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Labile plasma iron levels predict survival in patients with lower-risk Myelodysplastic syndromes

by Louise de Swart, Chloé Reiniers, Timothy Bagguley, Corine van Marrewijk, David Bowen, Eva Hellström-Lindberg, Aurelia Tatic, Argiris Symeonidis, Gerwin Huls, Jaroslav Cermak, Arjan A. van de Loosdrecht, Hege Garelius, Dominic Culligan, Mac Macheta, Michail Spanoudakis, Panagiotis Panagiotidis, Marta Krejci, Nicole Blijlevens, Saskia Langemeijer, Jacqueline Droste, Dorine W. Swinkels, Alexandra Smith, and Theo de Witte

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Title: Labile plasma iron levels predict survival in patients with lower-risk Myelodysplastic syndromes

Short title: Toxic iron species in lower-risk MDS

Key points:

Key point 1: Ineffective erythropoiesis in untransfused RS-MDS may lead to iron toxicity

Key point 2: Elevated labile plasma iron species are associated with impaired survival, especially in transfused MDS patients

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Abstract

Red blood cell transfusions remain one of the cornerstones in supportive care of lower-risk patients with myelodysplastic syndromes. We hypothesized that patients develop oxidant mediated tissue injury through the formation of toxic iron species, caused either by red blood cell transfusions or by ineffective erythropoiesis. We analyzed serum samples from 100 lower-risk patients with myelodysplastic syndromes at six-month intervals for transferrin saturation, hepcidin-25, growth differentiation factor 15, soluble transferrin receptor, non-transferrin bound iron and labile plasma iron in order to evaluate temporal changes in iron metabolism and presence of potentially toxic iron species and their impact on survival. Hepcidin levels were low in 34 patients with ringed sideroblasts compared to 66 patients without. Increases of hepcidin and non-transferrin bound iron levels were visible early in follow-up of all transfusion dependent patient groups. Hepcidin levels significantly decreased over time in transfusion independent patients with ringed sideroblasts. Increased soluble transferrin receptor levels in transfusion-independent patients with ringed sideroblasts confirmed the presence of ineffective erythropoiesis and suppression of hepcidin production in these patients. Detectable labile plasma iron levels in combination with high transferrin saturation levels occurred almost exclusively in patients with ringed sideroblasts and all transfusion dependent patient groups. Detectable labile plasma iron levels in transfusion dependent patients without ringed sideroblasts were associated with decreased survival. In conclusion: toxic iron species occurred in all transfusion dependent patients and in transfusion independent patients with ringed sideroblasts. Labile plasma iron appeared to be a clinically relevant measure for potential iron toxicity and a prognostic factor for survival in transfusion dependent patients. This trial was registered at www.clinicaltrials.gov as #NCT00600860.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired clonal hematopoietic stem cell disorders that are characterized by abnormal differentiation and maturation of hematopoietic cells, bone marrow failure and genetic instability with an enhanced risk of progression to acute myeloid leukemia.[1] The EUMDS registry is a prospective, observational registry established in 2007 to collect data on low and intermediate-1 risk MDS patients, which represents the lower-risk MDS population comprising approximately seventy percent of the overall MDS population.[2,3] The majority of lower-risk MDS patients (51% in the EUMDS Registry)[3] become transfusion dependent, usually early after diagnosis. With an expected median survival of 2.4 to 11.8 years, these patients are prone to long-term accumulation of iron due to red blood cell (RBC) transfusions.[4-8] Iron overload may also occur in MDS patients who do not receive RBC transfusions, due to the stimulation of intestinal iron absorption, mediated through suppression of hepcidin production in patients with ineffective erythropoiesis[9]. Patients with ringed sideroblasts (MDS-RS) are of special interest in this context, considering their pronounced ineffective erythropoiesis.[6,7,10,11]

The toxic effects of iron overload in other iron loading diseases, such as hereditary hemochromatosis[11] and the thalassemia syndromes[12], are well known, but the consequences in MDS remain to be elucidated. MDS patients are generally older than patients with other iron loading disorders[13]. Their exposure may not be long enough to develop classical tissue damage due to iron overload, but they may suffer from oxidative stress caused by toxic iron molecules. Moreover, iron toxicity might be restricted to specific subgroups of MDS patients: patients receiving RBC transfusions and a subgroup of patients with MDS-RS and increased ineffective erythropoiesis.[5,13]

A greater insight into the pathophysiology of iron metabolism in MDS, might be obtained through an optimized diagnostic work-up and monitoring by specific iron metabolism markers, including hepcidin, growth differentiation factor 15 (GDF15), soluble transferrin receptor (sTfR), and the recently introduced serum toxic iron species, namely non-transferrin bound iron (NTBI) and labile plasma iron (LPI).[14-18] The most important regulator of systemic iron metabolism is hepcidin, a 25-aminoacid peptide hormone, produced predominantly by the hepatocytes. Hepcidin triggers internalization and lysosomal degradation of ferroportin, a membrane bound cellular iron exporter present on macrophages and the basolateral site of enterocytes that releases iron into the circulation.[19,20] Hepcidin is suppressed in hypoxia and with increased erythropoietic iron demand and is upregulated in case of inflammation and increased circulating iron levels and elevated body iron stores.[5,20,21]
GDF15 is a protein produced by erythroid precursors and has been reported to be involved in the communication between bone marrow and liver in case of an increased erythroid demand, functioning as a suppressor of hepcidin synthesis, as shown for β-thalassemia.[5,9,22]

However, its role in MDS is still a matter of debate because of conflicting results.[5,11,22-25] Twisted gastrulation factor 1 (TWSG1) and Erythroferrone (ERFE) are also reported to have a suppressive function in hepatic hepcidin production, but validated human assays are not available.[9] Of additional interest in iron homeostasis is sTfR. The serum concentration of sTfR is proportional to the quantity of the Transferrin Receptors 1 (TfR1) on cellular membranes, especially on erythroid precursors, and is a valuable parameter of erythroid mass and iron supplies.[26,27] Among others, sTfR levels are elevated in case of high erythroid proliferation rates especially in combination with adequate iron supply,[27] as in diseases characterized by ineffective erythropoiesis such as β-thalassemia syndromes, and suppressed in case of decreased erythropoietic activity as in anemia of chronic disease, and diseases with erythroid hypoplasia.[20,25,28] Earlier studies showed that sTfR levels are increased in MDS-RS[5], including SF3B1-positive MDS, patients.[11]

NTBI concentrations are only sporadically present with transferrin saturations (TSAT) <70% and increase sharply when saturation of transferrin with iron exceeds 70%.[29] Chemically, NTBI consists of iron that is rather loosely bound to albumin or low molecular weight metal complexing groups.[30,31] The NTBI complexes may be taken up by specific NTBI transporters in liver, pancreas, and heart and contribute to oxidant-mediated cellular injury in these tissues.[17,32] LPI is thought to be the NTBI fraction that is mostly responsible for tissue injury, since it is readily available to participate in redox cycling causing oxidative damage to cellular membranes, proteins and DNA.[15,33] It has been proposed that plasma NTBI is an important early indicator of extra-hepatic iron toxicity in β-thalassemia major.[34,35]

Improved insights in levels and roles of key players of iron metabolism during treatment with transfusions in the various MDS subtypes may provide leads for novel diagnostic and iron reducing treatment strategies. The prospective study of the EUMDS registry has been initiated to provide a better understanding of the pathophysiology and prognostic value of iron overload and iron mediated oxidative stress as well as possibly important markers in iron homeostasis over time in MDS. To this end, we evaluated serum ferritin, iron, transferrin saturation, hepcidin-25, GDF15, sTfR, NTBI and LPI levels over time in lower-risk MDS patients and their relation with WHO 2001 subtype and transfusion history. We identified detectable LPI levels as a new important prognostic factor for survival in patients with MDS-RS or lower-risk MDS patients treated with regular RBC transfusions.

Methods

Study design and participants

Patients were eligible to be included in the EUMDS registry if they were newly diagnosed with MDS according to the WHO 2001 classification and a low or intermediate-1 score according to the IPSS prognostic system.[2] Patients with IPSS intermediate-2 or high risk, patients with secondary or therapy-related MDS were excluded from this registry. The ethics committees of all participating countries and centers have approved the protocol (ClinicalTrials.gov Identifier: NCT00600860). Patients were required to provide written informed consent.

Serum samples were collected prospectively, at registration and at 6-month intervals, from 109 patients included in six countries participating in this study from April 2008 to December 2010, but samples from nine patients had to be excluded due to technical reasons, see online supplemental information for details. The total number of analyzed serum samples was 454.

Biochemical assays

The iron parameters in this sub study were analyzed centrally at the department of Laboratory Medicine of the Radboudumc, Nijmegen, Netherlands. Detailed information these iron parameters is described in the online supplemental information.

