Iron, Inflammation, and Early Death in Adults with Sickle Cell Disease

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ABSTRACT

Rationale: Patients with sickle cell disease (SCD) have markers of chronic inflammation but the mechanism of inflammation and its relevance to patient survival are unknown.

Objective: To assess the relationship between iron, inflammation and early death in SCD.

Methods and Results: Using peripheral blood mononuclear cell transcriptome profile hierarchical clustering, we classified 24 patients and 10 controls in clusters with significantly different expression of genes known to be regulated by iron. Subsequent gene set enrichment analysis showed that many genes associated with the high iron cluster were involved in the toll-like receptor system (TLR4, TLR7 and TLR8) and inflammasome complex pathway (NLRP3, NLRC4, and CASP1). Quantitative PCR confirmed this classification and showed that ferritin light chain, TLR4 and interleukin-6 expression were more than 100-fold higher in patients than in controls (P<0.001). Further linking intracellular iron and inflammation, 14 SCD patients with a ferroportin Q248H variant that causes intracellular iron accumulation had significantly higher levels of interleukin-6 and C-reactive protein compared to 14 matched SCD patients with the wild type allele (P<0.05). Finally, in a cohort of 412 patients followed for a median period of 47 months, (IQR 24-82), C-reactive protein was strongly and independently associated with early death (HR 3.0, 95%CI 1.7-5.2, P<0.001).

Conclusions: Gene expression markers of high intracellular iron in patients with SCD are associated with markers of inflammation and mortality. The results support a model in which intracellular iron promotes inflammatory pathways such as the TLR system and the inflammasome, identifying important new pathways for additional investigation.

Keywords: Iron, gene expression, inflammation, mortality, sickle cell disease

Nonstandard Abbreviations and Acronyms:

- IL-6   Interleukin-6
- SCD   Sickle cell disease
- HbSS  Homozygous sickle cell anemia
- Q-PCR Quantitative real-time PCR
- PBMCs peripheral blood mononuclear cells
- TLR   Toll-like receptor
INTRODUCTION

Disturbances in iron metabolism and storage are increasingly recognized as important factors in the pathogenesis of disease. The plasma level of the iron storage protein ferritin is used as an estimate of body iron storage. However, elevated plasma ferritin is associated with inflammation as well. Cellular ferritin synthesis is tightly regulated by intracellular iron via the IRE/IRP system.1 Several studies have suggested a causal relation between intracellular “chelatable” iron levels in monocytes/macrophages and inflammation.2,7

Sickle cell disease (SCD) is one of the most prevalent mono-genetic diseases known and is responsible for high morbidity and mortality with an estimated median survival of 45-48 years in Western society.8 Clinical hallmarks of SCD are recurrent painful crises and chronic hemolytic anemia resulting in a systemic vasculopathy characterized by chronic organ damage including a high incidence of stroke, osteonecrosis, nephropathy, pulmonary disease, retinopathy and ultimately death.6,9-12

The pathophysiology of SCD is not undisputed,13,14 but there is general consensus that inflammation plays an important role.15-17 SCD patients in general have higher levels of C-reactive protein (CRP) or other markers of inflammation than healthy controls and these markers increase further during painful crisis.18,19 Although no direct association with CRP and mortality has been described, the long-standing recognition that an elevated leucocyte count predicts early death in SCD suggests a possible relationship of inflammation to mortality.8

Three mechanisms cause a high exposure of SCD peripheral blood mononuclear cells (PBMCs) to iron. First, frequent blood transfusions in many SCD patients lead to accumulation of high levels of body iron. Second, red cell survival, which in healthy controls continues for about 120 days, is decreased to approximately 25 days in SCD.16,20 Considering that during normal red cell turn-over the monocyte-macrophage system absorbs and releases 25 mg of iron from senescent erythrocytes each day, this rate should be dramatically increased in SCD.21,22 Third, in SCD a significant fraction of the hemolysis takes place intravascularly and some of the resulting hemoglobin and heme is taken up by circulating monocytes.

In the present study, we analyzed the SCD transcriptome to identify genes that are highly co-regulated in patients categorized by their iron-regulated gene expression profile. Next, we showed that SCD patients with the Q248H ferroportin gene mutation, which is reported to increase monocyte macrophage intracellular iron levels, have more inflammation than SCD patients with wild type ferroportin. Finally, to underline the importance of these findings we showed the association of inflammation with mortality in a large cohort of SCD patients.

METHODS

Subjects.
The National Heart, Lung, and Blood Institute’s Institutional Review Board approved the protocols. All subjects provided written informed consent. All patients presented in this paper are selected from a long term registry study evaluating the prevalence of secondary pulmonary hypertension and mortality in SCD (ClinicalTrials.gov identifier NCT00011648). We will refer to the group of patients included in this study as the “NIH SCD cohort”. Inclusion criteria for the sickle cell individuals were age 18 or older and chromatographic diagnosis of SCD. Only outpatients in stable condition were included; patients who had had a vaso-occlusive crisis within the previous two weeks or an episode of acute chest syndrome within the previous four weeks were evaluated at a later time. For more detailed description, refer to the online supplement.
CRP and interleukin-6 protein measurements.
CRP was measured by the NIH Clinical Center Department of Laboratory Medicine using nephelometry. All samples were directly measured at inclusion of the patient in the NIH SCD cohort. The lower limit of detection was 0.2 mg/dL. Interleukin-6 (IL-6) was measured using a high sensitivity IL-6 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's directions. IL-6 was measured on frozen plasma samples in a random group of 161 patients of the NIH SCD cohort. (ClinicalTrials.gov identifier NCT00011648).

Messenger RNA profiling.
To assess the role the pro-inflammatory profile in SCD, whole peripheral blood mononuclear cell transcriptome was evaluated in 24 adult SCD patients and 10 healthy controls. We prospectively defined a group of 33 genes, previously reported to be regulated by iron, as a gene signature for intracellular iron status which we refer to as the “pre-defined iron-regulated gene-set” (Supplemental Table I). To explore the correlation within patient and control samples, we applied unsupervised two-way hierarchical clustering analysis on this pre-defined iron-regulated gene-set.

Real time Quantitative PCR analysis.
Quantitative real-time PCR (Q-PCR) assays were carried out on 10 differentially expressed genes with the use of gene-specific double fluorescently labeled probes. Samples were analyzed in duplicate and the Ct values obtained were normalized to the housekeeping gene RNA18S5. Comparative CT (ΔΔCT) was used to achieve the relative fold change in gene expression between the high iron group and medium/low iron group.

