Biochemical, Radiologic, Ultrastructural, and Genetic Evaluation of Iron Overload in Acute Leukemia and Iron-chelation Therapy

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Summary: Iron overload in hereditary hemochromatosis and hematologic malignancy has unfavorable effects on morbidity. Herein, 53 children (age 108.4 ± 58.3 mo, 25 girls and 28 boys) with acute myeloblastic and lymphoblastic leukemia, who received 4 different chemotherapy protocols, were evaluated for iron overload throughout the whole course of chemotherapy; iron overload arose: (1) before chemotherapy, which was dependent on neither chemotherapy nor packed red blood cell transfusions and (2) after chemotherapy, which was dependent on the duration and nature of chemotherapy and partially on transfusion of packed red blood cells. Iron overload was documented in 75% of patients with a ferritin level >1000 ng/mL, by liver and heart magnetic resonance imaging, and they were administered iron-chelation therapy with success. Three of 10 radiologically iron-overloaded patients were heterozygous for H63D mutation. Aminolevulinic acid and porphobilinogen levels were normal. Light microscopic examination of the bone marrow revealed increased iron granules in erythroblasts, platelets, and megakaryocytes, iron-laden macrophages, free iron in the matrix, dysmatopoiesis, and apoptotic cells. Electron microscopic examination revealed iron-laden secondary lysosomes and autolysosomes in normoblasts and iron-laden primary granules in pro-myelocytes, irrelevant to the ferritin level, implying autophagia due to chemotherapy as a source of the excess iron. We think that iron overload, which is an important complication of acute leukemia, should be evaluated separately from “transfusion overloading,” and the management principles specific to leukemia should be implemented.

Key Words: acute lymphoblastic leukemia, acute myeloblastic leukemia, iron overload, autophagia, iron chelation, ultrastructure, iron-overload genes

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W e observed that our leukemia patients developed high serum iron, transferrin saturation, and high ferritin levels during chemotherapy with and without impaired liver function tests. In the literature, there are studies on hematologic malignancy patients who showed elevation in at least 1 among serum iron, transferrin saturation, ferritin, and non–transferrin-bound iron levels prior, during, close to the end or at the end of chemotherapy, after the cessation of chemotherapy, and after transplantation. The subject of a correlation between iron overload and packed red blood cell (PRBC) transfusions is controversial.

Iron overload in hereditary hemochromatosis and hematologic malignancy was reported to lead to increased bacterial and fungal infections, altered leucocyte characteristics as in iron-deficiency anemia (as dysfunction of neutrophils, monocytes, T and B lymphocytes, and altered T-lymphocyte number), and progressive organ damage, which are aggravated by chemotherapy. Growth of cancer cells, mucositis, and liver enzyme elevations. Elevated pretreatment serum ferritin level was strongly associated with lower survival in acute leukemia or myelodysplastic syndrome (MDS).

To the best of our knowledge, there has been no study examining the role of different leukemia types and different chemotherapy protocols in the development of iron overload or the efficacy of iron-chelation therapy in iron-overloaded leukemia patients. We also found no study evaluating iron overload in leukemia from radiologic, genetic, and ultrastructural perspectives.

The objectives of this study were: (1) to monitor the course of iron parameters like serum iron, serum iron–binding capacity, transferrin saturation, ferritin, and transfused total red blood cell volume throughout different leukemia chemotherapy protocols; (2) to confirm iron overload by liver-heart magnetic resonance imaging (MRI) and by light and electron microscopy; (3) to determine the efficacy of iron-chelation therapy in patients with radiologically documented iron overload; and (4) to evaluate the etiology of iron overload in leukemia from the perspectives of iron-overload genes and porphyria metabolism.

PATIENTS AND METHODS

Patients and Samples

Patients with acute lymphoblastic leukemia (ALL) (n = 44) (age: 100.4 ± 55.80 mo; 19 female [F] and 25 male [M] individuals) and acute myeloblastic leukemia (AML) (n = 9) (age: 155.2 ± 58.8 mo, 6F, 3M) who were followed up between March 2005 and March 2011 in our clinics were prospectively evaluated with respect to pretreatment iron
parameters (serum iron, serum iron–binding capacity, transferrin saturation, and ferritin), simultaneous hemogram, liver function tests, transfused cumulative PRBC volume, and iron per body weight during treatment. Hepatitis viruses A, B, and C and human immunodeficiency virus were serologically negative in all patients before and during chemotherapy. “Transfused cumulative PRBC volume and iron per body weight” represented cumulative PRBCs that had been transfused to a patient as of the time of evaluation, considering the patient’s weight at that time.

Characteristics of the Groups and Chemotherapy Protocols

The patients were allocated into 4 groups as ALL-1, ALL-2, ALL-3, and AML.

The ALL-1 group comprised 13 of the 44 ALL patients who received modified ALL St Jude TXIII therapy protocol. All were from the intermediate-risk group (age 107 ± 57.6 mo; 6F, 7M). The therapy was completed in all patients and all had been alive for 4 to 6 years.

The ALL-2 group comprised 26 ALL patients who received TRALL-BFM protocol (age 101 ± 54.8 mo; 10F, 16M). Of these, 18 were from medium-risk, 5 from standard-risk, and 3 from high-risk groups. Chemotherapy was completed in 12, and the others were continued on maintenance therapy.

The ALL-3 group comprised 5 patients who received COG 1962 ALL protocol, who could be evaluated only at the beginning and end of induction (age 86.4 ± 53 mo; 3F, 2M). Two patients were from the standard-risk group and 3 were from the high-risk group.

The AML group included 9 patients who received AML BFM 2004 protocol (age 155.2 ± 58.8 mo; 6F, 3M). Eight were from the high-risk group and 1 was from the standard-risk group. One patient developed remission failure; 3 developed bone marrow relapse during the maintenance phase, and all 3 were transplanted successfully from human leukocyte antigen–identical siblings. Chemotherapy in 1 was completed 8 months ago, and 1 is still on maintenance therapy. Of all leukemia patients, only 1 from the ALL-2 group had central nervous system involvement (from the medium-risk group).