Measurement of serum NTBI consisted of the chelation-ultrafiltration-detection approach based on the prior mobilization of serum NTBI by weak iron-mobilizing chelators such as nitrilotriacetate (NTA) at 80 mM. The chelated NTBI is separated from transferrin-bound iron by ultrafiltration and detected by colorimetry.[36] The lower limit of detection (LLOD) of the NTBI assay is 0.47 µmol/L. The LPI measurement was based on the measurement of the redox-active and readily chelatable fraction of NTBI. This assay measures iron-catalyzed radical generation in the presence of a low ascorbate concentration. Radical generation was measured with the fluorogenic redox sensitive probe dihydrorhodamine (DHR) 123, and iron-catalyzed radical generation was calculated by subtracting the radical generation in the presence of 50 µmol/L of the bidendate iron chelator deferiprone (DFO, the LPI DHR oxidation that is NOT iron dependent).[37] The LLOD of the LPI assay is 0.24 µmol/L.

Statistical analysis

Standard descriptive techniques were used to assess the association between the iron parameters including Spearman's rank correlation coefficients. Where NTBI or LPI was below LLOD, values were randomly drawn from a univariate distribution in the range from zero to the LLOD. Overall survival (OS) was defined as the time from date of diagnosis to death or for subjects still alive censored at the date of the last visit a sample was available. Cox proportional hazards regression models and Kaplan–Meier survival curves with time-dependent covariates[38] were used in time-to-event analyses to assess the impact of LPI level, NTBI and TSAT by transfusion status on survival. All variables were treated as time-

varying covariates in the model by assessing the levels of the parameters (LPI, NTBI: <LLOD vs elevated, TSAT <80% vs ≥80%) and transfusion status (transfused vs. not transfused) at each visit. LPI and NTBI levels >LLOD were considered abnormal. Once a subject had received a transfusion, they were classified as transfused for the remaining time. Hazard ratios (HR) and 95% confidence intervals (95% CI) are reported for both univariate and multivariate models. In the case of the multivariate analyses, the additional covariates included were age at diagnosis, IPPS-R category and usage of Erythroid Stimulating Agents (ESA). All analyses were undertaken in Stata 14 (StataCorp, College Station, TX).

Results

Patient Characteristics

The median age of all patients at registration was 73 years (range 43-95 years). The majority of the patients were male: 64% (n=64). The IPSS risk groups of the 100 patients in the study were low 47%, intermediate-1 41% and unknown 12% and the IPSS-R risk groups were very low 32%, low 41%, intermediate 8%, high 3% and unknown 16%. WHO2001 MDS-subtypes were RCMD (37%), RARS (30%), RA (18%), RAEB (7%), 5q-syndrome (4%) and RCMD-RS (4%). Fourteen percent of the patients were transfusion dependent (defined as any time after starting transfusions) at registration (n=14). No patients received iron chelation therapy at time of registration. Six patients received iron chelation therapy during this observation period (Table S1). The median number of samples available per patient is five samples (range 1-7), and median follow-up was 5.8 years. Overall survival and progression free survival in our study population were 4.8 and 4.6 years, respectively. Nineteen patients have died, including 5 patients after progression and 9 patients from causes possibly related to MDS (hemorrhage 2, infection 5, and cardiovascular 2 patients) (table S4).

Iron parameters

Median ferritin levels were elevated (>250 µg/l) at registration in all patient groups, but the highest median levels were observed in the transfusion dependent (TD) groups (Table 1). Median serum iron levels were within reference range (12-30 µmol/L) in all patient groups at registration. Overall, median TSAT was within reference range (<45%) at registration with the exception of TD MDS-RS patients (Table 2). Median hepcidin levels were within reference range in all patient groups at registration, but TD patients had significantly higher hepcidin levels compared to transfusion independent (TI) patients ($p<0.001$). Ferritin levels correlated significantly with hepcidin levels ($r=0.55$, $p<0.001$). The median GDF15 levels were elevated in the RS subgroup only. NTBI levels above LLOD (>0.47 µmol/L) occurred in all patient groups at registration with highest levels in MDS-RS patients. STfR levels were within reference range (0.8-1.8 mg/L) at registration, and the highest levels were observed in TI MDS-RS patients (Table 2). The median LPI levels were below LLOD in all patient groups at registration (<0.24 mol/L), except in TD MDS-RS patients. Median CRP levels were below the upper limit of the reference range (<10 mg/L) in all groups at all time-points (Table 1) and the majority of patients with CRP levels above 50 mg/L were transfusion-dependent. CRP levels correlated positively with hepcidin levels ($r = 0.30$, $p<0.001$) and ferritin levels ($r=0.22$, $p<0.001$).

Impact of MDS subtype and transfusions on iron parameters over time

The impact of transfusions and MDS subtype (RS versus nonRS) on TSAT, hepcidin, GDF15, NTBI and LPI levels over time is shown in Table 2. Both serum ferritin and serum iron levels increased significantly ($r=0.59$, $p<0.001$ and $r=0.32$, $p<0.001$, respectively) with cumulative number of transfused units over time in transfusion dependent patients (Table S2) as well as in RS patients (Table S3). TSAT remained stable and within reference range in the TI patients

with the exception of a minority of RS patients (Figure S2) and increased over time in the TD patients up to 94.9% in patients with >10 RBC units transfused (Table S2). Hepcidin levels increased with the number of units transfused, but in contrast, hepcidin levels significantly decreased over time in transfusion independent MDS-RS patients. (Table S3). GDF15 levels were not associated with transfusion status alone, but did increase over time in TD MDS-RS patients with a median of 2893 ng/L at registration compared to 5361 ng/L at two years follow up.

STfR levels increased significantly ($p<0.001$) over time both in TI- and TD MDS-RS patients ($p=0.01$) (Table 2). STfR levels did not change over time in non-RS MDS patients. The lowest sTfR levels were observed in patients who had received more than 10 units (Table S2). TD MDS-RS patients had the most elevated levels of NTBI and LPI over time (Table S2, S3).

Correlation between markers of iron overload

Both elevated NTBI and LPI levels (>LLOD) showed a threshold effect with TSAT of >70% and >80%, respectively (Figure 1A-B). Detectable LPI levels occurred almost exclusively in patients with MDS-RS and/or patients who had received transfusions. NTBI and LPI levels above the LLOD were mutually positively correlated ($r = 0.46$; $p<0.001$). Both NTBI and LPI showed a linear relationship ($P<0.001$) with ferritin, but no threshold levels could be detected (Figure 1C-D). The highest values were observed in transfusion dependent MDS patients; and subgroup analyses showed mainly a positive correlation in the transfusion dependent and/or RS subgroup (Figure 1C-D).

Prognostic impact of iron overload markers

Time-dependent, multivariate analysis of overall survival, adjusted for age and IPSS-R risk groups revealed no significant effect on overall survival for NTBI (HR=0.56, 95%CI=0.21-1.52; $p=0.26$) and for TSAT (HR=0.91, 95%CI=0.29-2.86; $p=0.88$) (table 3, figure 2B and Figure S1).

Ten out of 19 patients who died during this study had detectable LPI. The majority (7 patients) died from progression or MDS-related causes (table S4). Kaplan-Meier curves demonstrate prognostic impact on survival of detectable LPI levels by transfusion status (Figure 2), but no significant effect in the multivariate analysis adjusted for age and IPSS-R risk (HR=2.1, 95%CI 0.7-6.2; table 3). Once LPI is increased in both transfusion dependent and independent patients, survival time decreases, with greatest impact in patients who are transfusion dependent and have increased LPI levels (adjusted HR=3.0, 95%CI= 0.7-13.3). Since 41 patients were also treated with erythropoietin stimulating agents (ESA), we repeated the analyses adjusted for whether or not the patient had been treated with erythropoietin stimulating agents (ESA) at each visit (Figure 3). These adjustments did not significantly alter the magnitude of the risk estimates on overall survival (HR=3.0, 95%CI= 0.7-13.5) (Table 3).

Because the survival of patients with RS-MDS is usually considered better than in the nonRS MDS population, we repeated the analyses in the largest group of 66 nonRS patients (table 4). Detectable LPI levels had a remarkable impact on survival in the whole nonRS group, but

the impact was only significant in the TD subgroup (HR=17.0, 95%CI 2.0-146.6). TSAT levels had a borderline impact on survival in TI patients.

Six patients received iron chelation in this study (Table S3). LPI levels during treatment with deferasirox decreased below LLOD (four patients), even in patients with high TSAT. Only three patients have been treated with lenalidomide.
Ferritin levels and elevated CRP are time dependent variables, which correlate closely with transfusion burden/transfusion intensity, and presumably with infections (detailed data not available). Ferritin levels and elevated CRP predict survival when adjusted for age and IPSS-R group only, but the prognostic impact is less clear when transfusion intensity was added to the model (data not shown).

