Original Paper

Stimulation of Erythrocyte Cell Membrane Scrambling by C-Reactive Protein

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Key Words

Phosphatidylserine • Calcium • Cell volume • Eryptosis • Inflammation • C-reactive protein • Appendicitis

Abstract

Background: Eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phosphatidylserine-translocation, is triggered by fever and inflammation. Signaling includes increased cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$), caspase activation, and ceramide. Inflammation is associated with increased plasma concentration of C-reactive protein (CRP). The present study explored whether CRP triggers eryptosis. **Methods:** Phosphatidylserine abundance at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, ceramide abundance and caspase-3-activity utilizing FITC-conjugated antibodies. Moreover, blood was drawn from patients with acute appendicitis (9♀, 11♂) and healthy volunteers (10♀, 10♂) for determination of CRP, blood count and phosphatidylserine. **Results:** A 48h CRP treatment significantly increased the percentage of annexin-V-binding cells ($\geq 5\mu\text{g/ml}$), $[\text{Ca}^{2+}]_i$ ($\geq 5\mu\text{g/ml}$), ceramide (20 $\mu\text{g/ml}$) and caspase-activity (20 $\mu\text{g/ml}$). Annexin-V-binding was significantly blunted by caspase inhibitor zVAD (10 μM). The percentage of phosphatidylserine-exposing erythrocytes in freshly drawn blood was significantly higher in appendicitis patients ($1.83 \pm 0.21\%$) than healthy volunteers ($0.81 \pm 0.09\%$), and significantly higher following a 24h incubation of erythrocytes from healthy volunteers to patient plasma than to plasma from healthy volunteers. The percentage of phosphatidylserine-exposing erythrocytes correlated with CRP plasma concentration. **Conclusion:** C-reactive protein triggers eryptosis, an effect at least partially due to increase of $[\text{Ca}^{2+}]_i$, increase of ceramide abundance and caspase activation.

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Introduction

Upon injury, erythrocytes may undergo suicidal death or eryptosis, which is characterized by cell shrinkage [1] and translocation of phosphatidylserine from the cell interior to the erythrocyte surface [2-4]. Cellular mechanisms triggering eryptosis include oxidative stress [2], increased cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [2], ceramide [5], energy depletion [2], caspases [2, 6, 7] and the G-protein subunit $\text{G}\alpha_{i2}$ [8]. Moreover, eryptosis may

be stimulated following activation of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and/or p38 kinase [2]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, and PAK2 kinase [2]. Eryptosis is stimulated by a wide variety of small molecules [2, 9-63]. Moreover, eryptosis is enhanced in elderly individuals [64]. It is sensitive to erythrocyte age [65] and enhanced eryptosis is observed following erythrocyte storage [66, 67].

Eryptotic erythrocytes are bound to and subsequently engulfed by macrophages and thus rapidly cleared from circulating blood [2, 68]. Accordingly, excessive eryptosis may lead to anemia [2]. Moreover, eryptotic erythrocytes may bind to endothelial cells of the vascular wall [69], trigger blood clotting and induce thrombosis [70-72], thus interfering with microcirculation [5, 70, 73-76].

A variety of clinical conditions are paralleled by stimulated eryptosis [2-4, 77-84] including fever [85]. Fever is a common complication of appendicitis [86], which is paralleled by an increase of C-reactive protein abundance in plasma [87, 88]. Enhanced CRP plasma levels are valuable markers of inflammation [89, 90]. C-reactive protein contributes to the host reaction against pathogens and participates in the orchestration of inflammation by interaction with endothelial cells, endothelial progenitor cells, leukocytes and platelets [90]. The inflammatory response is accomplished by pentameric C-reactive protein (pCRP) [89]. C-reactive protein has been shown to trigger suicidal death or apoptosis of nucleated cells [91] and could thus, at least in theory, similarly stimulate eryptosis.

