DETECTION OF THE RED BLOOD CELL ADHESION TO THE VASCULAR ENDOTHELIUM USING MICROFLUIDIC CHIP IN BETA THALASSEMIA PATIENTS

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Koc University

Graduate School of Health Sciences

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and have found that it is complete and satisfactory in all respects,
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ABSTRACT

Detection of the red blood cell adhesion to the vascular endothelium using microfluidic chip in beta thalassemia patients

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Beta thalassemia is a hereditary genetic disorder affecting the hematopoietic system, primarily due to mutations in the beta globin gene. The disease is characterized by ineffective erythropoiesis resulting from reduced or absent beta globin production, leading to low or no production of hemoglobin A. This deficiency causes an accumulation of free alpha globin chains in the cytoplasm of erythroid cells, which disrupts their physiology. The free α-globin chains aggregate and precipitate, causing cytotoxic effects, oxidative stress, and decreased survival of erythroid cells, leading to high levels of hemolysis. The hemolysis of red blood cells (RBCs) releases hemoglobin and heme into plasma. Extracellular hemes are highly proinflammatory, activating immune and endothelial cells (ECs), contributing to the disease's pathogenesis. EC activation leads to the overexpression of adhesion molecules and increased RBC adhesion to ECs. Beta thalassemia patients exhibit altered RBC flow characteristics, causing microcirculatory issues. However, the mechanisms and molecules involved in RBC adhesion in beta thalassemia remain not fully understood. This study investigated RBC adhesion in beta thalassemia major (BTM) patients under flow conditions using a microfluidic chip with microchannels functionalized with 40 μM heme-activated HUVECs, fibronectin, and laminin. Increased RBC adhesion in BTM patients in microchannels functionalized with 40 μM heme-activated HUVECs and fibronectin was demonstrated compared to laminin. There were significant positive correlations between hemoglobin, WBC, platelet values, and adherent RBC number in microchannels functionalized with fibronectin. Application of Forskolin (activator of adenylyl cyclase) and SQ22536 (inhibitor of adenylyl cyclase) indicated that RBC adhesion could be altered by targeting the AC-cAMP-PKA signaling pathway. RBC deformability was also assessed, showing a slight decrease after shear stress at high shear stress levels for untreated patient blood, with Forskolin and SQ22536 impairing deformability compared to the vehicle after shear stress. Lastly, plasma

hemoglobin concentration was higher in beta thalassemia major patients' plasma samples than healthy controls. These data highlight the importance of VCAM-1 and fibronectin for RBC adhesion in BTM patients and the effects of targeting the AC-cAMP-PKA signaling pathway on RBC deformability and adhesion. This study has contributed to understanding the pathophysiology of beta thalassemia major patients and importance of targeting AC-cAMP-PKA signaling pathway. This underscores the need for these studies to be conducted with a larger patient cohort to gain more thorough insights.

OZETCE

Beta talasemi hastalarında mikroakışkan çip kullanılarak kırmızı kan hücrelerinin vasküler endotele yapışmasının tespiti

Neslihan Çilek

Hücresel ve Moleküler Tıp Doktora, 19 Temmuz, 2024

Beta talasemi, beta globin genindeki mutasyonların sebep olduğu hematopoietik sistemi etkileyen kalıtsal bir genetik bozukluktur. Hastalık, beta globin zincirinin üretiminin azalması veya yokluğu sonucu oluşan etkisiz eritropoez ile karakterize edilir ve hemoglobin A'nın düşük veya hiç üretilmemesine yol açar. Bu eksiklik, eritroid hücrelerin sitoplazmasında serbest alfa globin zincirlerinin birikmesine neden olur ve bu da fizyolojilerini bozar. Serbest alfa globin zincirleri kümelenir ve çöker, sitotoksik etkilere, oksidatif strese ve eritroid hücrelerin hayatta kalma oranının azalmasına neden olarak yüksek hemolize yol açar. Kırmızı kan hücrelerinin (RBC'ler) hemolizi, hemoglobin ve hem'i plazmaya salar. Hücre dışı hemler, bağışıklık ve endotel hücrelerini (EC'ler) aktive ederek hastalığın patogenezine katkıda bulunur ve oldukça proinflamatuardır. EC aktivasyonu, adezyon moleküllerinin aşırı ekspresyonuna ve EC'lere artan RBC adezyonuna yol açar. Beta talasemi hastaları mikrosirkülasyon sorunlarına neden olan değişmiş RBC akış özellikleri sergilerler. Ancak, beta talasemi hastalarında RBC adezyonunda yer alan mekanizmalar ve moleküller henüz tam olarak araştırılmamıştır. Bu çalışma, 40 μM hem aktiveli HUVEC'ler, fibronektin ve laminin ile işlevselleştirilmiş mikrokanallara sahip bir mikroakışkan çip kullanılarak akış koşulları altında beta talasemi majör (BTM) hastalarında RBC yapışmasını araştırmıştır. 40 μM hem aktiveli HUVEC'ler ve fibronektin ile işlevselleştirilmiş mikrokanallarda BTM hastalarında laminin ile karşılaştırıldığında artmış RBC adezyonu gösterilmiştir. Fibronektin ile fonksiyonlandırılmış mikrokanallarda hemoglobin, WBC, trombosit değerleri ve kırmızı kan hücresi adezyonu arasında anlamlı pozitif korelasyonlar gözlemlendi. Forskolin (adenil siklaz aktivatörü) ve SQ22536 (adenil siklaz inhibitörü) uygulaması, AC-cAMP-PKA sinyal yolunu hedefleyerek kırmızı kan hücresi adezyonunun değiştirilebileceğini gösterdi. Kırmızı kan hücresi deforme edilebilirliği de değerlendirildi ve hasta kanında yüksek kayma gerilimi seviyelerinde kayma geriliminden sonra hafif bir azalma gösterilmiştir; kayma gerilimiyle birlikte Forskolin ve SQ22536’ nun deforme

edilebilirliği değiştirdiği de gözlenmiştir. Son olarak, plazma hemoglobin konsantrasyonu, beta talasemi major hastalarının plazma örneklerinde sağlıklılara kıyasla hemoglobinin daha yüksek olduğunu göstermiştir. Bu veriler, BTM hastalarında RBC adezyonu için VCAM-1 ve fibronektinin önemini ve AC-cAMP-PKA sinyal yolunu hedeflemenin RBC deforme edilebilirliği ve adezyonu üzerindeki etkilerini vurgulamaktadır. Bu çalışma, beta talasemi majör hastalarının patofizyolojisini ve AC-cAMP-PKA sinyal yolunu hedeflemenin önemini anlamaya katkıda bulunmuştur. Bu, daha kapsamlı bilgiler elde etmek için bu çalışmaların daha büyük bir hasta kohortuyla yürütülmesi ihtiyacını vurgulamaktadır.

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ABBREVIATIONS

AC	Adenylyl cyclase
AMP	Adenosine monophosphate
APTES	3-aminopropyl triethoxysilane
ATP	Adenosine triphosphate
BTI	Beta thalassemia intermedia
BTM	Beta thalassemia major
cAMP	Cyclic adenosine 3',5'-monophosphate
Ca^{2+}	Calcium ions
CBC	Complete blood count
CO_2	Carbon dioxide
DFO	Deferoxamine
DFP	Deferiprone
DFX	Deferasirox
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSA	Double-sided adhesive
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EI	Elongation Index
EI_{max}	Maximal RBC elongation at infinite shear stress
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FN	Fibronectin
GDF15	Growth differentiation factor 15
GMBS	N-g-Maleimidobutyryloxy succinimide ester
GPCR	G protein-coupled receptor
Hb	Hemoglobin
HbA	Hemoglobin A
HbF	Hemoglobin F
HbS	Hemoglobin S
HBB	Beta globin gene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIFs	Hypoxia-inducible factors
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-4	Intercellular adhesion molecule 4
IgSF	Immunoglobulin superfamily
LDH	Lactate dehydrogenase
LN	Laminin
LORRCA	Laser-assisted optical rotational cell analyzer
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MOH	Ministry of Health
NO	Nitric oxide
NRBC	Nucleated red blood cell
NTDT	Non-transfusion-dependent thalassemia
OS	Oxidative stress
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
Plt	Platelet
PMMA	Polymethyl methacrylate
PS	Phosphatidylserine
P/S	Penicillin/Streptomycin
PV	Polycythemia vera
PVP	Polyvinylpyrrolidone
RAGE	Receptor for advanced glycation end-product
RBC	Red blood cell
RDW	Red cell distribution width
ROS	Reactive oxygen species
RT	Room temperature
RVO	Retinal vein occlusion
SCD	Sickle cell disease

SS	Shear stress
$SS_{1/2}$	Shear stress required for half-maximal deformation
std	Standard deviation
TDT	Transfusion-dependent thalassemia
TNF-α	Tumor necrosis factor alpha
TSP	Thrombospondin
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4
VOC	Vaso occlusive crises
WBC	White blood cell
β2AR	β2 adrenergic receptor

Chapter 1

INTRODUCTION

1.1 Thalassemia

Thalassemia is the world's most prevalent hemoglobinopathy, characterized by mutations in the globin genes that cause reduced or absent production of specific globin chains of hemoglobin (Hb). The structure and synthesis of the diverse hemoglobins produced during human life are determined and regulated by human globin genes. These globin genes are organized into two clusters: the α (alpha) globin genes on chromosome 16 and the β (beta) globin genes on chromosome 11. Hemoglobin in adult humans (hemoglobin A (HbA)) consists of two alpha and two beta globin chains. Mutations or deletions occurring in the alpha or beta globin genes of hemoglobin disrupt the effectiveness of globin chain production, resulting in distinct types of thalassemia, namely alpha and beta thalassemia (Weatherall, 2001; Weatherall et al., 2019).

The International Thalassemia Federation has adopted new terminology for the clinical classification of thalassemia, categorizing it into transfusion-dependent thalassemia (TDT) and non-transfusion-dependent thalassemia (NTDT). This classification is now widely used in clinical practice to determine transfusion requirements. TDT refers to patients who cannot produce sufficient hemoglobin to survive without regular blood transfusions, while NTDT describes patients who do not require lifelong regular transfusions (Shash, 2022).

Alpha thalassemias are the most common genetic disorders, found at high frequencies across the tropical belt from sub-Saharan Africa to the Middle East, South Asia, and Southeast Asia. In some parts of North India and Southeast Asia, the heterozygote frequency is approaching fixation, with more than 75% of the population affected. The more severe form, alpha^0 thalassemia, is less common but is found at high frequencies in parts of the Mediterranean and Southeast Asia. Beta thalassemias are less common in sub-Saharan Africa and have varying frequencies across the tropical belt. Each high-frequency region has its own specific beta thalassemia mutations, indicating that these mutations are relatively recent in evolutionary terms and have not yet spread evenly. Thalassemia is not prevalent in wealthier countries like Europe and the United States, but its frequency is rising due to increasing immigration from the tropical belt. This trend is expected to continue in Europe due to significant immigration from the Middle East (Weatherall, 2018; Williams & Weatherall, 2012). Thalassemia also has a high prevalence in Turkey, particularly along the Mediterranean coast. There are approximately 1,300,000 carriers and 4,000 patients (Arpaci et al., 2021).

Thalassemia could be prevented through carrier identification, prenatal diagnosis, and genetic counseling (Origa & Comitini, 2019). Genetic counseling provides evidence for the risk to both carriers and their offspring. Prenatal diagnosis can be performed by examining the fetal gene at around 4–5 months of development or by sampling chorionic villi at 11 weeks of gestation. Detecting fetal DNA in the mother's serum and investigating fetal cells in maternal blood can help identify paternal mutations. Fetal DNA sources include lymphocytes, trophoblasts, and nucleated red blood cells (NRBCs). Preventing the birth of homozygotes can significantly reduce the burden of thalassemia. Over the past two decades, many Mediterranean and Western countries have successfully reduced their homozygote populations. Countries such as Turkey, Canada, Egypt, Iran, Malaysia, Lebanon, Pakistan, and China have also implemented control programs for thalassemia (Ali et al., 2021).

Turkey is actively working to prevent congenital diseases, with a focus on identifying thalassemia carriers, as this is key to preventing such disorders. Thalassemia screening centers assist couples planning to marry, and the Ministry of Health (MOH) provides guidance for prenatal diagnosis and treatment based on protocols in their diagnostic and

treatment guides. Screening involves complete blood count (CBC) and high-performance liquid chromatography (HPLC) tests, with individuals referred to molecular centers if necessary. Mutation screening strategies are developed by these centers based on screening results or hematology clinic referrals (Arpaci et al., 2021; Çürük et al., 2013). In 2003, the MOH initiated a national hemoglobinopathy prevention program in provinces with high prevalence. The rate of premarital screening tests for couples increased from 30.0% in 2003 to 86.0% in 2013. The number of newborns with thalassemias and hemoglobinopathies decreased from 272 in 2002 to 25 in 2010, marking a 90.0% reduction in affected births over the decade (Canatan, 2014), and this number continues to decline.

1.2 Beta thalassemia

Beta thalassemia is an inherited blood disorder resulting from mutations in the beta globin gene (HBB) on chromosome 11, leading to the reduced or absent synthesis of beta globin chains. There are identified more than 200 beta thalassemia mutations which can be a single nucleotide substitution, small deletions, or insertions for the beta globin gene, classifying as β_+ (mild mutations resulting a relative reduction of the beta globin chain product) and β_0 (severe mutations resulting a complete absence of the beta globin chain product) (Cao & Galanello, 2010; Rao et al., 2024; Rund & Rachmilewitz, 2005; Weatherall, 1976). The clinical presentation of beta thalassemia is classified into three primary phenotypes: beta thalassemia minor (carriers), beta thalassemia intermedia (BTI), and beta thalassemia major (BTM) (Taher et al., 2021).

