Serum ferritin level changes in children with sickle cell disease on chronic blood transfusion are non-linear, and are associated with iron load and liver injury.

Author(s):

Institution(s):
From 1Morehouse School of Medicine, Atlanta, GA; 2Medical University of South Carolina, Charleston, SC; 3American University of Beirut, Beirut, Lebanon; 4Novartis Corporation, East Hanover, NJ; 5University of Toronto, Toronto, ON, Canada; 6Children's Hospital & Research Center Oakland, Oakland, CA; 7Johns Hopkins University, Baltimore, MD; 8University of Miami, Miami, FL; 9Columbia University, New York, NY; 10University of Mississippi Medical Center Children's Hospital, Jackson, MS; 11Medical College of Georgia, Augusta, GA; 12Children's Hospital of Philadelphia, Philadelphia, PA; 13Children's Mercy Hospital, Kansas City, MO; 14Children's Hospital Los Angeles, Los Angeles, CA; 15St. Jude Children's Research Hospital, Memphis, TN.

Thomas Adamkiewicz, MD, MsCR, FRCP(C)
NCMHD Southeastern Exploratory Sickle Cell Center of Excellence
Co-Director, MSM Hemoglobinopathy/Genomics Training Program
Assistant Professor
Department of Family Medicine, Division of Research
Morehouse School of Medicine
1513 E. Cleveland #100, 3rd Fl, Ste 300-A
East Point, Georgia, 30344
Office phone: 404-756-1230
Cell: 404-697-0726
FAX: 404-756-1229
Email: tadamkiewicz@msm.edu

Short title: Iron Overload in Children with Sickle Cell Disease on Transfusion.

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Abstract

Although chronic blood transfusion is increasingly indicated in patients with sickle cell disease, measuring resulting iron-overload remains a challenge. Children without viral hepatitis enrolled in two trials for stroke prevention were examined for iron-overload (STOP, STOP2; n=271). Most received desferrioxamine chelation. Serum ferritin (SF) changes appeared non-linear when compared to pre-chelation estimated transfusion iron load (TIL), or to liver iron concentrations (LIC). Averaged correlation coefficient between SF and TIL (patients/observations: 26/164) was (+/-standard deviation) r=0.70+/-0.09; between SF and LIC (patients/observations: 33/47) r=0.55+/-0.06. In mixed models, SF was associated with LIC (p=0.006), ALT (p=0.025) and weight (p=0.026). Most patients with SF in the range of ≥750-<1500 ng/ml had a TIL between ≥25-<100 mg/kg (72.8+/-5.9%; patients/observations: 24/50) or a LIC between ≥2.5-<10 mg/gm dry liver weight (75%+/-0%; patients/observations: 8/9). Most patients with SF≥3000 ng/ml had a TIL≥100 mg/kg (95.3+/-6.7%; patients/observations: 7/16) or a LIC≥10 mg/gm dry liver weight (87.7%+/-4.3%; patients/observations: 11/18). Although SF changes are non-linear, levels <1500 ng/ml indicated mostly acceptable iron-overload; levels ≥3000 ng/ml were specific for significant iron-overload and were associated with liver injury. However, to determine accurately iron-overload in patients with intermediately elevated SF levels, other methods of iron assessment are required. The trials described herein are registered with clinicaltrials.gov under identifiers NCT00000592 and NCT00006182.
Background

Increasing numbers of patients with sickle cell disease (SCD) are receiving chronic blood transfusions for the prevention or management of disease-related complications. As a result, these patients require treatment with either chelator drugs\(^1\) or exchange transfusion\(^2\) to prevent tissue injury from iron-overload. Serum ferritin is a non-invasive measure widely used to monitor iron load.\(^1\)-\(^3\) However, the relationship between serum ferritin to other iron load measures varies among studies.\(^4\)-\(^12\) Serum ferritin levels increase in inflammatory states, thus levels can be variable over relatively short time frames.\(^13\) In patients with thalassemia cured with bone marrow transplantation,\(^10\) liver iron concentrations (LIC) obtained from liver biopsy correlated more closely with iron stores measured by phlebotomy than serum ferritin. To better characterize measures of iron-overload in children with SCD, this study examined patients enrolled in two clinical trials in which blood transfusion was evaluated for stroke prevention.\(^14\), \(^15\) The relation of serum ferritin to iron load estimated from transfusion history and LICs was examined.