The present study addressed the impact of C-reactive protein on eryptosis. To this end, erythrocytes drawn from healthy individuals were exposed to C-reactive protein and $[Ca^{2+}]_i$, ceramide abundance and caspase activity were determined. Moreover, blood was drawn from patients with acute appendicitis or erythrocytes from healthy individuals exposed to plasma from appendicitis patients and eryptotic erythrocytes identified by determination of phosphatidylserine exposure.

Materials and Methods

Patients, erythrocytes and treatments

Blood was drawn from untreated patients diagnosed with acute appendicitis (11 ♀, 9 ♂, age range 22-76 years) and healthy volunteers (10 ♀, 10 ♂, age 22-69 years). The patients were recruited 2012 and 2013 from the Department of General, Visceral and Transplant Surgery, Tübingen, Germany. The study was approved by the ethics committee of the University of Tübingen (184/2003V). Both, patients and healthy volunteers provided written informed consent. Measurements were made in freshly isolated erythrocytes or in erythrocytes (O⁻ blood group) from healthy young individuals incubated *in vitro* with fresh plasma from either patients or healthy volunteers. Alternatively, fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. To isolate erythrocytes, blood was centrifuged at 120 g for 20 min at 23 °C, the platelets and leukocytes-containing supernatant was disposed, and the erythrocyte pellet washed once with Ringer solution. Erythrocytes were incubated *in vitro* for 24 hours at a hematocrit of 0.4% in plasma from patients or healthy volunteers or in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 CaCl₂; the pH was adjusted to 7.4 and the temperature kept at 37°C. The low hematocrit was chosen to minimize mutual erythrocyte interaction. Where indicated, erythrocytes were exposed to C-reactive protein (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental conditions, 150 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl₂ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Intracellular Ca^{2+}

After incubation under the respective experimental conditions, 150 μl cell suspension was washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 5 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Ceramide abundance

To determine ceramide abundance, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Confocal microscopy

For the visualisation of eryptotic erythrocytes, 20 μl of erythrocyte suspension were incubated in respective experimental conditions and stained with Annexin-V-FLUOS (1:100 dilution; Roche Life Sciences, Germany) in 200 μl Ringer solution containing 5 mM CaCl_2 . Then the erythrocytes were washed twice and finally resuspended in 100 μl Ringer solution. A volume of 40 μl cell suspension was smeared onto glass slides. After 15 min drying at RT, the slides were mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) and covered with a coverslip. Images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with water immersion Plan-Neofluar 40/1.3 NA DIC.

Caspase activity

Abundance of active caspase-3 was detected according to the manufacturer's instruction of CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA). Briefly, cells were treated as mentioned before including a negative control with 10 μM Z-VAD-FMK. Subsequently 150 μl cell suspensions were transferred to a 96-well plate, centrifuged at 1600 rpm for 4 min, and washed once with wash buffer solution followed by staining with 1 μl FITC-DVD-FMK for 1 h at 37°C. Stained cells were washed once, resuspended in 200 μl wash buffer and measured immediately with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Statistics

Data are expressed as arithmetic means $\pm$ SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored whether C-reactive protein could trigger eryptosis of erythrocytes drawn from healthy volunteers. As illustrated in Fig. 1A,B, a 24 h exposure of erythrocytes isolated from healthy volunteers to C-reactive protein increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 5 $\mu\text{g}/\text{ml}$ C-reactive protein. The exposure to C-reactive protein did not significantly modify erythrocyte forward scatter (Fig. 1C,D). In order to test whether C-reactive protein interferes with erythrocyte shrinkage following increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), erythrocytes

Fig. 1. Effect of C-reactive protein on phosphatidylserine exposure and forward scatter. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml C-reactive protein. B. Arithmetic means $\pm$ SEM (n = 16) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of C-reactive protein (1–20 µg/ml). C. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 20 µg/ml C-reactive protein. D. Arithmetic means $\pm$ SEM (n = 16) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) C-reactive protein (1–20 µg/ml). * (p < 0.05), *** (P < 0.001) indicate significant difference from the absence of C-reactive protein (ANOVA). E. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (solid line) presence of 20 µg/ml C-reactive protein alone, with (red dotted line) presence of 1 µM ionomycin alone and with (blue dotted line) presence of 20 µg/ml C-reactive protein and 1 µM ionomycin. F. Arithmetic means $\pm$ SEM (n = 16) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) C-reactive protein (20 µg/ml) in the absence (left bars) and presence (right bars) of ionomycin (1 µM). ** (p < 0.01), *** (P < 0.001) indicate significant difference from the absence of C-reactive protein, ### (P < 0.001) indicate significant difference from the absence of ionomycin (ANOVA).

