An update on recent studies of extracellular vesicles and their role in hypercoagulability in thalassemia (Review)

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Abstract. Thromboembolic events are a significant clinical concern in thalassemia and hemoglobinopathies, highlighting the need for new strategies to treat and detect these specific hematologic complications. In recent years, extracellular vesicles (EVs) have garnered interest due to their role in cell-to-cell communication, including angiogenesis, immune responses and coagulation activation. Their multifaceted role depends on the cellular origin and cargo, making them potential diagnostic biomarkers and therapeutic agents. The present review highlights recent advances in understanding the involvement of EVs in hypercoagulability in thalassemia, the characterization of circulating EVs and the potential for using EVs as predictive biomarkers. β-Thalassemia intermedia exhibits a high incidence of thromboembolic events, contributing to significant morbidity and mortality. Advanced technologies have enabled the profiling and characterization of circulating EVs in patients with β-thalassemia through various techniques, including flow cytometry, proteomic studies, reverse transcription-quantitative PCR, transmission electron microscopy, nanoparticle tracking analysis and western blot analysis. Microparticles from splenectomized β-thalassemia/hemoglobin E patients induce platelet activation and aggregation, potentially contributing to thrombus formation. The abundance of these microparticles, primarily released from platelets and damaged red cells, may have a role in thromboembolic events and other clinical complications in thalassemia. This suggests a promising future for EVs as diagnostic and predictive biomarkers in thalassemia management.

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Contents

1. Introduction

Thalassemia, an inherited disease, is characterized by deficient or absent α- or β-globin chains within red blood cells, leading to imbalanced globin chains and reduced red cell survival. Excess globin chains form hemichromes, causing oxidative stress and phosphatidylserine (PS) exposure on red cell membranes (1,2). Thalassemia varies in severity: Thalassemia minor, thalassemia intermedia (TI) and thalassemia major (TM). Patients with TM require regular blood transfusions commencing within the first 2 years of life. Untreated or inadequately transfused patients display jaundice, growth retardation, splenohepatomegaly and skeletal malformation from extramedullary hematopoiesis. Excessive iron from transfusions may lead to irreversible organ damage, with death often resulting from cardiovascular diseases, iron overload and infections (3-5).

Of note, patients with β-thalassemia have an increased risk of hypercoagulability and thrombosis, significantly impacting morbidity and mortality (6). This phenomenon involves interactions between damaged red cells, activated leukocytes and platelets, adhesive endothelial cells and coagulation factor dysregulation (7). The incidence of thrombosis may be minimized by regular blood transfusion, while splenectomy significantly potentiates the risk (8,9). Increased circulating cell-derived vesicles have been reported in splenectomized thalassemic patients, suggesting the role of the spleen in the clearance of these vesicles (10).

Extracellular vesicles (EVs) are submicron-sized, bioactive membrane vesicles released by cells under stress, activation or apoptosis. They are classified into three types: Apoptotic bodies, microparticles (MPs), which are large

EVs, and exosomes (which are small EVs), each differing in their biogenesis, properties and molecular markers. They have received extensive interest due to their important role as a conveyer of biological cargos. EVs are critical in cell communication, carrying proteins, lipids and nucleic acids to target cells (11,12). Their elevated levels in cardiovascular and hematological disorders make them potential diagnostic and prognostic biomarkers (12-14). Our group in Thailand was the first to characterize MPs in patients with β-thalassemia/hemoglobin (Hb)E following bone marrow transplantation. Lower levels of circulating PS-externalizing red blood cells, their MPs and procoagulant platelets were found in transplanted patients than in those receiving regular transfusions (15). These circulating MPs, along with leukocyte-platelet aggregates, may contribute to hypercoagulability in patients with β-thalassemia/HbE who have undergone bone marrow transplantation (16). Understanding the role of EVs in altering target cell phenotypes and inducing functional changes may provide insight into the disease's pathogenesis and complications.

2. β-Thalassemia

β-Thalassemia, an autosomal recessive anemia, arises from reduced (β^+) or absent (β^0) synthesis of the β-globin chain. Its severity varies, primarily depending on the excessive degree of α-globin chains, which accumulate in red blood cell precursors, causing ineffective erythropoiesis (17). Ineffective erythropoiesis features rapid expansion of early erythroid precursors and apoptosis of late-stage ones, resulting in low production of reticulocytes and mature red blood cells. This mechanism is primarily responsible for inherited anemia disorders such as β-thalassemia (18).

β-Thalassemia is categorized into β-TM, β-TI, thalassemia minor and the β-thalassemia trait. Patients with TM require regular blood transfusions for survival. Untreated or poorly transfused individuals exhibit growth retardation, pallor, jaundice, skin pigmentation, weak musculature, hepatosplenomegaly, leg ulcers, extramedullary hematopoiesis masses and skeletal changes due to marrow expansion.

Patients who do not receive blood transfusions usually succumb to heart failure. However, those receiving transfusions may develop iron overload, depending on their adherence to iron chelation therapy. Complications related to iron overload in children include growth retardation and failure of sexual maturation. In adults, complications may include liver fibrosis and cirrhosis, endocrine gland involvement (e.g., diabetes mellitus, and insufficiency of hormones released from the parathyroid, thyroid and pituitary glands), and cardiovascular diseases such as dilated myocardiopathy and arrhythmias (19).

Patients with TI have milder anemia, requiring infrequent or no transfusions. They may be asymptomatic until adulthood, with symptoms including pallor, mild jaundice, cholelithiasis, liver and spleen enlargement, bone changes, leg ulcers, extramedullary erythroid marrow masses, osteopenia, osteoporosis and thrombotic complications (17). Patients with TI often have an increased tendency to develop thrombosis, particularly when compared to those with TM, particularly in splenectomized patients. This increased risk may lead to thromboembolic conditions such as deep vein thrombosis, portal vein thrombosis, stroke and pulmonary embolism (20). The β-thalassemia trait is typically clinically asymptomatic, although mild anemia may occur in certain cases (17).

The high incidence of thromboembolic events in TI leads to a chronic hypercoagulable state, causing major morbidity and mortality. These events include cerebral thrombosis, deep venous thrombosis, pulmonary embolism and recurrent arterial occlusion (7). In addition, all studies of EVs in β-thalassemia classified the patients as TM, and TI according to severity, anemia, blood transfusion and complications due to the higher incidence of hypercoagulable state found in patients with TI. In the present review, the forms of β-thalassemia, such as TM, TI and β-thalassemia trait, were described.

