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The Role of Thalassaemic Red Cells in the Mechanism Process of Hypercoagulable State in Thalassaemia

Wardah Roslan [1,2], Rosnah Bahar [1,2,*], Mohd Nazri Hassan [1,2], Norsarwany Mohamad [2,3], Shafini Mohamed Yusoff [1,2], Salfarina Iberahim [1,2], Zefarina Zulkafli [1,2,*], Noor Haslina Mohd Noor [1,2], Marini Ramli [1,2], Razan Hayati Zulkeflee [1,2], Marne Abdullah [1,2] and Wan Suriana Wan Ab Rahman [1,4]

[1] Department of Haematology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian 16150, Malaysia; wardahroslan@gmail.com (W.R.); shafini@usm.my (S.M.Y.); salfarina@usm.my (S.I.); drhaslina@usm.my (N.H.M.N.); marini@usm.my (M.R.); rhayatiz@usm.my (R.H.Z.); marneabd@usm.my (M.A.); suriana@usm.my (W.S.W.A.R.)
[2] Hospital Pakar Universiti Sains Malaysia, Kubang Kerian 16150, Malaysia; sarwany@usm.my
[3] Department of Paediatric, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian 16150, Malaysia
[4] School of Dental Sciences, Universiti Sains Malaysia, Kubang Kerian 16150, Malaysia
* Correspondence: rosnahkb@usm.my (R.B.); zefarinazulkafli@gmail.com (Z.Z.)

Abstract

Background: Thromboembolic events (TEEs) are recognized complications in thalassaemia, arising from a hypercoagulable state driven by multiple proposed mechanisms. These concerns have prompted numerous investigations to better understand the underlying prothrombotic pathways. This study aimed to compare and correlate the levels of fragmented red blood cells (FRCs), hypercoagulable protein markers, and erythrocyte phosphatidylserine (PS) exposure in thalassaemia patients. This 12-month prospective case-control study at HPUSM recruited 34 thalassaemia patients (21 with thalassaemia major and 13 with thalassaemia intermedia) and 10 healthy controls. Methods: FRCs, erythrocyte PS, protein C, free protein S, and antithrombin III levels were measured. Results: A total of 44 subjects were included, with a mean age of 22.43 ± 10.45 years; most were male (61.4%) and Malay (86.4%). Median FRCs and PS exposure were significantly elevated in both thalassaemia groups compared to controls ($p < 0.001$ for FRCs; $p = 0.048$ for PS). Mean protein C and free protein S levels were significantly reduced in thalassaemia major (55.00 ± 10.20% and 65.77 ± 8.66%) and thalassaemia intermedia (61.23 ± 16.99% and 61.11 ± 14.65%) compared to controls (101.60 ± 18.97% and 95.12 ± 23.57%) ($p < 0.001$ for both), while antithrombin III levels were not significantly different. A significant negative correlation was observed between PS exposure and protein C ($p = 0.014$). Protein C was positively correlated with free protein S ($p = 0.021$) and antithrombin III ($p < 0.001$). Conclusions: Elevated PS exposure and reduced protein C and free protein S levels in thalassaemia patients indicate a potential procoagulant phenotype.

Keywords: thalassaemia major; thalassaemia intermedia; thromboembolisms; hypercoagulable markers

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1. Introduction

Thalassaemia syndromes represent a prevalent group of monogenic disorders globally, characterized by insufficient production of one or more globin chains, leading to imbalanced haemoglobin production and subsequent anaemia [1,2]. This autosomal recessive condition

affects a significant portion of the world's population (7%) [3] particularly in tropical and subtropical regions (Southeast Asia, Mediterranean, Indian subcontinent, and Africa), with estimated prevalence rates of 1–20% for beta thalassaemia and 12–50% for alpha thalassaemia [4]. Malaysia, located within this thalassaemia belt, is particularly impacted, with beta-thalassaemia emerging as a significant public health concern, notably among Malaysian Chinese and Malays.

While severe forms of thalassaemia were historically associated with complications such as iron overload and bone deformities, advances in treatment have improved life expectancy [5,6]. However, haemostasis-related complications, including thromboembolic events (TEEs), are increasingly recognized and contribute significantly to morbidity and mortality [5,7].