were treated with Ca^{2+} ionophore ionomycin in the absence and presence of C-reactive protein. As illustrated in Fig. 1E,F, ionomycin decreased forward scatter, an effect significantly blunted in the presence of C-reactive protein.

As illustrated in Fig. 2, the effect of C-reactive protein on phosphatidylserine exposure was paralleled by increase of $[\text{Ca}^{2+}]_i$. A 48 h exposure of erythrocytes isolated from healthy volunteers to C-reactive protein increased the Fluo3 fluorescence (Fig. 2A,B), an effect reaching statistical significance at 5 µg/ml C-reactive protein. The effect of C-reactive protein on annexin-V-binding was significantly blunted in the absence of extracellular Ca^{2+} (Fig. 2C,D).

Moreover, a 48 h exposure of erythrocytes isolated from healthy volunteers to C-reactive protein (20 µg/ml) increased the ceramide abundance at the erythrocyte surface (Fig. 3A,B).

As illustrated in Fig. 4A,B, a 48 h exposure of erythrocytes isolated from healthy volunteers to C-reactive protein (20 µg/ml) was paralleled by a slight, but significant increase

Fig. 2. Effect of C-reactive protein on erythrocyte Ca^{2+} activity and Ca^{2+} sensitivity of C-reactive protein induced Annexin V binding. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of C-reactive protein (20 $\mu\text{g}/\text{ml}$). B. Arithmetic means $\pm$ SEM ($n = 16$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) C-reactive protein (1-20 $\mu\text{g}/\text{ml}$). C. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (solid line) presence of C-reactive protein (20 $\mu\text{g}/\text{ml}$) and presence of extracellular Ca^{2+} as well as with (dotted line) presence of C-reactive protein (20 $\mu\text{g}/\text{ml}$) and absence of extracellular Ca^{2+} . D. Arithmetic means $\pm$ SEM ($n = 16$) of erythrocyte annexin-V-binding following incubation for 48 h to Ca^{2+} containing Ringer solution without (white bar) or with (black bar) C-reactive protein (20 $\mu\text{g}/\text{ml}$) as well as with (grey bars) C-reactive protein (20 $\mu\text{g}/\text{ml}$) in absence of extracellular Ca^{2+} . ***($P < 0.001$) indicate significant difference from the absence of C-reactive protein, ## ($P < 0.01$) indicate significant difference from the absence of extracellular Ca^{2+} (ANOVA).

of caspase-3 activity. The effect of 20 $\mu\text{g}/\text{ml}$ C-reactive protein on Annexin-V-binding was significantly blunted in the presence of 10 μM caspase inhibitor zVAD (Fig. 4C,D). However, even in the presence of zVAD, C-reactive protein still significantly increased Annexin-V-binding (Fig. 4D).

In order to test whether the stimulation of eryptosis by C-reactive protein is reflected by the respective modification of eryptosis in inflammatory disorders, blood was drawn from patients with acute appendicitis. As illustrated in Fig. 5, the percentage of erythrocytes exposing phosphatidylserine at the erythrocyte surface was significantly higher in freshly drawn blood from appendicitis patients than in freshly drawn blood from healthy volunteers. Thus, the percentage of phosphatidylserine exposing erythrocytes was enhanced in patients with acute appendicitis.

In order to determine whether the stimulation of phosphatidylserine translocation was due to a plasma-borne factor, erythrocytes isolated from healthy volunteers were exposed to plasma drawn from healthy volunteers or from patients. As shown in Fig. 6, the percentage of erythrocytes isolated from healthy volunteers was significantly higher following a 24 h exposure to plasma from appendicitis patients than following a 24 h exposure to plasma from healthy volunteers. Thus, a plasma-borne component contributed to or even accounted for the triggering of phosphatidylserine exposure during appendicitis.