Methods

Study population

STOP was a prospective randomized clinical trial of children with SCD who were identified as having risk of stroke by transcranial Doppler ultrasound (TCD).\(^14\) The children were randomly assigned to either chronic blood transfusions or observation and followed to assess the occurrence of stroke. In the follow-up study by the same investigators, STOP2, patients who were initially at risk for stroke and whose TCD velocities normalized after at least 30 months on chronic transfusion were randomly assigned either to continue or cease transfusion therapy.\(^15\)
During the course of both trials, enrolled patients were evaluated every three months, with history review, physical examination and laboratory tests. Both quarterly tests and annual viral hepatitis serology were performed at the trial core laboratory at Medical College of Georgia (Augusta, GA) on randomized patients, as described in detail elsewhere. Briefly, serum chemistries, including alanine transaminase (ALT), were measured by DuPont RXL Chemistry Analyzer and serum ferritin by Abbott AXSYM system immunoassay. Only blood chemistry measures assayed at the core laboratory were examined in the current study (collected only after patient randomization). To reduce possible acute phase reactant effect, laboratory measures obtained within 2 weeks before or after a documented infection or SCD-related complication or within 2 weeks after a surgical procedure were excluded. Transfusion intervals in patients who were enrolled in both trials were assumed to be the same between studies as during study periods. Exchange transfusion use was only documented after randomization during the course of the trials. Permission for the current study, which included collection of additional data pertaining to LIC measures, was obtained from the STOP2 Steering Committee, Data Safety Monitoring Board and Institutional Review Boards at Morehouse School of Medicine, MCG, and participating sites. Patient informed consent was obtained in accordance with the Declaration of Helsinki.

Transfusion iron load

Serum ferritin levels were compared to two benchmark measures of iron load: transfusion iron load (TIL) and liver iron concentration (LIC). TIL was estimated from the cumulative blood volume received in patients that only received simple transfusions, prior to start of chelation therapy. Transfusion volume data from the onset of the stroke prevention program was available only in the first STOP trial. Patients documented with more than 10
transfusions prior to randomization or that received exchange transfusion(s) were excluded from this analysis. For the comparison between serum ferritin and TIL, data were censored if transfused blood volume information was missing on >1 consecutive transfusion; otherwise missing transfusion volume was assumed to be equal to average patient transfusion dose. To calculate the amount of iron received, blood unit hematocrit was estimated to be 60%, based on blood preservatives data recorded during the study, and 1 ml of red blood cells was estimated to contain 1 mg of iron. Patient weights at each monthly transfusion and quarterly visit were recorded. Weights in between visits were estimated in relation to time elapsed from actual measurements (weighted averages). To calculate TIL, cumulative iron load received was divided by the estimated weight on the date of TIL assessment.

Liver iron concentration

LIC measurements were not mandated, but were instead performed at the discretion of investigators, as clinically indicated. As with all procedures, liver biopsy dates were recorded prospectively during the course of the trials. Permission to collect LIC-related data after the trials were completed was obtained from the STOP2 Steering Committee, Data Safety Monitoring Board, and Institutional Review Boards at Morehouse School of Medicine, MCG, and participating sites. Additional data collected included: LIC results, pathology reports, biopsy complications, and start dates of transfusion, exchange transfusion and chelation therapy. Possible liver biopsy complications were also identified by reviewing trial data. Only LICs assayed by inductively coupled plasma-mass spectrometry (ICPMS) analytical chemistry method, were considered for analysis of iron measures. For the purpose of this study, an acceptable iron load range was defined as TIL ≥25-<100 mg/kg or LIC ≥2.5-<10 mg/gm dry weight (dw). Liver fibrosis, as described
in clinical pathology reports, were grouped into 3 severity levels: no fibrosis (stage 0\(^1^9\)); mild fibrosis (stage\(^1^9\): minimal fibrosis or fibrous expansion of some portal areas, with or without short fibrous septa) or moderate to severe fibrosis (stages 2 to 6\(^1^9\): fibrous expansion of most portal areas to definitive cirrhosis).