3. α-Thalassemia

In α-thalassemia, the clinical phenotypes range from asymptomatic to lethal, depending on the number of non-functional copies of α-globin genes. Two main groups of α-thalassemia include α-thalassemia 1 and α-thalassemia 2. As a result of deficient production of α-globin, it leads to the formation of homotetramer of unaffected β-globin chains. One of the moderately severe symptomatic forms of α-thalassemia is HbH disease (β_4 tetramers). The non-deletion (ND) form of HbH disease ($\alpha\alpha^{ND/-}$) and Hb constant spring, which is caused by mutation in α-globin gene termination, are common in Asian countries. Patients in this group are severely anemic and have a risk of iron overload and splenomegaly. Therefore, splenectomy is recommended for patients with ND HbH, but they have a high rate of counteracting with the pro-thrombophilic complication (2,21). Similarly, abnormal externalization of PS on the red cell surface and their shedding MPs can stimulate the disturbance of coagulation. However, the coagulation parameters tested by Sirachainan *et al* (22) were not different among pediatric patients with α-thalassemia and normal controls. Further investigations on the effect of other factors, such as activation of platelet and endothelial cells and shedding EV, are required to confirm their contribution of hemostatic alteration in α-thalassemia.

4. EVs in patients with thalassemia

In patients with thalassemia, various studies have examined the profile and quantity of circulating MPs, focusing on their cellular origin and procoagulant properties. Among these studies, those involving patients who have undergone splenectomy revealed a notable presence of plasma MPs, primarily released from platelets and damaged red cells, which express negatively charged PS (23). In patients with β-thalassemia/HbE who have been splenectomized, these MPs are known to trigger platelet activation and aggregation, contributing to the formation of thrombi (24). The notably elevated levels of these MPs may partly contribute to thromboembolic events, hypercoagulable states and other clinical complications commonly observed in patients with thalassemia (25).

Discovery of EVs in thalassemia. The etiology of thromboembolic risk in thalassemic patients involves several factors. They include hyperaggregation and oxidative damage of red cells, chronic platelet activation and increased circulating cell-derived EVs. This complex interplay requires further

Table I. Surface antigens employed in the characterization of circulating extracellular vesicles using flow cytometry.

Antigen marker	Alternative name	Cell of origin
Phosphatidylserine	-	Apoptotic cell membrane
CD235a	Glycophorin A	Red cells
CD71	Transferrin receptor	Erythroid precursor
CD41a	Glycoprotein IIb	Platelets
CD42b	Glycoprotein Ibα	Platelets
CD31	Platelet endothelial cell adhesion molecule	Platelets, endothelial cells
CD144	Vascular endothelial cadherin (or cadherin-5)	Endothelial cells
CD146	Melanoma cell adhesion molecule	Endothelial cells
CD105	Endoglin	Mesenchymal stroma
CD45	Protein tyrosine phosphatase receptor type C	Leukocytes
CD11b	Integrin α-M	Activated leukocytes
CD62P	P-selectin or platelet activation-dependent granule membrane protein	Activated platelets
CD142	Coagulation factor III or tissue factor	Leukocytes and subendothelial cells, such as smooth muscle cells and fibroblasts

investigation to fully understand its underlying mechanisms. The initial functional exploration of the role of EVs in the pathogenesis of the hypercoagulable state in thalassemia involved coculturing recipient cells, such as endothelial cells and leukocytes, with isolated EVs. This was followed by analyzing the induced phenotypic changes in these target cells to gain insight into the impact of EVs.

Initial evidence of the procoagulant role of EVs and their influence on thromboembolic events and endothelial dysfunction came from observing endothelial cells interacting with PS-externalizing red cells. This exposure showed that PS-enriched lipid vesicles compete for PS-binding sites on endothelial cell monolayers, indicating the prothrombotic effects of both PS-exposed red cells and PS-enriched vesicles (26). Studies have documented significant evidence that red cells in thalassemic patients externalize PS on their outer surface (27,28). When these cells shed as PS-expressing red cell vesicles, they contribute to the initiation of chronic hypercoagulability, along with other procoagulant factors (29-31).

With advancements in flow cytometric analysis, Pattanapanyasat *et al* (32) and another study by the same group (33) successfully quantified the number of vesicles shed from red cells. These vesicles are phenotypically distinct, as identified by their reactivity to annexin-V coupled with glycophorin A (CD235a). They are also smaller than the platelet population, as observed in logarithmic forward- and side-scatter dot plots. These characteristics revealed that thalassemic patients have more circulating red cell vesicles than healthy individuals. Furthermore, patients who had undergone splenectomy were found to have significantly elevated levels of these vesicles.

These results align with those reported by other research groups, who found that platelets, rather than red cell-derived EVs, are predominantly responsible for procoagulant activity in splenectomized patients receiving blood transfusions (34). In addition, a marked increase in platelet-derived EVs has been identified as a predictive biomarker for thromboembolic events in both patients with TM and TI (35,36).

In addition to red blood cells and platelets, other cell types also release EVs. Flow cytometry may be employed to identify the cellular origin of these EVs using fluorochrome-conjugated antibodies specific to their parent cells (37). Common surface markers used to determine the cellular origin of EVs are listed in Table I. Beyond flow cytometry, several other techniques are utilized for characterizing circulating EVs, including proteomic studies for specific marker identification. Several common biomarkers for exosomes, including major histocompatibility complex, flotillin and the heat-shock 70-kDa protein (HSP70) are similarly present in all EV types. Proteins particularly enriched in small EVs and showing varying expression levels in different EV populations have been documented. Western blot analysis is used to assess the differential expression of CD9, CD63 and CD81. These transmembrane proteins are frequently observed in EVs from various cell types, providing a framework for identifying subtypes of EVs (38,39).

Transmission electron microscopy, a widely utilized technique (40), is crucial in assessing the quality and purity of samples containing EVs. This technique can distinguish individual EVs from particles of similar size that are not EVs (41).