1.2.1 Beta thalassemia minor (carriers)

Beta thalassemia minor, also known as beta thalassemia carrier or trait, is typically asymptomatic or experiences mild anemia. Characteristic hematological features including microcytosis (reduced red blood cell volume), hypochromia (reduced hemoglobin content in red blood cells), reduced HbA synthesis, and increased levels of HbA_2 (a minor component of adult hemoglobin composed of two alpha and two delta

chains) are exhibited in beta thalassemia minor (Cao & Galanello, 2010; Needs et al., 2024).

Individuals with beta thalassemia minor have one normal beta globin gene, allowing them to produce enough hemoglobin to meet the body's needs without significant erythroid hyperplasia. The deficit of hemoglobin A is frequently offset by increased levels of HbA_2. The hemoglobin pattern, determined by high-performance liquid chromatography (HPLC) or cellulose acetate electrophoresis, varies with the beta thalassemia types (Cao & Galanello, 2010). In beta thalassemia minor, the hemoglobin pattern includes 92-95% HbA, more than 3.8% HbA_2, and 0.5-4% HbF (a normal hemoglobin contains two alpha and two gamma chains produced by the fetus) (Needs et al., 2024; Rund & Rachmilewitz, 2005).

Beta thalassemia minor is often discovered during routine blood tests including blood smears revealed variations in red blood cell size and shape. While generally requiring no treatment, beta thalassemia minor may present with mild symptoms such as slight fatigue or pallor (Brancaleoni et al., 2016; Cao & Galanello, 2010; Weatherall, 1976).

1.2.2 Beta thalassemia intermedia

Beta thalassemia intermedia (BTI) contains a broad spectrum of clinical presentations, varying in severity from those resembling beta thalassemia minors to more severe cases related to beta thalassemia major. Symptoms commence early in childhood, characterized by growth and developmental delays, while adults exhibit symptoms in milder form (Cao & Galanello, 2010; Rund & Rachmilewitz, 2005). BTI is defined by hemoglobin levels ranging from 7 to 10 g/dl, mean corpuscular volume (MCV) between 50 and 80 fl, and mean corpuscular hemoglobin (MCH) between 16 and 24 pg (Origa, 2017).

The main symptoms include paleness, jaundice (yellowing of the skin), gallstones, enlargement of the spleen and liver, skeletal changes ranging from moderate to severe, leg ulcers, the presence of abnormal masses of hyperplastic erythroid marrow outside the bone marrow, a propensity for developing osteoporosis (brittle bones) and osteopenia (developing the bone density loss), and thrombotic complications due to an increased tendency for blood coagulation caused by the abnormal lipid membrane composition of

the red blood cells, especially in individuals who have undergone splenectomy (Needs et al., 2024; Origa, 2017). While BTI patients are not reliant on regular transfusions, intermittent transfusions are able to help mitigate complications and prevent severe anemia as part of the management protocol (Asadov et al., 2018; Graffeo et al., 2018). The clinical symptoms of the BTI patients are mainly caused by chronic anemia and iron overload primarily from increased absorption of iron in the intestines due to ineffective erythropoiesis, with severity contingent upon molecular defects. The BTI patient's clinical diversity reflects its genetic heterogeneity and the interplay of various genetic determinants and modifiers. (Asadov et al., 2018; Cao & Galanello, 2010; Thein, 2004).

The genetic basis of BTI which is compound heterozygous for beta thalassemia mutations involves the inheritance of one or two mutant alleles in combination with other genetic factors (Ali et al., 2021; Musallam et al., 2013). Point mutations and, less commonly, deletions in the HBB gene or its surrounding regions on chromosome 11 are the main genetic modifiers of beta thalassemia (Patrinos et al., 2004; Rao et al., 2024; Thein, 2004). The inheritance of milder HBB gene mutations (β^+ and silent), that still allow to produce beta globin chains, are the main reason of the reduced severity of BTI compared to BTM (Taher et al., 2009). Secondary and tertiary modifiers of BTI include variations in alpha globin inheritance patterns, HbF production, and genetic factors, linked to potential complications of BTI. BTI can also occur in individuals with two β_0 mutations, where increased HbF production compensates for the absence of beta globin chains. The inheritance of a single mutant allele is another mechanism for BTI (Asadov et al., 2013). With the diverse genetic basis, BTI patients produce enough beta globin chain to mitigate clinical symptoms.

The clinical presentation of BTI is influenced by three key factors: ineffective erythropoiesis, chronic anemia, and iron overload. Ineffective erythropoiesis, characterized by the precipitation of the unstable alpha chain within the erythroid progenitors forming inclusion bodies, causes the oxidative cell membrane damage and cell lysis, resulting in bone marrow hyperplasia, extramedullary erythropoiesis, and skeletal abnormalities (Haidar et al., 2010; Origa, 2017). Anemia in BTI is primarily caused by ineffective erythropoiesis, while secondary factors include peripheral hemolysis and decreased hemoglobin synthesis (Asadov et al., 2018).

Unlike BTM, where iron overload stems from continuous transfusions, BTI patients experience iron overload due to the increased iron absorption driven by heightened erythropoiesis and hemoglobinization (Musallam et al., 2012; Porter et al., 2017; Taher et al., 2013). Iron metabolism and absorption are regulated by factors like hypoxia-inducible factors (HIFs) under hypoxia conditions, which stimulate erythropoietin production and reduce hepcidin synthesis, allowing for increased iron absorption. Hepcidin and growth differentiation factor 15 (GDF15), inhibiting hepcidin expression, play crucial roles in regulating iron absorption, recycling, and storage. Reduced hepcidin levels lead to systemic iron overload, resulting in complications including heart failure and endocrine dysfunction such as diabetes and hypogonadism in BTI (De Domenico et al., 2007; Peyssonnaux et al., 2007; Rivella, 2012; Tanno et al., 2007).

In BTI patients, chelation therapy initiation is determined by the severity and progression of iron overload, as well as the duration of iron accumulation (Asadov et al., 2018).

1.2.3 Beta thalassemia major

Beta thalassemia major (BTM) patients present within the first two years of life and need regular blood transfusions for survival. Those diagnosed later, who do not need transfusions, are classified as having BTI. It is crucial to distinguish between BTM and BTI at the time of diagnosis to prevent unnecessary transfusions in BTI patients and to begin early transfusions in BTM patients (Rund & Rachmilewitz, 2005).

BTM, known as Cooley's anemia, is the most severe form of beta thalassemia, having severe mutations in the beta globin chain, causing a total lack of beta globin chains that results in a significant excess of alpha globin chains relative to gamma globin chains. However, there are some mutations resulting the reduction in beta globin chain synthesis (Galanello & Origa, 2010; Tari et al., 2018). BTM causes severe anemia, leading to complications such as heart failure, fatigue, and cachexia. In BTM, where beta globin chain synthesis is absent, the hemoglobin pattern presents absent HbA, HbF at 95–98%, and HbA_2 at 2–5%. In BTM, with some residual beta globin synthesis, the hemoglobin pattern presents HbA at 10-30%, HbF at 70-90%, and HbA_2 at 2-5% (Cao & Galanello, 2010). The complete blood count (CBC) reveals microcytic hypochromic anemia,

characterized by hemoglobin levels below 7 g/dl. The MCV presents between 50 and 70 fl, and the MCH ranges from 12 to 20 pg (Origa, 2017). The peripheral blood smear reveals not only microcytosis and hypochromia but also anisocytosis, poikilocytosis (including spiculated tear drop and elongated cells), and the presence of nucleated red blood cells (erythroblasts). Erythroblasts number correlates with the severity of anemia and significantly increases following a splenectomy (Cao & Galanello, 2010).

Infants with BTM often exhibit failure to grow and progressive pallor. BTM patients may also experience feeding difficulties, diarrhea, irritability, recurrent fevers, and abdominal enlargement due to splenomegaly. Initiating a regular transfusion program to maintain a hemoglobin concentration of 95–105 g/l is necessary to ensure normal growth and development until about 10–11 years of age. Beyond this age, the risk of severe complications from iron overload increases (Needs et al., 2024).

Regular blood transfusions, a common treatment, can lead to iron overload in various organs, causing diabetes, hypopituitarism, liver complications, endocrine issues like hypothyroidism, hypoparathyroidism, dark skin pigmentation, cardiac arrhythmias, cirrhosis, and myopathy, which contribute to a 71% mortality rate in patients with BTM (Lei et al., 2019). The resulting bone marrow expansion, an attempt to compensate for the loss of hemoglobin, leads to bone abnormalities, splenomegaly, and stunted growth. Severe hemolysis causes pulmonary hypertension, gallstones, and leg ulcers. The disorder is further complicated by hypercoagulability (Ali et al., 2021). Additional complications include prolonged hepatitis B and C, the human immunodeficiency virus (HIV) infection, osteoporosis, blood clots, and an increased risk of liver cancer in those with liver infections (Borgna-Pignatti et al., 2004).

Individuals who receive consistent transfusions and appropriate chelation therapy might live beyond 30 years. However, myocardial disease due to transfusional siderosis remains the most significant life-limiting complication in beta thalassemia due to the iron overload, accounting for 71% of deaths in BTM patients (Borgna-Pignatti et al., 2004; Cao & Galanello, 2010).

1.3 Treatment strategies for beta thalassemia patients

No treatment is necessary for individuals with beta thalassemia minor, but genetic counseling is advised due to the risk of passing the mutant gene to their offspring. Those with BTI experience mild anemia throughout life and generally lead normal lives but require regular monitoring and occasional blood transfusions. Iron supplementation is not recommended, but folic acid supplementation is often prescribed (Ali et al., 2021).

In BTI and BTM, splenomegaly results from severe hemolysis. Early initiation of regular blood transfusions can prevent splenomegaly in childhood, though hypersplenism may develop between ages 5 to 10. Splenectomy can protect against health complications and growth retardation by reducing the need for transfusions, increasing Hb levels, and lowering iron accumulation (Pecorari et al., 2008). Splenectomy is recommended when transfusion needs exceed 200 to 220 ml of packed red blood cells (RBCs) per kg per year with a hematocrit of 70%, or 250–275 ml/kg with a hematocrit of 60% (Ali et al., 2021).

Blood transfusion therapy in BTM aims to maintain Hb levels and correct anemia resulting from inadequate erythropoiesis. Transfusions should be initiated for severe anemia after confirming thalassemia diagnosis. However, transfusions should not be delayed beyond the second to third year to avoid complications like antibody formation and difficulty in finding suitable blood donors. The goal of transfusion therapy is to maintain pre-transfusion Hb levels around 9 to 10 g/dl, and post-transfusion levels at 13 to 14 g/dl to prevent organ damage, growth retardation, and bone deformities, thereby improving quality of life (Ali et al., 2021). The frequency of transfusions depends on factors such as hematocrit, Hb levels, and patient weight (Taher & Cappellini, 2018). Transfusion volumes should not exceed 15 to 20 ml/kg per day to avoid circulatory overload. Monitoring of transfusion efficacy is crucial through pre- and post-transfusion Hb and hematocrit levels to assess iron intake and RBC requirements (Galanello & Origa, 2010).

With regular blood transfusions, each red blood cell contains approximately 200 mg of iron, leading to an accumulation of 0.3–0.6 mg/kg of iron per day (Sarker et al., 2014). Chelation therapy, involving deferasirox (DFX), deferiprone (DFP), and deferoxamine (DFO), is crucial for managing iron overload resulting from frequent blood transfusions

(Adewoyin & Oyewale, 2015). Chelation typically begins after 20–25 RBC units have been transfused, starting between ages 2 to 4 (Taher & Cappellini, 2018). However, it is essential to adjust the dosage individually for each patient because long-term use may lead to adverse effects such as ocular and auditory toxicity, growth retardation, arthropathy, and gastrointestinal symptoms (Galanello & Origa, 2008; Pennell et al., 2006).

Alternative to traditional blood transfusion and chelation therapy, bone marrow transplantation remains the definitive treatment option for BTM patients (Ali et al., 2021). In addition, patients' stem cells are used for a potential cure for BTM in gene therapy, similar to treatments used for various other conditions including sickle cell disease (Angelucci et al., 2014).

Long-term manifestations of beta thalassemia can also be managed by inducing fetal hemoglobin production, which extends the lifespan of RBCs. Drugs like hydroxyurea stimulate fetal hemoglobin production and improve clinical outcomes in patients with BTI (Tari et al., 2018; Wilber et al., 2011).

1.4 Red blood cells and their flow properties

Red blood cells (RBCs) are specialized for oxygen transport during their formation before entering the bloodstream (Hoffman et al., 2013; Wang & Popel, 1993). Under normal conditions in a stress-free environment, RBC in a normal human is shaped like a biconcave disk, measuring about 8 micrometers in diameter and 2 micrometers in thickness which has an area of 140 μm^2 and a volume of 90 μm^3 (Figure 1.1) to pass through capillaries that are only 3–5 μm in diameter, thereby delivering oxygen to body tissues (Canham & Burton, 1968; Tomaiuolo, 2014). RBCs are filled with hemoglobin, which is a protein binding oxygen. As blood travels through the lungs, hemoglobin binds oxygen. Once in the systemic circulation, hemoglobin releases oxygen to the tissues.

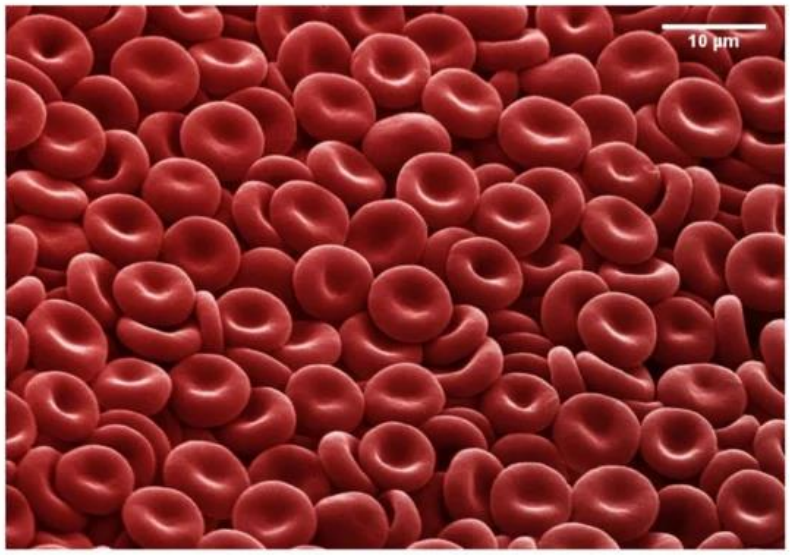


Figure 1.1 Image of RBCs biconcave disc shape on scanning electron microscopy (SEM) (Trejo-Soto et al., 2022).

