Pharmacotherapy of chronic pain: A synthesis of recommendations from systematic reviews. Gen Hosp Psychiatry 2009;31:206–219.
19. Becker M, Axelrod DJ, Oyesanmi O, et al. Hematologic problems in psychosomatic medicine. Psychiatr Clin North Am 2007;30:739–759.
20. Hennessy S, Leonard CE, Freeman CP, Deo R, Newcomb C, Kimmel SE, Strom BL, Bilker WB. Validation of diagnostic codes for outpatient-originating sudden cardiac death and ventricular arrhythmia in Medicaid and Medicare claims data. Pharmacoepidemiol Drug Saf 2010;19:555–562.
21. Chibnik LB, Massarotti EM, Costenbader KH. Identification and validation of lupus nephritis cases using administrative data. Lupus 2010;19:741–743.
22. Darbari DS, Minniti CP, Rana S, van den Anker J. Pharmacogenetics of morphine: Potential implications in sickle cell disease. Am J Hematol 2007;83:233–236.
23. Lanzkron S, Haywood C Jr, Segal JB, Dover GJ. Hospitalization rates and costs of care of patients with sickle-cell anemia in the state of Maryland in the era of hydroxyurea. Am J Hematol 2006;81:927–932.
24. Carroll CP, Haywood C Jr, Fagan P, Lanzkron S. The course and correlates of high hospital utilization in sickle cell disease: Evidence from a large, urban Medicaid managed care organization. Am J Hematol 2009;84:666–670.
25. Ballas SK. The cost of health care for patients with sickle cell disease. Am J Hematol 2009;84:320–322.
26. Ijaduola GT, Akinyanju OO. Chronic tonsillitis, tonsillectomy and sickle cell crises. J Laryngol Otol 1987;101:467–470.
27. Maddern BR, Reed HT, Ohene-Frempong K, Beckerman RC. Obstructive sleep apnea syndrome in sickle cell disease. Ann Otol Rhinol Laryngol 1989;98:174–178.

Examining the characteristics and beliefs of hydroxyurea users and nonusers among adults with sickle cell disease

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The attitudes of patients with sickle cell disease (SCD) toward the use of hydroxyurea (HU) therapy may contribute to the underutilization of HU in the United States, yet our understanding of these attitudes is limited. We examined the attitudes and beliefs of 94 adult SCD patients, comparing those who never used HU ($n = 37$), formerly used HU ($n = 23$), and were currently using HU ($n = 34$). Seventy percent of current HU users reported some level of improvement from the drug ("average" or "very much") and 80% reported little or no trouble from side effects. Fifty-seven percent of former users reported taking HU for less than 6 months, with "doctor's recommendation," or "not liking the way it made me feel" given as the most commonly reported reasons for stopping HU. Fifty percent of the never users reported receiving no information about HU from any source, and 85% of the never users thought that they would receive no improvement if they were to take HU. A deeper understanding of patient perspectives toward HU utilization is required as part of multipronged efforts to combat its underutilization in the treatment of SCD.

Sickle cell disease (SCD) is a serious, chronic, genetic disease that leads to the production of abnormal hemoglobin and is marked by many complications, including recurrent episodes of severe pain. Despite 100 years of SCD being a recognized clinical entity in Western medical literature, hydroxyurea (HU) is currently the only approved disease-modifying medication available for the treatment of SCD.

HU has been shown to be efficacious in the treatment of SCD [1]. Studies also suggest that treatment with HU may reduce the costs of health care for SCD, improve health outcomes, and decrease early mortality [2–10]. A recent independent panel convened by the National Heart, Lung, and Blood Institute concluded that the benefits of HU therapy for SCD outweigh its known risks [11–14].

Unfortunately, despite its potential benefits, the clinical literature suggests that HU is underutilized as a treatment for SCD. Although multiple studies have examined provider-level barriers to HU use, evidence on SCD patient beliefs and attitudes about HU is limited [15,16]. Lack of patient knowledge about HU, misperceptions about its side effects, and poor health literacy are hypothesized as patient-level barriers [17]. Hankins et al. found that the perceived safety and efficacy of therapies were shown to be the two most important factors affecting treatment preference among parents and their children with SCD [16]. The aim of our study was to address the gap in current knowledge regarding SCD patient attitudes and beliefs about HU by examining surveys administered to adults with SCD who were taking part in a larger study assessing their experiences with health care.

Ninety-four adult patients (age 18 years and older) participated in our study and provided data about their HU utilization and attitudes. The distribution of HU use was as follows: 39% never users, 24% former users, and 36% current users. Table I describes the characteristics of our sample by HU use. Compared with those with current or former use, the never users had fewer sickle cell-related comorbidities and were less likely to report being on disability. When combined, those with current or former use experienced more hospital visits in the prior 12-month period than the never users (mean visits = 3.7 vs. 2.0, $P = 0.054$).

Seventy percent of current HU users reported some level of improvement from the drug ("average" or "very much"), and 80% reported "not at all" or "not much" trouble from side effects. Approximately, 83% of the former users reported taking HU for 1 year or less, with 57% taking HU for less than 6 months. The most commonly reported reasons given for stopping HU were their "doctor's recommendation" (39%) and "not liking the way it made me feel" (35%). Thirty-six percent of the never users reported that their doctors had previously suggested that they take HU. Fifty percent of the never users reported receiving no information about HU from any source. Eighty-five percent of the never users thought that they would receive no improvement if they were to take HU.

Thirty-three respondents (eight never users, nine former users, and 16 current users) described their concerns about HU in an open-ended manner. We labeled these concerns as representing three general categories (Table II): no perceived benefit, lack of knowledge, and side effects. Examples of specific concerns raised in each category are as follows:

No perceived benefit

Some respondents raised concern over a lack of perceived benefit from HU, or raised doubts about its effectiveness:

- "I am concerned about why it didn't work, and I continued to experience pain crises."
- "Not sure if it works well because it's been 4 months and I'm still getting sick."
- "I am concerned that I still have crises and it has not helped keep me out of the hospital."

Lack of knowledge

Respondents expressed a lack of general knowledge concerning HU, or what type of medication it is:

- "Is HU a blood thinner?"
- "I have never heard about it."
- "I would like to know more about it."

Side effects

The majority of respondents raised concerns regarding perceived or potential side effects from HU:

- Nonspecific side effects: "I am concerned about the side effects."
- General safety: "There is no safety information past 10 years."
- Reproductive effects: "I was told that HU causes birth deformities."
- Cancer concerns: "My concerns are that it could lead to cancer, and it is dangerous for our bone marrow, it could harm us."

Efforts to understand and overcome the barriers to the use of HU in treating SCD are an important area of inquiry. In this study, we sought to describe some of the characteristics, attitudes, and beliefs about HU among adult SCD patients. Nearly 83% of respondents who were categorized as former users of HU reported being on the drug for 1 year or less, with 56.5% being on it for less than 6 months. Many of these former users (39%) reported that they stopped because their doctor suggested it. We were unable to discern the reason why a patient's doctor may have suggested that they stop taking HU. It is possible that the patient was told to temporarily stop taking HU after experiencing some of the well-known short-term toxicities of the drug, such as leukopenia. A patient may also be told to stop taking HU if that patient demonstrates an inability or unwillingness to comply with the treatment regimen. In their studies of provider barriers to the use of HU for SCD patients, both Zumberg et al. and Lanzkron et al. found that provider concerns over patient compliance with the HU treatment regimen were the most endorsed reasons for not prescribing HU when it is otherwise indicated [18,19].

Thirty-five percent of the former users in our study reported that they stopped taking HU because they did not like the way that it made them feel, whereas the majority (80%) of current HU users in our study reported little to no difficulties from side effects. It is possible that these are self-selecting groups. That is, the former users may have experienced more short-term toxicities from HU, on average, than the current users. It is known that a clinical response from HU can take a minimum of 3–6 months to manifest. It is possible that our former users did not stay on HU long enough to experience a clinical benefit from the treatment which may have outweighed any short-term difficulties they may have experienced. This suggests the need for adequate patient

TABLE I. Patient Characteristics by Self-Reported Hydroxyurea Use

Patient characteristics	Hydroxyurea use: Never (n = 37)	Former (n = 23)	Current (n = 34)	P-value
Age, mean(sd)	32.7 (11.9)	32.9 (8.8)	33.3 (10.1)	0.972
Female	59.5%	65.2%	52.9%	0.646
Sickle cell type				0.093
HbSS	54.1%	50.0%	82.4%	
HbSC	16.2%	27.3%	5.9%	
SbThal	24.3%	18.2%	8.8%	
Other/Unknown	5.4%	4.5%	2.9%	
Hospital visits, mean(sd)	2.0	3.7	3.6	0.203
Sickle comorbidities, mean(sd)	1.02 (0.99)	1.69 (0.97)	1.73 (1.1)	0.008
Education				0.774
Some HS	13.5%	13.0%	5.9%	
HS grad or GED	29.7%	43.5%	41.2%	
Some college	35.1%	21.7%	26.5%	
College or beyond	21.6%	21.7%	26.5%	
Unemployed	59.5%	69.6%	76.5%	0.303
On disability	43.2%	73.9%	67.6%	0.030
Household income				0.999
<10k	32.4%	31.8%	29.4%	
10k to –35k	32.4%	31.8%	32.4%	
35k+	35.3%	36.4%	38.2%	

pre-education about HU before the therapy is begun so that the patient may form realistic expectations for the course of therapy, particularly their expectations regarding when they might begin to experience benefit and what they might experience in the short-term before the benefits manifest.

Patient expectations may also explain why our respondents demonstrated doubts about the perceived benefits of HU. Many of our respondents expressed concern over the fact that they continued to experience pain crises or hospitalizations while on HU. If the outcome expectations of patients on HU are unrealistically high, then nonadherence with, or discontinuation of, the therapy would be unsurprising. Realistic expectations about HU must be promoted among this patient population.

Our study participants exhibited a lack of general knowledge about HU. LaVista et al. described the beneficial effects of a video-based educational intervention to raise patient interest in discussing HU with their physicians [17]. Similar interventions could be systematically used as part of any curriculum designed to educate SCD patients and their families about HU.

The most common concern about HU cited by our respondents was a concern over side effects. Although most respondents were nonspecific as to the nature of the bothersome side effects, the majority of those with a specific reason cited a concern over the general safety of HU. Although specific stated concerns over the potential reproductive effects of HU, or its potential carcinogenicity, were cited much less frequently in our study than are endorsed in studies of providers, it is possible that these concerns were encompassed in our respondent's nonspecific concerns over side effects and/or safety.

Care should be taken in attempting to generalize the results of our study to the wider adult SCD population in the United States. This was a small, single institution study involving patients receiving care at a comprehensive sickle cell center. Patients who receive their care outside of a specialty sickle cell center may have different attitudes and beliefs about HU, which may have important implications for the nature and type of HU educational interventions developed for SCD patients.

Barriers to a wider uptake of HU therapy among the SCD population exist at multiple levels, including at the level of the patient. Our study suggests that a general lack of knowledge about HU, doubts about its effectiveness, and concerns over its side effects are all patient-level barriers that need to be addressed. Furthermore, when patients do begin treatment with HU, it is important that their expectations about the drug and its effects are appropriately managed, particularly at the start of therapy, so that the patient and his or her medical team may give HU therapy an adequate chance to succeed.

Methods

Study design, setting, and sample

We conducted a cross-sectional study of 95 adults (age 18+) with SCD receiving care at an urban academic medical center from September 2006 to June 2007. Participants were recruited from the adult sickle cell and hematology outpatient clinics (49.5%) or those seeking acute care for the treatment of a vaso-occlusive sickle cell crisis from the Emergency Department (ED) or inpatient units (50.5%). Participating patients underwent a 15-min interview by a trained study team member and received $10 for interview completion. Health status information was collected by self-report and abstraction from the patient's medical record. The academic medical center's institutional review board reviewed and approved the study procedures, and all participating patients gave informed consent.

TABLE II. Number of Adult Sickle Cell Patients Describing Concerns About Hydroxyurea (n = 33)

Concern	No. (%)[a]
Side effects	25 (75.8)
Nonspecific	14 (56)
Safety	8 (32)
Reproductive effects	2 (8)
Carcinogenic	1 (4)
No perceived benefit	8 (24.2)
Lack of knowledge	4 (12.1)

[a]Concerns are not mutually exclusive.

Measures

HU history, beliefs, and attitudes. Patients who self-reported that they were not currently taking HU were categorized as "never" or "former" HU users. "Current" users were subsequently asked to assess how much they thought taking HU had helped them (not at all to very much) and the extent to which they have been bothered by side effects from taking HU (not at all to very much). Former users were asked to estimate the length of time they were on HU (<6 months to 5+ years) and to provide the reason that they stopped taking HU. Never users were asked to recall if a doctor had ever suggested that they take HU (yes/no), to gauge the amount of information they have received about HU (none to too much), and to postulate how they thought taking HU would make them feel relative to their current assessment of their condition (much worse to much better). Additionally, patients were given the opportunity to describe in their own words any concerns that they may have had about HU. Thirty-three of the 95 participating patients provided responses to this open-ended question.

Patient characteristics. We assessed a number of patient demographic and clinical characteristics by patient self-report, including the patient's level of education (<high school, high school/GED, some college, college or beyond), annual household income (<$10,000; $10,000–$35,000; and >$35,000+), current employment (employed/unemployed), and current receipt of disability benefits (yes/no).

Using the patient's medical record, we assessed the following demographic and clinical variables: patient age, patient sex, the patient's sickle cell genotype (HbSS/HbSC/SbThal/Other or Unknown), the number of sickle cell-related comorbidities ever experienced by the patient (out of five possible: acute chest syndrome, avascular necrosis, renal disease, pulmonary hypertension, and iron overload), and the number of hospital visits by the patient in the prior 12 months.

Statistical methods. Bivariate associations between patient characteristics and HU use status were conducted using the chi-square test, Fishers exact test, Student's t-test, and one-way analysis of variance as appropriate. Two investigators (CH and SL) reviewed the responses to the open ended questions about the reason for stopping HU use (among "former" users) or any concerns that any respondent expressed about HU, and coded these responses into smaller categories. Disagreements between the investigators regarding the coding were resolved through discussion. The frequencies with which these smaller categories were mentioned are presented as simple tabulations and percentages in Table II. Analyses of quantitative data were conducted using Stata 10 software [20].

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Contract grant sponsor: Johns Hopkins Blaustein Pain Research Fund; Contract grant sponsor: National Heart, Lung, and Blood Institute; Contract grant numbers:

5F31HL082037-02 and 5K23HL083089-02; Contract grant sponsor: Johns Hopkins Clinical Research Scholars award; Contract grant number: #5KL2RR025006-03; Contract grant sponsor: Agency for Healthcare, Research, and Quality; Contract grant number: 5K08HS013903-04.
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Conflict of interest: Nothing to report.
Received for publication 9 August 2010; Revised 9 September 2010; Accepted 10 September 2010
Published online 17 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21883

References

1. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. N Engl J Med 1995;332:1317–1322.
2. Moore RD, Charache S, Terrin ML, et al. Cost-effectiveness of hydroxyurea in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. Am J Hematol 2000;64:26–31.
3. Ballas SK, Barton FB, Waclawiw MA, et al. Hydroxyurea and sickle cell anemia: effect on quality of life. Health Qual Life Outcomes 2006;4:59.
4. Steinberg MH, Barton F, Castro O, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. JAMA 2003;289:1645–1651.
5. Steinberg MH, McCarthy WF, Castro O, et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. Am J Hematol 2010;85:403–408.
6. Carroll CP, Haywood C Jr, Fagan P, Lanzkron S. The course and correlates of high hospital utilization in sickle cell disease: Evidence from a large, urban medicaid managed care organization. Am J Hematol 2009;84:666–670.
7. Kauf TL, Coates TD, Huazhi L, et al. The cost of health care for children and adults with sickle cell disease. Am J Hematol 2009;84:323–327.
8. Ballas SK. The cost of health care for patients with sickle cell disease. Am J Hematol 2009;84:320–322.
9. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT. Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol 2009;84:618–625.
10. Olnes M, Chi A, Haney C, et al. Improvement in hemolysis and pulmonary arterial systolic pressure in adult patients with sickle cell disease during treatment with hydroxyurea. Am J Hematol 2009;84:530–532.
11. Strouse JJ, Lanzkron S, Beach MC, et al. Hydroxyurea for sickle cell disease: a systematic review for efficacy and toxicity in children. Pediatrics 2008;122:1332–1342.
12. Lanzkron S, Strouse JJ, Wilson R, et al. Systematic review: Hydroxyurea for the treatment of adults with sickle cell disease. Ann Intern Med 2008;148:939–955.
13. Haywood C, Jr, Beach MC, Lanzkron S, et al. A systematic review of barriers and interventions to improve appropriate use of therapies for sickle cell disease. J Natl Med Assoc 2009;101:1022–1033.
14. Brawley OW, Cornelius LJ, Edwards LR, et al. NIH consensus development statement on hydroxyurea treatment for sickle cell disease. NIH Consens State Sci Statements 2008;25:1–30.
15. Meyappan JD, Lampl M, Hsu LL. Parents' assessment of risk in sickle cell disease treatment with hydroxyurea. J Pediatr Hematol Oncol 2005;27:644–650.
16. Hankins J, Hinds P, Day S, et al. Therapy preference and decision-making among patients with severe sickle cell anemia and their families. Pediatr Blood Cancer 2007;48:705–710.
17. LaVista JM, Treise DM, Dunbar LN, et al. Development and evaluation of a patient empowerment video to promote hydroxyurea adoption in sickle cell disease. J Natl Med Assoc 2009;101:251–257.
18. Zumberg MS, Reddy S, Boyette RL, et al. Hydroxyurea therapy for sickle cell disease in community-based practices: A survey of Florida and North Carolina hematologists/oncologists. Am J Hematol 2005;79:107–113.
19. Lanzkron S, Haywood C, Jr, Hassell KL, Rand C. Provider barriers to hydroxyurea use in adults with sickle cell disease: A survey of the sickle cell disease adult provider network. J Natl Med Assoc 2008;100:968–973.
20. StataCorp.Stata statistical software: Release 10. StataCorp, 2007.

Evidence of persistent neurologic injury following thrombotic thrombocytopenic purpura

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Despite improvements in our understanding of the pathophysiology of thrombotic thrombocytopenic purpura (TTP), little data exist regarding the long-term sequelae following a diagnosis of TTP. We present the results of a comprehensive evaluation of neurologic injury that included a magnetic resonance imaging (MRI), a neurocognitive testing, and an evaluation of health-related quality of life. Twenty-seven patients with a history of idiopathic TTP functioning normally in their activities of daily living were recruited from existing patient cohorts at both the Ohio State University (n = 12) (Columbus) and the University College London Hospitals (n = 15) (London, UK). Nine of 23 (39%) of the MRI studies were abnormal; 17/27 (63%) patients demonstrated neurocognitive impairment, particularly in tests of visual learning and memory. Health-related quality of life scores were also significantly lower than age- and gender-matched US norms for both the composite mental component score and physical component score. These data suggest that the prevalence of neurologic findings in TTP patients in remission is quite high and is largely undetected by routine clinical evaluations. Further longitudinal study will be required to define the risk for neurologic injury and the long-term prognosis in patients previously diagnosed with TTP.

Magnetic Resonance Imaging

A total of 23 magnetic resonance imaging (MRI) exams were completed with 10 performed at the Ohio State University and 13 performed at the United College London Hospitals. Four patients did not undergo MRI testing due to either contraindications to MRI testing or patient refusal/inability to complete the study. Nine of 23 (39%) of the MRI studies were abnormal; seven of nine subjects with abnormal MRI scans showed mild, small vessel ischemic changes felt to be months to years in age. Two subjects had both large vessel infarctions and microvascular changes. Six of seven subjects with only small vessel ischemic changes had multiple lesions, with the majority being subcortical white matter abnormalities, consistent with lesions that would be expected to be seen in a microvascular thrombotic process.

Neurocognitive Function

Cognitive function was assessed in all 27 convalescent subjects from both sites. Only 18/27 (67%) subjects were able to successfully complete the Groton Maze Learning Test; therefore, for the remaining nine subjects no score was able to be imputed for this testing domain. Nineteen of the 27 (70%) subjects showed performance <1 standard deviation (SD) below age-matched norms on one of four domains, 14 of 27 (52%) showed performance <2 SD below age-matched norms on one of four domains, and 11 of 27 (41%) showed performance <1 SD below age-matched norms on at least two of the four domains. From these data, 17 of 27 (63%) patients were classified as having cognitive impairment (≥ 2 domains <1 SD below age-matched controls, or one domain <2 SD below controls). The distribution of performance data for all tests was skewed heavily toward subnormal performance, tests of visual learning, and memory showing the greatest negative skew (see online supplement).

