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Extracellular vesicles in thalassemia: Mechanisms, implications, and therapeutic potential

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ABSTRACT

Thalassemia is one of the most common inherited disorders of erythrocytes, caused by abnormalities in the production of globin chains. The clinical spectrum of thalassemia is broad, ranging from severe and persistent anemia that necessitates consistent blood transfusions to mild, asymptomatic conditions. Key contributors to thalassemia complications, particularly, in patients with β-thalassemia major, are ineffective erythropoiesis and iron overload. These complications can lead to a variety of severe health issues, including chronic inflammation, organ dysfunction, thrombosis, vascular abnormalities, and systemic iron overload. Extracellular vesicles (EVs) are tiny membrane-bound particles secreted from the plasma membranes of various cells during activation and cell death. Research has indicated that EVs are involved in numerous physiological and pathological processes, including inflammatory responses, clot formation, and vascular injury. Recently, the role of EVs has garnered interest of their potential as biomarkers, providing diagnostic and prognostic value of various disorders. In the context of thalassemia, elevated levels of EVs have been observed, highlighting their significance in the disease's cellular activities. The current review aims to examine the role of EVs in the pathogenesis of thalassemia, their implications, and their potential clinical applications. By exploring the involvement of EVs in the inflammatory and vascular complications associated with thalassemia, this review provides insights into their potential as therapeutic targets and diagnostic tools, offering a new perspective on managing this complex and multifaceted disorder.

1. Introduction

Thalassemia syndrome is a cluster of hereditary disorders of hemoglobin described by decreased or absent production of one or more chains of globin. Depending on the specific globin chain affected, thalassemia can be classified into alpha (α), beta (β), delta (δ), gamma (γ), delta-beta (δβ), and gamma-delta-beta (γδβ) thalassemia. The most common forms as β- and α-thalassemia, which result from defects in the genes of β- and α-globin chains, respectively(Angastiniotis and Lobitz, 2019). The chief pathophysiological issue in thalassemia syndrome is the reduced production of globin chains, leading to an imbalance in their ratio within hemoglobin. This imbalance causes the unstable unbound globin chains to attach the cytoplasmic surface of the erythrocyte membranes, resulting in the lysis of erythroid cells, ineffective erythropoiesis, and increased RBC aggregation and hemolysis in circulation. The major contributors to complications in β-thalassemia patients are iron overload and ineffective erythropoiesis from regular blood transfusions. These complications include cardiac problems, chronic pain, organ dysfunction, thrombosis, pulmonary hypertension, high ferritin levels, inflammation, and vascular dysfunction (Longo et al., 2021a). The excess unpaired chains that accumulated in erythroid precursor cells within the bone marrow induce oxidative stress and damage cell membranes, triggering apoptosis and leading to the early destruction of these precursors. This process, termed ineffective erythropoiesis, is central to the pathology of thalassemia, as it severely impairs the body's ability to produce sufficient mature RBCs, resulting in chronic anemia. The remaining RBCs, burdened with globin chain precipitates on their membranes, are more vulnerable to hemolysis which further intensifies the anemia (Longo et al., 2021a).

To counteract the chronic anemia, the body responds by expanding bone marrow activity in an attempt to increase RBC production a process known as erythroid hyperplasia. Unfortunately, this compensatory mechanism is often insufficient and can lead to skeletal deformities, particularly in the facial bones, as well as stunted growth in children

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(Messick, 2023; Sayani and Kwiatkowski, 2015). Additionally, elevated erythropoietin production stimulates extramedullary hematopoiesis, causing enlargement of organs like the liver and spleen. Iron overload is another major complication of thalassemia, arising both from the increased absorption of dietary iron-driven by ineffective erythropoiesis signaling the need for more iron- and the repeated blood transfusions used to manage anemia. Each transfusion introduces additional iron into the body, which, in the absence of an efficient excretion mechanism, accumulates in critical organs such as the heart, liver, and endocrine glands. This iron buildup promotes the formation of reaction oxygen species, leading to oxidative stress and progressive damage of these organs. Over time, this can results in serious complications, including heart disease, liver cirrhosis, diabetes, and hormonal imbalances like hypothyroidism and hypogonadism (Yadav and Singh, 2022). Patients with thalassemia are also at higher risk of developing thromboembolic events due to multiple factors. Chronic hemolysis releases free hemoglobin into the bloodstream, which depletes nitric oxide, a molecule essential for vascular relaxation and inhibition of platelet aggregation. The reduction in nitric oxide leads to vasoconstriction and increased platelet activation, creating a hypercoagulable state (Cappellini et al., 2012). Furthermore, elevated levels of circulating extracellular vesicles (EVs), particularly microparticles originating from platelets and erythrocytes, enhance the risk of blood clot formation by promoting coagulation and contributing to endothelial dysfunction (Zifkos et al., 2021). Vascular issues are particularly significant in β-thalassemia, where prolonged inflammation, damage to the endothelium, and oxidative stress contribute to complications such as pulmonary hypertension, leg ulcers, and other vascular problems. In the milder form of disease, β-thalassemia intermedia, patients may develop iron overload independent of transfusions, which exacerbates vascular and organ dysfunction (Hirsch et al., 2017).

Extracellular vesicles (EVs), small membrane-bound particles, are secreted into the extracellular space from membrane of different cells, including cancer cells, RBCs, platelets, endothelial cells, and leukocytes, during activation of apoptosis (Buzas, 2023). EVs are categorized by size into apoptotic bodies, exosomes, and microparticles. Exosomes, the tiniest EVs, form by the combination of membrane with the multivesicular bodies. Microparticles bud off from the plasma membrane, with 70–90% of circulating microparticles being platelet-derived. Apoptotic bodies are released during cell death. EVs consists of different molecules i.e., proteins, lipids, small interfering RNAs (siRNAs), messenger RNAs (mRNAs), microRNAs (miRNAs), and antigens from their cells. They play an important role in cell-to-cell communication and signal transmission (Wu et al., 2021). Research indicates that EVs are involved in cell signaling pathways, proinflammatory pathways, vascular damage, and clot formation (Yalameha et al., 2022). Recently, EVs have been explored as biomarkers for diagnosing clinical complications of various diseases (Bucciarelli et al., 2012; Li et al., 2021; Ma et al., 2021).

In healthy individuals, EV concentrations in the bloodstream are small but rise in conditions e.g., hematological diseases, infections, cardiovascular diseases, and infections (Gillespie and Doctor, 2021). In thalassemia, the buildup of unmatched globin chains on the RBC membrane leads to oxidative damage and membrane phospholipids disruption, causing the microparticles release into the extracellular space. Given their involvement in various cellular processes, elevated EV concentration, particularly those derived from the platelets and erythrocytes, may contribute to or aggravate the complications in patients with thalassemia (Sanchez-Villalobos et al., 2022). This review examines the significance of EVs in pathogenesis, implication and their clinical role in thalassemia.

2. Extracellular vesicles

The extracellular vesicles (EVs), membrane-bound structures fluctuating in size from 30-10,000nm, are secreted from cells to facilitate local and distinct intercellular communication. Initially identified over 50 years ago through electron microscopy, platelet-derived vesicles have only recently become a significant focus of research, revealing a broad spectrum of subtypes and activities. In 1980s, it was discovered that reticulocytes in sheep selectively secrete transferrin receptor in the EVs while red cell maturation, initially thought to be a method of expelling cellular waste (Doyle and Wang, 2019). However, recent studies have shown that EVs in the bone marrow play roles in regulating hematopoiesis, activating immune cells, and mediating hemostatic functions (Mittelbrunn et al., 2011; Goloviznina et al., 2017; Geddings et al., 2016). Hematologic malignancies, such as multiple myeloma and leukemia, as well as viral infections, can hijack EV mechanisms to promote tumor growth, invasion, relapse, metastasis, and chemotherapeutic resistance (Sousa-Pimenta et al., 2023). EVs can be categorized into subtypes based on size and release method: exosomes (30–150nm), microvesicles (50–1000 nm), large vesicles (more than 1000 nm), and apoptotic bodies (1000–5000nm) (Doyle and Wang, 2019). Separating these vesicle types is challenging, with no standardized method currently available. Common purification techniques rely on density or size, yet overlap exists among microvesicles and exosomes in function and composition, complicating efforts to achieve pure populations (Li et al., 2023a). Furthermore, due to size and function similarities with chylomicrons and very low-density lipoproteins in plasma, classifying EVs by size alone is arbitrary. A biologically informed classification would improve reproducibility, detection, and treatment strategies in the field.

Exosomes biogenesis starts with the interior budding of the plasma membrane to synthesize endosomes consisting of selectively bounded cytoplasmic constituents. Early endosomes, marked by Rab5 protein, mature into Rab7-containing late endosomes, which produce numerous intraluminal vesicles via tetraspanins and the endosomal sorting complex required for transport (ESCRT) proteins. Such proteins expedite additional inward budding and organization of endosome contents into intraluminal vesicles. The multivesicular bodies avoid lysosomal degradation and fuse combine with plasma membrane to secrete exosomes. The regulated mechanism results in relatively consistent exosome sizes and compared to larger vesicles (Skjeldal et al., 2021). Proteins such as ALG-2 interacting-protein X and tumor susceptibility gene 101 (ALIX and TSG1010) are involved in this process of endosomes and are commonly utilized as exosome markers. Different cell types release distinct exosome subpopulations, each with unique proteomic properties, membrane protein compositions, and RNA cargo (Sung et al., 2020).

