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Circulating Iron in Patients with Sickle Cell Disease Mediates the Release of Neutrophil Extracellular Traps

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Keywords
Sickle cell disease · Hemolysis · Iron · Neutrophils · Neutrophil extracellular traps

Abstract

Introduction: Neutrophils promote chronic inflammation and release neutrophil extracellular traps (NETs) that can drive inflammatory responses. Inflammation influences progression of sickle cell disease (SCD), and a role for NETs has been suggested in the onset of vaso-occlusive crisis (VOC). We aimed to identify factors in the circulation of these patients that provoke NET release, with a focus on triggers associated with hemolysis. ***Methods:*** Paired serum and plasma samples during VOC and steady state of 18 SCD patients (HbSS/HbSβ^0-thal and HbSC/HbSβ$^+$-thal) were collected. Cell-free heme, hemopexin, and labile plasma iron have been measured in the plasma samples of the SCD patients. NETs formation by human neutrophils from healthy donors induced by serum of SCD patients was studied using confocal microscopy and staining for extracellular DNA using Sytox, followed by quantification of surface coverage using ImageJ. ***Results:*** Eighteen patients paired samples obtained during VOC and steady state were available (11 HbSS/HbSβ^0-thal and 7 HbSC/HbSβ$^+$-thal). We observed high levels of systemic heme and iron, concomitant with low levels of the heme-scavenger hemopexin in sera of patients with SCD, both during VOC and in steady state. In our in vitro experiments, neutrophils released NETs when exposed to sera from SCD patients. The release of NETs was associated with high levels of circulating iron in these sera. Although hemin triggered NET formation in vitro, addition of hemopexin to scavenge heme did not suppress NET release in SCD sera. By contrast, the iron scavengers deferoxamine and apotransferrin attenuated NET formation in a significant proportion of SCD sera. ***Discussion:*** Our results suggest that redox-active iron in the circulation of non-transfusion-dependent SCD patients activates neutrophils to release NETs, and hence, exerts a direct pro-inflammatory effect. Thus, we propose that chelation of iron requires further investigation as a therapeutic strategy in SCD.

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Introduction

Sickle cell disease (SCD) is a monogenetic disease wherein a single point mutation in β-globin gives rise to sickle hemoglobin (HbS), affecting millions of people worldwide [1]. Deoxygenation results in intracellular hemoglobin (Hb) polymerization inducing the characteristic cellular shape of sickle erythrocytes. Vaso-occlusion and chronic hemolysis are predominating vascular events that contribute to the pathogenesis of SCD [2]. Vaso-occlusion is thought to trigger acute systemic painful vaso-occlusive crises (VOCs), which is the most common complication of SCD and the leading cause of hospitalization of SCD patients [1, 3]. A role for neutrophils in promoting vaso-occlusion in SCD mice was first demonstrated by Turhan et al. [4]. Later, activated neutrophils were shown to interact with the inflamed vessel wall in vivo, and to adhere to erythrocytes promoting VOC in SCD mice [5]. Also, circulating neutrophils were shown to be primed or constitutively activated in SCD patient [6].

Experimental evidence suggests that hemolysis in SCD mice triggers neutrophil activation and the formation of neutrophil extracellular traps (NETs) within the pulmonary microcirculation, which led to acute lung injury and death of these mice [7]. Earlier, we found levels of neutrophil elastase-α-1-antitrypsin complexes and nucleosomes to be elevated in plasma of SCD patients during VOC, suggesting that neutrophils release NETs in SCD patients [8]. More recently, circulating NET fragments have been shown to contribute to acute chest syndrome in SCD mice [9]. Remarkably, therapeutic neutralization of heme attenuated pulmonary NET release, prevented NET-associated hypothermia and rescued SCD mice from acute chest syndrome and death [7, 10]. In turn, SCD mice receiving heme developed acute chest syndrome, further supporting a central role of heme in systemic vaso-occlusion [10]. Altogether, these findings suggest that therapeutic administration of the heme-scavenging plasma protein hemopexin (Hpx) would protect SCD patients from vaso-occlusion. The therapeutic benefit of Hpx administration to improve the outcome of VOC has been shown in SCD mice [10, 11]. Yet, whether heme instigates clinically high-risk VOC, its cellular mediators herein, and hence, its full therapeutic value is still not clear. Here, we hypothesize a role for iron in SCD patients that had not previously been considered. Using therapeutic blockade by chelation of iron, we show that depletion of iron, and not heme, prevents the release of NETs in SCD patient's blood.