Ethnicity

Our patients were from the central (n: 15), southeast (n: 10), north central (n: 9), Marmara (n: 4), eastern (n: 4), northeast (n: 3), northwest (n: 2), Mediterranean (n: 2), Aegean (n: 2) regions of Turkey. Two foreign patients came from northern Iraq.

Control Group

The control group comprised 26 nonanemic and asymptomatic children (age: 119.53 ± 66.90 mo; 13F, 13M) who were admitted to our outpatient clinic for the evaluation of their iron status at the family’s request during their general or preoperative hematological check-up. The hemogram, serum iron, serum iron–binding capacity, transferrin saturation, and ferritin levels of these patients were used as “control” values.

Determination of the Sample Size and the Statistics

The required minimum patient number for comparisons between the subgroups was determined by GPOWER package program, and it was found to be 52, with 95% reliability, 80% power, and 0.4 sensitivity. The number of the volunteers in the control group was planned to be at least half of the total patients. As the number of the patients in the subgroups was low due to the limited number of patients who had received the different therapy protocols and were available for the study, we used the following nonparametric tests for statistical evaluation through the Statistical Package for the Social Sciences (SPSS) 15 package program. For the comparison of 2 groups, the Mann-Whitney U test was performed; for 3 to 4 group comparisons, the Kruskal-Wallis H test was performed; and for the comparison of dependent groups, the Wilcoxon test was performed. For the evaluation of the relationship between the variables, correlation analysis was performed. Significance level was accepted as 0.05.

Analysis of the Groups

The analysis was implemented in 10 different stages as follows: (1) analysis of the initial iron parameters of the groups; (2) intragroup evaluation of iron parameters during chemotherapy in ALL patients, AML patients, and in ALL-1, ALL-2, and ALL-3 groups, separately; (3) intergroup comparison of iron parameters of ALL-1, ALL-2, ALL-3, and AML groups during the postinduction phase (at the end of induction) and of ALL-1 and ALL-2 groups during the postmaintenance phase; (4) pretransplant iron evaluation in AML patients; (5) changes in reticulocyte count at the beginning and end of the chemotherapy protocols; (6) radiologic evaluation of iron in patients with ferritin level > 1000 ng/mL by liver and heart MRI; (7) efficacy of iron-chelation therapy; (8) evaluation of iron-overload genes in patients with radiologically documented iron overload; (9) microscopic and ultrastructural evaluation of blood cells; and (10) evaluation of porphyria metabolites in a few patients. The details are presented hereunder.

To evaluate the iron status during chemotherapy within each leukemia group, the iron parameters and transfused cumulative PRBC volume and iron per body weight attained during preinduction, postinduction, premaintenance, and postmaintenance (posttreatment) phases in 44 ALL patients and the same parameters attained before and after each chemotherapy block in 9 AML patients were established, and each value from each group was compared. In addition, the same parameters attained before and at the end of each chemotherapy block in ALL-1, ALL-2, and ALL-3 groups were compared (intragroup comparison) separately.

To evaluate the effect of different leukemia therapy protocols on iron parameters, the postinduction iron parameters and transfused cumulative PRBC volume and iron per body weight values of ALL-1, ALL-2, ALL-3, and AML groups were compared. As only the ALL-1 and ALL-2 groups were followed up until the end of maintenance, the postmaintenance (posttreatment) parameters could be compared only in these groups. The pretreatment (preinduction) and posttreatment values were compared with those of the control group.

To evaluate the iron status of AML patients attained after haM block when referral to the Bone Marrow Transplantation Unit was recommended, values were also compared with those of the control group.

Reticulocyte Count

Reticulocyte counts were determined in only 18 patients of the ALL-2 group, on 22 occasions. All the
phases were considered as “one phase” without discrimination. The reticulocyte values attained at the beginning and at the end of the same phase (prephase and postphase) and at the end of the previous phase and beginning of the consecutive phase (post–previous chemotherapy phase and the consecutive prechemotherapy phase) were compared.

Liver and Heart MRI

Patients who had >1000 ng/mL level of ferritin for at least 1 month were evaluated for iron overload by liver and heart MRI. Liver iron overload was evaluated by the T2* and R2 methods and heart iron overload by the T2* method. Patients with normal and abnormal MRI while having >1000 ng/mL ferritin level were compared with respect to iron parameters, transfused cumulative PRBC volume, and iron per body weight, hemoglobin, hematocrit, white blood cell count, absolute neutrophil count, thrombocyte count, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, total protein, albumin, total-direct bilirubin, total transfusion count, body weight, and body mass index. The relationship between the transfused cumulative iron and the above parameters was investigated in patients who had iron overload documented by MRI.

Iron-chelation Therapy

Iron-chelation therapy was administered to patients who had both >1000 ng/mL ferritin level and radiologically documented iron overload.

For chelation, desferrioxamine (40 mg/kg × 3 to 5/wk, as 12 h infusion) was administered when the patients were hospitalized, but deferasirox (30 mg/kg/d, per oral) was initiated after their discharge. Therapy was discontinued when serum ferritin levels declined to <3000 ng/mL.

For social reasons, 3 patients were given delayed iron-chelation therapy; 1 patient did not receive therapy because of her deteriorated condition; 1 ALL patient who was on induction therapy was hospitalization, but deferasirox (30 mg/kg/d, per oral) was initiated after their discharge. Therapy was discontinued when serum ferritin levels declined to <3000 ng/mL.

The therapy protocols, details about PRBC transfusions, methods of echocardiography, electron microscopy, MRI, tests of porphyrin metabolism, and iron-overload genes are described in the supplemental data, Supplemental Digital Content 6 (http://links.lww.com/JPHO/A50).