Intermediate-sized EVs, typically called microvesicles, oncosomes if tumor-derived, or ectosomes, form by direct budding and membrane cleavage. Microvesicles, ranging from 50 to 1000 nm in diameter, differ from exosomes in that they do not use the endosomal/multivesicular body pathway. In its place, their formation involved calcium influx and cortical cytoskeletal remodeling. Unlike exosomes, microvesicles do not form consistently across cell types, though specific cell types can produce uniform populations (Stahl and Raposo, 2019). For example, neutrophils reliably shed two diverse populations of vesicles (about 50 nm and 500 nm) from the limiting membrane (Timár et al., 2013).

Larger vesicles, also called oncosomes when tumor-derived, are up to 10 μm and consist intact cytoskeletal structures and organelles. Unlike apoptotic bodies, which are similar in size and composition but form during programmed cell death, large vesicles originate from living cells' cytoplasmic extensions (Ciardiello et al., 2020). They have been identified in B-cell acute lymphoblastic leukemia and prostatecancer, observed in patient samples and *in vitro* cancer cell cultures (Johnson et al., 2017; Vizio et al., 2012). Apoptotic bodies form during programmed cell death as the cytoplasm and plasma membrane fragment, enclosing cytosolic components, nuclear fragments, and organelles, which are later phagocytosed and degraded in phagolysosomes. These bodies can transfer DNA horizontally, as shown in studies where Epstein-Barr virus-infected B-lymphocytes produce apoptotic bodies

carrying viral DNA to uninfected cells (Yu et al., 2023).

Once released, EVs can take various paths. Some tumor cells release EVs that damage shortly following release, dispersing enzymes like vascular endothelial growth factor and matrix metalloproteinases to promote angiogenesis and cancer invasion (Möller and Lobb, 2020). EVs in the bloodstream have a short half-life, with studies showing that melanoma-derived EVs lose luciferase activity within minutes post-injection, redistributing rapidly into tissues (Takahashi et al., 2013). EV uptake by cells involves mechanisms like membrane fusion, receptor-mediated endocytosis, and phagocytosis, transporting EV cargo into the intracellular compartment. Uptake variability suggests that it depends on the EV type and the involved cells (Kwok et al., 2021). Lower temperatures prevent uptake, indicating an energy-dependent process. Blocking uptake with heparan sulfate or proteinase K highlights proteoglycans and surface proteins' roles, while drugs like dynasore and cytochalasin D inhibit uptake by disrupting cytoskeletal remodeling and endocytosis pathways (Christianson et al., 2013; Escrevente et al., 2011; Svensson et al., 2013).

Targeting mechanisms of EVs to particular cell types in hematopoietic niche are not well understood. Tetrespanins, a large family or cell-surface proteins, are abundant on EVs and play roles in intracellular signaling, morphogenesis, migration, and metastasis. These proteins vary by parent cell types but include common markers like CD151, CD82, CD81, CD63, and CD9 (Jankovičová et al., 2020). Hematopoietic-specific tetraspanins, such as CD53 and CD37, interact with targets like Src homology region 2 domain-containing phosphatase-1 and MHC-I/II, aiding in EV targeting within the hematopoietic niche (Beckwith et al., 2015). Once inside target cells, EVs enter the pathway of endosomes, moving rapidly via cytoplasm before hedging at the endoplasmic reticulum and ultimately merging with the lysosomes for degradation. The endoplasmic reticulum, a major translation site, likely plays a role in depositing mRNA and miRNA cargo from EVs, potentially altering the synthesis of protein and behavior or cell. The half-life of internalized EVs varies, with studies showing they can remain intact for hours to days, with significant fusion with lysosomes occurring by 48 h (Heusermann et al., 2016).

3. Functions of extracellular vesicles

Extracellular vesicles (EVs) can bind to activated coagulation factors due to the presence of phospholipids on their surface, implicating them in various diseases with enhanced coagulation states. They contribute to coagulation disorders by promoting intravascular clotting and supporting thrombin generation (Kumar et al., 2024). In healthy individuals, EVs support the coagulation system by generating low-level thrombin, which reduces bleeding risk. Notably, the coagulation activity on the surface of EVs derived from platelets is 50–100 times greater than that of activated platelets, highlighting the critical role of EVs in coagulation defense (Berckmans et al., 2001). Phosphatidylserine on EVs is crucial for generating tissue factors, which begins the coagulation process. The interaction of activated factor VII (VIIa) with tissue factors is vital for the extrinsic coagulation pathway, with EVs supporting this process through VIIa/tissue factor-dependent and independent pathways. Damage to blood vessels exposes tissue factors to the bloodstream, triggering the extrinsic coagulation pathway and resulting in formation of fibrin. Activated platelets facilitate the joining of tissue factors with EVs enhancing activity of coagulation (Weiss et al., 2024).

In atherosclerosis, the adhesion of monocytes to endothelial cells and subsequent relocation beneath the endothelium are key events. Studies have shown that high-shear-stress-induced EVs derived from platelets significantly increase inflammatory cytokine levels in monocytes and endothelial cells. Pretreating these cells with platelet-derived EVs before co-incubation enhances their interactions by increasing adhesion molecule expression (Nomura et al., 2001; Barry et al., 1998). Apoptotic cell death, common in the synthesis and progression of atherosclerotic plaques, contributes to the presence of numerous procoagulant EVs within plaques. When endothelial cells are activated by various stimuli, the quantity of EVs derived from endothelial cells enhances, and those EVs display tissue factors on the surface (Konkoth et al., 2021). Monocyte-derived EVs, which also have high tissue factor content, initiate clot formation with platelets and fibrin, accumulating in blood vessels. Various molecules, including P-selectin glycoprotein ligand-1 (PSGL-1), show important roles in clot formation. During this process, EVs derived from endothelial cells combine to P-selectin on activated platelets via PSGL-1, directing tissue factors to clot and increasing thrombin accumulation. The fusion of tissue factor-coated EVs with the membrane of activated platelets localizes tissue factors and coagulation factors, thereby advancing the extrinsic coagulation pathway and significantly contributing to clot formation (Koizume and Miyagi, 2022). Although EVs generally promote coagulation, they also possess anticoagulant properties. They can enhance the functionally of the tissue factor pathway inhibitor (TFPI), thrombomodulin or inhibiting protein C. The equilibrium between TFPI and tissue factors among EVs is crucial for initiating coagulation, and increased levels of tissue factor-covered EVs can supersede TFPI. The activated protein C, which has anticoagulant and anti-inflammatory functions, influences EV binding to endothelial cells. The endothelial protein C receptor (EPCR) is important in this process, and EVs derived from endothelial cells also contain EPCR, mirroring the protein-C related mechanisms on the endothelial cell surface. Activated protein C attached to the EPCR upholds anticoagulant activity and efficiently reduces synthesis of local thrombin (Hisada and Mackman, 2021).

EVs have become a principal focus in various biological research areas because of their function in cell-to-cell communication. EVs facilitate communication between cells. For example, one cell type can stimulate specific responses in another. Most cells can uptake EVs through mechanisms involving proteins like tetraspanins, which may help target EVs to specific tissues or microenvironments. Another mechanism involves the fusion of EVs with cell membranes, transferring proteins, signaling molecules, miRNA, and mRNA. Studies have found that miRNA is enriched in EVs secreted by various tumor cells and present in the body fluids, suggesting their potential as disease prognosis markers (van Niel et al., 2022).

4. Effect of Extracellular Vesicles in thalassemia

4.1. Cellular and molecular mechanisms connecting extracellular vesicles to thalassemia

In β-thalassemia, the generation of extracellular vesicles (EVs) is driven by disturbed erythropoiesis and iron dysregulation, which are characteristics of the disease. Ineffective erythropoiesis results in surplus unbound chains within erythroid precursors, causing oxidative damage. This oxidative stress activates apoptotic pathways, including caspase-3 and p53, leading to cellular stress and membrane alterations such as blebbing. Proteins like TSG101 and ALIX within the Endosomal Sorting Complex Required for Transport (ESCRT) system assist in sorting cellular contents into vesicles, which are later released as exosomes (Saad et al., 2022; Gurung et al., 2021). Additionally, microparticles are produced through direct budding of the membrane, regulated by calcium influx and cytoskeletal changes. These processes are dysregulated in thalassemic cells, leading to elevated EV release. EVs are derived from erythroid cells in thalassemia transport materials such as heme, free iron, reactive oxygen species, and membrane proteins, including ankyrin and band 3. These vesicles are rich in phosphatidylserine, which are externalized due to oxidative stress, a key indicator of apoptotic cell death (Fig. 1). The presence of phosphatidylserine on microparticles enables their interaction with coagulation factors, thereby promoting a hypercoagulable state (Thangaraju et al., 2021).