SF-36v2 Quality of Life Questionnaire

SF-36v2 surveys were completed by all 27 subjects. In aggregate, the subjects scored significantly lower than age- and gender-matched US norms for both the mental component score (MCS) and physical component score (PCS). In addition, these data were also compared with more common disease states (anemia, depression, cancer) to provide a relevant comparison. The PCS in thrombotic thrombocytopenic purpura (TTP) patients is quite similar to the compared disease states, with the MCS significantly lower in

TABLE I. Demographic Data for all 27 patients Studied in Convalescence from a Previous Episode of TTP

Subject #	Age	Sex	Pre-existing conditions prior to TTP diagnosis	ADAMTS13 activity[a]	Number previous episodes	Months since last episode	Abnormal neurocognitive testing	Abnormal MRI
US 1	60	M		<5%	1	6		X
US 2	32	F		9.8%	2	5	X	
US 3	30	M		<5%	9	4	X	
US 4	50	M	Hypertension	<5%	6	5	X	
US 5	23	F		98%	1	45	X	
US 6	39	F		8.4%	2	46		
US 7	53	F	Seizure disorder	<5%	1	12		
US 8	33	M		NA	5	1	X	
US 9	29	F		6.2%	2	51		
US 10	27	M		NA	12	7		
US 11	56	F		<5%	1	61		X
US 12	41	M	Sleep apnea	<5%	1	23	X	
UK 1	42	F		9%	1	5	X	X
UK 2	49	F		6%	1	19		
UK 3	45	F		<5%	1	43		X
UK 4	22	F		5%	1	42	X	X
UK 5	49	M		<5%	1	27		X
UK 6	45	F		32%	1	5	X	X[c]
UK 7	58	F		<5%	1	66	X	
UK 8	62	M		<5%	1	16	X	
UK 9	51	F		NA	1	2	X	
UK 10	18	F		<5%[b]	8	1	X	
UK 11	40	M		NA	2	16	X	
UK 12	55	F		NA	3	6	X	
UK 13	52	F		40%	1	13	X	X
UK 14	31	M		<5%[b]	1	40		
UK 15	45	F		NA	2	50	X	X[c]

Subjects with abnormal neurocognitive testing and MRI imaging are also shown in the columns to the right. The shaded subjects represent those patients with residual neurologic deficits at the time of enrollment.

[a]ADAMTS13 activity reported was the value measured at the time of presentation of the most recent acute episode.

[b]ADAMTS13 activity not available (**NA**) for most recent episode, reported ADAMTS13 activity represents value at initial presentation.

[c]Indicates subjects with both large and small vessel abnormalities on MRI.

TTP patients than in patients with anemia and cancer, and on par with patients diagnosed with depression (see online supplement).

Correlation Between MRI, Neurocognitive Findings, and Time Since Most Recent Episode and Number of Previous Episodes of TTP

In the nine patients with abnormal MRIs, there was no significant difference in the presence of MRI abnormalities between the subjects studied within 1 year of their last acute episode of TTP and those greater than 1 year since their last acute episode. Although counterintuitive, there was a significantly higher rate of MRI abnormalities in patients with only one episode of TTP compared with those with greater than one episode of TTP (62 vs. 13%, $P = 0.017$). With respect to neurocognitive function, there was a significantly higher rate of impairment in patients whose most recent episode of TTP was <1 year compared with those >1 year from their last episode of TTP (73 vs. 31%, $P = 0.035$). However, there was no significant difference in the rate of cognitive impairment between patients with one versus more than one episode of TTP (38 vs. 55%, $P = 0.401$).

The potential for neurologic injury as a result of an acute episode of TTP is widely known, but it has been recognized only recently that chronic neurocognitive deficits may be present and persist long after recovery from an acute episode of TTP [1]. Using complementary methodologies to evaluate neurologic injury and neurocognitive function, we have attempted to objectively document the prevalence and severity of neurologic abnormalities in a cohort of clinically stable TTP patients. Although these data would be strengthened by the inclusion of a control group, the relatively young median age of this cohort (45 years, range 18–62) and the lack of comorbid conditions in nearly all subjects suggest that there should be a low incidence of neurologic abnormalities independent of their diagnosis of TTP, supporting our hypothesis that these findings presented are likely related to their previous history of TTP.

These data presented demonstrate a high rate of silent cognitive impairment (63%) and MRI abnormalities (39%) in a cohort of clinically stable without demonstrable acute neurologic signs or symptoms, suggesting that the true prevalence of neurologic injury in patients with a previous history of TTP is likely to be much greater than what would be predicted by the presence of symptoms alone. Although two subjects had residual neurologic deficits that dated back to their initial presentation, all patients studied were functioning normally, and either gainfully used or functioning normally in their activities of daily living at the time of their studies. None of the 27 patients demonstrated clinically apparent cognitive difficulties. The higher rate of neurocognitive deficits compared with MRI abnormalities suggests that neurocognitive testing may be a more sensitive marker of neurologic sequelae in TTP patients than imaging studies such as MRI. It is also interesting to note that four patients with MRI abnormalities did not demonstrate neurocognitive deficits. Although it is possible that the number or location of the lesions documented by MRI may not have been sufficient to result in demonstrable neurocognitive deficits in these four subjects, it is also possible that the abnormalities seen in these four patients may not be related to their previous acute episodes of TTP. Additional prospective study will be required to better understand the structure–function relationship between imaging abnormalities and functional deficits in patients with a previous history of TTP.

In the only previous report in which patients were studied in convalescence from an acute episode of TTP, Fiorani et al. reported normal MRI studies in five TTP patients who recovered after initially presenting with neurologic signs and symptoms [2]. The MRIs performed in our study utilized diffusion-weighted imaging, a sensitive technique that can detect the microvascular lesions commonly seen in TTP patients. Given that abnormalities on diffusion-weighted imaging (DWI) are typically only seen after an acute injury for up to 2 weeks [3–5], the demonstration of new findings by DWI in this study could be evidence for new injury that developed in convalescence and likely not related to their previous acute episodes of TTP. This finding supports the hypothesis that patients with a history of TTP may be at risk for progressive neurologic injury in the absence of clinically overt TTP.

The first published data reporting neurocognitive deficits in TTP patients were from the Oklahoma TTP-hemolytic uremic syndrome (HUS) registry who detailed their study of cognitive function in 24 patients with normal physical and mini-mental state examinations and no evidence of active TTP [1]. As a group, the patients performed significantly worse on four of the 11 cognitive domains tested compared with standardized data from normal individuals, with performance in 75% of subjects at least 1 SD below normal in one of these four domains. These data are consistent with the data presented here that demonstrated that 63% of subjects had neurocognitive impairment. This impairment was most frequent in the domains of visual learning, attention, and psychomo-

tor function and suggests that in convalescent TTP patients there exists a persistent impairment in their ability to attend, learn, and make simple decisions quickly. The nature of the cognitive impairments presented in this study are also consistent with those observed by Kennedy et al. who also found that impairments were more frequent in the domains of psychomotor speed, attention/vigilance, and visual learning. These combined data suggest that chronic neurocognitive deficits in patients with a previous history of TTP are far more common than realized previously.

The marked abnormalities in the Health-related quality of life (HRQoL) presented in this article are also consistent with previously published data by Lewis et al. [6]. Our cohort shows significant differences in all eight HRQoL domains and the combined physical and MCS compared with race- and gender-adjusted US norms, consistent with the published data from the Oklahoma TTP-HUS registry and suggest that patients with a previous history of TTP have a decreased quality of life.

Although these data support the fact that patients with a previous history of TTP are at a markedly increased risk for chronic neurocognitive deficits, it is not clear if these deficits improve over time. In our cohort of patients, there was a significant difference in the prevalence of neurocognitive deficits in patients tested within 1 year of their last episode of TTP compared with those greater than 1 year from their last acute episode, suggesting that there may be improvement over time as patients get further away from their last acute episode of TTP. The data presented by Kennedy et al., however, suggest that abnormalities of cognitive function were not related to the time since their most recent TTP episode. Consistent with the data from the Oklahoma TTP-HUS registry, the risk for neurocognitive deficits in our cohort of patients was also not related to the number of previous TTP episodes. Ultimately, a prospective study with serial evaluations of neurocognitive function at defined time points will be required to definitively determine the risk factors for neurologic injury and the long-term prognosis of these deficits in TTP patients.

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

Conflict of interest: Nothing to report

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Grant sponsor: Archemix Corp

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Published online 17 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/ajh.21881

References

1. Kennedy AS, Lewis QF, Scott JG, et al. Cognitive deficits after recovery from thrombotic thrombocytopenic purpura. Transfusion 2009;49:1072–1101.
2. Fiorani L, Vianelli N, Gugliotta L, et al. Brain MRI and SPET in thrombotic thrombocytopenic purpura. Ital J Neurol Sci 1995;16:149–151.
3. Lovblad KO, Laubach HJ, Baird AE, et al. Clinical experience with diffusion-weighted MR in patients with acute stroke. AJNR Am J Neuroradiol 1998;19:1061–1066.
4. Fiebach JB, Schellinger PD, Jansen O, et al. CT and diffusion-weighted MR imaging in randomized order: diffusion-weighted imaging results in higher accuracy and lower interrater variability in the diagnosis of hyperacute ischemic stroke. Stroke 2002;33:2206–2210.
5. Provenzale JM, Sorensen AG. Diffusion-weighted MR imaging in acute stroke: theoretic considerations and clinical applications. AJR Am J Roentgenol 1999;173:1459–1467.
6. Lewis QF, Lanneau MS, Mathias SD, et al. Long-term deficits in health-related quality of life after recovery from thrombotic thrombocytopenic purpura. Transfusion 2009;49:118–124.

Activation of mononuclear phagocytes and its relationship to asplenia and phosphatidylserine exposing red blood cells in hemoglobin E/β-thalassemia patients

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Aged or abnormal red blood cells with exposed phosphatidylserine (PS-RBCs) are cleared from the circulation by splenic macrophages. In asplenic patients, other mononuclear phagocytic cells in tissues and in circulation may function in this capacity. To better understand these changes and the relationship among splenic status, PS-RBCs, blood monocytes, and serum tumor necrosis factor (TNF-α), a product of mononuclear phagocyte activation, patients with hemoglobin E/β-thalassemia (E/β-Thal) were studied. Whole blood of 20 nonsplenectomized, 20 splenectomized E/β-Thal patients, and 20 healthy subjects was assayed for PS-RBCs; for monocytes, activated monocytes, and monocyte response to lipopolysaccharide stimulation; and serum was assayed for TNF-α. Asplenic E/β-Thal patients had significantly increased ($P < 0.05$) amounts of PS-RBCs, monocytes, activated monocytes, and levels of serum TNF-α. The amount of PS-RBCs correlated with levels of serum TNF-α, but the amount of activated monocytes did not correlate with either the amount of PS-RBCs or levels of serum TNF-α. Monocyte response to lipopolysaccharide stimulation in asplenic patients was not as efficient as in the other patients or in normals (77 vs. 404, and 304 folds increment, respectively). The results suggest that splenectomy in E/β-Thal patients led to an increased amount of PS-RBCs and activation in the mononuclear phagocytic system.

Thalassemia (Thal), an autosomal recessive hereditary hemolytic anemia, is the most common known human genetic disorder. It is caused by mutations of the globin gene clusters resulting in varying degrees of decreased globin chain synthesis. It is classified into α and β according to the globin chain involved [1]. It can be co-inherited with an abnormal hemoglobin (Hb), such as Hb E, resulting in Hb E/β-Thal (E/β-Thal), which is the most common form of the symptomatic thalassemic syndromes globally [2,3]. The produced defective red blood cells (RBC) are cleared prematurely by various mechanisms during their passages through the spleen [4]. One important mechanism is through splenic macrophages' recognition of phosphatidylserine (PS)-exposing cells [5]. From overwork, the spleen may become very large, and splenectomy is usually performed for hypersplenism and/or symptomatic splenomegaly. These defective and pathologic RBCs must then be cleared by other mononuclear phagocytic or reticuloendothelial (RE) cells, such as Kupffer cells [6], whose function is not as effective as shown by an increased amount of such cells in circulation in splenectomized E/β-Thal patients [5,7].

Monocyte activation has previously been reported in both Thal [8–10] and sickle cell disease (SCD) [11,12]. Increased antibody-dependent cellular cytotoxicity [8] and up-regulation of Fcγ receptor I (FcγRI or CD64) expression [9] of monocytes in response to their clearance of thalassemic RBCs were reported in both α- and β-Thal. Macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor (TNF-α) also play roles as inducers and effectors of this activation [8,9]. Increased serum TNF-α, interleukin-6, and interferon-γ levels were reported in E/β-Thal patients, particularly after splenectomy [13,14]. Increased amounts of TNF-α and interleukin-1β per cell, indicative of monocyte activation, and increased serum C-reactive protein levels were also reported in SCD [11], who invariably have functional asplenia.

TABLE I. Characteristics and Results of Studied Blood Profiles Expressed as Means (SD) or Median (Range) in Each Group of Subjects

Characteristic	Hemoglobin E/β-thalassemia		Normal controls (n = 20)
	Splenectomized (n = 20)	Nonsplenectomized (n = 20)	
Age (year)	26.0 (20.0–48.0)[a]	33.0 (19.0–57.0)[b]	24.0 (18.0–44.0)
Gender (male:female)	9:11	10:10	9:11
Total packed red blood cells transfusion (unit)	83.5 ± 111	22.4 ± 41.4	0
Hematologic parameters			
RBC (10^{12}/L)	2.8 ± 0.4[c,d]	3.8 ± 1.0[c]	4.7 ± 0.6
Hb (g/L)	60 (45–80)[a,b]	71 (55–117)[b]	131 (106–176)
Hct (proportion of 1.0)	0.209 (0.164–0.288)[a,b]	0.229 (0.193–0.368)[b]	0.396 (0.324–0.547)
MCV (fL)	75.4 ± 7.0[c,d]	65.3 ± 7.8[c]	86.5 ± 5.9
MCH (fmol/cell)	1.4 ± 0.1[c]	1.3 ± 0.2[c]	1.8 ± 0.2
MCHC (mmol/L)	18.0 ± 1.6[c]	19.2 ± 1.2[c]	20.3 ± 1.9
Reticulocyte (10^9/L)	239.1 (154.0–509.6)[a,b]	75.9 (48.4–193.2)[b]	41.2 (8.5–76.7)
NRBC/100 WBC	598.0 (61.0–1,418.0)[a,b]	4.0 (0.0–66.0)[b]	0
WBC (10^9/L)	10.2 ± 2.2[c,d]	7.6 ± 2.2[c]	5.7 ± 1.2
Monocyte (10^6/L)	976.0 (223.0–2,650.0)[a,b]	295.0 (100.0–909.0)	372.0 (180.0–990.0)
Platelet (10^9/L)	735.0 (450.0–1,056.0)[a,b]	227.5 (31.0–423.0)	280.0 (163.0–381.0)
Absolute amount of annexin V + RBCs (10^9/L)	96.3 ± 44.9[c,d]	46.9 ± 21.6	40.2 ± 19.3
Absolute amount of CD14+-expressing intracellular TNF-α (10^6/L)	4.3 (1.0–67.9)[a,b]	0.5 (0.1–10.5)	1.2 (0.1–2.9)
MFI of CD14+/CD11b+	65.7 ± 28.2[c,d]	44.1 ± 12.0	51.4 ± 10.8
Serum TNF-α (ng/L)	11.3 ± 3.6[c,d]	8.4 ± 3.3	7.1 ± 1.6

Kruskal–Wallis with corrected Mann–Whitney test: [a]significant difference compared with nonsplenectomized group ($P < 0.05$); [b]significant difference compared with normal controls ($P < 0.05$).
One-way analysis of variance with Bonferroni test: [c]significant difference compared with normal controls ($P < 0.05$); [d]significant difference compared with nonsplenectomized group ($P < 0.05$).

TABLE II. Percentage of CD14+-Expressing Intracellular TNF-α Before and After Lipopolysaccharide (LPS) Stimulation in Each Group of Subjects

Type of subjects	N	Percentage of CD14+-expressing intracellular TNF-α, median (range)		P-value[a]
		Before stimulation	After LPS stimulation	
Splenectomized hemoglobin E/β-thalassemia	20	0.45 (0.15–3.42)[b]	34.65 (0.31–67.16)[c]	<0.001
Nonsplenectomized hemoglobin E/β-thalassemia	20	0.21 (0.02–3.50)	84.93 (42.94–94.83)	<0.001
Normal controls	20	0.26 (0.02–1.23)	78.93 (55.25–93.45)	<0.001

[a]Wilcoxon signed rank test.
[b]Significant difference from normal controls and nonsplenectomized patients ($P < 0.05$) (Mann–Whitney U test).
[c]Significant difference from normal controls and nonsplenectomized patients ($P < 0.01$) (Mann–Whitney U test).

To better understand the inter-relationship among splenic status, PS-exposing RBCs (PS-RBCs), monocyte activation, and serum TNF-α, a product of mononuclear phagocyte activation, we studied these parameters in E/β-Thal patients with and without intact spleen, and compared them with those of normal controls (NC).

In total, 40 ambulatory and well E/β-Thal patients who attended the adult hematology clinic at Ramathibodi Hospital were studied. Their age ranged from 19 to 57 years, and 19 were men. They were free from medication and blood transfusion for at least four preceding weeks. Half of them had undergone splenectomy, with a median interval since splenectomy of 13.5 years (range, 5–35). In nonsplenectomized (NS) patients, median vertical measurement from splenic tip on palpation to left costal margin was 3.5 cm (range, 0.5–12.5). Blood transfusion and iron chelation therapy were usually modest, and at the discretion of the attending physicians. Controls were 20 consenting healthy subjects, who had not taken any drugs for at least four preceding weeks. Study protocol was approved by the institutional ethics committee for studies in humans (11-46-31). Written informed consents were obtained from all patients.

Patients' characteristics, mean (standard deviation) or median (range) of hematological data, absolute amounts of PS or annexin V (AV)+ RBCs, activated monocytes (CD14+ expressing intracellular TNF-α and CD14+/CD11b+), and levels of serum TNF-α of the patients and normal controls (NC) are shown in Table I. Total amount of packed RBCs transfusion in the two patients' groups almost reached statistically significant difference ($P = 0.055$, independence t-test). Values of platelet count in the NS group had a wide variation because of the varied spleen sizes. Splenectomized (S) patients had significantly lower amounts of RBCs, and significantly higher amounts of reticulocytes and NRBCs than the others ($P < 0.05$), indicating more severe hemolysis. They also had significantly higher amounts of white blood cells (WBCs), monocytes, platelets, PS-RBCs, activated monocytes, and levels of serum TNF-α than the others ($P < 0.05$). Similar to the amount of monocytes, values of the latter three in the NS and NC groups were not significantly different, suggesting an influential role of the spleen on these parameters.

Correlation among absolute amounts of activated monocytes, PS-RBCs, and levels of serum TNF-α were assessed by multiple linear regression analysis after controlling for types of subjects. In consideration of skewness, data were first transformed to a log-scale. The only statistically significant correlation (coefficient = 0.914, standard error = 0.188, $P < 0.001$) was between levels of serum TNF-α and absolute amounts of PS-RBCs in the S group.

Values of median (range) of percentage of CD14+-expressing intracellular TNF-α before and after lipopolysaccharide (LPS) stimulation in each group of subjects are shown in Table II. Before stimulation, the S group had a significantly larger number of such cells than the others ($P < 0.05$). A significant increase in amounts of such cells after LPS stimulation was shown in all groups ($P < 0.001$). However, the increment in the S was less than those in the NS and NC groups (77 vs. 404, and 304 folds increment, respectively).

The increased amount of circulating PS-RBCs in the S group (Table I) is in agreement with previous reports [5,7] and likely due to the less-efficient function of other RE cells than splenic macrophages in clearing these pathologic cells. However, until we can do pre- and postsplenectomy studies on the same patient, there will always be an argument that the finding could also be due to a more severe disease in the splenectomized one. Findings of a nonstatistically significant difference between the amounts of PS-RBCs in the NS and NC groups (Table I) reinforce a recognition of the better performance by the spleen [4]. Taken together, these findings in the S and NS groups in the context of overlapping disease severity between them suggest that the final amount of circulating PS-RBCs is determined more by status of the spleen than the inherent RBC defects alone.

Monocytosis in the S group could be due to reactive production and/or inability to home to the spleen. However, it was not found in another study despite elevated serum macrophage colony-stimulating factor levels, which were unrelated to splenic status [8]. This discrepancy requires further studies.

Monocyte activation in the S group (Table I) was similar to previous reports in SCD patients [11,12], who invariably have autosplenectomy, suggesting its close relationship with splenic absence in these two hereditary hemolytic anemia. The smaller amounts, but not statistically different, of activated monocytes in the NS compared with the NC groups (Table I) is quite interesting. It could be misleading because of a small number of patients causing skewness of data, or because of ancillary monocytes having limited roles in the presence of more efficiently functioning (in clearing defective RBCs) and abundant splenic macrophages in an enlarged hyperplastic spleen. Compared with the S group, the higher increment of CD14+-expressing intracellular TNF-α after an *ex vivo* LPS stimulation in the NS one (Table II) with reserved capacity would lend some support to the latter possibility. It also suggests that the impaired response of monocytes in splenectomized patients could be due to "overwork," at least to help clear the now abundant circulating pathologic RBCs in the absence of splenic macrophages. Because chronic iron overload can also contribute to this impairment [15,16], we searched for data on serum ferritin levels near the time of studies and found no statistically significant difference between the S and NS groups [median (range) = 2,599 (424–7,180) ng/mL and 1,150 (266–4,450) ng/mL, respectively. $P = 0.136$, Mann-Whitney *U* test].

Levels of serum TNF-α were significantly increased in the S compared with the other two groups (Table I). Their correlation with the amounts of activated monocytes and PS-RBCs were assessed by multiple linear regression analysis after controlling for types of subjects. The only correlation among the three variables in the three groups was between serum TNF-α levels and amounts of PS-RBCs in the S group (coefficient = 0.914, standard error = 0.188, $P < 0.001$). The results suggest that, after splenectomy, an abnormally large amount of PS-RBCs activated many types of RE cells, all of which can produce TNF-α [6]. Finding hepatosplenomegaly in Thal disease and chronic hemolytic anemia suggests the Kupffer cell being the prominent one.