Discussion

This study among 100 European lower-risk MDS patients showed that both red blood cell transfusions and presence of RS increased the occurrence of the toxic iron species NTBI and LPI in serum. Our data on iron parameters over time suggest that body iron accumulation and toxic iron species (NTBI and LPI) in RS-MDS patients occur along the axis of ineffective erythropoiesis, characterized by elevated sTfR, increased GDF15, low hepcidin, and increased circulating and parenchymal iron levels (Figure 4a). Interestingly, we found detectable LPI, but not NTBI, to be associated with a significantly decreased overall survival in the nonsideroblastic MDS patients.

Hepcidin levels were significantly elevated in all transfusion dependent (TD) patient categories, immediately after initiation of transfusions and remained elevated during transfusion dependency, confirming recent studies in transfused MDS patients and illustrated in Figure 4b.[5,7] However, the elevated hepcidin levels showed a tendency to decrease during continued exposure to transfusions. In addition, sTfR levels decreased over time in TD patients, compatible with previously reported suppression of erythropoiesis by continued transfusions.[20,25] Interestingly, GDF15 increased over time in TD MDS patients and especially in TD RS-MDS patients. Increased GDF15 has previously been associated with ineffective erythropoiesis, but not with TD-mediated suppression of erythropoiesis.[5] This suggests that TD-mediated suppression of ineffective erythropoiesis may be less effective during prolonged transfusions. This is supported by the gradual decline over time of the initially elevated hepcidin levels during prolonged transfusions. These data show that previous conflicting observations on the relationship of GDF15 and hepcidin can be explained by the impact of transfusions on GDF15 and hepcidin levels, especially in RS-MDS patients.[5]

Hepcidin levels decreased over time in TI patients of the RS subtype. An earlier study in 107 untransfused patients observed generally elevated hepcidin levels in MDS, but they observed low hepcidin/ferritin ratios in the RS subtypes, compatible with the low hepcidin levels in the RS patients of our study.[39] In addition, RS patients showed elevated sTfR levels and decreased hepcidin levels compared to TI non-RS at all time points. These observations confirm the previously reported association between sTfR and ineffective erythropoiesis, resulting in increased uptake of dietary iron and iron release by macrophages, subsequently leading to increased circulating iron levels, elevated parenchymal iron stores and toxic iron species.[7] Interestingly, recently developed hepcidin agonists prevented low-hepcidin induced toxicity pre-clinically showing the potential of these compounds to prevent iron loading erythropoietic activity in MDS, especially in RS-MDS.[25,40] Altogether, our data suggest worsening over time of the ineffective erythropoiesis in RS patients and lower hepcidin levels in these patients.[41,42]

Elevated NTBI levels could be demonstrated in our study early in follow up of all patient groups. In iron loading anemias, such as thalassemia syndromes, iron species, like NTBI and LPI, have been suggested to serve as early indicators of iron toxicity and as measures for the

effectiveness of iron chelation therapy in reducing potentially toxic iron molecules in the plasma.[7,43] Excess toxic iron species catalyze the cellular generation of ROS. Oxidative stress, and high TSAT, as in combination with subsequent decrease in cellular antioxidants, may lead to oxidation of lipids, proteins and DNA causing cell and tissue damage.[44,45] Biomarkers of oxidative stress have been found to be increased in patients with MDS and iron overload.[3,46-49] The combination of high serum ferritin levels as well as the presence of NTBI and LPI, was noted more frequently in RS patients compared to non-RS patients in our study. Here, it is important to realize that in general practice, including our study, serum samples are collected immediately prior to transfusions. LPI levels are usually elevated for a few days after transfusion (except when transferrin is highly saturated) in contrast to the more stable NTBI which have been reported to have a longer half-life.[50,51] These free iron molecules are easily translocated intracellularly and cause oxidative stress as shown in thalassemia.[33] Oxidative stress may explain why elevated LPI levels are associated with an increased risk of dying prematurely, too early to die from causes related to classical iron overload in lungs, liver and heart as observed in young thalassemia patients after long-term transfusions.

Less is known about pathophysiology and tissue toxicity of iron overload caused by ineffective erythropoiesis in MDS. We observed that high NTBI and LPI levels also occurred in RS patients not receiving transfusions, indicating that iron toxicity (oxidative stress) may also occur in this category of MDS patients (Figure 4), similar to transfusion independent β-thalassemia intermedia, α-thalassemia (Hb-H disease), and X-linked sideroblastic anemia.[52,53]

Previously, we reported that detectable LPI occurred almost exclusively in samples with TSAT >80%.[29] Interestingly, in the current study survival of patients with TSAT >80% was not different from the survival of patients with a TSAT below this level (Figure S1). The lowest hepcidin levels have been observed in RS patients,[5] similar to our observations. Elevation of LPI in TI patients occurred exclusively in RS patients as expected in view of the low hepcidin levels leading to increased serum iron levels, through increased intestinal iron absorption and increased iron release from macrophages. Non-RS patients with SF3B1 mutations may show a similar iron pathophysiology since they appear to have a similar outcome compared to RS-MDS patients with SF3B1 mutations.[54] In addition, significant relationships were found between SF3B1 mutations and marrow erythroblasts (P=0.001) or soluble transferrin receptor factor 15 (P=0.033).[11] Our data show that elevated LPI levels - in contrast to elevated NTBI levels and TSAT - associate with decreased survival. The risk of dying prematurely in patients with detectable LPI levels occurred too early in this study to explain this risk by classical iron overload due to organ toxicity (lungs, liver and heart) after long term transfusions, but this indicates a direct effect associated with elevated LPI levels. The impact of detectable LPI was only significant in the large nonRS group, but the same tendency was observed in the smaller RS subpopulation. This effect was independent of ESA treatment indicating that the effect of LPI on outcome is not simply an effect of the

interaction of LPI with ESA - as a previously described outcome modifier.[55,56] The widely used parameter TSAT cannot serve as a parameter to predict survival. However, TSAT can be used as a pre-screening method to identify patients who are at risk to develop detectable LPI levels and associated poor prognosis. This approach may reduce the number of LPI determinations considerably.

Ferritin levels have been reported as a prognostic indicator in MDS, but ferritin as a marker of iron toxicity may be compromised by the stage of MDS, the cumulative transfusional load and its properties as an acute phase protein.[57,58,59] Moreover, the level of ferritin does not indicate whether iron is stored in parenchymal cells or in the reticulo-endothelial system (RES), of which the former is considered to be a more toxic form of iron overload. The foregoing is reflected by the weaker correlation of ferritin levels with LPI when compared with the correlation between TSAT and LPI levels. The positive correlation between CRP and hepcidin in our study suggests that inflammation also influences iron homeostasis in some MDS patients, as reported for patients with other inflammatory diseases.[4] Similar to ferritin, CRP has a significant impact on survival, potentially reflecting the impact of infections and autoimmune diseases on survival in this patient group. Finally, we could show in the limited number of patients treated with iron chelators in this study that LPI levels decreased below LLOD, even in patients with high TSAT during treatment with deferasirox. These data corroborate with the post-hoc data from a large chelation study in MDS.[43]

In conclusion, we demonstrated a disturbed iron homeostasis both in transfusion dependent MDS patients and in the subgroup of transfusion independent RS patients. This is the first clinical study that identifies LPI as a relevant marker for the potentially toxic fraction of iron species and its impact on overall survival. Increased LPI levels were restricted to patients with TSAT percentages exceeding 80%. However, TSAT exceeding 80% alone was not prognostic for survival. Therefore, we propose TSAT as a screening parameter to assess risk for detectable LPI. Additional studies are warranted to show that intervention with iron chelation improves survival, co-morbidities and quality of life in lower-risk MDS patients by lowering LPI levels.

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Authorships

All authors contributed actively to the study. TB and AS performed the statistical analyses. All authors contributed to the preliminary versions and the final version of the manuscript. All authors approved the final version of the manuscript. The authors personally, without help of a medical writer, have written this manuscript.

