Original Paper

Reduced Erythrocyte Survival in Uremic Patients Under Hemodialysis or Peritoneal Dialysis

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Key Words

Anemia • End-stage renal disease • Eryptosis • Hemodialysis • Peritoneal dialysis

Abstract

Background/Aims: Recent observations in end-stage renal disease (ESRD)-patients on hemodialysis revealed that anemia is, in part, due to stimulated suicidal erythrocyte death or eryptosis leading to accelerated clearance of circulating erythrocytes. The present study explored whether eryptosis is similarly enhanced in patients on peritoneal dialysis (PD). **Methods:** Measurements were made in freshly drawn erythrocytes from healthy volunteers (n=38), and ESRD patients on hemodialysis (HD; n=18) or on PD (n=22). Both, HD patients and PD patients suffered from anemia despite increased reticulocyte numbers. **Results:** The percentage of phosphatidylserine-exposing erythrocytes was significantly higher in HD patients than in healthy volunteers and significantly higher in PD patients than in healthy volunteers and HD patients. In PD patients, the percentage of phosphatidylserine-exposing erythrocytes was positively correlated with dialysis volume. The increase in phosphatidylserine exposure was in both, HD and PD patients, paralleled by increase of reactive oxygen species and ceramide abundance. In both, HD and PD patients, a positive correlation was observed between the percentage of phosphatidylserine-exposing erythrocytes and both, erythropoietin dosage and the percentage of reticulocytes. **Conclusions:** Similar to HD patients, PD patients suffer from enhanced eryptosis, which is paralleled by oxidative stress and enhanced ceramide abundance contributing to the anemia of uremic patients.

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Introduction

In end-stage renal disease (ESRD), the decreased renal erythropoietin (EPO) release leads to a subsequent decrease of erythropoiesis [1-9] and thus to anemia [10, 11]. In ESRD, erythropoiesis is further compromised by iron deficiency [4, 6, 12-15].

As shown in patients on hemodialysis (HD) [16-18], anemia in ESRD results in part from stimulation of suicidal erythrocyte death or eryptosis, leading to accelerated clearance of circulating erythrocytes [19]. Eryptosis is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface [20, 21]. Defective and aged erythrocytes are cleared from circulating blood [20, 22]. Thus, accelerated erythrocyte death may lead to anemia [20]. Cellular mechanisms involved in the triggering of eryptosis include increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), oxidative stress and ceramide formation [20, 21, 23]. The enhanced eryptosis in patients on HD was, at least in part, due to a plasma borne component increasing $[\text{Ca}^{2+}]_i$, reactive oxygen species (ROS), and ceramide abundance [18].

Peritoneal dialysis (PD) is an equivalent yet under-utilised modality of renal replacement therapy with similar outcome compared to HD. The prevalence and severity of renal anemia tends to be lower in PD patients [24] and was associated with a lower EPO dose to maintain similar Hb values compared to HD patients [25]. However, the relevance of eryptosis in the aggravation of renal anemia in PD patients remained illdefined. The present study explored, whether eryptosis similarly contributes to anemia in patients on peritoneal dialysis (PD) and whether the rate and mechanisms of eryptosis differ between HD and PD.

Patients and Methods

Patients, erythrocytes and treatments

Eighteen patients (11 male, 7 female) on hemodialysis (HD) and 22 patients (11 male, 11 female) on peritoneal dialysis (PD) have been enrolled in the study. Clinical details of the patients are compiled in Table 1. The patients were recruited from the Division of Nephrology at the Department of Internal Medicine, University Hospital Tübingen. The blood from HD patients was drawn prior to a 4-h hemodialysis session on a Fresenius 5008 dialysis machine. Blood from PD patients was drawn at a regular outpatient visit between 9 and 12 am. Blood samples for HD and PD patients as well as blood from healthy volunteers were drawn on the same day. The study was approved by the ethics committee of the University of Tübingen (184/2003V) and has been executed in accordance with the Declaration of Helsinki. Both, volunteers and patients provided informed written consent. Eryptosis measurements were made in freshly isolated erythrocytes from the three groups HD, PD and healthy volunteers in parallel. For all measurements using FACS analysis, 50,000 cells were counted.