Ferroportin Q248H genotyping.
Ferroportin Q248H genotyping was performed as described in the online supplement. In short, exon 6 of ferroportin was amplified by PCR from genomic DNA using a set of primers encompassing portions of the introns that flank the exon. The resulting DNA fragments were resolved on 2.5% agarose gel and detected with ethidium bromide staining. Ferroportin Q248H mutations included the GT and/or TT genotype. Hepcidin decreases ferroportin expression. Because the ferroportin Q248H is associated with less sensitivity for hepcidin, and hepcidin levels are strongly associated with ferritin levels in SCD selected only cases and controls with a ferritin level lower than 1000 ng/mL. IL-6 data were log 10 transformed to improve normality, and between group differences were tested using unpaired and paired student’s t-test.

Mortality analysis.
The association of CRP with mortality was studied using data from 412 subjects with sickle cell disease from a larger cohort for whom follow-up information and CRP levels were available (ClinicalTrials.gov identifier: NCT00011648). Patients were divided in three groups; a high CRP group at the 75th percentile level of CRP which was 0.8 mg/dL and a low CRP group with a CRP level under the limit of detection of the assay, and an intermediate group which had CRP level in between 0.8mg/dL and 0.2mg/dL. Subjects who were not known to be dead were censored at the date of last contact with study staff or at a follow-up time of 100 months. The cumulative incidence curves of mortality for the three CRP groups were estimated by the Kaplan-Meier method and compared by the log-rank test. Cox Proportional Hazards regression models were used to examine the association of CRP while adjusting for other factors previously published to be associated with mortality: age, gender, NT-proBNP, tricuspid regurgitant jet flow velocity, glomerular filtration rate (BSA adjusted MDRD), white blood cell count, transferrin, systolic blood pressure, and fetal hemoglobin.
Statistics.
Continuous data are expressed as medians with corresponding inter-quartile ranges. Between group differences were tested with the Mann-Whitney U or Kruskal-Wallis test or otherwise as stated. Baseline tables were not tested for statistical differences. Categorical data are presented as percentages or fractions. Differences between groups of categorical data were tested with the \( \chi^2 \) test. When needed, parameters were log10 transformed to normalize their distributions. For correlation studies, Spearman’s rank correlation coefficient was determined otherwise as stated. \( P \) values of 0.05 or less were considered statistically significant. Statistical analyses were performed using SPSS Statistics 17.0.2 (IBM, USA) and SAS version 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

A subgroup of SCD patients has high PBMC expression of iron regulated genes.

In order to categorize patients by iron regulated gene expression, we profiled the PBMC transcriptome of 24 patients with SCD in steady state (median age 36 years) and also 10 healthy controls of similar ethnicity (median age 37 years). A pre-defined set of 33 iron regulated genes (see Supplemental Data Table I) was used to classify subjects according to their characteristics of iron-regulated gene expression. Two way hierarchical clustering of the 34 included subjects and 33 iron regulated genes yielded three clusters of subjects (Figure 1). Detailed laboratory characteristics of the subjects in these clusters are shown in supplemental table II. Serum levels of bilirubin were associated with the expression of heme oxygenase-1 (HO-1), the gene for the enzyme that cleaves heme to the bilirubin precursor biliverdin (Spearman rho=0.387, \( P =0.034 \)), and also with the rank of the cluster in which patients and controls were grouped based upon the expression of their iron regulated genes (ordered from low to high iron, rho=0.358, \( P =0.044 \)). One of the sources of free intracellular iron in monocytes/macrophages in SCD is likely iron released from heme by HO-1, the first committed biochemical step in conversion of heme to bilirubin. The group with the highest differential expression of iron regulated genes contained only SCD patients. Thus, clustering by iron-regulated genes readily identifies a subgroup of patients with internally consistent and highly differentially expressed iron genes.

The expression of iron regulated genes is associated with co-expression of genes involved in inflammasome formation.

In our gene discovery experiment, we identified other genes that were differentially expressed among the three groups and found 98 genes which were consistently and significantly differentially expressed. Using Ingenuity Pathway Analysis, we found that the majority of involved canonical pathways were associated with innate immunity and inflammasome formation (each \( P <0.004 \), Table 1). Highly expressed inflammasome and innate immunity related genes in the high iron-regulated gene expression patients included TLR4, TLR7, TLR8, NLRP3, NLRC4, PRKCA, CLEC7A and CASP1, IL-1 and IL-18. To help validate these results, we tested nine representative regulated genes found on microarray with confirmatory Q-PCR and the two results were strongly correlated (R=0.72, \( P =0.023 \), Figure 2AB).

One of the most highly differentially expressed genes between the three iron clusters was Toll-like receptor 4 (TLR4) gene. The expression of the TLR4 gene was highly correlated to ferritin light chain (FTL) gene expression in both patients (R=0.930, \( P =0.001 \)) and controls (R=0.943, \( P =0.005 \); Figure 2C). TLR4 activation results in the secretion of several pro-inflammatory cytokines such as IL-6 by monocytes/macrophages and plays a key role in innate immunity, which prompted us to also measure IL-6 gene expression by QPCR, as a surrogate marker of TLR4 activation. In SCD patients, IL-6 expression correlated closely to expression of both TLR4 (R=0.897, \( P <0.001 \)) and FTL (R=0.909,
In concordance with the clustering of SCD patients in the high iron group, each of the three genes was expressed over two hundred fold more highly in SCD patients than in controls \( (P<0.001; \text{Figure 2D}) \). Together these results suggest that several iron regulated genes are expressed in SCD patients at much higher levels than in controls and are co-expressed with genes involved in inflammation, and especially members of the inflammasome pathway.