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Table 1 Frequency, median and quartiles of iron substudy parameters overall, by transfusion status and MDS subtype at first sample

			Transfusion				Ring Sideroblasts			
	Total		Independent		Dependent		No		Yes	
	N	Median *(p10-p90)*	N	Median *(p10-p90)*	N	Median *(p10-p90)*	N	Median *(p10-p90)*	N	Median *(p10-p90)*
Hemoglobin (g/dl)	100	10.2 (8.3 - 12.4)	85	10.3 (8.6 - 12.6)	15	9.3 (6.4 - 10.9)	66	10.4 (8.5 - 12.5)	34	9.9 (7.3 - 12.1)
White blood cells (10^9/L)	100	4.8 (2.4 - 8.7)	85	5.1 (2.5 - 8.7)	15	3.8 (2.3 - 10.7)	66	3.9 (2.3 - 7.4)	34	6.0 (3.9 - 11.4)
Platelets (10^9/L)	99	212 (94 - 475)	84	218 (97 - 475)	15	158 (87 - 463)	66	168 (89 - 341)	33	316 (169 - 501)
Serum Iron (µmol/L)	100	20 (12 - 38)	85	19 (12 - 34)	15	26.0 (4.0 - 47.0)	66	17 (10 - 26)	34	30 (16 - 45)
Ferritin (µg/L)	100	287 (48 - 982)	85	264 (49 - 692)	15	634 (20 - 1897)	66	246 (36 - 665)	34	376 (127 - 1242)
Transferrin saturation (%)	100	36 (19 - 87)	85	35 (19 - 81)	15	52 (13 - 93)	66	31 (17 - 61)	34	59 (25 - 93)
Hepcidin (nmol/L)	99	4.5 (1.1 - 21.7)	84	4.2 (1.2 - 13.8)	15	6.8 (0.5 - 53.7)	66	4.7 (1.1 - 24.2)	33	4.2 (1.2 - 10.3)
Soluble transferrin receptor (mg/L)	100	1.3 (0.7 - 2.8)	85	1.3 (0.8 - 2.8)	15	0.9 (0.6 - 3.0)	66	1.2 (0.7 - 2.7)	34	1.5 (0.8 - 3.1)
C-reactive protein (mg/L)	100	5.0 (4.0 - 11.5)	85	5.0 (4.0 - 11.0)	15	5.0 (4.0 - 139.0)	66	5.0 (4.0 - 13.0)	34	5.0 (4.0 - 10.0)
Non transferrin bound iron (µmol/L)	100	0.7 (0.1 - 3.0)	85	0.6 (0.1 - 2.9)	15	1.0 (0.1 - 3.4)	66	0.5 (0.1 - 1.8)	34	1.2 (0.3 - 3.8)
Labile plasma iron (µmol/L)	100	0.1 (0.0 - 0.2)	85	0.1 (0.0 - 0.2)	15	0.1 (0.0 - 0.3)	66	0.1 (0.0 - 0.2)	34	0.1 (0.0 - 0.3)
Growth differentiation factor 15 (ng/L)	100	2193 (952 - 5663)	85	2140 (921 - 6084)	15	2823 (1232 - 5026)	66	1844 (921 - 4828)	34	2888 (1026 - 10361

Table 2 Frequency, median and quartiles of iron parameters by transfusion status per MDS subtype at registration, 1 year and 2 years follow-up

	Registration		1 year follow-up		2 years follow-up	
	N	**Median(p10-p90)**	**N**	**Median(p10-p90)**	**N**	**Median(p10-p90)**
Transferrin saturation (%)	100	35.6 (19.0 - 87.4)	78	34.4 (16.4 - 92.9)	64	37.5 (22.2 - 94.3)
MDS non-RS: TI	56	32.8 (17.1 - 55.6)	32	28.4 (17.4 - 59.1)	26	30.1 (18.8 - 54.2)
MDS non-RS: TD	10	28.7 (8.5 - 77.9)	21	36.8 (14.0 - 89.1)	17	39.3 (20.4 - 97.7)
MDS-RS: TI	29	48.8 (24.6 - 92.5)	16	36.4 (20.8 - 86.4)	9	35.6 (23.9 - 92.6)
MDS-RS: TD	5	90.0 (53.1 - 120.4)	9	93.6 (42.1 - 110.6)	12	93.1 (71.7 - 97.6)
Hepcidin (nmol/L)	99	4.5 (1.1 - 21.7)	78	5.6 (1.2 - 19.6)	65	5.2 (1.0 - 19.6)
MDS non-RS: TI	56	4.5 (1.7 - 22.1)	32	4.3 (1.5 - 11.8)	26	4.6 (0.9 - 13.6)
MDS non-RS: TD	10	4.9 (0.5 - 75.9)	21	17.3 (0.5 - 29.2)	17	9.2 (1.3 - 28.4)
MDS-RS: TI	28	3.8 (1.0 - 8.7)	16	3.4 (0.5 - 5.8)	9	2.9 (0.8 - 12.2)
MDS-RS: TD	5	10.3 (3.8 - 15.9)	9	9.2 (3.8 - 14.4)	13	5.2 (1.0 - 14.6)
Growth differentiation factor 15 (ng/L)	100	2193 (952 - 5663)	77	2479 (1016 - 7982)	63	2576 (1045 - 7746)
MDS non-RS: TI	56	1777 (731 - 4658)	32	1653 (615 - 5684)	26	1685 (633 - 5736)
MDS non-RS: TD	10	2306 (1218 - 4927)	20	2583 (1725 - 7166)	17	2998 (1398 - 8037)
MDS-RS: TI	29	2619 (996 - 11083)	16	2694 (1223 - 10303)	8	2780 (1331 - 9554)
MDS-RS: TD	5	2893 (2113 - 5370)	9	3866 (830 - 15167)	12	5361 (1053 - 8399)

Soluble transferrin receptor (mg/L)	100	1.3 (0.7 - 2.8)	78	1.4 (0.7 - 3.0)	62	1.3 (0.8 - 2.7)
MDS non-RS: TI	56	1.2 (0.8 - 2.7)	32	1.4 (0.9 - 2.8)	26	1.2 (0.9 - 2.7)
MDS non-RS: TD	10	1.0 (0.6 - 2.8)	21	1.1 (0.4 - 3.1)	16	1.2 (0.6 - 2.2)
MDS-RS: TI	29	1.6 (0.8 - 3.3)	16	2.0 (1.1 - 2.8)	8	2.2 (1.0 - 2.8)
MDS-RS: TD	5	0.9 (0.4 - 3.1)	9	1.2 (0.6 - 3.1)	12	1.4 (0.4 - 3.6)
Non transferrin bound iron (µmol/L)	100	0.65 (0.14 - 3.03)	77	0.59 (0.15 - 3.64)	65	0.64 (0.14 - 5.42)
MDS non-RS: TI	56	0.41 (0.10 - 1.51)	31	0.42 (0.03 - 0.91)	26	0.50 (0.18 - 1.78)
MDS non-RS: TD	10	0.80 (0.05 - 2.73)	21	0.69 (0.16 - 3.64)	17	1.00 (0.12 - 7.25)
MDS-RS: TI	29	0.88 (0.26 - 3.99)	16	0.70 (0.16 - 3.52)	9	0.52 (0.05 - 5.42)
MDS-RS: TD	5	3.03 (1.90 - 3.40)	9	3.60 (0.15 - 8.64)	13	2.86 (0.46 - 7.57)
Labile plasma iron (µmol/L)	100	0.09 (0.02 - 0.22)	77	0.13 (0.03 - 0.38)	65	0.13 (0.02 - 0.38)
MDS non-RS: TI	56	0.10 (0.03 - 0.19)	31	0.10 (0.02 - 0.17)	26	0.11 (0.01 - 0.30)
MDS non-RS: TD	10	0.06 (0.01 - 0.18)	21	0.17 (0.06 - 0.38)	17	0.14 (0.02 - 1.08)
MDS-RS: TI	29	0.10 (0.02 - 0.32)	16	0.09 (0.05 - 0.24)	9	0.10 (0.03 - 0.17)
MDS-RS: TD	5	0.08 (0.00 - 0.35)	9	0.47 (0.06 - 1.26)	13	0.19 (0.08 - 1.39)

RS: ring sideroblastic; TI = Transfusion Independent, TD = Transfusion Dependent

Table 3 Cox model of overall survival by labile plasma iron, non-transferrin bound iron and transferrin saturation along with transfusion status as time varying variable for all patients (n=100)

