The effect of iron loading and iron chelation on the innate immune response and subclinical organ injury during human endotoxemia: a randomized trial

Lucas T. van Eijk, Suzanna Heemskerk, Rob W. van der Pluijm, Susanne M. van Wijk, Wilbert H.M. Peters, Johannes G. van der Hoeven, Matthijs Kox, Dorine W. Swinkels, and Peter Pickkers

Introduction

Systemic inflammation is accompanied by profound changes in iron distribution, mainly under the influence of hepcidin, leading to sequestration of iron in macrophages of the reticuloendothelial system, and ultimately anemia of inflammation. This redistribution of iron may represent an immune modulatory effect of both iron and iron chelation: Iron sucrose has been shown to potentiate the inflammatory response in murine models of inflammation and sepsis. The immune modulatory effects of iron supplements and chelators are mainly attributed to their ability to potentiate or reduce the formation of reactive oxygen species (ROS). A subfraction of non-transferrin bound catalytically active iron, labile plasma iron (LPI), is thought to be responsible, as this free iron is able to easily donate or accept electrons, thereby fueling redox reactions. Oxidative stress is associated with propagation of the immune response, and contributes to the organ damage that occurs during systemic inflammation. In agreement with this, anti-oxidants exert anti-inflammatory effects. As such, iron chelation has been suggested to be a valuable adjuvant therapy during infection for two distinct reasons: 1) inhibition of bacterial growth; and 2) protection of organs against inflammation-induced oxidative stress.

Effects of iron status on the immune response have up till now mainly been investigated in vitro and in animal models, often using supra-therapeutic dosages of iron donors or iron chelators. Data on the effect of iron loading and iron chelation during systemic inflammation in humans are lacking. The objectives of the present study were to investigate the acute effect of therapeutic dosages of iron loading and iron chelation therapy on iron homeostasis, oxidative stress, the innate immune response and subclinical organ injury during experimental human endotoxemia. The administration of iron sucrose induced a profound increase in plasma malondialdehyde 1 h after administration (433±37% of baseline; P<0.0001), but did not potentiate the endotoxemia-induced increase in malondialdehyde, as was seen 3 h after endotoxin administration in the placebo group (P=0.34) and the iron chelation group (P=0.008). Endotoxemia resulted in an initial increase in serum iron levels and transferrin saturation that was accompanied by an increase in labile plasma iron, especially when transferrin saturation reached levels above 90%. Thereafter, serum iron decreased to 51.6±9.7% of baseline at T=8 h in the placebo group versus 84±15% and 60.4±8.9% of baseline at 24 h in the groups treated with iron sucrose and deferasirox, respectively. No significant differences in the endotoxemia-induced cytokine response (TNF-α, IL-6, IL-10 and IL-1RA), subclinical vascular injury and kidney injury were observed between groups. However, vascular reactivity to noradrenalin was impaired in the 6 subjects in whom labile plasma iron was elevated during endotoxemia as opposed to those in whom no labile plasma iron was detected (P=0.029). In conclusion, a single dose of iron sucrose does not affect the innate immune response in a model of experimental human endotoxemia, but may impair vascular reactivity when labile plasma iron is formed. (Clinicaltrials.gov identifier:01349699)
immune response, and subclinical organ injury during systemic inflammation induced by experimental endotoxemia in humans in vivo.

Methods

Design

This double-blind, randomized, placebo-controlled trial (Clinicaltrials.gov identifier:01349699) was approved by the local ethics committee, and carried out according to standards of Good Clinical Practice and the Declaration of Helsinki. After written informed consent, 30 healthy non-smoking male subjects were randomly treated with either iron loading (iron sucrose), or iron chelation (deferasirox) or placebo, prior to the intravenous administration of 2 ng/kg purified E.Coli endotoxin, as described in detail in the Online Supplementary Methods.