Materials and Methods

Patients

A full description of the patients in our study has been published previously [12]. In brief, consecutive adult patients with SCD admitted for VOC at the Academic Medical Center Amsterdam or the Slotervaart Hospital, Amsterdam, The Netherlands, were approached for inclusion. Patients diagnosed with sickle cell anemia or a compound heterozygous state HbSβ^0-thalassemia, HbSβ$^+$-thalassemia, or HbSC was eligible. Patients were excluded when on hydroxyurea therapy. The study protocol was approved by the Medical Ethical Committee of the participating centers and conducted in agreement with the Helsinki declaration. Written consent was obtained from each participant or their legal guardian. Serum samples and neutrophils of healthy donors (controls) have been obtained from Sanquin. Blood samples were taken by venipuncture. Blood serum tubes of SCD patients were centrifuged for 15 min at 3,000×g at 4°C to obtain serum and stored in aliquots at −80°C until further analysis.

Laboratory Analysis

Total serum heme was measured with a colorimetric method according to the manufacturer's instruction (Quantichrom heme assay kit, Fischer Scientific). Serum Hpx (purified from human plasma with high-performance liquid chromatography) and IgM were determined by ELISA (in-house, see online suppl. Data; see www.karger.com/doi/10.1159/000526760 for all online suppl. material). Purified Hpx was used as standard for the measurement of serum Hpx. Labile plasma iron (LPI) was measured by dihydrorhodamine (Sigma Aldrich) fluorescence as described previously (see online suppl. Data). The differences in the rate of dihydrorhodamine oxidation with and without the iron-chelator deferoxamine (DFO; Sigma Aldrich) represent the fraction of plasma non-transferrin-bound-iron that is redox active. Neutrophil isolation, stimulation, and inhibition of NET formation, analysis of NET formation by neutrophils, immunostaining of NET components, and determination of reactive oxygen species (ROS) production are described in detail in the online supplementary data [13, 14].

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software (version 6.0). Data are presented as mean ± SD of independent experiments. Paired student's *t* test or Wilcoxon matched-pairs signed rank test was used to compare two groups. For comparing more than two groups, one-way ANOVA with Tukey's multiple comparisons post hoc test or Kruskal-Wallis with Dunn's post hoc testing was used where appropriate. Spearman correlation coefficients were calculated to correlate two variables. A p value of 0.05 or less was considered statistically significant.

Results

From 18 patients, paired samples obtained during VOC and steady state were available (11 sickle cell anemia/HbSβ^0-thalassemia and 7 HbSC/HbSβ$^+$-thalassemia). Patients with SCD show reduced levels of the plasma scavenger for heme, Hpx – both in steady state and VOC – compared to healthy donors (shown in Fig. 1a). High systemic levels of heme were found in patients with SCD. Upon comparison of cell-free heme levels in healthy do-