Mature RBCs have a lifespan of about 120 days, during which they mainly travel through the microcirculation. Throughout this period, RBCs are estimated to cover approximately 400 kilometers, completing 170,000 circuits through the vascular system. These circulating RBCs possess unique physiological adaptations that allow them to withstand significant biochemical and biomechanical stresses. As RBCs mature, their energy and antioxidant systems degrade, causing oxidative damage to critical proteins such as hemoglobin and lipids. At the cellular level, maturing is marked by a reduction in MCV because of the loss of cell membrane fragments, which in turn causes an increase in the intracellular Hb concentration (MCHC) and cell density (Huisjes et al., 2018; Sudnitsyna et al., 2020; Willekens et al., 2008). These damages impair their rheology, adhesion, gas transport, and vascular signaling. Aged RBCs display signs of senescence and are either removed by the spleen or undergo eryptosis, a cell death process specific to RBCs and resembling apoptosis. This degradation process is accelerated during critical illness, potentially limiting oxygen delivery, and contributing to the progression and outcome of organ failure (Barshtein et al., 2023; Rogers & Doctor, 2020).

To perform this oxygen delivery to tissues, RBCs possess unique flow-affecting properties that significantly influence blood flow across all types and sizes of blood

vessels. Blood flow is significantly affected by the flow properties of RBCs, which are deformability, aggregability and adherence to the endothelial cells (EC). These characteristics are crucial for haemodynamics, especially in microcirculation, and their dysfunction is associated with microcirculatory disorders in various pathological conditions (Barshtein et al., 2007; Baskurt & Meiselman, 2003; Hovav et al., 1999).

1.4.1 Red blood cell deformability

RBC deformability is essential for maintaining proper blood flow in microcirculation, allowing them to alter their shape in response to varying flow conditions, thereby minimizing flow resistance. The biconcave disc shape of RBCs maximizes their surface area relative to their volume, enabling elongation when subjected to external forces in the circulation and navigating through capillaries narrower than diameter of RBCs (Barshtein et al., 2023; Baskurt & Meiselman, 2003; Chien, 1987; Mohandas & Chasis, 1993). Intracellular viscosity, membrane and cytoskeletal organization, and the ionic content of cytoplasm are the other factors that affect the deformability of RBCs (Baskurt et al., 1998; Cilek et al., 2024; Huisjes et al., 2018). Metabolic processes regulating adenosine triphosphate (ATP) levels and the balance of redox reactions, mainly through second messengers such as calcium ions (Ca^{2+}) and nitric oxide (NO), also influence the deformability of RBC (Bogdanova et al., 2013; Grau et al., 2013). In addition, mechanical stress affects RBC deformability by raising intracellular Ca^{2+} levels through Piezo1, which is the mechanosensitive cation channel (Figure 1.2) (Kleinbongard et al., 2006; Kuck et al., 2019).

Membrane fragment shedding in RBCs, involving changes in the cytoskeleton and loss of membrane lipids and proteins, leads to a lower area-to-volume ratio and decreased deformability (Orbach et al., 2017; Salzer et al., 2008). The RBC membrane skeleton is composed of proteins, primarily spectrin, keeping the cell's unique geometry under mechanical stress by folding and unfolding. Integral proteins, e.g., band 3, glycophorin C, and the junctional protein Protein 4.1, facilitate the connection between the spectrin network and the lipid layer of the membrane. Two main cytoskeletal interaction points are the actin, spectrin, and Protein 4.1 network assembly, and the band 3/ankyrin complex

anchoring this network to the membrane. These interactions, regulated by posttranslational modifications such as protein phosphorylation and dephosphorylation, determine the stability of RBC membrane and adjust RBC deformability when subjected to shear stress. To illustrate, tyrosine phosphorylation of band 3's cytoplasmic domain disrupts the band 3-ankyrin bridge, affecting cytoskeletal connections and metabolic processes by inhibiting anion transport (Ferru et al., 2011; Mohandas & Gallagher, 2008; Tao, 1988).

Phosphorylation of the membrane protein is a fundamental mechanism in cellular physiology, influencing processes, e.g., cytoskeletal regulation, signal transduction, transport, metabolism, and cell volume. Phosphorylation involves adding a phosphate group to the amino acids' polar group, converting the protein from hydrophobic and apolar to hydrophilic and polar. This modification enables the protein to alter its shape, facilitating interactions with other proteins and the assembly or disassembly of protein complexes (Ardito et al., 2017; Boivin, 1988; Ferru et al., 2011; Gauthier et al., 2011). In RBCs, cytoskeletal proteins are phosphorylated at serine, tyrosine, or threonine residues by various kinases and dephosphorylated by different phosphatases. The phosphorylation state of a protein is regulated by the balance between the activities of these phosphatases and kinases. Notable protein kinases in RBCs include protein kinase A (PKA), protein kinase C (PKC), casein kinases I and II, Lyn, Syk, Hck-Fgr, and Fyn (George et al., 2010; Pantaleo et al., 2010). Enhancing kinase activity, such as by increasing the levels of intracellular cyclic adenosine 3',5'-monophosphate (cAMP), has been shown to increase the phosphorylation of cytoskeletal proteins and improve RBC deformability (Ugurel et al., 2022). Various intracellular signaling pathways control the functions of kinases and phosphatases, thus arranging the cytoskeletal network (Barvitenko et al., 2005; Cilek et al., 2024; Minetti & Low, 1997).

The AC-cAMP-PKA signaling pathway (Figure 1.2) begins with the activation of membrane-bound adenylyl cyclase (AC) by G protein-coupled receptors (GPCRs). Subsequently, AC catalyzes the conversion of ATP into cAMP, a crucial second messenger that regulates cellular responses to external stimuli. In RBCs, the cAMP concentration is firmly controlled through a dynamic balance between its production by ACs and its degradation by phosphodiesterases (PDEs), which provide local negative

feedback loops to terminate the signal (Adderley et al., 2010; Bender & Beavo, 2006; Wong & Scott, 2004). PDEs manage cAMP levels by breaking down the phosphodiester bonds in cyclic nucleotides, thereby converting cAMP to AMP. Inhibition of PDEs maintains the activity and integrity of cyclic nucleotides (Adderley et al., 2011; Huisjes et al., 2018). PKA, activated by elevated cAMP levels, phosphorylates target proteins. In RBCs, PKA also phosphorylate and activate PDEs, regulating intracellular signals. Prior research has shown that PKA phosphorylates proteins, e.g., β-spectrin, Protein 4.1, adducin, and dematin in the membrane of RBC (Chen et al., 2013; Cohen & Gascard, 1992). The activation of PKA by cAMP reduces the mechanical stability of the RBC membrane, mainly through the phosphorylation of dematin, which weakens its interaction with spectrin and F-actin. This phosphorylation alters the cytoskeleton-membrane interactions, enhancing RBC deformability (Chen et al., 2013; Koshino et al., 2012; Ugurel et al., 2022)

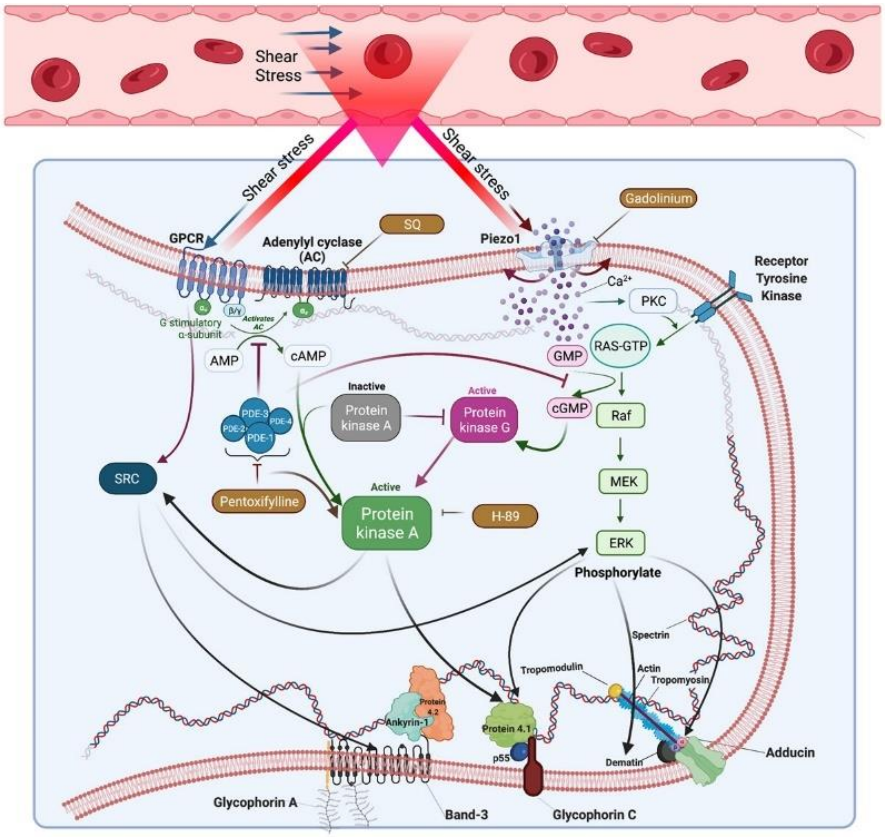


Figure 1.2 A schematic representation of two key cytoskeletal junctions, the role of phosphorylation events through cyclic AMP (cAMP) and extracellular signal-regulated kinase (ERK) signaling pathways, and mechanisms for sensing shear stress. The lower section of the figure illustrates cytoskeleton-membrane junctions, specifically the band 3-ankyrin bridge and the Glycophorin C-Protein 4.1 connection. The upper section depicts shear stress sensing mechanisms involving Piezo1 and GPCR. Activation of adenylate cyclase (AC) by G protein-coupled receptors (GPCRs) increases cAMP levels, which in turn activates protein kinase A (PKA). PKA then phosphorylates membrane proteins like Protein 4.1, adducin, and dematin, and can also activate SRC family members, including Syk and Lyn, to further phosphorylate the band 3 protein. When shear stress is applied, Piezo1 induces Ca^{2+} influx, potentially influencing protein kinase C activity, Ca^{2+}-ATPase, and the Gardos channel (not shown). The MEK/ERK pathway, regulated by Raf proteins, may target membrane proteins of red blood cell (RBC), affecting cytoskeletal interactions. Inhibitory effects are shown by closed lines, and phosphorylation events are indicated by black arrows. PDE stands for phosphodiesterase, and PKC for protein kinase C (Ugurel et al., 2022).

Blood flow and oxygen delivery to peripheral tissues are impaired by reduced deformability, and capillaries are blocked directly by rigid cells. Deformability is also vital for RBCs to pass through the splenic vasculature; decreased deformability causes increased sequestration and destruction of RBCs in the spleen (Mohandas & Chasis, 1993; Mohandas & Gallagher, 2008; Warkentin et al., 1990). Thus, diminished RBC deformability is associated with circulatory disorders and anemias in various conditions, e.g., sickle cell disease (SCD), thalassemia, cerebral malaria, diabetes, cardiovascular diseases, sepsis, and stroke (Barshtein et al., 2023).

In RBCs, targeting the cAMP/PKA pathway is crucial for developing new treatments for hemoglobinopathies. Research aims to increase fetal hemoglobin levels in SCD by decreasing cAMP level, particularly using hydroxyurea (Bartolucci et al., 2010; Lebensburger et al., 2010). Forskolin, another agent, enhances RBC deformability by elevating cAMP levels through adenylyl cyclase activation directly (Semenov et al., 2019). Conversely, inhibiting AC with SQ22536 notably improves RBC deformability in SCD patients, with more pronounced effects than Forskolin (Goksel et al., 2023).

RBC deformability can be evaluated by observing how RBCs move through cylindrical pores that are narrower than RBCs. Typically, the transit time through these pores under constant pressure is measured, with longer transit times suggesting decreased deformability (Baskurt et al., 1996). Another method to measure RBC deformability involves examining the shape changes of the cells under applied fluid forces, either through direct microscopic observation or by analyzing laser-diffraction patterns produced by the deformed cells (Hardeman et al., 1994).

1.4.2 Red blood cell aggregability

Under normal conditions, RBCs behave like fluid droplets display high flexibility under shear stress and align with the flow of the bloodstream. However, RBCs tend to clump into linear stacks, known as rouleaux, during inflammatory stress due to presence of plasma proteins like fibrinogen and acute phase reactants such as C-reactive protein, haptoglobin, serum amyloid A, and ceruloplasmin. RBC aggregation significantly

increase blood viscosity and, consequently, the distribution and volume of oxygen delivery, especially in microcirculation under low-shear conditions found in critical illnesses or abnormal vessel tone (McHedlishvili et al., 2002). Oxidative stress, prevalent in critical illness and sepsis, disrupts this hemorheological physiology. This disruption is linked to increased intracellular 2,3-DPG levels, intracellular free calcium, and reduced intraerythrocytic ATP, which lowers sialic acid content in RBC membrane (Rogers & Doctor, 2020). Increased interaction between RBCs and white blood cells (WBCs), coupled with the release of reactive oxygen species (ROS) during sepsis, further changes RBC membrane properties, eventually increasing RBC aggregability (Baskurt & Meiselman, 1998).

1.4.3 Red blood cell adhesion to the endothelial cells

Under normal circumstances, circulating RBCs are distributed singly, have insignificant adhesion to the endothelial cells (ECs), and have sufficient deformability to allow them to move through capillaries with sufficient ease. However, in infectious, thrombotic, and inflammatory conditions, especially under oxidative stress (OS), RBC adherence to the ECs is increased, and RBC deformability is decreased. These perturbations from normal function have the potential to cause circulatory diseases and impact hemodynamics, especially in microcirculation. Various research has been carried out over the past 20 years to determine the molecular and physical characteristics involved in this interaction between RBCs and ECs (Chiang & Frenette, 2005; Hebbel, 2000; Yedgar et al., 2008). Recently, the significance of RBC adhesion to ECs in developing thrombotic complications has been recognized.