Our findings of monocyte activation in splenectomized E/β-Thal patients help explain certain clinical features pertaining to this group. Monocyte activation together with elevated serum TNF-α levels could activate vascular endothelial cells, leading to an expression of adhesion molecules and tissue factor on their surfaces [11]. Together with increased amounts of thrombogenic PS-RBCs [5,7] and activated platelets [17], this could further facilitate the formation of thrombotic pulmonary arteriopathy, the basis of pulmonary arterial hypertension, which is more prevalent after splenectomy [18]. The blunted inflammatory cytokine response to LPS, in keeping with previous reports of reduced monocyte phagocytic activity because of a "compensatory" increased erythrophagocytic activity in splenectomized β-Thal patients [19,20], may affect host defense against infection, which is known to be more prevalent and severe among these patients [21–23].

In conclusion, our studies suggest role of spleen in controlling the amount of PS-RBCs and mononuclear phagocytic activity in E/β-Thal patients. Validation in a larger cohort and preferably on the same patients before and after splenectomy would strengthen our conclusion. In the meantime, splenectomy must be judiciously applied in E/β-Thal patients to avoid the undesirable consequences.

Methods

After an overnight fast, venous blood was drawn from the antecubital vein into a plastic syringe. Seven, 3, and 3 mL each of blood were then transferred into plain (Becton Dickinson Bioscience (BDB), Franklin Lakes, NJ), potassium ethylenediaminetetraacetic acid (EDTA)- (BDB), and sodium heparin- (Greiner Bio-One, Monroe, NC) containing vacutainer tubes, respectively. Blood anticoagulated with EDTA was used for determination of complete blood count, reticulocyte count, Hb typing, and monocyte surface expression of CD11b. Blood anticoagulated with sodium heparin was used for determination of monocytes expressing intracellular cytokine and PS-RBCs. Serum was used for the TNF-α assay.

Hematologic and serum TNF-α assay. Complete blood count and reticulocyte count were done by automated hematological analyzer (Technicon H*3; Bayer Diagnostics, Tarrytown, NY). Hb typing was done by high-performance liquid chromatography (Variant II; Bio-Rad Laboratories, Hercules, CA). Serum TNF-α assay was done by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN).

Flow cytometric measurement of monocyte activation. Monocyte surface expression of CD11b. Blood samples were kept at 4°C before processing. One hundred microliters of EDTA blood was lysed with 2 mL of fluorescence-activated cell sorting (FACS) lysing solution (BDB) for 10 min at 4°C. After washing, the remaining WBCs were incubated with 2 μL of allophycocyanin-conjugated anti-CD14 and 5 μL of phycoerythrin (PE)-conjugated anti-CD11b (BDB) for 20 min at 4°C in the dark. The stained cells were washed and resuspended in 350 μL of 1% paraformaldehyde. Monocyte activation was measured as mean fluorescence intensity of CD11b+ by BDB FACSCalibur flow cytometer (BDB). A total of 5,000 cells were collected.

Monocytes expressing intracellular cytokine. Five hundred microliters of sodium heparinized whole blood was incubated with 10 μL of Brefeldin A (BFA) (Sigma-Aldrich, St. Louis, MO) under 5% CO_2 for 4 hr at 37°C with and without 1 μg/mL of LPS (Sigma-Aldrich). BFA prevents cytokine from leaking out of cytoplasm [24]. Two hundred microliters each of unstimulated and LPS-stimulated blood samples were lysed with 2 mL of FACS lysing solution for 10 min at room temperature. After centrifuging and decanting the supernatant, WBCs were washed and stained with 2 μL of allophycocyanin-conjugated anti-CD14 for 15 min. Samples were then washed and incubated with 500 μL of FACS permeabilizing solution (BDB) for 10 min. After washing, cells were incubated with 5 μL of fluorescein isothiocyanate (FITC)-conjugated anti-human TNF-α (BDB) for 30 min. The last three steps were done at room temperature in the dark. FITC-conjugated anti-mouse IgG1 (BDB) was used as a negative marker. The amount of stained cells was determined by BDB FACSCalibur flow cytometer. A total of 5,000 cells were collected.

Flow cytometric measurement of PS-RBCs. Two microliters of sodium heparinized whole blood was incubated with 2 μL of FITC-conjugated AV (BDB), 2 μL of PE-conjugated glycophorin A (DAKO, Hamburg, Germany), and 94 μL of 1× AV binding buffer (BDB) at room temperature for 15 min in the dark. FITC-conjugated anti-mouse IgG1 was used to set a negative control. After a similar incubation process, 300 μL of 1× AV binding buffer was added to both samples. The amount of stained cells was determined by BDB FACSCalibur flow cytometer. A total of 100,000 cells were collected.

Acquisition and data analysis were performed by CellQuest Software (BDB). Stained cells were excited with 488-nm light from a 15-mW argon ion laser. Logarithmic green and orange-red fluorescence of FITC for PS expression and PE for RBCs were measured through 530/30 and 585/42 band pass filters, respectively. The RBCs were gated on the basis of their logarithmic amplification of the forward scatter and 90° light scatter signals.

Statistical analysis. Data were described by means (standard deviation) or median (range) where appropriate. Multiple linear regression analysis was applied to determine the correlation between absolute amounts of CD14+/CD11b+- or CD14+-expressing intracellular TNF-α and those of AV+ RBCs, and levels of serum TNF-α, respectively in each group. Data were transformed to be log-scale where appropriate. Assumptions of linear regression, normality, and constant variances of residuals were checked. All analyses were performed using STATA 10.0 (Stata Corp., College Station, TX). *P* value less than 0.05 was considered to be statistically significant.

Author Contributions

V.A., P.B. (W.B.'s preceptor for her M.S. thesis), K.P., W.B., and S.C. designed the research; W.B. and N.A. performed the research; W.B. and A.T. performed statistical analyses; W.B., P.B., A.T., and V.A. wrote the article.

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Grant sponsor: Thailand Research Fund-Senior Research Scholar

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Conflict of interest: Nothing to report.
Published online 17 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21884

References

1. Olivieri N, Weatherall D. Thalassemias. In: Lilleyman J, Hann I, Blanchette V, editors. Pediatric Hematology, 2nd ed. New York: Churchill Livingstone; 1999. pp307–326.
2. Fucharoen S, Winichagoon P. Thalassemia and abnormal hemoglobin. Int J Hematol 2002;76(Suppl 2):S83–S89.
3. Fucharoen S, Winichagoon P. Clinical and hematologic aspects of hemoglobin E/β-thalassemia. Curr Opin Hematol 2000;7:106–112.
4. Goodman J, Newman MI, Chapman WC. Disorders of the spleen. In: Greer JP, Foerster J, Lukens JN, et al., editors.Wintrobe's Clinical Hematology,11th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004. pp 1893–1909.
5. Kuypers FA, Yuan J, Lewis RA, et al. Membrane phospholipid asymmetry in human thalassemia. Blood 1998;91:3044–3051.
6. Weinberg JB. Mononuclear phagocytes. In: Greer JP, Foerster J, Lukens JN, et al., editors. Wintrobe's Clinical Hematology, 11th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004. pp 349–386.
7. Atichartakarn V, Angchaisuksiri P, Aryurachai K, et al. Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/β-thalassaemic patients. Br J Haematol 2002;118: 893–898.
8. Wiener E, Wanachiwanawin W, Chinprasertsuk S, et al. Increased serum levels of macrophage colony-stimulating factor (M-CSF) in α- and β-thalassaemia syndromes: Correlation with anaemia and monocyte activation. Eur J Haematol 1996;57:364–369.
9. Wiener E, Allen D, Porter RJ, et al. Role of FcγRI (CD64) in erythrocyte elimination and its up-regulation in thalassemia. Br J Haematol 1999;106:923–930.
10. Kyriakou DS, Alexandrakis MG, Kyriakou ES, et al. Activated peripheral blood and endothelial cells in thalassemia patients. Ann Hematol 2001;80: 577–583.
11. Belcher JD, Marker PH, Weber JP, Hebbel RP. Activated monocytes in sickle cell disease: Potential role in the activation of vascular endothelium and vaso-occlusion. Blood 2000;96:2451–2459.
12. Wun T, Cordoba M, Rangaswami A, et al. Activated monocytes and platelet-monocyte aggregates in patients with sickle cell disease. Clin Lab Haematol 2002;24:81–88.
13. Chuncharunee S, Archararit N, Hathirat P, et al. Levels of serum interleukin-6 and tumor necrosis factor-α in postsplenectomized thalassemic patients. J Med Assoc Thai 1997;80(Suppl 1):S86–S91.
14. Wanachiwanawin W, Wiener E, Siripanyaphinyo U, et al. Serum levels of tumor necrosis factor-α, interleukin-1, and interferon-γ in β^0-thalassaemia/HbE and their clinical significance. J Interferon Cytokine Res 1999;19:105–111.
15. Ballart IJ, Estevez ME, Sen L, et al. Progressive dysfunction of monocytes associated with iron overload and age in patients with thalassemia major. Blood 1986;67:105–109.
16. Gordeuk VR, Ballou S, Lozanski G, Brittenham GM. Decreased concentrations of tumor necrosis factor-α in supernatants of monocytes from homozygotes for hereditary hemochromatosis. Blood 1992;79:1855–1860.
17. Atichartakarn V, Angchaisuksiri P, Aryurachai K, et al. In vivo platelet activation and hyperaggregation in hemoglobin E/β-thalassemia: A consequence of splenectomy. Int J Hematol 2003;77:299–303.
18. Atichartakarn V, Likittanasombat K, Chuncharunee S, et al. Pulmonary arterial hypertension in previously splenectomized patients with β-thalassemic disorders. Int J Hematol 2003;78:139–145.
19. Wiener E. Impaired phagocyte antibacterial effector functions in β-thalassemia: A likely factor in the increased susceptibility to bacterial infections. Hematology 2003;8:35–40.
20. Pittis MG, Estevez ME, Diez RA, et al. Decreased phagolysosomal fusion of peripheral blood monocytes from patients with thalassemia major. Acta Haematol 1994;92:66–70.
21. Wanachiwanawin W. Infections in E-β-thalassemia. J Pediatr Hematol Oncol 2000;22:581–587.
22. Vento S, Cainelli F, Cesario F. Infections and thalassaemia. Lancet Infect Dis 2006;6:226–233.
23. Bisharat N, Omari H, Lavi I, Raz R. Risk of infection and death among post-splenectomy patients. J Infect 2001;43:182–186.
24. Jung T, Schauer U, Heusser C, et al. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 1993;159:197–207.

Quality of life in Thalassemia: A comparison of SF-36 results from the Thalassemia longitudinal cohort to reported literature and the US norms

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Thalassemia is a chronic, inherited blood disorder, which, in its most severe form, causes life-threatening anemia. Advances in treatment have led to increased life expectancy however the need for chronic blood transfusions and chelation therapy remains a significant burden for patients. Our study compared health related quality of life (HRQOL) from the Thalassemia Clinical Research Network's (TCRNs) Thalassemia Longitudinal Cohort (TLC) study to US norms and assessed association with clinical variables. There were 264 patients over age 14 who completed the Medical Outcomes Study 36-Item Short Form Health Survey version 2 (SF36v2) baseline assessment. When compared to US norms, TLC patients had statistically significant ($P < 0.05$) worse HRQOL on five of the eight subscales (physical functioning, role-physical, general health, social functioning, and role-emotional) and on both summary scales (physical component summary and mental component summary). Women, older patients, and those with more disease complications and side effects from chelation reported lower HRQOL. In general, adolescents and adults with thalassemia report worse HRQOL than the US population, despite contemporary therapy. The SF-36 should become a standard instrument for assessing HRQOL in thalassemia to determine predictors of low HRQOL which may be better addressed by a multidisciplinary team.

Although medical advances in the treatment of thalassemia have led to increased survival patients still suffer disease complications. These, along with the significant burden from chronic treatment with transfusions and chelation, can adversely impact patient's quality of life [1,2]. Health Related Quality of Life (HRQOL) refers to the patient's perception of their physical and mental health. Measuring HRQOL is important to assess the impact that a chronic disease has on a patient's everyday life, to compare different groups of patients, and to measure the effect of an intervention [3].

There are several main gaps in the literature of HRQOL in thalassemia which our study will help fill. Most HRQOL studies have focused on children, whereas adult studies have been relatively small and included non-thalassemia patients [4,5]. While lower HRQOL has been described in thalassemia patients, it is not known what clinical factors have a significant impact on HRQOL, nor have there been prior studies which follow patients over time. Our study will fill some of these gaps in the literature by focusing on the baseline data in a large adult population of thalassemia patients with robust clinical data as well as HRQOL evaluations.

The goal of our study is to define the HRQOL in adolescents and adults with thalassemia by describing the quality of life in a cohort of North American and UK patients with thalassemia, comparing those results with US norms, and evaluating the association between HRQOL and clinical factors. We hypothesized that patients with thalassemia will report lower HRQOL than the standard US population. Furthermore we hypothesized that HRQOL will be lower in (1) patients with more severe disease since those patients will require more regular transfusion, and are more likely to suffer from dis-

TABLE I. Patient Demographics

Variable	TLC $n = 264$
Gender	
Male	127 (48%)
Female	137 (52%)
Age (years)	Mean = 29 (14-58)
14–17	40 (15%)
18–24	72 (27%)
25–34	72 (27%)
35–44	57 (22%)
45–54	20 (7.6%)
55–64	3 (1%)
Race	
White	144 (56%)
Asian	104 (40%)
Other	11 (4%)
Chelator	
None	22 (8.4%)
Deferoxamine (DFO, Desferal)	57 (22%)
Deferasirox (DFX, Exjade)	136 (52%)
Deferiprone (L1)	9 (3.4%)
DFO/L1	21 (8%)
DFO/Exjade	17 (6.5%)
Thalassemia Diagnosis	
B-thal regularly transfused[a]	204 (78%)
B-thal intermittently transfused[b]	24 (9%)
B-thal non-transfused[c]	2 (0.8%)
HbH CS	4(1.5%)
EB-thal regularly transfused[a]	20 (7.6%)
EB-thal intermittently transfused[b]	6 (2.3%)
EB-thal non-transfused[c]	1 (0.4%)
alpha-thal	2 (0.8%)
Transfusion Status[d]	
Transfused	224 (87%)
Nontransfused	33 (13%)
Location	
US	193 (73%)
Canada	36 (14%)
UK	35 (13%)
Ferritin	Median 1371 (range 67–23,712)
Number of secondary complications	Mean 1.7 (range 0–8)
0	90 (34%)
1	60 (23%)
2	39 (15%)
3	38 (14%)
4	15 (5.7%)
5	14 (5.3%)
6	5 (1.9%)
7	2 (0.76%)
8	1 (0.38%)

[a]At least 8 transfusions in the past year.
[b]1–7 transfusions in the past year.
[c]No transfusions in the past year.
[d]In the past year.

ease and transfusion related complications, (2) patients on non-oral chelation, since there is some literature to suggest that oral chelation is associated with greater patient satisfaction [6], and (3) patients who report problems with chelation adherence.

Our study included 264 patients over age 14 with self-reported SF-36 scores. Basic patient demographics are shown in Table I. As expected TLC patients reported significantly lower HRQOL compared with the US norm in seven domains; physical functioning, role-physical, general health, social functioning, role-emotional and both the physical and mental summary scores (see Table II). Effect size calculations demonstrated a clinically significant but small effect in role-physical, social functioning, role-emotional, and the physical summary score, and a large effect on general health. When limited to US patients HRQOL remained significantly lower in five domains; role-physical, general health, social functioning, role-emotional and the physical summary score with a clinically significant effect size in general health (large), and the physical summary score (small).

In univariate analysis lower HRQOL was associated with female gender, older age, receiving treatment in the UK, having a higher number of complications, and having a higher number of chelation side effects (for patients on oral chelator). Higher HRQOL was associated with being transfused (general health domain only) and being on an oral chelator. There was no association between any measure of compliance and HRQOL for patients on DFO alone. Race and ferritin were not associated with differences in SF-36 scores. In multivariate analysis (controlling for gender, race, chelator choice, frequency of side effects from chelation, country, number of complications, ferritin and transfusion status), older age, greater number of side effects, country (UK), and number of complications were the major factors associated with lower HRQOL, with race and gender showing a more limited effect, and chelator choice and ferritin showing no association with HRQOL in any SF-36 domain (Table III).

This is one of only a few published reports of HRQOL in thalassemia patients, and the first to look at clinical associations with HRQOL. As expected, we found that adolescent and adult patients with thalassemia had impaired HRQOL compared with US norms. After controlling for demographic and clinical variables, we found that older age, greater number of side effects, country (UK), greater number of complications, and to a lesser extent female gender, Asian race, and not being transfused were associated with lower HRQOL.

Older age and female gender were associated with lower HRQOL in the TLC patients, but are known to be associated with lower HRQOL in the general population as well. After controlling for population differences in HRQOL we found no effect of gender over and above that of the general population; however HRQOL is lower in the older TLC patients than would be expected in the general population. This difference was primarily seen in areas of patient reported physical health rather then mental health and remained even after removing UK patients from the analysis.

This conclusion, that HRQOL is lower than expected in older TLC patients, must be interpreted with caution. Since this initial evaluation is a cross-sectional analysis, older TLC patients were diagnosed in an earlier era and may have been receiving transfusion and chelation therapy for longer. Also oral chelation is a relatively new therapeutic option in the US, which may give a generational effect on HRQOL. It will be enlightening to follow TLC patients over time and see if this age effect on HRQOL persists.

Our multivariate results are quite striking, especially since chelator choice was not associated with HRQOL in any domain after adjusting for other variables. This is in contrast with some literature which suggests oral chelation is associated with higher patient satisfaction. This may be due to the fact that patients are free to choose their own chelation, or may reflect the importance of adding clinical variables to the analysis.

Country remained a significant factor across domains, even after controlling for multiple variables, with UK patients reporting lower HRQOL compared with US or Canadian patients; however, the number of UK patients is too small to make conclusions about this finding.

We have attempted to demonstrate which HRQOL differences are clinically relevant to the population. There is a growing literature on minimal clinically important difference (MCID) which can be loosely defined as the smallest change that is important, or seen by the patient as an improvement. A difference in two to five points on the normalized (mean of 50) SF-36 scores is often used as an approximation of the MCID [7,8]. By that measure we can see that many factors (such as age, gender, side effects, and country) have a large effect which is likely to be clinically relevant.

Our findings show some differences from other published literature, although interestingly two Italian thalassemia studies showed contrasting results as well. Messina et al. showed striking impairments in social functioning, role-emotional and the mental component summary [9]. However Scalone et al. showed SF-36 scores for thalassemia patients which were close to country norms, and in some cases even higher [10]. By contrast our results show HRQOL lower than US norms with the greatest effect in general health and the physical domains. It is unclear why the Messina study showed such extremely low scores for social functioning, role-emotional, and the mental summary score, although this may be in part because of the higher average age of the patients in that study. Our findings are however similar to two studies by Payne et al. In a UK study they report SF-36 scores that were lower than age- and gender-matched country-specific norms for all domains and both summary scores, with much lower scores for physical functioning and general health [5]. In a US study they found that patient reported lower HRQOL than US norms with general health showing the most impact [4].

It is important to keep in mind that HRQOL is in many ways a social construct, since it relies on a person's expectation of health. Thus SF-36 norms vary by country with Italian norms being lower than US. For a disease such as thalassemia, which occurs in different populations, and is seen increas-

TABLE II. Comparison Between TLC SF-36 Scores and US Norms

SF-36 measure	US norm	TLC (n = 264)	P-value	Effect size[b]	US patients only	P-value	Effect size#
Physical functioning	50	48.18[a]	0.004	0.18	49.32	0.35	0.068
Role-physical	50	47.33[a]	<0.0001	0.27 (small)[c]	48.52[a]	0.042	0.15
Bodily pain	50	49.41	0.35	0.059	50.19	0.80	−0.019
General health	50	41.5[a]	<0.0001	0.85 (large)[c]	41.27[a]	<0.0001	0.87 (large)[c]
Vitality	50	48.89	0.08	0.11	49.65	0.63	0.035
Social functioning	50	46.79[a]	<0.0001	0.32 (small)[c]	48.01[a]	0.006	0.20
Role-emotional	50	46.78[a]	<0.0001	0.32 (small)[c]	48.15[a]	0.01	0.18
Mental health	50	49.04	0.12	0.096	49.38	0.40	0.062
Physical summary	50	46.8[a]	<0.0001	0.32 (small)[c]	47.57[a]	<0.0001	0.24 (small)[c]
Mental summary	50	47.95[a]	0.002	0.20	48.73	0.11	0.13

[a]One sample T test shows significant difference between population and US norm ($P < 0.05$).
[b]Effect size = difference in mean/population SD
[c]Clinically significant effects defined as: 0.2–0.49 = "small", 0.50–0.79 = "moderate", >0.80 = "large" (8).

TABLE III. Magnitude of Effect for Significant Factors Associated with HRQOL ($P < 0.05$) from Multivariate Model

	Age (decade)	Asian	Side effects	Country (US ref)	Complications	Female	Transfused
Physical Functioning	**−2.28**	**−2.64**		Canada −1.69 **UK −9.42**		**−2.63**	
Role−Physical	−1.93		−1.63	**Canada −2.14 UK −9.45**			
Bodily Pain			**−2.97**	Canada 0.79 **UK −8.96**	−1.16		
General Health	**−2.22**		−1.62		−1.17		**5.46**
Vitality	−1.57	**−3.29**	**−2.37**	Canada 0.87 **UK −6.86**	−0.98		
Social Functioning	**−2.25**		**−2.37**	Canada −1.99 **UK −6.58**			
Role-Emotional	−1.92			**Canada −2.05 UK −10.20**		**−3.13**	
Mental Health			−1.67	Canada 0.59 **UK −4.83**	−1.10		
Physical Summary	**−2.12**		−1.80	Canada −0.09 **UK −8.03**	−0.87		
Mental Summary			−1.79	Canada −0.41 **UK −5.91**			

Highlighted effects (greater than 2 points) may be considered clinically relevant.

ingly in immigrants in the US, these cultural differences are an important area for further study.