EVs play a crucial role in the disturbance of iron homeostasis in thalassemia. Exosomes, in particular, carry proteins such as transferrin receptors, ferritin, and other iron-regulatory molecules. Ferritin,

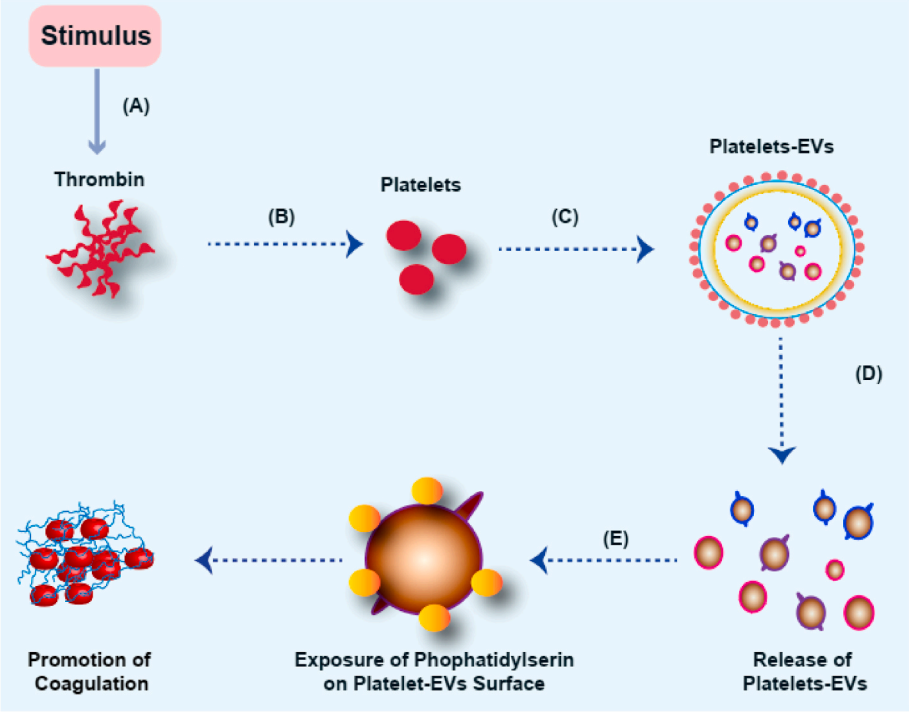

Fig. 1. Relationship between Platelet Activation and Release of Extracellular Vesicles (EVs)
(A) Stimulus: Various physiological and pathological stimuli, such as tissue injury or inflammation, lead to the activation of thrombin, a key enzyme in the coagulation cascade.
(B) Thrombin Activation: Thrombin, activated through plasminogen activator inhibitor-1 (PAI-1), binds to platelets under hypoxic conditions, initiating platelet activation and promoting coagulation.
(C) Formation of Platelets-EVs: Activated platelets begin to shed extracellular vesicles (EVs) from their membrane, encapsulating specific proteins, lipids, and nucleic acids.
(D) Release of Platelet EVs: Once formed, platelet-derived EVs are released into the bloodstream, where they can interact with other cells and modulate hemostatic and inflammatory responses.
(E) Exposure: Platelet EVs expose phosphatidylserine on their outer surface. a hallmark of EVs, which play a crucial role in promoting coagulation.

responsible for iron storage, is often enclosed in EVs originating from erythroid cells and macrophages in thalassemia. Due to abnormal erythropoiesis, iron overload ensues, and ferritin-loaded EVs are released into the bloodstream, contributing to systemic iron excess. This circulating iron exacerbates oxidative damage in distant organs, such as the heart and liver (Palsa et al., 2023). At the cellular level, macrophages internalize iron-rich EVs via receptor-mediated endocytosis, primarily through scavenger receptors like CD163, which recognize hemoglobin-haptoglobin complexes on the EV surface. Once inside, the iron released from ferritin can engage in the Fenton reaction, producing reactive oxygen species, which leads to cellular damage and inflammation. EVs also modulate the expression of hepcidin, the primary regulator of iron absorption, thus influencing systemic iron distribution and metabolism (Garton et al., 2017). One of the key mechanisms by which EVs contribute to the complications of thalassemia involves their interaction with endothelial cells. EVs released from erythrocytes and platelets in thalassemia are enriched in phosphatidylserine, which binds to receptors on endothelial cells, triggering activation. This activation leads to the release of pro-inflammatory cytokines like TNF-α and IL-6 through the NF-$_{K}$B pathway. These cytokines, in turn, stimulate the expression of adhesion molecules, including E-selectin, ICAM-1, and VCAM-1, on endothelial cells, promoting leukocyte adhesion and migration, hallmark processes in vascular inflammation (Abdolalian et al., 2023). Furthermore, EVs carrying oxidative stress indicators such as malondialdehyde (MDA) and 4-hydroxynanenal (HNE) negatively affect endothelial function by disruption nitric oxide signaling. Nitric oxide is essential for vasodilation, and its depletion due to EV-induced oxidative stress results in vasoconstriction and increased vascular resistance. This endothelial dysfunction, combined with the procoagulant properties of EVs, significantly raises the risk of thrombosis, a frequent complication in β-thalassemia (Chiaradia et al., 2021; Chapple et al., 2013).

The cellular stress resulting from ineffective erythropoiesis and iron overload in thalassemia activates molecular pathways that are linked to EV biogenesis. For example, the accumulation of reactive oxygen species within erythroid cell activates the JNK and p38 MAPK pathways, which drive the expression of genes involves in inflammation and apoptosis, such as Bax and caspase-3. This activation enhances EV production and alters the cargo these vesicles carry (Yue and López, 2020). Molecularly, EVs from thalassemic patients contain non-coding RNAs, particularly microRNAs (miRNAs) that influence gene expression in recipient cells. For instance, miR-144–3p, present in these EVs, is known to suppress the transcription factor NRF2, which is central to antioxidant defense. The reduction in NRF2 exacerbates oxidative stress, further advancing the pathological processes associated with thalassemia. These miRNA-mediated effects highlights the intricate molecular mechanisms EVs participate in (Srinoun et al., 2019). Phosphatidylserine-expressing EVs play a vital role in activating coagulation at the molecular level. These EVs provide a platform for the assembly of prothrombinase complexes, accelerating the conversion of prothrombin to thrombin, a crucial enzyme in blood clotting. This process is especially relevant in β-thalassemia, where elevated levels of phosphatidylserine -positive EVs are associated with increased thrombin production and a higher risk of venous thromboembolism (VTE). (Zhang et al., 2023).

4.2. *Inflammation and endothelial dysfunction*

The organization and functionality of endothelial cells are vital for

integrity of blood vessels and proper circulatory activity. Endothelial cells takes part as a key role in hemostasis, regulating blood flow and vascular tone via synthesis of nitric oxide, prostaglandin, and endothelin (Fig. 2). Moreover, endothelial cells are involved in both anti-inflammatory and pro-inflammatory responses by producing cytokines and expressing adhesion molecules (Neubauer and Zieger, 2022). Owing to their diverse functions, endothelial dysfunction is linked to the pathological mechanisms of various diseases, such as hemoglobinopathies i.e., thalassemia syndrome and sickle cell anemia (Trimm, 2023). Studies have shown that patients with thalassemia often exhibit increased activation of endothelial cells and higher levels of activation markers like tumor necrosis factor – α (TNF-α), E-selectin, IL-1β, soluble intercellular adhesion molecule-1 (sICAM-1), and soluble vascular adhesion molecule-1 (sVCAM-1). Elevated levels of pro-inflammatory cytokines, e.g., adhesion molecules and IL-6, are also observed in β-thalassemia major patients, indicating the significant role of inflammation and dysfunction of endothelial cells in the disease's progression (Aggeli et al., 2005; Butthep et al., 2002; Kyriakou et al., 2001). Aggeli et al. highlighted that β-thalassemia major patients exhibit impaired endothelial function, evidenced by decreased nitric oxide bioavailability (Aggeli et al., 2005). This dysfunction is linked to iron overload, which reduced nitric oxide production through direct inhibition of endothelial NO synthase (eNOS) or indirectly through oxidative stress. Despite therapy with desferrioxamine, iron accumulation remains high, contributing to vascular issues even in asymptomatic patients. These patients show elevated levels inflammatory mediators such as IL-6, sVCAM-1, and sICAM01, indicating ongoing endothelial activation and systemic inflammation. The study correlates these elevated markers with ferritin levels, suggesting that both inflammation and endothelial dysfunction play a role in disease progression. Compared to healthy controls, patients with β-thalassemia major had lower levels of LDL, ApoA, ApoB, and Lp(a). The altered lipid profile may be due to erythropoiesis-induced increased LDL receptor expression or inflammation-driven hypercholesterolemia (Aggeli et al., 2005).