The study was approved by the Local Ethical Committee, and informed consent was obtained from the parents.

RESULTS

The Initial Iron Parameters

The initial transferrin saturation (514.0% ± 1534.1% and 169.0% ± 370.4% vs. 31.1% ± 12.3%, P = 0.0001 and 0.001) and ferritin (437.8 ± 565.5 and 971.7 ± 2102.0 vs. 38.9 ± 30.7 ng/mL, P = 0.0001 and 0.0001) levels in the ALL and AML groups and serum iron levels in the ALL group as a whole (ALL-1 + 2 + 3) (134.1 ± 76.5 vs. 81.5 ± 17.9 μg/dL, P = 0.049) were significantly higher, and the serum iron–binding capacity in ALL as a whole and in AML (171.4 ± 108.6 and 200.6 ± 99.8 vs. 2777.6 ± 52.9 μg/dL, P = 0.0001 and 0.001) was significantly lower than in the control group (Supplemental Table 1S, Supplemental Digital Content 5, http://links.lww.com/JPHO/A49).

The initial serum iron level, serum iron–binding capacity, transferrin saturation, and ferritin level in the ALL and AML groups were similar (P = 0.32, 0.72, 0.34, 0.28, respectively). The initial hemoglobin and transfused PRBCs per body weight concentrations before initiation of chemotherapy are presented in Supplemental Table 2S, Supplemental Digital Content 5, http://links.lww.com/JPHO/A49.

Changes in the Iron Parameters During Chemotherapy Within Each Leukemia Group

When all patients with ALL were evaluated together, it was found that, although transfused cumulative PRBC volume and iron per body weight progressively and significantly increased throughout the chemotherapy, no changes were noted in serum iron or transferrin saturation in ALL. However, serum iron–binding capacity first decreased until the end of the postinduction phase and then started to progressively increase until the end of treatment (postmaintenance phase). The ferritin value progressively increased until the beginning of the maintenance phase, and it significantly declined until the end of therapy (maintenance), attaining values less than those of the postinduction phase. The posttreatment (maintenance) iron parameters were still comparable to the pretreatment parameters (Fig. 1A).

Evaluation of ALL-1 and ALL-2 groups separately (Supplemental Figs. 1–3S, Supplemental Digital Content 1, http://links.lww.com/JPHO/A45, Supplemental Digital Content 2, http://links.lww.com/JPHO/A46, Supplemental Digital Content 3, http://links.lww.com/JPHO/A47) showed that transfused cumulative PRBC volume and iron per body weight progressively and significantly increased, but no significant changes were noted in serum iron, transferrin saturation, and ferritin levels from the beginning to the end of treatment, although some fluctuations were observed. Only serum iron–binding capacity increased until the end of the maintenance phase. In the ALL-3 group, who could be followed up until the end of the induction phase, serum iron and transferrin saturation levels declined, although serum iron–binding capacity did not change (Figs. 1–3S, Supplemental Digital Content 1, http://links.lww.com/JPHO/A45, Supplemental Digital Content 2, http://links.lww.com/JPHO/A46, Supplemental Digital Content 3, http://links.lww.com/JPHO/A47).

In the AML group, transfused cumulative PRBC volume and iron per body weight progressively and
significantly increased until post-HAM, whereas serum iron, transferrin saturation, and ferritin levels significantly increased and serum iron–binding capacity significantly decreased from preinduction to post-AI block, after which they showed no significant change. During intervals when transfused cumulative PRBC volume and iron per body weight increased, a temporary increment in the serum iron, transferrin saturation, and ferritin levels and a decrement in serum iron–binding capacity were noted (Fig. 1B).

Reticulocyte Counts
In the ALL-2 group, the reticulocyte counts in the beginning of chemotherapy phases declined significantly at the end of the phases and increased significantly at the beginning of the consecutive phase (Supplement Table 3S, Supplemental Digital Content 5, http://links.lww.com/JPHO/A49). In addition, hypochromic erythrocytes were noted in the peripheral blood smear in all patients during chemotherapy.

The Effect of Different Leukemia Treatments on Iron Parameters

Postinduction Parameters
The ALL-1 group was transfused with a significantly greater amount of PRBCs than in the ALL-2 and ALL-3 groups until the end of the induction phase. However, serum iron and transferrin saturation levels in ALL-1 were significantly less compared with those of the ALL-2 group, and ferritin levels of the 2 groups were comparable, whereas the serum iron–binding capacity of the ALL-1 group was significantly higher compared with that of ALL-2. In addition, serum iron level, serum iron–binding capacity, transferrin saturation, and ferritin level in ALL-1 and ALL-3 groups were comparable.

Transfused cumulative PRBC volume and iron per body weight values of the AML group were comparable to those of the ALL-1 group. However, in the AML group, serum iron, transferrin saturation, and ferritin levels were significantly higher, and serum iron–binding capacity was lower than that in ALL-1.

Transfused cumulative PRBC volume and iron per body weight of the AML group were significantly higher than those in ALL-2 and ALL-3 groups. In the AML group, serum iron, transferrin saturation, and ferritin levels were significantly higher than those in ALL-2 and ALL-3, whereas serum iron–binding capacity was lower than that in ALL-3 and comparable to that in ALL-2 (Table 1).

The interval during which the induction of ALL was administered to our patients was 42.8 ± 8.5 days for ALL-1 versus 36.8 ± 4.9 for ALL-2 versus 28.6 ± 1.3 for ALL-3 (P = 0.000, ALL-1 > ALL-2; ALL-1 > ALL-3; ALL-2 > ALL-3).

Postmaintenance (Posttherapy) Parameters
Transfused cumulative PRBC volume and iron per body weight at the beginning of the maintenance phase were significantly higher in ALL-2 than in ALL-1 (76.9 ± 36.8 mL/kg and 84.8 ± 39.4 mg/kg vs. 40.9 ± 19.5 mL/kg and 43.3 ± 20.5 mg/kg; P = 0.009 and 0.005, respectively).