As we continue to make medical advances and improve life expectancy in thalassemia, HRQOL become an even more important marker of treatment success. The fact that the SF36 is widely validated and accepted in many countries makes it a good choice to be a standard assessment in thalassemia. Poor HRQOL in TLC patients is due to a complex combination of living with a chronic disease, medical complications, and side effects from chelation therapy. By identifying specific factors associated with lower HRQOL, we can help patients and health care providers focus on those areas likely to have the largest impact. As we follow our patient cohort over time we will also be able to examine associations between clinical changes and changes in HRQOL, which will add valuable information to how we can maximize HRQOL while striving to reduce the clinical burden of disease.

Methods

Our study involved patients enrolled in the Thalassemia Longitudinal Cohort (TLC), part of the Thalassemia Clinical Research Network (TCRN). The TCRN is an NIH/NHLBI funded research network composed of 6 core centers in the US, Canada, and the UK and their associated satellite sites. Patients with thalassemia who required regular medical monitoring and were followed at TCRN and satellite sites were recruited to become part of the TLC study. The goal of the TLC is to measure the prevalence and incidence of complications of thalassemia and their treatment [11]. The core data for TLC includes a medical history interview, medical record review, blood collection, and questionnaires on quality of life, nutritional status, and medication adherence done for each participant at baseline and at yearly follow-up visits.

Demographics (age, race, gender, country of enrollment) and medical data (ferritin, method of chelation, and frequency of transfusions) were collected through self-report and medical chart review. Race was dichotomized into Asian vs. non-Asian because of the small number of patients of 'other' race. Country was used as a three-level predictor and was also dichotomized into US/Canada vs. UK given the similarity in results among North American patients. We divided chelator choice into deferoxamine (DFO) only, oral chelator only, combination chelator, and no chelation. Patients on no chelation where excluded from some analyses that focused on chelator choice.

Because there is no universally agreed upon definition of disease severity beyond diagnosis and transfusion status, we used several proxy measures to get a more nuanced picture of disease burden. Measures we examined included ferritin, transfusion status, self-reported problems related to chelation, and number of disease or treatment related complications. Ferritin levels were obtained from the patient's medical record and used as a continuous variable but log transformed for analysis because of the skew of the data. Transfusion status was defined by the number of transfusions received in the past year; nontransfused (no transfusions) or transfused (including intermittently transfused patients requiring one to seven transfusions and regularly transfused at least eight transfusions per year).

A complication summary score was created for each patient by summing the "yes" responses to a list of 14 potential complications in the questionnaire, for a possible score of 0–14. If data on a complication was missing, it was counted as zero. Potential complications included cardiac complications (congestive heart failure, ventricular arrhythmia, low cardiac T2* by MRI), endocrine complications (diabetes Type I, diabetes Type II, growth hormone deficiency, hypothyroidism, hypoparathyroidism, hypogonadotropic hypogonadism), liver disease (cirrhosis), and transfusion related complications and infections (alloimmumization, active hepatitis C, chronic active hepatitis B, HIV). The list of complications included in the questionnaire was developed by group consensus among the site PIs for the study, all of whom are experienced clinicians caring for thalassemia patients. The summary score does not take into account the fact that some complications are more significant than others; however, the number of patients with each complication was too small to analyze each one individually.

To evaluate adherence to chelation patients were asked 'in the past month have you had problems' "remembering," " preparing," or "taking" their chelator. Patients on deferoxamine were also asked how often they had problems "sticking yourself" and "wearing the pump". Answers were on a 5-point Likert scale where 1 = never, 2 = rarely, 3 = sometimes, 4 = often, and 5 = a lot. Therefore a higher score indicated more problems with that aspect of adherence. Patients were also asked to rank how often they "felt successful" taking their chelator on the same 5-point Likert scale as above; in that case a higher score was better, indicating more success with chelation. For the final question about compliance, patients were asked to rate how many side effects they felt they had from their chelator. Each question was examined separately, and as part of a total score, to determine which aspect of chelation contributed the most to adherence difficulties and HRQOL

HRQOL was assessed by self-report with the Medical Outcomes Study 36-Item Short Form Health Survey version 2 (SF-36v2) for all patients over age 14. This is a generic measure of health related functional status and

well-being, which has been well-validated in many different populations and disease states. It generates 8 subscale scores; physical functioning, role limitations due to physical health, bodily pain, general health, vitality, social functioning, role limitations due to emotional problems, and mental health. These domains are combined into two summary scores; a physical component summary (PCS) and mental component summary (MCS). Possible scores for each domain range from 0 (worst) to 100 (best), and can be normalized to a mean of 50 and standard deviation of 10 [12]. Published norms for the US population by age and gender were used for comparison. Other reported values for patients with thalassemia were obtained from published studies [4,5,9,10,13-15]. There were a total of 276 patients over age 14 in the TLC. Twelve were excluded for not having the SF-36 filled out completely, which resulted in a total sample of 264.

Mean values and standard deviation for subscales and component summaries of the SF-36 are presented. Comparisons between the US subset of the study population and US norms were done using *t*-test. Comparison was also made with age and gender matched norms to determine if the effects on age and gender in TLC patients were different than that seen in the general population.

To determine which differences were clinically as well as statistically significant we used measures of effect size and minimal clinically important difference (MCID). Effect size was calculated for each SF-36 subscale by dividing the difference between TLC scores and the US norm by the US standard deviation. Clinically significant effects was defined as an effect size >0.2, where 0.2–0.49 = "small," 0.50–0.79 = "moderate" and >0.80 = "large" [7,8]. A difference in at least 2 points on the normalized (mean of 50) SF-36 scores was used as an approximation of a clinically relevant MCID.

Descriptive statistics were calculated for demographic variables (age, gender, race, country), transfusion status (transfused versus nontransfused), chelation choice (deferoxamine, oral chelator, combination therapy or no chelation), complications (as a continuous measure), ferritin (log transformed) and measures of adherence, as well as the SF-36 component scores and summary scores. Because of the differences noted in the UK patients, analyses were re-run with North American patients only. Bivariate analysis was done on each of the above mentioned variables with the SF-36 scores to determine associations and for consideration of inclusion in the final multivariate model.

Multivariate analysis was done including factors that were significant on bivariate screen (age, gender, country, complications, chelator choice, side effects, ferritin, transfusion status). Race was kept in the model despite not being significant on univariate screen because it was felt that it was important to include in this population. Since the only measure of adherence with a significant association with SF-36 scores was "side-effect," it was the only adherence measure included in the final model. The final multivariate model included gender, race (Asian vs. non-Asian), chelator choice (only for patients on chelator), self-reported frequency of side effects from chelation, country, number of complications (as a continuous measure), ferritin (in log scale) and transfusion status (transfused versus not-transfused).

Statistical analyses were performed with SAS 9.2 for Windows (SAS Institute Inc., Cary, NC). QualityMetric Health Outcomes™ Scoring Software 2.0 (QualityMetric Inc., Lincoln, RI) was used to score the SF36 data.

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Contract grant sponsor: NIH-NHLBI; Contract grant number: U01 HL065238. Contract grant sponsor: National Center for Research Resources; Contract grant number: UL1RR024131-01
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Conflict of interest: Ellis Neufeld is the recipient of research grants from Novartis. Published online 30 September 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/ajh.21896

References

1. Borgna-Pignatti C, Cappellini MD, De Stefano P, et al. Survival and complications in thalassemia. Ann NY Acad Sci 2005;1054:40–47.
2. Caro JJ, Ward A, Green TC, et al. Impact of thalassemia major on patients and their families. Acta Haematol 2002;107:150–157.
3. CDC. National Center for Chronic Disease Prevention and Health Promotion [DONE]Health-Related Quality of Life http://www.cdc.gov/hrqol/ June 3, 2010.
4. Payne KA, Desrosiers MP, Caro JJ, et al. Clinical and economic burden of infused iron chelation therapy in the United States. Transfusion 2007;47:1820–1829.
5. Payne KA, Rofail D, Baladi JF, et al. Iron chelation therapy: Clinical effectiveness, economic burden and quality of life in patients with iron overload. Adv Ther 2008;25:725–742.
6. Cappellini M, Bejaoui M, Agaoglu L, et al. Prosepective evaluation of patient-reported outcomes during treatment with deferasirox or deferoxaminen for iron overload in patients with beta-thalassemia. Clin Ther 2007;29:909–917.
7. Kazis LE, Anderson JJ, Meenan RF. Effect sizes for interpreting changes in health status. Med Care 1989;27:S178–S189.
8. Klassen AF, Miller A, Fine S. Health-related quality of life in children and adolescents who have a diagnosis of attention-deficit/hyperactivity disorder. Pediatrics 2004;114:e541–e547.
9. Messina G, Colombo E, Cassinerio E, et al. Psychosocial aspects and psychiatric disorders in young adult with thalassemia major. Intern Emerg Med 2008;3:339–343.
10. Scalone L, Mantovani LG, Krol M, et al. Costs, quality of life, treatment satisfaction and compliance in patients with beta-thalassemia major undergoing iron chelation therapy: The ITHACA study. Curr Med Res Opin 2008;24:1905–1917.
11. NHLBI. Evaluating People With Thalassemia: The Thalassemia Longitudinal Cohort (TLC) Study. Available at: www.clinicaltrials.gov/ct2/show/NCT00661804.
12. Ware JE, Kosinski M, Bjorner JB, et al. User's Manual for the SF-36v2 Health Survey Second Edition.Quality Metric Incorporated; 2007.
13. Apolone G, Mosconi P. The Italian SF-36 Health Survey: Translation, validation and norming. J Clin Epidemiol 1998;51:1025–1036.
14. Hopman WM, Towheed T, Anastassiades T, et al. Canadian normative data for the SF-36 health survey. Canadian Multicentre Osteoporosis Study Research Group. CMAJ 2000;163:265–271.
15. Jenkinson C, Stewart-Brown S, Petersen S, Paice C. Assessment of the SF-36 version 2 in the United Kingdom. J Epidemiol Commun Health 1999;53:46–50.

APPENDIX 1

The following institutions and researchers contributed to the Thalassemia Clinical Research Network Thalassemia Longitudinal Cohort data reported in this paper.

Children's Hospital, Boston (N = 38): Ellis Neufeld, MD, PhD, Principal Investigator, Jennifer Braunstein, NP, Research Nurse, Amber Smith, Study Coordinator, Latoya Lashley, Study Coordinator; Satellite: University of Texas Southwestern Medical Center at Dallas (N = 12), Charles Quinn, MD, MS, Principal Investigator, Deborah Boger, RN, MSN, PNP, Study Coordinator, Leah Adix, Study Coordinator, Sandra Richardson, Study Coordinator; Children's Healthcare of Atlanta (N = 16), Jeanne Boudreaux, MD, Principal Investigator, Leann Hassen, Study Coordinator; Baylor College of Medicine (N = 6), Brigitta Mueller, MD, Principal Investigator, Bogden Dino, Study Coordinator. Weill Medical College of Cornell University (N = 59): Patricia Giardina, MD, Principal Investigator, Elizabeth Evans, Study Coordinator; Satellite: Winthrop University Hospital (N = 6), Mark Weinblatt, MD, Principal Investigator, Linda Skelly, Study Coordinator. The Children's Hospital of Philadelphia (N = 59): Janet Kwiatkowski, MD, Principal Investigator, Marie Martin, RN, Research Nurse, Owen Beams, Study Coordinator; Satellite: Children's Memorial Hospital, Chicago, IL (N = 39), Alexis Thompson, MD, Principal Investigator, Janice Beatty, RN, Research Nurse, Tiffany Drinkwater, Study Coordinator. Children's Hospital at Oakland (N = 52): Elliott Vichinsky, MD, Principal Investigator, Dru Foote, NP, Research Nurse, Nancy Sweeters, Study Coordinator, Olivia Vega, Study Coordinator; Satellites: Children's Hospital of Los Angeles (N = 12), Thomas Coates, MD, Principal Investigator, Susan Carson, RN, Research Nurse, Eun Ha Pang, Study Coordinator, Rachna Khanna, Study Coordinator; Stanford Hospital (N = 5), Michael Jeng, MD, Principal Investigator, Kokil Bakshi, Clinical Research Associate; Children's and Women's Health Center of British Columbia (N = 4), John Wu, Principal Investigator, Heather McCartney, RN, Research Nurse, Colleen Fitzgerald, Study Coordinator, Stephanie Badour, Study Coordinator. Toronto General Hospital, Toronto, Ontario, Canada (N = 5): Nancy F. Olivieri, MD, Principal Investigator, Vivek Thayalasuthan, Study Coordinator; Satellite: Hospital for Sick Children (N = 64), Isaac Odame, MD, Principal Investigator, Manuela Merelles-Pulcini, RN, Study Coordinator. University College London (N = 15), John Porter, MD, Principal Investigator, Cindy Bhagwandin, Study Coordinator; Satellite: Whittington Hospital (N = 24), Farrukh Shah, MD, Principal Investigator. NHLBI oversight, Kathryn Hassell, MD. Data Coordinating Center: New England Research Institutes, Sonja McKinlay, PhD, Principal Investigator, Lisa Virzi, RN, MS, MBA, Project Director, Felicia Trachtenberg, PhD, Senior Statistician.

International working group for myelofibrosis research and treatment response assessment and long-term follow-up of 50 myelofibrosis patients treated with thalidomide-prednisone based regimens

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We have previously reported the benefits of combination therapy with thalidomide and prednisone for the improvement of cytopenias and splenomegaly for patients with myelofibrosis (MF); both primary and those arising from an antecedent polycythemia vera (PV) and essential thrombocythemia (ET) [1]. We report here the overall efficacy of three different thalidomide-prednisone based regimens (alone or in combination with either oral cyclophosphamide for three months or continuous weekly etanercept) when assessed by the IWG-MRT response criteria [2] which were developed after completion of these studies. Additionally we report on the durability of response and long-term follow-up. We observed an overall response rate of 28% by IWG-MRT criteria (mostly clinical improvement—22% anemia, 8% splenomegaly) with a median duration of response of 8.5 months. Toxicities primarily consisted of neuropathy (Grade 3 or higher neuropathy in 6%) and cytopenias (Grade 3 or higher in 20%). After a median follow-up of three years, fourteen patients (28%) had expired from MF at the time of this analysis with median survival of 36 months.

MF is a chronic, clonal myeloproliferative neoplasm characterized by progressive anemia, leukoerythroblastic peripheral blood smear picture, splenomegaly and the potential transformation to acute leukemia [3]. Clinically, patients experience a heterogeneous course with problems of significant constitutional symptoms [4], progressive anemia, and splenomegaly. The treatment of patients with MF remains unsatisfactory. Allogeneic stem cell transplantation is a potentially curative option [5], but its wide applicability is limited by significant toxicity, lack of suitable donors, and the advanced age of many MF patients. Currently available medical therapies have been only of modest efficacy and used mainly for palliation of MF-associated cytopenias (erythropoietin [6], androgens like danazol [7,8]) or splenomegaly (hydroxyurea [9], interferon [10]). Median survival of patients with MF has not improved with medical therapy.

Thalidomide, originally developed as a sedative, hypnotic agent in the 1950s exhibits potent anti-angiogenic properties by inhibiting basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [11,12]. Initial tests of thalidomide in MF [13,14] demonstrated improvement in cytopenias without much impact on splenomegaly, and none on the histologic manifestations of the disease. Tolerance at doses in excess of 100 mg/day was poor (100–800 mg) with significant neurotoxicity which was dose limiting. We performed a subsequent Phase 2 study demonstrating that low dose thalidomide (50 mg) with a tapering dose of prednisone had a much better tolerance with improvement in cytopenias with modest efficacy for splenomegaly [1]. Among objective responses based upon the criteria of that trial 62% of patients experienced improvement in anemia, 40% achieved transfusion independence and 19% had a reduction in their spleen size.

Subsequently we tried to augment the efficacy seen with thalidomide-prednisone by the addition of low dose oral cyclophosphamide (as there was some evidence of synergy when it was used with thalidomide and steroids in multiple myeloma [15]). Additionally, a second combination trial of thalidomide-prednisone with the TNF-alpha inhibitor etanercept (demonstrated single agent symptomatic benefit [16]) was undertaken. In this report we assess the combined long term follow-up on our three thalidomide-prednisone based clinical trials. Finally, we analyzed the outcomes from Thal-pred based MF therapy in light of the IWG-MRT prognostic and response criteria both of which were developed subsequent to these trials [2,17].

A total of 50 patients with MF were enrolled between the three trials (See Table I for demographic information) with demographic characteristics typical for the disorder. Median age at enrollment was 68 years (range, 46–85 years) and 14 were females (28%). Thirty-four (68%) had PMF and the remainder had evolved from pre-existing PV and ET. Forty-five (90%) had Intermediate-2 or higher risk disease by IWG-MRT prognostic criteria. Thirty-one patients were transfusion-dependent prior to the study (62%). The median spleen size was 17.2 cm (range, 0–34 cm). Overall, 78% patients reached the three month juncture on the trials (at which point prednisone was stopped), with 40% reaching six months. Ninety-five percent patients were able to complete the first three months of treatment with thali-

TABLE I. Demographic Characteristics for 50 Patients with Myelofibrosis Treated on Thalidomide-Prednisone Based Regimens

Demographics	TPC ($N = 14$)	TPE ($N = 15$)	TP ($N = 21$)	Totals ($N = 50$)
Median age (years)	72.5 (range 46–85)	67 (54–78)	65 (43–78)	68 (43–85)
Sex	M - 7 (50%)	M −13 (86%)	M −16 (76%)	M-36 (72%)
Type of MF	Primary −11 (79%)	9 (60%)	14 (67%)	34 (68%)
	Post-PV-3 (21%)	2 (13%)	2 (9%)	7 (14%)
	Post-ET-0	4 (27%)	5 (24%)	9 (18%)
IWG-MRT prognostic group [17]	Low-0	0	1 (5%)	1 (2%)
	Intermediate1–1 (7%)	1 (7%)	2 (9%)	4 (8%)
	Intermediate2–1 (7%)	7 (47%)	8 (38%)	16 (32%)
	High risk-12 (86%)	7 (47%)	10 (48%)	29 (58%)
Performance Status (ECOG)				
0	0	1	1	2
1	9	10	16	35
2	5	4	4	13
Transfusion dependent anemia[a]	8/14 (57%)	7/15 (47%)	16/21 (76%)	31 (62%)
Median platelets (×10 9/L)	76 (11–229)	127 (9–512)	154 (23–448)	117 (11–512)
Median WBC (×10 9/L)	11(2.8–70.1)	8.15 (2.6–116)	9.6 (1.6–23.5)	11 (1.6–116)
Abnormal karyotype at enrollment	5/14 (35%)	8/15 (53%)	12/21 (57%)	25 (50%)
Median spleen size[b]	17.2 (0–29)	19.7 (0–23)	16.8 (0–34)	17.2 (0–34)

TP, thalidomide prednisone; TPC, thalidomide prednisone cyclophosphamide; TPE, thalidomide, prednisone, etanercept; M: male.

[a]Requiring more than 2 units of red cells in a month prior to enrollment.

[b]The cm below the left costal margin in mid-clavicular line.

TABLE II. Efficacy and Toxicity Observed in Three Thalidomide-Prednisone Based Regimens for Myelofibrosis

Toxicity	TPC ($N = 14$)	PET ($N = 15$)	TP ($N = 16$)	Totals ($N = 50$)
Toxicity observed ($\geq$ Grade 3)				
Anemia	2	1	0	3
Neutropenia	2	1	0	3
Thrombocytopenia	3	1	0	4
Neurological	1	1	1	3
Constipation	2	1	2	5
Fluid retention/edema	1	0	1	2
Thrombosis	0	0	1	1
Somnolence	0	0	2	2
Efficacy and outcomes				
Median duration of follow up (Months)	22.5 (3–57)	58 (6–75)	42 (6–106)	36 (3–106)
Completion of 3 cycles	8/14 (57%)	11/15 (73%)	20/21 (95%)	39 (78%)
Reason for stopping therapy	No response ($N = 7$)	($N = 8$)	($N = 8$)	23 (46%)
	Toxicity ($N = 4$)	($N = 1$)	($N = 2$)	7 (14%)
Response by IWG criteria [2]				
Complete response	0	0	0	0
Partial response	0	0	1	1
Clinical improvement				
• Anemia	1	4	6	11
• Splenomegaly	0	0	4	4
Stable disease	9	6	9	24
Progressive disease	3	5	2	10
Relapse	0	0	1	1
Median time to response (weeks)	4	4	8	8
Median duration of response (months)	6 (only one response)	9.5 (6–16)	8 (3–42)	7 (3–42)
Median time to next therapy (months)	2 (1–4)	3 (1–45)	7 (2–50)	3 (1–50)

domide-prednisone alone whereas it dropped to 73% and 57% for thalidomide-prednisone-etanercept and thalidomide-prednisone-cyclophosphamide, respectively.

In general across the trials there was good tolerance except for the thalidomide-prednisone-cyclophosphamide regimen (Table II). Toxicity during active therapy included myelosuppression with three cases of Grade 3 anemia, three cases of Grade 3 neutropenia, and four cases of Grade 3 thrombocytopenia (mostly in the cyclophosphamide containing regimen). Initial neuropathy was uncommon with only three cases of Grade 3 neuropathy observed. There was no treatment-attributable mortality. When responses were assessed BY IWG-MRT criteria [2], none of the patients were able to achieve complete response (CR) and there was only one partial remission (PR). Fourteen (28%) obtained clinical improvement (CI) by the IWG-MRT response assessment criteria and the majority (48%) of responses were stable disease (SD). Among the achievers of CI, 11 had response for anemia (22%) and 4 (8%) had response for splenomegaly (one patient in Thalidomide-Prednisone regimen had combined response to anemia and splenomegaly).