RBC adhesion to EC can disrupt circulation in multiple ways, depending on the size of the vessel. In large vessels, adherent RBCs can create irregular flow patterns, causing flow disturbances that activate ECs (Barshtein et al., 2007; Hebbel, 2000; Rattan et al., 1997). In microcirculation, RBC adhesion decreases blood flow, prolonging RBC residence time and impeding flow. In capillaries, adherent RBCs can directly block the vessel, interrupting blood flow.

The adhesive interaction between RBC and EC in blood vessels is a significant factor leading to vaso occlusive crises (VOC) in various pathological conditions, including polycythemia vera (PV), retinal vein occlusion (RVO), diabetic mellitus, hypertension, and stroke (Wautier et al., 1985; Wautier et al., 1981; Wautier & Wautier, 2013; Wautier et al., 2007; Wautier et al., 2011). In addition, this interaction is also thought to be a powerful effector of circulatory problems, and the enhancement of this interaction has been linked to the pathophysiology of many diseases, including hemoglobinopathies, such as thalassemia, SCD, and malaria in that continuous hemolysis and excessive iron overload causes the oxidative stress (Hebbel et al., 1980; Piriyakhuntorn et al., 2020; Wautier & Wautier, 2020).

Erythroid cells have numerous cell adhesion molecules, some of which remain on mature RBCs, typically non-adhesive under normal conditions (Spring & Parsons, 2000). These adhesion molecules are involved in erythropoiesis, self-recognition, and RBC turnover through interactions with macrophages in the bone marrow or reticuloendothelial system. In the pathophysiology of diseases, including SCD, diabetic mellitus, malaria, and vascular disorders, they play important roles by abnormally adhering to the vascular endothelium (Cartron & Elion, 2008; Wautier & Wautier, 2020; Wautier et al., 1981; Wautier et al., 2007).

The most studied adhesion molecules on RBCs include members of the immunoglobulin superfamily (IgSF) like ICAM-4, Lu/BCAM, CD47, Neurothelin, Sema7A, and LFA-3. Except for CD47, most are expressed at low levels on RBCs, while some, like CD36 and α4β1 integrin, are present on stress reticulocytes in SCD and hemolytic anemias (Aricescu & Jones, 2007; Joneckis et al., 1993; Springer, 1990; Swerlick et al., 1993). These adhesion molecules, found on various cells, including endothelial, epithelial, hematopoietic, adipocyte, neuronal, or muscular cells, indicate their complex interactions and diverse roles in health and disease. Expression level, clustering, and activation state are crucial for their adhesive function (Cartron & Elion, 2008; Telen, 2000).

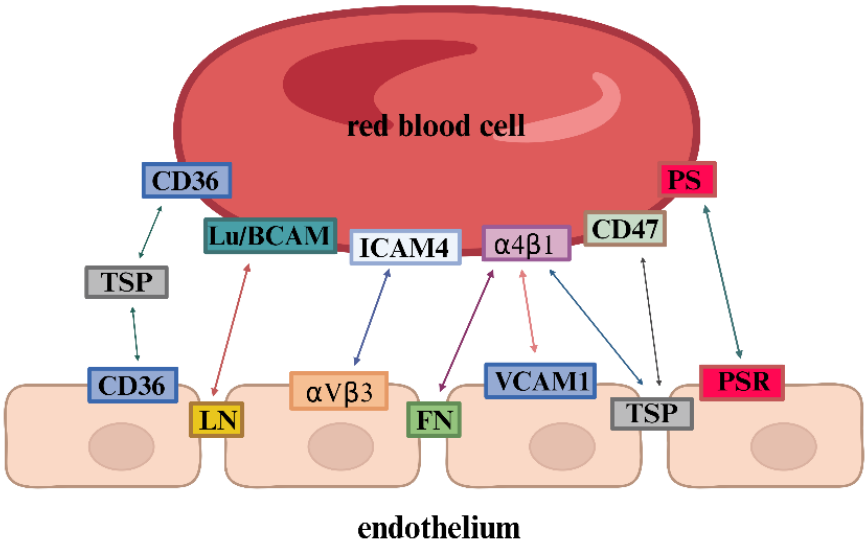


Figure 1.3 Adhesive interactions between RBC and endothelial cell (Produced by BioRender).

SCD was the first disease to discover abnormal RBC adherence with the adhesion molecules in RBC, including α4β1, Lu/BCAM, and intercellular adhesion molecule (ICAM)-4. These abnormal interactions between sickle cell RBCs and the endothelium can be triggered by the activation of β2 adrenergic receptors (β2AR) by epinephrine, which is the stress hormone. The intracellular PKA pathway is activated by this stimulation. β2ARs, which are typical G-coupled receptors, signal partly through the activation of stimulatory GTP-binding proteins (Gs proteins). These proteins activate AC, resulting in the cAMP production and subsequent PKA activation (Zennadi et al., 2012). It is demonstrated that Lu/BCAM which is a receptor for laminin is the most important receptor providing hemoglobin S (HbS, occurring with a mutation causing valine to substitute glutamic acid at beta globin) RBC adhesion to laminin under flow and static conditions, which is upregulating by PKA, downstream of β2 adrenergic receptors (Bartolucci et al., 2010). Very late antigen-4 (VLA-4), also known as α4β1 integrin, is a member of the integrin family binding to the vascular cell adhesion molecule (VCAM)-1, the extracellular matrix protein fibronectin, and thrombospondin (TSP) on endothelium (Figure 1.3). It is found that expression of VLA-4 is increased highly on circulating HbS RBCs while their expression is lost during RBC maturation (Gee & Platt, 1995).

More recently, in diabetic mellitus, increased red blood cell (RBC) adhesion correlates with the vascular complications severity, where glycated RBC band 3 interacts with the endothelial receptor for advanced glycation end products (RAGE), leading to endothelial dysfunction (Grossin et al., 2009). In PV, the most common myeloproliferative disorder, continuous phosphorylation of RBC Lu/BCAM increases adhesion to the laminin (Wautier et al., 2007). RVO, causing permanent vision loss, is seen in over 25% of patients with spontaneous erythroid precursor growth, enhanced RBC adhesion, correlated to the expression of phosphatidylserine (PS) on RBCs (Wautier et al., 2011). Blocking the PS receptor reduces RVO RBC adhesion, indicating the involvement of PS endothelial cell receptors (Wautier & Wautier, 2013).

Increased RBC adhesion to the endothelium can disrupt circulation by altering flow patterns in large vessels or causing direct blockages in microcirculation and capillaries under pathological disruptions associated with thrombotic conditions. Patients with beta thalassemia also have been seen to have circulatory irregularities, which can be characterized by stroke, transient ischemic episodes, venous and arterial thrombosis, and microcirculatory blockage that results in the development of chronic leg ulcers (Michaeli et al., 1992; Wong et al., 1990). Patients with beta thalassemia diseases have RBCs with altered flow characteristics, leading to microcirculatory problems. These include increased RBC aggregability in BTM (Chen et al., 1996), much higher RBC adherence to EC in BTM, and even higher RBC adherence to EC in BTI (Hovav et al., 1999), along with decreased deformability, which has been found to be connected with anemia severity (Ramot et al., 2008). However, underlying mechanism and molecules that play roles in the RBC adhesion for beta thalassemia patients are not fully explained.

1.5 Evaluation of the red blood cell flow properties

Most relevant studies to evaluate red blood cell flow properties have been conducted under in vitro conditions. The range of in vitro techniques available for characterizing the rheological properties of RBCs is extensive, including methods such as laser ektacytometry, laser tweezers, atomic force microscopy, and microfluidics (Barabino et al., 2010; Guizouarn & Barshtein, 2020).

Traditionally, RBC membrane viscoelastic mechanical properties, including deformability, volume loss, and rupture, have been evaluated using micropipette aspiration (Baskurt & Meiselman, 2003). This method applies external pressure to a localized area of the membrane, causing it to deform. Measuring this deformation enables determining the membrane's elastic modulus by the Law of Laplace. Nevertheless, the context dependent mechanics of the RBC are not taken into consideration by micropipette aspiration. Therefore, more advanced techniques, such as ektacytometry, are required to measure the mechanical response of RBC to shear stress (Jani et al., 2020). Ektacytometry gives an overall measurement, but its capability to assess the RBC deformability of individual cells is restricted (Parrow et al., 2018). Optical tweezers and atomic force microscopy offer increased spatial resolution by mapping stress along the RBC membrane.

Later, flow chambers with gaskets featuring inlet, outlet, and vacuum ports, combined with glass slides coated with biomolecules or endothelial cells are used to determine the interaction between RBCs and vascular endothelium by simulating physiological shear stress (1 dyne/cm^2) in blood circulation (Barabino et al., 1987; Montes et al., 2002). However, the clinical application of flow chambers is limited due to the need for complex equipment and specialized personnel. Recently, microfluidic devices have advanced the study of disease pathophysiology at molecular and cellular levels by providing insights into single-cell behavior and tissue responses, facilitating in-vitro experiments, and mimicking physiological systems (Aich et al., 2020).

1.5.1 Microfluidics for RBC adhesion

The advancements in microfluidics have played a pivotal role in investigating the RBC membrane characteristics and understanding their rheological aspects. Integrating biological research with microfluidic techniques has been crucial in exploring the biomechanical attributes of RBCs in various health conditions and diseases (Trejo-Soto et al., 2022).

RBC adhesion to the vascular endothelium is a critical precursor to enabling VOC in vivo. Nevertheless, these interactions are regulated by complex interactions between RBCs,

endothelium, platelets and leukocytes in addition to the adhesion proteins expression on activated RBCs and endothelial cells. To distinguish the role of interactions between RBCs, endothelium, platelets, WBCs, and immune cells in the pathobiology of diseases including SCD, two main methods for modifying the microfluidic devices' channels have been developed: (1) using endothelialized channels, and (2) using functionalized channels with adhesion molecule (Aich et al., 2020).

Microfluidic models with endothelialized microchannels enable the replication of in vivo endothelial barrier function and a detailed examination of the interactions between blood and endothelium within a more physiological microenvironment than previously possible. These platforms offer precise control over geometry, inputs including media or whole blood, and the shear forces applied to the endothelium. Moreover, they enable low-interference, high-resolution, real-time observation of cell-cell interactions by using transparent substrates. Additionally, because these devices are designed to avoid the interspecies variations seen in animal models, they have the potential to complement one another and reduce reliance on animal models, which would eventually lower costs and simplify ethical issues (Hesh et al., 2019).

A microscale approach for investigating key biophysical phenomena, specifically the adhesion and deformation of each RBC within the microvasculature, relevant to SCD and potentially other disorders, is presented in this thesis. The microfluidic chip presented here, designed for single use and disposability, is straightforward and cost-effective while still enabling detailed analysis of single-cell biophysical properties. The results discussed in this thesis lay the foundation for a method that could help beta thalassemia patients by offering a functional blood test in a variety of resource-constrained settings through a new, clinically adoptable microfluidic technology.

Hypothesis

RBC adhesion and deformability are altered in beta thalassemia patients, defining the clinical status and the requirements for the blood transfusion. In addition, RBC deformability is modulated by AC-cAMP-PKA signaling pathway of RBCs, and RBC adhesion is mediated by abnormal expression of adhesion molecules both on endothelium and RBCs and enhanced by signaling molecules of AC-cAMP-PKA signaling pathway.

Aims

Our study aims to examine RBC adhesion and deformability in beta thalassemia patients and determine the degree of adhesion under flow conditions using a microfluidic chip functionalized with endothelial cells and different extracellular matrix protein. In addition, we aim to examine the changes in RBC adhesion by blocking or activating AC-cAMP-PKA signaling pathway.

Objectives

- Fabrication of the microfluidic chip, and functionalization of the microchannels using endothelial cells, fibronectin and laminin.
- Blood collection from beta thalassemia patients and healthy subjects, and their preparation.
- Measurement of RBC deformability.
- Determination of the degree of RBC adhesion in functionalized microchannels.
- Examination of the changes in RBC adhesion in functionalized microchannels by targeting AC-cAMP-PKA signaling pathway.
- Measurement of plasma hemoglobin.

Chapter 2

MATERIALS AND METHODS

2.1 Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs grown in Dulbecco's Modified Eagle Medium (DMEM) High Glucose with Pyruvate supplemented with 10% fetal bovine serum (FBS, Sigma, F7524) and 1% Penicillin/Streptomycin (P/S, Gibco, 10000 U/ml) were used at passages 10 to 16. After reaching 70-80% confluency, the trypan blue exclusion test was made to assess the viability of cultured HUVECs microscopically.

2.2 Fabrication of the microfluidic chip

The microfluidic chip was fabricated with a glass surface using a lamination technique by mimicking the physiological conditions of the microcirculation as previously designed (Akkaya et al., 2019; Alapan et al., 2016; Kucukal et al., 2018). For this, a laser micro-machined (VersaLASER, Universal Laser Systems Inc., Scottsdale, AZ) double-sided adhesive (DSA, iTapestore, Scotch Plains, NJ) layer was placed in between a top polymethyl methacrylate (PMMA, McMaster-Carr, Elmhurst, IL) cover and a bottom microscope glass slide coated with 3-aminopropyl triethoxysilane (APTES) (Electron Microscopy Sciences, Hatfield, PA) to obtain desired microchannel geometry (4 mm width and 25 mm length) (Figure 2.1.A). The thickness of the DSA layer determined the microchannel height as approximately 50 μm. As final, the microfluidic chip had a

uniform flow domain that mimics the size scale of post-capillary venules with inlet and outlet holes, having tubing adhered with epoxy (Figure 2.1.B). The microfluidic chips were designed to be used once and then discarded, owing to an economical approach that utilized commonly accessible materials and manufacturing techniques. This disposable use of the microfluidic chips provided avoids the risk of cross-contamination between different blood samples.