Thrombosis, the fifth most common complication and affecting 14% of patients, is influenced by various factors such as age, splenectomy, and serum ferritin levels [1,8]. Notably, TEEs are more prevalent in thalassaemia intermedia (TI) patients and those who have undergone splenectomy [1,9]. Due to the spleen's beneficial role in scavenging these procoagulant platelets and red blood cells (RBCs), these abnormalities become more evident after splenectomy [10].

While the exact mechanisms underlying TEEs remain unclear, factors such as increased platelet activation, endothelial activation, and RBC membrane abnormalities leading to the coagulation cascade's activation and the coagulation protein level changes are implicated. They have been ascribed to numerous factors, typically a combination of these factors that lead to TEE [5,9,11].

Some data suggested that abnormal RBCs in thalassaemia contribute to a hypercoagulable state by exposing phosphatidylserine (PS) to their membranes [12]. PS exposure leads to premature cell death, promoting ineffective erythropoiesis and chronic haemolysis, consequently producing thrombogenic RBCs and microparticles, which increase the risk of thrombosis [13]. Erythrocyte PS exposure also stimulates platelets and triggers coagulation pathways, increasing the risk of thrombosis, particularly in TI patients who have undergone splenectomy [14]. Following blood transfusion, these abnormalities (PS) had reduced to the normal range, which could partly explain why patients with TI who are infrequently transfused had a higher incidence of thrombotic events than those who undergo regular transfusion. Additionally, deficiencies in anticoagulant proteins like protein C and protein S have been observed in thalassaemia patients, further contributing to the hypercoagulable state [15,16].

Pertaining on this data, the study pursues to characterize red blood cell abnormalities in thalassaemia and their involvement to thrombotic pathways. It assesses the proportion of fragmented RBCs (FRCs) and evaluates basic hypercoagulable biomarkers including erythrocyte PS exposure, protein C, free protein S, and antithrombin III (ATIII) in thalassaemia patients compared with controls. The findings may benefit in predicting thrombotic risk and support the development of customized prophylactic strategies for individual patients.

2. Materials and Methods

This prospective case-control study was conducted at the Paediatric Daycare and Clinical Haematology (Medical daycare/ward) Hospital Pakar Universiti Sains Malaysia (HPUSM), Kelantan, from 2022 until 2023. The required sample size was calculated to be 105 participants using G*Power software version 3.1. However, due to the limited number of eligible patients during the study period, a total of 44 participants were recruited, comprising 21 patients with thalassaemia major (TM), 13 patients with thalassaemia intermedia (TI), and 10 healthy blood donors as the control group.

According to the 3rd edition Guidelines for the Management of Transfusion-dependent thalassaemia (TDT), the patient was divided into TM and TI groups by Cappellini et al. TM is defined as transfusion-dependent thalassaemia (TDT) in which blood transfusion is required every two to five weeks. TI was distinguished from TM when the blood transfusion requirement was less than that and considered non-transfusion-dependent thalassaemia (NTDT). Thalassaemia patients were also further categorized into splenectomized and non-splenectomized groups.

All eligible patients who fulfilled the inclusion and exclusion criteria were recruited. The inclusion criteria for cases include all TM and TI patients who seek treatment in HUSM and are more than 9 years old. Healthy controls were selected from healthy blood donors who attended the blood bank HUSM for donation and had no known history of blood diseases, chronic systemic illness, or blood transfusion. The exclusion criteria for both cases and the control group were (1) pregnant women, (2) autoimmune disease, (3) liver disease, (4) other comorbid, diabetes mellitus (DM), hypertension (HPT), chronic kidney disease (CKD), and (5) history of taking anticoagulant in the preceding four weeks. None of the subjects had a history of thrombosis in the past. The last blood transfusion received was at least four weeks before the blood sample collection. For every subject, a complete history and preferred investigations were performed.