In addition, microRNAs (miRNAs) associated with EVs have gained attention as potential biomarkers for various human diseases. To detect these EV-miRNAs, reverse transcription-quantitative PCR (RT-qPCR) is commonly employed (42). Recent advancements include the development of a simplified and cost-effective approach, single-step RT-qPCR, which allows for the direct detection of EV-miRNAs without the need for RNA purification (43).

Nanoparticle tracking analysis is another critical technology used for quantifying and determining the size of EVs. It offers reproducibility and accuracy in measuring the particle concentration and size distribution of EVs isolated from diverse sources through various methods. To enhance reproducibility

in future EV research, standardization of nanoparticle tracking analysis methods is essential (44).

EVs as predictive biomarkers in thalassemia. Proteomic analysis of plasma vesicles from β-thalassemia/HbE patients has revealed an accumulative increase in proteins associated with oxidative damage in red blood cells and platelets. Notable among these proteins are peroxiredoxin 6 and HSP90 (45). In addition, changes in the levels of specific proteins in EVs from thalassemic patients, such as increased α-Hb-stabilizing protein and decreased haptoglobin, hemopexin and cathepsin S, suggest their potential as biomarkers for hemolysis and inflammation. Mass spectrometry has been utilized to quantify these markers in patients with β-thalassemia/HbE (46). Ferru *et al* (47) explored hemichromes, composed of denatured α-globin within thalassemic red cell EVs, that bind to band 3 protein and trigger phosphorylation of p27Syk kinase. This process promotes red cell membrane instability and subsequent EV shedding. They also identified a set of HSP70 and peroxiredoxin 2 proteins that are enriched in these EVs (47). Levin *et al* (48) used nanoparticle tracking analysis to show increased levels of thalassemic EVs following splenectomy. These EVs have high concentrations of HSP70, which contributes to ineffective erythropoiesis, hemolysis and disease severity (48). Further studies indicate that stored thalassemic blood units show overexpression of caspase-3 and molecular chaperones such as HSP70 and DJ-1 in the EV fraction. This finding suggests that oxidative stress affects the physiology and aging of stored thalassemic red cells (49).

Increased amounts of circulating EVs have been reported to be associated with clinical complications commonly observed in patients with β-thalassemia. This increase has been observed to positively correlate with the levels of hypoxia-inducible factor α, a marker for tissue hypoxia, particularly in pediatric patients with thalassemia (50). This relationship underscores the connection between oxidative stress and the formation of EVs, which in turn has a role in the thromboembolic phenomena frequently observed in these patients. Significant increases in circulating $CD146^+$ endothelial EVs, endothelial progenitor cells and von Willebrand factor have also been identified. These changes are linked to the incidence of cardiovascular complications in young patients with β-thalassemia (51). However, elevated levels of circulating EVs have not been associated with pulmonary arterial hypertension in splenectomized thalassemic patients. This finding is attributed to the use of antiplatelet drugs aimed at reducing platelet activation levels (52).

Classifying β-thalassemia subtypes is crucial for effective treatment and EVs have emerged as potential biomarkers for screening the disease and differentiating among its subtypes. Research conducted by Li *et al* (53) has shed light on the proteomics profiles of plasma-derived EVs in patients with TM, TI and healthy donors. They identified the top six proteins for the diagnosis of patients with β-thalassemia: Complement C1s subcomponent (C1S), clusterin (CLU), lactotransferrin, complement C1r subcomponent, C4b-binding protein β chain and C4b-binding protein α chain. Their study revealed distinct proteomic patterns in patients with TI and TM. The top six proteins that showed potential in distinguishing patients with TM from those with TI were serotransferrin (TF), Hb subunit α (HBA1), immunoglobulin heavy constant γ 4 (IGHG4), HBB, plasma kallikrein B1 and apolipoprotein M. Furthermore, they proposed a model using five proteins (TF, C1S, IGHG4, CLU and HBA1) to discriminate among the three groups. The findings suggest the potential of EVs in subtyping and highlight the ability of plasma-derived EVs to aid in diagnosing β-thalassemia patients (53).

Placenta-derived EVs have emerged as promising tools for the diagnosis of thalassemia. These EVs are detectable in maternal circulation as early as 6 weeks of gestation, and their release can be induced by conditions such as hypoxia and hyperglycemia. Changes in the quantity and composition of EVs have been observed in placental-related complications such as preeclampsia and fetal growth restriction, highlighting their potential as a diagnostic tool for identifying such complications in asymptomatic pregnant women. Furthermore, in cases of Bart's hydrops fetuses, placental hypoxia may prompt alterations in the number or function of placenta-derived EVs. This characteristic may offer a valuable means for early, noninvasive prenatal diagnosis. However, standardized detection methods are needed to effectively use placenta-derived EV detection in diagnosing, monitoring and treating Bart's hydrops fetalis and similar placental disorders (54).

Pathologic function of thalassemic EVs. In thalassemic patients, an increased number of plasma EVs has been linked to a hypercoagulable state; however, their specific role in thalassemia is not well defined. Various research groups have examined the biological effects of plasma EVs isolated from thalassemic patients on different types of recipient cells. EVs, primarily MPs, from splenectomized patients have shown a significant capacity to activate platelets and cause microaggregation of leukocyte platelets compared to vesicles isolated from healthy individuals or non-splenectomized patients (24).

The prothrombotic function of thalassemic EVs is further evident in their contribution to endothelial cell dysfunction. When endothelial cells internalize EVs derived from splenectomized patients, there is an enhanced induction of endothelial cell activation markers and proinflammatory mediators, as well as an increased binding ability of endothelial cells to the human monocytic leukemia cell line THP-1 (55,56). In addition, thalassemic EVs, particularly exosomes containing ferritin and hemichrome, have been found to promote the hyperproliferation of cardiac cells, potentially leading to cardiomyopathic complications commonly seen in thalassemic patients (57).

Fig. 1 illustrates the prothrombotic potential of thalassemic EVs in contributing to hyperthrombophilic phenomena. Thalassemic EVs, which are mainly released from activated platelets and damaged red cells, facilitate the formation of microemboli: Complexes of platelets, red cells, leukocytes and coagulation factors. These microaggregates adhere to activated endothelial cells, triggering a hypercoagulable state in thalassemic patients.