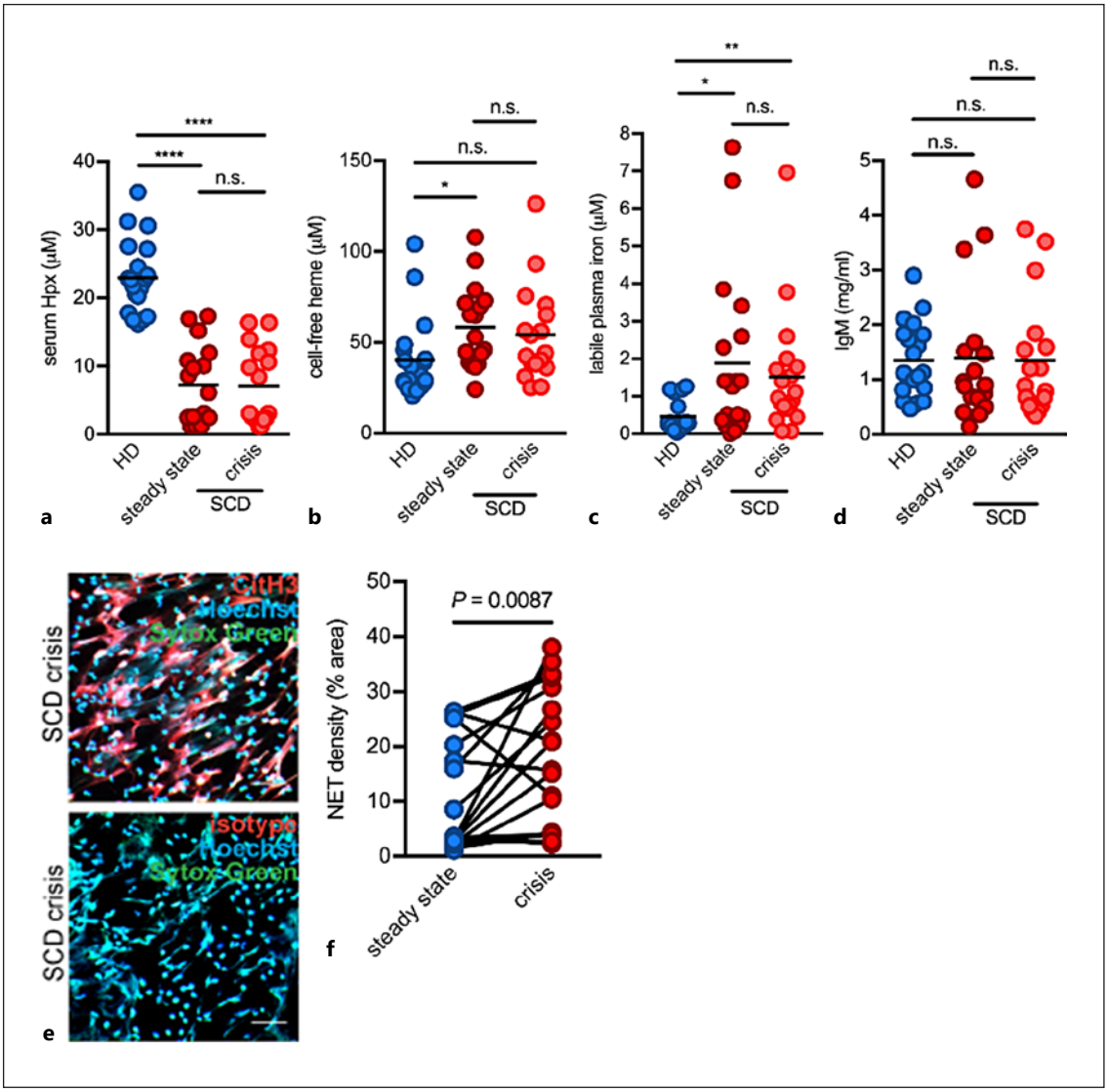

Fig. 1. Neutrophils release more NETs when exposed to sera of patients obtained during VOC compared to the steady state. **a** Serum levels of Hpx in healthy control (HD; $n = 20$) and SCD patients in the steady state and VOC ($n = 17$/group). One-way ANOVA with Tukey's multiple comparisons post hoc test was used. ****$p < 0.0001$. **b** Levels of serum-free heme in HD ($n = 20$) versus patients with SCD in the steady state and VOC ($n = 17$ in each group). A Kruskal-Wallis test with Dunn's post hoc testing was used. *$p < 0.05$. n.s. indicates not significant. **c** Levels of LPI in HD ($n = 19$) versus patients with SCD in the steady state and VOC ($n = 19$ for each group). We used a Kruskal-Wallis test with Dunn's post hoc testing for statistical analysis. *$p < 0.05$, **$p < 0.01$. n.s. indicates not significant. **d** Levels of IgM in serum of HD ($n = 20$) and SCD patients in the steady state and VOC ($n = 17$/group). **e** Immunostaining for citrullinated histone H3 (CitH3) after exposure to SCD serum. Neutrophils from a HD were exposed to serum from a SCD patient in VOC for 180 min. Then, extracellular DNA was stained with Sytox Green (green). Immunostaining was performed on NETs induced by 3 different SCD sera. Representative images are shown. Scale bar, 50 μm. Original magnifications ×40. **f** Quantification of extracellular DNA release in response to sera from SCD patients. A Wilcoxon matched-pairs signed rank test was used for statistical analysis. Incubations of neutrophils with paired SCD sera ($n = 18$) were performed with neutrophils from 3 different HD for each subject group (steady state and crisis). **$p = 0.0087$.

nor plasma and in plasma of SCD patients tested, cell-free heme levels were significantly increased in steady state SCD, though failed to reach significance in VOC as compared to healthy controls (shown in Fig. 1b). In addition, levels of LPI were significantly elevated in samples from SCD patients in steady state and VOC as compared to samples from healthy donors (shown in Fig. 1c), although no significant differences have been observed between LPI levels in VOC and steady state, respectively. Differences in levels of Hpx, heme, and LPI that are caused by hemodilution due to therapeutic hyperhydration during VOC have been excluded by the measurement of IgM levels (shown in Fig. 1d). To determine whether circulating plasma factors in samples obtained from patients with