Fig. 3. Effect of C-reactive protein on ceramide abundance. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of C-reactive protein (20 µg/ml). B. Arithmetic means $\pm$ SEM (n = 16) of ceramide abundance (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) C-reactive protein (20 µg/ml). * (p<0.05), ** (P<0.01), *** (P<0.001) indicates significant difference from the absence of C-reactive protein (ANOVA).

Fig. 4. Effect of C-reactive protein on erythrocyte caspase-3 activity and the influence of caspase inhibitor zVAD on stimulation of annexin-V-binding by C-reactive protein. A. Original histogram of Caspase-3 activity in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of C-reactive protein (20 µg/ml). B. Arithmetic means $\pm$ SEM (n = 16) of the caspase-3 activity (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) C-reactive protein (20 µg/ml). *** (P<0.001) indicates significant difference from the absence of C-reactive protein. C. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with presence of 20 µg/ml C-reactive protein alone (solid line) or together with caspase inhibitor Z-VAD-FMK (dotted line). D. Arithmetic means $\pm$ SEM (n = 16) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with presence of C-reactive protein (20 µg/ml) alone (black bar) and together with caspase inhibitor Z-VAD-FMK (grey bar). ** (P<0.01), *** (P<0.001) indicates significant difference from the absence of C-reactive protein, # (p<0.05), indicates significant difference from the absence of caspase inhibitor Z-VAD-FMK (ANOVA).

Fig. 5. Phosphatidylserine-exposure of erythrocytes drawn from patients with appendicitis and healthy volunteers. A. Confocal images of human erythrocytes stained with Annexin-V-FLUOS (upper panels) and corresponding light microscopy images (lower panels). The specimens were obtained from a healthy volunteer (left panels) and from a patient with appendicitis (right panels). B. Original histogram of Annexin V-binding of erythrocytes in freshly drawn blood from healthy volunteers (grey area) and from patients (black line). C. Arithmetic means ± SEM (n = 9 ♀, 11 ♂) of the percentage of Annexin V-binding erythrocytes in freshly drawn blood from healthy volunteers (Control, white bar) and patients (black bar). *** (p<0.001) indicates significant difference from healthy volunteers.

As illustrated in Fig. 7, a highly significant (p<0.001) correlation was observed between C-reactive protein concentration in plasma and the percentage of phosphatidylserine exposing erythrocytes. This observation suggests that C-reactive protein triggers phosphatidylserine translocation or correlates with another parameter stimulating phosphatidylserine translocation.

Accordingly, hemoglobin concentration (Fig. 8A), erythrocyte count (Fig. 8B) and hematocrit (Fig. 8C), but not erythrocyte volume (Fig. 8D) were slightly but significantly lower in appendicitis patients with high levels of C-reactive protein than in appendicitis patients with normal levels of C-reactive protein.

Discussion

The present observations disclose that C-reactive protein triggers phosphatidylserine translocation in erythrocytes, a key feature of eryptosis, the suicidal erythrocyte death. Exposure of erythrocytes drawn from healthy volunteers to C-reactive protein at concentrations prevailing in plasma from patients with acute appendicitis is followed by significant stimulation of phosphatidylserine translocation.

The C-reactive protein induced phosphatidylserine translocation is paralleled by and at least in part due to increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i). The increase of [Ca²⁺]_i were further expected to activate Ca²⁺ sensitive K⁺ channels, K⁺ exit, cell membrane hyperpolarization, Cl⁻ exit and thus cellular loss of KCl with osmotically obliged water [1]. However, C-reactive protein did not significantly modify erythrocyte forward scatter. Possibly, C-reactive protein, besides increasing [Ca²⁺]_i, interferes with cell volume regulation of erythrocytes. Along those lines, the cell shrinkage following exposure to Ca²⁺ ionophore ionomycin was significantly blunted in the presence of C-reactive protein.