Exosomes have a critical role in intercellular communication by shuttling biological cargos, such as miRNAs, to recipient cells. This transfer leads to modifications in the functional properties of target cells. In the case of β-thalassemia,

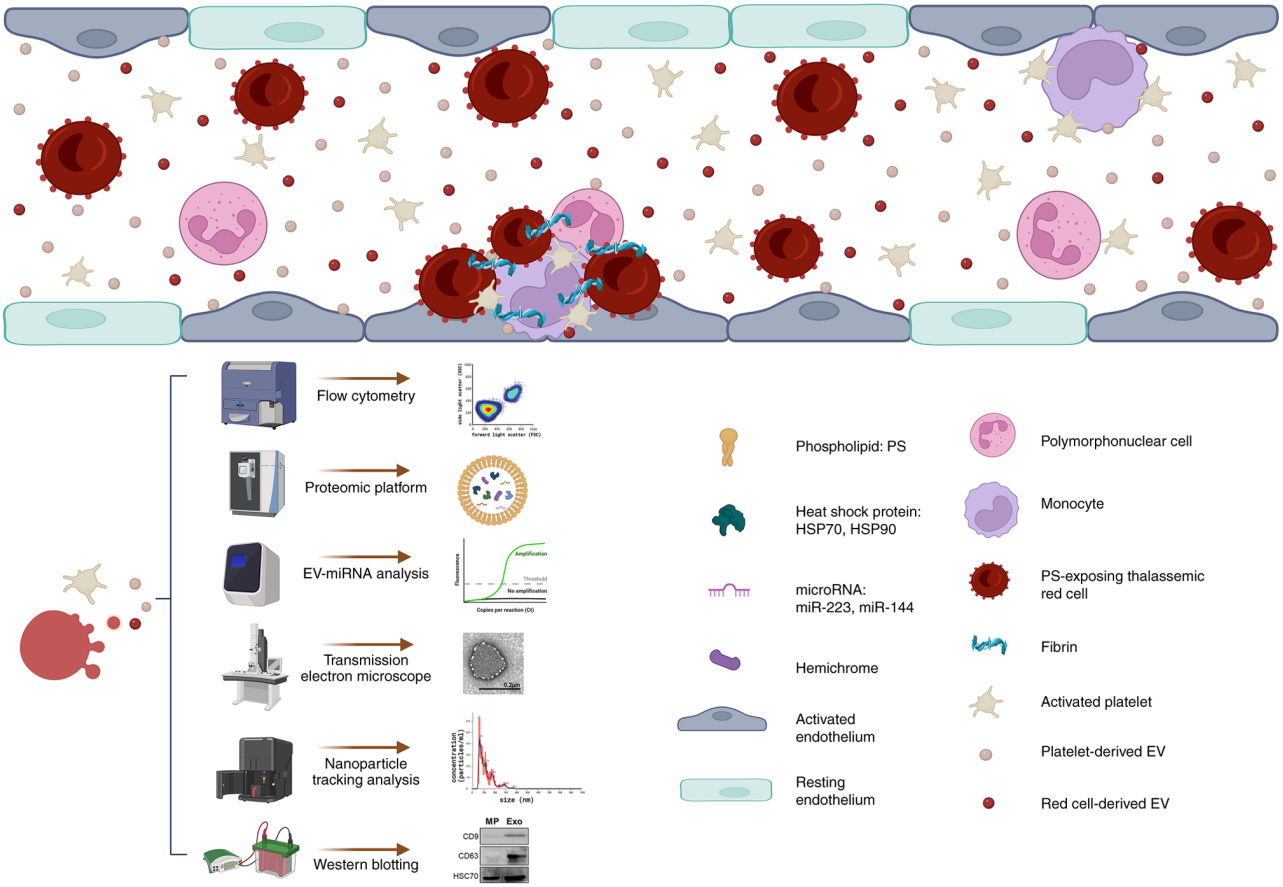

Figure 1. Role of thalassemic EVs in thromboembolic events. The schematic illustrates how thalassemic EVs, predominantly released by activated platelets and damaged red cells, contribute to the formation of microemboli. These microemboli, composed of platelets, red cells, leukocytes and coagulation factors, adhere to activated endothelial cells, thereby inducing a hypercoagulable state in thalassemic patients. The characterization of these circulating EVs involves various advanced techniques: Flow cytometry (to determine size and cell of origin); proteomic analysis (to identify biological content); reverse transcription-quantitative PCR (for EV miRNA profiling); transmission electron microscopy (to assess the quality and purity of EV-containing samples); nanoparticle tracking analysis (to measure size and concentration); and western blot analysis (to detect HSP70 and EV-specific protein markers, such as the tetraspanin proteins CD9 and CD63). The figure was designed using BioRender.com. EV, extracellular vesicle; miRNA/miR, microRNA; HSP, heat shock protein.

where genes that encode γ-globin are silent, thalassemic exosomes and miR-223-3p have been reported to suppress γ-globin expression by downregulating LIM-only protein 2, the globin gene regulator (58). Furthermore, differential expression of miRNA profiles in EVs from patients with β-thalassemia compared to those from healthy individuals has been observed.

When various recipient cells, such as endothelial cells, hepatocytes, pancreatic cells and mesenchymal stromal cells, are exposed to thalassemic EVs containing miR-144-3p, they exhibit dysfunctional phenotypes. These include reduced cell viability and induction of cell apoptosis. The mechanism behind β-thalassemia EV-induced apoptosis in endothelial cells is associated with the MAPK/JNK signal transduction pathway. By contrast, EVs from splenectomized patients with β-thalassemia induce the proliferation of bone marrow mesenchymal stromal cells. This finding suggests that EVs contribute to organ damage and complications in β-thalassemia (59).