2.1. Study Procedures

A total of 10 mL blood samples were collected from the patients and controls. Three ml of blood were collected into an ethylenediaminetetraacetic acid (EDTA) container, and another seven ml in two 3.2% trisodium citrate containers (3.5 mL each) with blood: anticoagulant ratio of 9:1. For patients with transfusion-dependent thalassaemia, peripheral blood samples were collected immediately before their scheduled blood transfusion, while blood samples from the control group were obtained immediately before blood donation. All samples were collected in EDTA tubes and processed within 2–4 h of collection according to a standardized laboratory protocol. Samples were handled under identical conditions, and flow cytometric analysis was performed on fresh specimens to minimise pre-analytical variation. The blood samples collected in an EDTA bottle were analysed for the fragmented RBC (FRC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), haematocrit (HCT), white blood cell (WBC), platelet, reticulocyte, and nucleated RBC (NRBC) count using a Sysmex XN 1000 (Sysmex corporation, Kobe, Japan) analyser. The FRC was measured using the fluorescence flow cytometry method in the reticulocyte (RET) channel of the automated haematology analyser. The analyser identifies FRC separately within the RET channel according to size and fluorescence/scatter characteristics.

The coagulation samples were analysed using ACL TOP CTS-300 (Werfen, Bedford, MA, USA). Chromogenic assays measured protein C and ATIII levels, while free protein S was via latex ligand immunoassay. Plasma was separated by centrifugation at 2500× *g* for 15 min and frozen at −80 °C.

Meanwhile, the remaining EDTA-anticoagulated blood sample was used to evaluate erythrocyte PS exposure by flow cytometry. Erythrocyte PS exposure was assessed using fluorescein isothiocyanate (FITC) conjugated Annexin V (AV) protein (Becton Dickinson, Franklin Lakes, NJ, USA). AV is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS exposed on the outer leaflet of the erythrocyte membrane. Erythrocytes were identified using phycoerythrin (PE)-conjugated anti-glycophorin A antibody, and the percentage of PS-expressing erythrocytes was determined as the proportion of Annexin V-positive events within the glycophorin A-positive erythrocyte population.

Flow cytometric acquisition and analysis were performed using a FACS Lyric flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with BD FACSDiva software version 9.0. Erythrocytes were initially gated based on their characteristic forward scatter (FSC) and side scatter (SSC) properties, followed by confirmation of glycophorin A positivity to exclude non-erythroid events and debris. The percentage of Annexin V-positive erythrocytes was then quantified within the glycophorin A-positive gate.

All samples were processed using a standardized protocol under identical instrument settings. Instrument quality control was performed according to the manufacturer's recommendations before sample acquisition. Blood samples were analysed promptly after collection to minimise pre-analytical variation.

2.2. Statistical Analysis

The data was analysed using IBM Statistical Package for the Social Sciences (SPSS) software version 29. Descriptive statistics were used to represent demographic data, with continuous variables presented as mean ± standard deviation (SD) or median ± interquartile range (IQR). Categorical data was described in frequency (n) and percentage (%). Statistical comparisons of haematological parameters and laboratory features among three groups utilized one-way analysis of variance (ANOVA) for parametric data and the Kruskal-Wallis test for non-parametric data. Additionally, a comparison between splenectomized and non-splenectomized groups was conducted using an independent *t*-test. Pearson correlation test was employed to examine correlations between quantitative variables. Significance was considered at a probability of error less than 5% ($p < 0.05$).

3. Results

A total of 44 subjects, 21 TM patients, 13 TI patients, and 10 healthy controls were recruited. Among thalassaemia patients, 35.3% had undergone splenectomy, while 64.7% were not.

The median percentage of FRC and erythrocyte PS exposure differed significantly among the three groups (FRC: $p < 0.001$; erythrocyte PS exposure: $p = 0.048$). Following Bonferroni correction, both the TM and TI groups had significantly higher FRC% than the control group (both $p < 0.001$), whereas no significant difference was observed between the TM and TI groups ($p = 0.700$). For erythrocyte PS exposure, the TM group showed a significantly higher percentage than the control group ($p = 0.014$). However, no significant differences were found between the TI and TM groups ($p = 0.487$) or between the TI and control groups ($p = 0.096$). Although erythrocyte PS exposure was higher in the TM group than in the TI group, the difference did not reach statistical significance (Table 1).

Table 1. Comparison of fragmented red blood cells and erythrocyte phosphatidylserine exposure among the 3 study groups ($n = 44$).