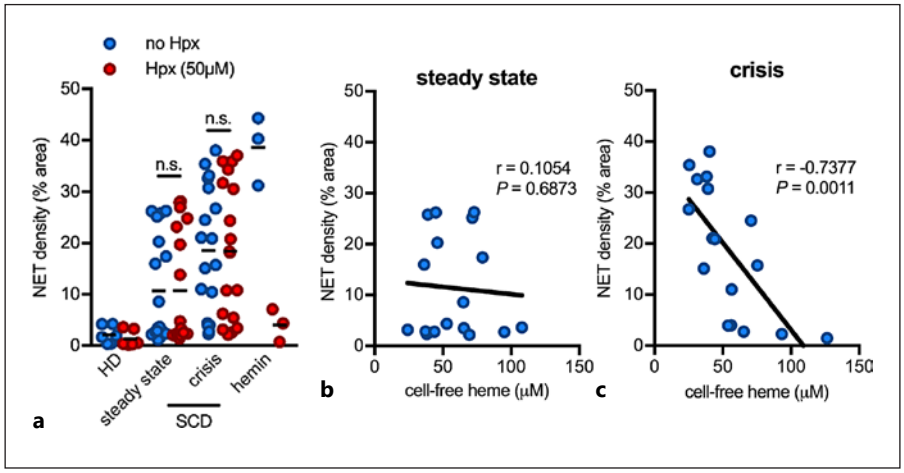

Fig. 2. Plasma-purified hemopexin does not prevent the release of NETs in sera of patients with SCD. **a** Quantification of NET release in response to sera from SCD patients in the presence of plasma-derived Hpx. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green+ pixels divided by the total number of pixels × 100) were determined for paired sera from patients with SCD in the presence or absence of 50-μM plasma-purified Hpx. A Wilcoxon matched-pairs signed rank test was used to compare NET release in response to SCD sera in the presence or absence of Hpx. Incubations of neutrophils with HD (n = 6) or SCD sera (n = 18) were performed with neutrophils from 3 different HD for each subject group. n.s. indicates not significant. **b**, **c** Correlations between levels of circulating heme and NET release in sera from SCD patients (n = 17) in the steady state (**b**) and crisis (**c**). A Spearman test was used to calculate correlation coefficients. p = 0.6873 and $^{**}p$ = 0.0011 in the steady state and crisis, respectively.

SCD induce NET release, we exposed healthy donor neutrophils to sera samples. After 3 h, NETs were detected with a cell-impermeable DNA-binding dye (Sytox Green) and imaged with fluorescence microscopy as described previously [15]. No extracellular DNA was released upon incubation of neutrophils with control serum from healthy donor as evidenced by the absence of Sytox Green staining (shown in online suppl. Fig. S1). By contrast, thread-like NET structures that stained positive for Sytox Green became visible upon incubation of neutrophils with SCD serum (shown in Fig. 1e, suppl. Fig. S3). Here, immunostaining of the NET component citrullinated histone H3 confirmed the identity of NETs. NET release induced by factors in SCD sera was highly reproducible, even when neutrophils from different donors were used (shown in online suppl. Fig. S2). In our hands, priming of neutrophils with TNF-α, as was used by Chen and coworkers [7], was not required to trigger NET formation with SCD serum. The surface area covered by NETs was quantified in paired sera obtained during steady state and VOC (shown in Fig. 1f) as previously described [15, 16]. While NETs were also released in some SCD sera obtained during steady state, NET formation was significantly enhanced in sera obtained during VOC (p = 0.0087).

It was recently suggested that the release of NETs in response to free heme may contribute to vaso-occlusion in a mouse model of SCD [7], and the administration of Hpx improved the outcome of TNF-α induced VOC. To evaluate the effect of Hpx on heme and NET formation in human samples, we purified Hpx from human plasma. Plasma-purified Hpx was functional as it reversed hemin-induced cytotoxicity of HEK-293 cells as determined by standard lactate dehydrogenase release (see suppl. Data and online suppl. Fig. S5a). Similarly, Hpx abolished hemin-induced generation of ROS in neutrophils as measured by the conversion of luminol chemiluminescence (see online suppl. Data, online suppl. Fig. S5b). Then, healthy neutrophils were incubated with hemin, and indeed NETs were formed (shown in online suppl. Fig. S5c). Again, positive immunostaining of the NET components neutrophil elastase and citrullinated histone H3 was found within these NETs (shown in online suppl. Fig. S4). In inhibitor studies, we observed minimal release of extracellular DNA from neutrophils stimulated with hemin when equimolar amounts of plasma-derived Hpx were added (shown in online suppl. Fig. S5c). Albumin also binds to heme, although it binds heme with lower affinity than Hpx [17]. When we added hemin in the presence of 1% normal human serum or 0.5% human serum albumin (HSA), the release of NETs was almost completely impaired (shown in online suppl. Fig. S5b, d). NET release induced by hemin or PMA in the presence or absence of either 1% normal human serum or 0.5% HSA was quantified, and we observed that each negated hemin-induced NET formation, while PMA-induced NET formation was unaffected by the presence of albumin (shown in online suppl. Fig. S5e). Thus, it appears that the presence of albumin is sufficient to prevent the interaction of heme with neutrophils and NET forma-