**IL-6 is associated with CRP in SCD.**

Because of the association of the iron regulated genes with genes involved in inflammation we planned further analysis of inflammation in the NIH SCD cohort. In this cohort we measured plasma levels of IL-6 in a subgroup of 161 consecutive patients that had frozen plasma samples available. Detailed baseline characteristics of the patients are provided in the supplementary table III. Plasma levels of IL-6 were correlated to CRP (Spearman \( \rho=0.496, P<0.001 \)), endothelin-1 (\( \rho=0.396, P<0.001 \)), leukocyte count (\( \rho=0.262, P=0.001 \)), and monocyte count (\( \rho=0.191, P=0.017 \)). IL-6 plasma level was negatively correlated with serum transferrin (\( \rho=-0.244, P=0.006 \)). These results were qualitatively similar when restricted to HbSS patients \((n=116)\). The strong correlation between CRP and IL-6 in SCD patients is consistent with published evidence that hepatic cell expression of CRP is induced principally by IL-1 and IL-6 in addition to recently found IL-17.\(^27\) Thus CRP expression is a useful and well accepted clinical marker of IL-6 and associated pathways. These results were qualitatively similar when restricted to HbSS patients.

**Relation between intracellular iron and inflammation in SCD.**

The common genetic mutation of African hemochromatosis, the Q248H mutation in the cellular iron exporter ferroportin, results in intracellular iron accumulation in monocytes and macrophages.\(^28\) We hypothesized that this defect in iron trafficking in monocytes/macrophages would promote the cross-talk between intracellular iron and inflammatory activation marked by IL-6 and CRP expression.

The patients that were included in our gene expression study had ferroportin Q248H mutation status available and therefore were used to explore the association between this mutation and inflammation. Indeed, patients with a ferroportin Q248H mutation (GT and/or TT genotype, \( n=5 \)) had significantly higher plasma levels of CRP than patients with wild type ferroportin alleles (GG genotype, \( n=19; P<0.05 \)), and also \( \log_{10} \text{IL-6} \) trended higher \( (P=0.08; \text{Figure 3AB}) \). We further confirmed this result in a case control study based on patients from the NIH SCD cohort. In the NIH SCD cohort ferroportin Q248H status was available in 359 patients, and of these 28 \((5.3\%)\) had the Q248H ferroportin variant. Fourteen SCD patients with a ferroportin Q248H mutation had a ferritin level less than 1000 mg/dL \( (n=14, \text{HbSS}=10, \text{HbSC}<=4) \) and were included as cases in the case-control study. These cases were compared to SCD patients with wild type ferroportin Q248H status matched for ferritin and genotype. Again, we found that plasma levels of IL-6 and CRP were significantly lower in the cases than in the controls \( (P<0.05, \text{Figure 3CD}) \). We found qualitatively similar trends in subgroup analysis restricted to HbSS patients only \( (P=0.07 \text{ and } P=0.17).\)

**Inflammation is associated with mortality.**

Association of CRP with mortality was examined using data from 412 subjects from another cohort (ClinicalTrials.gov identifier: NCT00011648) for whom follow-up information and CRP levels were available. Patients were divided in high, intermediate and low CRP groups. Detailed baseline characteristics of the patients are provided in Supplementary Table IV. Kaplan-Meier survival curves show that mortality rate significantly increased according to CRP group \( (P=0.0017, \text{Figure 4}) \). Univariate regression showed that each increase in \( \log_{10} \text{transformed CRP} \) was associated with a hazard ratio of 3.0 for early death \((95\% \text{ CI 1.7-5.2, } P<0.0001)\). This mortality effect was even more prominent when the...
population was restricted to patients with sickle cell anemia (HbSS) (hazard ratio (HR) 3.4, 95% CI 1.9-6.3, P<0.0001). We did not evaluate the CRP-mortality relationship in the smaller subgroup of non-HbSS patients alone because of the small group size and low number of events. Five year mortality percentages for the low, intermediate and high CRP groups were respectively 12.4%, 20.5% and 25.8%. CRP was independently associated with mortality in a Cox proportional hazards regression model after adjustment for other covariates previously published as associated with mortality. This adds to previous published models, which have reproducibly identified renal dysfunction, and higher TRV as predictors of death in SCD.

Polytransfused patients had higher serum ferritin and CRP levels compared to non-polytransfused patients (data not shown). Exclusion of polytransfused patients did not change the results of the mortality analysis. Surprisingly, the survival of polytransfused patients did not differ significantly from non-polytransfused patients (data not shown). Analyzing polytransfused patients only in a Cox proportional hazards regression model showed that CRP was significantly associated with mortality in this group as well (HR 3.7 (95%CI 1.5-8.7); P=0.003).

The observation of an independent relationship of CRP with mortality in SCD provides strong new support for an important role of inflammation in SCD pathophysiology.

**DISCUSSION**

The results of this study show that: (1) iron regulated genes such as ferritin light chain are highly up-regulated in SCD PBMCs compared to healthy control PBMC; (2) up-regulation of iron regulated genes is associated with genes involved in inflammation and innate immunity such as TLR4 and IL-6; (3) genetic evidence for a causal relationship between intracellular PBMC iron and inflammation suggested by higher levels of CRP and IL-6 in patients with the ferroportin Q248H mutation compared to patients with wild type ferroportin; and (4) the inflammatory marker CRP is independent predictor of early mortality in SCD.

The clustering of SCD patients exclusively in the high iron group is consistent with the hypothesis that PBMC intracellular iron trafficking is high in SCD patients. This thesis is supported strongly by the two log difference in FTL expression between SCD patients and controls in the QPCR assay. The transcriptional and post-transcriptional regulation of FTL gene expression is well characterized and mainly dependent on intracellular free iron. By combining a set of 33 genes that are described to be regulated by iron, we increased the power to discover genes associated with the common regulating factor, i.e., intracellular iron. Therefore it is very likely that the clustering and the subsequent identification of associated canonical pathways are a relevant reflection of intracellular iron exposure.

Our findings in the gene discovery assay provide the most comprehensive evidence to date for a role of the inflammasome in SCD as suggested by Wanderer. Many of the genes that were the most differentially expressed are involved in the inflammasome complex pathway. Inflammasomes are key signaling platforms that detect pathogenic microorganisms and sterile stressors, and that activate expression of the highly pro-inflammatory cytokines interleukin-1β (IL-1β), IL-18, and IL-6. Activation of the TLR system leads to cellular priming and up-regulation of NLRP3 and subsequently its activation. Thus, TLR4 can act as a co-factor of inflammasome activation or induce pro-inflammatory cytokines such as IL-6 via NF-κB activation. Interestingly, the PBMC transcriptome of SCD patients resembles that of healthy volunteers exposed to the TLR4 ligand lipopolysaccharide, highlighting the same pathways we find associated with high iron trafficking in SCD. Pentraxin-3, the product of the PTX3 gene that is also highly expressed in our high iron trafficking group, is a humoral pattern
recognition molecule related to CRP\textsuperscript{39} that is associated with the duration of SCD painful crisis episodes.\textsuperscript{19} Using the microarray-based gene discovery assay, we have linked several important inflammation and innate immunity genes for the first time to excessive iron trafficking.