A B

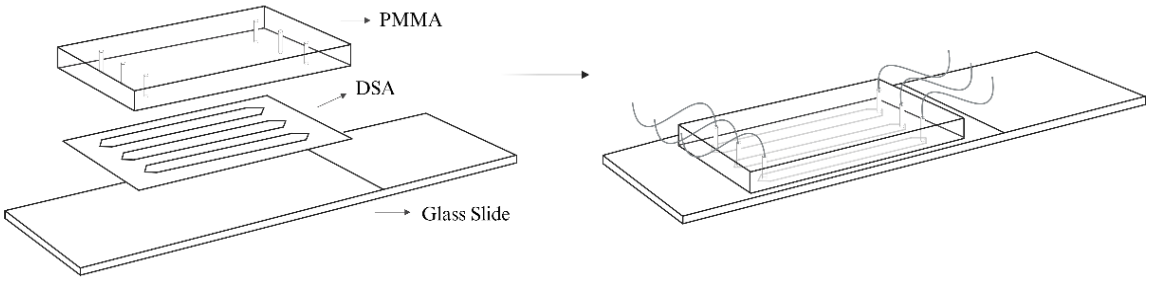


Figure 2.1 The microfluidic chip (**A**) The assembly of the microfluidic chip consisted of a top polymethyl methacrylate (PMMA) cover featuring micromachined inlets and outlets, a double-sided adhesive (DSA) layer that determines the shape and height of the microchannels, and a glass slide base (4 mm × 25 mm × 0.05 mm (width × length × height)). (**B**) The assembled microfluidic chip with inlet and outlet tubing (GH06419-00 Cole Parmer tubing) (Produced by BioRender).

2.3 Surface chemistry of the microfluidic chip

After assembly, the microchannels were washed with phosphate buffered saline (PBS) and 100% ethanol twice, respectively. Then, a cross-linker agent N-g-Maleimidobutyryloxy succinimide ester (GMBS, Thermo Fisher, Waltham, MA, USA) stock solution was prepared by dissolving 25 mg of GMBS in 0.25 ml dimethyl sulfoxide (DMSO), and this stock solution was then diluted with 100% ethanol to produce a working solution with a concentration of 0.28% v/v GMBS. After the washing step, the GMBS working solution was injected into the microchannels and incubated for 20 min at room temperature to obtain the surface chemistry inside the microchannels (Figure 2.2), followed by washing twice with 100% ethanol and PBS, respectively.

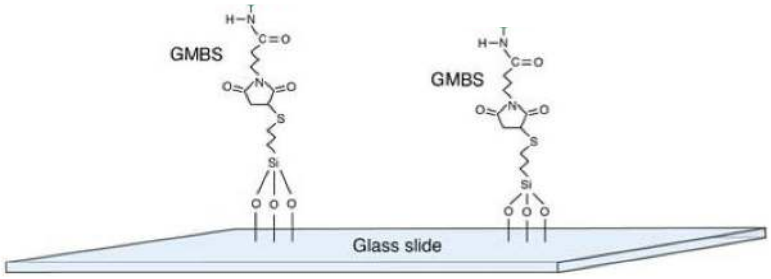


Figure 2.2 Surface chemistry inside the microchannels (Akkaya et al., 2019).

2.4 Functionalization of the microchannels with fibronectin

After the last washing, fibronectin (FN, fibronectin from human plasma, Sigma Aldrich, St Louis, Mo) solution was injected into the microchannels at a concentration of 0.1 mg/ml (in PBS) and incubated for 1 hour at 37ºC to obtain the immobilization of protein on the GMBS-functionalized surface (Figure 2.3).

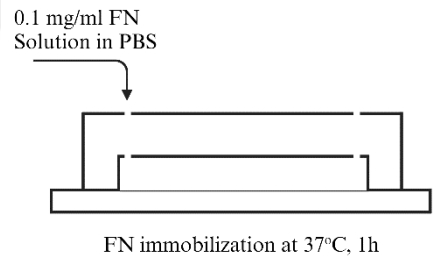


Figure 2.3 Experimental methodology for functionalization with fibronectin (Produced by BioRender).

2.5 Functionalization of the microchannels with laminin

After the last washing, laminin (LN, laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, Sigma Aldrich, St Louis, Mo) solution was injected into the microchannels at a concentration of 0.1 mg/ml (in PBS) and incubated for 1.5 hour at room temperature (RT) to obtain the immobilization of protein on the GMBS-functionalized surface (Figure 2.4).

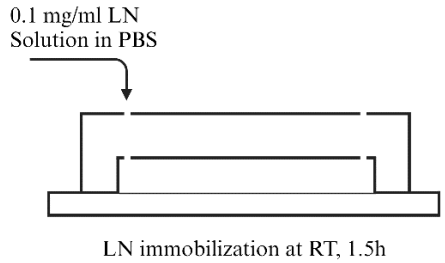


Figure 2.4 Experimental methodology for functionalization with laminin (Produced by BioRender).

2.6 Functionalization of the microchannels with HUVECs

To functionalize microchannels with HUVECs, HUVECs were seeded into fibronectin-coated microchannels at a density of $8x10^6$ cells/ml and incubated for 24 hours at 37°C and 5% CO_2 to allow cell attachment and spreading. The culture medium was replaced in the microchannels every 4 hours (Figure 2.5).

2.7 Activation of the HUVECs in the microchannels

After 24 hour incubation of HUVECs in microchannels, the HUVECs in the microchannels were activated with 40 μM TNF-α or 20 μM and 40 μM Heme for 6 hours to increase the expression of VCAM-1 adhesion molecule (Kong et al., 2018; Wagener et al., 1997) (Figure 2.5).

Tumor necrosis factor alpha (TNF-α, Sigma Aldrich, St Louis, Mo) was prepared by diluting stock TNF-α solution with DMEM High Glucose with Pyruvate (FBS and antibiotics-free) to the final working concentration of 40 μM.

40 mM Heme stock solution was prepared by dissolving bovine hemin (Sigma Aldrich, St Louis, Mo) in 0.1 M NaOH solution. Then, the stock solution was diluted using DMEM High Glucose with Pyruvate (FBS and antibiotics-free) to the final working concentrations of 20 and 40 μM, followed by adjusting the pH of both Heme solutions with 0.1 M HCl to the pH of the human body ranges between 7.35 to 7.45.

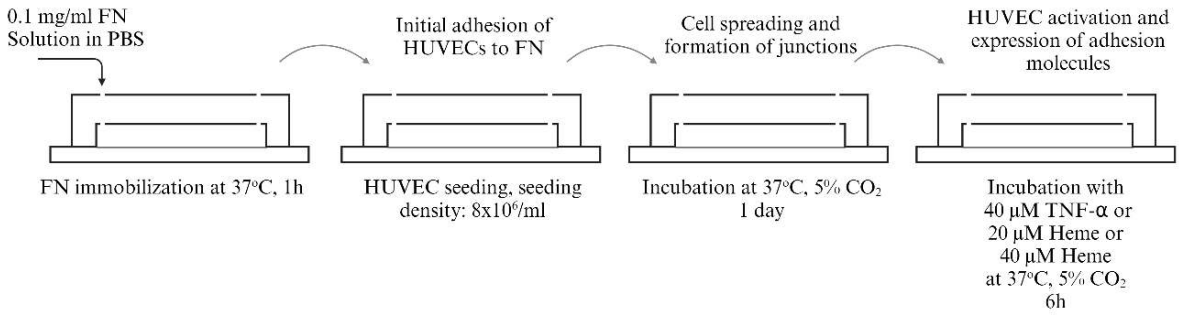


Figure 2.5 Experimental methodology for functionalization with HUVECs (Produced by BioRender).

2.8 Immunofluorescence Staining and Imaging

Following activation of HUVECs with 40 µM TNF-α or 20 µM and 40 µM Heme, all the non-activated and activated HUVECs seeded into the microchannels were fixed with 4% PFA for 20 minutes at room temperature. After the fixation process was completed, fixed HUVECs were washed three times for 5 minutes with PBS and incubated with PBS+ solution (contained PBS, 0.1% Tween20, 1% BSA, 22,52 mg/ml glycine) for 30 min to block unspecific binding of antibodies. After blocking, HUVECs were incubated with the diluted VCAM-1 antibody (1:250, Recombinant Anti-VCAM1 antibody [EPR5047] (ab134047)) in 1%BSA in PBST (contains PBS, 0.1% Tween20) overnight at 4ºC. Then, HUVECs, whose primary antibody incubation was completed, were washed three times for 5 minutes with PBS and incubated with secondary antibody (10 µg/ml, Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488, Invitrogen A32731) in PBS- solution (contained 1% BSA) for 1 hour at room temperature in the dark. After the secondary antibody incubation, HUVECs were washed three times for 5 minutes with PBS, and cell nuclei were stained with DAPI for 20 minutes in the dark. Fluorescent images of labeled VCAM-1 adhesion molecules were obtained at multiple locations under appropriate channels using Live Cell Microscopy (Leica, DMi8).

2.9 Subjects and blood sample collection

Peripheral blood samples were collected from patients diagnosed with beta-thalassemia (beta-thalassemia major and/or beta-thalassemia intermedia) before blood transfusion

followed in the Hematology Polyclinic of Istanbul University, Istanbul Faculty of Medicine and Pediatric Hematology Polyclinic of Hacettepe University Hospital, under the Ethics Committee of Koç University School of Medicine (2023.229.IRB2.049). As the control group, individuals who are not beta-thalassemia patients, do not have hemoglobinopathy, and whose total hemoglobin (Hb) and plasma iron levels are within the normal reference range were included, considering the same age range and gender ratio as the patient group. Clinical phenotypes of individual patients are shown in Table 3.1 and Table 3.2. All blood samples from subjects were collected in 9 ml ethylenediaminetetraacetic acid (EDTA)-containing tubes.

2.10 Blood sample preparation and blood perfusion into the microchannels

After blood sample collection, the blood sample was centrifuged at 500g for 10 minutes at room temperature to isolate RBCs, ensuring they were free from EDTA and plasma containing cytokines. The reason of this was that EDTA and patient cytokines affect endothelial cell adhesion negatively. Then, isolated RBCs were washed with PBS three times and resuspended in fresh basal cell culture medium (DMEM high glucose with pyruvate) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at a hematocrit between 20% and 25% (Figure 2.6).

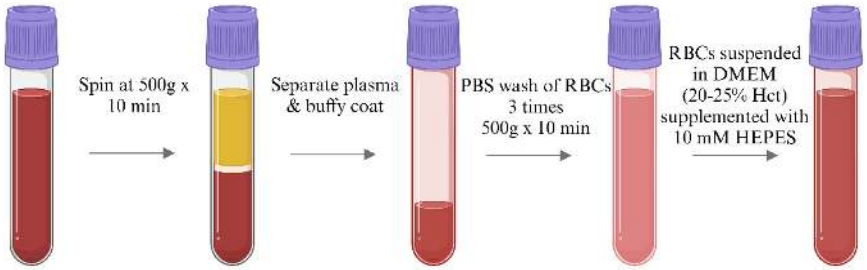


Figure 2.6 Experimental methodology for blood sample preparation (Produced by BioRender).

Before blood perfusion, microchannels were rinsed with HEPES buffered basal cell culture medium. Then, 1 ml syringes containing RBCs suspension were attached to the inlet lines of the microfluidic chip and the sample-containing syringes were placed on a multichannel syringe pump (Legato® 210/210P Syringe Pump, KD Scientific Inc.,

Holliston, MA). Thereafter, the samples were perfused into the microchannels functionalized with activated and non-activated HUVECs, fibronectin and laminin at a flow rate of 1 dyne/cm^2 for a total of 15 µl (Figure 2.7), and the microchannels were rinsed with HEPES-buffered basal cell culture medium at a rate of 1 dyne/cm^2 for a total of 180 µl after blood sample perfusion to remove unbound RBCs. Finally, image analysis for RBCs adhesion into the microchannels was made by scanning 32 mm^2 field of view in the middle of the microchannel under 10x objectives using an inverted microscope (Nikon Eclipse Ts2 Inverted Biological Microscope).

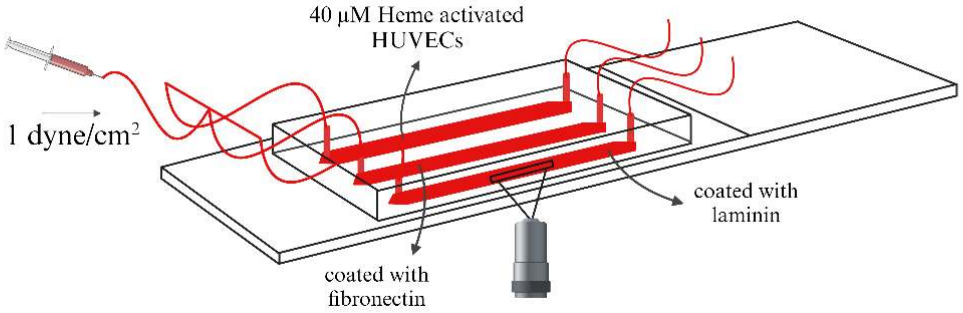


Figure 2.7 Experimental methodology for blood perfusion into the microchannels (Produced by BioRender).

2.11 Treatment of blood samples with Forskolin and SQ22536, and blood perfusion into the microchannels

After blood sample collection, whole blood samples were incubated with Forskolin (final concentrations in blood: 10 µM) and SQ22536 (final concentrations in blood: 100 µM) (Sigma-Aldrich, St. Louis, MO, USA) at 37ºC for 15 min for the stimulation and the inhibition of adenylyl cyclase, respectively. Forskolin and SQ22536 were prepared as stock solutions in DMSO (dimethyl sulfoxide, Sigma-Aldrich, USA) according to supplier's recommendations, and then they were diluted in PBS before the administration with blood samples. The chemical effect was examined based on the vehicle (PBS and DMSO) used in their preparation. The vehicle was applied to the whole blood sample and incubated under same conditions as the chemical agent, including volume (v/v), duration, and conditions.