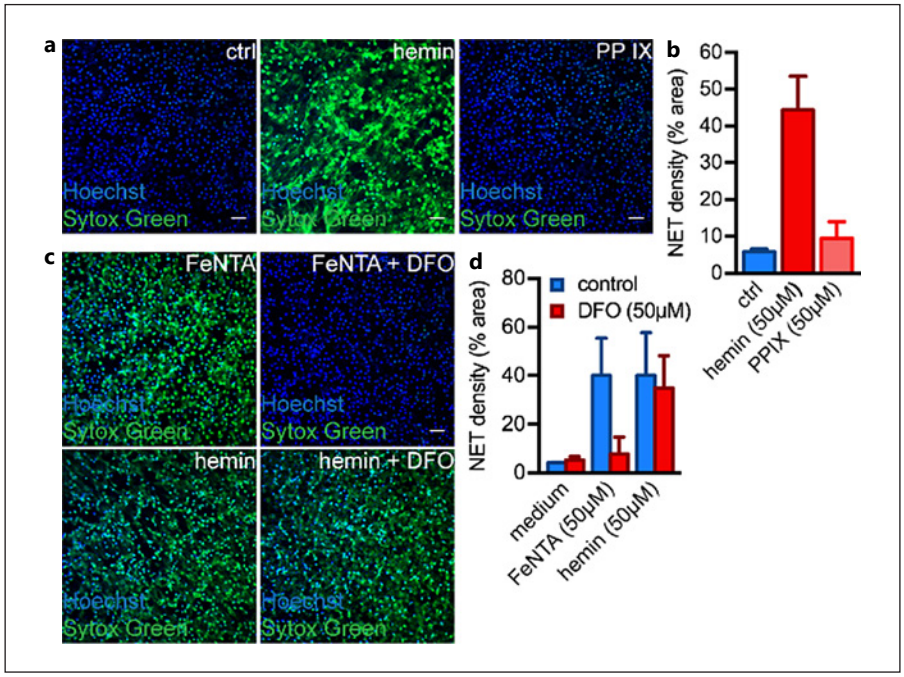

Fig. 3. Iron triggers NET formation, and iron-mediated NET release is blocked by chelation with deferoxamine. **a** Neutrophils isolated from HD were incubated with medium alone (ctrl) or challenged with 50-μM hemin or protoporphyrin IX (PPIX) for 180 minutes. Release of NETs (green in these images) was detected by fluorescence imaging with confocal microscopy using a mixture of 2 DNA-labeling dyes, one cell impermeable (Sytox Green, green) and the other cell permeable (Hoechst 33,342, blue). Depicted are merged images of green and blue fluorescence. All images are representative of 2 independent experiments using neutrophils from different HD. Scale bars, 50 μm. **b** NET formation was quantified after exposure to hemin or PPIX. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green+ pixels divided by the total number of pixels × 100) were determined after the challenge with hemin or PPIX and depicted as mean NET density ± SD in 2 separate experiments. **c** In 2 independent experiments, neutrophils from a HD were exposed to 50-μM FeNTA or hemin in the presence or absence of equimolar amounts of deferoxamine (DFO). After 180 minutes, NETs (green in these images) were visualized with confocal fluorescence microscopy as in panel **a**. Depicted are representative images in which Sytox Green (green) and Hoechst 33,342 (blue) fluorescence are merged. Scale bars, 50 μm. **d** NET release was quantified as in panel b and depicted as mean NET density ± SD (n = 2). Original magnifications ×20 for panels **a**, **c**.

tion. Strikingly, when SCD serum obtained in VOC was preincubated with relatively high levels of Hpx (50 μm) before exposure to neutrophils, NET release was not affected (shown in online suppl. Fig. S5f).