	Unadjusted		Adjusted[1]		Adjusted[2]		Adjusted[3]	
	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
LPI (µmol/L) <LLOD[1]	1	-	1	-	1	-	1	-
≥LLOD	2.2 (0.8 – 6.2)	0.14	2.0 (0.7 – 6.0)	0.21	2.0 (0.7 – 5.8)	0.23	2.0 (0.7 – 6.2)	0.20
LPI<LLOD, TI	1	-	1	-	1	-	1	-
LPI≥LLOD, TI	4.6 (0.5 – 42.4)	0.18	3.2 (0.3 – 30.2)	0.31	3.3 (0.4 – 31.1)	0.30	3.2 (0.3 – 30.4)	0.31
LPI <LLOD, TD	4.1 (1.2 – 13.6)	0.02	2.0 (0.5 – 7.1)	0.30	2.2 (0.6 – 8.1)	0.24	2.0 (0.5 – 7.1)	0.31
LPI ≥LLOD, TD	4.7 (1.1 – 19.7)	0.03	3.0 (0.7 – 13.3)	0.15	3.0 (0.7 – 13.5)	0.14	3.0 (0.7 – 13.4)	0.15
NTBI (µmol/L) <LLOD[1]	1	-	1	-	1	-	1	-
≥LLOD	0.7 (0.3 – 1.7)	0.39	0.6 (0.2 – 1.6)	0.27	0.5 (0.2 – 1.5)	0.24	0.6 (0.2 – 1.5)	0.26
NTBI<LLOD, TI	1	-	1	-	1	-	1	-
NTBI≥LLOD, TI	0.6 (0.1 – 3.8)	0.61	0.7 (0.1 – 4.0)	0.65	0.7 (0.1 – 4.2)	0.67	0.6 (0.1 – 4.0)	0.62
NTBI<LLOD, TD	4.7 (1.1 – 19.0)	0.03	2.6 (0.6 – 11.6)	0.22	3.1 (0.7 – 14.4)	0.14	2.5 (0.6 – 11.5)	0.22
NTBI≥LLOD, TD	2.2 (0.5 – 8.6)	0.27	1.1 (0.3 – 5.0)	0.86	1.2 (0.3 – 5.4)	0.80	1.1 (0.3 – 4.9)	0.89
TSAT <80%	1	-	1	-	1	-	1	-
>80%	1.3 (0.4 – 3.6)	0.66	0.9 (0.3 – 2.9)	0.88	0.9 (0.3 – 2.8)	0.85	1.0 (0.3 – 3.1)	0.97
TSAT <80%, TI	1	-	1	-	1	-	1	-
TSAT≥80%, TI	2.5 (1.0 – 6.2)	0.04	2.3 (0.9 – 5.7)	0.08	2.5 (1.0 – 6.5)	0.05	2.3 (0.9 – 5.9)	0.10
TSAT <80%, TD	1.9 (1.2 – 3.0)	0.003	1.6 (1.0 – 2.5)	0.05	1.7 (1.1 – 2.7)	0.03	1.6 (0.99 – 2.5)	0.053
TSAT≥80%, TD	1.3 (0.9 – 2.0)	0.19	1.1 (0.7 – 1.7)	0.70	1.1 (0.7 – 1.7)	0.67	1.1 (0.7 – 1.7)	0.70

LLOD = lowest level of detection, LPI = Labile Plasma Iron, TI = Transfusion Independent, TD = Transfusion Dependent, NTBI = NonTransferin Bound Iron, TSAT = Transferin SATuration

[1] Adjusted for age at diagnosis and IPSS-R; [2] Adjusted for age at diagnosis, IPSS-R and ESA treatment status at each visit; [3] Adjusted for age, IPSS-R and RS status

Table 4 Cox model of overall survival by labile plasma iron, non-transferrin bound iron and transferrin saturation along with transfusion status as time varying variable for non-ring sideroblast patients only (n=66)

	Unadjusted		Adjusted[1]		Adjusted[2]	
	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	P
LPI (µmol/L) <LLOD[1]	1	-	1	-	1	-
Elevated	4.9 (1.4 – 16.8)	0.01	5.4 (1.5 – 19.6)	0.01	9.3 (2.0 – 43.3)	0.004
LPI<LLOD, TI	1	-	1	-	1	-
LPI≥LLOD, TI	10.2 (0.9 – 115.4)	0.06	5.3 (0.4 – 68.9)	0.20	5.9 (0.4 – 86.2)	0.19
LPI <LLOD, TD	4.6 (0.9 – 23.5)	0.07	2.0 (0.3 – 12.0)	0.47	1.4 (0.2 – 8.9)	0.70
LPI ≥LLOD, TD	11.8 (1.9 – 74.0)	0.008	10.3 (1.3 – 79.5)	0.03	17.0 (2.0 – 146.6)	0.01
NTBI (µmol/L) <LLOD[1]	1	-	1	-	1	-
Elevated	0.6 (0.2 – 1.9)	0.37	0.6 (0.2 – 2.0)	0.38	0.6 (0.2 – 2.2)	0.46
NTBI<LLOD, TI	1	-	1	-	1	-
NTBI≥LLOD, TI	0.6 (0.1 – 6.9)	0.70	1.1 (0.1 – 14.4)	0.92	1.1 (0.09 – 14.3)	0.92
NTBI<LLOD, TD	5.7 (1.1 – 30.3)	0.04	5.7 (0.8 – 42.2)	0.09	5.4 (0.7 – 43.7)	0.11
NTBI≥LLOD, TD	2.1 (0.4 – 12.3)	0.39	1.4 (0.2 – 8.2)	0.74	1.4 (0.2 – 8.2)	0.74
TSAT <80	1	-	1	-	1	-
Elevated	2.1 (0.6 – 7.8)	0.28	1.1 (0.2 – 5.4)	0.90	1.5 (0.3 – 8.6)	0.63
TSAT <80, TI	1	-	1	-	1	-
TSAT≥80, TI	3.8 (1.1 – 12.7)	0.03	3.7 (0.98 – 13.8)	0.053	3.7 (0.99 – 14.1)	0.052
TSAT <80, TD	1.9 (1.1 – 3.2)	0.02	1.7 (0.9 – 3.2)	0.13	1.6 (0.8 – 3.2)	0.21
TSAT≥80, TD	1.5 (0.9 – 2.5)	0.12	1.1 (0.6 – 1.9)	0.80	1.1 (0.6 – 1.9)	0.77

LLOD = lowest level of detection, LPI = Labile Plasma Iron, TI = Transfusion Independent, TD = Transfusion Dependent

[1] Adjusted for age at diagnosis and IPSS-R; [2] Adjusted for age at diagnosis, IPSS-R and ESA treatment status at each visit

Legends to figures

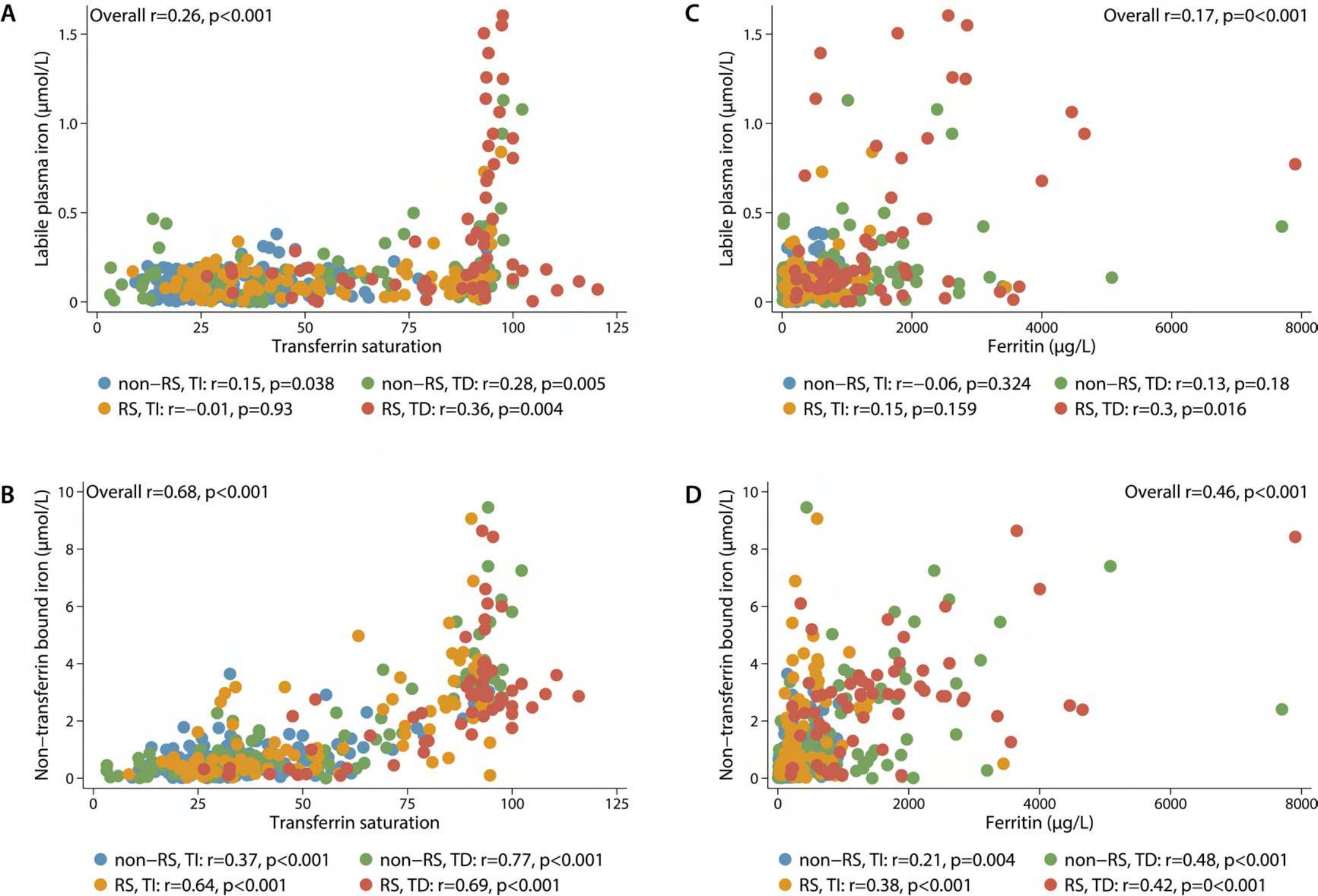
Figure 1 LPI and NTBI correlated to TSAT and Ferritin in different patient groups. A) relation between LPI and TSAT. B) relation between NTBI and TSAT C) relation between LPI and ferritin. D) relation between NTBI and ferritin. Each dot represents one sample (median: 5 samples/patient).
RS = ring sideroblastic, TI = Transfusion Independent, TD = Transfusion Dependent