Annexin V-binding and forward scatter

In order to determine annexin-V-binding, 2 μl of freshly drawn blood were mixed in 500 μl Ringer solution containing 5 mM CaCl_2 , subsequently stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in Ringer solution containing 5 mM CaCl_2 at 37°C for 15 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for healthy erythrocytes and erythrocytes from patients on hemodialysis and peritoneal dialysis. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters.

Intracellular Ca^{2+}

In order to quantify intracellular Ca^{2+} , 2 μl freshly drawn blood was mixed in 500 μl Ringer solution containing 5 mM CaCl_2 , stained with Fluo-3/AM (5 μM ; Biotium, Hayward, USA) and incubated at 37°C for 30 min. Then Ca^{2+} -dependent fluorescence intensity was measured in FL-1 channel with an excitation

Table 1. Characteristics of the patients. Values are given as median with interquartile range (IQR, 25th-75th percentile).

	Hemodialysis	Peritoneal dialysis	P-value*
Number and gender of patients	18 (11 ♂, 7 ♀)	22 (11 ♂, 11 ♀)	0.3023
Age	68.5 (55.8; 76.5) years (range: 47-79)	49.0 (38.8; 63.8) years (range: 31-87)	0.0077**
Body mass index	23.2 (21.3; 28.8)	24.6 (21.1; 28.6)	0.9892
Cause of CKD	Diabetic-Hypertensive nephropathy (n=5) Hypertension (n=3) Glomerulonephritis (n=1) Polycystic kidney disease (n=1) Other/unknown (n=8)	Diabetic nephropathy (n=1) Diabetic-Hypertensive nephropathy (n=3) Hypertension (n=3) Glomerulonephritis (n=6) Polycystic kidney disease (n=3) Other/unknown (n=7)	
Time on dialysis (months)	33.0 (7.50; 78.0)	20.0 (12.75; 35.0)	0.4611
Mode of dialysis	22% HD, 78% HDF 4 hours; 280-320 ml/min	41% CAPD, 59% APD Median dialysate volume 10.5 l (4.5; 11.2); Median glucose concentration 2.03 g/l (1.7; 2.3)	
ESA dose (I.U.)	3125 (401; 6000)	4583 (1114; 6579)	0.3904
Plasma creatinine concentr. (mg/100 ml)	6.75 (5.12; 8.42)	7.62 (5.22; 9.95)	0.4967
Plasma c-reactive protein (mg/100 ml)	1.15 (0.42; 2.79)	0.9 (0.34; 2.14)	0.4629
Leucocytes (/μl)	7229 (5544; 8638)	8688 (6580; 9001)	0.1458
Plasma urea concentration (mg/100 ml)	104.3 (74.9; 127.4)	102.5 (84.38; 124.7)	0.8597
Plasma uric acid concentr. (mg/100 ml)	6.55 (5.38; 7.70)	6.1 (5.05; 6.69)	0.3510
Plasma iron concentration (μg/100 ml)	53.75 (40.50; 84.0)	59.5 (47.25; 89.63)	0.5772
Plasma ferritin concentration (μg/100 ml)	44.5 (24.5; 68.0)	18.5 (9.15; 41.13)	0.0051**
Transferrin (mg/100 ml)	168.3 (138.6; 197.8)	223 (194.0; 245.6)	0.0020**
Transferrin saturation (%)	25.6 (15.58; 33.7)	19.1 (15.58; 27.15)	0.2369
Blood count			
hematocrit	31.31 (27.2; 33.32)	32.04 (30.26; 34.91)	0.2264
hemoglobin	10.07 (9.17; 11.22)	10.89 (10.16; 11.61)	0.0794
reticulocytes (%)	2.66 (1.95; 3.42)	2.23 (1.56; 3.29)	0.5229
Erythropoietin/hemoglobin	331.5 (47.5; 540.0)	429 (98.5; 731.0)	0.4213
Residual diuresis (ml/24 h)	450 (75; 1188)	904.2 (200; 1513)	0.1237
Concurrent medication (Dialysis specific medication)	beta-blockers (n=13), AT-1 blockers (n=5), ACE- inhibitors (n=5), calcium antagonists (n=7), anticoagulants (n=5), loop diuretics (n=11), Vitamin D (n=18), phosphate binders (n=14), antidepressants (n=6), proton-pump inhibitors (n=10)	beta-blockers (n=16), AT-1 blockers (n=2), ACE- inhibitors (n=9), calcium antagonists (n=14), anticoagulants (n=5), loop diuretics (n=19), Vitamin D (n=22), phosphate binders (n=18), antidepressants (n=2), proton-pump inhibitors (n=9)	