Although transfused cumulative PRBC volume and iron per body weight at the end of the maintenance phase in
the ALL-1 and ALL-2 groups were similar, in ALL-2, the serum iron level and transferrin saturation were significantly higher than that in ALL-1, and serum iron-binding capacity was lower than that in ALL-1; ferritin level was comparable between the 2 groups.

In ALL-2, serum iron, transferrin saturation, and/or ferritin levels remained elevated, and serum iron–binding capacity remained declined in comparison with the controls when chemotherapy was discontinued (Table 2).

The interval during which the whole leukemia therapy was administered was shorter in ALL-2 than in ALL-1 (817.3 ± 145.5 vs. 1077.8 ± 73.8 d; P = 0.004).

Iron parameters in AML Before Bone Marrow Transplantation

According to the protocol, patients are recommended to undergo bone marrow transplantation after the block haM. The AML patients who had just received haM block and had not previously received chelation therapy had higher serum iron (168.3 ± 133.2 vs. 81.5 ± 17.9 μg/dL, P = 0.026), transferrin saturation (5138.7% ± 8110.8% vs. 31.1% ± 12.4%, P = 0.001), and ferritin levels (1620.8 ± 702.0 vs. 38.9 ± 30.7 ng/mL, P = 0.0001) and a lower serum iron–binding capacity (13.7 ± 12.6 vs. 277.7 ± 52.9, P = 0.0001) than those of the controls. Unfortunately, the 2 patients who underwent bone marrow transplantation could not be evaluated by liver-heart MRI with respect to iron overload and did not receive chelation but successfully underwent transplantation. However, the one who received chelation after haM block, until referral to the Bone Marrow Transplantation Unit, developed graft versus host disease.

Patients With Ferritin Level >1000 ng/mL

Three patients (from ALL-2 [high risk], ALL-2 [medium risk], AML [high risk]) had >1000 ng/mL ferritin level at admission (1361, 1052, and 6163 ng/mL; transferrin saturation: 46.3%, 9600%, 41.5%, respectively). Their ferritin level decreased shortly after chemotherapy but later increased again. The point at which ferritin level started to re-increase was accepted as the time when ferritin level increased after the initiation of chemotherapy.

During chemotherapy, 20 leukemia patients (9/9 AML [100%] and 11/26 ALL [42.3%]) attained a ferritin level >1000 ng/mL. All ALL patients were from the ALL-2 group (2 standard risk, 7 medium risk, and 2 high risk). Transferrin saturation in those with a ferritin level >1000 ng/mL was 5214% ± 6202.74% (range, 33.6% to 9600%).

The time elapsed between the initiation of chemotherapy and establishment of a ferritin level of >1000 ng/mL...
was 110 ± 101.9 and 51 ± 16 days for ALL and AML patients, respectively (Fig. 2). Five ALL patients were at the end of P1P1, 3 at the end of P1P2, 1 at the beginning of P-M, 1 at the end of P2P1, and 1 in the sixth month of maintenance therapy; 2 AML patients were in pre-HAM block, 3 in post-HAM block, 3 in pre-AI, and 1 in pre-haM block.

**MRI T2* Results**

Sixteen of 20 patients with a ferritin level of >1000 ng/mL were evaluated by MRI 22 times for liver iron overload (6 were evaluated twice, 10 were evaluated once; 11 with T2* and 8 with R2). Heart MRI could be performed in 11 patients 13 times (2 were evaluated twice) by T2*.

Among patients with ferritin levels >1000 ng/mL (Fig. 2), only 10/16 (62.5%) and later 12/16 (75%) had documented iron overload in liver MRI, whereas 6/16 (37.5%) and later 4/16 (25%) had no iron overload. The R2 and liver T2* values are presented in Table 3. No iron overload was detected by heart MRI (T2* 27.3 ± 3.69 ms) (Fig. 2).

Accordingly, the 14 liver MRIs (from 8 ALL and 4 AML patients) showed mild (n = 10) and moderate (n = 4) iron overload. Eight MRIs (from 3 AML and 3 ALL patients) showed no liver iron overload (Table 3). One AML and 1 ALL patient from this group were found to have iron overload as documented by liver MRI performed 55 and 163 days later, respectively, and, therefore, they were included in the iron-overloaded group.

**Comparison of Patients With Normal and Abnormal MRI Despite Having Ferritin Levels >1000 ng/mL**

In patients with ferritin levels >1000 ng/mL who showed iron overload on MRI, the total PRBC transfusion number (9.8 ± 5.3 vs. 5.3 ± 4.0, \(P = 0.042\)), transfused cumulative PRBC volume per body weight (92.90 ± 42.0 vs. 32.10 ± 40.7, \(P = 0.020\)), and the transfused cumulative iron per body weight (100.90 ± 44.50 vs. 34.90 ± 44.20, \(P = 0.020\)) were significantly higher than in those with ferritin levels >1000 ng/mL but who did not have radiologic iron overload. There was no significant difference in other parameters like serum iron, serum iron-binding capacity, transferrin saturation, ferritin, hemoglobin, hematocrit, white blood cell count, absolute neutrophil count, thrombocyte count, aspartate aminotransferase, alanine aminotransferase, \(\gamma\)-glutamyl transpeptidase, total protein, albumin, total bilirubin, direct bilirubin, transfused cumulative iron, body mass index, and body weight.