Fig. 6. Phosphatidylserine-exposure of erythrocytes drawn from healthy volunteers and exposed 24 hours to plasma from either patients with appendicitis or healthy volunteers. A. Confocal images of human erythrocytes stained with Annexin-V-FLUOS (upper panels) and corresponding light microscopy images (lower panels). The erythrocytes were obtained from a healthy volunteer and exposed 24 hours to plasma from a healthy volunteer (left panels) and to plasma from a patient with appendicitis (right panels). B. Original histogram of Annexin V-binding of erythrocytes in blood drawn from healthy volunteers exposed for 24 h to plasma from healthy volunteers (grey area) or to plasma from patients (black line). C. Arithmetic means $\pm$ SEM ($n = 9$ ♀, 11 ♂) of the percentage of Annexin-V-binding erythrocytes drawn from healthy volunteers and exposed for 24 h to plasma from healthy volunteers (Control, white bar) or from patients (black bar). ***($p < 0.001$) indicates significant difference from healthy volunteers.

Fig. 7. Annexin-V-binding as a function of C-reactive protein concentration. The percentage annexin-V-binding erythrocytes as a function of C-reactive protein concentration in freshly drawn blood from healthy volunteers (white circles) and patients (black circles).

The effect of C-reactive protein on phosphatidylserine translocation was further paralleled by increase of ceramide abundance, which triggers cell membrane scrambling even at normal $[Ca^{2+}]_i$ [2]. Moreover, C-reactive protein slightly triggers caspase activation. The partial inhibition of phosphatidylserine translocation by caspase inhibitor zVAD suggests that the effect of C-reactive protein on cell membrane scrambling is partially but not fully due to caspase activation.

Considering the *in vitro* effects of C-reactive protein, it must be kept in mind that *in vivo* C-reactive protein may interact with other plasma proteins thus modifying its effects. Nevertheless, acute appendicitis is associated with increase of annexin-V-binding reflecting erythrocyte cell membrane scrambling with phosphatidylserine translocation from the cell interior to the erythrocyte surface. The paper further reveals that eryptosis is triggered by a plasma borne component, as it is stimulated by exposure of erythrocytes drawn from healthy

Fig. 8. Blood hemoglobin concentration, erythrocyte count, hematocrit and erythrocyte volume in blood drawn from female and male appendicitis patients with normal or high C-reactive protein. A-D. Arithmetic means $\pm$ SEM ($n = 249$ ♀, 224 ♂) of the hemoglobin concentration (A), erythrocyte number (B), hematocrit (C) and erythrocyte volume (D) in female (left bars) and male (right bars) patients with low (white bars) and high (black bars) levels of C-reactive protein (CRP) in plasma. * ($p < 0.05$), ** ($P < 0.01$), indicates significant difference from low levels of C-reactive protein.

volunteers to plasma drawn from patients with acute appendicitis. Moreover, the percentage of phosphatidylserine exposing erythrocytes significantly correlated with the plasma concentration of C-reactive protein. Collectively those observations strongly suggest that C-reactive protein contributes to the stimulation of eryptosis in appendicitis patients.

Eryptosis serves to clear defective erythrocytes in order to prevent hemolysis with release of hemoglobin, which may be filtered at renal glomerula, precipitate in the acidic lumen of renal tubules and thus occlude affected nephrons [92]. To the extent that eryptosis exceeds the formation of new erythrocytes eryptosis may lead to anemia [2]. Moreover, phosphatidylserine exposing erythrocytes may interfere with microcirculation by binding to the vascular wall [69], triggering of blood clotting and induction of thrombosis [70-72], thus interfering with microcirculation [5, 70, 73-76].

In conclusion, appendicitis is paralleled by erythrocyte cell membrane scrambling with phosphatidylserine exposure to the erythrocyte surface. The effect is at least partially due to increase C-reactive protein, which increases cytosolic Ca^{2+} activity and ceramide formation in erythrocytes. As several inhibitors of eryptosis are available [2-4, 93, 94], eryptosis and subsequent development of anemia may be prevented.

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Disclosure Statement

The authors have declared no conflict of interest.

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