EVs in bone marrow transplantation. Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the only established, potentially curative therapy for patients with TM. However, it is limited by the availability of matched donors and each patient's medical condition. Human leukocyte antigen (HLA)-matched stem cells, sourced from cord blood or bone marrow, have yielded excellent outcomes in HSCT. Therefore, it is recommended that patients with TM, particularly young patients, should consider HSCT at an early stage of the disease if they have HLA-identical sibling donors. This approach aims to prevent the development of severe clinical complications due to iron overload and multiorgan failure. Anurathapan *et al* (60,61) have utilized haploidentical-related donors as a source of hematopoietic stem cells for pediatric patients with TM. Following matched-donor or haploidentical HSCT, certain patients with thalassemia exhibited increased endothelial activation and a heightened risk of thromboembolic events. These complications are attributed to several factors, including the conditioning regimen, high-dose chemotherapy, immune response and graft-versus-host disease (62). However, evidence indicates that HSCT can effectively correct hemostatic alterations in patients with TM. Sirachainan *et al* (63) found that plasma levels of coagulation markers (thrombin-antithrombin complex, prothrombin fragment and D-dimer) and anticoagulation factors (protein C, protein S and antithrombin activity) returned to normal levels following successful HSCT. This signified a positive therapeutic outcome.

Several studies have noted an increase in the number of circulating EVs following HSCT in hematological disorders. Trummer *et al* (64), for instance, reported a rise in plasma P-selectin glycoprotein ligand-1-expressing MPs and linked this increase to the risk of refractory disease and relapse in patients. Research by our group focusing on EV profiling in HSCT for thalassemia, flow cytometric analysis was utilized to measure plasma MPs in children with TM undergoing HSCT. The randomized study by our group specifically measured PS-expressing red cells and $CD235a^+$ red cell MPs. The levels of these two factors decreased after transplantation (15). Conversely, in another study by our group, an increase in other MP populations from platelets ($CD41a^+$ MP), leukocytes ($CD45^+$ MP) and endothelial cells ($CD146^+$ MP) in the plasma of transplanted patients was observed. These procoagulant MPs were associated with a higher incidence of monocyte-platelet microaggregates, suggesting a complex interaction and potential clinical implications in the post-transplant setting (16).

5. EVs in other hemoglobinopathies

In the realm of common hemoglobinopathies, sickle cell disease (SCD) serves as the prototype. This inherited disorder arises from a point mutation in the β-globin gene, leading to the formation of hemoglobin S. SCD is often linked to chronic vascular inflammation and hypercoagulability. These conditions result from the combined effects of procoagulant sickle red cells, activated platelets and cell-derived EVs. Collectively, these components induce endothelial cell activation, leading to cardiovascular complications (25,65,66). Two core mechanisms have been identified in this process: The transfer of heme to endothelial cells (67) and the increased adhesiveness of sickle red cells to endothelial cells (68). These interactions highlight the complex pathophysiology of SCD and the significant role of EVs in its progression and complications.

In SCD, numerous studies have reported the presence of large numbers of EVs, particularly those derived from red blood cells and platelets. EVs are found in patients both during steady-state and sickling crises, and their levels are significantly higher than those in healthy individuals (69,70). These sickle cell-derived EVs initiate hemostatic abnormalities, such as prolonged thrombin generation (71). With regard to abnormal thrombin generation in SCD, the amplification was presumably mediated by PS exposed on sickle cell MPs that activates the intrinsic pathway of the coagulation cascade through factor XI (69), and the extent of prothrombotic PS relies on the size of red cell MPs, particularly macrovesicles (72). Of note, treatments with hydroxyurea (73) or hydroxycarbamide (74) have been shown to ameliorate these effects.

Beyond their role as bioeffectors in SCD, these sickle cell-derived EVs also serve as biomarkers for predicting disease-related complications. These complications include oxidative stress related to vaso-occlusive crises (75), osteonecrosis (76), leg ulcers and pulmonary hypertension (77). A notable aspect of SCD-EVs is their content of functional miRNAs, specifically miR-124-3p, miR-2278 and miR-4763-5p. These miRNAs are reported to be enriched in SCD-plasma EVs and could contribute to the initiation and progression of the disease (78). This finding highlights the multifaceted role of EVs in the pathophysiology and potential diagnostic and therapeutic approaches in SCD.

6. Conclusions and future perspectives

In both normal and disease conditions, EVs, including MPs and exosomes, are produced by most, if not all, cell lineages. The study of MPs has garnered increased attention in exploring the pathogenesis of common complications in patients with thalassemia. While certain studies have demonstrated the regulatory role of exosomes and exosomal miRNAs in contributing to physiological and pathophysiological processes in thalassemia biology, further research is needed. The phenotypic characterization and functional study of EVs require precise and accurate methodologies and techniques due to the challenges posed by their small size. Understanding the molecular mechanisms that govern the production and clearance of EVs, as well as those involved in intercellular communication, is crucial. This knowledge could pave the way for novel therapeutic approaches to reduce clinical complications in thalassemia, offering new insight and potential breakthroughs in the treatment of thalassemia.