*P-value (Mann-Whitney-test), **($p < 0.01$); ESA erythropoiesis-stimulating agent, CKD chronic kidney disease, HD hemodialysis, HDF online hemodiafiltration, CAPD continuous ambulatory peritoneal dialysis, APD automated peritoneal dialysis.

wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Subsequently, the geomean of the Ca^{2+} dependent fluorescence was determined.

Reticulocyte count

For determination of the reticulocyte count, heparinized whole blood (2 μl) was added to 500 μl Retic-COUNT® (thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature in the dark and flow cytometry was performed according to the manufacturer's instructions. Forward scatter (FSC), side scatter (SSC) and thiazole orange-fluorescence intensity (in FL-1) of the blood cells were determined. The number of Retic-COUNT positive reticulocytes was expressed as the percentage of the total gated erythrocyte population. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using CellQuest software.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). A volume of 4 μ l erythrocytes was mixed in 1 ml Ringer. From the resulting cell suspension, 150 μ l were centrifuged (1600 rpm for 3 min at RT). Cells were stained with DCFDA (10 μ M; Sigma, Schnellendorf, Germany) in Ringer solution at 37°C for 30 min and then washed three times in 150 μ l Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution and ROS-dependent fluorescence intensity was measured in FL-1 channel at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Afterwards, the geomean of the ROS-dependent fluorescence was determined.

Ceramide abundance

To determine ceramide abundance, a monoclonal antibody-based assay was used. Four μ l erythrocytes were mixed in 1 ml Ringer. From the resulting cell suspension, 100 μ l were centrifuged (1600 rpm for 3 min at RT) and the erythrocytes were pelleted. Subsequently, cells were stained for 1 h at 37°C with 1 μ g/ml anti-ceramide antibody (1:10 dilution; clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). After two washing steps with 100 μ l PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (1:50 dilution; BD Pharmingen, Hamburg, Germany) in PBS-BSA. Unbound secondary antibody was removed by repeated washing with 50 μ l PBS-BSA. The samples were resuspended in 200 μ l PBS-BSA and then analyzed in FL-1 channel by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Finally, the geomean of the ceramide dependent fluorescence was determined.

Statistics

Data are expressed as median with interquartile range (IQR, 25th-75th percentile). Mann-Whitney test or One-way-ANOVA-test with Tukey's test as post-test were performed as appropriate to determine statistical significance between the two or three groups, respectively, using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. For all correlations, Spearman nonparametric analysis was used. n denotes the number of individuals. $p < 0.05$ was considered significant.

Results

The present study explored whether eryptosis is stimulated in patients with end-stage renal disease (ESRD) and under peritoneal dialysis (PD). Comparison was made to healthy individuals and ESRD patients under treatment with hemodialysis (HD). Eryptotic erythrocytes exposing phosphatidylserine at their surface were identified utilizing annexin-V-binding. As illustrated in Fig. 1, the percentage of annexin V-binding erythrocytes was significantly higher in patients with ESRD under either PD or HD treatment than in healthy volunteers. The percentage of annexin V-binding erythrocytes was significantly higher in patients treated with PD than in patients treated with HD. As illustrated in Fig. 2, a significant correlation was observed in PD patients between volume of the dialysate and the percentage of annexin V-binding erythrocytes.