The effect of Hpx supplementation on NET formation was then quantified for all paired SCD sera in our study. Unexpectedly, the addition of high concentrations of Hpx (the normal plasma Hpx concentration is ~ 20 μM) did not alter NET release in the SCD patient sera tested (shown in Fig. 2a). In line with this, high levels of cell-free heme did not positively correlate with the extent of NET formation induced by SCD sera in our cohort (shown in Fig. 2b, c).

In order to investigate the role of iron in the induction of NETs, we compared NET induction of heme to protoporphyrin IX (PPIX) – a porphyrin without iron moiety. Interestingly, PPIX did not induce NET formation suggesting that the iron moiety is required for this process (shown in Fig. 3a, b). We further explored the involvement of iron in NET formation using ferric nitrilotriacetate (Fe^{3+}-NTA). Indeed, incubation of neutrophils with FeNTA triggered NET formation (shown in Fig. 3c, d). In inhibitor studies, FeNTA-induced NET release was prevented with equimolar amounts of iron chelator DFO. Concentrations of 300-μM DFO have previously been shown to provoke NET release by human neutrophils [18]; however, in clinical use, DFO achieves plasma levels of 10 μm in transfusion-dependent patients who receive chelation therapy [19]. At the concentration of 50-μm DFO that we used to supplement sera, we have not observed the formation of NETs. Likewise, DFO had no effect on hemin-induced NET formation since it is not able to remove iron directly from hemin [20, 21] (shown in Fig. 3c, d).

To determine whether iron in the SCD samples in our study may provoke NET release, we tested whether preincubation with DFO or the specific iron-binding protein apotransferrin (apoTf) would affect NET release in SCD sera. We supplemented SCD sera with DFO and apoTf at a concentration of 50 μM, thus, in large excess over the

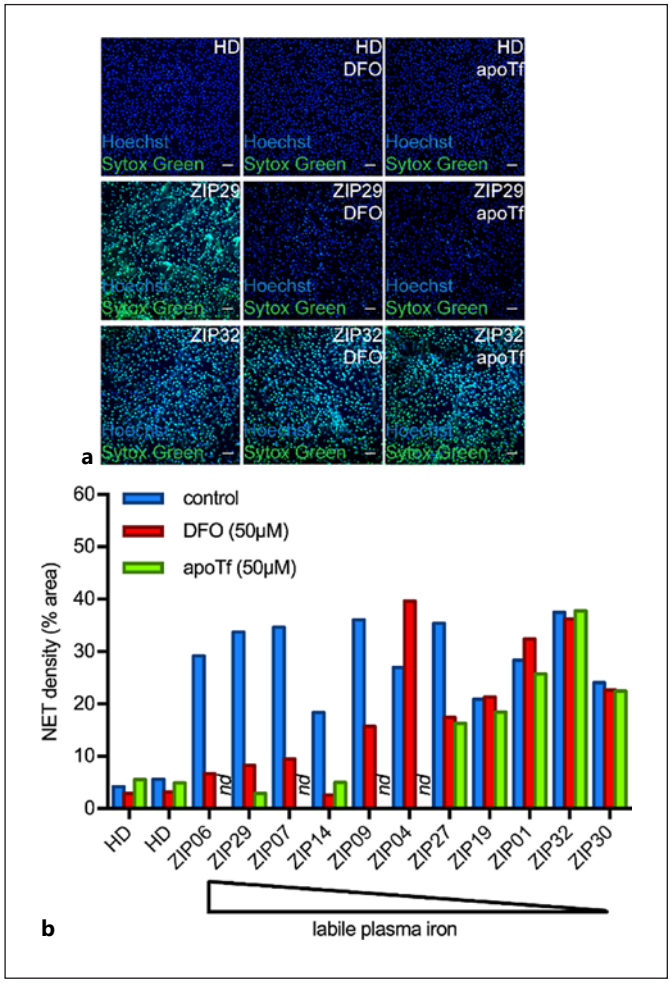

Fig. 4. Iron chelation abrogates NET release in sera from SCD patients. **a** Neutrophils from a HD were exposed to serum from a nonautologous HD or patients with SCD during VOC for 180 minutes in the presence or absence of deferoxamine (DFO, 50 μM) or apotransferrin (apoTf, 50 μM). Release of NETs (green in these images) was visualized with confocal fluorescence microscopy using 2 DNA-labeling dyes, one cell impermeable (Sytox Green), and the other cell permeable (Hoechst 33342). Depicted are merged images of Sytox Green (green) and Hoechst 33342 (blue) fluorescence. All images are representative of experiments performed with sera from 11 different patients. Scale bars, 50 μm. Original magnifications. ×20. **b** Quantification of NET release in response to sera from SCD patients in the presence of iron chelators. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green$^+$ pixels divided by the total number of pixels × 100) were determined for sera from patients with SCD during VOC in the presence or absence of 50-μM DFO or apoTf. Incubations of neutrophils with SCD sera (n = 11) were performed with neutrophils from 3 different HD for each subject group. n.d. indicates not determined.