Iron loading and iron chelation therapy

At T = -2 h (i.e. 2 h before endotoxin administration), iron chelation therapy (deferasirox (Exjade®), Novartis, The Netherlands) or placebo (starch powder) was administered orally. A commonly applied dose of 30 mg/kg of deferasirox was dissolved in water and was stirred until the moment of intake. Subjects drank the suspension within 1 min. The time point of deferasirox administration was chosen so that maximal plasma levels were achieved between 0 and 2 h after endotoxin administration, just before and during the expected peak of the pro-inflammatory cytokine response. At T = 1 h, a generally applied therapeutic dose of 1.25 mg/kg iron sucrose (Venofer®, Vifor Nederland BV, The Netherlands), or placebo (NaCl 0.9%), was intravenously administered through blinded syringes and lines. Because the two therapies had different administration routes, a double dummy was used to ensure adequate blinding.

Iron homeostasis analysis

Serum iron, ferritin, transferrin and soluble transferrin receptor (sTfR), hepcidin, GDF-15 and LPI were determined as described in the Online Supplementary Design and Methods.

Oxidative stress and cytokine measurements

Oxidative stress (malondialdehyde) and plasma levels of pro-inflammatory cytokines TNF-α and IL-6, the anti-inflammatory cytokines IL-10 and IL-1ra, and markers of endothelial activation Vascular Cell Adhesion Molecule (VCAM) and Intercellular Adhesion Molecule (ICAM) were determined as described in the Online Supplementary Design and Methods.

Subclinical organ injury

Subclinical endothelial injury was assessed by measuring the changes in response of the forearm vasculature to the infusion of vasoactive medication into the brachial artery, using venous occlusion plethysmography. For the determination of renal tubular damage, urinary Glutathione S-Transferase Alpha-1-1 (GSTA1-1) and Glutathione S-Transferase-Pi-1-1 (GSTP1-1) were determined. A detailed description is provided in the Online Supplementary Design and Methods.

Statistical analysis

Data are expressed as mean±SEM, or median (25th-75th percentile), depending on their distribution. P<0.05 was considered statistically significant. For all outcome variables, the intervention groups were compared to the placebo group in two separate analyses. Group means or medians were tested using Student’s t-test for non-parametric data. Changes over time for each treatment group were analyzed by one-way ANOVA-repeated measures and post hoc testing was performed with Dunnett’s Multiple Comparison Test. Differences over time between groups were analyzed using two-way ANOVA repeated measures (interaction term: time*treatment). Peak levels of cytokines were correlated to oxidative stress levels using Spearman’s correlation coefficients. The analysis of forearm blood flow (FBF) and a power calculation are described in the Online Supplementary Design and Methods.

Results

No significant differences between the intervention groups with respect to demographic data and anti-oxidative status were found (Table 1).

Symptoms and hemodynamic sequelae

Iron sucrose and deferasirox treatment were well tolerated. Experimental endotoxemia resulted in flu-like symptoms (shivering, headache, backache, muscle ache and nausea) that gradually declined to base-line values at the end of the experiment (T=8 h). There were no differences between the treatment groups, measured by a cumulative subjective symptom scoring list that has been described previously (data not shown). Body temperature in the three treatment groups increased by 2.2±0.1°C; no differences were observed between groups. Also, the hemodynamic response to endotoxemia (increase in heart rate of 25.6±1.7 bpm and decrease in mean arterial pressure of 11.5±1.5 mmHg) was not influenced by iron loading or iron chelation treatment (Table 2).

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<th>Table 2. Hemodynamic changes during experimental endotoxemia.</th>
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Online Supplementary Methods

Clinicaltrials.gov identifier:01349699

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Iron parameters

Administration of iron sucrose resulted in a big increase in serum iron to 218±17% of baseline (Figure 1A). Serum iron levels initially increased during LPS-induced systemic inflammation in all three groups, reaching peak levels at approximately 3 h after LPS administration, followed by a gradual decrease, reaching its lowest values at T = 8 h in the placebo group (51.6±9.7% of baseline) and 24 h in the iron sucrose- and deferasirox-treated groups (84±15% and 60.4±8.9% of baseline, respectively). Transferrin satura-