The QPCR results indicate that PBMC cytokine gene expression is associated with high iron and is dramatically increased in SCD. The high correlation of TLR4, FTL and IL-6 in SCD PBMC gene expression by QPCR validates and extends the results of the gene discovery assay (Figure 2C). The two log difference in mean expression of IL-6 between SCD patients and controls reinforces that high iron and high expression of pro-inflammatory genes are both associated with expression of known downstream inflammatory cytokines (Figure 2D). Plasma levels of IL-6 are high in SCD\textsuperscript{20-22,40} and increase further during acute complications.\textsuperscript{25-27,40} Most nucleated cells are capable of expressing and synthesizing IL-6, but activated monocytes and macrophages, notable constituents of the PBMC cell population in our experiments,\textsuperscript{27} are the likeliest source of IL-6 detected in our assays. Monocyte TLR4 signaling has been reported to be augmented by intracellular iron,\textsuperscript{5,6,41} and comparable mechanisms may be involved in the augmentation of TLR4 signaling in SCD. Our results combined with published data indicate that robust iron trafficking in SCD is associated with increased expression of important inflammatory cytokines.

A relationship between iron and inflammation is supported by our observation that SCD patients with an inborn defect of cellular iron export have more inflammation compared to other SCD patients. Ferroportin is the only known mammalian iron exporter and is essential for cellular iron export.\textsuperscript{42} The q248h mutation has an allele frequency of 2.2-13.4\% in African populations and results in a significant increase of cellular iron accumulation compared to wild type ferroportin \textsuperscript{43,28}. Two independent data sets show clearly higher IL-6 and CRP levels in the SCD patients with the ferroportin q248h mutation, suggesting a role of monocyte and macrophage intracellular iron in inflammation.

For this study, we combined several observations in different subgroups of patients from a 12-year study evaluating the prevalence and outcome of secondary pulmonary hypertension in SCD (\textit{ClinicalTrials.gov} identifier NCT00011648). The specimens from patients in the various subgroups were chosen based on availability of plasma or DNA biospecimens, primarily influenced by the year of enrollment. Plasma specimens tended to be depleted due to prior research utilization from those patients enrolled early in the 12-year study relative patients enrolled later. The IL-6 assays were performed on a subgroup of specimens from patients enrolled later in the study, with consequently shorter follow-up time and fewer events, and therefore IL-6 mortality analysis was not performed; conversely, the related inflammatory biomarker CRP was available for the entire cohort with long term follow up. Microarray specimens were obtained in a subgroup of patients who volunteered for this additional study. It is possible that the composition of these subgroups, such as inclusion of HbSC double heterozygote patients for improved statistical power, may have affected our results, but where applicable, our sensitivity analysis restricted to only HbSS patients showed qualitatively similar results.

To our knowledge this is the first study showing a strong link between a molecular marker of inflammation and mortality in SCD. Inflammation is generally accepted to be very active in SCD, but its pathogenesis has been poorly understood. CRP is a sensitive but non-specific marker of inflammatory pathways, produced by the liver in response to pro-inflammatory cytokines IL-1, IL-6 and IL-17,\textsuperscript{27} and associated with TLR signaling and inflammasome activation. Our finding of an independent association of CRP with early death suggests the importance of inflammation in the pathophysiology of SCD. These results could explain how high intracellular iron could contribute to risk of early death.

The association of the hemolytic marker bilirubin with high iron cluster in our gene expression experiment suggests that hemolysis is one of the causes of high SCD PBMC iron. In SCD vast amounts of heme are released during intravascular hemolysis\textsuperscript{44,45}; heme augments TLR signaling in monocytes and macrophages probably by inducing intracellular ROS.\textsuperscript{41,46} Injection of heme induces an acute fatal
inflammatory vasculopathy in a SCD mouse model involving the TLR4 pathway.\textsuperscript{47,48} Recently it was shown that next to TLR4 activation, inflammasome related proteins NLRP3 and caspase-1, which also showed up in our gene discovery experiment, are required for heme induced inflammation in wildtype mice.\textsuperscript{49} In humans with beta-thalassemia, another hemoglobinopathy associated with a high rate of hemolysis, monocyte TLR4 expression and CRP is increased as well.\textsuperscript{50,51} Interventions that blockade the hemolysis-iron-inflammation axis, i.e. heme scavenging by recombinant hemopexin, iron chelation, TLR4 or downstream pro-inflammatory cytokine inhibition all are effective in animal models of SCD.\textsuperscript{47-48,51-53} Because we now show comparable pathways are activated in human SCD as in the animal models, these interventions seem promising in human SCD as well.

Conclusion.

The SCD PBMC transcriptome reflects high intracellular iron exposure, which is associated with inflammation and mortality. The associations found in this study support a hypothetical model in which PBMC iron promotes clinically significant inflammatory pathways such as TLR system and the inflammasome, identifying important targets for potential pharmacological intervention.

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E.J. van Beers and G.J. Kato had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors edited the content of the manuscript.

DISCLOSURES

There are no potential conflicts of interest, including relevant financial interests, activities, relationships, and affiliations.
REFERENCES


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FIGURE LEGENDS

Figure 1. A subgroup of SCD patients cluster together in a group with very high expression of iron regulated genes. PBMC transcriptome profiling in 24 SCD patients and 10 healthy controls identified 3 clusters of subjects with high, low and intermediate expression of iron regulated genes. patients (ClinicalTrials.gov identifiers: NCT00072826). The Heat map shows unsupervised two-way hierarchical clustering of the pre-defined set of iron regulated genes on the y-axis (for a tabular version of this list, see supplemental data) and the clustering of study participants on the x-axis. None of the controls was found to be in the high iron cluster. “S” means SCA patient; “C” means control.