After a median follow up of 36 months across this cohort, we observed an overall median duration of response of 8.5 months (range, 3–42 months). Median time to institution of next therapy was three months (range, 1–50 months). There were patients with periods of prolonged stabilization after cessation of therapy. At the time of this analysis, 14 patients (28%) had expired from MF. Median survival across the entire cohort was 36 months (range, 3–106).

Although comparison of three independent trials is not an ideal way to compare and contrast regimen effectiveness, this report suggests greater toxicity, inferior response rate, duration of response, and time to next therapy with the cyclophosphamide containing regimen. There were more females in the regimen containing cyclophosphamide than the other two. More patients had transfusion dependent anemia in the thalidomide-prednisone alone regimen whereas more patients were thrombocytopenic in the cyclophosphamide containing regimen. The median spleen size was comparable across the three studies but more patients had less chromosomal abnormalities in the cyclophosphamide containing. Eastern cooperative group (ECOG) performance status was comparable among these three studies but significantly higher percentage of patients turned out to be high risk by the new IWG-MRT risk assessment criteria in the cyclophosphamide and etanercept containing studies. Despite reported synergy of cyclophosphamide in multiple myeloma and activity of etanercept in alleviating constitutional symptoms in MF, addition of these agents to thalidomide and prednisone does not seem to augment the efficacy. This could partly be secondary to inability of patients to complete the planned total duration of treatment due to toxicity and intolerance. It could also be secondary to more patients belonging to a higher risk prognostic category (as suggested by IWG-MRT criteria) in the other two regimens. Nevertheless, thalidomide-prednisone based regimens seem to be active in a subset of patients with MF, with modest response rates by IWG-MRT criteria but limited by neuropathy.

The therapeutic landscape in MF is changing fast. The discovery of JAK2-V617F mutation, and other molecular insights into the pathophysiology of myeloproliferative neoplasms, has opened an era of potential new therapies for MF. Over a dozen JAK2 inhibitors are in development [18]. Epigenetic changes are being targeted with agents like decitabine [19,20]. Reduced intensity allogeneic transplantation is being explored which may broaden its applicability to more patients with curative potential [21,22]. Newer Jak-2 inhibitors seem to have their main effect on splenomegaly with potential worsening of cytopenias [23,24]. Pomalidomide, a distinct IMiD® immunomodulatory drug , is being tested in MF [25,26]. Published response rates of pomalidomide appear equal or exceed that of thalidomide-prednisone based regimens without treatment limiting neuropathy.

Methods and Study Design

The patients were enrolled in all the three studies after approval from the Mayo Clinic Institutional Review Board. We retrospectively analyzed the data taking into account IWG-MRT response and prognostic criteria. Patients with primary MF and those with MF secondary to Polycythemia Vera (PV) and Essential Thrombocythemia (ET) were eligible. All patients had a pretreat-

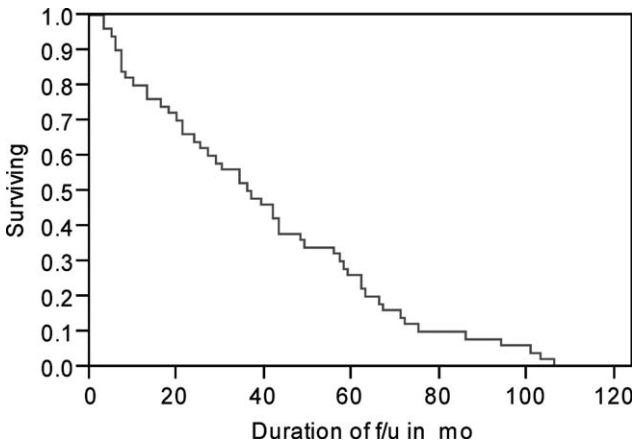

Figure 1. Survival estimate in 50 patients with myelofibrosis treated on thalidomide-prednisone based regimens

ment physical examination, baseline laboratory assessment, assessment of circulatory hematological parameters and bone marrow examination with cytogenetics and fluorescent in situ hybridization (FISH) to exclude occult chronic myelogenous leukemia. Thalidomide was administered per the Celgene System for Thalidomide Education and Prescribing Safety (STEPS) program. In the thalidomide-prednisone study; patients received thalidomide 50-mg orally daily. Prednisone was given in a tapering fashion; 0.5 mg/kg/d for first month, 0.25 mg/kg/d for second month, and 0.125 mg/kg/d for the third month. Patients continued to receive additional three months of thalidomide at the 50 mg daily dose if they showed any response for cytopenias and organomegaly. In the thalidomide-prednisone-cyclophosphamide study; patients received oral cyclophosphamide 25 mg daily for three months along with thalidomide 50 mg daily and prednisone taper as before. Thalidomide was continued as maintenance treatment if there was a response. In the thalidomide-prednisone-etanercept study; 25 mg dose of etanercept was administered subcutaneously twice-a-week along with thalidomide-prednisone as in the aforementioned studies. Patients remained on etanercept and thalidomide if they had any response per protocol. The National Cancer Institute Common Toxicity Criteria (NCI CTC versions 3.0) was used to evaluate toxicity. The descriptive statistics were calculated using means, standard deviations, medians, and ranges for continuous variables; frequencies and relative frequencies for categorical variables. The survival was calculated using Kaplan-Meier survival statistics.

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Conflict of interest: Nothing to report.
Published online 30 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21892

References

1. Mesa RA, Verstovsek S, Cervantes F, et al. Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the international working group for myelofibrosis research and treatment (IWG-MRT). Leuk Res 2007;31:737–740.
2. Tefferi A, Barosi G, Mesa RA, et al. International Working Group (IWG) consensus criteria for treatment response in myelofibrosis with myeloid metaplasia, for the IWG for Myelofibrosis Research and Treatment (IWG-MRT). Blood 2006;108:1497–1503.
3. Tefferi A. Myelofibrosis with myeloid metaplasia. New England Journal of Medicine 2000;342:1255–1265.
4. Mesa RA, Schwager S, Radia D, et al. The Myelofibrosis Symptom Assessment Form (MFSAF): an evidence-based brief inventory to measure quality of life and symptomatic response to treatment in myelofibrosis. Leuk Res 2009; 33:1199–1203.
5. Guardiola P, Anderson JE, Bandini G, et al. Allogeneic stem cell transplantation for agnogenic myeloid metaplasia: a European Group for Blood and Marrow Transplantation, Societe Francaise de Greffe de Moelle, Gruppo Italiano per il Trapianto del Midollo Osseo, and Fred Hutchinson Cancer Research Center Collaborative Study. Blood 1999;93:2831–2838.
6. Rodriguez JN, Martino ML, Muniz R, Prados D. Recombinant human erythropoietin for the treatment of anemia in myelofibrosis with myeloid metaplasia [letter]. Am J Hematol 1995;48:135–136.
7. Cervantes F, Hernandez-Boluda JC, Alvarez A, Nadal E, Montserrat E. Danazol treatment of idiopathic myelofibrosis with severe anemia. Haematologica 2000;85:595–599.
8. Besa E, Nowell P, Geller N, Gardner F. Analysis of the androgen response of 23 patients with agnogenic myeloid metaplasia: the value of chromosomal studies in prediciting response and survival. Cancer 1982;49:308.
9. Lofvenberg E, Wahlin A. Management of polycythaemia vera, essential thrombocythaemia and myelofibrosis with hydroxyurea. European Journal of Haematology 1988;41:375–381.
10. Cervantes F, Alcorta I, Escoda L, Montserrat E, Rozman C. Alpha interferon treatment of idiopathic myelofibrosis. Med Clin (Barc) 1993;101:498–500.
11. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proceedings of the National Academy of Sciences of the United States of America 1994;91:4082–4085.
12. Kenyon BM, Browne F, D'Amato RJ. Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization. Experimental Eye Research 1997;64:971–978.
13. Barosi G, Grossi A, Comotti B, Musto P, Gamba G, Marchetti M. Safety and efficacy of thalidomide in patients with myelofibrosis with myeloid metaplasia. Br J Haematol 2001;114:78–83.
14. Canepa L, Ballerini F, Varaldo R, et al. Thalidomide in agnogenic and secondary myelofibrosis. Br J Haematol 2001;115:313–315.
15. Mesa RA, Steensma DP, Pardanani A, et al. A phase 2 trial of combination low-dose thalidomide and prednisone for the treatment of myelofibrosis with myeloid metaplasia. Blood 2003;101:2534–2541.
16. Garcia-Sanz R, Gonzalez-Porras JR, Hernandez JM, et al. The oral combination of thalidomide, cyclophosphamide and dexamethasone (ThaCyDex) is effective in relapsed/refractory multiple myeloma. Leukemia 2004;18:856–863.
17. Steensma DP, Mesa RA, Li CY, Gray L, Tefferi A. Etanercept, a soluble tumor necrosis factor receptor, palliates constitutional symptoms in patients with myelofibrosis with myeloid metaplasia: results of a pilot study. Blood 2002;99: 2252–2254.
18. Cervantes F, Dupriez B, Pereira A, et al. New prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. Blood 2009;113:2895–2901.
19. Mesa RA. How I treat symptomatic splenomegaly in patients with myelofibrosis. Blood 2009;113:5394–5400.
20. Vannucchi AM, Guglielmelli P, Rambaldi A, Bogani C, Barbui T. Epigenetic therapy in myeloproliferative neoplasms: evidence and perspectives. J Cell Mol Med 2009;13:1437–1450.
21. Danilov AV, Relias V, Feeney DM, Miller KB. Decitabine is an effective treatment of idiopathic myelofibrosis. Br J Haematol 2009;145:131–132.
22. Snyder DS, Palmer J, Gaal K, et al. Improved outcomes using tacrolimus/sirolimus for graft-versus-host disease prophylaxis with a reduced-intensity conditioning regimen for allogeneic hematopoietic cell transplant as treatment of myelofibrosis. Biol Blood Marrow Transplant 2006;16:281–286.
23. Kroger N, Holler E, Kobbe G, et al. Allogeneic stem cell transplantation after reduced-intensity conditioning in patients with myelofibrosis: a prospective, multicenter study of the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Blood 2009;114:5264–5270.
24. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and Efficacy of INCB018424, a JAK1 and JAK2 Inhibitor, in Myelofibrosis. New England Journal of Medicine 2010;363:1117–1127.
25. Mesa RA, Pardanani AD, Hussein K, et al. Phase1/-2 study of Pomalidomide in myelofibrosis. Am J Hematol 2010;85:129–130.
26. Tefferi A, Verstovsek S, Barosi G, et al. Pomalidomide is active in the treatment of anemia associated with myelofibrosis. J Clin Oncol 2009;27:4563–4569.

Methotrexate-induced subacute neurotoxicity in a child with acute lymphoblastic leukemia carrying genetic polymorphisms related to folate homeostasis

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Subacute methotrexate neurotoxicity (MTX-NT) may occur days to weeks after systemic or intrathecal (IT) MTX administration and is often manifest by stroke-like symptoms. The pathogenesis of MTX-NT has mainly been associated with cerebral folate homeostasis, but the specific mechanism leading to the development of this complication is mostly unknown and is likely to be multifactorial. Most of studies aimed to determine putative genetic determinants of this syndrome have been focused on the methylenetetrahydrofolate reductase (*MTHFR*) C677T single nucleotide polymorphism (SNP). However, there are other functional polymorphisms that have also been identified in

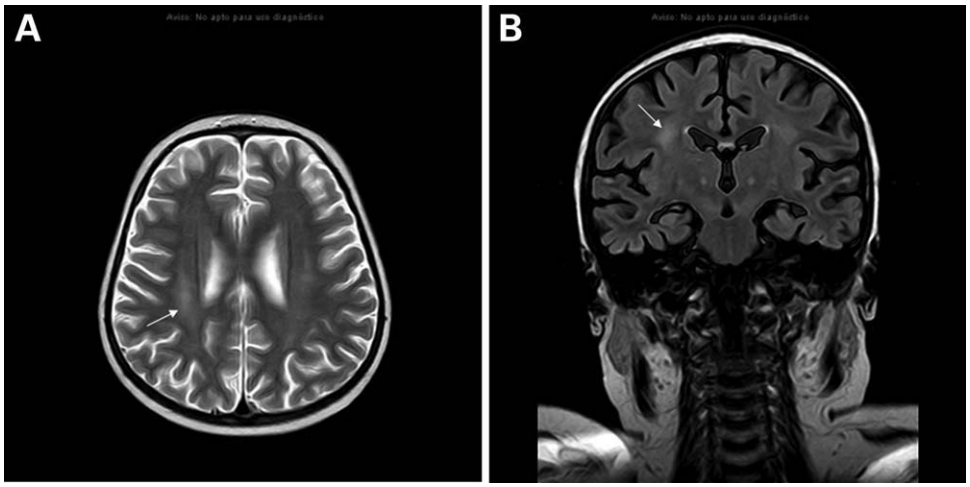

Figure 1. MR imaging findings six weeks after neurologic event. Axial T2 (A) and coronal FLAIR (B) sequences show deep and periventricular white matter hyperintensity, especially in the right hemisphere.

TABLE I. Single Nucleotide Polymorphisms (SNPs) Analyzed in the Patient

Polymorphism	SNP effect	Patient's genotype (%)[a]	Correlation genotype-toxicity
Enzymes			
MTHFR C677T	Reduced enzyme activity	TT (10-20)	+++ [8,9]
MTHFR A1298C	Reduced enzyme activity	AC (25-30)	Controversial [15,26].
DHFR ins/del 19bp	Increased transcription rate	del/del (NA)	++ [17]
DHFR C-1610G/T, C-680A, A-317G	Decreased transcription rate	CC/AA/GG (NA)	NS, association with ALL outcome [27]
TS 2R/3R	Increased transcription rate	2R/3R (30)	++[b] [16]
TS UTR-6bp ins/del	Lower mRNA expression	ins/del (45)	++[b] [16]
CCND1 A870G	Increased expression	AG (65)	+ [28]
MTRR A66G	Reduced enzyme activity	AG (35)	++ [15]
MS A2756G	Reduced enzyme activity	AA (60-90)	−[b] [29]
SHMT C1420T	Reduced enzyme activity	CC (85)	+++[c,d] [10,11]
Transporters			
RFC1 G80A	Decreased MTX uptake	GA ([b])	++ [20]
ABCG2 C421A	Decreased transport activity	AA (8)	+++ [14]
ABCB1 C3435T	Decreased expression	CT (40-60)	+ [14]
ABCB1 G2677T/A	?	GT (25)	NS
ABCB1 C1236T	?	CT (45)	++[c] [22]
ABCC2 C-24T	?	CC (60)	−[c] [30]
ABCC2 IVS 23+56 T>C	?	CC (NA)	++[c] [22]
ABCC2 G1058A	?	GG (100)	−[c] [22]
ABCC2 G1249A	?	GA (15)	+[c] [22]

The patient's genotype for each SNP, its frequency in the Asian (Chinese) population and the reported correlation with MTX toxicity is also shown. NA, frequency not available in Asians; NS, no significant association with toxicity reported for this polymorphism; -, Association with toxicity reported for the SNP but not for the patient's genotype; +, Association with toxicity reported for homozygous but not heterozygous carriers; ++, Association with toxicity reported for the patient's genotype; +++, Severe neurotoxicity reported for the patient's genotype.

[a]Frequency of the genotype carried by the patient in Asian (Chinese) populations. Data have been retrieved from the HapMap database (www.hapmap.org) or from population studies when available.

[b]Frequency variability higher than 50% between studies

[c]Evidences from studies carried out in diseases other than leukemia

[d]In vitro data.

enzymes and transporters related to MTX and folate homeostasis. In this context, we carried out an extensive genetic analysis through the screening of 21 SNPs in 11 relevant genes in a five-year-old girl with acute lymphoblastic leukemia (ALL) who developed MTX-NT. The analysis revealed the presence of numerous genetic variants that may have accounted for the neurotoxicity observed. We discuss the putative role of MTX pharmacogenetics in the pathogenesis of MTX-NT.

MTX is a valuable drug in the treatment of childhood malignancy and is included in both antileukemic and antisolid tumor protocols. However, the drug also has a significant toxic effect on the central nervous system (CNS) and can potentially lead to severe neurologic morbidity [1].

Subacute methotrexate neurotoxicity is an uncommon complication of MTX therapy that generally develops within 5–14 days after the administration of intrathecal or high-dose intravenous (HD-IV) MTX and is manifest by abrupt onset of transitory focal neurological deficits, such as aphasia or hemiparesis. Current knowledge on MTX-NT is based upon spinal fluid analysis and neuroimaging techniques. At the onset of the symptoms, conventional CT scans, T1 or T2 weighted magnetic resonance (MR) imaging and angiography typically show no abnormalities, whereas diffusion-weighted imaging is able to show restricted diffusion of water in the brain that clears after resolution of the clinical symptoms [2]. Follow-up MR imaging shows variable abnormal T2 and FLAIR signal intensity in the deep white matter, with no detectable neurological sequelae in most patients [2].

We present the case of a five-year-old girl with acute lymphoblastic leukemia (ALL) who developed MTX-NT and was found to carry numerous allelic variants in relevant genes of the folate pathways.

A five-year-old Chinese girl presented with a two-month history of fatigue, pallor, and ecchymosis. Work-up revealed pancytopenia, hepatosplenomegaly and more than 90% lymphoblasts in the bone marrow. She was diagnosed with high risk B-precursor ALL, with aberrant expression of myeloid markers and pathological karyotype t (7,17) (q32,q21). Pre-treatment study revealed no CNS affectation by leukemia and thrombophilia tests were negative. In accordance with the protocol of the Spanish Society of Pediatric Hematology and Oncology [3], the patient was treated with induction therapy

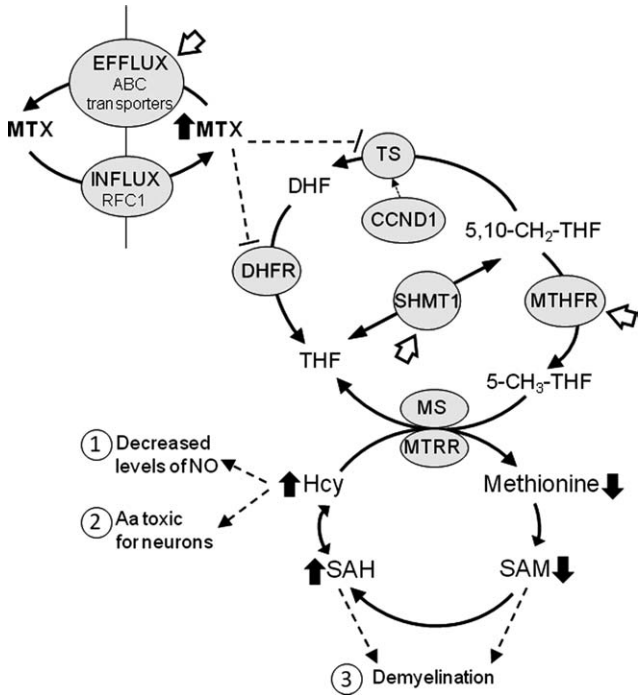

Figure 2. Overview of methotrexate (MTX) mechanisms of action in the folate metabolic pathway. Blank arrows mark the location of the genotypes carried by the patient that most likely could have contributed to the reported complications. Solid arrows mark the predicted consequences of these mutations: Three mechanisms underlying the onset of neurotoxicity are proposed.

that included prednisone, daunorubicin, vincristine, cyclophosphamide, asparaginase and triple IT therapy (TIT) (12 mg MTX, 30 mg cytarabine, and 20 mg hydrocortisone on days +1, +8, and +15). The patient tolerated induction therapy without complications and was a rapid early responder, without evidence of residual disease at day +36.

One week later (day +43), consolidation treatment was started with oral mercaptopurine (40 mg/m^2 daily), TIT and HD-IV MTX (3 g/m^2) over 24-hr continuous infusion, followed 36 hr later by 15 mg/m^2 of folinic acid every 6 hr. No other drugs were administered during this time. MTX clearance was normal and the treatment was well tolerated except for mild thrombopenia. However, 10 days after the first course of HD-IV MTX (day +53), the patient developed two intermittent episodes of moderate dysarthria without aphasia, followed 12 hr later by left hemiparesis and paresthesias that lasted less than 2 hr with subsequent complete recovery. On physical examination, blood pressure was normal; the patient had no fever and was awake and alert with left hemiparesis and speech disability. Ocular movements and fundoscopic and sensitive examinations were normal. An emergency CT scan was negative and the analyses of CSF revealed normal protein and glucose and no lymphoblasts. Microbiologic analyses and PCR testing for neurotropic virus were also negative. Hemogram, coagulation studies, acute phase reactants, electrolytes, and viral serology were all normal. Plasma homocysteine (Hcy) concentration was within normal range (5.94 μM/L). Prophylactic treatment with low-molecular-weight heparin and dexamethasone was implemented.

MR imaging, including angiography and diffusion-weighted sequences, was performed four days after the neurological events (day +57) showing no abnormalities, which could be attributable to the patient being asymptomatic at that time [4]. However, electroencephalogram (EEG) revealed δ-waves activity on right parieto-temporal region without epileptiform discharges. In any case, the patient was scheduled to receive prophylactic antiepileptic therapy with levetiracetam for one year. MR images were repeated six weeks after the clinical event revealing higher signal intensity on FLAIR images in the deep white matter (see Fig. 1). These abnormalities disappeared three months later, which is consistent with previous reports [5]. At that time the EEG had become normal as well.