Forward scatter was taken as a measure of cell volume. Forward scatter was not significantly different between healthy individuals (508.1 [497.1; 521.4], $n = 38$), patients on HD treatment (522.9 [507.6; 553.2] $n = 18$) and patients on PD treatment (518.2 [492.8; 545.8], $n = 22$).

Further experiments were performed in order to identify cellular mechanisms underlying eryptosis in ESRD patients. A powerful stimulator is an increase in erythrocyte Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was thus employed to estimate $[\text{Ca}^{2+}]_i$. As a result, Fluo3 fluorescence tended to be slightly higher in patients on HD treatment (20.78 [17.59; 24.26]), $n = 18$) and patients on PD treatment (19.50 [15.62; 24.69], $n = 22$) than in healthy individuals (19.93 [18.44; 20.59], $n = 38$), a difference, however, not reaching statistical significance.

Triggers of eryptosis include oxidative stress, which is enhanced in ESRD [17]. The abundance of reactive oxygen species (ROS) was thus determined utilizing

Fig. 1. Phosphatidylserine-exposing erythrocytes in freshly drawn blood from HD patients, PD patients and healthy volunteers. A. Original representative histogram of annexin V binding of erythrocytes in freshly drawn blood from healthy volunteers (grey shadow), from patients on hemodialysis (blue line) and from patients on peritoneal dialysis (red line). M1 indicates the annexin V fluorescence defining the percentage of annexin V binding erythrocytes. B. Individual values and median with interquartile range (IQR, 25th-75th percentile) of the percentage of annexin V-binding erythrocytes in freshly drawn blood from healthy volunteers (control, white circles), from patients on hemodialysis (HD, grey circles) and from patients on peritoneal dialysis (PD, black circles). **($p<0.01$), *** ($p<0.001$) indicate significant difference from healthy volunteers, #($p<0.05$) indicates significant difference between HD and PD (ANOVA).

Fig. 2. Phosphatidylserine-exposing erythrocytes as a function of dialysate volume in PD. Percentage of annexin V-binding of erythrocytes in freshly drawn blood from patients on peritoneal dialysis as a function of dialysate volume ($p=0.0261$, $R^2=0.2241$). * ($p<0.05$) indicates significant correlation.

2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. As shown in Fig. 3, DCFDA-fluorescence was significantly higher in erythrocytes from both, HD patients and PD patients than in erythrocytes from healthy individuals. No significant difference was observed between erythrocytes from HD patients and erythrocytes from PD patients.

A further known trigger of eryptosis is ceramide, which sensitizes erythrocytes to the effects of cytosolic Ca^{2+} . Thus, ceramide abundance was determined by FITC-labeled anti-ceramide antibodies. As illustrated in Fig. 4, ceramide abundance was significantly higher in erythrocytes from both, HD patients and PD patients than in erythrocytes from healthy individuals. Again, no significant difference was observed between erythrocytes from HD patients and erythrocytes from PD patients.

To correct anemia, ESRD patients are treated with erythropoietin or other erythropoiesis-stimulating agents (ESA). The rate of effective stimulation of erythropoiesis is reflected by the number of reticulocytes. As illustrated in Fig. 5, the reticulocyte number was higher in blood of both HD and PD patients than in healthy individuals, a difference reaching statistical significance in HD patients. Thus, the ESA treatment apparently rather over-compensated

Fig. 3. Reactive oxygen species in freshly drawn blood from HD patients, PD patients and healthy volunteers. A. Original representative histogram of DCFDA dep. fluorescence of erythrocytes in freshly drawn blood from healthy volunteers (grey shadow), from patients on hemodialysis (blue line) and from patients on peritoneal dialysis (red line). B. Individual values and median with interquartile range (IQR, 25th-75th percentile) of the DCFDA fluorescence in freshly drawn erythrocytes from healthy volunteers (control, white circles), from patients on hemodialysis (HD, grey circles) and from patients on peritoneal dialysis (PD, black circles). *** ($p<0.001$) indicates significant difference from healthy volunteers (ANOVA).