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Authors' contributions

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Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Rund D and Rachmilewitz E: Beta-thalassemia. N Engl J Med 353: 1135-1146, 2005.
2. Piel FB and Weatherall DJ: The α-thalassemias. N Engl J Med 371: 1908-1916, 2014.
3. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, Romeo MA, Forni GL, Gamberini MR, Ghilardi R, *et al*: Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica 89: 1187-1193, 2004.
4. Zurlo MG, De Stefano P, Borgna-Pignatti C, Di Palma A, Piga A, Melevendi C, Di Gregorio F, Burattini MG and Terzoli S: Survival and causes of death in thalassaemia major. Lancet 2: 27-30, 1989.
5. Sleiman J, Tarhini A, Bou-Fakhredin R, Saliba AN, Cappellini MD and Taher AT: Non-Transfusion-Dependent Thalassemia: An update on complications and management. Int J Mol Sci 19: 182, 2018.
6. Cappellini MD, Robbiolo L, Bottasso BM, Coppola R, Fiorelli G and Mannucci AP: Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia. Br J Haematol 111: 467-473, 2000.
7. Eldor A and Rachmilewitz EA: The hypercoagulable state in thalassemia. Blood 99: 36-43, 2002.
8. Atichartakarn V, Angchaisuksiri P, Aryurachai K, Chuncharunee S and Thakkinstian A: In vivo platelet activation and hyperaggregation in hemoglobin E/beta-thalassemia: A consequence of splenectomy. Int J Hematol 77: 299-303, 2003.
9. Atichartakarn V, Chuncharunee S, Chandanamattha P, Likittanasombat K and Aryurachai K: Correction of hypercoagulability and amelioration of pulmonary arterial hypertension by chronic blood transfusion in an asplenic hemoglobin E/beta-thalassemia patient. Blood 103: 2844-2846, 2004.
10. Atichartakarn V, Angchaisuksiri P, Aryurachai K, Onpun S, Chuncharunee S, Thakkinstian A and Atamasirikul K: Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/beta-thalassaemic patients. Br J Haematol 118: 893-898, 2002.
11. Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, *et al*: Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4: 27066, 2015.
12. Loyer X, Vion AC, Tedgui A and Boulanger CM: Microvesicles as cell-cell messengers in cardiovascular diseases. Circ Res 114: 345-353, 2014.
13. Westerman M, Pizzey A, Hirschman J, Cerino M, Weil-Weiner Y, Ramotar P, Eze A, Lawrie A, Purdy G, Mackie I and Porter J: Microvesicles in haemoglobinopathies offer insights into mechanisms of hypercoagulability, haemolysis and the effects of therapy. Br J Haematol 142: 126-135, 2008.
14. Aharon A, Rebibo-Sabbah A, Tzoran I and Levin C: Extracellular vesicles in hematological disorders. Rambam Maimonides Med J 5: e0032, 2014.
15. Klaihmon P, Vimonpatranon S, Noulsri E, Lertthammakiat S, Anurathapan U, Sirachainan N, Hongeng S and Pattanapanyasat K: Normalized levels of red blood cells expressing phosphatidylserine, their microparticles, and activated platelets in young patients with β-thalassemia following bone marrow transplantation. Ann Hematol 96: 1741-1747, 2017.
16. Klaihmon P, Lertthammakiat S, Anurathapan U, Pakakasama S, Sirachainan N, Hongeng S and Pattanapanyasat K: Activated platelets and leukocyte activations in young patients with β-thalassemia/HbE following bone marrow transplantation. Thromb Res 169: 8-14, 2018.
17. Origa R: β-Thalassemia. Genet Med 19: 609-619, 2017.
18. Cazzola M: Ineffective erythropoiesis and its treatment. Blood 139: 2460-2470, 2022.
19. Galanello R and Origa R: Beta-thalassemia. Orphanet J Rare Dis 5: 11, 2010.
20. Taher AT, Otrock ZK, Uthman I and Cappellini MD: Thalassemia and hypercoagulability. Blood Rev 22: 283-292, 2008.
21. Vichinsky EP: Clinical manifestations of alpha-thalassemia. Cold Spring Harb Perspect Med 3: a011742, 2013.
22. Sirachainan N, Chuansumrit A, Kadegasem P, Sasanakul W, Wongwerawattanakoon P and Mahaklan L: Normal hemostatic parameters in children and young adults with α-thalassemia diseases. Thromb Res 146: 35-42, 2016.
23. Pattanapanyasat K, Gonwong S, Chaichompoo P, Noulsri E, Lerdwana S, Sukapirom K, Siritanaratkul N and Fucharoen S: Activated platelet-derived microparticles in thalassaemia. Br J Haematol 136: 462-471, 2007.
24. Klaihmon P, Phongpao K, Kheansaard W, Noulsri E, Khuhapinant A, Fucharoen S, Morales NP, Svasti S, Pattanapanyasat K and Chaichompoo P: Microparticles from splenectomized β-thalassemia/HbE patients play roles on procoagulant activities with thrombotic potential. Ann Hematol 96: 189-198, 2017.
25. Tantawy AA, Adly AA, Ismail EA, Habeeb NM and Farouk A: Circulating platelet and erythrocyte microparticles in young children and adolescents with sickle cell disease: Relation to cardiovascular complications. Platelets 24: 605-614, 2013.
26. Manodori AB, Barabino GA, Lubin BH and Kuypers FA: Adherence of phosphatidylserine-exposing erythrocytes to endothelial matrix thrombospondin. Blood 95: 1293-1300, 2000.
27. Zahedpanah M, Azarkeivan A, Aghaieepour M, Nikogoftar M, Ahmadinegad M, Hajibeigi B, Tabatabaiee MR and Maghsudlu M: Erythrocytic phosphatidylserine exposure and hemostatic alterations in beta-thalassemia intermediate patients. Hematology 19: 472-476, 2014.
28. Mahdi ZN, Al-Mudallal SS and Hameed BM: Role of red blood cells 'annexin V' and platelets 'P-selectin' in patients with thalassemia. Hematol Oncol Stem Cell Ther 12: 15-18, 2019.
29. Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS and Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arterioscler Thromb Vasc Biol 27: 414-421, 2007.
30. Willekens FL, Were JM, Groenen-Döpp YA, Roerdinkholder-Stoelwinder B, de Pauw B and Bosman GJ: Erythrocyte vesiculation: A self-protective mechanism? Br J Haematol 141: 549-556, 2008.
31. Camus SM, Gausserès B, Bonnin P, Loufrani L, Grimaud L, Charue D, De Moraes JA, Renard JM, Tedgui A, Boulanger CM, *et al*: Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease. Blood 120: 5050-5058, 2012.
32. Pattanapanyasat K, Noulsri E, Fucharoen S, Lerdwana S, Lamchiagdhase P, Siritanaratkul N and Webster HK: Flow cytometric quantitation of red blood cell vesicles in thalassemia. Cytometry B Clin Cytom 57: 23-31, 2004.
33. Lamchiagdhase P, Nitipongwanich R, Rattanapong C, Noulsri E, Lerdwana S and Pattanapanyasat K: Red blood cell vesicles in thalassemia. J Med Assoc Thai 87: 233-238, 2004.
34. Agouti I, Cointe S, Robert S, Judicone C, Loundou A, Driss F, Brisson A, Steschenko D, Rose C, Pondarré C, *et al*: Platelet and not erythrocyte microparticles are procoagulant in transfused thalassaemia major patients. Br J Haematol 171: 615-624, 2015.
35. Youssry I, Soliman N, Ghamrawy M, Samy RM, Nasr A, Abdel Mohsen M, ElShahaat M, Bou Fakhredin R and Taher A: Circulating microparticles and the risk of thromboembolic events in Egyptian beta thalassemia patients. Ann Hematol 96: 597-603, 2017.
36. Habib A, Kunzelmann C, Shamseddeen W, Zobairi F, Freyssinet JM and Taher A: Elevated levels of circulating procoagulant microparticles in patients with beta-thalassemia intermedia. Haematologica 93: 941-942, 2008.
37. Nielsen MH, Beck-Nielsen H, Andersen MN and Handberg A: A flow cytometric method for characterization of circulating cell-derived microparticles in plasma. J Extracell Vesicles 3: 2014.
38. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M and Théry C: Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci USA 113: E968-E977, 2016.
39. Kowal EJK, Ter-Ovanesyan D, Regev A and Church GM: Extracellular Vesicle Isolation and Analysis by Western Blotting. Methods Mol Biol 1660: 143-152, 2017.
40. Tijssen MR, Cvejic A, Joshi A, Hannah RL, Ferreira R, Forrai A, Bellissimo DC, Oram SH, Smethurst PA, Wilson NK, *et al*: Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. Dev Cell 20: 597-609, 2011.