Then, the blood samples incubated with drug was centrifuged at 500g for 10 minutes at room temperature to isolate RBCs, and isolated RBCs were washed with PBS three times and resuspended in fresh basal cell culture medium (DMEM high glucose with pyruvate) supplemented with 10 mM HEPES at a hematocrit between 20% and 25%.

Before blood perfusion, microchannels were rinsed with HEPES-buffered basal cell culture medium. Then, 1 ml syringes containing RBCs suspension were attached to the inlet lines of the microfluidic chip and the sample-containing syringes were placed on a multichannel syringe pump. Thereafter, the samples were perfused into the microchannels functionalized with 40 μM Heme activated HUVECs and fibronectin at a flow rate of 1 dyne/cm^2 for a total of 15 μl, and the microchannels were rinsed with HEPES-buffered basal cell culture medium at a rate of 1 dyne/cm^2 for a total of 180 μl after blood sample perfusion to remove unbound RBCs. Finally, image analysis for RBCs adhesion into the microchannels was made by scanning 32 mm^2 field of view in the middle of the microchannel under 10x objectives using an inverted microscope.

2.12 RBCs Deformability Measurements

After blood sample collection, the hematocrit level of whole blood samples was adjusted to 40%, and blood samples with adjusted hematocrit were incubated with Forskolin (final concentrations in blood: 10 μM), SQ22536 (final concentrations in blood: 100 μM) and their vehicles at 37ºC for 15 min. Then, 12.5 μl of the blood sample was diluted with 2.5 ml iso-osmolar polyvinylpyrrolidone (PVP) solution (360 kDa, 29.9 ± 0.5 mPa s, Mechatronics, Netherlands), and applied into the measuring chamber between the two cylinders of the LORRCA MaxSis (the laser-assisted optical rotational cell analyzer, Mechatronics, Netherlands) (Figure 2.8). These two cylinders of the laser integrated device are the rotating and static cylinders made of glass, generating shear stresses. After applying blood into the measuring chamber, RBC deformability was evaluated by the LORRCA MaxSis at 37°C by applying 10 different shear stresses which are 0.30, 0.53, 0.94, 1.65, 2.91, 5.15, 9.09, 16.04, 28.32, and 50 Pa (Hardeman et al., 1994). After measurement, an Elongation Index (EI) was obtained from the diffraction pattern generated by a diode laser integrated into the inner cylinder and transversed through the blood sample, and it was projected on a screen recorded by a CCD-video camera of the

LORRCA. The Elongation Index (EI) was determined using an integrated computer with the formula EI = (a − b)/(a + b), where “a” represents the vertical axis and “b” represents the horizontal axis of the diffraction pattern.

Deformability curves were plotted by measuring the Elongation Index (EI) against applied shear stress (SS) levels. Key parameters, EI_{max} (maximal RBC elongation at infinite shear stress) and $SS_{1/2}$ (shear stress required for half-maximal deformation), were determined using the Lineweaver-Burke model (Baskurt et al., 2009). $SS_{1/2}$ serves as an index of RBC deformability, while the $SS_{1/2}$:EI_{max} ratio normalizes this measure (Baskurt & Meiselman, 2013). Deformability measurements, conducted at 37°C, were taken before and immediately after the physiological shear stress (5 Pa) application for 300 seconds to blood sample.

Figure 2.8 LORRCA MaxSis (the laser-assisted optical rotational cell analyzer, Mechatronics, Netherlands (Hardeman et al., 1994).

2.13 Plasma Hemoglobin Measurement

The hemoglobin concentration of donors’ plasma sample was determined using Drabkin’s Assay (Whitehead et al., 2019). To make Drabkin’s Solution, one vial of Drabkin’s Reagent (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1000 ml of distilled water. Then, 0.5 ml of 30% Brij L23 Solution (Catalog Number B4184) was added to the 1000 ml of prepared Drabkin’s Reagent and mixed well. After that, 10 mg/ml hemoglobin standard solution was prepared by adding 0.01 gr Hb (Human hemoglobin (Sigma-Aldrich)) to 1 ml PBS, and working standards of Hb with different concentration were prepared by making serial dilutions with PBS. After 50 µl of each working standards were mixed with 200 µl Drabkin’s Solution in 96 well plate, the plate was put at dark for 15

min and the absorbance values were read at 540 nm. A calibration curve was plotted with absorbance values versus hemoglobin concentration (mg/ml). Then, 50 µl plasma sample of each patient was mixed with 200 µl Drabkin in 96 well plate, the plate was put at dark for 15 min and the absorbance values were read at 540 nm. The total hemoglobin concentration (mg/ml) of plasma sample of each patient was determined directly from the calibration curve.

2.14 Statistical Analysis

Data obtained in each experiment were presented as mean ± standard deviation unless otherwise stated. Data were tested for normality with the D'Agostino and Pearson omnibus normality test, and correlation analysis was made with nonparametric Spearman correlation. Comparison among groups was performed using one-way analysis of variance (ANOVA)/Kruskal–Wallis/Dunn's multiple comparison tests together with two-way ANOVA test. All statistical analyses were performed using the GraphPad Prism 10.0.3 statistical software (GraphPad Software, Inc., San Diego, CA, USA).

2.15 Study Summary

Our study is focused on the determination of RBC deformability and adhesion for beta thalassemia patients. RBC adhesion was examined under flow conditions using microfluidic chip functionalized with endothelial cells, fibronectin, and laminin. The effects of the activation or inhibition of AC-cAMP-PKA pathway on RBC deformability and adhesion was also determined (Figure 2.9).

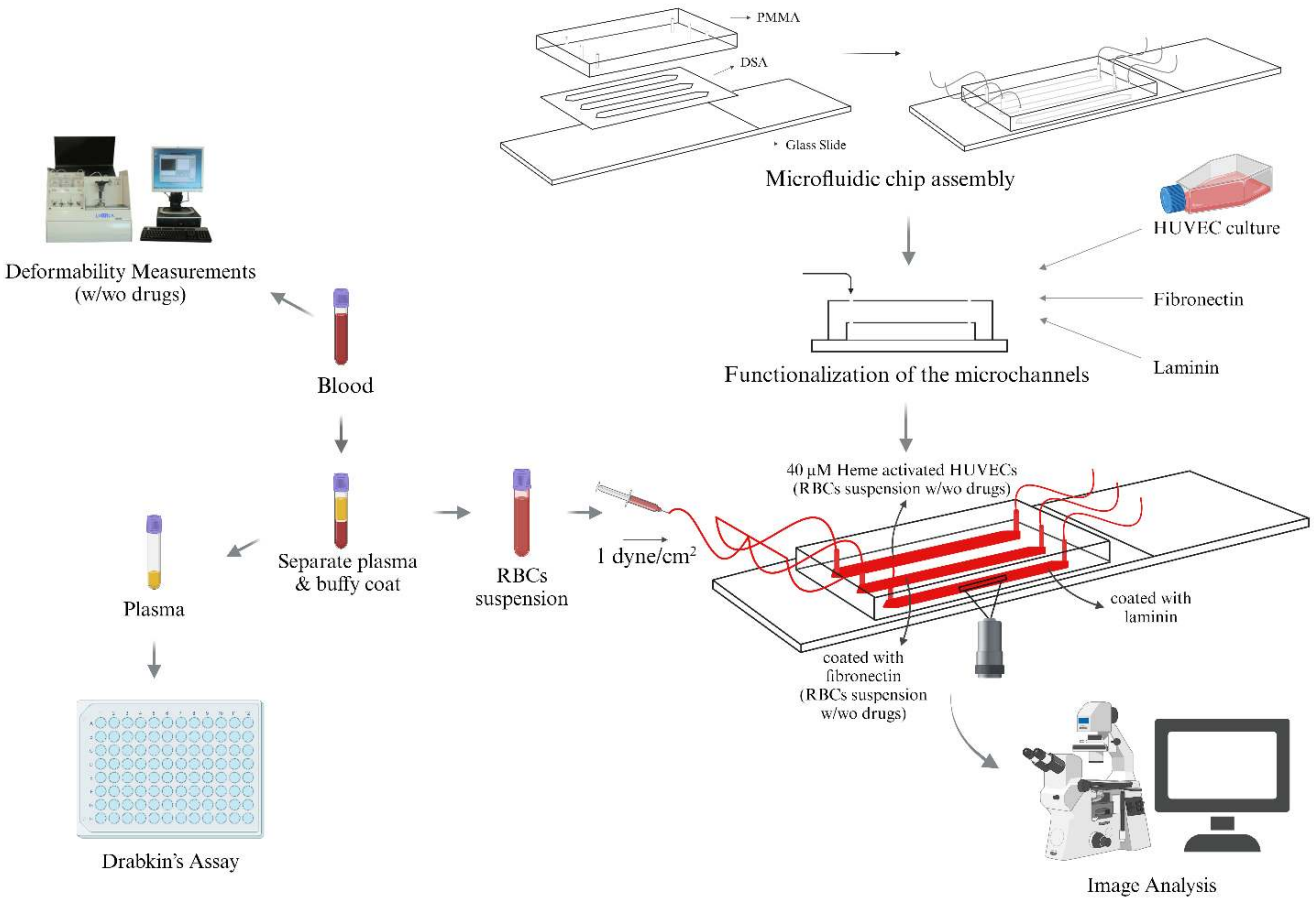


Figure 2.9 The study design (Produced by BioRender).

Chapter 3

RESULTS

3.1 HUVEC Culture

In T75 flask, HUVECs were seeded and checked regularly and when they reached sufficient confluency, they were repassaged (Figure 3.1).

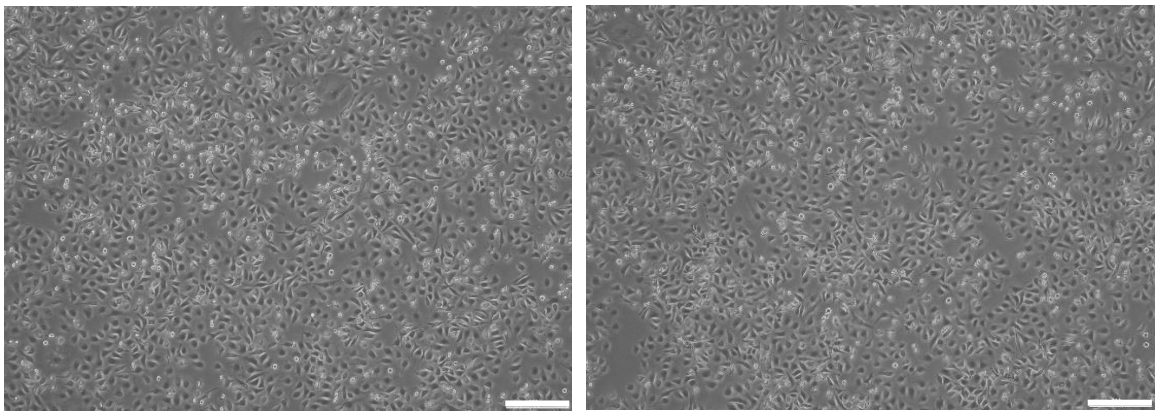


Figure 3.1 Morphological appearance of HUVEC. The image was taken by inverted phase contrast microscopy at 4X magnification (Scale bar = 300 μm).

3.2 Functionalization of microchannels with HUVECs

Microchannels were functionalized with HUVECs at a density of $8x10^6$ cells/ml and incubated for 24 hours at 37°C and 5% CO2 to allow cell attachment and spreading. Images showing their morphological appearance in microchannels were obtained by inverted phase contrast microscopy (Figure 3.2).

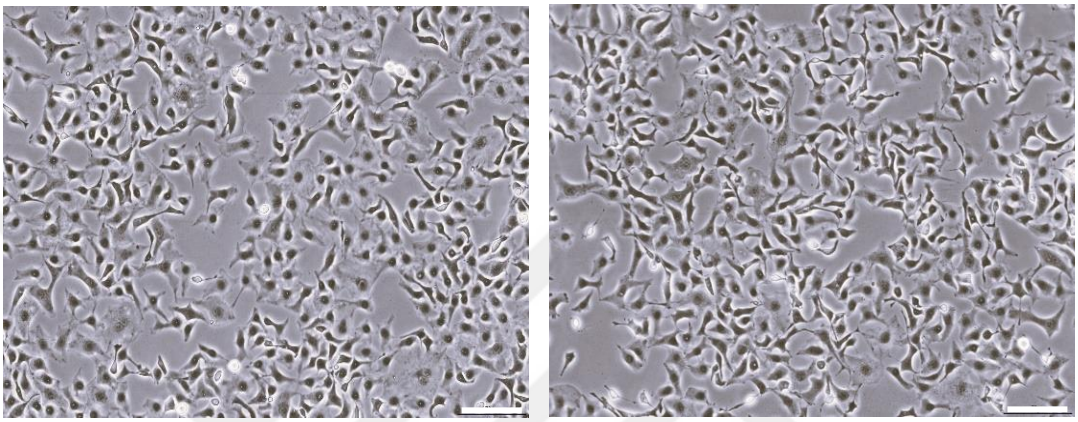


Figure 3.2 Morphological appearance of HUVEC in microchannels. The image was taken by inverted phase contrast microscopy at 10X magnification (Scale bar=100 μm).

3.3 Expression of VCAM-1 on activated HUVECs in microchannels

After 24 hours incubation of HUVECs in microchannels, the HUVECs in the microchannels were activated with 40 μM TNF-α (Figure 3.3, Figure 3.4) or 20 μM and 40 μM Heme (Figure 3.5) for 6 hours to increase the expression of VCAM-1 adhesion molecule.