Figure 1. Iron parameters. IS: iron sucrose; DFX: deferasirox; Plac: placebo; NS: not significant. Arrows indicate the time points of deferasirox and iron sucrose administration. Changes over time for each treatment group were analyzed by one-way ANOVA-repeated measures followed by Dunnett’s post hoc test: †Significant change from baseline in iron sucrose group; *Significant change from baseline in deferasirox group. #Significant change from baseline in placebo group. Differences between groups were analyzed using two-way ANOVA-repeated measures (interaction term: time*treatment). The intervention groups were compared to the placebo group in separate analyses. Significant interactions between treatments are depicted within each graph. Data are represented as mean±SEM.
tion showed the same profile (data not shown), peaking at T = 3 h. Iron chelation did not reduce serum iron levels, which can be explained by the fact that in the serum iron analysis, the total of both chelated and unchelated iron is determined.16 When serum iron was elevated, transferrin saturations of over 90% were reached in 5 out of 10 iron sucrose-treated subjects, whereas this was observed in none of the deferasirox-treated and in 1 of the placebo-treated subjects. Ferritin was increased at 3 h after LPS administration, after which it reached a plateau in the placebo- and deferasirox-treated group, while further increasing in the iron sucrose-treated group (Figure 1B). TIBC was increased at T = 24 h (Figure 1C), as was sTfR (Figure 1D). GDF-15 peaked at 6 h post LPS (Figure 1E). These last three parameters were not influenced by iron loading or chelation treatment. Hepcidin was markedly induced by LPS-induced inflammation, reaching its peak levels at 6 h following LPS administration (Figure 1F). Again, no differences between the treatment groups were observed. Treatment with iron sucrose did not result in measurable LPI levels at T = 0 (Figure 2A). However, at T = 2 and 3 h, simultaneously to the endotoxin-induced rise in serum iron and transferrin saturation, LPI was increased in 5 out of 10 iron sucrose-treated subjects, compared to 1 placebo-treated subject (Figure 2A and B). Importantly, LPI formation most notably occurred when transferrin saturation was over 90% (Figure 2C).

Oxidative stress

The administration of iron sucrose induced a profound increase in plasma lipid peroxidation products (MDA) at T = 0 h (433±37% of baseline; P<0.0001) (Figure 3). In this group, MDA remained elevated in comparison with the other two groups until 3 h after LPS administration. Three hours after endotoxin administration, a similar inflammation-induced increase in MDA was observed in the placebo group (P=0.54) and the iron chelation group (P=0.008). Iron administration did not potentiate the endotoxemia-associated increase in oxidative stress at T = 3 h. MDA increases were not related to LPI formation in any way.

Plasma cytokines

Endotoxemia resulted in increased plasma levels of pro-inflammatory cytokines (Figure 4A and B). The TNF-α response was similar in the different groups. The increase in IL-6 tended to be potentiated in the iron sucrose-treated group compared with the other treatment groups (P=0.052). No correlations between iron parameters and pro-inflammatory cytokines were found. In particular, no correlation was found between LPI or MDA and pro-inflammatory cytokine production.

The anti-inflammatory response, illustrated by plasma levels of IL-10 and IL-1ra, was similar in all three groups (Figure 4C and D). For the three groups combined, plasma levels of ICAM and VCAM gradually increased after the endotoxin challenge from 84±5 and 164±7 pg/mL to peak levels of 161±12 (P<0.0001) and 259±10 ng/mL (P<0.0001), respectively at T = 24 h (data not shown). Again, no differences were observed between the treatment groups and no correlations were found with iron parameters.

Subclinical organ injury

Vascular reactivity as measured by changes in forearm blood flow in response to intrabrachial infusion of vasoactive drugs was reduced after endotoxemia (Figure 5). Elevated MDA levels caused by infusion of iron sucrose prior to endotoxemia were not associated with the inflammation-induced vascular hyporesponsiveness, and iron chelation had no ameliorating effects. However, subjects in whom elevated LPI levels were found 2 or 3 h after endotoxin administration (5 subjects in the iron sucrose group and 1 in the placebo group) did show a more pronounced inflammation-induced reduction of vascular reactivity to noradrenaline compared with those who showed no LPI increase (48.5%, range 19.7-80.8%) of base-line.