Figure 2. QPCR results confirm the relation between iron regulated genes and genes involved in inflammatory and anti-inflammatory pathways. To validate the microarray results we performed qantitative polymerase chain reaction (QPCR) normalized to the housekeeping gene RNA18S5 on the same samples. (ClinicalTrials.gov identifiers: NCT00072826) (A) This diagram shows a good correlation (R=0.72, P=0.023) between the fold change as found by microarray and QPCR. (B) Bars show the fold change in expression of the group of patients with highly expressed iron regulated genes over the other groups. (C) The number of PCR cycles to reach threshold compared to the housekeeping gene RNA18S5 (∆CT) of FTL was highly correlated to TLR4 in both patients (R=0.930, P<0.001) as controls (R=0.943, P=0.005) although mean expression of both genes was much higher in patients than in controls (P<0.001). TLR4 was also highly correlated to ALDH1A1, GAPDH, SAT2 and HMOX expression (data not shown). To get an better impression of actual gene expression X-and Y-axis are reversed and originate 20 ∆CT. (D) Bars show the fold change in expression of selected genes in SCD patients compared to healthy controls.

- Black bars represent genes that were included in the pre-defined set of iron regulated genes (see Supplemental table I).
- White bars represent genes that were identified as significantly differentially expressed between the three iron clusters or were included to validate the microarray data.


Figure 3. Patients with the ferroportin Q248H mutation which is associated with high retention of intracellular iron have higher levels of CRP and interleukin-6. To assess relationship between intracellular iron and inflammation we used available ferroportin Q248H mutation status. Patients were analyzed previously for ferroportin mutation status. In the gene-expression part of the study (ClinicalTrials.gov identifier: NCT00072826) the five patients with a ferroportin Q248H mutation (GT and/or TT genotype) had a significant (p<0.05) higher plasma level of CRP and log 10 IL-6 trended (P=0.08) to be higher compared to 19 patients with wild type ferroportin (GG genotype). As a sensitivity analysis we repeated this in a case-control study in selection of patients of the NIH SCD cohort (ClinicalTrials.gov identifier: NCT00011648). Fourteen patients with Q248H mutation were matched on genotype and ferritin level. We found that the 14 patients with the mutation had a significant higher CRP and log10 il6 than the matched controls. Because the ferroportin Q248H is also associated with less sensitivity for hepcidin, and hepcidin levels are strongly associated with ferritin levels in SCD we decided to perform this last analysis only in patients with a ferritin level smaller than 1000 mg/dL. * P<0.05
Figure 4. Kaplan Meier curve showing a significant difference in survival between patients with low and high CRP. SCD Patients (n=412) of the NIH pulmonary hypertension screening cohort (ClinicalTrials.gov identifier: NCT00011648) were divided in a high CRP group and a low CRP group at the 75th percentile level of CRP (0.8 mg/dL, (upper limit of normal)). Median follow up time was 47 months, (IQR 24-82, range 2-132). After 100 months patients were censored. Log rank test showed that patients with CRP above 0.8 mg/dL had significantly lower (p=0.0035) survival rate by time from enrollment. Five year mortality percentages for the low, intermediate and high CRP groups were respectively 12.4%, 20.5 and 25.8%. In a multivariate analysis, CRP was an independent predictor of mortality (see Table 2).
### Table 1. Top 10 canonical pathways associated with increased expression of the pre-defined iron regulated gene set. (p<0.01)

<table>
<thead>
<tr>
<th>Name</th>
<th>Differential regulated genes</th>
</tr>
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<tbody>
<tr>
<td>Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses</td>
<td>TLR4, NLRP3, NLRC4, TLR8, TLR7, PTX3, PRKCA, CLEC7A CASP1, OAS1.</td>
</tr>
<tr>
<td>IL-10 Signaling</td>
<td>CCR1, HMOX1, BLVRA, CD14, IL18, SP1.</td>
</tr>
<tr>
<td>Altered T Cell and B Cell Signaling in Rheumatoid Arthritis</td>
<td>TLR4, TNFSF13, IL15, IL18, TLR7, TLR8, FCER1G, CD86.</td>
</tr>
<tr>
<td>Communication between Innate and Adaptive Immune Cells</td>
<td>TLR4, FCER1G, CD86, IL15, IL18, TLR7, TLR8, TNFSF13.</td>
</tr>
<tr>
<td>Phospholipase C Signaling</td>
<td>CAMK4, CD247, FCER1G, HMOX1, GNB4, MARCKS, PRKCA, PLA2G4A, NFATC2, PLD3, RHOU, RRAS.</td>
</tr>
<tr>
<td>TREM1 Signaling</td>
<td>CASP1, CD86, IL18, JAK2, TLR4, TLR7, TLR8.</td>
</tr>
<tr>
<td>Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis</td>
<td>CAMK4, CEBPA, FCGR1A, Fzd1, IL15, IL18, JAK2, LEF1, LRP1, LTBR, NFATC2, PRKCA, RRAS, TLR4, TLR7, TLR8, VEGFA.</td>
</tr>
<tr>
<td>Dendritic Cell Maturation</td>
<td>CD86, CD1D, FCER1G, FCGR1A, FCGR1B, IL15, IL18, JAK2, LTBR TLR4.</td>
</tr>
<tr>
<td>SLE Signaling</td>
<td>CAMK4, CD86, CD247, FCER1G, FCGR1A, FCGR1B, IGKC, IL18, NFATC2, RRAS, TLR7.</td>
</tr>
<tr>
<td>Heme Degradation</td>
<td>HMOX1, BLVRA.</td>
</tr>
</tbody>
</table>
Table 2. Output of Cox regression model: the association between log 10 transformed CRP and mortality, unadjusted and adjusted for covariates.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% Confidence Interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exp (β)</td>
<td>95% Confidence Interval</td>
<td>P value</td>
</tr>
<tr>
<td>Unadjusted</td>
<td>3.01</td>
<td>1.74-5.19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Adjusteda Log 10 CRP</td>
<td>2.82</td>
<td>1.03-4.20</td>
<td>0.041</td>
</tr>
<tr>
<td>TRV ≥3.0 m/s</td>
<td>2.00</td>
<td>1.02-3.90</td>
<td>0.043</td>
</tr>
<tr>
<td>Log 10 proBNP</td>
<td>1.96</td>
<td>1.19-3.22</td>
<td>0.008</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.04</td>
<td>1.01-1.06</td>
<td>0.005</td>
</tr>
<tr>
<td>WBC</td>
<td>1.09</td>
<td>1.003-1.18</td>
<td>0.042</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.997</td>
<td>0.991-1.003</td>
<td>0.29</td>
</tr>
</tbody>
</table>

*aOther covariates used which were no significant contributors to the model: systolic blood pressure, gender, age, fetal hemoglobin, GFR estimated by the 4-variable MDRD equation (ml/min/1.73 m²).
Novelty and Significance

What Is Known?