Variables	Groups	N	Median (IQR)	F-Statistic (df) [a]	*p*-Value
Fragmented red blood cells % (FRC %)	Thalassaemia Major	21	5.68 (6.99)	22.82 (2)	<0.001 [b]
	Thalassaemia Intermedia	13	6.32 (5.55)		
	Control	10	0.10 (0.37)		
Erythrocyte Phosphatidyl serine (PS) exposure (%)	Thalassaemia Major	21	0.73 (1.80)	6.09 (2)	0.048 [b]
	Thalassaemia Intermedia	13	0.22 (1.74)		
	Control	10	0.15 (0.13)		

[a] Kruskal Wallis Test. Results are calculated by using median ± interquartile range (IQR). $p < 0.05$ is statistically significant. [b] Bonferroni correction of *p*-value was conducted to test for the differences in the median scores between each independent group.

Table 2 showed a significant mean difference in protein C and free protein S levels among the groups ($p < 0.001$). Further analysis using the post-hoc Tukey tests indicated that both TM and TI patients exhibited significantly lower levels of protein C and free protein S compared to the control group ($p < 0.001$ for all comparisons). However, there was no statistically significant difference between TM and TI patients for either protein C ($p = 0.456$) or free protein S ($p = 0.651$). Regarding ATIII levels, no significant difference was observed among the three groups ($p = 0.164$).

Table 2. Comparison of Protein C, free Protein S, and Antithrombin III among the 3 study groups ($n = 44$).

Group	Thalassaemia Major Mean (SD)	Thalassaemia Intermedia Mean (SD)	Control Mean (SD)	F-Statistic (df) [a]	*p*-Value
Protein C (%)	55.00 (10.20)	61.23 (16.99)	101.60 (18.97)	35.98 (2.41)	<0.001 [b]
Free Protein S (%)	65.77 (8.66)	61.11 (14.65)	95.12 (23.57)	17.30 (2.41)	<0.001 [b]
Anti-thrombin III (%)	92.71 (13.69)	92.46 (14.68)	101.60 (6.88)	1.89 (2.41)	0.164

[a] One-way ANOVA test, Results are calculated using mean ± standard deviation (SD). $p < 0.05$ is statistically significant. [b] Post-hoc Tukey test was carried out for significant *p*-value.

Based on the results in Table 3, a significant linear correlation exists between PS and protein C ($p = 0.014$). The correlation coefficient (r) for protein C was −0.37, indicating a fair correlation. Protein C exhibited a significant positive correlation with free protein S ($p = 0.021$) and ATIII ($p < 0.001$), with correlation coefficients of 0.35 and 0.48, respectively. No significant correlation was observed between erythrocyte PS exposure and free protein S or ATIII.

Table 3. Correlation between erythrocyte PS exposure and other hypercoagulable parameters.

	Erythrocyte PS Exposure %	Protein C %	Free Protein S %	Antithrombin III %
Erythrocyte PS exposure %				
r	1	−0.37	−0.16	−0.24
p-value		0.014	0.286	0.112
Protein C %				
r	−0.37	1	0.35	0.48
p-value	0.014		0.021	<0.001
Free Protein S %				
r	−0.16	0.35	1	0.11
p-value	0.286	0.021		0.465
Antithrombin III%				
r	−0.24	0.48	0.11	1
p-value	0.112	<0.001	0.465	

Pearson correlation was applied. Correlation is significant at *p*-value < 0.05. Abbreviations: PS, Phosphatidylserine; r, correlation coefficient.

Splenectomy patients have significantly lower Hb and RBC, and higher WBC, platelet, MCV and NRBC than non-splenectomy patients. Reticulocyte, FRC, and erythrocyte PS exposure percentages were also significantly elevated in splenectomy patients compared to non-splenectomy patients. Other hypercoagulable parameters, protein C free protein S, and ATIII showed no significant difference between the two groups (Table 4).

Table 4. Comparison of haematological and haemostatic parameters between splenectomy and non-splenectomy groups (n = 34).