Fig. 4. Ceramide abundance in freshly drawn blood from HD patients, PD patients and healthy volunteers. A. Original representative histogram of ceramide dep. fluorescence of erythrocytes in freshly drawn blood from healthy volunteers (grey shadow), from patients on hemodialysis (blue line) and from patients on peritoneal dialysis (red line). B. Individual values and median with interquartile range (IQR, 25th-75th percentile) of the ceramide abundance in freshly drawn erythrocytes from healthy volunteers (control, white circles), from patients on hemodialysis (HD, grey circles) and from patients on peritoneal dialysis (PD, black circles). *** ($p<0.001$) indicates significant difference from healthy volunteers (ANOVA).

the lack of endogenous erythropoietin. The dosage of erythropoietin provided was similar in patients on HD treatment [3125 [401; 6000], $n = 18$] and patients on PD treatment [4583 [1114; 6579]; $n = 22$].

Several correlations were calculated in order to disclose the relationships between erythropoietin dosage, reticulocyte numbers, blood hemoglobin concentration and eryptosis. The dosage of erythropoietin was adjusted to the plasma hemoglobin concentration, i.e. the dosage was increased in patients with severe anemia leading to a negative correlation between blood hemoglobin concentration and dosage of erythropoietin treatment (Fig. 6A).

Fig. 5. Reticulocyte count in freshly drawn blood from HD patients, PD patients and healthy volunteers. A. Original representative histogram of Thiazole Orange fluorescence of erythrocytes in freshly drawn blood from healthy volunteers (grey shadow), from patients on hemodialysis (blue line) and from patients on peritoneal dialysis (red line). B. Individual values and median with interquartile range (IQR, 25th-75th percentile) of the percentage of reticulocytes in freshly drawn blood from healthy volunteers (control, white circles), from patients on hemodialysis (HD, grey circles) and from patients on peritoneal dialysis (PD, black circles). * ($p < 0.05$) indicates significant difference from healthy volunteers (ANOVA).

Fig. 6. The dosage of erythropoietin as a function of blood hemoglobin concentration and reticulocyte number in HD and PD patients. A. The dosage of erythropoietin as a function of hemoglobin concentration in freshly drawn blood from patients on hemodialysis (HD) ($p = 0.7379$, $R^2 = 0.0072$) and peritoneal dialysis (PD) ($p = 0.0214$, $R^2 = 0.2377$). * ($p < 0.05$) indicates significant correlation. B. The dosage of erythropoietin as a function of reticulocyte number in freshly drawn blood from patients on hemodialysis (HD) ($p = 0.0026$, $R^2 = 0.4430$) and peritoneal dialysis (PD) ($p = 0.1725$, $R^2 = 0.0910$). ** ($p < 0.01$) indicates significant correlation.

The correlation reached statistical significance in PD patients. A positive correlation was observed between dosage of erythropoietin treatment and reticulocyte number (Fig. 6B). The correlation reached statistical significance in HD patients.

The percentage of annexin-V-binding erythrocytes was positively correlated with the reticulocyte number (Fig. 7A) and with erythropoietin dosage (Fig. 7B). The correlations were statistically significant for both HD patients and PD patients.