- Both oxidant stress and inflammation have been shown to contribute to the pathogenesis of several forms of vasculopathy.
- The burden of a natural oxidant, iron, has been consistently associated in adults with sickle cell disease with vasculopathy, such as pulmonary arterial hypertension.

What New Information Does This Article Contribute?

- Immune cells that show signs of high intracellular iron turnover or iron burden strongly express genes known to be part of the innate immune system.
- The activated genes include those encoding proteins involved in the inflammasome pathway, which is an innate immune pathway that has evolved to recognize Danger-Associated Molecular Patterns (DAMPs), such as heme-bound iron.
- These results highlight a previously underappreciated role for excess heme iron in the pathobiology of vasculopathy.

Sickle cell disease is associated with massive turnover of heme iron in macrophages, as revealed by signature changes in iron-regulated genes. We have found that in patients with sickle cell disease immune cell expression of iron-regulated genes is linked to increased expression of pro-inflammatory genes. The expression of two of these genes (the TLR4 and IL-6 genes) is more than hundred times higher in sickle cell patients than in healthy controls. Furthermore, patients with a mutation causing iron retention in macrophages expressed higher levels of IL-6 protein, suggesting a causal relationship between iron and inflammation. Finally, high levels of C-reactive protein, an inflammatory marker, strongly correlated to interleukin-6 levels and independently predict mortality in sickle cell disease. These findings suggest an intrinsic link between heme iron and activation of inflammation in patients with sickle cell disease.
Figure 1

Gene expression heat map with clustering of genes and clusters labeled as 'Intermediate iron cluster', 'High iron cluster', and 'Low iron cluster'.
Figure 2

A

Microarray fold change (log2)

QPCR fold change (log2)

R = 0.72
P = 0.023

B

Fold change (Standard Error)

GAPDH
ALDH1A1
FTL1
HMOX1
SAT2
TLR4
IL6
ECE1
ST13

C

FTL expression on QPCR (Δ CT)

TLR4 expression on QPCR (Δ CT)

Controls, P = 0.026
Patients, P < 0.001

D

Fold change (Standard Error)

GAPDH
ALDH1A1
FTL1
HMOX1
SAT2
TLR4
IL6
ECE1
ST13

***
**

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Figure 4

Cumulative incidence of mortality

log-rank test for three groups, P=0.0017

No. at risk
CRP ≥ 0.8: 102 72 37 20 5
0.2 < CRP < 0.8: 112 78 43 22 3
CRP ≤ 0.2: 198 164 124 88 28

Follow-up (years)
Iron, Inflammation, and Early Death in Adults with Sickle Cell Disease
Eduard van Beers, Yanqin Yang, Nalini Raghavachari, Xin Tian, Darlene T Allen, James S Nichols, Laurel Mendelsohn, Sergei Nekhai, Victor R Gordeuk, James G Taylor and Gregory J Kato

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Methods

Subjects

The National Heart, Lung, and Blood Institute’s Institutional Review Board approved the protocols. All subjects provided written informed consent. All patients presented in this paper are selected from a long follow-up study evaluating the prevalence of secondary pulmonary hypertension and mortality in SCD ([ClinicalTrials.gov identifier NCT00011648](https://clinicaltrials.gov/ct2/results?cond=Secondary%20Pulmonary%20Hypertension&art=disease&recr=Patient&trt=Alcohol&status=All&phase=)). We will refer to the group of patients included in this study as the “NIH SCD cohort”. Inclusion criteria for the sickle cell individuals were age 18 or older and chromatographic diagnosis of SCD. Only outpatients in stable condition were included; patients who had had a vaso-occlusive crisis within the previous two weeks or an episode of acute chest syndrome within the previous four weeks were evaluated at a later time.

The patients in the interleukin-6 analysis were chosen from the NIH SCD cohort based on availability of plasma. In addition to this, the gene expression experiment included 24 patients and 10 age and gender matched African-American controls that had gene expression data available as a baseline measurement from a clinical trial restricted to SCA patients with above-median plasma level of soluble vascular adhesion molecule-1 ([ClinicalTrials.gov identifier NCT00072826](https://clinicaltrials.gov/ct2/results?cond=Soluble%20Vascular%20Adhesion%20Molecule-1&art=disease&recr=Patient&trt=Alcohol&status=All&phase=)). Only patients with homozygous hemoglobin S disease were included in this study and no blood transfusions in the previous three months were allowed. Ferroportin Q248H mutation status was available for most of these gene expression study patients, and they were used in the first analysis of the association between this variant and inflammation. Additional analysis included another 359 patients in the NIH SCD cohort patients with ferroportin Q248H mutation status determined in a previous study of hepcidin sensitivity. From this second
group, all SCD patients with a ferroportin Q248H mutation without significant iron overload (serum ferritin level <1000 mg/dL) were included as cases in a case-control study, and compared to SCD patients with wild type ferroportin Q248H status, matched for ferritin and genotype.

**mRNA profiling**

To assess the role the pro-inflammatory profile in SCD, whole peripheral blood mononuclear cell transcriptome was evaluated in 24 adult SCD patients and 10 healthy controls. Total RNA from PBMCs was used to prepare biotinylated target RNA, according to the manufacturer’s recommendations for overnight hybridization to Human Genome U133 Plus 2.0 oligonucleotide arrays. Arrays were washed and stained using the Affymetrix Fluidics Station 450 and then scanned using an Affymetrix GeneArray 3000 scanner. Signal intensity values were achieved by background correction, quantile normalization and median polish summarization. We were interested in genes which would be associated with high intracellular PBMC iron exposure. Therefore, we selected all genes that were reported to be differentially expressed according to iron status from a series of studies exploring iron regulated genes in various human cell types. This prospectively defined group of 33 genes were used as a gene signature for cellular iron status which we refer to as the “pre-defined iron-regulated gene-set.” All genes included in the pre-defined iron-regulated gene-set are listed in supplemental table I. To explore the correlation within patient and control samples, we applied unsupervised two-way hierarchical clustering analysis on this pre-defined iron-regulated gene-set. Various combinations of the resulting clustering of groups were used to find differentially expressed genes with the filter of a change greater than 40% between the clustering groups and 10% false discovery rate (FDR). Functional analysis was performed on Ingenuity Pathway Analysis (IPA) System. Array data were deposited at the Gene Expression Omnibus (National Center for Biotechnology Information) with accession number GSE53441.
Real time Q-PCR analysis