Statistical considerations
STOP, STOP2 and LIC data sets were merged. Data software included Excel 2000 (Microsoft Corporation) and SAS version 9.1 (SAS Institute). Differences in proportions were assessed by two-tailed Fisher’s exact method, differences in continuous variables by Wilcoxon rank test and differences in variances distribution by F test.\(^2^0\) Relation of serum ferritin to TIL or LIC were estimated by Pearson correlation coefficient, sensitivity, specificity and receiver operating characteristic (ROC) area under the curve (AUC).\(^2^1\) To account for repeated measures in individual patients, data from a group of patients under consideration were sampled randomly one thousand times (one measure per patient). Each set of sampled measures was analyzed separately, after which results were averaged.\(^2^2\) Factors that may affect serum ferritin or ALT change were examined using mixed models.\(^2^3\)

Results
Study population
Of the 277 enrolled patients that had clinical trial study visits and documented viral hepatitis serology, 6 (2\%) were excluded (hepatitis C, n=4; hepatitis B, n=1; hepatitis B and C, n=1). Of the remaining 271 patients, 163 were randomized subjects (86 in STOP only, 38 in STOP2 only, 39 in both) and 108 were STOP2 patients observed on transfusion that were not randomized in either study. Fifty-four percent of patients (n=89)
were female, 1% (n=2) were diagnosed with hemoglobin (Hb) S beta-0 thalassemia, the remainder with Hb SS. Twenty-four of percent of patients (n=39) participated in both STOP and STOP2. Average age at start of transfusion therapy was (+/-standard deviation) 8.5+-3.4 years. For the most part (97% of the time, median 28 days), transfusions were administered within < 60 days of each other. The average volume of simple transfusion was 11.0+-3.6 cc/kg. Chelation therapy was documented in 60% (n=98) of patients, starting on average after 33+/20 transfusions; 1% (n=2) initially received deferasirox, the remainder desferrioxamine. Chelation therapy was administered on average 60+-27% of the time while patients where on transfusion, according to quarterly visit records. Eleven percent (n=18) of patients had a splenectomy, 50% (n=9) of these occurred after start of transfusion therapy. Exchange transfusion use was reported in 42% (n=68), 54% (n=37) received ≥10 exchanges.

Liver iron concentration

LIC information was obtained in 103 liver biopsies from 60 patients enrolled in STOP/STOP2. These included 83% (77/93) of patients initially identified from the database as having had a liver biopsy. The remainder were either liver biopsies done during the trials but not initially identified (n=12), or biopsies done within 10+/9 months outside of trial active observation periods (n=14). Complications included pain after biopsy (n=2, one requiring hospitalization for 5 days) and a liver abscess requiring surgical drainage 5 months after biopsy in one patient. LIC was assayed by ICPMS (n=85), colorimetric methods (n=8), atomic absorption spectroscopy (n=7) or undetermined methods (n=3). Only LIC measured by ICPMS in patients without viral hepatitis were further considered for analysis (n=83). Seventeen-percent (n=14) were obtained from 1998 to 2000; the remainder from 2001 to 2006. Sixty-four percent (n=53) were from 3
centers with previously published LIC experience.\textsuperscript{9, 12, 17} Twenty-three percent (n=19) of patients had a second analyzable LIC, 11% (n=9) a third, and 1% (n=1) a fourth one. The mean age at first LIC was 11\(\pm\)4 years (median 11, range 4 to 19). Mean first LIC was 13.6\(\pm\)8.5 mg/gm dw (median 12.1, range 1.8 to 41.3). In patients with multiple LICs, last LIC was on average 15.9\(\pm\)10.1 mg/gm dw (median 12.8, range 4.3 to 37.1).

Iron measure comparisons

Median serum ferritin change with increasing number of transfusions, TIL and LIC are presented in Figure 1 and Table 1. TIL data included 164 observations in 26 patients, after an average of 22\(\pm\)15 transfusions (range 1 to 64; volume data was missing in 3%). LIC that had corresponding serum ferritin measures included 47 observations in 33 patients, after an average of 69\(\pm\)30 transfusions (range 14 to 126). Serum ferritin levels were obtained on average 31\(\pm\)30 days (range 0 to 117) from the time of LIC.