**Relationship Between Various Parameters and Cumulative Iron in Patients With Radiologically Documented Iron Overload (n = 12)**

Cumulative iron was inversely correlated with thrombocyte count (\(P < 0.042\)) and aspartate aminotransferase (\(P < 0.035\)) and positively correlated with body weight (\(P < 0.002\)). Cumulative iron did not demonstrate any relationship with transfused cumulative PRBC volume and

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**TABLE 1. Comparison of Postinduction Parameters of the Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>(P)</th>
<th>Groups With Significant Difference in Double Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI (postinduction) (µg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: ALL-1</td>
<td>12</td>
<td>65.2</td>
<td>24</td>
<td>0.000</td>
<td>B-A</td>
</tr>
<tr>
<td>B: ALL-2</td>
<td>24</td>
<td>132.2</td>
<td>75.8</td>
<td>0.000</td>
<td>B-C</td>
</tr>
<tr>
<td>C: ALL-3</td>
<td>5</td>
<td>54</td>
<td>21.6</td>
<td>0.000</td>
<td>D-A</td>
</tr>
<tr>
<td>D: AML</td>
<td>6</td>
<td>202</td>
<td>49.3</td>
<td>0.000</td>
<td>D-B</td>
</tr>
<tr>
<td>D-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SIBK (postinduction) (µg/dL)</td>
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</tr>
<tr>
<td>A: ALL-1</td>
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<td>179.8</td>
<td>49.3</td>
<td>0.000</td>
<td>B-A</td>
</tr>
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<td>71.5</td>
<td>59.8</td>
<td>0.000</td>
<td>B-C</td>
</tr>
<tr>
<td>C: ALL-3</td>
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<td>205.4</td>
<td>35.3</td>
<td>0.000</td>
<td>D-A</td>
</tr>
<tr>
<td>D: AML</td>
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<td>126.8</td>
<td>174.4</td>
<td>0.000</td>
<td>D-B</td>
</tr>
<tr>
<td>D-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TS (postinduction) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: ALL-1</td>
<td>12</td>
<td>40</td>
<td>19.6</td>
<td>0.000</td>
<td>B-A</td>
</tr>
<tr>
<td>B: ALL-2</td>
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<td>488</td>
<td>624.6</td>
<td>0.000</td>
<td>B-C</td>
</tr>
<tr>
<td>C: ALL-3</td>
<td>5</td>
<td>25.8</td>
<td>6.7</td>
<td>0.000</td>
<td>D-A</td>
</tr>
<tr>
<td>D: AML</td>
<td>5</td>
<td>643.3</td>
<td>748.4</td>
<td>0.000</td>
<td>D-B</td>
</tr>
<tr>
<td>D-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ferritin (postinduction) (ng/mL)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: ALL-1</td>
<td>9</td>
<td>598.4</td>
<td>17.7</td>
<td>0.004</td>
<td>D-A</td>
</tr>
<tr>
<td>B: ALL-2</td>
<td>20</td>
<td>702.8</td>
<td>20.4</td>
<td>0.004</td>
<td>D-B</td>
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<tr>
<td>C: ALL-3</td>
<td>5</td>
<td>329.2</td>
<td>9.6</td>
<td>0.004</td>
<td>D-C</td>
</tr>
<tr>
<td>D: AML</td>
<td>6</td>
<td>1553</td>
<td>34.2</td>
<td>0.004</td>
<td>D-C</td>
</tr>
<tr>
<td>Transf cum PRBC per wt (postinduction) (mL/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: ALL-1</td>
<td>13</td>
<td>45</td>
<td>35.5</td>
<td>0.021</td>
<td>A-B</td>
</tr>
<tr>
<td>B: ALL-2</td>
<td>26</td>
<td>30.3</td>
<td>23.3</td>
<td>0.021</td>
<td>A-C</td>
</tr>
<tr>
<td>C: ALL-3</td>
<td>5</td>
<td>31.4</td>
<td>24.2</td>
<td>0.021</td>
<td>A-D</td>
</tr>
<tr>
<td>D: AML</td>
<td>9</td>
<td>60.4</td>
<td>36.6</td>
<td>0.021</td>
<td>A-D</td>
</tr>
<tr>
<td>D-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transf cum Fe per wt (postinduction) (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: ALL-1</td>
<td>13</td>
<td>47.6</td>
<td>33.3</td>
<td>0.023</td>
<td>A-B</td>
</tr>
<tr>
<td>B: ALL-2</td>
<td>26</td>
<td>33.2</td>
<td>21.6</td>
<td>0.023</td>
<td>A-C</td>
</tr>
<tr>
<td>C: ALL-3</td>
<td>5</td>
<td>31.4</td>
<td>21.2</td>
<td>0.023</td>
<td>A-D</td>
</tr>
<tr>
<td>D: AML</td>
<td>9</td>
<td>65.7</td>
<td>36.7</td>
<td>0.023</td>
<td>A-D</td>
</tr>
</tbody>
</table>

SI indicates serum iron; SIBK, serum iron binding capacity; TS, transferrin saturation.
The Results of Iron-chelation Therapy

By the end of the observation time, 7 patients had been receiving only desferrioxamine, 1 with only deferasirox, and 2 patients had been receiving desferrioxamine first (for 104 and 97 days, respectively) and then deferasirox later (for 425 and 203 days, respectively). In the iron-chelated group,
although the transfused cumulative PRBC volume and iron per body weight significantly increased, serum iron—binding capacity increased and ferritin level decreased, whereas those who were not chelated showed no change in any of the iron parameters or transfused cumulative PRBC volume and iron per body weight (Table 4).

In 6 patients, chelation was discontinued as ferritin level decreased to < 500 ng/mL. These patients received chelation for 342 ± 243.24 (range, 49 to 529) days as desferrioxamine only (n = 1), deferasirox only (n = 2), and desferrioxamine followed by deferasirox (n = 3).

A patient who developed mild liver iron overload in the sixth month of maintenance therapy and refused chelation attained a ferritin level < 500 ng/mL within 12 months during maintenance.

Only ferritin-1 was higher in the treated than in the nontreated patients (P = 0.037). The other first and second parameters were similar in chelated and unchelated patients (serum iron-1 and -2; serum iron—binding capacity-1 and -2; transferrin saturation-1 and -2; ferritin-2; transfused cumulative PRBC volume per body weight-1 and -2; and transfused cumulative iron per body weight-1 and -2, P = 0.08 to 0.95).