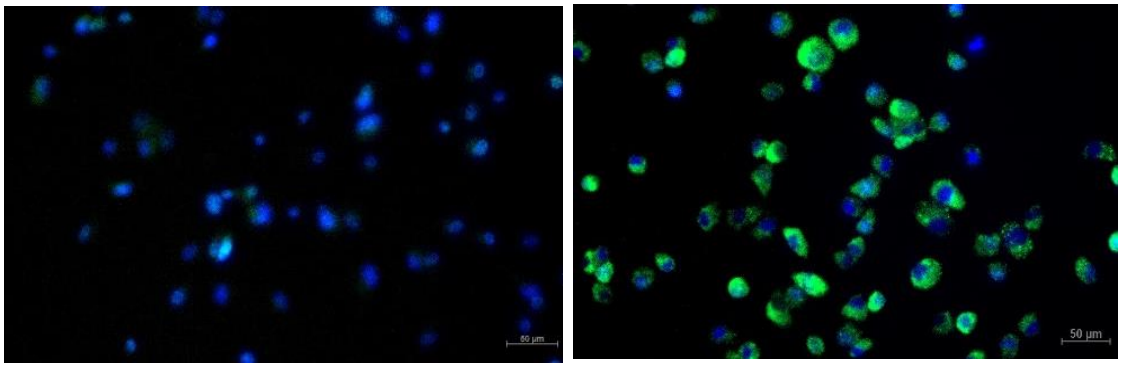


Figure 3.3 Immunofluorescence staining of VCAM-1 (green) in nonactivated (left) and 40 μM TNF-α activated (right) HUVECs at 20X (Scale bar=50 μm) magnification. DAPI (blue) was used for nuclear staining.

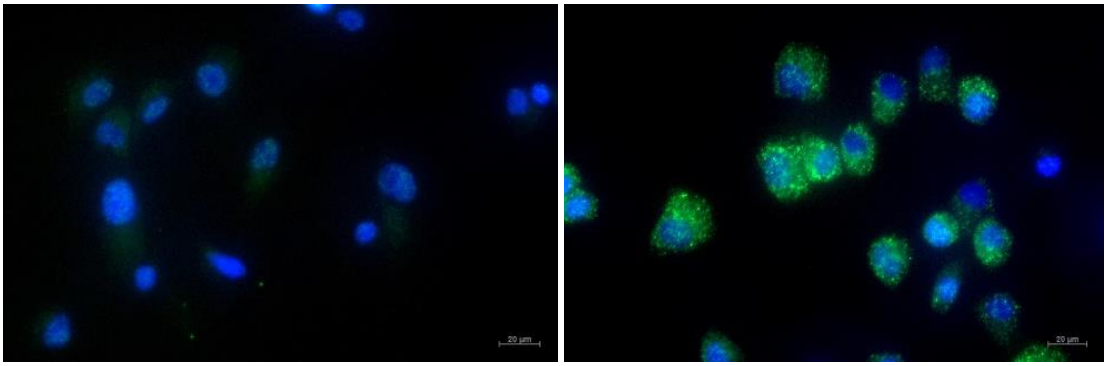


Figure 3.4 Immunofluorescence staining of VCAM-1 (green) in nonactivated (left) and 40 μM TNF-α activated (right) HUVECs at 40X (Scale bar=20 μm) magnification. DAPI (blue) was used for nuclear staining.

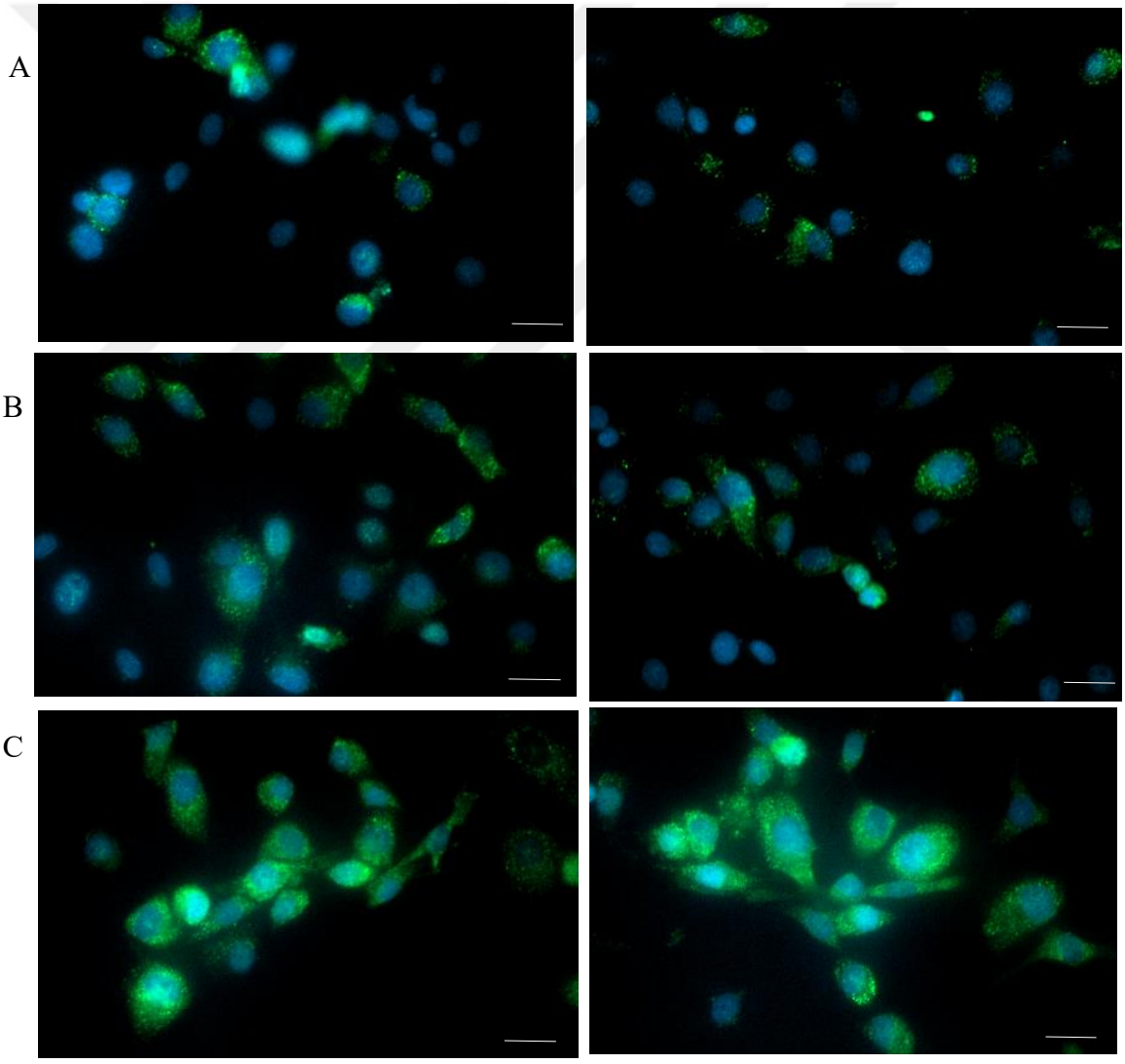


Figure 3.5 Immunofluorescence staining of VCAM-1 (green) in nonactivated (A) and 20 μM (B) and 40 μM (C) Heme activated HUVECs at 40X (Scale bar=20 μm) magnification. DAPI (blue) was used for nuclear staining.

Heme activated HUVECs were chosen to study RBC adhesion for beta thalassemia patients due to mimicking disease conditions in microchannels, and 40 μM Heme concentration was selected due to the increased expression level of VCAM-1 as seen Figure 3.5.

3.4 Study population

Two female and two male beta thalassemia intermedia patients between the ages of 29 and 42 (mean: 34) (Table 3.1), and ten female and twelve male beta thalassemia major patients between the ages of 8 and 42 (mean: 22,86) (Table 3.2) were included in the study. Their first transfusion year, frequency of transfusion, whether a splenectomy has been performed, and the active ingredients of the drugs that patients use were recorded. While splenectomy had been performed for one patient in beta thalassemia intermedia patients, there were five people in beta thalassemia major patients. It has been observed that most of the drugs used by patients are iron chelators such as DFX and DFP.

Table 3.1 Clinical phenotype of beta thalassemia intermedia patients included in this study.

	Birth Year	**Sex**	**Splenectomy (Yes/No)**	**Active ingredients of the drugs**
Patient 1	1982	M	NO	Mitapivat
Patient 2	1989	F	NO	Folic Acid, Vitamin D3, Allopurinol
Patient 3	1995	F	YES	Deposilin - Once in a week
Patient 4	1994	M	NO	Fludrocortisone, Prednisolone, Folic Acid

Table 3.2 Clinical phenotype of beta thalassemia major patients included in this study.

	Birth Year	**Sex**	**First Transfusion Year**	**Frequency of Transfusion**	**Splenectomy (Yes/No)**	**Active ingredients of the drugs**
Patient 1	1999	M	1999	Once in 3 weeks	YES - 2011	DFX
Patient 2	2012	M	2012	Once in 3 weeks	NO	DFX
Patient 3	1990	M	1990	Once in 3 weeks	NO	DFX
Patient 4	2013	M	2019	Once in a month	NO	Enalapril maleate, DFX, Folic Acid
Patient 5	2009	F	2011	Once in 3 weeks	NO	DFP, Folic Acid, Zinc
Patient 6	2015	F	2015	Once in 3 weeks	NO	DFX
Patient 7	1984	M	1985	Once in a month	YES - 2000	DFX, Zinc, Folic Acid, Bvit, Dvit, Prednisolone
Patient 8	2006	M	2006	Once in a month	NO	DFX, Folic Acid, Evit, Dvit
Patient 9	2003	F	2004	Once in 3 weeks	NO	DFP, Zinc, Folic Acid, Dvit, Bvit
Patient 10	2016	M	2019	Once in 3 weeks	NO	DFX, Dvit, Bvit
Patient 11	2012	F	2016	Once in a month	NO	DFX
Patient 12	2016	F	2017	Once in a month	NO	DFX
Patient 13	1993	F	1995	Once in 3 weeks	NO	DFX, Folic Acid, Bvit
Patient 14	2006	M	2010	Once in 3 weeks	YES - 2015	DFP
Patient 15	1987	M	1996	Once in 3 weeks	NO	DFP
Patient 16	1982	F	1983	Once in 3 weeks	YES - 1989	DFP
Patient 17	2006	F	2011	Once in 3 weeks	NO	DFX
Patient 18	2016	M	2016	Once in 3 weeks	NO	Folic Acid, DFX
Patient 19	1987	M	1987	Once in 3 weeks	YES - 2006	DFX
Patient 20	1990	M	1990	Once in 2 weeks	NO	DFX
Patient 21	1982	F	1984	Once in 3 weeks	NO	DFX
Patient 22	1998	F	1998	Once in 2 weeks	NO	DFX

Beta thalassemia major patients' hematological (RBC count, Hb amount, Hct, MCV, MCH, MCHC (RBC's hemoglobin amount in relation to its size), RDW (the size and volume variation of RBCs), WBC, Plt, and NRBC count), and biochemical parameters (LDH, albumin, Total bilirubin, ferritin) are listed in Table 3.3 and Table 3.4 respectively.

Table 3.3 Beta thalassemia major patients' hematological parameters.

	RBC	**Hb**	**Hct**	**MCV**	**MCH**	**MCHC**	**RDW**	**WBC**	**Plt**	**NRBC**
Patient 1	3,12$^-$	8,70$^-$	26,20$^-$	84,00	27,90	33,20	15,90$^+$	15,66$^+$	1118$^+$	1,38$^+$
Patient 2	3,57$^-$	9,70$^-$	30,40$^-$	85,20	27,20	31,90$^-$	14,40	11,19	348	0,15$^+$
Patient 3	2,54$^-$	6,80$^-$	19,80$^-$	78,00	26,80	34,30	23,80$^+$	7,28	228	0,27$^+$
Patient 4	3,27$^-$	8,70$^-$	25,70$^-$	78,60	26,60	33,90	21,20$^+$	7,72	333	0,03$^+$
Patient 5	3,54$^-$	9,30$^-$	28,50$^-$	80,50	26,30	32,60$^-$	14,60	7,76	313	0,14$^+$
Patient 6	3,17$^-$	8,60$^-$	26,20$^-$	82,60	27,10	32,80$^-$	12,70	7,91	468$^+$	0,02$^+$
Patient 7	3,89$^-$	9,20$^-$	29,40$^-$	75,60$^-$	23,70	31,30$^-$	30,50$^+$	13,85$^+$	725$^+$	11,75$^+$
Patient 8	3,63$^-$	8,00$^-$	26,00$^-$	71,60$^-$	22,00$^-$	30,80$^-$	27,60$^+$	8,09	335	0,48$^+$
Patient 9	2,84$^-$	7,60$^-$	23,30$^-$	82,00	26,80	32,60$^-$	13,70	5,91	306	0,10$^+$
Patient 10	3,36$^-$	9,00$^-$	27,30$^-$	81,30	26,80	33,00	15,90$^+$	9,16	453$^+$	0,01
Patient 11	5,24$^+$	10,00$^-$	34,20$^-$	65,30$^-$	19,10$^-$	29,20$^-$	27,30$^+$	9,63	450$^+$	0,28$^+$
Patient 12	4,31$^-$	8,50$^-$	28,80$^-$	66,80$^-$	19,70$^-$	29,50$^-$	30,30$^+$	7,75	200	1,01$^+$
Patient 13	3,70$^-$	10,00$^-$	31,10$^-$	84,10	27,00	32,20$^-$	23,00$^+$	11,28$^+$	551$^+$	0,02$^+$
Patient 14	3,84$^-$	9,80$^-$	30,40$^-$	79,20	25,50	32,20$^-$	18,50$^+$	11,55$^+$	708$^+$	8,34$^+$
Patient 15	3,42$^-$	9,10$^-$	28,10$^-$	82,20	26,60	32,40$^-$	17,80$^+$	13,84$^+$	807$^+$	1,76$^+$
Patient 16	4,11$^-$	10,70$^-$	33,50$^-$	81,50	26,00	31,90$^-$	20,90$^+$	8,26	324	1,86$^+$
Patient 17	2,95$^-$	7,90$^-$	22,30$^-$	75,60$^-$	26,80	35,40	19,00$^+$	8,98	470$^+$	0,42$^+$
Patient 18	2,54$^-$	6,90$^-$	19,60$^-$	77,20	27,20	35,20	12,20	4,11	119$^-$	0,25$^+$
Patient 19	3,50$^-$	9,60$^-$	27,90$^-$	79,70	27,40	34,40	17,90$^+$	19,86$^+$	527$^+$	11,71$^+$
Patient 20	2,20$^-$	6,10$^-$	17,40$^-$	79,10	27,70	35,10	14,60	5,35	114$^-$	0,11$^+$
Patient 21	2,96$^-$	8,30$^-$	23,80$^-$	80,40	28,00	34,90	14,00	3,98$^-$	265	0,03$^+$
Patient 22	2,33$^-$	6,80$^-$	19,10$^-$	82,00	29,20	35,60	14,60	7,03	269	0,14$^+$

RBC: Red blood cell (4,44-5,06x10^6/µl), Hb: Hemoglobin (13,5-16,9 g/dl), Hct: Hematocrit (35-45%), MCV: Mean corpuscular volume (76-90 fl), MCH: Mean corpuscular hemoglobin (23-31 pg), MCHC: Mean corpuscular hemoglobin concentration (33-36 g/dl), RDW: Red cell distribution width (11-15%), WBC: White blood cell (4,1-11,2x10^9/l), Plt: Platelet count (150-400x10^9/l), NRBC: Nucleated red blood cell (0.001-0.015).
($^-$ below average, $^+$ above average)

Table 3.4 Beta thalassemia major patients' biochemical parameters.