After these neurological events, the administration of MTX (IV or IT) was terminated and the consolidation phase was resumed with HD-IV cytarabine. The patient completed the rest of the therapy for ALL, which included TIT without MTX and oral MTX. Oral doses were adjusted to 7 mg/m^2 because severe neutropenia was observed when higher doses of MTX were administered. Presently, she remains in complete remission, asymptomatic and with normal neurological examination.

The neurological syndrome experienced by this patient is consistent with MTX-NT. Indeed, the stroke-like symptoms occurred 10 days after MTX administration and resolved completely within 24 hr. In addition, FLAIR images performed six weeks later showed abnormal hyperintense signals involving right subcortical white matter in a distribution according to the neurological deficits [2]. Moreover, further studies ruled out additional processes that could have explained the symptoms, such as CNS infiltration as well as metabolic, infectious or vascular events.

The pathogenesis of MTX neurotoxicity is poorly understood and no specific risk factors for the development of this complication have been identified to date. Pharmacokinetic data in our patient did not support the occurrence of neurotoxicity, although MTX-NT is not necessarily associated with pharmacokinetic parameters [6]. In search of a mechanistic explanation for the adverse effects observed, we focused on MTX pharmacogenetics.

There is a growing body of evidence indicating that MTX toxicity in ALL and other patients, including CNS affectation, can be related to the presence of SNPs [7]. However, most of the available studies are focused in one or, less commonly, a few significant SNPs. In the present work, we have aimed to identify a wider genetic signature that could have accounted for the observed toxicity.

The patient was found to carry functional allelic variants in most of the genes involved in MTX and folate pathways (Table I). On the basis of data from previous association studies, three detected genotypes could have played a major role in the described complications. Most importantly, the patient was homozygous for the methylenetetrahydrofolate reductase (*MTHFR*) C677T SNP, a genotype present in 10–20% of Chinese subjects. This genotype has been consistently associated with MTX adverse effects and suggested to induce MTX-NT in young ALL patients [8,9]. Second, the patient carried the wildtype CC genotype in position 1420 of the serine hydroxymethyltransferase (*SHMT1*) gene, which codes for a pivotal enzyme of the folate pathway. This genotype has been linked to increased citotoxicity in leukemic cells of children with ALL [10] and to MTX-induced neurotoxicity in rheumatoid arthritis (RA) patients [11].

Third, the patient was homozygous for the ATP-binding cassette (*ABC*)*G2* 421A minor allele, a genotype carried by less than 10% of Chinese individuals that decreases the ability to pump substrates such as MTX out of the cell [12,13]. Indeed, the 421AA genotype has been related to the occurrence of MTX adverse effects in children with ALL [14]. Moreover, the combined presence of the *ABCG2* 421A and *ABCB1* 3435T minor alleles detected in the patient has also been related with higher risk of encephalopathy [14].

The patient carried other genotypes that could also account for MTX-induced toxicity, although probably playing a less important role. For instance, the methionine synthase reductase (*MTRR*) 66AG heterozygous genotype has been recently related to increased risk of developing toxicity in children with ALL treated with HD MTX [15]. In addition, the patient was heterozygous for the two SNPs analyzed in the thymidylate synthase (*TS*) gene, which codes for a pharmacological target of MTX. While there is as yet no evidence of a link between *TS* SNPs and toxicity in the leukemia setting, a study in psoriasis patients did find an association with increased occurrence of MTX side effects [16]. Interestingly, the authors showed that even heterozygous individuals were also at higher risk for toxicity [16].

The patient was also homozygous for three of the four polymorphisms analyzed in the dihydrofolate reductase (*DHFR*) gene, which codes for another major MTX target enzyme. Of these SNPs, the 19bp del/del genotype has been associated with increased toxicity in leukemia patients treated with MTX [17].

With regard to SNPs in genes involved in MTX or folate transport, the patient was heterozygous for the G80A polymorphism in the reduced folate carrier 1 (*RFC1*, *SLC19A1*). The 80A minor allele has been associated to increased toxicity in children with ALL treated with MTX [18,19], even in heterozigosity [20], although it should be remarked that the functional role of this SNP is still controversial [21].

In addition, data from the RA setting regarding polymorphisms in efflux transporters indicate that two minor alleles detected in the patient (*ABCC2* IVS 23 + 56C and 1249A) might lead to higher risk of MTX toxicity [22]. Furthermore, the observed heterozygous *ABCB1* 1236GT and *ABCC2* IVS 23 + 56TT genotypes have also been associated with increased MTX side effects in the same population [22].

It should be noted that large studies on the association between MTX toxicity and genetics in childhood ALL are still scarce. Because of this, some of the conclusions for the SNPs analyzed (especially regarding ABC transporters) were extrapolated from other diseases, particularly RA, and therefore should be taken cautiously.

According to the scheme depicted in Fig. 2, the excess of MTX and the decreased functionality of folate enzymes caused by the described genetic alterations could lead to increased Hcy intracellular levels [23]. Toxic effects of Hcy may be mediated by cerebrovascular ischemia via oxidative stress [24], which is consistent with the stroke-like symptoms developed by our patient. This underlying mechanism has also been proposed for patients with MTX-NT carrying the MTHFR 677T variant [8,9]. It should be noted that Hcy plasma concentrations in the patient were normal. In fact, it has been suggested that other biological markers in the CSF could correlate better with the MTX-NT [25].

In summary, the genetic analysis shows that the patient carried numerous genetic variants previously associated with MTX side effects, including neurotoxicity, which may have accounted for some of the symptoms described. However, the current scenario of MTX pharmacogenetics is extremely complex. The wide array of applications of this drug has probably hampered the drawing of definitive conclusions with respect to the clinical impact of genetic polymorphisms, as research efforts have been divided into study designs with greatly different populations, therapies, etc. Larger, prospective studies are therefore needed to fully elucidate the genetic determinants of MTX-induced toxicity in childhood ALL patients.

Methods

Twenty-one SNPs affecting 11 genes involved in the MTX and folate intracellular pathways were analyzed in this study (Table I). SNPs were chosen on the basis of a previous reported association with MTX toxicity. In a few cases no direct evidence for this association was evident in the ALL setting but a relation with toxicity had been shown for other diseases (e.g., RA and psoriasis) (Table I). No direct association with MTX toxicity has been reported for four of the SNPs analyzed, namely *ABCB1* G2677T/A and *DHFR C-1610G/T, C-680A* and *A-317G*. The *ABCB1* G2677T/A SNP is a nonsynonimous polymorphism with the potential to alter the transporting ability of P-glycoprotein, a transmembrane protein likely involved in MTX transport. On the other hand, *DHFR* SNPs have been shown to decrease the transcription rate of the gene and to modify the outcome of ALL. For these reasons, a putative association with toxicity seems plausible and the SNPs were included in the analysis.

All polymorphisms were screened for by means of PCR-RFLP and direct sequencing.

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Contract grant sponsor: Junta de Extremadura, Consejería de Economía, Comercio e Innovación, Merida, Spain; Contract grant number: PRI08A008
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Conflict of interest: Nothing to report.
Published online 30 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21897

References

1. Shuper A, Stark B, Kornreich L, et al. Methotrexate-related neurotoxicity in the treatment of childhood acute lymphoblastic leukemia. Isr Med Assoc J 2002;4:1050–1053.
2. Rollins N, Winick N, Bash R, et al. Acute methotrexate neurotoxicity: Findings on diffusion-weighted imaging and correlation with clinical outcome. AJNR Am J Neuroradiol 2004;25:1688–1695.
3. Badell I, Munoz A, Estella J, et al. Long-term results of two consecutive trials in childhood acute lymphoblastic leukaemia performed by the Spanish Cooperative Group for Childhood Acute Lymphoblastic Leukemia Group (SHOP) from 1989 to 1998. Clin Transl Oncol 2008;10:117–124.
4. Kuker W, Bader P, Herrlinger U, et al. Transient encephalopathy after intrathekal methotrexate chemotherapy: Diffusion-weighted MRI. J Neurooncol 2005; 73:47–49.
5. Inaba H, Khan RB, Laningham FH, et al. Clinical and radiological characteristics of methotrexate-induced acute encephalopathy in pediatric patients with cancer. Ann Oncol 2008;19:178–184.
6. Rubnitz JE, Relling MV, Harrison PL, et al. Transient encephalopathy following high-dose methotrexate treatment in childhood acute lymphoblastic leukemia. Leukemia 1998;12:1176–1181.
7. Gervasini G. Polymorphisms in methotrexate pathways: What is clinically relevant, what is not, and what is promising. Curr Drug Metab 2009;10:547–566.
8. Mahadeo KM, Dhall G, Panigrahy A, et al. Subacute methotrexate neurotoxicity and cerebral venous sinus thrombosis in a 12-year old with acute lymphoblastic leukemia and methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: Homocysteine-mediated methotrexate neurotoxicity via direct endothelial injury. Pediatr Hematol Oncol 2010;27:46–52.
9. Strunk T, Gottschalk S, Goepel W, et al. Subacute leukencephalopathy after low-dose intrathecal methotrexate in an adolescent heterozygous for the MTHFR C677T polymorphism. Med Pediatr Oncol 2003;40:48–50.
10. de Jonge R, Hooijberg JH, van Zelst BD, et al. Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia. Blood 2005;106:717–720.
11. Weisman MH, Furst DE, Park GS, et al. Risk genotypes in folate-dependent enzymes and their association with methotrexate-related side effects in rheumatoid arthritis. Arthritis Rheum 2006;54:607–612.
12. Vlaming ML, van Esch A, Pala Z, et al. Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. Mol Cancer Ther 2009;8:3350–3359.
13. Mizuarai S, Aozasa N, Kotani H. Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2. Int J Cancer 2004;109:238–246.
14. Erdilyi DJ, Kamory E, Csokay B, et al. Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy. Pharmacogenomics J 2008;8:321–327.
15. Huang L, Tissing WJ, de Jonge R, et al. Polymorphisms in folate-related genes: Association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Leukemia 2008;22:1798–1800.
16. Campalani E, Arenas M, Marinaki AM, et al. Polymorphisms in folate, pyrimidine, and purine metabolism are associated with efficacy and toxicity of methotrexate in psoriasis. J Invest Dermatol 2007;127:1860–1867.
17. Ongaro A, De Mattei M, Della Porta MG, et al. Gene polymorphisms in folate metabolizing enzymes in adult acute lymphoblastic leukemia: Effects on methotrexate-related toxicity and survival. Haematologica 2009;94:1391–1398.
18. Imanishi H, Okamura N, Yagi M, et al. Genetic polymorphisms associated with adverse events and elimination of methotrexate in childhood acute lymphoblastic leukemia and malignant lymphoma. J Hum Genet 2007;52:166–171.
19. Shimasaki N, Mori T, Samejima H, et al. Effects of methylenetetrahydrofolate reductase and reduced folate carrier 1 polymorphisms on high-dose methotrexate-induced toxicities in children with acute lymphoblastic leukemia or lymphoma. J Pediatr Hematol Oncol 2006;28:64–68.
20. Kishi S, Cheng C, French D, et al. Ancestry and pharmacogenetics of antileukemic drug toxicity. Blood 2007;109:4151–4157.
21. Whetstine JR, Gifford AJ, Witt T, et al. Single nucleotide polymorphisms in the human reduced folate carrier: Characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. Clin Cancer Res 2001;7:3416–3422.
22. Ranganathan P, Culverhouse R, Marsh S, et al. Methotrexate (MTX) pathway gene polymorphisms and their effects on MTX toxicity in Caucasian and African American patients with rheumatoid arthritis. J Rheumatol 2008;35:572–579.
23. Gellekink H, Blom HJ, van der Linden IJ, et al. Molecular genetic analysis of the human dihydrofolate reductase gene: Relation with plasma total homocysteine, serum and red blood cell folate levels. Eur J Hum Genet 2007;15:103–109.
24. Stamler JS, Osborne JA, Jaraki O, et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308–318.
25. Vezmar S, Schusseler P, Becker A, et al. Methotrexate-associated alterations of the folate and methyl-transfer pathway in the CSF of ALL patients with and without symptoms of neurotoxicity. Pediatr Blood Cancer 2009;52:26–32.
26. Pakakasama S, Kanchanakamhaeng K, Kajanachumpol S, et al. Genetic polymorphisms of folate metabolic enzymes and toxicities of high dose methotrexate in children with acute lymphoblastic leukemia. Ann Hematol 2007;86:609–611.
27. Dulucq S, St-Onge G, Gagne V, et al. DNA variants in the dihydrofolate reductase gene and outcome in childhood ALL. Blood 2008;111:3692–3700.
28. Costea I, Moghrabi A, Laverdiere C, et al. Folate cycle gene variants and chemotherapy toxicity in pediatric patients with acute lymphoblastic leukemia. Haematologica 2006;91:1113–1116.
29. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum 2006;54:3095–3103.
30. Rau T, Erney B, Gores R, et al. High-dose methotrexate in pediatric acute lymphoblastic leukemia: Impact of ABCC2 polymorphisms on plasma concentrations. Clin Pharmacol Ther 2006;80:468–476.

Demonstration of additional benefit in adding lenalidomide to azacitidine in patients with higher-risk myelodysplastic syndromes

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Lenalidomide and azacitidine are active in MDS patients, and may complement each other by targeting the bone marrow microenvironment and the malignant clone. A recent Phase I trial testing the lenalidomide and azacitidine combination yielded encouraging results; however, lenalidomide's contribution was unclear. In this study, 18 higher-risk MDS patients were treated with the combination for seven cycles, after which lenalidomide was discontinued in eight patients who achieved a complete response, with azacitidine monotherapy continuing until disease progression. We report on three patients who relapsed on monotherapy with excess blasts at 12, 19, and 24 months, in whom lenalidomide was then resumed in combination with azacitidine. Each patient, one with normal cytogenetics at relapse; one with a +8 abnormality; and one with del(4q25), recaptured a complete response that was sustained for 5, 7, and 7+ months. We conclude that the addition of lenalidomide to azacitidine provides additional clinical benefit over azacitidine monotherapy.

The myelodysplastic syndromes (MDS) comprise a spectrum of bone marrow disorders associated with cytopenias, cytogenetic abnormalities, and in higher-risk subtypes (patients with excess myeloblasts or an International Prognostic Scoring System (IPSS) risk score >1.0), a high likelihood of transformation to acute myeloid leukemia (AML) [1–5]. Three drugs, azacitidine, decitabine and lenalidomide, are approved for the treatment of MDS [6–8]. Lenalidomide selectively suppresses deletion 5q MDS clones through inhibition of haplodeficient cell cycle regulatory targets coded within the common deleted region (CDR) [9,10] complemented by effects on the bone marrow microenvironment. Azacitidine and decitabine exert their effects via DNA methyltrasferase inhibition and also direct cytotoxicity, [11] and azacitidine monotherapy improves overall survival in higher-risk MDS patients compared to conventional care [12].

We recently reported results from a Phase I study of combination lenalidomide and azacitidine in 18 higher-risk MDS patients, in which a Phase II dose was established: azacitidine administered at 75 mg/m^2 daily for five consecutive days, and lenalidomide 10 mg daily for 21 days, of a 28-day cycle [13]. The overall response rate was 67%, including a complete response (CR) rate of 44%. Patients were treated with combination therapy for seven cycles, after which patients achieving a CR were continued on azacitidine monotherapy, administered for five to seven consecutive days at 75 mg/m^2 daily, repeated every four to six weeks. As this was a single-arm study, it was unknown whether combination therapy provided additional benefit over single-agent azacitidine.

We report herein three patients with normal cytogenetics who achieved a CR with a lenalidomide and azacitidine combination regimen; continued on azacitidine monotherapy until disease relapse, with two patients demonstrating distinct cytogenetic abnormalities; and recaptured their CR status with the reinitiation of lenalidomide combined with azacitidine (see Fig. 1).

This multicenter, single-arm, open-label, Phase I study of combination therapy with lenalidomide and azacitidine received local Institutional Review Board approval from all participating sites and from the Data Safety and Monitoring Board of the Rare Diseases Branch of the National Institutes of Health, and was registered with http//:clinicaltrials.gov (NCT00352001). The study opened in May, 2005 and the last subject was enrolled in May, 2008. Responses were defined according to 2006 International Working Group criteria [14].

Case 1

This 70-year-old man with newly diagnosed RAEB-2 (15% blasts), normal cytogenetics, no somatic lesions by single nucleotide polymorphism array (SNP-A) karyotyping, and an IPSS score of 1.5, started combination therapy with lenalidomide (5 mg daily for 21 days) and azacitidine (75 mg/m^2 daily for 5 days) in May, 2007 (Table I). He achieved a CR in September, 2007, and completed the study in December, 2007. Maintenance therapy was initiated with azacitidine monotherapy, 75 mg/m^2 daily for six days, repeated every four to six weeks. Nineteen months later (April, 2009) he relapsed, with decreased peripheral blood counts and 7% blasts, with normal cytogenetics. Lenalidomide was resumed at a daily dose of 10 mg for 21 days combined with azacitidine. His counts normalized, and a bone marrow biopsy in August, 2009, confirmed CR recovery, with 1% blasts and normal peripheral counts. A microdeletion of 4q25, spanning nucleotides 109226732-109731065, was detected using SNP-A. He remained in a CR until February, 2010, when he progressed to AML with persistence of the 4q25 SNP-A-detected microdeletion. He is undergoing cytotoxic induction therapy.

Case 2

This 72-year-old man presented with newly-diagnosed RAEB-1 (7% blasts), normal cytogenetics, with no additional somatic lesions by SNP-A-based karyotyping, and an IPSS score of 1.0. He started combination therapy in September, 2007, with lenalidomide (5 mg daily for 14 days) and azacitidine (50 mg/m^2 daily Days 1–5 and 8–12). He achieved CR in January, 2008, and completed the study in April, 2008, then continuing azacitidine monotherapy, 75 mg/m^2 daily for six days, repeated every four to six weeks. He relapsed nine months later, in January, 2009, with decreased platelet and neutrophil counts and 8% bone marrow blasts, with a new +8 cytogenetic abnormality. He continued on azacitidine, with lenalidomide added at 10 mg daily for 21 days. His peripheral blood counts normalized, and a second CR was confirmed by bone marrow biopsy in May, 2009, with 3% blasts and normal cytogenetics. SNP confirmed no somatic lesions. He progressed to AML in January, 2010, and achieved a third CR following remission induction chemotherapy.

Case 3

This 60-year-old man presented with newly diagnosed RAEB-2 (16% blasts), normal cytogenetics, and an IPSS score of 2.0. He began combination therapy with lenalidomide (10 mg daily for 21 days) and azacitidine (75 mg/m^2 daily for 5 days) in September, 2007. A CR was documented in November, 2007, and he completed the combination study in March, 2008, continuing therapy with azacitidine monotherapy, at a dose of 75 mg/m^2 daily for five to seven days, repeated every four to six weeks. Karyotyping using SNP-A identified no somatic lesions. Twenty months later, in November, 2009, he developed neutropenia and thrombocytopenia, and a bone marrow biopsy confirmed relapsed disease, with 6% blasts and normal cytogenetics, and no additional somatic lesions identified by SNP-A analysis. He continued on azacitidine at the same dose and schedule, with the addition of lenalidomide at 10 mg daily for 21 days. A bone marrow biopsy performed in April, 2010, demonstrated a reduction in blast percentage, to 2%, and no somatic lesions detected by SNP-A karyotyping. He continues on the combination therapy.

Discussion

Our Phase I study was the first trial to combine two FDA-approved drugs for MDS, lenalidomide, and azacitidine. Here we report evidence supporting the additive benefit of lenalidomide in this combination strategy. Three patients served as their own internal controls, achieving a CR with the combination regimen; relapsing after prolonged azacitidine monotherapy maintenance; and then recapturing CR with lenalidomide readministration. In so doing, we believe we satisfied Koch's postulates for causality, [15] when applied to disease in general, as agents were introduced and evoked a response; one was withdrawn, with loss of that response; and was then reintroduced, with the same response resulting.

The combination design of the initial Phase I study was intended to complement the antiproliferative effects of azacitidine on the MDS clone with the modulatory effects of lenalidomide on the bone marrow microenvironment. These

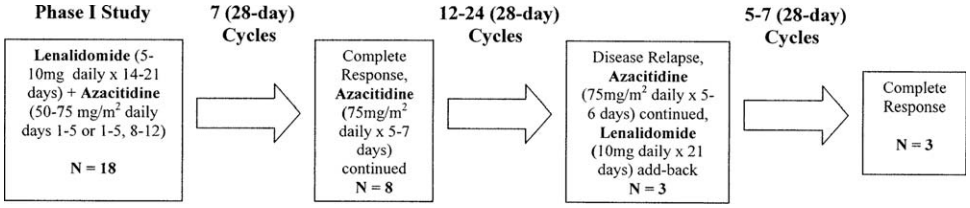

Figure 1. Treatment course.