Microscopic Evaluation

Bone marrow smears showed large iron aggregates within the bone marrow matrix both before and during chemotherapy. Sideroblasts and siderocytes containing 1 to 15 fine and coarse iron-containing granules, iron-laden macrophages, thrombocytes, megakaryocytes, and dyserythropoiesis were evident. No ringed sideroblasts were seen (Supplemental Figure 4S, Supplemental Digital Content 4, http://links.lww.com/JPHO/A48).

DISCUSSION

Iron overload denotes excess iron deposition in tissues, defined as elevated serum iron, transferrin saturation ([ > 45%; > 50%]19,20), and ferritin levels (> 300 ng/mL). Total iron-binding capacity may be reduced in advanced states.20

In this study, the increased iron parameters in both ALL and AML even before chemotherapy was initiated and PRBCs were administered show that active leukemia

### TABLE 3. The Liver MRI Values of Patients With Ferritin Level >1000 ng/mL

<table>
<thead>
<tr>
<th>Patients, n</th>
<th>Liver R2 Value</th>
<th>Liver T2* Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate iron overload (R2: 7.0-15 mg Fe/g dry weight)</td>
<td>4 (3 ALL, 1 AML)</td>
<td>11.07 ± 1.83 (n = 5)</td>
</tr>
<tr>
<td>Mild iron overload (R2: 3.2-7.0 mg Fe/g dry weight) (T2*: 2.7-6.3 ms)</td>
<td>10 (7 ALL, 3 AML)</td>
<td>4.26 ± 1.26 (n = 5)</td>
</tr>
<tr>
<td>No iron overload (0.6-1.2 mg Fe/g dry weight) (T2*: &gt; 6.3 ms)</td>
<td>8 (3 AML, 4 ALL)</td>
<td>1.36 ± 0.75 (n = 2)</td>
</tr>
</tbody>
</table>

### TABLE 4. Among Patients Who Had Radiologically Documented Iron Overload, Comparison Between Those Who Did and Did Not Receive Iron-chelation Therapy in a Comparable Time Period

<table>
<thead>
<tr>
<th>Patients Who Received Therapy (n = 10)</th>
<th>Patients Who Did Not Receive Therapy (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin-1 (ng/mL)</td>
<td>1919.5 ± 834.4</td>
<td>1277.0 ± 117.6</td>
</tr>
<tr>
<td>Ferritin-2 (ng/mL)</td>
<td>1109.3 ± 631.4</td>
<td>1600.2 ± 811.5</td>
</tr>
<tr>
<td>Transf cum PRBC per wt-1 (mL/kg)</td>
<td>101.1 ± 40.9</td>
<td>68.4 ± 31.5</td>
</tr>
<tr>
<td>Transf cum PRBC per wt-2 (mL/kg)</td>
<td>127.7 ± 46.8</td>
<td>95.2 ± 23.0</td>
</tr>
<tr>
<td>Transf cum Fe per wt-1 (mg/kg)</td>
<td>111.3 ± 42.4</td>
<td>74.3 ± 34.2</td>
</tr>
<tr>
<td>Transf cum Fe per wt-2 (mg/kg)</td>
<td>139.2 ± 48.3</td>
<td>103.5 ± 25.0</td>
</tr>
<tr>
<td>SI-1 (µg/dL)</td>
<td>158.6 ± 86.8</td>
<td>168.2 ± 57.8</td>
</tr>
<tr>
<td>SI-2 (µg/dL)</td>
<td>148.3 ± 75.5</td>
<td>163.4 ± 68.5</td>
</tr>
<tr>
<td>SIBC-1 (µg/dL)</td>
<td>86.1 ± 92.2</td>
<td>129.2 ± 77.5</td>
</tr>
<tr>
<td>SIBC-2 (µg/dL)</td>
<td>216.4 ± 194.1</td>
<td>90.4 ± 93.3</td>
</tr>
<tr>
<td>TS-1 (%)</td>
<td>574.5 ± 710.5</td>
<td>257.2 ± 326.2</td>
</tr>
<tr>
<td>TS-2 (%)</td>
<td>844.7 ± 2146.5</td>
<td>603.7 ± 586.6</td>
</tr>
</tbody>
</table>

PRBC indicates packed red blood cell; SI, serum iron; SIBC, serum iron binding capacity; TS, transferrin saturation.

During electron microscopic evaluation of patients with/without radiologic iron overload, it was observed that normoblasts showed abnormal mitochondria, increased endoplasmic reticulum, numerous free ribosomes, and secondary lysosomes, which included iron deposits and autolysosomes, and promyelocytes showed enlarged nuclear pores, granulated endoplasmic reticulum sacs, numerous free ribosomes, and primary granules, which contained iron deposits (Fig. 3).

### Genetic Iron-overload Genes

Three patients with radiologically documented iron overload (1 moderate, 2 mild) were heterozygous for the H63D mutation. The ferritin values at admission were 1361, 1052, and 87.2 ng/mL, respectively, and transferrin saturation values were 46.3%, 9600%, and 40.1%, respectively. The other 7 did not show any of the 18 screened mutations.

### Porphyrin Metabolites

The mean values of aminolevulinic acid and porphobilinogen were 1.85 ± 1.27 mg/24 h (N: 1.5 to 7.5) and 1.35 ± 0.06 mg/24 h (N: 0 to 3.4), respectively.
itself leads to iron overload; it is aggravated by chemotherapy and is partially independent of PRBC transfusions.

In the English literature, information about pretreatment iron parameters in leukemia is restricted to elevated levels of serum iron and low–molecular mass iron complexes in AML, and high levels of ferritin and hepcidin together with hypoxia-inducible factor-1, which reached its maximum during agranulocytosis after chemotherapy and normalized after recovery of hematopoiesis. The normal pretreatment serum iron levels in children with solid tumors point to an association between iron overload and hematological malignancies.