Figure 2 Survival according to LPI (2A) or NTBI (2B) and transfusion status. LPI, NTBI and transfusion status were analyzed as time dependent factors, implicating that patients may switch groups over time according to the LPI/NTBI and transfusion status at each specific time point.
LLOD = Lower Limit of detection, TI = Transfusion Independent, TD = Transfusion Dependent

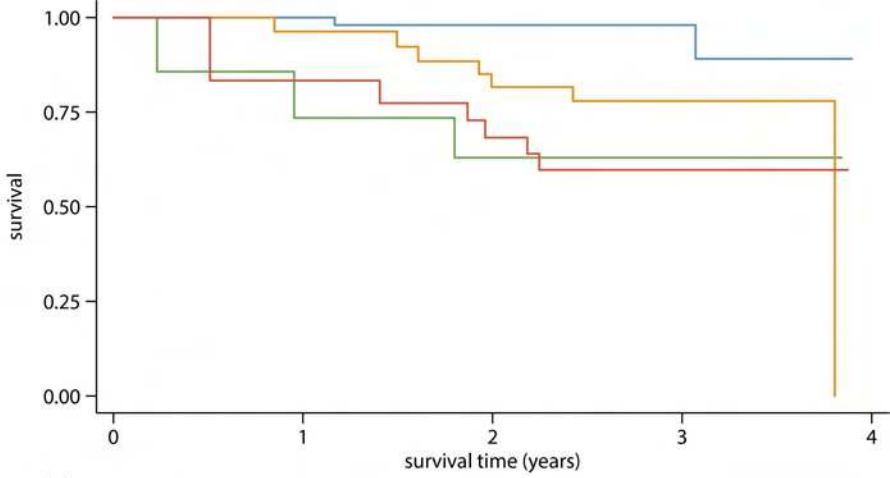
Figure 3 Flow diagram of patients treated with transfusions and erythropoietin stimulating agents (ESAs). In total, 10 patients became transfusion independent after starting ESA treatment

Figure 4 Proposed pathogenesis of iron toxicity in lower-risk MDS: the impact of ineffective erythropoiesis (4a) and of transfusions (4b)

Ineffective erythropoiesis (IE), especially in ring sideroblastic MDS, results in increased bone marrow production of GDF15 and possibly twisted gastrulation 1(TWSG1) and erythroferrone (ERFE). These factors inhibit hepcidin production by the hepatocytes. Low hepcidin levels increase iron absorption from intestinal mucosa and increase iron release from the macrophages. Finally, this may lead to toxic levels of NTBI and LPI causing damage in solid organs, immune system and the marrow. During transfusions hepcidin levels increase, despite higher GDF15 levels, leading to lower iron absorption in the gut. However, transfusions cause massive iron loading of RES-macrophages leading to elevated circulating, stored iron levels and toxic iron species - despite elevated hepcidin levels - and subsequent toxicities. Figure adapted from M. Cuijpers, et al [6]


A
Overall r=0.26, p<0.001
Labile plasma iron (µmol/L)
Transferrin saturation
non−RS, TI: r=0.15, p=0.038
non−RS, TD: r=0.28, p=0.005
RS, TI: r=−0.01, p=0.93
RS, TD: r=0.36, p=0.004
C
Overall r=0.17, p=0<0.001
Labile plasma iron (µmol/L)
Ferritin (µg/L)
non−RS, TI: r=−0.06, p=0.324
non−RS, TD: r=0.13, p=0.18
RS, TI: r=0.15, p=0.159
RS, TD: r=0.3, p=0.016
B
Overall r=0.68, p<0.001
Non−transferrin bound iron (µmol/L)
Transferrin saturation
non−RS, TI: r=0.37, p<0.001
non−RS, TD: r=0.77, p<0.001
RS, TI: r=0.64, p<0.001
RS, TD: r=0.69, p<0.001
D
Overall r=0.46, p<0.001
Non−transferrin bound iron (µmol/L)
Ferritin (µg/L)
non−RS, TI: r=0.21, p=0.004
non−RS, TD: r=0.48, p<0.001
RS, TI: r=0.38, p<0.001
RS, TD: r=0.42, p=0<0.001