EVs can lead to dysfunction of endothelial cells through impairing vasorelaxation and promoting the vascular inflammation through increased levels of inflammatory cytokines, adhesion molecules, and reactive oxygen species (Chatterjee et al., 2020). Boulanger et al. found that microparticles cause endothelial cell dysfunction in myocardial infarction patients by disrupting nitric oxide-mediated relaxation (Boulanger et al., 2001). Fu et al. showed that phosphatidylserine + microparticles takes part in inflammatory process and dysfunction of endothelium in valvular cardiac disease through inhibition of eNOS, thereby reducing production of nitric oxide and increasing superoxide anion synthesis (Fu et al., 2015). Research on EVs in myocardial infarction demonstrates their role in endothelial dysfunction and pro-coagulant activity. These findings are highly relevant to thalassemia, where EVs derived from erythrocytes and platelets induce similar vascular complications (Fu et al., 2015). Li et al. reported that microparticles from valvular heart disease patients contain elevated levels of pro-inflammatory proteins, which can trigger a systemic inflammatory response (Li et al., 2021). Toeh et al. demonstrated that microparticles induce migration of neutrophils, dysfunction of endothelial cells, activation of JNK and NK-$_K$B pathways, and leukocyte interactions, leading to enhanced expression of E-selectin, P-selectin, VCAM-1, and ICAM-1, and mediating inflammatory responses (Teoh et al., 2014). The findings highlight that microparticles, released shortly after ischemic reperfusion, are derived from sinusoidal endothelial cells, platelets, macrophages, and other inflammatory cells. These microparticles promote inflammation, platelet activation, and direct hepatocyte injury by triggering mitochondrial dysfunction and reactive oxygen species production. Microparticles activated neutrophil migration and triggered pro-inflammatory pathways, such as NF-$_K$B and JNK, which are associated with up regulation of adhesion molecules and pro-inflammatory gene expression. Additionally, annexin V-homodiner (Diannexin) was found to inhibit microparticle formation by binding to phosphatidylserine on microparticle surfaces, thereby preventing their pro-inflammatory and hepatocyte-damaging effects (Teoh et al., 2014). Another study investigated the role of EVs from β-thalassemia patients, particularly those with iron overload, in promoting cardiac cell proliferation (Atipimonpat et al., 2021). It shows that EVs, including microparticles and exosomes, carry iron-containing proteins such as ferritin

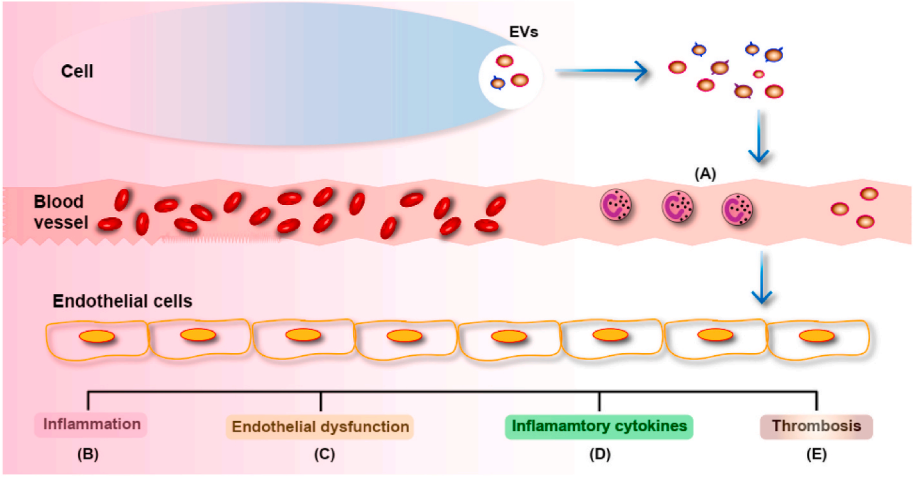

Fig. 2. Effect of Extracellular Vesicles (EVs) on Endothelium
(A) Migration of Neutrophils and Leukocyte-leukocyte Interaction: Extracellular vesicles (EVs) facilitate neutrophil migration and promote leukocyte interactions, contributing to immune cell recruitment in vascular regions.
(B) Inflammation: EVs released from various cells enhance the inflammatory response by activating endothelial cells and other immune cells, amplifying inflammation within the vascular microenvironment.
(C) Endothelial Dysfunction: EVs induce endothelial dysfunction by disrupting the normal functioning of endothelial cells, impairing vascular integrity and homeostasis.
(D) Inflammatory Cytokines: EVs stimulate the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, further aggravating vascular inflammation and endothelial injury.
(E) Thrombosis: EVs contribute to thrombosis by promoting the activation of platelets and coagulation pathways, leading to the formation of blood clots and increasing the risk of thrombotic events.

and hemichromes, which may contribute to cardiovascular complications in β-thalassemia patients. EVs in these patients were derived mainly from platelets, but β-thalassemia patients with iron overload had significantly higher numbers of RBC-derived microparticles. These RBC-derived microparticles showed abnormal red blood cell morphology, suggesting increased microvesicular budding in β-thalassemia. Exosomes from thalassemia patients, especially those with iron overload, carried higher ferritin content compared to microparticles. Elevated ferritin levels were also correlated with higher serum ferritin levels in these patients, suggesting a link between iron overload and EV-mediated ferritin transport. Both microparticles and exosomes from thalassemia patients promoted cardiac cell proliferation, but exosomes from iron-overloaded thalassemia patients had a significantly greater impact. The study demonstrates that these exosomes induced higher proliferation and angiogenesis compared to controls and thalassemia patients without iron overload. Furthermore, cardiac cells rapidly internalized EVs within two hours of treatment. The exosomes and microparticles were found to deliver ferritin and hemichromes into cardiac cells, contributing to increased iron content and cell proliferation. The study also suggests that endocytosis is the likely mechanism for EV uptake by cardiac cells.

The association among EVs and dysfunction of endothelial cells in patients with thalassemia has also been studied. Kheansaard et al. found that microparticles from spelenectomized β-thalassemia/HbE patients experience activation of endothelial cells and promote adhesion of monocytes to endothelial cells (Kheansaard et al., 2018). This investigation also indicated that EV-induced dysfunction of endothelial cells enhance the synthesis of pro-inflammatory cytokines i.e., IL-6, IL-8 etc., and enhances VCAM-1, ICAM-1, P-selectin, and E-selectin expression in endothelial cells. This heightened endothelial cells activity and cytokine production in thalassemia patients contribute to chronic inflammation, a common complication of the disease (Kheansaard et al., 2018). Additionally, the increase in EV levels can disrupt vascular homeostasis, affecting the balance of procoagulant and anticoagulant factors and potentially leading to events of thrombosis. Tantawy et al. found a significant association between the levels of microparticles from platelets and RBCs and the pulmonary artery pressure and aortic stiffness index in thalassemia patients (Tantawy et al., 2013). Elevated levels of platelet-derived microparticles and erythrocyte-derived microparticles were detected in β-thalassemia major patients compared to healthy controls, with a marked increase in patients with a history of thrombosis, pulmonary hypertension risk, splenectomy, and high serum ferritin levels. Patients exhibited increased aortic stiffness and pulmonary artery pressure, alongside reduced aortic strain and distensibility, correlating with elevated microparticle levels. Likewise, the hypercoagulable state in β-thalassemia major patients were linked to microparticles, which promote thrombus formation through exposure to phosphatidylserine and activation of coagulation factors, exacerbating thrombotic events. Microparticles were positively correlated with serum ferritin levels and hemolysis markers, indicating a connection between iron overload and vascular dysfunction. Compliance with iron chelation therapy was associated with lower microparticle levels, suggesting a protective effect against cardiovascular complications. Furthermore, splenectomized patients had significantly higher microparticle levels, attributed to increased procoagulant activity from damaged red blood cells and platelet activation, which can exacerbate thrombotic events (Tantawy et al., 2013). This suggests that higher microparticle levels contribute to dysfunction of vessels and increase the risk to develop aortic stiffness and pulmonary hypertension in thalassemia patients. Klaihmon et al. reported that microparticles from platelets and activated leukocytes increase platelet-neutrophil aggregation among splenectomized β-thalassemia/HbE individuals, a prompt biomarker of injury in cardiac tissues, thalassemia, and stroke (Klaihmon et al., 2017a). Thus, microparticles may exacerbate thalassemia complications by promoting aggregation of platelets and neutrophils.