First-strand cDNA was synthesized using 500 ng of RNA and random primers in a 20 μl reverse transcriptase reaction mixture using Invitrogen’s Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer’s directions. Quantitative real-time PCR assays were carried out on 10 differentially expressed genes with the use of gene-specific double fluorescently labeled probes in a 7900 Sequence Detector (PE Applied Biosystems, Norwalk, CT). Probes and primers were obtained from Applied Biosystems. In brief, PCR amplification was performed in a 384 well plate with a 20-μl reaction mixture containing 300 nm of each primer, 200 nm probe, 200 nm dNTP in 1x real time PCR buffer and passive reference (ROX) fluorochrome. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C. Samples were analyzed in duplicate and the Ct values obtained were normalized to the housekeeping gene RNA18S5. The comparative CT (ΔΔ CT) method which compares the differences in CT values between groups was used to achieve the relative fold change in gene expression between the high iron group and medium/low iron group.

Ferroportin Q248H (rs11568350) genotyping

Exon 6 of ferroportin was amplified by PCR from genomic DNA using a set of primers encompassing portions of the introns that flank the exon (forward primer: 5’-CAT CGC CTG TGG CTT TAT TT-3’; reverse primer: 5’-GCT CAC ATC AAG GAA GAG GG-3’). PCR reactions were performed in 25 μL volumes in a standard PCR buffer containing 1.5 mM MgCl2, 200 μM dNTP, 20 nM Primers and 0.5U Taq DNA polymerase. After initial denaturation at 95°C for 5 min, a PCR was performed in a thermocycler (Bio-Rad) for 38 cycles of heating at 95°C for 15 s, annealing at 55°C 15 s and extension at 72°C for 1 min. A final cycle of 10 min at 72°C was also
Supplemental material: methods and tables    E.J. van Beers et al.

added. Ten microliters of PCR product (392 bp) were digested with PvuII enzyme (MBI Fermentas, Hanover, MD, USA) for 2 h at 37°C, and the resulting DNA fragments (252 bp and 140bp) were resolved on 2.5% agarose gel and detected with ethidium bromide staining.

Hepcidin decreases ferroportin expression. Because the ferroportin Q248H is associated with less sensitivity for hepcidin, and hepcidin levels are strongly associated with ferritin levels in SCD we decided to select only cases and controls with a ferritin level smaller than 1000 mg/dL. IL-6 data were log 10 transformed to improve normality, between group differences were tested using unpaired and paired student’s t-test.
### Table I. Gene Symbols of genes included in the predefined ‘iron regulated’ gene set.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Description</th>
<th>Regulated by*</th>
<th>Cell type**</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C2</td>
<td>Aldo-keto reductase family 1</td>
<td>DFO↓</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>Aldehyde dehydrogenase 1</td>
<td>DFO↓</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td>ARID4A</td>
<td>Retinoblastoma binding protein 1 (RBBP1)</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td>CALR</td>
<td>calreticulin</td>
<td>iron↑</td>
<td>ACC (1)</td>
</tr>
<tr>
<td>ENO2</td>
<td>Enolase 2</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DFO↓</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td>FTL</td>
<td>Ferritin L chain</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iron↑</td>
<td>ACC (1)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
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<td>GAS1</td>
<td>Gas-1</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
<tr>
<td>GLRX</td>
<td>Glutaredoxin (thioltransferase)</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
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<tr>
<td>GPX1</td>
<td>glutathione peroxidase-1</td>
<td>iron↑</td>
<td>ACC (1)</td>
</tr>
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<td>HIF1A</td>
<td>Hif-1</td>
<td>iron↓</td>
<td>HeLa (4)</td>
</tr>
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<td>HMOX1</td>
<td>Heme Oxygenase-1</td>
<td>iron↑</td>
<td>HeLa (4)</td>
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<td>HSPA1A</td>
<td>Heat shock 70kDa protein 1a</td>
<td>iron↑</td>
<td>HeLa (4)</td>
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<tr>
<td>HSPA1L</td>
<td>Heat shock 70kDa protein 1-like</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
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<td>HSPA4</td>
<td>Heat shock 70kDa protein 4</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
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<td>HSPA9</td>
<td>Heat shock 70kDa protein 9</td>
<td>iron↑</td>
<td>HeLa (4)</td>
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<td>HSPH1</td>
<td>Hsp105</td>
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<td>HeLa (4)</td>
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<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
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<td>Gene</td>
<td>Function</td>
<td>Regulation</td>
<td>Cell Type</td>
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<tr>
<td>-------</td>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
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<td>KLHL4</td>
<td>Kelch-like 4</td>
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<td>LOX</td>
<td>lysyl oxidase</td>
<td>iron↓</td>
<td>HeLa (4)</td>
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<tr>
<td>MAPK9</td>
<td>c-jun</td>
<td>iron↓</td>
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<td>MAT1A</td>
<td>Methionine adenosyltransferase 1</td>
<td>DFO↓</td>
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<td>MTNR1B</td>
<td>Mt-2</td>
<td>iron↓</td>
<td>HeLa (4)</td>
</tr>
<tr>
<td>MYC</td>
<td>c-myc</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
<tr>
<td>PMP22</td>
<td>Gas-3</td>
<td>iron↑</td>
<td>HeLa (4)</td>
</tr>
<tr>
<td>PSAP</td>
<td>prosaposin</td>
<td>iron↑</td>
<td>ACC (1)</td>
</tr>
<tr>
<td>RPS9</td>
<td>ribosomal protein S9 mRNA</td>
<td>iron↑</td>
<td>ACC (1)</td>
</tr>
<tr>
<td>SAT2</td>
<td>Spermidine/spermine N1-acetyltransferase</td>
<td>DFO↑</td>
<td>HC (2)(3)</td>
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<td>SEC61B</td>
<td>sec61-complex beta-subunit</td>
<td>iron↑</td>
<td>ACC (1)</td>
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<td>SH2D4A</td>
<td>Hypothetical protein FLJ20967</td>
<td>DFO↑</td>
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<td>SLC11A2</td>
<td>DMT-1</td>
<td>iron↓</td>
<td>HeLa (4)</td>
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<td>STMN1</td>
<td>Stathmin 1/oncoprotein</td>
<td>DFO↓</td>
<td>HC (2)(3)</td>
</tr>
<tr>
<td>TFRC</td>
<td>Transferrin receptor</td>
<td>iron↓</td>
<td>HeLa (4)</td>
</tr>
</tbody>
</table>