A

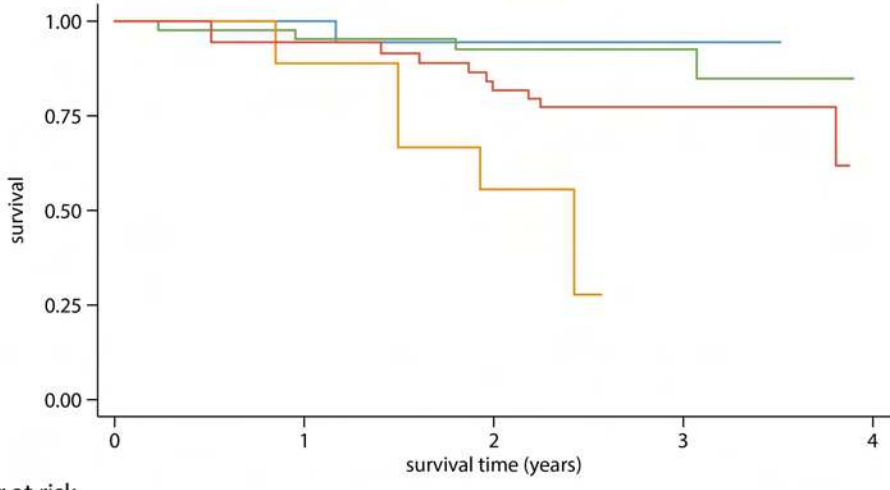


Number at risk

LPI<LLOD, TI	77	53	33	13	0
LPI>=LLOD, TI	9	6	7	5	0
LPI<LLOD, TD	12	26	24	4	0
LPI>=LLOD, TD	2	11	15	10	3

LPI<LLOD, TI
LPI>=LLOD, TI
LPI<LLOD, TD
LPI>=LLOD, TD

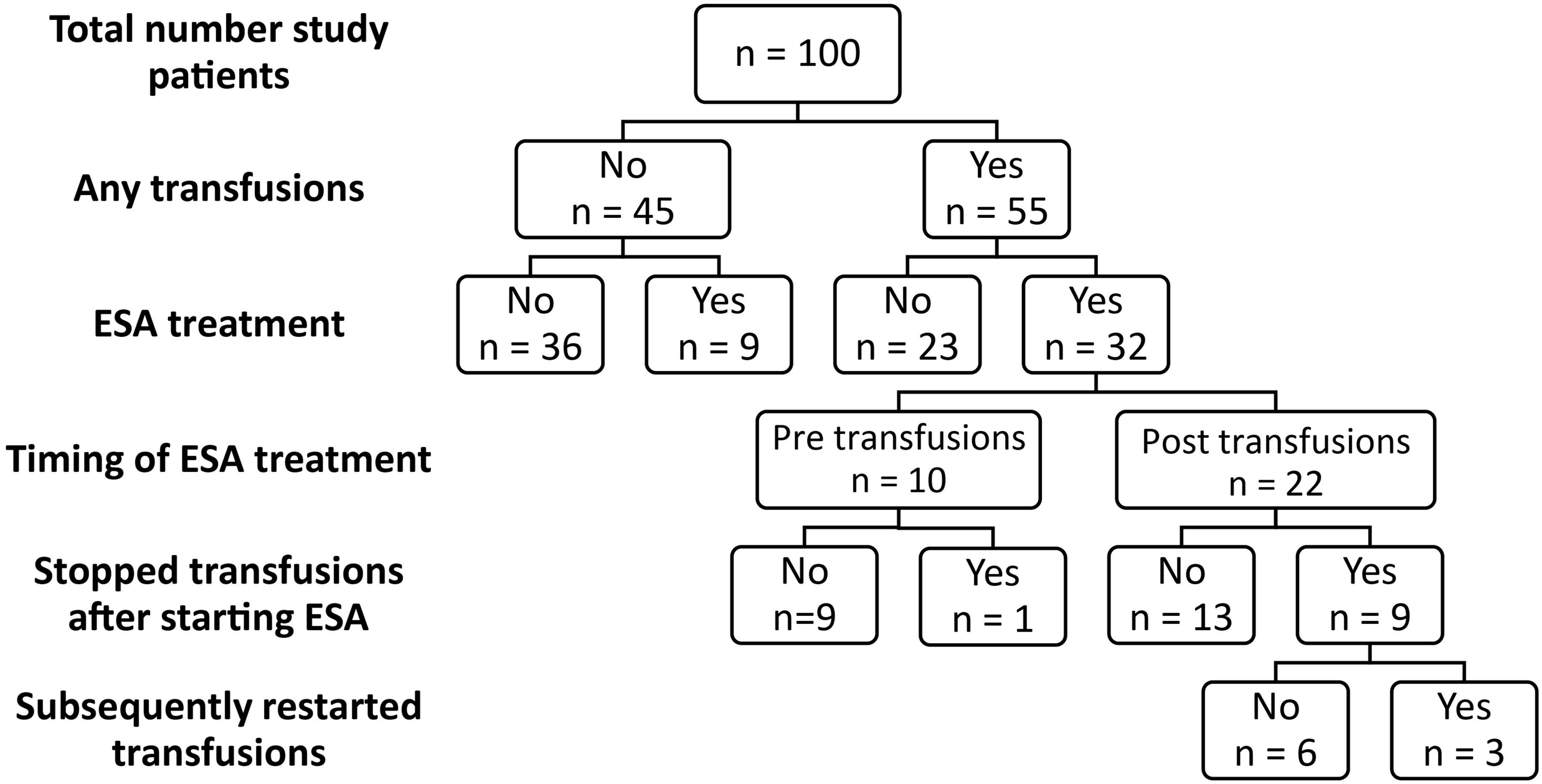
B



Number at risk

NTBI<LLOD, TI	40	19	8	1	0
NTBI>=LLOD, TI	46	40	32	17	0
NTBI<LLOD, TD	6	7	4	0	0
NTBI>=LLOD, TD	8	30	35	14	3

NTBI<LLOD, TI
NTBI>=LLOD, TI
NTBI<LLOD, TD
NTBI>=LLOD, TD


Total number study patients
Any transfusions
ESA treatment
Timing of ESA treatment
Stopped transfusions after starting ESA
Subsequently restarted transfusions
n = 100
No n = 45
Yes n = 55
No n = 36
Yes n = 9
No n = 23
Yes n = 32
Pre transfusions n = 10
Post transfusions n = 22
No n=9
Yes n = 1
No n = 13
Yes n = 9
No n = 6
Yes n = 3

A

Bone marrow

GDF15 sTFR

Liver

Hepatocytes

EPO

?

Kidney

Toxic iron

Hepcidin

NTBI

LPI

Fe

Stomach/ duodenum

Enterocyte

TSAT
>75-80%

Less anemic

Ferroportin

Erythrocytes

Transfusions

Macrophages

Hepcidin

B

Ineffective
erythropoiesis

Bone marrow

GDF15

sTFR

RS-only

Liver

Hepatocytes

EPO

Kidney

Toxic iron

Hepcidin

NTBI

LPI

Fe

Stomach/ duodenum

Enterocyte

TSAT
>75-80%

Fe-transferrin

Ferroportin

Anemia

Hepcidin

RES - macrophage

Online supplemental information

Methods

Study design and participants

Serum samples were collected just prior to transfusion in transfusion dependent patients, stored at -80C and shipped on dry ice to the central Laboratory in Nijmegen, the Netherlands. The overall analyses had to be restricted to 100 patients, since samples from three centers showed consistently elevated NTBI/LPI levels (even in samples with low transferrin saturation), due to technical issues. Clinical Information was collected at registration and 6-monthly intervals thereafter via a bespoke web-based database on: concomitant diseases, detailed red cell transfusion history, other treatment modalities, peripheral blood values, bone marrow pathology, progression of MDS or acute myeloid leukemia (AML), and loss to follow-up. Additionally analyzed parameters included the conventional iron parameters ferritin, serum iron, TSAT, the less standard iron parameters: hepcidin, GDF15, sTfR, NTBI, LPI and the inflammation parameter CRP.

Biochemical assays

Serum ferritin, iron, transferrin, and CRP were measured with routine methodologies. The serum hepcidin-25 assay was based on a combination of weak cation exchange chromatography and time-of-flight mass spectrometry using an stable hepcidin-25 isotope for quantification at nM level as previously reported[40] (www.hepcidinanalysis.com). The lower limit of detection of this method was 0.5 nM. The median reference value of serum hepcidin-25 (Dutch population) is 4.5 nM for men, 2.0 nM for premenopausal women, and 4.9 nM for postmenopausal women (www.hepcidinanalysis.com).[41]

GDF15 levels were measured with DuoSet (R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay for human GDF15 following the manufacturer's protocol. Serum concentration of sTfR was measured immunonephelometrically with the use of polystyrene particles coated with monoclonal antibody specific to human sTfR on a BN II System (Dade Behring Marburg GmbH, Marburg, Germany).

Table S1 Treatment with iron chelation and corresponding LPI and follow-up

Patient nr	Nr Visits	Max LPI levels	First visit LPI>LLOD	Max TSAT	Max Ferritin	Chelation/months	Survival (years)/AML
1	5	0.38	1	96	1954	Deferasirox/7.5	5.2+
2	4	-	-	>100	3560	Deferoxamine/6.5	1.8
3	7	0.24	1	>100	1237	Deferasirox/26	6.1+
4	7	0.92	3	100	2563	Deferasirox/10+	3.5/AML
5	6	0.81	1	>100	1840	Deferasirox/36	5.0+
6	10	1.25	8	98	4857	Deferoxamine/14	4.7/AML

1. Good response; LPI low all the time with the exception of visit 1 before start of chelation.
2. No LPI levels available
3. LPI levels low all the time with the exception of one determination
4. Only first LPI positive
5. Only visit 6 LPI positive, the starting date of chelation. No LPI levels measured after this date.
6. LPI levels became positive during treatment (last visit).

Table S2 Frequency, median and percentiles of parameters by transfusion status at registration, 1-year follow up and 2-years follow up

	At registration		1-yr follow-up(3)		2-yr follow-up(5)	
	N	**Median (p10-p90)**	**N**	**Median (p10-p90)**	**N**	**Median (p10-p90)**
Serum Iron (µmol/L)	100	20.0 (12.0 - 37.5)	78	18.5 (8.0 - 41.0)	64	21.5 (11.1 - 43.0)
0 units	88	20.0 (12.0 - 36.0)	52	17.0 (11.0 - 32.0)	40	17.0 (11.1 - 40.0)
<=10 units	11	23.0 (4.0 - 45.0)	12	22.0 (7.0 - 43.0)	15	22.0 (11.0 - 47.0)
>10 units	1	16.0 (16.0 - 16.0)	14	38.0 (5.0 - 47.0)	9	38.0 (18.0 - 47.5)
Ferritin (µg/L)	100	287 (48 - 982)	78	285 (57 - 1573)	64	341 (59 - 2387)
0 units	88	272 (49 - 819)	52	207 (56 - 662)	40	237 (51 - 777)
<=10 units	11	408 (20 - 1897)	12	593 (192 - 901)	15	590 (61 - 870)
>10 units	1	1885 (1885 - 1885)	14	1528 (829 - 2217)	9	2085 (591 - 7904)
Transferrin saturation (%)	100	35.6 (19.0 - 87.4)	78	34.4 (16.4 - 92.9)	64	37.5 (22.2 - 94.3)
0 units	88	36.1 (19.0 - 85.7)	52	31.8 (17.4 - 73.3)	40	31.6 (21.0 - 93.0)
<=10 units	11	29.8 (12.9 - 90.0)	12	37.4 (14.0 - 93.5)	15	59.5 (22.2 - 92.9)
>10 units	1	32.0 (32.0 - 32.0)	14	87.6 (11.1 - 102.3)	9	94.1 (31.6 - 102.2)
Hepcidin (nmol/L)	99	4.5 (1.1 - 21.7)	78	5.6 (1.2 - 19.6)	65	5.2 (1.0 - 19.6)
0 units	87	4.2 (1.2 - 13.8)	52	4.3 (1.2 - 10.4)	41	3.9 (0.9 - 13.6)
<=10 units	11	5.1 (0.5 - 53.7)	12	9.3 (3.8 - 19.6)	15	5.2 (1.0 - 19.6)
>10 units	1	39.1 (39.1 - 39.1)	14	15.9 (4.9 - 39.4)	9	10.5 (2.9 - 48.5)