The increased risk to develop cardiovascular disorders, which are essential reasons of mortality and morbidity in thalassemia patients, has been documented (Table 1). The inhibitors of eNOS, leading to reduced nitric oxide production and increased superoxide anion formation, appears to be a primary mechanism through which EVs cause inflammation and dysfunction of endothelial cells. Activation of JNK and NF-$_{K}$B pathways, along with enhanced adhesion molecule expression, inflammatory cytokine production, and leukocyte recruitment, are other mechanisms by which EVs may worsen inflammation and endothelial cell dysfunction in thalassemia patients (Aessopos et al., 2007; Wang et al., 2011). In thalassemia, endothelial dysfunction is closely associated with several clinical and molecular abnormalities. Patients with β-thalassemia major typically show increased levels of markers of endothelial activation, including VCAM-1, ICAM-1, E-selectin, IL-6, and TNF-α, indicating that chronic inflammation and endothelial activation are key features of the disease. One of the main factors contributing to this is iron overload, which is a common consequence of repeated blood transfusions in these patients. Excess iron induces oxidative stress and interferes with nitric oxide synthesis by impairing eNOS activity, leading to reduced nitric oxide availability. Nitric oxide is crucial for maintaining vascular tone and overall vascular health (Caprari et al., 2022). Research has revealed that heightened systemic inflammation in β-thalassemia patients is associated with endothelial dysfunction and cardiovascular risk indicators such as aortic stiffness and increased pulmonary artery pressure (Stoyanova et al., 2012). These findings collectively underscore the significant connection between endothelial dysfunction and the progression of thalassemia.

The mechanism driving endothelial dysfunction in thalassemia involve a combination of iron overload, inflammatory responses, and oxidative stress. Iron overload hampers nitric oxide production by either directly inhibiting eNOS or by promoting the generation of superoxide anions, which further depletes nitric oxide levels (Voskou et al., 2015). This decline in nitric oxide disrupts vasodilation, leading to vascular complications. Additionally, circulating EVs and microparticles are elevated in individuals with thalassemia, contributing to endothelial inflammation. These particles contain pro-inflammatory cytokines, adhesion molecules, and iron-binding proteins like ferritin, which further aggravate the inflammatory state of the endothelium. Microparticles, in particular, activate signaling pathways such as JNK and NF-$_{K}$B, which lead to increased expression of adhesion molecules and inflammatory cytokines (Abdolalian et al., 2023). These processes promote platelet aggregation, recruitment of leukocytes, and chronic inflammation, all of which contribute to the heightened risk of thrombosis observed in patients with β-thalassemia.

4.3. *Iron homeostasis*

Iron balance is essential for maintaining the body's physiological equilibrium, given the substantial presence of reactive iron that could pose risks if not properly managed. This equilibrium is carefully regulated at systemic and cellular levels. At cellular levels, the metabolism of iron undergoes processing through transcriptional and post-transcriptional mechanisms, while systemically, hepcidin governs the metabolism of iron (Sawicki et al., 2023). EVs participate in intercellular communication by facilitating the transfer of genetic materials, proteins, and lipids (van Niel et al., 2022). Given the complexity of iron metabolism, it is plausible that EVs play a function in its regulation and maintenance, although their specific contributions remain uncertain. Ferritin acts as an intracellular iron reservoir, with its synthesis and breakdown rates dictated by cellular iron levels. Ferritin is distributed across various cellular compartments, including the mitochondria, cytoplasm, and nucleus (Sudarev et al., 2023). Studies have detected ferritin within EVs, particularly in urine-derived exosomes (Principe et al., 2013). Furthermore, the secretion of ferritin via EVs is reported among several studies (Yanatori et al., 2021; Hurwitz et al., 2016). It is been suggested that iron may regulate the expression of CD63, a protein involved in EV secretion, thereby influencing the release of

Table 1
Role of EVs in thalassemia complications.

Thalassemia complication	Type of involved EV	Pathogenesis	Ref.
Cardiac complications	Ferritin loaded Exos	Elevated levels of Exos containing ferritin as linked to enhanced proliferation of cardiac cells in β-thalassemia	Atipimonpat et al. (2021)
	MPs	MPs exacerbate inflammation and EC dysfunction by suppressing the function of eNOS, leading to NO production and heightened generation of superoxide anions	(Aggeli et al., 2005; Fu et al., 2015)
		MPs trigger platelet-neutrophil aggregation in individuals who have undergone splenectomy due to β-thalassemia or HbE	(Klaihmon et al., 2017a; Keawvichit et al., 2012; Furman et al., 2001; Marquardt et al., 2002)
	PMPs RMPs	Elevated levels of PMPs and RMPs cause vascular dysfunction in patients with β-thalassemia	Tantawy et al. (2013)
Inflammation	EVs	EVs stimulate neutrophil migration, activation of NK-$_{\kappa}$B pathways, elevated expression of VCAM-1, ICAM-1, P-selectin, E-selectin, increased leukocyte interactions, and cytokine production	(Aggeli et al., 2005; Teoh et al., 2014; Kheansaard et al., 2018)
Thromboembolic complications	PS-EVs	Engagement between PS and gamma-carboxyglutamic acid domains within tenase and prothrombinase complexes stimulates the coagulation process, leading to enhanced thrombin production	(Westerman et al., 2008; Perez-Pujol et al., 2007)
	PMPs-MPs	Elevated levels of MPs trigger platelet aggregation and activation in individuals with β-thalassemia or HbE, thereby enhancing thrombus formation	Klaihmon et al. (2017a)
	PS-RMPs	Correlation between the rise in markers indicating thrombin generation, such as TAT and PF1.2, and the elevation in concentration of PS-RMPs	Westerman et al. (2008)
	PS-MPs	Elevated levels of PS-MPs are linked to platelet activation markers like P-selectin and CD63 in thalassemia	(Pattanapanyasat et al., 2004, 2007)
	EPCR-EVs	Alteration in the hemostatic equilibrium of the activated protein C system occurs after decrease in EVs labelled with EPCR in individuals with β-thalassemia major	Levin et al. (2018)
	PMPs RMPs	Rise in EVs leads to EC dysfunction and disrupts the regulation of anticoagulant and procoagulant factor expression	(Aggeli et al., 2005; Kheansaard et al., 2018)
Hemochromatosis	LFR-Exos TFR-Exos	Exos containing TRF and LFR transport transferrin and lactoferrin to mammalian cells	Malhotra et al. (2016)
Ineffective erythropoiesis	BM-derived Exos	Exos derived from BM stimulates hepcidin expression by activating the SMAD1/5/8 pathway in patients with β-thalassemia	(Mattera et al., 2020; Ruiz-Martinez et al., 2019)
	HSP70-EVs	Higher concentrations of HSP70-EVs are linked to a notable rise in markers of ineffective erythropoiesis, such as reticulocyte count, erythropoietin concentration and LDH levels in patients with β-thalassemia major	(Levin et al., 2018; Arlet et al., 2014)
Organ dysfunction	miR-144-3p-EVs	EVs containing miR-144–3p trigger apoptosis in hepatic and pancreatic cells	Levin et al. (2021)
	T-cell derived MPs	Increased expression of fibrinolytic genes in liver cells	Kornek et al. (2011)
	$CD63^{+}$-EVs $CD81^{+}$-EVs	EVs elevate the number of apoptotic cells and caspase 3/7 activity while reducing cell viability in endothelial, pancreatic, and liver cells	Levin et al. (2021)

CD, cluster of differentiation; EVs, extracellular vesicles; EPCR, endothelial protein C receptor; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; HSP70, heat-shock protein 70; ICAM1, intercellular adhesion molecule-1; LDH; lactate dehydrogenase; LFR; lactoferrin receptor; miR; microRNA, MPs, microparticles; NO, nitric oxide; PS, phosphatidylserine; PF1.2; prothrombin fragments 1.2; PMPs, platelet-derived MPs, RMPS, red blood cell-derived MPs; TAT, thrombin-antithrombin complex; TF; transferrin; TFR, transferrin receptor; VCAM1, vascular adhesion molecule-1.

CD63-positive EVs (Yanatori et al., 2021). This implies a potential correlation between iron levels and EV production. Some studies have observed elevated ferritin and EV levels in thalassemia patients, particularly those originating from red blood cells and activated platelets (Thiengtavor et al., 2020; Kittivorapart et al., 2018a). These ferritin-laden exosomes might exacerbate cardiac failure and hypertrophy in β-thalassemia patients. However, the precise pathophysiology

and their effects on the symptoms and quality of life of thalassemia patients remain uncertain.