TABLE I. Characteristics of Subjects

Characteristic	Patient 1	Patient 2	Patient 3
Age at study entry (years)	66	72	62
WHO diagnosis	RAEB-2	RAEB-1	RAEB-2
IPSS score	1.5	1.0	2.0
Baseline: Hgb (g/dl)	8.1	12	9.2
Plt ($\times 10^3$/ml)	243	94	37
ANC ($\times 10^3$/ml)	4.28	0.88	0.32
Blast %	15	7	16
Cytogenetic lesions	ND	ND	ND
Initial combination regimen:	AZA 75mg/m^2 days 1-5; LEN 5mg days 1-21	AZA 50mg/m^2 days 1-5, 8-12; LEN 5mg days 1-14	AZA 75mg/m^2 days 1-5; LEN 10mg days 1-21
Time to initial CR (months)	4	4	4
1st CR duration (months)	19	12	24
Cytogenetic lesions at 1st CR	ND	ND	ND
Cytogenetic lesions at relapse	Del(4q25)	+8	ND
2nd CR Duration (months)	5	7	7+ (NR)
Cytogenetic lesions at 2nd CR	Del(4q25)	ND	ND

WHO, World Health Organization; IPSS, International Prognostic Scoring System; Hgb, hemoglobin; Plt, platelet; ANC, absolute neutrophil count; AZA, azacitidine; LEN, lenalidomide; CR, complete response; NR, not reached yet; ND, none detected.

cases provide unambiguous evidence for the contribution of lenalidomide to the disease modifying effect of the combination regimen, and show that remissions were not maintained by treatment with azacitidine alone. All three patients had normal cytogenetics at diagnosis, confirmed at the molecular level by 250k SNP-A analysis, with specific molecular testing for lesions involving *c-Cbl, b-Cbl, JAK2*, and *TET2* genes. Two patients acquired new genetic lesions during relapse; one involving trisomy 8 (Case 2), which disappeared upon achieving a second CR; the second involving a microdeletion 4q25 (Case 3) detected by SNP-A karyotyping, which persisted at the time of pathologic CR recapture. Second CRs were not durable, and two patients progressed to AML. It is possible that such cryptic genetic lesions involving the *TET2* gene may be linked to lenalidomide responsiveness, as has been speculated recently as lenalidomide relates to histone methylation in Namalwa cell lines [16]. The latter will be investigated in a comparative study of azacitidine vs. the combination to verify the benefit of this novel combination regimen.

In conclusion, the combination of azacitidine and lenalidomide produced clinical benefit compared with single agent azacitidine alone, when patients were used as their own controls.

Author Contributions

M.A.S. designed the research, performed the research, analyzed the data, and wrote the manuscript. C.O. analyzed data and edited the manuscript. A.F.L. designed the research, performed the research, and edited the manuscript. K.P. analyzed the data and edited the manuscript. M.A. performed the research and edited the manuscript. R.E. performed the research and edited the manuscript. J.P.M. designed the research, performed the research, analyzed the data, and wrote the manuscript.

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Contract grant sponsor: NIH; Contract grant number: U54RR19397-03

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Conflict of interest: Celgene Corp - Advisory Board, honoraria.
Published online 30 September 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21891

References

1. List AF, Vardiman J, Issa JP, DeWitte TM. Myelodysplastic syndromes. Hematology (Am Soc Hematol Educ Program) 2004:297–317.
2. Harris NL, Jaffe ES, Diebold J, et al. The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. Ann Oncol 1999;10:1419–1432.
3. Vardiman J, Thiele J, Arber D, et al. The 2008 Revisions of the WHO classification of myeloid neoplasms and acute leukemia: Rationale and important changes. Blood 2009;114:937–951.
4. Steensma DP. The changing classification of myelodysplastic syndromes: what's in a name? Hematology 2009;2009:645–655.
5. Sekeres MA, Schoonen WM, Kantarjian H, et al. Characteristics of US patients with myelodysplastic syndromes: Results of six cross-sectional physician surveys. J Natl Cancer Inst 2008;100:1542–1551.
6. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: A study of the cancer and leukemia group B. J Clin Oncol 2002;20:2429–2440.
7. Kantarjian H, Issa JP, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: Results of a phase III randomized study. Cancer 2006;106:1794–1803.
8. List A, Dewald G, Bennett J, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. N Engl J Med 2006;355:1456–1465.
9. Wei S, Chen X, Rocha K, et al. A critical role for phosphatase haplodeficiency in the selective suppression of deletion 5q MDS by lenalidomide. Proc Natl Acad Sci 2009;106:12974–12979.
10. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. Nature 2008;451:335–339.
11. Jiang Y, Dunbar A, Gondek LP, et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. Blood 2009;113:1315–1325.
12. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: A randomised, open-label, phase III study. Lancet Oncol 2009;10:223–232.
13. Sekeres MA, List AF, Cuthbertson D, et al. Phase I combination trial of lenalidomide and azacitidine in patients with higher-risk myelodysplastic syndromes. J Clin Oncol 2010;28:2253–2258.
14. Cheson BD, Greenberg PL, Bennett JM, et al. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. Blood 2006;108:419–425. Epub 2006 Apr 2011.
15. Koch R. Über den augenblicklichen Stand der bakteriologischen Choleradiagnose. Zeitschrift für Hygiene und Infectionskrankheiten 1893;14:319–333.
16. Escoubet-Lozach L, Lin IL, Jensen-Pergakes K, et al. Pomalidomide and lenalidomide induce p21 WAF-1 expression in both lymphoma and multiple myeloma through a LSD1-mediated epigenetic mechanism. Cancer Res 2009;69:7347–7356.

Effect of chronic red cell transfusion therapy on vasculopathies and silent infarcts in patients with sickle cell disease

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Regular, chronic red cell transfusions (CTX) have been shown to be effective prophylaxis against stroke in sickle cell disease (SCD) in those at risk. Because serial brain imaging is not routinely performed, little is known about the impact of CTX on silent infarcts (SI) and cerebral vascular pathology. Thus, we retrospectively evaluated the magnetic resonance imaging reports of a cohort of SCD patients who were prescribed CTX for either primary or secondary stroke prophylaxis. Seventeen patients with Hb SS were included (mean age 15 years, mean follow-up 4.3 years). Eight patients were on CTX for primary prophylaxis. New SI occurred in 17.6% of patients corresponding to an SI rate of 5.42 per 100 patient-years. Vasculopathy of the cerebral arteries was present in 65% of patients and progressed in 63% of these patients. Those who developed progressive vasculopathy were on CTX for an average of 8 years before lesions progressed. Patients on CTX for secondary prophylaxis had more SIs and evidence of progressive vascular disease than patients on CTX for primary prophylaxis. We conclude that adherence to CTX does not necessarily prevent SI or halt cerebral vasculopathy progression, especially in those with a history of overt stroke.

Cerebrovascular disease is a major complication of sickle cell disease (SCD) and can manifest as an overt stroke, defined as abnormal magnetic resonance imaging (MRI) changes with corresponding clinically apparent neurologic symptoms [1], or a silent infarction (SI), MRI changes occurring without any symptoms. The majority of children with overt strokes have cerebral vasculopathy (injury to intracranial arterial walls) involving the anterior portion of the Circle of Willis [2,3]. The pathophysiology of vasculopathy in SCD has been attributed to multiple molecular and mechanical mechanisms [4]. Chronic hemolysis in SCD causes decreased nitric oxide availability, which may lead to oxidative damage of vessels. Chronic inflammation in SCD causes increased leukocyte activation, which may also lead to endothelial damage. Finally, damage to arterial walls from high blood flow velocity due to anemia may cause noninflammatory intimal hyperplasia of intracranial arteries, leading to progressive vascular stenosis, establishing a focus for thrombosis, infarction, and overt stroke [5,6].

The role of cerebral vasculopathy in the development of SI is still unclear. One hypothesis is that stenosis of medium to large cerebral vessels may set up hemodynamic conditions in which perfusion to distal microvascular beds is impaired, leading to neuronal death around affected microvasculature. This theory may be supported by the finding that those with elevated transcranial Doppler ultrasound (TCD) velocities (representing stenosis of medium to large cerebral vessels) and previous SI have a high risk of developing new or progressive SI [1].

Prophylaxis with regular, chronic erythrocyte transfusions (CTX) for cerebral vascular accident (CVA) prevention is currently recommended for children found to have abnormally high mean blood flow velocities in either the proximal middle cerebral artery or distal internal carotid artery as measured by TCD (termed primary CVA prophylaxis) [7] and in patients who have suffered from an overt CVA (termed secondary CVA prophylaxis). Overall, CTX for primary and secondary CVA prophylaxis reduces the relative risk of stroke in both groups by 85% [8].

Few studies have evaluated the longitudinal effects of primary and secondary CVA prophylaxis on SI or vasculopathy in SCD. There is a paucity of data on the effect of CTX on SI incidence, though a multi-center trial is currently being conducted [15]. Older studies have reported inconsistent findings on the effects of CTX on vasculopathy [3,5,9–12], while more recent work seems to suggest that CTX does not prevent progressive cerebrovascular disease [13,14]. Thus, we retrospectively examined the prevalence and incidence of SI and changes in radiological cerebral vasculopathies in a cohort of SCD patients on primary or secondary stroke prophylaxis who had serial surveillance MRI and magnetic resonance angiography (MRA) of the brain.

Patients. Seventeen patients met inclusion criteria, with eight patients on primary stroke prophylaxis and nine patients on secondary stroke prophylaxis. There were nine males and eight females, with a mean patient age of 15 years (range: 6–23 years). The median follow-up for each patient was 1,184 days (3.2 years), with a mean of 1,580 days (4.3 years ± 3.9), and a range of 275 to 5,819 days. A total of 73.83 patient-years were available for analysis, with an average of 2.7 MRI/MRA studies per patient (range 2–6). The average time interval between the first and last MRI/MRA was 4.3 years. Table I outlines the MRI and MRA findings of the 17 patients.

White matter disease/SI. No patient in this study was found to have had an overt stroke during the observation period. Four of eight (50%) patients on primary CVA prophylaxis had evidence of focal deep white matter lesions on initial imaging, with only one of these patients (patient 15) developing new white matter disease. The rest of the patients on primary prophylaxis did not progress. All nine patients prescribed secondary prophylaxis had white matter disease (WMD) on their initial imaging, with two patients (patients 7 and 9) developing new and/or progressive WMD over the course of approximately 3 and 4 years, respectively. Overall, we found three progressive and one new white matter lesions in three patients, all of which were asymptomatic based on clinical reports. Thus, in this cohort, we observed a rate of 5.42 silent infarcts per 100 patient-years while on CTX, and a prevalence of 17.6%.

Vasculopathy. Overall, 11 of 17 (65%) patients had evidence of cerebral macrovascular pathology, either at initial or subsequent imaging. Of these patients, 63% (7 of 11) had progressive vasculopathy while on CTX. Patients who developed progressive vascular disease were receiving transfusions for an average of 8 years (median = 9.1 years) before their imaging studies revealed progressive vasculopathy. The median time interval between studies for those patients with evidence of progressive vasculopathy and those without evidence of progression was 3.8 years (mean: 6 years) and 3.1 years (mean: 3 years), respectively. Progressive lesions were primarily characterized by increased degree of stenosis of vessels initially affected, rather than involvement of previously unaffected vessels.

Primary prophylaxis. Eight subjects were on primary stroke prophylaxis with two (25%) patients having abnormal macrovascular pathology. One (patient 15) had normal initial imaging, but developed de novo 50% stenosis of the right internal carotid artery (RICA) after a total of 10 years, while the other (patient 16) had vasculopathy on baseline imaging which remained stable over 4 years of observation. The other remaining six (75%) patients had no evidence of vasculopathy (Table II).

Secondary prophylaxis. A total of nine patients were on secondary stroke prophylaxis and eight (89%) had evidence of initial vasculopathy. Six of the nine (67%) patients had evidence of progressive vasculopathy, with the remaining three patients (33%) exhibiting stable vasculopathy. Five patients had evidence of moyamoya and based on clinical reports, the moyamoya had developed in these patients while receiving CTX (Table II).

Our results suggest that CTX does not necessarily halt the progression of vasculopathy in children with SCD on secondary stroke prophylaxis. These observations are consistent with those of Brousse et al. who used a standardized scoring system to grade vascular lesions [13]. Their results demonstrated that in a group of 18 patients on primary and secondary CVA prophylaxis, vasculopathy progressed in patients on secondary prophylaxis, but did not progress in patients receiving primary CVA prophylaxis. A post-hoc analysis of data from the Stroke Prevention Trial in Sickle Cell Anemia (STOP) also found that vasculopathy is not likely to progress in those without a history of overt stroke [16].

Other studies have attempted to assess cerebral vascular changes in those with SCD on CTX for CVA prophylaxis. Two small studies reported progression of vasculopathy in 35–47% of patients [12,17]; however these findings are confounded by nonadherence with CTX in 33–50% of those observed to have progressive vasculopathy. Though these rates of vasculop-

TABLE I. MRA/MRI Findings of Each Study Subject. All Subjects with Hgb SS Disease

Patient	Age (years)/ gender	Transfusion indication	Initial finding		Follow-up finding		Overall changes	Study interval (years)
			Vascular	WMD	Vascular	WMD		
1	20/F	Ischemic stroke	L/ICA/O; R/SC-ICA/S; small caliber L MCA and ACA	L/PL/E; BL/CS/DWMI	Generally progressive stenoses	Stable WMD	Progressed vasculopathy; stable WMD	11
2	11/F	Ischemic stroke	L/ACA,MCA/S; R/PCA/S	BL/FL/WML; L/PL/DWMI; L/PVR/DWMI w/CC	Stable	Stable WMD	Stable vasculopathy; stable WMD	3.4
3	13/F	Ischemic stroke	L/ACA,MCA, SC-ICA/S; R/MCA/O; R/SC-ICA/S; moyamoya	R/MCA/I	L/MCA/O	Stable WMD	Progressed vasculopathy w/moyamoya; stable WMD	3.8
4	21/F	Ischemic stroke	L/SC-ICA/S; L/MCA/S,O; L/ACA/H; R/ACA/S,O; moyamoya	L/MCA/OI; L/PL, TL/E; R/FH/WML	L/MCA/CO; R/ACA/CO	Stable WMD	Progressed vasculopathy w/moyamoya; stable WMD	5.6
5	16/M	Ischemic stroke	L/ICA, PCA/N; L/ACA,MCA/O; R/ICA/S; R/MCA/O; R/ACA/N,O; moyamoya	L/MCA/OI,E; R/MCA/OI, E	Stable	Stable WMD	Stable vasculopathy w/moyamoya; stable WMD	1.25
6	23/M	Ischemic stroke	L/ICA/N; L/MCA,ACA/O; R/MCA/N; R/ICA/O; moyamoya	BL/FL/E; BL/PL/WML; BL/FL, CS, IC/WML; R/T/WML; CCB thinning	Stable	Stable WMD	Stable vasculopathy w/moyamoya; stable WMD	4.2
7	8/M	Ischemic stroke	L/SC-ICA,ACA/S; R/SC-ICA, ACA, MCA/S	R,WSD/FL/AI; R/PVR/WML; L/FL/WML	L/SC-ICA/O; R/MCA/O; R/ACA,SC-ICA/WS	R/FL/IWMD	Progressed vasculopathy; Progressed WMD	2.9
8	7/M	Ischemic stroke	L/ACA,MCA/S; R/ACA,SC-ICA/S; moyamoya	L/FL, P, CN/OI; R/PVN/WML; L/PL/AI; R/CS/AI	R/ACA/O	Stable WMD	Progressed vasculopathy w/moyamoya; stable WMD	1.9
9	21/F	Ischemic stroke	NEV	R/PL/E,WMD; R/FL,CS/WML	L/ACA/S; R/SC-ICA/S	R/CS/IWM; R/PL/NII	Progressed vasculopathy; progressed WMD	16
10	14/F	Abnormal TCDs	NEV	L/FL/WML	NEV	Stable WMD	NEV; Stable WMD	1.5
11	8/M	Abnormal TCDs	NEV	BL/FL/DWMI; R/OL, PL/DWMI	NEV	Stable WMD	NEV; Stable WMD	2.5
12	20/F	Abnormal TCDs	NEV	BL/PL/DWMI; L/FL/DWMI	NEV	Stable WMD	NEV; Stable WMD	1.7
13	21/M	Abnormal TCDs	NEV	NEWMD	NEV	NEWMD	NEV; NEWMD	7.25
14	6/M	Abnormal TCDs	NEV	NEWMD	NEV	NEWMD	NEV; NEWMD	0.75
15	16/F	Abnormal TCDs	NEV	NEWMD	R/ICA/S	L/FL, PVR/WML	New vasculopathy; new WMD	1.6
16	17/M	Abnormal TCDs	L/SC-ICA, MCA, ACA/S; R/SC-ICA/S	BL/CS/WML	Stable	Stable WMD	Stable vasculopathy; stable WMD	3.25
17	16/M	Abnormal TCDs	NEV	NEWMD	NEV	NEWMD	NEV; NEWMD	5.25

L, left; R, right; ICA, internal carotid artery; SC-ICA, supraclinoid internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; PCA, posterior cerebral artery; O, occlusion; S, stenosis; H, hypoplasia; CO, complete occlusion; N, narrowing; WS, worsened stenosis; NEV, no evidence of vasculopathy; PL, parietal lobe; FL, frontal lobe; TL, temporal lobe; OL, occipital lobe; PVR, periventricular region; CS, centrum semiovale; IC, internal capsule; T, thalamus; P, putamen; CN, caudate nucleus; CCB, corpus callosal body; WSD, watershed distribution; E, encephalomalacia; BL, bilateral; DWMI, deep white matter infarct; WMD, white matter disease; WML, white matter lesions; IWMD, increased white matter disease; CC, cystic change; I, infarct; OI, old infarct; AI, acute infarct; NII, new interval infarct; and NEWMD, no evidence of white matter disease.

TABLE II. Comparison of Patients on Primary Stroke Prophylaxis and Secondary Stroke Prophylaxis

	Number of subjects	Progressive vasculopathy	New silent infarcts
Total	17	7	3
Primary prophylaxis	8	1	1
Secondary prophylaxis	9	6	2

Patients on secondary stroke prophylaxis are at higher risk for development of progressive vasculopathy, and possibly silent infarct.

athy progression are similar to our finding of progression in 41% (7 of 17) of patients, our patients are known to be adherent to CTX as documented in their medical records. The significance of progressive vasculopathy, however, remains unclear. No patients in our cohort had an overt stroke during the period in which they were observed. It has been suggested that even if vasculopathy persists, smoothing of vascular luminal surfaces due to CTX may prevent recurrent stroke in affected patients [9]. In our analysis, vasculopathy may progress even after a decade of CTX therapy. Further long-term follow up is needed to better determine the impact of CTX on SCD vasculopathy.

The Cooperative Study of Sickle Cell Disease (CSSCD) sampled an unselected group of 266 children with HbSS and found a SI prevalence of 21.8% and an incidence rate of 7.06 per 100 patient-years [11]. In our cohort, we found a 17.6% SI prevalence and an incidence rate of 5.42 per 100 patient-years. Due to our small sample size, we were unable to calculate whether this is a statistically meaningful difference. Thus, the role of CTX in SI prophylaxis remains unclear. However, King et al. have recently reported that CTX is a feasible treatment option for prophylaxis of progression of SI and are conducting a large multicenter study (the Silent Cerebral Infarct Transfusion trial, or SIT trial) to assess the efficacy of CTX in preventing progression of SI in patients with SI at baseline [18].

Technological advances in imaging techniques over the period of observation in our study may represent another study limitation. Our initial imaging techniques may not have picked up true WMD due to low resolution, leading to the conclusion of development of new WMD once higher resolution MRI scanners were employed over a long observation period. In one published study, a higher SI prevalence in SCD patients was found when compared with historical controls using a higher Tesla MRI scanner [19]. Other limitations of our study include its retrospective design and the subjective nature of radiologic reading.

In conclusion, in SCD patients receiving stroke prophyaxis, new vasculopathy may develop and progress despite CTX. Patients on primary prophylaxis had less progression than those on secondary prophylaxis, consistent with other published reports. This supports the clinical observation that early detection and intervention are key components to maintaining normal or stable cerebral vasculature in the setting of SCD. In addition, SIs can occur in high risk SCD patients despite CTX, although our study could not determine any effects of CTX on SI development due to small sample size. The SIT trial will be better able to clarify the efficacy of this intervention. Our study highlights MRI/MRA as an important, noninvasive tool in monitoring cerebral pathology in patients with SCD and stroke risk. Further longitudinal studies are needed to understand the significance of progressive vasculopathy in SCD.

Methods

An IRB-approved retrospective chart review was performed on patients at Lucile Packard Children's Hospital and Children's Hospital Oakland with the diagnosis of hemoglobin SS who were receiving either primary or secondary stroke prevention as per the National Institute of Health/National Heart, Lung, and Blood Institute (NIH/NHLBI) guidelines. Inclusion criteria included adherence with transfusion therapy as documented by the medical records and hemoglobin S levels, and undergoing at least two MRI and MRA images taken while receiving CTX. Age at study entry, age at CTX initiation, duration of CTX, history of stroke and neurologic studies, and signs and symptoms suggestive of stroke were recorded. MRI and MRA images and reports were centrally reviewed by a board-certified neuroradiologist at Lucile Packard Children's Hospital blinded to all clinical data.

Acknowledgments

We would like to thank the staff of the Sickle Cell Program at Children's Hospital and Research Center at Oakland for all of their help on this project. We would also like to acknowledge the Stanford University Medical Scholars program, which provided funding for this study.