GDF15 (ng/L)	100	2193 (952 - 5663)	77	2479 (1016 - 7982)	63	2576 (1045 - 7746)
0 units	88	2165 (921 - 6084)	52	2268 (1014 - 5909)	39	1986 (664 - 6737)
<=10 units	11	2823 (1232 - 4987)	12	2417 (1923 - 11543)	16	3235 (1053 - 7488)
>10 units	1	1856 (1856 - 1856)	13	3210 (1765 - 7071)	8	3780 (1247 - 9474)
Soluble transferrin receptor (mg/L)	100	1.3 (0.7 - 2.8)	78	1.4 (0.7 - 3.0)	62	1.3 (0.8 - 2.7)
0 units	88	1.3 (0.8 - 2.8)	52	1.5 (0.9 - 3.0)	39	1.3 (0.9 - 2.8)
<=10 units	11	1.1 (0.6 - 2.6)	12	1.3 (1.0 - 2.6)	15	1.4 (0.7 - 2.3)
>10 units	1	0.6 (0.6 - 0.6)	14	0.9 (0.4 - 1.7)	8	1.1 (0.4 - 5.4)
Non transferrin bound iron (µmol/L)	100	0.65 (0.14 - 3.03)	77	0.59 (0.15 - 3.64)	65	0.64 (0.14 - 5.42)
0 units	88	0.63 (0.14 - 2.97)	51	0.54 (0.16 - 2.62)	41	0.52 (0.18 - 2.86)
<=10 units	11	0.97 (0.05 - 3.36)	12	0.71 (0.20 - 4.01)	15	1.00 (0.09 - 2.91)
>10 units	1	0.81 (0.81 - 0.81)	14	3.21 (0.08 - 5.03)	9	3.78 (0.12 - 8.42)
Labile plasma iron (µmol/L)	100	0.09 (0.02 - 0.22)	77	0.13 (0.03 - 0.38)	65	0.13 (0.02 - 0.38)
0 units	88	0.09 (0.02 - 0.21)	51	0.10 (0.02 - 0.27)	41	0.11 (0.01 - 0.30)
<=10 units	11	0.09 (0.01 - 0.32)	12	0.16 (0.06 - 1.14)	15	0.13 (0.07 - 0.20)
>10 units	1	0.02 (0.02 - 0.02)	14	0.17 (0.06 - 0.47)	9	0.18 (0.02 - 1.39)

Table S3 Frequency, median and percentiles of parameters by ring sideroblast status at registration, 1 year follow-up and 2 years follow-up

	Registration		1-yr follow-up		2-yr follow-up	
	N	**Median (p10-p90)**	**N**	**Median (p10-p90)**	**N**	**Median (p10-p90)**
Serum Iron (µmol/L)	100	20.0 (12.0 - 37.5)	78	18.5 (8.0 - 41.0)	64	21.5 (11.1 - 43.0)
Non-RS	66	17.0 (10.0 - 26.0)	53	16.0 (7.0 - 35.0)	43	18.0 (11.0 - 38.0)
RARS/RCMD-RS	34	29.5 (16.0 - 45.0)	25	30.0 (12.0 - 44.0)	21	33.0 (13.0 - 47.0)
Ferritin (µg/L)	100	287 (48 - 982)	78	285 (57 - 1573)	64	341 (59 - 2387)
Non-RS	66	246 (36 - 665)	53	279 (56 - 1367)	43	283 (54 - 1970)
RARS/RCMD-RS	34	376 (127 - 1242)	25	287 (149 - 2217)	21	590 (215 - 2560)
Transferrin saturation (%)	100	35.6 (19.0 - 87.4)	78	34.4 (16.4 - 92.9)	64	37.5 (22.2 - 94.3)
Non-RS	66	31.2 (17.1 - 61.2)	53	30.2 (14.0 - 76.1)	43	31.7 (20.4 - 92.7)
RARS/RCMD-RS	34	58.5 (25.0 - 93.0)	25	46.2 (22.2 - 95.1)	21	85.0 (26.2 - 95.5)
Hepcidin (nmol/L)	99	4.5 (1.1 - 21.7)	78	5.6 (1.2 - 19.6)	65	5.2 (1.0 - 19.6)
Non-RS	66	4.7 (1.1 - 24.2)	53	7.4 (1.2 - 23.7)	43	6.3 (1.0 - 22.4)
RARS/RCMD-RS	33	4.2 (1.2 - 10.3)	25	4.9 (1.4 - 9.8)	22	3.4 (1.0 - 14.2)
Growth differentiation factor 15 (ng/L)	100	2193 (952 - 5663)	77	2479 (1016 - 7982)	63	2576 (1045 - 7746)
Non-RS	66	1844 (921 - 4828)	52	2195 (1016 - 5909)	43	2113 (982 - 5995)
RARS/RCMD-RS	34	2888 (1026 - 10361)	25	3148 (1223 - 10303)	20	3661 (1192 - 8977)

Soluble transferrin receptor (mg/L)	100	1.3 (0.7 - 2.8)	78	1.4 (0.7 - 3.0)	62	1.3 (0.8 - 2.7)
Non-RS	66	1.2 (0.7 - 2.7)	53	1.3 (0.5 - 3.0)	42	1.2 (0.8 - 2.2)
RARS/RCMD-RS	34	1.5 (0.8 - 3.1)	25	1.8 (0.9 - 2.8)	20	1.5 (0.5 - 3.2)
Non transferrin bound iron (µmol/L)	100	0.65 (0.14 - 3.03)	77	0.59 (0.15 - 3.64)	65	0.64 (0.14 - 5.42)
Non-RS	66	0.46 (0.08 - 1.75)	52	0.51 (0.08 - 2.78)	43	0.52 (0.14 - 3.31)
RARS/RCMD-RS	34	1.22 (0.30 - 3.85)	25	1.61 (0.16 - 5.20)	22	2.02 (0.16 - 6.00)
Labile plasma iron (mg/L)	100	0.09 (0.02 - 0.22)	77	0.13 (0.03 - 0.38)	65	0.13 (0.02 - 0.38)
Non-RS	66	0.09 (0.02 - 0.19)	52	0.12 (0.02 - 0.31)	43	0.12 (0.01 - 0.30)
RARS/RCMD-RS	34	0.09 (0.02 - 0.32)	25	0.16 (0.06 - 0.87)	22	0.14 (0.04 - 0.94)

Table S4 Cause of death by LPI status

	Total n (%)	LPI status N (%)	
		LPI<LLOD	LPI≥LLOD
Total	19	9	10
AML	3 (15.8)	2 (22.2)	1 (10.0)
Cardiovascular	2 (10.5)	1 (11.1)	1 (10.0)
Hemorrhage	2 (10.5)	-	2 (20.0)
Infection	5 (26.3)	2 (22.2)	3 (30.0)
Myelodysplastic	2 (10.5)	2 (22.2)	-
Not Known	2 (10.5)	-	2 (20.0)
Other	2 (10.5)	1 (11.1)	1 (10.0)
Pulmonary	1 (5.3)	1 (11.1)	-

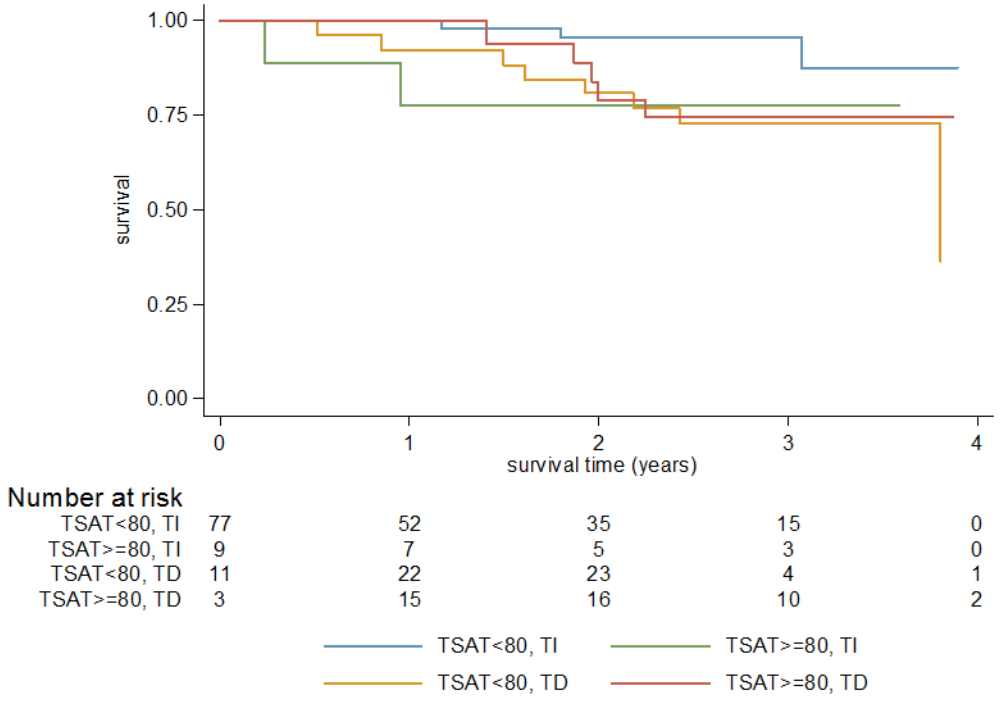


Figure S1 Survival according to transferrin saturation (TSAT) and transfusion status.