41. Rikkert LG, Nieuwland R, Terstappen Lwmm and Coumans FAW: Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent. J Extracell Vesicles 8: 1555419, 2019.
42. El Andaloussi S, Mäger I, Breakefield XO and Wood MJA: Extracellular vesicles: Biology and emerging therapeutic opportunities. Nat Rev Drug Discov 12: 347-357, 2013.
43. Lee H, He X, Le T, Carnino JM and Jin Y: Single-step RT-qPCR for detection of extracellular vesicle microRNAs in vivo: A time- and cost-effective method. Am J Physiol Lung Cell Mol Physiol 318: L742-l749, 2020.
44. Comfort N, Cai K, Bloomquist TR, Strait MD, Ferrante AW Jr and Baccarelli AA: Nanoparticle tracking analysis for the quantification and size determination of extracellular vesicles. J Vis Exp: Mar 28, 2021 (Epub ahead of print). doi: 10.3791/62447.
45. Chaichompoo P, Kumya P, Khowawisetsut L, Chiangjong W, Chaiyarit S, Pongsakul N, Sirithanaratanakul N, Fucharoen S, Thongboonkerd V and Pattanapanyasat K: Characterizations and proteome analysis of platelet-free plasma-derived microparticles in β-thalassemia/hemoglobin E patients. J Proteomics 76 Spec No.: 239-250, 2012.
46. Kittivorapart J, Crew VK, Wilson MC, Heesom KJ, Siritanaratkul N and Toye AM: Quantitative proteomics of plasma vesicles identify novel biomarkers for hemoglobin E/β-thalassemic patients. Blood Adv 2: 95-104, 2018.
47. Ferru E, Pantaleo A, Carta F, Mannu F, Khadjavi A, Gallo V, Ronzoni L, Graziadei G, Cappellini MD and Turrini F: Thalassemic erythrocytes release microparticles loaded with hemichromes by redox activation of p72Syk kinase. Haematologica 99: 570-578, 2014.
48. Levin C, Koren A, Rebibo-Sabbah A, Koifman N, Brenner B and Aharon A: Extracellular Vesicle Characteristics in β-thalassemia as Potential Biomarkers for Spleen Functional Status and Ineffective Erythropoiesis. Front Physiol 9: 1214, 2018.
49. Tzounakas VL, Anastasiadi AT, Dzieciatkowska M, Karadimas DG, Stamoulis K, Papassideri IS, Hansen KC, D'Alessandro A, Kriebardis AG and Antonelou MH: Proteome of Stored RBC membrane and vesicles from heterozygous beta thalassemia donors. Int J Mol Sci 22: 3369, 2021.
50. Elsayh KI, Zahran AM, El-Abaseri TB, Mohamed AO and El-Metwally TH: Hypoxia biomarkers, oxidative stress, and circulating microparticles in pediatric patients with thalassemia in Upper Egypt. Clin Appl Thromb Hemost 20: 536-545, 2014.
51. Adly AA, El-Sherif NH, Ismail EA, El-Zaher YA, Farouk A, El-Refaey AM and Wahba MS: Vascular dysfunction in patients with young β-thalassemia: Relation to cardiovascular complications and subclinical atherosclerosis. Clin Appl Thromb Hemost 21: 733-744, 2015.
52. Manakeng K, Prasertphol P, Phongpao K, Chuncharunee S, Tanyong D, Worawichawong S, Svasti S and Chaichompoo P: Elevated levels of platelet- and red cell-derived extracellular vesicles in transfusion-dependent β-thalassemia/HbE patients with pulmonary arterial hypertension. Ann Hematol 98: 281-288, 2019.
53. Li N, Wu B, Wang J, Yan Y, An P, Li Y, Liu Y, Hou Y, Qing X, Niu L, *et al*: Differential proteomic patterns of plasma extracellular vesicles show potential to discriminate β-thalassemia subtypes. iScience 26: 106048, 2023.
54. Chaemsaithong P, Luewan S, Taweevisit M, Chiangjong W, Pongchaikul P, Thorner PS, Tongsong T and Chutipongtanate S: Placenta-Derived extracellular vesicles in pregnancy complications and prospects on a liquid biopsy for hemoglobin Bart's Disease. Int J Mol Sci 24: 5658, 2023.
55. Kheansaard W, Phongpao K, Paiboonsukwong K, Pattanapanyasat K, Chaichompoo P and Svasti S: Microparticles from β-thalassaemia/HbE patients induce endothelial cell dysfunction. Sci Rep 8: 13033, 2018.
56. Klaihmon P, Khuhapinant A, Kheansaard W and Pattanapanyasat K: Internalization of cell-derived microparticles triggers endothelial pro-inflammatory responses. Asian Pac J Allergy Immunol: Apr 18, 2021 (Epub ahead of print).
57. Atipimonpat A, Siwaponanan P, Khuhapinant A, Svasti S, Sukapirom K, Khowawisetsut L and Pattanapanyasat K: Extracellular vesicles from thalassemia patients carry iron-containing ferritin and hemichrome that promote cardiac cell proliferation. Ann Hematol 100: 1929-1946, 2021.
58. Sun KT, Huang YN, Palanisamy K, Chang SS, Wang IK, Wu KH, Chen P, Peng CT and Li CY: Reciprocal regulation of γ-globin expression by exo-miRNAs: Relevance to γ-globin silencing in β-thalassemia major. Sci Rep 7: 202, 2017.
59. Levin C, Koren A, Rebibo-Sabbah A, Levin M, Koifman N, Brenner B and Aharon A: Extracellular Vesicle MicroRNA That Are Involved in β-Thalassemia complications. Int J Mol Sci 22: 9760, 2021.
60. Anurathapan U, Hongeng S, Pakakasama S, Sirachainan N, Songdej D, Chuansumrit A, Charoenkwan P, Jetsrisuparb A, Sanpakit K, Rujkijyanont P, *et al*: Hematopoietic stem cell transplantation for homozygous β-thalassemia and β-thalassemia/hemoglobin E patients from haploidentical donors. Bone Marrow Transplant 51: 813-818, 2016.
61. Anurathapan U, Hongeng S, Pakakasama S, Songdej D, Sirachainan N, Pongphitcha P, Chuansumrit A, Charoenkwan P, Jetsrisuparb A, Sanpakit K, *et al*: Hematopoietic stem cell transplantation for severe thalassemia patients from haploidentical donors using a novel conditioning regimen. Biol Blood Marrow Transplant 26: 1106-1112, 2020.
62. Lertthammakiat S, Sitthirat P, Anurathapan U, Songdej D, Pakakasama S, Chuansumrit A, Putawornsub N, Sirasittikarn S, Wantanawijarn S, Kadegasem P, *et al*: No differences in hemostatic and endothelial activations between haploidentical and matched-donor hematopoietic stem cell transplantation in thalassemia disease. Thromb J 18: 21, 2020.
63. Sirachainan N, Thongsad J, Pakakasama S, Hongeng S, Chuansumrit A, Kadegasem P, Tirakanjana A, Archararit N and Sirireung S: Normalized coagulation markers and anticoagulation proteins in children with severe β-thalassemia disease after stem cell transplantation. Thromb Res 129: 765-770, 2012.
64. Trummer A, De Rop C, Stadler M, Ganser A and Buchholz S: P-selectin glycoprotein ligand-1 positive microparticles in allogeneic stem cell transplantation of hematologic malignancies. Exp Hematol 9: 1047-1055, 2011.
65. Ataga KI, Cappellini MD and Rachmilewitz EA: Beta-thalassaemia and sickle cell anaemia as paradigms of hypercoagulability. Br J Haematol 139: 3-13, 2007.
66. Garnier Y, Ferdinand S, Garnier M, Cita KC, Hierso R, Claes A, Connes P, Hardy-Dessources MD, Lapouméroulie C, Lemonne N, *et al*: Plasma microparticles of sickle patients during crisis or taking hydroxyurea modify endothelium inflammatory properties. Blood 136: 247-256, 2020.
67. Camus SM, De Moraes JA, Bonnin P, Abbyad P, Le Jeune S, Lionnet F, Loufrani L, Grimaud L, Lambry JC, Charue D, *et al*: Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. Blood 125: 3805-3814, 2015.
68. An R, Man Y, Cheng K, Zhang T, Chen C, Wang F, Abdulla F, Kucukal E, Wulftange WJ, Goreke U, *et al*: Sickle red blood cell-derived extracellular vesicles activate endothelial cells and enhance sickle red cell adhesion mediated by von Willebrand factor. Br J Haematol 201: 552-563, 2023.
69. van Beers EJ, Schaap MC, Berckmans RJ, Nieuwland R, Sturk A, van Doormaal FF, Meijers JC and Biemond BJ; CURAMA study group: Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica 94: 1513-1519, 2009.
70. Nebor D, Bowers A, Connes P, Hardy-Dessources MD, Knight-Madden J, Cumming V, Reid M and Romana M: Plasma concentration of platelet-derived microparticles is related to painful vaso-occlusive phenotype severity in sickle cell anemia. PLoS One 9: e87243, 2014.
71. Gerotziafas GT, Van Dreden P, Chaari M, Galea V, Khaterchi A, Lionnet F, Stankovic-Stojanovic K, Blanc-Brude O, Woodhams B, Maier-Redelsperger M, *et al*: The acceleration of the propagation phase of thrombin generation in patients with steady-state sickle cell disease is associated with circulating erythrocyte-derived microparticles. Thromb Haemost 107: 1044-1052, 2012.
72. Smith RA, Mankelow TJ, Drizou D, Bullock T, Latham T, Trompeter S, Blair A and Anstee DJ: Large red cell-derived membrane particles are major contributors to hypercoagulability in sickle cell disease. Sci Rep 11: 11035, 2021.
73. Nouraie M, Lee JS, Zhang Y, Kanias T, Zhao X, Xiong Z, Oriss TB, Zeng Q, Kato GJ, Gibbs JS, *et al*: The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe. Haematologica 98: 464-472, 2013.
74. Garnier Y, Ferdinand S, Connes P, Garnier M, Etienne-Julan M, Lemonne N and Romana M: Decrease of externalized phosphatidylserine density on red blood cell-derived microparticles in SCA patients treated with hydroxycarbamide. Br J Haematol 182: 448-451, 2018.