Cells have an internal low–molecular mass iron pool. When the rapid cell growth of the leukemia cells declines due to cell loss like exfoliation necrosis and nutritional depletion, leukemia cells are destructured to give rise to release of some or all of their iron pool. The low-normal hemoglobin ([17.9 ± 2.08] [range, 4.2 to 12.9]) and low-high reticulocyte counts ([2.88% ± 2.17%] [range, 0.27% to 6.17%]) in our patients at presentation (data not shown) suggest that cessation of erythropoiesis in some patients, if not all, may cause accumulation of unused iron for erythropoiesis. Elevation of iron, through these routes, causes elevation in transferrin saturation and ferritin level and decrease in serum iron–binding capacity. Decreased serum iron–binding capacity and/or low levels of serum transferrin are additive factors that yield elevation in transferrin saturation. Although a high hepcidin level at presentation reported reduces iron entry into the plasma compartment by reducing dietary absorption in the duodenum, reduces the release of recycled iron from macrophages, and reduces the release of stored iron from hepatocytes, its effect seems inadequate to counter the elevation in iron, through the aforementioned ways. The absence of hyperbilirubinemia in our patients with high reticulocyte counts at presentation rules out coexistent hemolytic anemia as the cause for elevated iron and ferritin levels.

Our findings showed that the quantity of transfused cumulative PRBC volume and iron per body weight was determinant of iron overload in AML (up until the end of AI block) but not in ALL in either part of chemotherapy. In this study, the different patterns of changes in iron parameters throughout chemotherapy in each group, which received different chemotherapy protocols, the elevation in iron parameters after certain blocks in leukemia subgroups (eg, P1P1, P1P2, P2P1 in ALL-2 and AIE, HAM, AI in

**FIGURE 3.** Electron microscopic evaluation of the bone marrow cells. A, Normoblasts from an ALL patient with a ferritin level >1000 ng/dL. Mitochondrial (m) hypertrophy, severe degree of intracristal swelling, and numerous free ribosomes are seen (arrow). The matrix chambers of the mitochondria present as electron-dense bands (×12,930). B, A normoblast from an AML patient whose ferritin level was <1000 ng/dL. Numerous secondary lysosomes (SL), which include iron deposits (arrow) and an autolysosome (arrowheads) are seen (×16,700). C, A promyelocyte from another ALL patient with a ferritin level >1000 ng/dL. Enlarged nuclear pore (†), enlargement of granulated endoplasmic reticulum sacs (thick arrows), numerous free ribosomes, and primary granules with their iron deposits (arrows) are seen (×7750). D, Higher magnification of the same cell (C) (×16,700) (arrow: iron deposits, †: dilated nuclear pore, G: Golgi complex, thick arrow: dilated granulated endoplasmic reticulum, m: mitochondrion, N: nucleus, SL: secondary lysosome, arrow head: autophagosome). ALL indicates acute lymphoblastic leukemia; AML, acute myeloblastic leukemia.
A high value for initiation of iron chelation, being dependent or not dependent directly to the already increased intravascular free iron (non—transferrin-bound iron) (Supplemental Tables 1, 2, JPHO/A45, Supplemental Digital Content 1, http://links.lww.com/JPHO/A45, Supplemental Digital Content 2, http://links.lww.com/JPHO/A46, Supplemental Digital Content 3, http://links.lww.com/JPHO/A47), and the sustained post-treatment elevations in iron parameters in ALL-2, as in some reports,7,8 but not in ALL-1, suggest that the intensity and character of the chemotherapy protocol is another determinant of iron overload. Hence, serum iron levels in ALL patients who were in the high-risk group and received more intense chemotherapy protocol were higher than in the standard-risk group and medium-risk group patients.7

It was also reported that the high ferritin level in ALL children correlated with the transfused PRBC quantity, but those who had the largest iron overload were those who received the heaviest chemotherapy.9,10

The high values of iron parameters in high-risk AML patients established after haM block (at the time of referral to Bone Marrow Transplantation Unit) show that these patients are at risk for adverse complications of bone marrow transplantation due to iron overload, if they are not given chelation therapy.

Our findings showed that patients who received chemotherapy in short-lasting blocks as in AML were more prone to iron overload. It seems that patients who received induction and the whole therapy (from beginning to end) in shorter periods (as in ALL-2 vs. ALL-1 groups) were also more liable to develop iron overload, suggesting that the time interval during which excess iron is introduced may be a variant of iron overload.20 Because of the low number of patients in the ALL-3 group, we reserve for further clarification the finding that the postinduction iron parameters of the ALL-3 group were less than or comparable to those of ALL-1 and ALL-2 groups, despite the short induction period (the shortest of all).

Carmine et al4 reported that chemotherapy injury could cause release of the cellular iron pool first. Iron in hemoglobin is released later with simultaneous hyperbilirubinemia in ALL patients (standard-risk and medium-risk groups).4

Given the microscopic findings, we think that chemotherapy damages the cellular elements activating the lysosomes to engulf them, after which the cellular iron is released (autophagia)20 (Figs. 3A–D). The cellular damage and dyshematopoiesis, like in other disorders,27 may also be due directly to the already increased intravascular free iron (non—transferrin-bound iron) (Supplemental Tables 1, 2, Supplemental Digital Content 5, http://links.lww.com/JPHO/A49), as increased transferrin saturation, as in our patients, is considered as a marker of non—transferrin-bound iron,12 the threshold of which is 45% and 75%,28,29 despite exceptions.3,5,12,25 The enlarged nuclear pores and autophagic vacuoles were also reported in sideroblastic anemia30 and MDS.31

In addition, in our patients, serum iron—bind capacity was lower than that in the controls, like in advanced hemochromatosis20 or sideroblastic anemia,32 both before and during chemotherapy. The fact that patients with most iron overload had the least serum iron—bind capacity suggests that synthesis of iron—bind proteins is impaired before, during, and after chemotherapy. Hence, Halonen et al7 reported that in patients with the highest liver iron levels, serum iron, transferrin saturation, and ferritin levels were the highest, but transferrin levels were the lowest, whereas soluble transferrin receptor levels were comparable to levels in nonoverloaded patients.