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Conflict of interest: Nothing to report.
Published online 13 October 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21901

References

1. Wong WY, Powars DR. Overt and incomplete (silent) cerebral infarction in sickle cell anemia: Diagnosis and management. Hematol Oncol Clin North Am 2005;19:839–855, vi.
2. Debaun MR, Derdeyn CP, McKinstry RC III. Etiology of strokes in children with sickle cell anemia. Ment Retard Dev Disabil Res Rev 2006;12:192–199.
3. Goldberg HI, Zimmerman RA. Central nervous system. Semin Roentgenol 1987;22:205–212.
4. Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT. Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol 2009;84:618–625.
5. Gerald B, Sebes JI, Langston JW. Cerebral infarction secondary to sickle cell disease: Arteriographic findings. AJR Am J Roentgenol 1980;134:1209–1212.
6. Morris CR. Mechanisms of vasculopathy in sickle cell disease and thalassemia. Hematol Am Soc Hematol Educ Prog 2008:177–185.
7. Adams RJ, McKie VC, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. N Engl J Med 1998;339:5–11.
8. Buchanan GR, DeBaun MR, Quinn CT, Steinberg MH. Sickle cell disease. Hematol Am Soc Hematol Educ Program 2004:35–47.
9. Russell MO, Goldberg HI, Hodson A, et al. Effect of transfusion therapy on arteriographic abnormalities and on recurrence of stroke in sickle cell disease. Blood 1984;63:162–169.
10. Moser FG, Miller ST, Bello JA, et al. The spectrum of brain MR abnormalities in sickle-cell disease: A report from the cooperative study of sickle cell disease. AJNR Am J Neuroradiol 1996;17:965–972.
11. Pegelow CH, Macklin EA, Moser FG, et al. Longitudinal changes in brain magnetic resonance imaging findings in children with sickle cell disease. Blood 2002;99:3014–3018.
12. Moritani T, Numaguchi Y, Lemer NB, et al. Sickle cell cerebrovascular disease: Usual and unusual findings on MR imaging and MR angiography. Clin Imaging 2004;28:173–186.
13. Brousse V, Hertz-Pannier L, Consigny Y, et al. Does regular blood transfusion prevent progression of cerebrovascular lesions in children with sickle cell disease? Ann Hematol 2009;88:785–788.
14. Mirre E, Brousse V, Berteloot L, et al. Feasibility and efficacy of chronic transfusion for stroke prevention in children with sickle cell disease. Eur J Haematol 2010;84:259–265.
15. Casella JF, King AA, Barton B, et al. Design of the silent cerebral infarct transfusion (SIT) trial. Pediatr Hematol Oncol 2010;27:69–89.
16. Abboud MR, Cure J, Granger S, et al. Magnetic resonance angiography in children with sickle cell disease and abnormal transcranial Doppler ultrasonography findings enrolled in the STOP study. Blood 2004;103:2822–2826.
17. Minniti CP, Gidvani VK, Bulas D, et al. Transcranial Doppler changes in children with sickle cell disease on transfusion therapy. J Pediatr Hematol Oncol 2004;26:626–630.
18. King AA, Noetzel M, White DA, et al. Blood transfusion therapy is feasible in a clinical trial setting in children with sickle cell disease and silent cerebral infarcts. Pediatr Blood Cancer 2008;50:599–602.
19. Steen RG, Emudianughe T, Hankins GM, et al. Brain imaging findings in pediatric patients with sickle cell disease. Radiology 2003;228:216–225.

Hepcidin in anemia of chronic heart failure

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Anemia is a common finding among patients with chronic heart failure (HF). Although comorbidities, such as kidney failure, might contribute to the pathogenesis of anemia, many patients with HF do not have any other obvious etiology for their anemia. We investigated whether anemia in HF is associated with an elevation in hepcidin concentration. We used time-of-flight mass spectrometry to measure hepcidin concentration in urine and serum samples of patients with HF and in control subjects. We found that the concentration of hepcidin was lower in urine samples of patients with HF compared with those of control subjects. Serum hepcidin was also reduced in HF but was not significantly lower than that in controls. There were no significant differences between hepcidin levels in patients with HF and anemia compared with patients with HF and normal hemoglobin level. We concluded that hepcidin probably does not play a major role in pathogenesis of anemia in patients with chronic HF.

Between 20% and 40% of patients with heart failure (HF) have anemia [1,2]. There are several possible confounding factors that might contribute to the high frequency of anemia among patients with HF, including concurrent kidney failure. However, because of an upregulation of several inflammatory cytokines in HF [3], it is a reasonable hypothesis that anemia in HF can be an anemia of inflammation. Over the past 8 years, hepcidin has been identified as the molecule contributing to anemia of inflammation [4,5]. Initially discovered as an antimicrobial molecule [6,7], hepcidin has an important role in regulating iron metabolism, particularly during infection and inflammation [8–10]. Hepcidin is mainly synthesized in the liver and released to the plasma. During an acute inflammatory response, hepcidin concentration in plasma increases several folds in a short period of time, which results in a rapid decline in the plasma iron concentration [4]. Hepcidin decreases export of iron absorbed from intestinal mucosa to blood and also decreases release of iron from macrophages recycling iron from senescent erythrocytes [9]. This results in a deprivation of erythroid progenitor cells from necessary iron for erythropoiesis. If the stimulus for production of hepcidin continues, such as in a chronic inflammatory condition, abnormal erythropoiesis would result in chronic anemia. We hypothesized that elevation in serum hepcidin mediates anemia in patients with HF. To study this hypothesis, we measured hepcidin in urine and serum samples of anemic and nonanemic patients with HF. Additionally, we measured hepcidin in a control group consisted of individuals without any clinical evidence of HF, who have been evaluated in various outpatient clinics.

Baseline characteristics of patients with HF and control subjects are summarized in Table I. We studied 36 patients with HF and anemia, 61 patients with HF and no anemia, and 38 control subjects. Patients in the anemic group had lower hemoglobin level (11.64 ± 0.19 g/dL) compared with those in the nonanemic group (14.25 ± 0.15 g/dL) or control subjects (14.14 ± 0.27 g/dL). There was no significant difference in the serum concentration of ferritin or creatinine among the three groups.

We conducted a multivariate analysis to detect the effect of age, sex, presence of coronary artery disease, history of coronary artery bypass graft surgery, hypertension, diabetes mellitus, New York Heart Association class, hemoglobin concentration, serum ferritin, left ventricle ejection fraction (LVEF), and the etiology of HF (ischemic vs. nonischemic) on serum and urine hepcidin concentration in patients with HF. Among these factors, only serum ferritin affected hepcidin concentration in both serum ($P < 0.001$) and urine ($P < 0.002$). Importantly, in anemic and nonanemic HF patients, there were no significant independent interactions between hepcidin and hemoglobin, LVEF, or etiology of HF (all $P > 0.05$).

Over all, patients with HF had a lower urine hepcidin compared with those of control subjects (0.9 ± 0.2 and 2.0 ± 0.5, respectively, $P = 0.002$) (Fig. 1). There was a similar trend in the serum hepcidin concentrations of HF patients and controls, which did not reach a statistical significance (4.0 ± 0.4 and 5.3 ± 0.6, respectively, $P = 0.054$).

Lower level of hepcidin in HF patients was reflected in both anemic and nonanemic subgroups. The serum hepcidin in patients with HF and anemia was significantly lower than that in control subjects ($P = 0.022$) (Fig. 2A). On the other hand, serum hepcidin in nonanemic HF patients was not significantly different from that of control subjects or anemic HF patients ($P = 0.251$ and 0.172, respectively). Urine hepcidin in both anemic and nonanemic HF patients was lower than that in control subjects ($P = 0.019$ and 0.003, respectively) (Fig. 2B). There was no statistically significant difference between anemic and nonanemic patients regarding their urine or serum hepcidin ($P = 0.74$ and 0.172, respectively).

Hepcidin plays an important role in iron metabolism and in the pathogenesis of anemia of inflammation. Hepcidin mediates anemia of inflammation [8,11,12] by binding to and internalizing ferroportin, a membrane iron transporter responsible for exit of iron from intestine epithelial cells and macrophages, resulting in degradation of ferroportin [13,14]. Inflammatory cytokines, such as IL-6, increase synthesis of hepcidin in the liver [15]. On the other hand, iron excess, anemia, and hypoxia decrease hepcidin synthesis and its plasma level [16,17]. The level of hepcidin increases in various infectious or inflammatory conditions and causes rapid change in plasma iron level.

Progression of HF is associated with a sustained elevation of several proinflammatory cytokines, including tumor necrosis factor-α, the interleukin (IL)-1 family, and IL-6 [18]. Among these cytokines, IL-6 has been shown to increase synthesis of hepcidin in the liver. We studied whether anemia of HF is associated with an elevation in hepcidin concentration.

A previous study on anemia in patients with HF showed that a relative increase in the plasma volume rather than a decrease in the red blood cell mass was the main finding in these patients [19]. However, this study only examined patients with HF and did not include control subjects without HF.

Recently, Matsumoto et al. [20] studied serum hepcidin level in patients with chronic HF. They compared serum hepcidin between 36 patients with HF and anemia, 16 patients with HF and no anemia, and 16 patients with no HF and no anemia. They found that serum hepcidin was lower in patients with HF and anemia compared with that of other groups and concluded that anemia of inflammation is a minor cause for anemia of HF. Our study confirmed this result in a larger number of patients using both urine and serum hepcidin.

Anemia in patients with HF is a multifactorial disease. In this study, we investigated whether anemia in HF is associated with an elevated hepcidin concentration. We found that patients with HF and anemia had both lower urine and serum hepcidin compared with those in control subjects. It is important to mention that our control subjects are from individuals visiting different outpatient clinics at Baylor College of Medicine and might be different from a group of healthy controls. In our previous study, median of serum hepcidin among healthy individuals was found to be 4.2 nM, with a range of 0.5–13.9 nM [21], which is lower than the serum hepcidin of controls in this study. The main goal of this study was to investigate the role of hepcidin in anemia of HF, and the comparison between hepcidin concentration in HF patients with and without anemia did not show a significant difference. These findings are not consistent with the presence of a major role for hepcidin in the pathogenesis of anemia in HF patients. However, one should be cautious about interpreting our results, because several factors might affect hepcidin level in HF, some of them in opposite directions; liver synthetic defect, anemia of dilution, and elevated erythropoietin [20] would decrease hepcidin. On the other hand, inflammatory cytokines can increase the hepcidin level. Chronic HF is associated with elevation of several cytokines; among them, tumor necrosis factor-α has been shown to decrease iron absorption from intestine and iron release from macrophages [22] and might contribute to anemia of HF. Once HF patients become anemic, it is likely that their anemia downregulates synthesis of hepcidin and causes lower level of hepcidin. This is a possible explanation for the lower concentration

TABLE I. Baseline Characteristics

	Anemic HF (n = 36)	Nonanemic HF (n = 61)	Controls (n = 38)	*P* value	
				Overall	Anemic vs. nonanemic
Male sex	34 (94.4)	57 (93.4)	30 (78.9)	0.056	1.000
Race				0.088	0.255
Caucasian	22 (61.1)	39 (67.2)	16 (42.1)		
African American	9 (25)	16 (27.6)	17 (44.7)		
Hispanic	5 (13.9)	2 (3.4)	4 (10.5)		
Asian or Pacific Islander	0 (0)	1 (1.7)	1 (2.6)		
CAD	28 (82.4)	36 (64.3)	11 (28.9)	0.0001	0.093
CABG	8 (30.8)	3 (10.7)	1 (2.6)	0.003	0.095
HTN	32 (91.4)	44 (75.9)	31 (81.6)	0.175	0.060
DM	19 (54.3)	19 (32.8)	20 (52.6)	0.061	0.041
COPD	5 (14.3)	10 (17.5)	3 (7.9)	0.398	0.681
NYHA				0.0001	0.646
1	0	0	0		
2	15 (45.5)	32 (55.2)	0 (0)		
3	17 (51.5)	24 (41.4)	0 (0)		
4	1 (3.0)	2 (3.4)	0 (0)		
Ejection fraction (%)	27.6 ± 1.5	25.0 ± 1.1	NA	NA	0.316
BNP (pg/mL)	914.9 ± 184.6	288.9 ± 40.8	78.8 ± 16.8	<0.001	<0.001
Age	67.7 ± 1.7	60.6 ± 1.2	63.6 ± 2.2	0.01	0.001
Weight (Kg)	84.6 ± 4.0	89.7 ± 2.9	95.9 ± 4.2	0.127	0.165
Height (m)	1.72 ± 0.01	1.70 ± 0.03	1.74 ± 0.02	0.528	0.400
WBC ($10^3/\mu L$)	9.9 ± 3.0	7.8 ± 0.30	8.04 ± 2.82	0.558	0.083
Hemoglobin (g/dL)	11.6 ± 0.2	14.3 ± 0.2	14.1 ± 0.3	<0.001	<0.001
Platelet ($10^3/\mu L$)	234 ± 14	248 ± 13	261 ± 12	0.460	0.874
Creatinine (mg/dL)	1.4 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.073	0.306
Iron (μg/dL)	56.4 ± 4.2	77.7 ± 4.0	85.1 ± 9.0	0.005	0.001
TIBC (μg/dL)	350.9 ± 15.1	355.2 ± 8.2	337.2 ± 11.1	0.484	0.586
Ferritin (ng/mL)	160.6 ± 45.4	146.1 ± 15.8	156.5 ± 38.4	0.938	0.302

CAD, coronary artery disease; CABG, coronary artery bypass graft surgery; HTN, hypertension; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease.
The values within parentheses represent percentages and the rest of the results are as mean ± standard deviation.

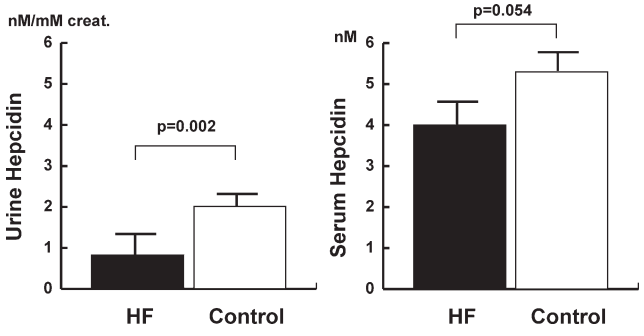

Figure 1. Hepcidin and HF. Bar graphs demonstrate serum and urine hepcidin levels in all patients with HF compared with control subjects. Left, Comparison of urine hepcidin levels in patients with HF and controls. Right, Comparison of serum hepcidin levels in patients with HF and controls. Error bars represent standard error of the mean.

of hepcidin in anemic HF patients. The other possible explanation for our results is that, in patients with HF, elevated erythropoietin [20] might decrease hepcidin level and overrides the effect of inflammatory cytokines.

Methods

This study was conducted according to the protocol for human subject study approved by the Institutional Review Board of Baylor College of Medicine. All of the subjects signed a consent form to participate in this study. Ninety-seven patients with chronic HF (LVEF less than 40% and New York Heart Association class II–IV symptoms) were recruited from hospitals affiliated with Baylor College of Medicine in Houston, Texas. According to their hemoglobin level, patients with HF were divided into anemic (hemoglobin of less than 13 g/dL for men and 12 g/dL for women) and nonanemic subgroups. Thirty eight patients without a history of HF were selected during outpatient clinic visits as the control subjects. Exclusion criteria included presence of kidney failure (serum creatinine above 1.5 mg/dL), history of cardiac transplant, and history of gastrointestinal or severe menstrual bleeding.

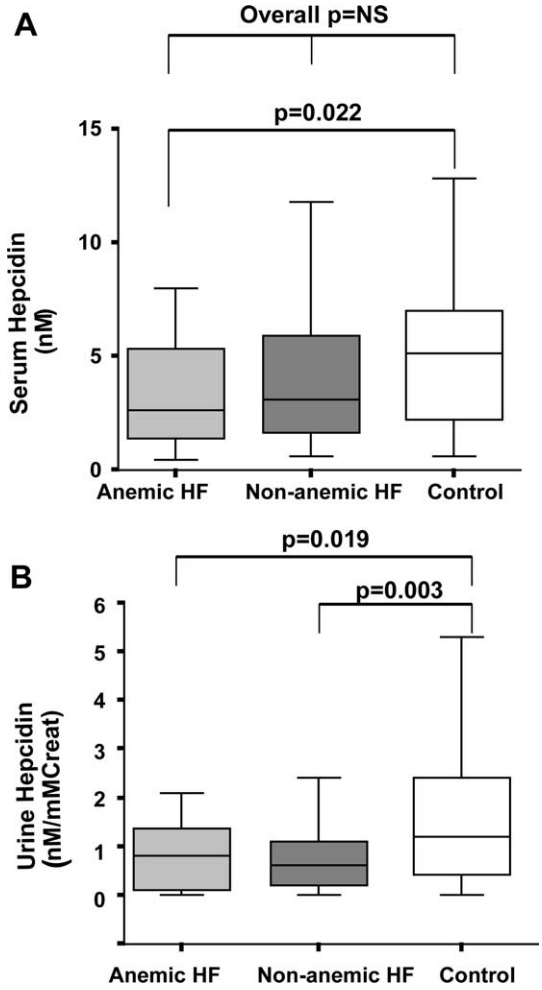

Figure 2. Hepcidin and anemia in HF. Bar-and-whisker plots representing hepcidin levels in patients with HF and anemia, patients with HF and no anemia, and control subjects. (A) Serum hepcidin levels (nM) and (B) urine hepcidin levels (nM/mM of creatinine).

Hepcidin comprises three isoforms that contains 20 (hepcidin-20), 22 (hepcidin-22), or 25 amino acids (hepcidin-25). Hepcidin-25 is the biologically active isoform. We performed urine and serum hepcidin-25 measurements using a combination of weak cation exchange chromatography and surface-enhanced laser desorption ionization–time-of-flight mass spectrometry, as previously reported [23,24]. An internal standard (synthetic hepcidin-24; Peptide International) was used for quantification. Peptide spectra were generated on the time-of-flight mass spectrometry platform of a PBS IIc mass spectrometer (Purchased from Ciphergen Biosystems). This method reproducibly detect elevated hepcidin levels in inflammatory conditions such as infection and rheumatoid arthritis and decreased hepcidin level in iron-deficiency anemia [23,24]. Serum hepcidin levels were expressed in nM, and urine levels in nM/mM creatinine (normalized to urine creatinine concentration). For serum, the intrarun coefficient of variation was 3.9% at 7.3 nM (n = 8) and 3.1% at 3.4 nM (n = 8), and the inter-run coefficient of variation was 7.5% at 4.0 nM (n = 8). Intrarun variation of hepcidin measured in urine is 3.0% both at 3.3 nM (n = 8) and 9.9 nM (n = 8). Inter-run variation for urine varies between 12.6% at 1.5 nM (n = 8) and 10.2% at 9.1 nM (n = 8). Lower limit of detection for serum was 0.5 nM and for urine was 50 pM.

Statistical analysis

All values are expressed as mean ± standard error of mean. Differences in baseline characteristics between the three groups (anemic HF, nonanemic HF, and control) were tested using the γ^2 test or Fisher's exact test for categorical variables and analysis of variance for continuous variables. Overall

differences in biomarker levels were tested using one-way analysis of variance or Kruskal–Wallis test (for non-Gaussian variables). Tukey's test was used for *post-hoc* testing where appropriate. HF patients as a group were compared with control subjects using the Student's *t*-test or Mann–Whitney *U* test. Multivariate regression analysis was performed on serum and urine hepcidin using the following predictors: age, sex, presence of coronary artery disease, history of coronary artery bypass graft surgery, hypertension, diabetes mellitus, New York Heart Association class, LVEF, ferritin, hemoglobin, and etiology of HF (ischemic vs. nonischemic). All data analysis was performed using SPSS 13 (SPSS, Chicago, IL). A *P*-value <0.05 was considered statistically significant.

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Contract grant sponsor: National Institutes of Health; Contract grant numbers: RO1 HL58081, RO1 HL61543, and RO1 HL42250 (to D.L.M.)
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Conflict of interest: Nothing to report.
Published online 13 October 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ajh.21902

References

1. Al-Ahmad A, Rand WM, Manjunath G, et al. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. J Am Coll Cardiol 2001;38:955–962.
2. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: Insights from a cohort of 12 065 patients with new-onset heart failure. Circulation 2003;107:223–225.
3. Mann DL. Inflammatory mediators and the failing heart: Past, present, and the foreseeable future. Circ Res 2002;91:988–998.
4. Ganz T. Hepcidin in iron metabolism. Curr Opin Hematol 2004;11:251–254.
5. Fleming RE, Sly WS. Hepcidin: A putative iron-regulatory hormone relevant to hereditary hemochromatosis and the anemia of chronic disease. Proc Natl Acad Sci U S A 2001;98:8160–8162.
6. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147–150.
7. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806–7810.
8. Nemeth E, Valore EV, Territo M, et al. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003;101:2461–2463.
9. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037–1044.
10. Kemna E, Pickkers P, Nemeth E, et al. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood 2005;106:1864–1866.
11. Maes K, Nemeth E, Roodman GD, et al. In anemia of multiple myeloma, hepcidin is induced by increased bone morphogenetic protein 2. Blood, 2010 [Epub ahead of print].
12. Weinstein DA, Roy CN, Fleming MD, et al. Inappropriate expression of hepcidin is associated with iron refractory anemia: Implications for the anemia of chronic disease. Blood 2002;100:3776–3781.
13. Yeh KY, Yeh M, Glass J. Hepcidin regulation of ferroportin 1 expression in the liver and intestine of the rat. Am J Physiol Gastrointest Liver Physiol 2004; 286:G385–G394.
14. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306: 2090–2093.
15. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271–1276.
16. Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. Annu Rev Nutr 2006;26:323–342.
17. Kemna EH, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: From discovery to differential diagnosis. Haematologica 2008;93:90–97.
18. Diwan A, Tran T, Misra A, Mann DL. Inflammatory mediators and the failing heart: A translational approach. Curr Mol Med 2003;3:161–182.
19. Adlbrecht C, Kommata S, Hulsmann M, et al. Chronic heart failure leads to an expanded plasma volume and pseudoanaemia, but does not lead to a reduction in the body's red cell volume. Eur Heart J 2008;29:2343–2350.
20. Matsumoto M, Tsujino T, Lee-Kawabata M, et al. Iron regulatory hormone hepcidin decreases in chronic heart failure patients with anemia. Circ J 2010; 74:301–306.
21. Kroot JJ, Hendriks JC, Laarakkers CM, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: Implications for clinical studies. Anal Biochem 2009;389:124–129.
22. Laftah AH, Sharma N, Brookes MJ, et al. Tumour necrosis factor alpha causes hypoferraemia and reduced intestinal iron absorption in mice. Biochem J 2006;397:61–67.
23. Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 2008;3:e2706.
24. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 2010;56:1570–1579.