Transferrin facilitates the transport of ferric iron (Fe^{3+}) in the bloodstream and interacts with two specific receptors, TFR1 and TFR2. While TFR1 facilitates cellular iron uptake, TFR2 deficiency can lead to systemic overload of iron and hemochromatoris due to downregulation of hepcidin (Silva et al., 2021). Studies have demonstrated TFR1 presence in EVs derived from the human plasma and mice (Mattera et al., 2020). Mattera et al. explores the potential of using EVs as nanocarriers to deliver apotransferrin to the central nervous system, aiming to develop less invasive treatments for demyelinating diseases (Mattera et al., 2020). The study highlighted the advantage of EVs as delivery systems due to their long circulating time, reduced toxicity, and ability to traverse the blood-brain barrier. Moreover, the intranasal route of EVs is considered a promising alternative to intracranial injection, bypassing the blood-brain barrier and minimizing side effects. Biophysical analysis confirmed the stability and integrity of the EVs. The incorporation of an apotransferrin onto the EV surface did not significantly alter their size or charge, supporting the feasibility of using EVs as stable nanocarriers. The researchers further verified that apotransferrin binding occurred specifically through TfR1, which holds potential for targeting oligodendrocytes in the central nervous system (Mattera et al., 2020). Additionally, exosomes have been shown to transport lactoferrin and transferrin to the mammalian cells, suggesting a possible role in exacerbating iron overload in thalassemia patients (Malhotra et al., 2016). An investigation by Malhotra et al. focused on utilizing exosomes as tunable nano vehicles to deliver iron carrier protein transferrin and lactoferrin into specific intracellular compartments (Malhotra et al., 2016). Exosomes were stable in suspension with a zeta potential around −28 mV. Loading with transferrin and lactoferrin did not significantly affect this stability, ensuring their suitability for drug delivery. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), present on exosome surface, was identified as a receptor for transferrin and lactoferrin. Exosomes with GAPDH were shown to effectively capture and deliver these proteins into various mammalian cells, enhancing delivery by 2–3 folds compared to traditional receptor-mediated uptake. Approximately 90% of the transferrin and lactoferrin cargo delivered via exosomes localized to late endosomes and lysosomes, promoting efficient intracellular assimilation. This compartmentalized delivery is crucial for diseases related to endosomal and lysosomal dysfunction, such as lysosomal storage disorders, Alzheimer's disease, and cancer. Furthermore, by modulating the GAPDH content in exosomes, the study demonstrated the ability to enhance or reduce the amount of transferrin and lactoferrin delivered, showcasing the tenability of exosomes as delivery vehicles (Malhotra et al., 2016). Hepcidin, a pivotal regulator in uptake and release of iron, is predominantly synthesized by liver cells and is influenced by iron stores in the body, inflammation, and erythrocyte production. During active erythropoiesis, erythropherone inhibits hepcidin production, leading to increased iron absorption (Ginzburg, 2023). In β-thalassemia, ineffective erythropoiesis suppresses hepcidin, resulting in iron overload (Longo et al., 2021b). Recent research suggests that exosomes derived from bone marrow could influence the expression of hepcidin and metabolism of iron, potentially worsening complications in β-thalassemia patients (Ruiz-Martinez et al., 2019). These exosomes may induce hepcidin expression in hepatocytes, affecting various signaling pathways and exacerbating ineffective erythropoiesis (Ruiz-Martinez et al., 2019). Ruiz-Martinez et al. hypothesized that exosomes derived from bone marrow play a significant role in regulating iron metabolism through modulation of hepcidin (Ruiz-Martinez et al., 2019). Their preliminary findings reveal that phlebotomy increases serum exosome concentration, and exosomes from β-thalassemic mice are also elevated. Additionally, *in vitro* experiments show that serum exosomes induce hepcidin in a dose-dependent manner. In the study involving serum samples from β-thalassemia major patients and age- and gender-matched controls, no differences were observed in the number or size of exosomes; however, the exosomal protein content per serum volume was significantly lower in patients. Hepatocyte treatments with sera from patients showed decreased hepcidin induction compared to controls, suggesting that hepcidin suppression is linked to the exosome-free portion of serum. Notably, only exosomes from β-thalassemic patients could induce hepcidin expression in hepatocyte cultures. Furthermore, exosomes from these patients did not affect ERK1/2 and STAT3 signaling in hepatocytes but enhanced SMAD1/5/8 signaling while decreasing AKT signaling. These findings imply that exosomes can increase hepcidin expression through enhanced SMAD signaling, potentially influencing multiple pathways in an autocrine manner in response to exosome presence, while counterbalancing hepcidin suppressive factors in the serum of β-thalassemia patients (Ruiz-Martinez et al., 2019). However, the precise mechanisms underlying these effects remain elusive. Further proteomic analysis of bone marrow-derived exosomes among β-thalassemia patients could provide insights in their role in regulating hepcidin expression.

4.4. *Ineffective erythropoiesis*

Ineffective erythropoiesis described a condition where the bone marrow fails to produce an adequate number of red blood cells despite having immature erythroid precursors. This condition is a key pathological feature of β-thalassemia major (Longo et al., 2021b). The precise mechanism causing ineffective erythropoiesis is still not properly understood. Under physiological circumstances, the chaperone heat shock protein 70 (HSP70) plays an essential role in erythrocyte production by moving into the erythroid precursor nucleus. There, it safeguards transcription factor GATA1 that is crucial for differentiation of erythrocytes, from being broken down by caspase-3 (Mathangasinghe et al., 2021). In erythroblasts affected by thalassemia, HSP70 binds to free α-globin chains and fails to enter the nucleus, thus not protecting GATA1 (Noulsri and Lerdwana, 2023). This results in the GATA1 degradation, leading to maturation arrest and increased cell death, which exacerbates ineffective erythropoiesis. Research indicates that the HSP70 can be secreted into the bloodstream via EVs derived from various cells (Hu et al., 2022). An investigation by Levin et al. found that patients with β-thalassemia major have significantly higher levels of EVs-HSP70 compared to normal individuals (Levin et al., 2018). Furthermore, there is a noteworthy rise in markers of ineffective erythropoiesis such as levels of erythropoietin, reticulocyte count, and lactate dehydrogenase (LDH). Consequently, assessing EVs-HSP70 concentrations could help predict the severity of ineffective erythropoiesis and might be considered as potential therapeutic targets of future thalassemia treatment.

4.5. *Thrombosis*

Thrombotic events, such as deep vein thrombosis, pulmonary thrombosis, and cerebral thrombosis, are serious complications among patients of β-thalassemia, particularly who have had a splenectomy (Vasilopoulou et al., 2022). Microparticles play a role in blood clotting by displaying tissue factor and phosphatidylserine. The presence of negatively charged phospholipids like phosphatidylserine on EVs promotes the assembly of tenase and prothrombinase complexes on the EV membrane. This interaction speeds up the coagulation cascade, leading to increased thrombin generation, which raise the risk of thrombotic events (Zhao et al., 2022). Recent research has shown that high microparticle concentration is an essential thrombotic risk factor in conditions such as venous thromboembolism (VTE), hemoglobinopathies, and antiphospholipid syndrome (APS) (Bucciarelli et al., 2012; MacEy et al., 2011; Dignant-George et al., 2004; Chirinos et al., 2005; Westerman et al., 2008) Westerman et al. highlighted the role of circulating RBC-derived vesicles in hemoglobinopathies like thalassemia intermedia and sickle cell anemia. The study found increased levels of vesicles in both conditions, with a strong correlation to intravascular hemolysis as indicated by plasma hemoglobin levels. Vesicle levels were six times higher in sickle cells anemia and four times higher in

thalassemia intermedia compared to healthy controls. In thalassemia intermedia, a higher proportion of plasma hemoglobin was contained within vesicles, contributing significantly to plasma hemoglobin level and potentially affecting nitric oxide availability. The study also identified that phosphatidylserine + RBCs are significant source of phosphatidylserine + vesicles, which promote thrombin generation. Markers of coagulation, such as thrombin-antithrombin complex, were significantly elevated in both sickle cell anemia and thalassemia intermedia, especially in splenectomized thalassemia intermedia patients. Splenectomy exacerbates the levels of vesicles, phosphatidylserine + RBCs, plasma hemoglobin, and thrombin generation markers, highlighting the spleen's role in regulating vesicle clearance and hypercoagulability (Westerman et al., 2008). In β-thalassemia/HbE patients, especially those who underwent splenectomy, damaged erythrocytes and chronic activation of platelets cause elevated microparticle levels in the circulation (Klaihmon et al., 2017b). These patients have increased levels of erythrocyte-derived microparticles, and platelet-derived microparticles, which promote platelet aggregation and increase P-selectin expression on platelets, as seen through flow cytometry. The elevated microparticle levels contribute to a hypercoagulable state, increasing formation of thrombus and the risk of thromboembolism (Klaihmon et al., 2017b).

About half of severe β-thalassemia patients have the β-thalassemia/HbE genotype (Modell and Darlison, 2008). Severe β-thalassemia/HbE, particularly in splenectomized patients, is characterized by higher platelet-derived microparticle levels, platelet aggregation, and platelet activation in contrast to individuals having milder disease or normal individuals (Klaihmon et al., 2017a; Pattanapanyasat et al., 2007). Pattanpanyasat et al. highlighted the role of microparticles in thrombotic complications in of β-thalassemia patients, especially those who have undergone splenectomy (Pattanapanyasat et al., 2007). Using flow cytometry, the study demonstrates that splenectomized of β-thalassemia/HbE patients have significantly higher levels of phosphatidylserine -bearing microparticles than non-splenectomized patients and healthy individuals. This increase correlates with elevated platelet activation, evidences by markers like CD41a, CD62P, and CD36, and heightened platelet factor-3 like activity. The study also emphasized the potential exacerbation of risks following splenectomy, raising concerns about its use as a therapeutic option in of β-thalassemia (Pattanapanyasat et al., 2007). The findings suggest that excess microparticles may act as a circulating platelet compartment, promoting adhesive interaction and prothrombotic conditions in the blood. Analysis by flow cytometry in patients of β-thalassemia major who receive regular blood transfusions show higher microparticle levels from red blood cells and platelets in contrast to healthy controls (Tantawy et al., 2013; Agouti et al., 2015; Chaichompoo et al., 2012). Studies utilizing the coagulation time method based on microparticles reveal that β-thalassemia patients have shorted clotting times than healthy individuals, mainly due to the procoagulant activity of platelet-derived microparticles. Although regular blood transfusions dilute the procoagulant potential of thalassemic erythrocyte-derived microparticles, the enhanced procoagulant platelet-derived microparticle levels in splenectomized β-thalassemia individuals still enhance thrombin generation, increasing the risk of thrombosis (Agouti et al., 2015).