Averaged correlation between serum ferritin and TIL was \(r=0.70 \pm 0.09\); when TILs <50 mg/kg were excluded, \(r=0.47 \pm 0.14\). Averaged correlation between serum ferritin and LICs was \(r=0.55 \pm 0.06\). Serum ferritin ROC AUC are presented in Figure 2. Predictive value of serum ferritin ranges compared to iron load ranges are presented in Table 2. All serum ferritin measures \(\geq 4000\) ng/ml were associated with LIC \(\geq 10\) mg/gm dw (8 patients, 11 observations).

Relation between TIL and LIC obtained within a month of start of chelation therapy was estimated in 15 patients on simple transfusion (STOP2 transfusion volume data in 8 patients that were never randomized, and in 5 patients prior to randomization, was estimated to be the same as the average volume administered in the study). Correlation
coefficient was \( r=0.64 \). Linear regression relation\(^{10}\) was expressed as: TIL (mg/kg) = 9.0 x LIC (mg/gm dw), assuming that the amount of blood received prior to the trials was negligible.

To examine if trends in serum ferritin levels can be used as a qualitative measure of increasing or decreasing iron-overload, serum ferritin linear regression trends over time were compared to LIC pair trends (all serum ferritin measures within 4 months before first or after last LIC were included). Fifteen LIC pairs in 10 patients were examined. These were obtained on average 1.6+/−1.3 years apart (range 0.5 to 4.7). Serum ferritin and LIC trends were significantly more likely to be in the same direction (87%, increasing n=8, decreasing n=5; \( p=0.007 \)), than in the opposite direction (13%, increasing serum ferritin and decreasing LIC, n=2).

Liver injury and serum ferritin

Of patients on transfusions on whom ALT levels were available, 57% (85/150) had at least one ALT measure ≥40 IU/L. For these, peak ALT elevation was on average 73+/−52 IU/L (range 40-329), and occurred on average after 43+/−32 transfusions. Relation between serum ferritin and ALT are presented in Figure 3. Averaged correlation of serum ferritin with ALT within the first 50 transfusions was \( r=0.09+/−0.10 \); after 50 transfusions \( r=0.53+/−0.08 \). Multivariate mixed models that examined variables that affected serum ferritin or ALT changes are presented in Table 3.

The presence or absence of liver fibrosis was described in 90% of biopsies (n=75; trichrome stain documented in 69). In these, fibrosis was absent in 56% (n=42), mild in 37% (n=28) and moderate to severe in 7% (n=5). When liver biopsies with highest degree
of fibrosis were selected, average serum ferritin in patients without or with mild liver fibrosis was 2415+/-1097 ng/ml (n=34); in patients with moderate to severe fibrosis average serum ferritin was 3571+/-2158 ng/ml (n=4, p=0.234). Respective values for LIC were 14.0+/-8.2 mg/gm dw (n=45) and 25.5+/-15.6 mg/gm dw (n=4, p=0.097). In univariate mixed models, degree of fibrosis was associated with ALT (p=0.018) and approached significance with gamma glutamyl transferase levels (p=0.051) and with number of transfusions (p=0.081).

Discussion
The natural history of iron-overload was examined in chronically transfused children with SCD and without viral hepatitis, who were observed for up to 10 years in two consecutive stroke prevention trials. These trials were conducted at a time when most patients received chelation as injectable desferrioxamine, which can be difficult to administer.¹ This allowed an analysis over a wide range of iron-overload. Serum ferritin level changes appeared non-linear when compared to increasing iron load iron measured by TIL or LIC. After an initial rapid rise, serum ferritin rate of change seemed to slow after reaching approximately 1500-2500 ng/ml, despite evidence of increasing iron load. After further iron-overload, patients developed high levels of serum ferritin (≥3000 ng/ml). Serum ferritin measures above this level appeared to be associated with both increased LIC and liver injury, as estimated by ALT levels.