concentrations of LPI. For these experiments, we focused on SCD patient sera obtained during VOC that had given rise to NETs shown in Figures 1 and 2. The addition of DFO largely abolished (ZIP06, 07, 14, 29) or partly inhibited (ZIP09, 27) the release of NETs in more than half of the sera of SCD patients tested (shown in online suppl. Fig. S5a, b). When DFO was added to the other sera, no effect was observed. Thus, it appears that iron provokes NET release in a significant proportion of SCD patient sera but not all. Indeed, upon ranking the patients according to the level of LPI, it became apparent that DFO addition affected NET release in those sera with high iron (shown in Fig. 4a). As DFO may bind both extra- and intracellular iron, and intracellular iron may directly influence ROS generation and potentially NET formation; we also tested the effect of apoTf on NET formation in SCD sera as it acts on extracellular iron. Unfortunately, we did not have sufficient serum left of all patients in our study to also screen for the effect of apoTf addition. Interestingly, for the samples that have some material left, we observed that apoTf addition appeared to match the effect of DFO on NET release (shown in Fig. 4b). These results indicate that, at least in a subset of patients with SCD, NET release may be induced through the presence of extracellular iron in the blood.

Conclusion

In this study, we reveal a novel role for iron in the circulation of patients with SCD. Our study highlights that the systemic redox-active iron may form an important trigger for neutrophil activation and NET formation in SCD. In our experiments, supplementation of sera from SCD patients with the heme-scavenger Hpx did not prevent the release of NETs from healthy donor neutrophils. By contrast, the addition of DFO or apoTf to scavenge-free iron abolished NET release in a significant proportion of SCD sera tested.

In a number of murine models of hemolytic diseases, including SCD and β-thalassemia, heme induced inflammation and tissue damage, an effect that was prevented by injection of heme scavengers [7, 10, 11, 22]. As such, the administration of Hpx has previously shown great potential as novel therapeutic drug. Vinchi et al. [22] showed a beneficial effect of Hpx administration in SCD mice, as it reduced endothelial activation induced by heme. Moreover, in another study, it was shown that hemin injection in SCD mice induced the development of acute chest syndrome and that both TLR4 inhibition and Hpx administration prevented acute chest syndrome development [10]. Of interest, Vinchi et al. [22] have recently shown that hemin-challenged Hpx knockout mice showed signs of heme accumulation in macrophages, and phenotype switching to a pro-inflammatory M1-like phenotype was observed. The latter effect was also found in SCD mice and prevented by Hpx administration [11]. Worth noting, in SCD animal models signs of VOC are often induced by injection of exogenous free hemin. However,

during in vivo hemolysis it is uncertain whether heme, which is a highly hydrophobic molecule, exists as a free form in plasma as it is rapidly sequestered by plasma proteins and lipids [23]. It thus seems plausible that Hpx administration shows greatest benefit in models with administered free hemin where concentrations of free heme are a least transiently increased. By contrast, administration of Hpx to TNF-α-treated SCD mice to lower plasma heme levels prevented pulmonary NET release and ameliorated-associated hypothermia [7]. However, we and others [7] have shown that albumin can efficiently prevent NET formation in response to hemin in vitro, and it remains to be elucidated whether treatment of TNF-α-treated mice with albumin would have similar effects on the phenotype of these mice. Indeed, studies on mice lacking Hpx reinforce the concept of redundancy, overlap, and backup in heme transport provided by Hpx, haptoglobin, and albumin. In the presence of normal albumin levels, Hpx-null mice exhibit a healthy state and a lack of general organ damage [24]. Another important consideration is that heme induces various pro-inflammatory effects other than neutrophil activation and NET formation through interactions with other cells, including effects of heme on macrophages and endothelial cells. Secondary anti-inflammatory effects of Hpx that go beyond the scavenging of heme have also been described [25]. Here, we show that increased levels of circulating heme in patients with SCD do not directly promote NET release but that NET release requires the iron moiety.