Figure 2. Labile plasma iron and transferrin saturation. (A) Mean labile plasma iron levels in the 3 treatment groups. Changes over time for each treatment group were analyzed by one-way ANOVA-repeated measures followed by Dunnett’s post hoc test: ‘Significant change from baseline in iron sucrose group. No significant changes were detected in the other treatment groups. IS: iron sucrose. DFX: deferasirox. Data are expressed as means±SEM. (B) Transferrin saturation of the subjects in the iron sucrose-treated group. Open dots: samples in which no labile plasma iron was detected, black dots: samples in which labile plasma iron was detected. Note that in general, labile iron was only positive in subjects in whom transferrin saturation increased above 90%. (C) Labile plasma iron in relation to transferrin saturation in iron sucrose-treated subjects at T = 2 h and T = 3 h.
response in LPI positive subjects versus 84.3% (range 61.7-121.1%) in LPI negative subjects (P=0.029). No correlation between MDA and vascular dysfunction was observed.

The excretion of markers of subclinical kidney injury GSTA1-1 (proximal tubule injury) and GSTP1-1 (distal tubule injury) was increased after endotoxin administration in all three groups. Treatment with either iron sucrose or deferasirox had no effect on the urinary excretion of GSTA1-1 and GSTP1-1 in comparison to the placebo group. Furthermore, excretion of these markers was not related to LPI or oxidative stress. For the three groups combined, median GSTA1-1 excretion increased from 254 ng/mmol (range 87-452 ng/mmol) creatinine at baseline to 1123 ng/mmol (611-2194 ng/mmol) creatinine at T = 12 h after LFS treatment, and returned to base-line levels at T = 24 h (189 ng/mmol, range 52-309 ng/mmol). GSTP1-1 increased from 85 ng/mmol (range 32-209 ng/mmol) creatinine at baseline to 1120 ng/mmol (414-1442 ng/mmol) creatinine at T = 12 h and also normalized at T = 24 (154 ng/mmol, range 84-338 ng/mmol).

Discussion

This study shows that administration of a single dose of iron sucrose results in an increase in oxidative stress, while it does not significantly modulate the acute innate immune response or inflammation-induced end-organ dysfunction in humans in vivo. In line with these findings, iron chelation with a single dose of deferasirox did not alter acute effects of experimental endotoxiaemia in healthy volunteers with regard to inflammation-induced oxidative stress, the innate immune response, and subclinical end-organ dysfunction. However, when transferrin becomes saturated following iron sucrose infusion, formation of redox active LPI during endotoxiaemia is associated with a more pronounced inflammation-induced reduction in vascular reactivity to noradrenalin, indicating that in a subgroup of subjects infused with iron sucrose, iron does exert deleterious vascular effects during inflammation.

In accordance with previous studies, the administration of iron sucrose resulted in an increase in oxidative stress, as reflected by the markedly elevated amount of lipid peroxidation product MDA. In the present study, we found that, while systemic inflammation elicited by endotoxiaemia increased oxidative stress, iron administration did not potentiate the inflammation-induced oxidative stress response or release of pro-inflammatory cytokines, despite the increment in LPI that was observed in a subgroup of iron sucrose-treated individuals. These findings during systemic inflammation in humans in vivo are in contrast with previous findings in animals. Iron sucrose administered together with heat-killed E.Coli in rats was shown to significantly enhance cytokine production and was associated with increased mortality. It was speculated that the iron sucrose-mediated increase in oxidative stress was responsible for amplification of the inflammatory response. Importantly, supra-pharmacological doses of iron sucrose were used in the animals, being approximately 40 times higher than the dose generally administered in humans and used in the present study. Furthermore, the dose used in rats elicited a significant increase in TNF-α in the absence of E. Coli. Therefore, the difference in iron dosages may explain the difference in results. Although we found a trend towards increased endotoxiaemia-induced IL-6 peak levels in iron-treated subjects, no effect on the production of TNF-α was observed, indicating that pharmacological doses of iron do not have any relevant influence on the innate immune response in humans in vivo. Our findings correspond to clinical observations of patients suffering from inflammatory bowel disease who are dependent on intravenous iron supplementation to restore inflammation-associated anemia. The majority of these patients experience no effects or even a beneficial effect on inflammation-related disease activity. However, since experimental endotoxiaemia is a model of acute and short-lived systemic inflammation for which we treated the subjects with a single bolus of iron sucrose or deferasirox, we do acknowledge that the absence of an acute immune-modulating effect of iron in the present study does not necessarily exclude a possible effect of iron in chronically iron loaded patients, such as those suffering from sickle cell disease, thalassemia or chronic kidney disease. Furthermore, our study does not rule out a possible immune-modulating role of hepcidin during these chronic diseases.