Variables	Group Mean (SD)		Mean Difference (95%CI)	T-Statistic(df) [a]	*p*-Value
	Splenectomized N = 12	Non-Splenectomized N = 22			
RBC ($\times 10^6/\mu L$)	2.93 (0.49)	3.56 (0.68)	0.63 (0.17,1.08)	2.81 (32)	0.008
HB (g/dL)	6.62 (0.93)	7.57 (1.16)	0.95 (0.16,1.75)	2.44 (32)	0.021
HCT (%)	22.78 (3.10)	25.36 (4.67)	2.59 (−0.48,5.66)	1.72 (32)	0.095
WBC ($\times 10^3/\mu L$)	18.23 (5.22)	7.95 (2.85)	−10.28 (−13.08, −7.48)	−7.47 (32)	<0.001
Platelet ($\times 10^3/\mu L$)	685.50 (293.88)	252.64 (100.52)	−432.86 (−622.79, −242.94)	−4.95 (12.42)	<0.001
MCV (fL)	78.08 (5.55)	71.69 (6.17)	−6.39 (−10.75, −2.03)	−2.99 (32)	0.005
MCH (pg)	22.73 (2.42)	21.58 (2.66)	−1.16 (−3.04, 0.73)	−1.25 (32)	0.222
MCHC (g/dL)	29.13 (2.32)	30.09 (2.50)	0.96 (−0.82, 2.74)	1.10 (32)	0.282
RDW (%)	27.72 (6.46)	25.64 (6.15)	−2.08 (−6.66, 2.50)	−0.93 (32)	0.361
NRBC ($\times 10^3/\mu L$)	52.53 (34.43)	0.34 (0.39)	−52.19 (−74.07, −30.31)	−5.25 (11.0)	<0.001
Reticulocyte (%)	33.27 (17.50)	5.53 (4.97)	−27.74 (−38.98, −16.49)	−5.37 (11.98)	<0.001
Erythrocyte PS exposure (%)	2.62 (1.91)	0.43 (0.56)	−2.19 (−3.42, −0.96)	−3.88 (12.03)	0.002
FRC (%)	11.26 (5.92)	5.21 (2.95)	−6.05 (−9.13, −2.97)	−4.00 (32)	<0.001
Protein C (%)	52.33 (11.77)	60.14 (13.56)	7.80 (−1.68,17.28)	1.68 (32)	0.103
Free Protein S (%)	68.51 (9.51)	61.53 (11.71)	−6.98 (−15.02,1.07)	−1.77 (32)	0.087
Antithrombin III (%)	86.92 (16.21)	95.73 (11.63)	8.81 (−0.97, 18.60)	1.83 (32)	0.076

[a] Independent sample T-test. Results are calculated by using mean ± standard deviation (SD). $p < 0.05$ is statistically significant.

4. Discussion

This study evaluated haemostatic abnormalities in patients with thalassaemia, focusing on biomarkers associated with a procoagulant phenotype. Hypercoagulability and thromboembolic events (TEEs) are well recognized complications of both thalassaemia major (TM) and thalassaemia intermedia (TI) and contribute substantially to morbidity and mortality. This study findings are consistent with previous reports demonstrating increased erythrocyte PS exposure and reduced protein C and free protein S levels in patients with thalassaemia [7,17]. Early recognition of these haemostatic abnormalities may help improve the understanding of thrombotic risk in our thalassaemia patients, although further studies are required to establish their clinical significance [11,18].

Previous studies have shown an association between FRC and erythrocyte PS exposure, although the contribution of PS-expressing FRCs to thrombotic risk remains unclear [19,20]. In this study, erythrocyte PS exposure was significantly higher in thalassaemia patients compared with healthy controls, consistent with previous reports linking increased PS expression to a procoagulant state in thalassaemia [21–23]. However, no significant difference was observed between TM and TI patients, which is in agreement with previous findings [14,21].

Regular RBC transfusions may reduce PS-exposed erythrocytes by replacing abnormal red cells with healthy donor erythrocytes, which may partly explain the lack of difference between TM and TI patients. However, the higher erythrocyte PS expression observed in TM patients in this study differs from some previous reports and may be influenced by factors such as transfusion adequacy, ineffective erythropoiesis, or splenectomy status [24]. Factors such as splenectomy may also contribute to the elevated PS levels observed in TM compared to TI patients. Further research with a larger sample size is needed to validate this finding.

The excess production of α-globin chains in TM and TI leads to the formation of haemichromes, resulting in oxidative damage to the red blood cell (RBC) membrane.

This damage promotes the externalization of anionic phospholipids, particularly phosphatidylserine (PS), which may enhance thrombin generation and contribute to a procoagulant environment. PS-exposed RBCs exhibit increased adhesion and aggregation, which may further promote thrombotic tendency in thalassaemia. The extent and proportion of PS-expressing cells are important determinants of their procoagulant potential [14,20,21,25].