Intra-cellular ferritin is a hollow protein shell made of 24 heavy (H) or light (L) subunits that stores iron.²⁴ Synthesis is differentially regulated at a post-transcriptional level, mediated by iron binding proteins.²⁵ L-subunits contain iron storage facilitating ferroxidase enzymatic activity,²⁴ and are approximately 3 to 4 times more abundant than H-subunits in
the liver of normal individuals. Cardiac ferritin is composed principally of H-subunits. During iron-overload, liver ferritin content increases more than fourfold and is further enriched in L-subunits. However, liver hemosiderin, which is formed from degraded ferritin in the lysome, is constituted predominantly of H-subunits. L-subunits are actively secreted in response to iron and inflammatory cytokines, by an unknown mechanism. In patients with thalassemia major on chronic transfusion, glycosylated serum ferritin levels reached a plateau after 100 units, consistent with a rate limited active secretion process. Rapid serum ferritin level changes at low iron load, and slower rate of change at moderate iron load levels was also described in patients with hemochromatosis undergoing phlebotomy, and was noted in a previous analysis of STOP data. Following a flattened response, a near exponential increase in serum ferritin was observed at high LIC levels in patients with a variety of hematological conditions or hemacromatosis. In patients with thalassemia major, un-glycosylated (and thus presumably not actively secreted) serum ferritin continued to rise with further transfusions. Authors proposed that serum ferritin changes beyond the flattened phase was due to leakage from damaged hepatocytes. An association between ALT and serum ferritin levels was observed in an iron-overload rat model and in patients on chronic transfusion for thalassemia major or acquired anemias. It can also occur in patients with acute liver injury without iron-overload.

In the current study, most patients developed elevated ALT levels during the course of transfusion therapy. However, peak ALT elevations and degree of liver fibrosis were mostly mild, consistent with previous observations. ALT level changes were significantly associated with iron load estimated both by TIL and LIC. A modest but significant association between fibrosis and LIC noted in a previous report was not evident in the present study, probably because only a small number of patients with moderate to severe
fibrosis were observed, or because of other factors. The association between ALT and serum ferritin was evident in patients with iron load assessed by LIC, and in general in patients that received more than 50 transfusions, suggesting that in children with SCD, this phenomenon occurs after prolonged exposure to chronic transfusion.

Weight at start of transfusion therapy was also associated with increased serum ferritin levels in the multivariate analysis. Serum ferritin changes were not significantly associated with other variables, such as gender \(^4\) or splenectomy. Possible other reasons for low serum ferritin levels, such as low ascorbic acid levels \(^32\) or asymptomatic mutations of the ferritin L-subunit gene \(^33\), were not examined.

Utility of serum ferritin as a measure of iron load has been questioned. \(^3, 12\) Poor predictability of serum ferritin can be in part attributed to when data is sampled in relation to degree of iron load. Indeed, serum ferritin levels correlate poorly with iron load when data points are obtained in the flattened part of the response curve. This effect can be simulated with TIL, by removing data points with TIL <50 mg/kg, as illustrated in Figure 2. An analysis comparing serum ferritin ranges to iron level ranges best summarizes when serum ferritin may be useful as a clinical tool to monitor iron, and when it is of limited value (see Table 2).

This study also examined LIC performed in a clinical setting as a measure of iron-overload. Complications from liver biopsy requiring hospitalization were observed in 2%. One patient developed a liver abscess, similar to a previous report. \(^34\) No patients died from the procedure. \(^35\) In a smaller group of patients, the linear regression equation between TIL and LIC assayed by ICPMS, which was the predominant method used, was
similar to that observed in patients with thalassemia major following bone marrow transplantation. In that study, TIL in mg/kg measured by phlebotomy was equal to 10.6 times LIC in mg/gm dw, assayed by atomic absorption spectroscopy. In the present study, the correlation coefficient between LIC and TIL was more modest. LIC specimen weight or transportation medium (paraffin block vs. other) was not evaluated, as it is not usually reported in commercially assayed LICs, and may have affected variability of results.