We determined vascular reactivity and excretion of markers of tubular damage in the urine as parameters of inflammation-induced subclinical end-organ dysfunction. In accordance with previous studies by our group and by others, experimental endotoxiaemia resulted in vascular hyporeactivity. Overall, iron sucrose treatment did not exert deleterious vascular effects during inflammation.

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directly exacerbate inflammation-induced endothelial dysfunction. In a previous study in human volunteers in the absence of systemic inflammation, it was found that endothelial dysfunction of the brachial artery occurred following iron sucrose administration, but had been restored at 4 h after administration.\(^8\) Therefore, in our study, the endothelial damage that may have occurred due to the oxidative stress caused by iron sucrose itself could have subsided by the time inflammation-induced endothelial dysfunction occurred.\(^23\) However, iron sucrose treatment contributed to the saturation of transferrin that occurred at T = 2-3 h after endotoxin administration, thereby contributing to LPI formation. LPI formation was shown to be associated with increased vascular dysfunction caused by inflammation. We confirmed our previous findings that endotoxemia induces subclinical renal damage of both the proximal tubule and the distal tubule.\(^24\) Iron loading before LPS administration did not modify the urinary excretion of the tubular injury markers, indicating that iron does not exert a direct toxic effect on tubular cells, and does not exacerbate inflammation-induced tubular damage.

Interestingly, LPI levels did not correlate with MDA. Possibly this is the result of a large difference in plasma half life whereby LPI only exists for a short time, whereas MDA products are eliminated more gradually. Therefore, as LPI was not measured directly following iron sucrose

Figure 4. Cytokine production during endotoxemia. Data are represented as median with interquartile range. Changes over time for each treatment group were analyzed by one-way ANOVA-repeated measures followed by Dunnett’s post hoc test: Changes over time were significant in all subgroups for all parameters. †Significant change from baseline in iron sucrose group; *Significant change from baseline in deferasirox group; #Significant change from baseline in placebo group. Differences between groups were analyzed using two-way ANOVA-repeated measures. The intervention groups were compared to the placebo group in separate analyses. No significant interactions between treatments were detected. As there was a trend towards a higher IL-6 in the iron sucrose group, peak levels for this parameter were additionally compared to the placebo group using the Mann Whitney U test; P=0.052.
We cannot exclude the possibility that there is a correlation between LPI and MDA levels directly following iron sucrose infusion.

Beneficial effects of iron chelator deferrioxamin on both inflammatory parameters and outcome in rodent models of sepsis have been reported. Moreover, iron chelation therapy has been shown to work synergistically with antibiotics in the treatment of murine sepsis. Several explanations for the differences with our findings in humans can be put forward. First of all, the majority of studies in which a beneficial effect of iron chelation therapy was found were performed in rodents or in vitro. Besides the fact that extrapolation to humans may be problematic, there are important differences in routes of administration and dosages administered. In animals, the iron chelator deferrioxamin is usually given as bolus injection, whereas in humans it needs to be administrated as a slow infusion over 8-24 h to avoid possible toxicity associated with high plasma levels. For this proof of principle study, we aimed to reach a high concentration of iron chelator between 0 and 2 h after endotoxin administration, the time window in which the inflammatory cascade is being activated and pro-inflammatory cytokines reach their peak levels. Therefore, we chose to administer deferasirox, a relatively new iron chelator that can be administered orally at once, has a good bioavailability of approximately 70%, with peak plasma levels between 2 and 4 h after ingestion, and has been shown to protect against oxidative stress-induced damage in patients. We chose a relatively high dosage of 30 mg/kg. Although deferasirox has roughly the same characteristics as deferrioxamin with regard to the chelation of iron, there might be a difference in anti-oxidative capacity between the two compounds. While deferasirox exerts anti-oxidative effects in animals models and patients, deferrioxamin exerts anti-oxidative effects even after pre-saturation of the deferrioxamin-molecule with iron, suggesting an anti-oxidative effect of the molecule itself, while this has not yet been established for deferasirox. The fact that deferasirox may not exert anti-oxidative effects by itself may in part explain why no effect on the inflammation-induced oxidative stress was observed. The fact that inflammation-induced oxidative stress was not attenuated by deferasirox may also explain why no effect on inflammation-induced endothelial dysfunction was found. Furthermore, LPI formation could only be shown in 1 subject of the placebo group, so apparently there was not much catalytically active iron present in the plasma for