75. Hierso R, Lemonne N, Villaescusa R, Lalanne-Mistrih ML, Charlot K, Etienne-Julan M, Tressières B, Lamarre Y, Tarer V, Garnier Y, *et al*: Exacerbation of oxidative stress during sickle vaso-occlusive crisis is associated with decreased anti-band 3 autoantibodies rate and increased red blood cell-derived microparticle level: A prospective study. Br J Haematol 176: 805-813, 2017.
76. Marsh A, Schiffelers R, Kuypers F, Larkin S, Gildengorin G, van Solinge W and Hoppe C: Microparticles as biomarkers of osteonecrosis of the hip in sickle cell disease. Br J Haematol 168: 135-138, 2015.
77. Olatunya OS, Lanaro C, Longhini AL, Penteado CFF, Fertrin KY, Adekile A, Saad STO and Costa FF: Red blood cells microparticles are associated with hemolysis markers and may contribute to clinical events among sickle cell disease patients. Ann Hematol 98: 2507-2521, 2019.
78. Khalyfa A, Khalyfa AA, Akbarpour M, Connes P, Romana M, Lapping-Carr G, Zhang C, Andrade J and Gozal D: Extracellular microvesicle microRNAs in children with sickle cell anaemia with divergent clinical phenotypes. Br J Haematol 174: 786-798, 2016.