The observed role of plasma iron in neutrophil activation is in line with previous in vitro studies, although none of these studies were performed in the context of SCD or investigated LPI in the circulation. Interestingly, Kono et al. [26] have shown that the addition of deferasirox, an iron chelating agent, to neutrophils prevented PMA- or fMLP-mediated ROS production and NET formation in vitro [26, 27]. Saha et al. [27] have shown that this suppressive effect of deferasirox is through chelation of intracellular labile iron that is required for neutrophil oxidative responses and NET release, and can be mimicked by enterobactin, a siderophore expressed by *Escherichia coli* [28]. Previous results had indicated that hemin induces TLR4 signaling to drive pro-inflammatory responses [29, 30]. Recently, however, hemin was shown to provoke NET release in a manner that depends on NADPH oxidase activity and ROS generation but does not require TLR4 signaling [31]. Taken together, these results suggest that heme does not interact with TLR4 to activate neutrophils for NET release but rather that the redox activity of the iron moiety in heme underlies heme-induced NET formation. Indeed, we show that exposure of neutrophils to hemin but not PPIX that triggered the release of NETs, and that the addition of exogenous iron sources, such as FeNTA leads to NET formation.

The recent literature suggests that free, redox-active iron is readily available in the circulation of patients with SCD. Levels of systemic iron were recently found to be elevated in patients with SCD, even in steady state. Consistently, we observed high levels of circulating heme and redox-active iron, which is associated with low Hpx in patients with SCD in steady state. Iron overload is well known to occur in chronically transfused SCD patients who receive prophylactic red-cell transfusions [32–35]. However, patients who have received a blood transfusion in 3 months prior to the development of VOC were excluded from inclusion in our study cohort. Nevertheless, high LPI was found in several SCD patients in our cohort. Thus, it appears that increased LPI levels are caused by continuous hemolysis and thus may also occur in patients who are not on a chronic transfusion scheme [35]. Recently, however, it was shown that chronic hemolysis in SCD mice maintained enhanced iron export and higher levels of circulating iron compared to normal mice [36]. Indeed, excessive release of heme facilitates the export of cellular iron by ferroportin [37–39], and intracellular iron levels are decreased in peripheral blood mononuclear cells from SCD patients [40].

Our results show that the ex vivo addition of the iron scavengers DFO and apoTf limited the NET-inducing effect of labile iron present in a significant proportion of SCD sera tested. The concentration of DFO used was based on a previous report where levels of up to 10-μm DFO were detected in vivo [19]. Clearly, iron chelation did not prevent NET release in all samples from SCD patients, and it is possible that other plasma factors could be involved in NET formation in DFO-insensitive patients. Complement activation, IL-8, and urate crystals are established NET inducers that we hypothesize to play a role in these DFO-insensitive SCD patient samples [15, 41–47].

Our study harbors some limitations that need to be addressed: first, the observation on iron as a trigger of NET formation in SCD is restricted to a small patient group. Second, it also remains unclear why specific iron neutralization only prevented NET formation in a part and not all of the sickle cell patients investigated. This suggests that besides iron other inflammatory mediators (e.g., complement activation products, IL-8, or urate crystals) present in the serum are responsible for NET induction of these patients. Investigating the NET-forming capacity of the serum of sickle cell patients before starting therapeutic iron chelation compared to serum samples when on therapy may give more insights on the clinical relevance of our findings. The effect of iron neutralization on NET formation should further be explored in a mouse model. Further research is required to determine which stimuli induced NET release in patient sera in which targeting iron was less or not effective.

In summary, we show that labile iron plays a role in NET formation in a subset of sera from a cohort of SCD patients and that iron chelation prevents NET formation. Extrapolation to a larger study requires experimental validation. Future studies aimed at validating the therapeutic efficacy of iron chelation therapy as potential novel therapy for VOC in patients with SCD are warranted. As DFO is widely used in patients with chronic iron overload disorders and transfusional iron overload, its use would form a readily available treatment strategy to prevent neutrophil activation, dampen formation of NETs, and possibly the development of VOC.

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Statement of Ethics

The study protocol was approved by the Medical Ethical Committee of the participating centers and conducted in agreement with the Helsinki declaration. Written consent was obtained from each participant or their legal guardian.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

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Author Contributions

Kristof van Avond, Brenda Luken, and Sacha Zeerleder conceived and designed the study, and performed data analysis and interpretation. Kristof van Avond, Ingrid Bulder, and Gerard van Mierlo performed experiments. Marein Schmimmel, Erfan Nur, and Bart Biemond coordinated the collection of patient material. Robin van Bruggen and Sacha Zeerleder obtained grant funding. Kristof van Avond wrote the manuscript. Marein Schimmel, Erfan Nur, Bart Biemond, Brenda Luken, Robin van Bruggen, and Sacha Zeerleder critically reviewed the manuscript. All authors approved the final version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to authors.

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