Decreased capacity of the liver to absorb non—transferrin-bound iron from the circulation3 and free iron from heme compounds after the lysis of red cells in the PRBC suspension,33,34 and after apoptosis (Supplemental Figures 4S-P, Supplemental Digital Content 4, http://links.lww.com/JPHO/A48) are other sources of excess iron. In contrast to reported cases,33–37 our findings showed no abnormality in the porphyrin pathway due to chemotherapy that would give rise to cellular iron overload.

The fact that the reticulocyte quantity declined significantly after the chemotherapy phase and increased until the consecutive phase, when serum iron, transferrin saturation, and ferritin levels nonstatistically decreased, implies a suspension of erythropoietic activity due to chemotherapy, as was reported previously.3 The elevated iron parameters at admission and hypochromic erythrocytes in peripheral blood may also be due to the suspended erythropoietic activity.

Genetic Aspects

The fact that not all patients who received the same chemotherapy protocol, but only a few, could develop radiologically documented iron overload and that both overloaded and nonoverloaded patients develop the same ultrastructural damage led us to investigate a genetic propensity for aggravated iron overload, as in thalassemia patients.38 We found that 3 of 10 patients with documented iron overload had heterozygosity of the H63D mutation, which already yields elevation in serum iron and transferrin saturation values.39 However, we cannot deduce that the untested mutations had no role, and those who were not genetically evaluated, as they had a ferritin level of <1000 ng/mL, did not have any of those mutations. In Turkey, allele frequency of H63D mutation was reported as 14%40 in volunteers living in central Turkey (Ankara) and the carrier frequency as 5.6% in eastern Turkey,39 whereas the C282Y mutation is absent.38–40 In our study, these 3 patients were from the northeast, north central, and southeastern parts of Turkey, suggesting that the H63D mutation does not seem to be restricted to any certain geographic region of Turkey.

Liver Iron Overload and Iron-chelation Therapy

Although our patients were irregularly transfused, chemotherapy caused iron release and overload, documented by elevated transferrin saturation levels, ferritin levels,41 liver MRI, and light microscopic findings consistent with stage IV and V iron storage,42 and iron granules in not only erythroid precursors and macrophages as in untreated AML43 and iron-overload conditions42 but also in promyelocytes, megakaryocytes, and platelets. To avoid the adverse effects of iron overload on immunity, organ viability, and outcome of chemotherapy,2–5,11–16 and to reduce iron overload before referral to Bone Marrow Transplantation Units, we treated iron overload. We avoided therapeutic phlebotomy unlike in reported leukemia cases8,9,44 in order to not aggravate the post-chemotherapy cytophenia. No therapy guideline for iron overload in leukemia is available. We thus modified the guidelines of iron overload in thalassemia45 and MDS,46 which accept a ferritin level of >1000 ng/mL as a critical value for initiation of iron chelation, being dependent or independent of MRI findings and transfusion history.
Therefore, we evaluated those with ferritin levels >1000 ng/mL with respect to iron overload in the liver and heart by MRI.

Iron overload (medium to heavy) in 63% of ALL patients at the end stage of chemotherapy or after the cessation of chemotherapy in liver biopsy and iron overload in liver/heart MRI associated with liver biopsy in a few selected leukemia patients were reported.

Total iron score, which reflected the liver iron, was reported to correlate positively with serum ferritin, transferrin saturation levels. However, in our study, the comparability of transferrin saturation and ferritin levels in radiologically iron-overloaded and non-overloaded patients and the radiologically documented iron overload in 1 patient despite transferrin saturation of <45% (33.6%) suggest that the threshold of 45% and 50% for transferrin saturation and 1000 ng/mL for ferritin level to define iron overload are unreliable to show iron overload as reportedly accepted. The finding that 25% to 37.5% of patients with a ferritin level >1000 ng/mL did not have liver iron overload shows once more the insufficiency of the criterion of a ferritin level >1000 ng/mL. Hence, children with leukemia may also have excess liver iron even while their ferritin level is <1000 ng/mL.

Thus, specific guidelines to define iron overload in leukemia and therefore chelation therapy are required.

Our results showed that chelation therapy was useful in children during chemotherapy. In addition, deferoxamine is known to diminish the injurious side effects of some chemotherapeutics and augment their effectiveness, despite some adverse effects as well.

We could not establish any correlation between liver enzyme elevations and cumulative iron overload in patients with ferritin levels >1000 ng/mL. Hence, ferritin level does not seem to be a good indicator of iron-mediated liver dysfunction compared with non-transferrin-bound iron.

It remains controversial as to whether iron accumulation in platelets has an additive role of shortening thrombocytopenia life span as in advanced hemochromatosis, given that an inverse correlation existed between thrombocyte count and transfused cumulative iron in patients with radiologically documented iron overload, and we found inconsistent correlations as well (such as an inverse correlation of cumulative iron with aspartate aminotransferase).

Other Coexistent Complications of Chemotherapy in Our Patients

Osteoporosis may accompany iron-overload conditions. However, studies from our clinic, partly involving the same patients from ALL-1 and ALL-2 groups of this study, showed that osteopenia/osteoporosis was encountered in radiologically iron-overloaded and non-iron-overloaded patients, at comparable rates (Öztürkmen et al, unpublished data), and the incidence of osteoporosis was comparable in patients who received modified ALL St Jude TXIII protocol, none of whom developed iron overload, and those that received TRALL-BFM 2000 protocol (Akyay et al, unpublished data).

This study shows that iron overload in leukemia is a major complication before and during chemotherapy. Further investigations are needed to determine the most appropriate follow-up and therapeutic approach.

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