Recommendations for routine use of LIC by biopsy are tempered by small but finite risk of severe complications, lack of cross-validation of assays and generally unremarkable liver histology. Safety and reproducibility of LIC by biopsy could be improved if prophylactic antibiotics were administered in stable patients at time of ultrasound guided biopsy, and if samples weighing the equivalent of 1 gm dry weight were sent in same transport media (all embedded in paraffin block, for example) only to laboratories that perform routinely LICs by validated assays. Adequacy of sample size could be determined if total dry weight was reported with LIC result.

In light of these observations, an approach to monitor iron-overload can be proposed, utilizing a combination of methods. Prior to start of chelation, determination of iron load from transfusion history, either by TIL or just by counting the number of plain transfusions, appears to be the simplest and most accurate measure of iron load to determine when to start chelation therapy (for example: TIL 75 mg/kg ≈ 90-130 cc/kg of blood ≈ 9-13 transfusions at 10 cc/kg). Serum ferritin levels <1500 ng/ml (e.g., before the response curve flattens), indicate acceptable iron load in most patients. However, approximately 15% to 25% of patients may be “high” or “low” serum ferritin responders. These can be detected by comparing serum ferritin to TIL prior to start of chelation. “Low responders,” once detected, may require a lower serum ferritin threshold, as seemingly adequate
ferritin levels in such patients may give a false sense of security despite significant iron-overload. Observing serum ferritin trends with frequent measures (e.g., at each transfusion) overcomes much of the variability seen over time in individual patients, and can also help avoid over chelation, another potential source of toxicity. Serial ALT measures can also be informative, as levels change in response to iron-overload.31, 37

In contrast, >3/4 of patients with serum ferritin levels ≥2250 ng/ml, 88% of those ≥3000 ng/ml and all measures ≥4000 ng/ml were associated with LICs ≥10 mg/gm dw. Clearly, patients with repeated high serum ferritin measures require intensified iron removal therapy, as such levels indicate significant and potentially toxic iron-overload in most.

Thus, maintaining a lower serum ferritin threshold (e.g., ≥750-<1250 ng/ml) in all patients may result in adequate iron load control, as 83% of patients with such serum ferritin levels have an acceptable iron load as assessed by TIL. However safety and effectiveness of such strategies would need to be evaluated prospectively long term. Pitfalls of such a strategy may include possible fluctuations of ferritin trends unrelated to iron load (e.g. from chronic inflammation or other reasons), possibility of missing "low responders" (e.g. TIL not available), and serum ferritin level changes that differ between chelation agents (different iron compartment mobilization).

However, there is no way of knowing with precision if a person with serum ferritin levels in the ≥1500-<3000 ng/ml range who was on transfusion and chelation therapy for a period of time, has an acceptable iron load or has developed significant iron-overload. For these reasons, optimal iron load assessment should also include periodic (yearly) tissue iron
determination, especially in patients with intermediately elevated serum ferritin levels (≥1500-<3000 ng/ml).

Non-invasive methods of tissue iron assessment include the investigational superconducting interference device susceptometer (SQUID)\textsuperscript{7, 41} and MRI.\textsuperscript{42-45} Clinically meaningful correlations between MRI R2,\textsuperscript{42, 43} R2*\textsuperscript{43, 45} and biopsy derived LICs were reported in patients with transfusion iron-overload and are currently the most accessible non-invasive methods. Cardiac MRI should be considered in patients with a prolonged history of transfusions, as heart iron-overload seems to follow that of the liver.\textsuperscript{44} Elucidating how tissue iron levels are measured by MRI\textsuperscript{46} may help improve the method further and allow its precise calibration and standardization, without need of tissue biopsies.