In thalassemia intermedia patients, higher levels of phosphatidylserine + -erythrocyte-derived microparticles are significantly associated with increased markers of thrombin generation, including thrombin-antithrombin complex, and prothrombin fragments 1,2 (PF1.2), highlighting the procoagulant outcome of phosphatidylserine + erythrocyte-derived microparticles (Westerman et al., 2008). There is a positive correlation between the phosphatidylserine + -platelets and phosphatidylserine + -RBCs in thalassemia patients. Microparticles expressing the phosphatidylserine and higher levels of markers for platelet activation i.e., CD62P, CD63 etc., are directly linked to thrombotic tendencies (Pattanapanyasat et al., 2004, 2007). Elevated platelet factor-3-like activity among β-thalassemia/HbE individuals further supports the platelet-derived microparticles role in activating the coagulation system (Pattanapanyasat et al., 2007). Pattanapanyasat et al. explored the role of RBC vesicles in thalassemia, particularly, their involvement in thrombotic risk. RBC vesicles, formed through oxidative damage and membrane instability, contribute to circulatory disturbances in thalassemia patients (Pattanapanyasat et al., 2004). RBC vesicles were present in both normal and thalassemic individuals, but their levels were significantly elevated in thalassemia, especially in splenectomized patients with β-thalassemia/hemoglobin E. RBC vesicles were annexin V-positive, indicative of membrane phospholipid asymmetry, contributing to procoagulant activity and enhanced erythrophagocytosis. The number of vesicles inversely correlated with the severity of thalassemia, indicating a potential link between RBC destruction and disease progression. Furthermore, elevated RBC vesicle levels, along with phosphatidylserine exposure, were associated with hypercoagulability and might contributed to the higher thromboembolic risk observed in thalassemia (Pattanapanyasat et al., 2004). The endothelial protein C receptor (EPCR) regulates generation of thrombin through activating protein C, playing an important function in anticoagulant pathway and coagulation maintenance (Wojtukiewicz et al., 2019). Levin et al. found that EVs labeled with EPCR are considerably decreased in β-thalassemia subjects in contrast to controls (Levin et al., 2018). This reduction in EPCR-labeled EVs disrupts the hemostatic balance, potentially contributing to a hypercoagulable state among β-thalassemia patients (Levin et al., 2018). β-thalassemia patients had significantly higher EV concentration than healthy controls, with the smallest and least numerous found in patients with hypersplenism. The hypersplenism was associated with lower EV counts and size, while splenectomized patients had elevated EV levels, particularly of platelet-derived EVs. Likiwse, splenectomized patients exhibited a higher tissue factor to tissue pathway inhibitor (TFPI) ratio, suggesting a hypercoagulable state and increased thrombotic risk. Furthermore, elevated HSP70 levels in EVs correlated with markers of ineffective erythropoiesis, as well as disease severity. Lower levels of RBC and monocyte-derived EVs were observed in patients, with RBC-derived EVs associated with hematocrit and reduced monocyte-derived EVs potentially indicating an increased infection risk (Levin et al., 2018). Likewise, Arlet et al. identified the role of heat shock protein 70 (HSP70) in β-thalassemia major (Arlet et al., 2014). In contrast to healthy erythroblasts, β-thalassemia erythroblasts exhibit cytoplasmic retention of HSP70 and reduced nuclear GATA-1 expression. GATA-1 is crucial for erythroid differentiation, and its protection from caspase-3 cleavage is dependent on HSP70 localization. *In vitro* analysis demonstrated accelerated differentiation in β-thalassemia erythroblasts, with increased numbers of polychromatophilic cells and higher apoptosis rates. Despite, accelerated differentiation, terminal maturation was halted, reflected by a significantly lower terminal maturation index in β-thalassemia major cells compared to controls. HSP70 directly interacts with free α-globin chains, leading to its cytoplasmic sequestration and loss of protective function for GATA-1. This interaction was confirmed through co-immunoprecipitation and confocal microscopy. Furthermore, the introduction of nuclear-targeted HSP70 mutant or a caspase-3 uncleavable GATA-1 mutant restored terminal maturation of β-thalassemia major erythroblasts, suggesting potential therapeutic strategies targeting this mechanisms (Arlet et al., 2014). The study by Keawvichit et al. on β-thalassemia/HbE patients with nucleated erythrocytosis highlights an increased risk of hypercoagulable complications, particularly in patient who have undergone splenectomy (Keawvichit et al., 2012). Using a novel four-color flow cytometry protocol, researchers assessed platelet activation and platelet-leukocyte aggregation. This method effectively bypassed technical difficulties posed by immature red blood cells in thalassemia patients. The findings revealed significant higher levels of circulating activated platelets (CD42a+/CD62P+) in β-thalassemia/HbE patients compared to healthy individuals. Additionally, platelet-neutrophil and platelet monocyte aggregates were significantly elevated, particularly in splenectomized patients, though platelet-lymphocyte aggregates were not affected. Notable,

platelet-monocyte aggregates were more frequent than platelet-neutrophil aggregates, indicating the platelet-monocyte interactions could serve as a more sensitive marker of platelet activation. The study also suggests that platelet activation and enhanced platelet-leukocyte interactions, particularly splenectomized patients, may contribute to an increased risk of thrombotic events in thalassemia patients. This is consistent with observations in other diseases with heightened thromboembolic risks, such as coronary artery disease and myocardial infarction (Keawvichit et al., 2012). The four-color flow cytometry method, requiring minimal blood volume, offers a reliable tool for assessing platelet function in both pediatric and adult patients with thalassemia. Similarly, Perez-Pujol et al. focused on the characterization of platelet-derived microparticles based on their activation mechanism using a new digital flow cytometer, highlighting the complexity of platelet microparticles and their significant role in coagulation and inflammation (Perez-Pujol et al., 2007). Microparticles exhibited substantial heterogeneity depending on the platelet activation mechanism. Two different reagents, thrombin receptor activating peptide (TRAP) and ionophore, were used to generate microparticles, resulting in distinct populations with variable expression on surface markers. Both TRAP and ionophore microparticles showed comparable representation of membrane glycoproteins (GPs) such as GP IIb-IIIa and Gp Ib. However, TRAP-induced microparticles exhibited higher P-selectin (CD62P) expression, while ionophore-induced microparticles had significantly elevated phosphatidylserine expression, making them as more procoagulant. The findings emphasize the importance of considering microparticles heterogeneity in clinical settings and their possible roles in both protective and pathogenic processes.

5. Extracellular vesicles as biomarkers in thalassemia

The proteomic analysis of plasma vesicles from patients with β-thalassemia has shown an escalation in proteins linked to oxidation damage in RBCs and platelets, including HSP90 and peroxiredoxin 6 (Chaichompoo et al., 2012). Additionally, alterations in the concentrations of particular proteins among EVs, e.g., high α-Hb-stabilizing protein and low levels of haptoglobin, cathepsin, and hemopexin, postulate their significance as inflammation and hemolysis biomarkers. Such markers have been employed for quantification through mass spectrometry (Kittivorapart et al., 2018b). Ferru et al. studied hemichromes, which are consisting of damaged α-globin chains among erythrocyte EVs in thalassemia (Ferru et al., 2014). These hemichromes attach to band 3 protein and activate the p27Syk kinase phosphorylation, leading to instability of red cell membrane and shedding of EVs. It was also found that peroxiredoxin 2 and HSP70 are supplemented in such EVs. Likewise, Levin et al. utilized nanoparticle tracking analysis to demonstrate high concentrations of EVs in thalassemia after splenectomy, which contain high concentration of HSP70, contributing to hemolysis, disease severity, and ineffective erythropoiesis (Levin et al., 2018). Additional investigations have shown that stored blood units of thalassemia patients have caspase-3 and molecular chaperones i.e., DJ-1 and HSP70, overexpression in the fraction of EVs, indicating that the oxidative stress disturbs the physiology and life of stored red cells in thalassemia patients (Tzounakas et al., 2021).

High EVs in circulation have been linked to serious clinical complications among β-thalassemia patients. This escalation is correlated with hypoxia-inducible factor α levels, a tissue hypoxia marker, especially in children (Elsayh et al., 2014). This connection between oxidative stress and EV formation plays a part in the thromboembolism often seen in such patients. Substantial enhancement in CD146+ endothelial EVs, von Willebrand factor, and endothelial progenitor cells have also been associated with cardiovascular complications in young β-thalassemia patients (Adly et al., 2015). However, higher circulating EVs concentrations have not been linked to pulmonary arterial hypertension among spelenectomized individuals, likely due to the utilization of antiplatelet drugs that reduce activation of platelets (Manakeng et al., 2019).