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**Figure 5.** Vascular dysfunction. Vascular dysfunction measured by Forearm Blood Flow (FBF) measurements before endotoxin administration (open circles) and 4-6 h after endotoxin administration (closed circles). In panels A, B and C dose response curves to Achetylcholine (Ach), Nitroglycerin (NTG), Norepinephrine (NE) are presented, respectively. Increases and decreases in FBF were expressed as percentage change from baseline. The results of all 30 subjects are combined in these three graphs, showing that the vascular responsiveness to Ach, NTG and NE were significantly attenuated after endotoxin administration. Data are presented as mean±SEM. In panels D, E and F, the relative response after LPS administration is expressed as a percentage of the response before endotoxin administration by comparing the area under the curve (AUC) before and after endotoxin to enable the evaluation of differences between the treatment groups. Data in these graphs are expressed as median and interquartile range. No differences in relative decrease in vascular responsiveness were present between the 3 groups.
deferaxirox to chelate during experimental endotoxemia. Finally, oxidative stress is only one of the factors that may lead to vascular hyporeactivity during endotoxemia. Other, local factors that are not influenced by anti-oxidant therapy may also play a role, such as the upregulation of inducible nitric oxide synthase and the activation of potassium channels on vascular smooth muscle cells.

It is generally known that inflammation results in a reduction of serum iron. Here we report for the first time that serum iron initially increases in the acute phase of systemic inflammation, which is accompanied by LPI formation once serum transferrin becomes saturated. Although we did not investigate the mechanism underlying the increase in serum iron in the present study, we speculate that the acute release of pro-inflammatory cytokines depresses the erythropoiesis in the bone marrow, while an iron flux from storage cells to the plasma is still present, as hepcidin is not yet elevated. Previous studies indicated that pro-inflammatory cytokines, mainly TNF-α, are able to suppress hematopoiesis in vitro and in vivo. As the utilization of iron is attenuated, serum iron increases and only decreases after the induction of hepcidin and the decrease of TNF-α. In accordance with this, the rate by which serum iron increased in the present study roughly resembles the rate by which iron is normally utilized by the bone marrow (5 μmol/L/h = 22 mg iron per day for an average person). GDF-15 has been reported both as a marker of innate immunity and as a humoral factor connecting increased or ineffective erythropoiesis in the bone marrow to a reduction of hepatocyte hepcidin production. We observed a peak of GDF-15 at 6 h post LPS, that is most likely attributed to the endotoxin-related inflammation. Interestingly, and in agreement with recent findings in mice suggesting that GDF-15 is not essential for systemic iron homeostasis, this increase in GDF-15 did not prevent the increase of hepcidin after LPS administration. Surprisingly, iron loading had no effect on serum hepcidin levels, despite a clear effect of serum iron and transferrin saturation. This finding suggests that during the acute inflammatory phase, transferrin-bound iron-induced hepcidin production does not add to cytokine-dependent hepcidin induction.

In conclusion, a single dose of iron sucrose or deferaxirox does not exert relevant effects on the innate immune response in a model of experimental human endotoxemia. Acute systemic inflammation is associated with an initial rise in serum iron and transferrin saturation that is associated with LPI formation once transferrin saturations over 90% are reached. LPI formation during experimental human endotoxemia is associated with more pronounced vascular dysfunction but not with other measures of subclinical end organ injury.

Acknowledgments
The authors would like to thank Faustin Albuoi for the measurement of MDA, Trees Jansen for cytokine measurements, Henkie Roelofs for the GSTA1-1 and GSTP1-1 measurements, Erwin Wiesengrund and Coby Laarakkers, for hepcidin measurements, Rian Roelofs and Aferrix Ltd. (Tel-Aviv, Israel) for labile plasma iron measurements and Siem Klaver for the coordination of the measurement of the other iron parameters.

Authorship and Disclosures
Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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