In patients with thalassemia major,\textsuperscript{3} serum ferritin levels exceeding 2500 ng/ml were associated with decreased survival from iron-overload-related heart complications. Although in patients with SCD, the relationship between iron measures and outcome needs to be further defined, in a recent study, transfused patients with serum ferritin ≥2000 ng/ml or LIC≥10 mg/gm dw had similar risk of death as those with thalassemia major with equivalent iron load and transfusion history.\textsuperscript{47} In the current study, most patients became iron-overloaded during the course of the trials, as most developed serum ferritin levels >2500 ng/ml after approximately 30 transfusions, reflecting difficulties in administering desferrioxamine. Studies of populations at risk, including surveys utilizing TIL and tissue iron assessments, will help ascertain novel iron removal methods\textsuperscript{1} and help validate simpler, cheaper but equally effective iron monitoring strategies that may be more generally applicable.
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Author contribution, study design: Dr. Thomas Adamkiewicz with assistance from Dr. Miguel Abboud, Dr. Carole Paley, Dr. Elliott Vichinsky and Dr. Robert Adams. Data collection: Dr. Thomas Adamkiewicz, Dr. Nancy Olivieri, Dr. Melanie Kirby-Allen, Dr. Elliott Vichinsky, Dr. James Casella, Dr. Ofelia Alvarez, Dr. Julio Barredo, Dr. Margaret Lee, Dr. Rathi Iyer, Dr. Abdullah Kutlar, Dr. Kathleen McKie, Dr. Virgil McKie, Nadine Odo, Dr. Beatrice Gee, Dr. Janet Kwiatkowski, Dr. Gerald Woods, Dr. Thomas Coates; data preparation and analysis: Dr. Thomas Adamkiewicz and Dr. Nadine Odo; analysis review
and manuscript preparation: Dr. Thomas Adamkiewicz, Dr. Miguel Abboud, Dr. Carole Paley, Dr. Nancy Olivieri, Dr. Elliott Vichinsky, Dr. James Casella, Dr. Ofelia Alvarez, Dr. Margaret Lee, Nadine Odo, Dr. Janet Kwiatkowski, Dr. Gerald M. Woods, Dr. Thomas Coates, Dr. Winfred Wang and Dr. Robert Adams;

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References


Table 1. Iron load measures and corresponding serum ferritin levels.*

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<th>Iron load</th>
<th>Patients, n</th>
<th>Serum ferritin, ng/ml</th>
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<tr>
<td></td>
<td></td>
<td>mean (SD)</td>
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<tr>
<td>Pre transfusion</td>
<td>95</td>
<td>139 (126)</td>
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<tr>
<td>TIL (mg/kg)</td>
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<td></td>
</tr>
<tr>
<td>50</td>
<td>26</td>
<td>914 (581)</td>
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<tr>
<td>150</td>
<td>20</td>
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</tr>
<tr>
<td>250</td>
<td>9</td>
<td>2259 (991)</td>
</tr>
<tr>
<td>350</td>
<td>3</td>
<td>2697 (912)</td>
</tr>
<tr>
<td>LIC (mg/gm dw)</td>
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<tr>
<td>5</td>
<td>13</td>
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</tr>
<tr>
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<td>16</td>
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<td>7</td>
<td>3276 (2366)</td>
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<td>3</td>
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* Closest serum ferritin obtained prior to start of transfusion, or closest to reference TIL value indicated (+/- 50 mg/kg) or LIC (+/- 5 mg/gm dw). Serum ferritin variance was significantly greater for LICs, compared to TILs at equivalent iron load (distribution ratio F test: p< 0.005). Abbreviations: dw: dry liver weight, LIC: liver iron concentration load, SD: standard deviation, TIL: transfusion iron load.
Table 2: Percent of patients within given serum ferritin ranges, that have low, moderate and increased iron load.*

<table>
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<tr>
<th>Serum ferritin, ng/ml</th>
<th>Patients/observations, n</th>
<th>Iron load</th>
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<tr>
<td></td>
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<td>0-&lt;25 mg/kg</td>
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<td>0-&lt;750</td>
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<td>51.2 (7.9)</td>
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<th>≥10 mg/gm dw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (SD)</td>
<td>% (SD)</td>
<td>% (SD)</td>
</tr>
<tr>
<td>0-&lt;750</td>
<td>1/1</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>≥750-&lt;1500</td>
<td>8/9</td>
<td>-</td>
<td>75.0</td>
</tr>
<tr>
<td>≥1500-&lt;2250</td>
<td>8/10</td>
<td>-</td>
<td>37.5</td>
</tr>
<tr>
<td>≥2250-&lt;3000</td>
<td>9/9</td>
<td>-</td>
<td>22.2</td>
</tr>
<tr>
<td>≥3000</td>
<td>11/18</td>
<td>-</td>
<td>12.1 (4.3)</td>
</tr>
<tr>
<td>1000+/-250</td>
<td>4/4</td>
<td>-</td>
<td>75.0</td>
</tr>
</tbody>
</table>