*↓ denotes downregulated, ↑ denotes upregulated, DFO denotes deferoxamine

**ACC denotes astrocytome cells, HC denotes hepatocarcinoma cells, and HeLa denotes HeLa cells.
<table>
<thead>
<tr>
<th>Iron cluster</th>
<th>Low (n=13)</th>
<th>Intermediate (n=13)</th>
<th>High (n=8)</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36 (30-42)</td>
<td>33 (24-42)</td>
<td>45 (30-51)</td>
</tr>
<tr>
<td>SCD Patients (fraction)</td>
<td>9/13</td>
<td>7/13</td>
<td>8/8</td>
</tr>
</tbody>
</table>

Hemolysis related parameters:
- Hemoglobin (mg/dL) 10.9 (8.2-11.5) 8.4 (7-12.6) 8.5 (7.4-9.3)
- Reticulocytes (%) 3.5 (1-5.6) 7.7 (1.4-12.3) 10.4 (8.5-12.5)
- Lactate dehydrogenase (IU/L) 232 (175-346) 216 (161-422) 325 (279-424)
- Total bilirubin (mg/dL) 1.3 (1-3.1) 2.1 (0.7-2.9) 2.9 (2.4-5.9)

Inflammation related parameters:
- CRP (mg/dL) 0.6 (0.4-0.9) 0.4 (0.4-0.7) 0.4 (0.4-1)
- WBC (10^9/L) 6.0 (4.7-8.8) 7.3 (4.4-13.9) 7.3 (6.9-10.2)
- Monocytes (10^9/L) 0.44 (0.36-0.63) 0.49 (0.45-0.63) 1.0 (0.86-1.46)

Iron metabolism related parameters:
- Ferritin (ng/mL) 243 (118-409) 143 (95-371) 396 (126-916)
- Transferrin (mg/dL) 206 (178-232) 186 (171-220) 177 (156-189)
- Iron saturation (%) 29 (22-88) 34 (26-39) 44 (34-76)

Numbers are medians (interquartile range)
* All SCD patients included in this study have homozygous hemoglobin S disease
### Table III. Baseline characteristics of patients included in the Interleukin-6 study.

<table>
<thead>
<tr>
<th>SC patients (n=161)</th>
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</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td><strong>Females (percentage)</strong></td>
</tr>
<tr>
<td><strong>SCD Phenotype (percentage)</strong></td>
</tr>
<tr>
<td>HbSS</td>
</tr>
<tr>
<td>HbSC</td>
</tr>
<tr>
<td>Other*</td>
</tr>
<tr>
<td><strong>Hemolysis related parameters:</strong></td>
</tr>
<tr>
<td>Hemoglobin (mg/dL)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (IU/L)</td>
</tr>
<tr>
<td>Total bilirubin(mg/dL)</td>
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<tr>
<td><strong>Inflammation related parameters:</strong></td>
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<tr>
<td>CRP (mg/dL)</td>
</tr>
<tr>
<td>WBC (10^9/L)</td>
</tr>
<tr>
<td>Monocytes (% of WBC)</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
</tr>
<tr>
<td><strong>Iron metabolism related parameters:</strong></td>
</tr>
<tr>
<td>Iron (mg/dL)</td>
</tr>
<tr>
<td>Iron saturation (%)</td>
</tr>
<tr>
<td>Transferrin (mg/dL)</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
</tr>
</tbody>
</table>

Numbers are medians (interquartile range)

*includes HbS-Beta+- thalassemia (5.6%), HbS-Beta0- thalassemia (1.9%) and unspecified (3.1%)
Table IV. Characteristics of patients in the different CRP-groups

<table>
<thead>
<tr>
<th>CRP group</th>
<th>≤0.2 (n=198)</th>
<th>0.2-0.8 (n=112)</th>
<th>≥0.8 (n=102)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32 (25-44)</td>
<td>33 (25-47)</td>
<td>33 (25-45)</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.2 (0.2-0.2)*</td>
<td>0.55 (0.46-0.66)</td>
<td>1.37 (1.02-2.23)</td>
</tr>
<tr>
<td>SCD Phenotype (percentage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbSS</td>
<td>71.2</td>
<td>73.2</td>
<td>78.4</td>
</tr>
<tr>
<td>..HbSC</td>
<td>20.2</td>
<td>19.6</td>
<td>12.7</td>
</tr>
<tr>
<td>..Other**</td>
<td>8.6</td>
<td>7.1</td>
<td>8.8</td>
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<tr>
<td>Polytransfused (percentage)</td>
<td>31.3</td>
<td>36.6</td>
<td>49.0</td>
</tr>
</tbody>
</table>

Hemolysis related parameters:
- Hemoglobin (mg/dL) 9.8 (8-10.9) 9.5 (8.2-10.5) 9.0 (8-10.2)
- Reticulocytes (%) 7.0 (3.6-11.3) 7.3 (4.0-11.7) 8.4 (4.4-12.5)
- Lactate dehydrogenase (IU/L) 310 (226-409) 318 (237-422) 333 (256-436)
- Total bilirubin (mg/dL) 2.3 (1.5-3.9) 2.2 (1.5-3.4) 2.5 (1.6-3.4)

Inflammation related parameters:
- Interleukin-6 (pg/mL) 0.89 (0.36-1.48) 1.33 (0.66-2.6) 2.61 (1.48-5.67)
- WBC (10⁹/L) 9.1 (7.2-11.4) 9.9 (8.1-12.1) 11.5 (9-14)
- Monocytes (10⁹/L) 0.76 (0.49-1.14) 0.85 (0.53-1.24) 0.87 (0.59-1.22)

Iron metabolism related parameters:
- Ferritin (ng/mL) 225 (105-761) 524 (130-1392) 907 (319-1863)
- Transferrin (mg/dL) 200 (169-243) 194 (166-229) 181 (153-222)
- Iron saturation (%) 34 (23-50) 33 (25-57) 28 (20-50)

Numbers are medians (interquartile range).
*0.2 mg/dL was the lower limit of detection.
**includes HbSD, HbS-betathalassemia and unspecified.
References