Discussion

The present study confirms a previous report [18] showing enhanced percentage of phosphatidylserine-exposing erythrocytes reflecting eryptosis in patients with end-stage renal

Fig. 7. Reticulocyte number and erythropoietin dosage as a function of the percentage of annexin-V- binding erythrocytes in HD and PD patients. A. The percentage of reticulocytes in freshly drawn blood from patients on hemodialysis (HD) ($p=0.0037$, $R^2=0.4190$) and peritoneal dialysis (PD) ($p=0.0171$, $R^2=0.2528$) as a function of the percentage of annexin V-binding erythrocytes. * ($p<0.05$) and ** ($p<0.01$) indicate significant correlation. B. The dosage of erythropoietin administration as a function of the percentage of annexin V-binding erythrocytes in freshly drawn blood from patients on hemodialysis (HD) ($p=0.0063$, $R^2=0.3817$) and peritoneal dialysis (PD) ($p=0.0260$, $R^2=0.2243$). * ($p<0.05$) and ** ($p<0.01$) indicate significant correlation.

disease (ESRD) treated with hemodialysis (HD). More importantly, the present study reveals that eryptosis is more prominent in patients on peritoneal dialysis (PD) than in patients on HD. In patients on PD, the percentage of phosphatidylserine-exposing erythrocytes is correlated with the dialysate volume. Possibly, glucose-based components of the dialysate contribute to the stimulation of eryptosis. A previous study revealed that eryptosis in ESRD patients on HD is at least partially stimulated by a plasma borne component [18], which could be an uremic toxin, such as vanadate [20], acrolein [26], methylglyoxal [20] and/or phosphate [27]. Eryptosis is further stimulated by a variety of clinical conditions [18, 26-36] and by a myriad of xenobiotics [32, 37-79], which may further aggravate the anemia of ESRD.

Similar to what has been observed earlier in HD patients [18], eryptosis in HD patients is paralleled by oxidative stress and enhanced ceramide formation, which are both well known triggers of eryptosis [20]. In an earlier study, eryptosis was shown to be paralleled by increase of cytosolic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$) [18]. In the present study $[\text{Ca}^{2+}]_i$ similarly tended to be higher in erythrocytes from HD patients than in erythrocytes from healthy individuals, a difference, however, not reaching statistical significance. Needless to say, the present observations do not rule out the contribution of further cellular mechanisms to the stimulation of eryptosis in ESRD.

The percentage of phosphatidylserine-exposing erythrocytes was twice as high in freshly drawn blood from ESRD patients on PD as in blood from healthy volunteers. In view of the rapid clearance of phosphatidylserine-exposing erythrocytes from circulating blood [20], a doubling of phosphatidylserine-exposing erythrocytes in circulating blood points to a decrease of erythrocyte life span to half [80]. At constant erythropoiesis, a decrease of erythrocyte life span to half should reduce the number of circulating erythrocytes by 50%. The accelerated eryptosis thus explains the persistence of anemia despite adequate replacement of erythropoietin and normalization of erythropoiesis in ESRD patients.

In addition to its impact on anemia, eryptosis may interfere with microcirculation, as phosphatidylserine-exposing erythrocytes adhere to the vascular wall [81]. Phosphatidylserine-exposing erythrocytes further stimulate blood clotting [20]. Excessive eryptosis could thus contribute to the known increase of cardiovascular risk in uremic patients [82, 83]. Attempts to normalize blood count in ESRD patients by stimulation of erythropoiesis were expected to increase erythrocyte turnover and thus the number of phosphatidylserine-exposing erythrocytes in circulating blood. Along those lines, the susceptibility of erythrocytes to triggers of eryptosis is increased by chronically enhanced erythropoietin levels [84] and excessive eryptosis could, at least in theory, contribute to the untoward side effects following

uncritical use of erythropoietin or other erythropoiesis-stimulating agents [5, 83, 85, 86]. In view of our observations we advocate a therapeutic approach combining inhibition of eryptosis and replacement of erythropoietin.

Conclusion

Similar to patients on HD, patients on PD suffer from marked stimulation of eryptosis leading to an approximate doubling of suicidal erythrocytes. Eryptosis is paralleled by oxidative stress and enhanced ceramide abundance. The enhanced eryptosis presumably leads to accelerated erythrocyte loss and thus substantially contributes to the anemia of the patients.

Disclosure Statement

The authors confirm that there are no conflicts of interest to be stated.

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