*averages from 1000 random data samplings (one observation within serum ferritin range per patient selected at each sampling; same patient may be represented in >1 serum ferritin range). All LICs in this analysis were > 2.5 mg/gm dw. SD: sampling standard deviation, dw: dry liver weight.
Table 3. Multivariate mixed models examining serum ferritin or alanine aminotransferase changes during chronic transfusion.*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Iron load measure method</th>
<th>TIL</th>
<th>P value</th>
<th>LIC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ferritin</td>
<td>Iron load</td>
<td>147.79</td>
<td>&lt;.001</td>
<td>15.66</td>
<td>0.006</td>
</tr>
<tr>
<td>ALT</td>
<td>0.37</td>
<td>0.542</td>
<td></td>
<td>8.02</td>
<td>0.025</td>
</tr>
<tr>
<td>Gender</td>
<td>0.41</td>
<td>0.523</td>
<td></td>
<td>1.32</td>
<td>0.288</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>0.48</td>
<td>0.490</td>
<td></td>
<td>0.14</td>
<td>0.715</td>
</tr>
<tr>
<td>Weight</td>
<td>0.51</td>
<td>0.478</td>
<td></td>
<td>7.97</td>
<td>0.026</td>
</tr>
<tr>
<td>ALT</td>
<td>Iron load</td>
<td>13.62</td>
<td>&lt;.001</td>
<td>10.43</td>
<td>0.012</td>
</tr>
<tr>
<td>Gender</td>
<td>0.03</td>
<td>0.858</td>
<td></td>
<td>4.28</td>
<td>0.072</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>0.03</td>
<td>0.859</td>
<td></td>
<td>0.26</td>
<td>0.622</td>
</tr>
<tr>
<td>Weight</td>
<td>2.10</td>
<td>0.149</td>
<td></td>
<td>0.15</td>
<td>0.709</td>
</tr>
</tbody>
</table>

Splenectomy before start of transfusion therapy, weight at start of transfusion therapy. Model degrees of freedom (numerator/denominator) TIL: 1/136; LIC: 1/7. When lactose dehydrogenase or white cell count or hemoglobin S concentration were included in models, these did not reach significance (data not shown).

Abbreviations: ALT: alanine aminotransferase, TIL: transfusion iron load, LIC: liver iron concentration load.
Figure Legends

Figure 1. Serum ferritin changes in relation to number of transfusions in all randomized STOP and STOP2 patients (a). Serum ferritin changes in relation to transfusion iron load (TIL, b) or liver iron concentration load (LIC, c). Thick lines represent median change, grey lines 10th and 90th percentiles and dashed lines change in individual patients.

Figure 2. Serum ferritin (SF) receiver operating characteristic area under the curve (ROC AUC) for iron load determined by transfusion iron load (TIL, a), TIL limited to measures > 50 mg/kg (b) or liver iron concentration (LIC, c). Circles represent averages of 1000 random data sampling, bars indicate standard deviations. ROC AUC represents the area under the curve of sensitivity plotted against 1-specificity for all possible SF values for a given iron load level (The closer ROC AUC equals 1, the better SF is, as a discriminator of iron; the closer ROC AUC equals 0.5, the closer SF is to random21).

Figure 3. Percent of patients within given serum ferritin that have increased alanine aminotransferase level (ALT). Serum ferritin was +/- 1000 ng/ml of level indicated (except for last level indicated left of chart). Percentages are averages from 1000 random data samplings, bars indicate sampling standard deviation (one observation within serum ferritin range per patient selected at each sampling; same patient may be presented in >1 strata.
Figure 1.
Figure 2.
Figure 3.
Serum ferritin level changes in children with sickle cell disease on chronic blood transfusion are non-linear, and are associated with iron load and liver injury


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