Categorizing the subtypes of β-thalassemia is essential for effective therapy, and EVs have appeared as significant markers for diagnosing the disease and distinguishing between the subtypes. An investigation by Li et al. has revealed the proteomic profiles of EVs derived from plasma in transfusion-dependent and independent thalassemia patients and healthy normal donors (Li et al., 2023b). This study recognized six proteins for diagnosing β-thalassemia: Lactotransferrin, C4b-binding protein α chain, C4b-binding protein β chain, complement C1as subcomponent, complement C1r subcomponent, and clusterin. This study also found different proteomic patterns among thalassemia intermedia and major patients. The top six proteins that distinguished thalassemia major from intermedia were apoplipoprotein M, plasma kallikrein B1, serotransferrin, HBB, immunoglobulin heavy constant γ4 (IGHG4), and Hb subunit α (HBA1). They projected a model utilizing these proteins to differentiate among three groups, underscoring the potential of EVs derived from plasma in subtyping and diagnosing β-thalassemia (Li et al., 2023b). In their study, the diagnostic potential of the proposed biomarkers was evaluated using Receiver Operating Characteristic curve analysis. This method calculates the Area Under the Curve, which quantifies how well a biomarker distinguish between patients and healthy individuals. Proteins like ferritin light chain, ferritin heavy chain, cathepsin S, and PLA2G7 showed high Area Under the Curve values, meaning they have strong diagnostic power, as they are highly effective at differentiating β-thalassemia patients from non-affecting individuals. However, while these results are promising, the study emphasizes the need for further validation.

6. Extracellular vesicles in bone marrow transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the merely proven, possibly curative therapy for transfusion-dependent thalassemia major patients. However, its use is considered by the accessibility of compatible donors and the overall health of patient. Human leukocyte antigen (HLA)-matched stem cells from bone marrow or cord blood have demonstrated exceptional results in HSCT. Thus, it is endorsed that young thalassemia major children consider undergoing HSCT early in disease course if they contain HLA-matched sibling donors, aiming to prevent dangerous complications such as multiorgan failure and iron overload (Farmakis et al., 2021). Research has observed an increase in circulating EVs after HSCT in various hematological diseases including thalassemia. For instance, Trummer et al. described an increase in plasma levels of P-selectin glycoprotein ligand-1-expresing microparticles, associating such increase with the risk of developing relapse and refractory disease (Trummer et al., 2011). Another group has focused on profiling of EVs in HSCT for patients of thalassemia, using flow cytometric analysis to quantify microparticles in thalassemia major children undergoing HSCT (Klaihmon et al., 2017c). This randomized investigation precisely examined CD235a + red cell microparticles and phosphatidylserine-expressing red cells, finding that their concentrations declined following transplantation. Likewise, another group observed an enhancement in other populations of microparticles derived from platelets i.e., CD41a + microparticles, endothelial cells (CD146+ microparticles), and leukocytes ($CD45^{+}$ microparticles) in patients following transplantation (Klaihmon et al., 2018). Such procoagulant microparticles were linked to an increased occurrence of monocyte-platelet microaggregates, indicating a multifaceted interplay and significant clinical implications after transplantation.

7. Limitations and Projections

The role of extracellular vesicles (EVs) in thalassemia-related complications is gaining recognition; however, our understanding of the precise mechanisms through which these vesicles contribute to disease progression remains incomplete. Although EVs are known to be involved in processes such as endothelial dysfunction and promoting a

hypercoagulable state, the exact signaling pathway and interactions are not yet fully elucidated. This incomplete knowledge restricts the development of targeted EV-based therapeutic approaches and limits the applications of EVs as reliable biomarkers in clinical settings. Another significant limitation in EV research is the lack of standardized protocols for their isolation and characterization. Various techniques, including ultracentrifugation and size exclusion chromatography, yield different results, making it challenging to achieve consistent and reproducible data across studies. This inconsistency hampers the potential for developing EV-based diagnostic tools and therapeutic interventions. Therefore, it is crucial to establish robust and standardized methods for EV analysis to facilitate their application in clinical practice.

While the potential of EVs as biomarkers for assessing severity of thalassemia complications is promising, their clinical utility remains in its infancy. There is currently a limited number of studies that evaluate the sensitivity and specificity of EVs in predicting or diagnosing clinical outcomes. As a result, further clinical trials are needed to validate the diagnostic value of EVs and integrate them into routine medical practice for thalassemia patients. Furthermore, the heterogeneity of thalassemia as a disease presents as additional challenge. The condition spans a broad clinical spectrum, from mild cases to transfusion-dependent thalassemia major, with significant variations in genetic background, transfusion history, and treatment regiments. This diversity complicates the study of EVs in thalassemia, as these factors can influence EV release and function, making it difficult to draw definitive conclusions that apply across different patient populations. Another limitation to consider is the potential influence of confounding factors such as infection, oxidative stress, and splenectomy, which are common in thalassemia patients. These factors can independently affect EV release, complicating efforts to attribute specific roles to EVs in disease progression. Longitudinal studies that control for these variables are needed to gain a clearer understanding of the role of EVs in thalassemia.

Looking forward, the field of EV research in thalassemia holds several exciting possibilities. One promising avenue is the development of EV-based therapeutics. With their ability to carry molecular cargo, such as proteins and miRNAs, EVs are being explored as potential vehicles for targeted drug delivery. In the future, it may be possible to engineer EVs to delivery therapeutic agent directly to affected tissues, offering a novel treatment strategy for managing complications such as iron overload, inflammation, and endothelial dysfunction in thalassemia. Additionally, EVs show great promise as diagnostic tools. As research into their role in thalassemia progresses, EVs could become valuable non-invasive biomarkers for monitoring disease progression and predicting the onset of complications, such as thromboembolic events or organ dysfunction. The incorporation of EV analysis into clinical practice could revolutionize the management of thalassemia by enabling earlier detection of complications and facilitating more personalized treatment approaches. The impact of blood transfusion on EV release and function is another area ripe for exploration. Since regular transfusions are a cornerstone of thalassemia management, understanding how transfusion-derived EVs contribute to complications such as iron overload and chronic inflammation could to more effective strategies to mitigate these risks. Moreover, the use of EVs in gene therapy offers another potential breakthrough in treating thalassemia. As gene-editing technologies advance, EVs could serve as vehicles for delivering gene-editing tools or therapeutic genetic material to correct hemoglobin defects. This would provide a more targeted and less invasive alternative to current gene therapy techniques, potentially transforming the treatment landscape of thalassemia.

8. Conclusion

Extracellular vesicles (EVs) can play a significant role in the onset and exacerbation of complications in individuals with thalassemia, including organ dysfunction, iron overload, inflammation, thrombotic events, ineffective erythropoiesis, cardiovascular conditions, and vascular complications. As a result, EVs have potential as biomarkers for assessing the severity of these complications, determining transfusion needs, and managing the clinical aspects of thalassemia. Their capacity to transport various bioactive molecules positions EVs as essential players in thalassemia's inflammatory and vascular responses. Elevated levels of circulating EVs correlate strongly with disease severity in thalassemia patients. This suggests their potential utility as diagnostic biomarkers for monitoring disease progression and predicting complications. Furthermore, the involvement of EVs in cellular communications and pathogenesis provides significant insights into novel therapeutic avenues aimed at addressing the multifaceted challenges faced by thalassemia patients. Looking ahead, future research should focus on elucidating the specific molecular mechanism through which EVs mediate the pathological processes associated with thalassemia. Investigating how these vesicles influence iron metabolism, reactive oxygen species generation, and the development of thrombotic events will be crucial in developing targeted interventions. Additionally, exploring the heterogeneity of EVs, particularly in terms of their origin and content, will enhance our understanding of their role in disease pathogenesis.

The lack of standardized methods for isolation and characterization of EVs is another crucial area that requires attention. Establishing robust protocols will enhance the reproducibility of research findings, facilitate comparisons across studies, and ultimately support the clinical application of EVs as biomarkers and therapeutic agents. Future investigations should also consider the impact of confounding factors on EV release and function, ensuring a comprehensive understanding of their roles in thalassemia. From clinical perspective, the incorporation of EV analysis into routine practice could revolutionize the management of thalassemia. EVs hold promise not only as non-invasive biomarkers for monitoring disease progression and complications but also as therapeutic agents capable of delivering targeted therapies. Furthermore, exploring the use of EVs in combination with current treatment modalities may enhance therapeutic outcomes. Research should also investigate the potential of EVs as a means to delivery iron-chelating agents or anti-inflammatory drugs directly to affected tissues, thereby improving the efficacy of existing therapies.

Patient consent statement

Not applicable. This review article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

The data that supports the findings are available in the article.

Ethics approval statement

Ethical approval was not required for this review article as it does not involve human or animal subjects.

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