Protein C and free protein S levels were significantly lower in thalassaemia patients compared with healthy controls, consistent with previous studies reporting reduced natural anticoagulants in TM and TI [9,17,26]. Reduced levels of these proteins may contribute to a procoagulant phenotype. However, no significant difference was observed between TM and TI, suggesting that thrombotic risk is influenced by multiple factors beyond natural anticoagulant levels. Potential mechanisms include impaired hepatic synthesis related to iron overload, chronic coagulation activation, and increased consumption. In this study, iron status, liver iron concentration, and chelation therapy were not fully assessed, limiting further interpretation.

The significant correlation between erythrocyte PS exposure and protein C may suggest an interaction between abnormal RBC surfaces and natural anticoagulant proteins. The relationship between protein C and protein S is consistent with their functional roles in coagulation regulation [26,27]. However, the lack of correlation between protein S and PS may be related to the limited sample size. Further studies incorporating functional haemostatic assays and adjustment for clinical confounders are needed to clarify these relationships.

Splenectomized patients in this study demonstrated higher FRC levels and erythrocyte PS exposure compared with non-splenectomized patients, consistent with previous reports [23,25]. The spleen plays an important role in removing PS-expressing erythrocytes through the reticuloendothelial system by recognizing PS as an "eat-me" signal [20]. Therefore, splenectomy may reduce the clearance of PS-expressing erythrocytes and RBC-derived vesicles, resulting in their accumulation in circulation. However, other factors, including transfusion status and iron overload, may also contribute to these findings.

Splenectomized patients also demonstrated higher platelet counts, WBCs, NRBCs, reticulocytes, and ferritin levels, consistent with previous reports [5,28–30]. Splenectomy may increase circulating abnormal RBCs and platelets due to reduced splenic clearance, contributing to a more procoagulant phenotype [30–37]. However, no significant differences in protein C or protein S levels were observed between splenectomized and non-splenectomized patients, likely due to the small sample size.

Although splenectomy is generally expected to improve haemoglobin levels by reducing splenic sequestration and haemolysis, our findings demonstrated lower haemoglobin levels in the splenectomized group. This may reflect differences in the underlying disease severity rather than the effect of splenectomy itself. Patients who undergo splenectomy often have more severe thalassaemia, greater transfusion requirements, or longstanding disease, which may contribute to persistently lower haemoglobin levels despite splenectomy. In addition, haemoglobin levels may be influenced by differences in transfusion regimen, iron overload, chelation therapy, and the interval between the last transfusion and blood sampling, variables that were not fully controlled in our study [30–37].

This study has several limitations, including the small sample size, single-center design, and the absence of global haemostatic assays such as thrombin generation testing and thromboelastometry. Important clinical factors, including iron status, chelation therapy, transfusion regimen, thalassaemia genotype and splenectomy status, were not fully assessed and may have influenced the observed findings. Iron overload, in particular, is known to affect haemostatic balance in patients with thalassaemia and may have contributed to the observed changes. Therefore, the findings should be interpreted as evidence

of an association with a hypercoagulable state rather than direct evidence of an increased thrombotic risk. Future multicenter studies with larger sample sizes, comprehensive clinical characterization, detailed assessment of iron status, and global haemostatic testing are warranted to validate and expand upon these findings.

5. Conclusions

In conclusion, thalassaemia patients demonstrated increased FRC and erythrocyte phosphatidylserine exposure, together with reduced protein C and free protein S levels compared with healthy controls. These findings suggest an imbalance between procoagulant cellular changes and natural anticoagulant mechanisms, which may contribute to the procoagulant phenotype observed in thalassaemia.

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Data Availability Statement: Data are available from the authors upon reasonable request due to privacy considerations.

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Abbreviations

The following abbreviations are used in this manuscript:

RBC	Red Blood Cell
HB	Haemoglobin
HCT	Haematocrit
WBC	White Blood Count
MCH	Mean Corpuscular Volume
MCV	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
RDW	Red Cell Distribution Width
NRBC	Nucleated Rbc
PS	Phosphatidylserine
FRC	Fragmented RBC

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