	LDH	**Albumin**	**Total Bilirubin**	**Ferritin**
Patient 1	--	--	--	--
Patient 2	176	4,34	--	--
Patient 3	212	$4,60^{+}$	$3,78^{+}$	4400^{+}
Patient 4	191	$4,88^{+}$	$1,62^{+}$	772^{+}
Patient 5	179	$4,67^{+}$	$1,36^{+}$	--
Patient 6	181	4,30	$1,23^{+}$	1801^{+}
Patient 7	196	$4,57^{+}$	$2,90^{+}$	--
Patient 8	--	$5,15^{+}$	--	--
Patient 9	--	--	--	--
Patient 10	187	$4,76^{+}$	$2,17^{+}$	1780^{+}
Patient 11	377^{+}	4,41	$2,67^{+}$	513^{+}
Patient 12	380^{+}	4,40	$2,01^{+}$	586^{+}
Patient 13	172	$4,72^{+}$	1,04	--
Patient 14	142^{-}	$5,03^{+}$	$3,82^{+}$	2042^{+}
Patient 15	178	$4,61^{+}$	--	1440^{+}
Patient 16	310^{+}	3,96	0,52	--
Patient 17	--	--	--	1306^{+}
Patient 18	267	4,29	$1,25^{+}$	--
Patient 19	--	--	--	644^{+}
Patient 20	--	--	--	2186^{+}
Patient 21	--	--	--	173^{+}
Patient 22	212	$4,89^{+}$	$3,38^{+}$	--

LDH: Lactate Dehydrogenase (150-300 U/l), Albumin (2,9-4,5 g/dl), Total Bilirubin (0,2-1,2 mg/dl), Ferritin (30-150 ng/ml).
($^{-}$ below average, $^{+}$ above average)

Beta thalassemia intermedia patients' hematological parameters (RBC count, hemoglobin amount, hematocrit (in percentage), platelet (Plt) count) and their adherent RBC number for microchannel functionalized with HUVEC are listed in Table 3.5.

Table 3.5 Beta thalassemia intermedia patients' hematological parameters and their adherent RBC number for HUVEC coated microchannel.

	RBC	**Hb**	**Hct**	**Plt**	**Plasma Hemoglobin Concentration**	**Adherent RBC Number for HUVEC Coated Microchannel**
Patient 1	$6{,}06^{+}$	$11{,}40^{-}$	38,80	217^{+}	4,26	0
Patient 2	$3{,}70^{-}$	$8{,}20^{-}$	$25{,}90^{-}$	289^{+}	2,70	0
Patient 3	$3{,}60^{-}$	$9{,}90^{-}$	$29{,}10^{-}$	754^{+}	3,45	5175
Patient 4	$3{,}59^{-}$	$9{,}80^{-}$	$27{,}90^{-}$	736^{+}	3,88	0

RBC: Red blood cell (4,44-5,06x10^{6}/µl), Hb: Hemoglobin (13,5-16,9 g/dl), Hct: Hematocrit (35-45%), Plt: Platelet count (150-400x10^{9}/l).
($^{-}$ below average, $^{+}$ above average)

3.5 Determination of the adherent RBC numbers for microchannels functionalized with endothelial cells, fibronectin and laminin

After blood perfusion, the microchannels were rinsed with HEPES-buffered basal cell culture medium, and image analysis for RBCs adhesion into the microchannels was made by scanning a 32 mm^2 field of view in the middle of the microchannel under 10x objectives using an inverted microscope.

It was observed that there were not any adherent RBCs for microchannel functionalized with non-activated HUVECs for both patient and healthy blood, and no adherent RBCs were seen for healthy donors for all microchannels functionalized with 40 µM heme activated HUVECs, fibronectin, and laminin.

Representative images of adherent RBCs for microchannels functionalized with 40 µM heme activated HUVECs (Figure 3.6), fibronectin (Figure 3.7) and laminin (Figure 3.8) were recorded.

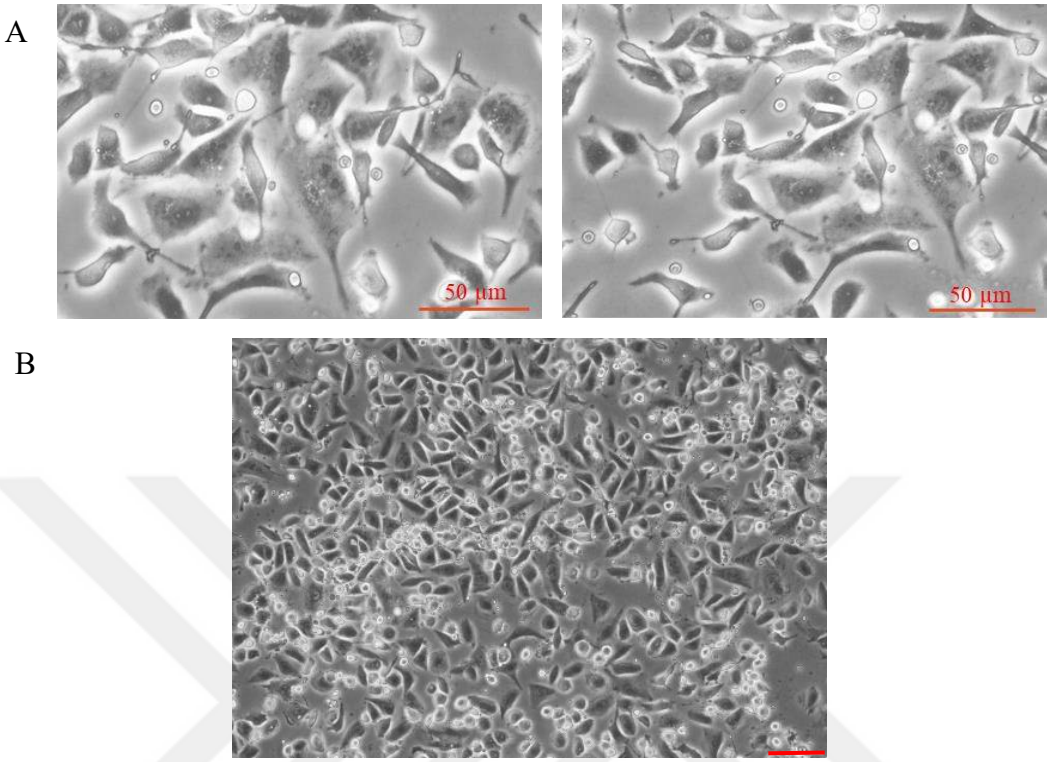


Figure 3.6 Representative images of adherent RBCs for microchannel functionalized with 40 µM heme activated HUVECs (Scale bar=50 µm (A), Scale bar=100 µm (B)).

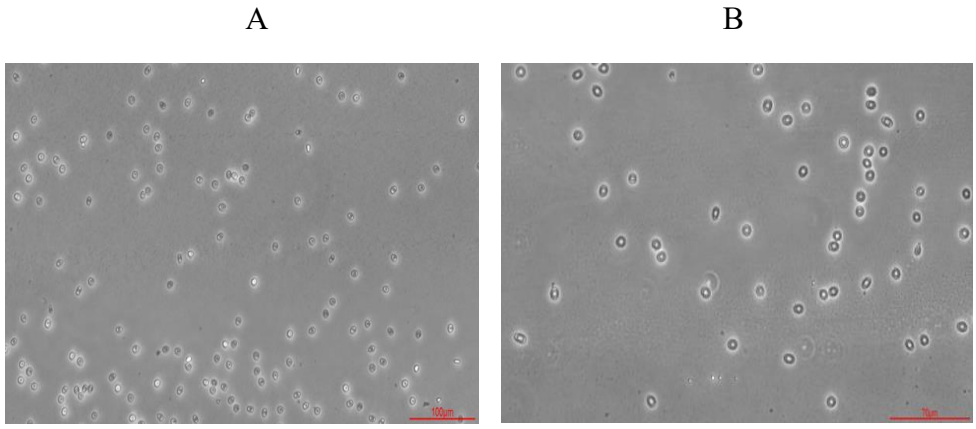


Figure 3.7 Representative images of adherent RBCs for microchannel functionalized with fibronectin (Scale bar=100 µm (A), Scale bar=70 µm (B)).

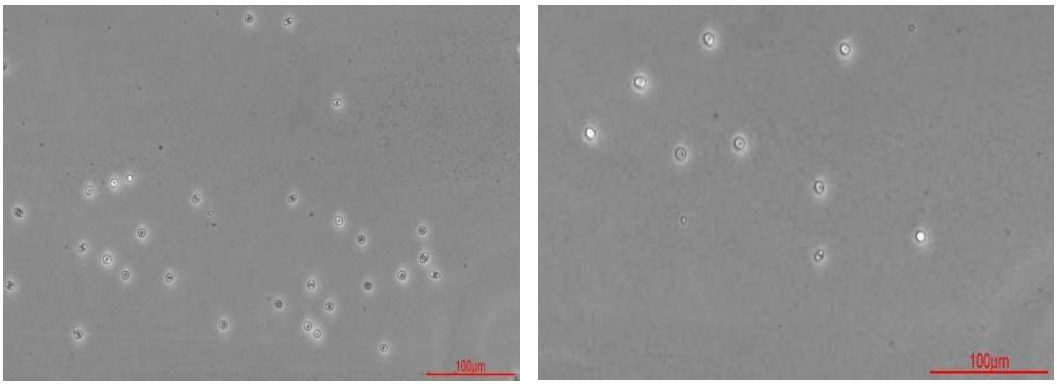


Figure 3.8 Representative images of adherent RBCs for microchannel functionalized with laminin (Scale bar=100 µm).

Adherent RBC numbers for microchannels functionalized with 40 µM heme activated HUVECs, fibronectin and laminin are determined and listed in Table 3.6 for beta thalassemia major patients. Beta thalassemia intermedia patients' adherent RBC numbers for microchannel functionalized with HUVECs are listed in Table 3.5.

For beta thalassemia major patients, 15 of 22 patients had adherent RBCs in the microchannel functionalized with 40 µM heme activated HUVECs. 19 patients were studied for microchannels functionalized with fibronectin and laminin. 11 of 19 patients had adherent RBCs for microchannel functionalized with fibronectin, while 4 of 19 patients had adherent RBCs for microchannel functionalized with laminin.

For beta thalassemia intermedia patients, four patients were studied for microchannel functionalized with 40 µM heme activated HUVECs. One of 4 patients had adherent RBCs for microchannel functionalized with 40 µM heme activated HUVECs. It was observed that this patient having adherent RBC had splenectomy, which is different than other beta thalassemia intermedia patients.

Table 3.6 Beta thalassemia major patients' adherent RBC numbers for microchannels functionalized with endothelial cells, fibronectin and laminin.

	Adherent RBC Number for HUVEC Coated Microchannel	**Adherent RBC Number for Fibronectin Coated Microchannel**	**Adherent RBC Number for Laminin Coated Microchannel**
Patient 1	2593	--	--
Patient 2	2087	--	--
Patient 3	3528	--	--
Patient 4	0	0	0
Patient 5	256	0	0
Patient 6	440	14936	2366
Patient 7	344	2467	2308
Patient 8	1783	8405	0
Patient 9	0	0	0
Patient 10	1329	30309	0
Patient 11	3001	3523	0
Patient 12	0	0	0
Patient 13	308	190	0
Patient 14	0	5790	0
Patient 15	1023	547	335
Patient 16	0	274	322
Patient 17	244	181	0
Patient 18	222	0	0
Patient 19	697	6122	0
Patient 20	6996	0	0
Patient 21	0	0	0
Patient 22	0	0	0

After obtaining all data, descriptive statistics were made for beta thalassemia major patients. Data were listed as minimum and maximum values, with their means and standard deviations (std) in Table 3.7. For each group of microchannel functionalized with 40 μM heme activated HUVECs, fibronectin, and laminin, two types of statistics were made, including patients with and without adherent RBCs.

For microchannel functionalized with 40 μM heme activated HUVECs, the minimum adherent RBC number was zero and the maximum adherent RBC number was 6996 (mean: 1130, std: 1697). When patients with zero RBC adhesion were not included, the minimum adherent RBC number was 222 and the maximum adherent RBC number was 6996 (mean: 1657, std: 1839). For microchannel functionalized with fibronectin, the minimum adherent RBC number was zero and the maximum adherent RBC number was

30309 (mean: 3829, std: 7542). When patients with zero RBC adhesion were not included, the minimum adherent RBC number was 181 and the maximum adherent RBC number was 30309 (mean: 6613, std: 9062). For microchannel functionalized with laminin, the minimum adherent RBC number was zero and the maximum adherent RBC number was 2366 (mean: 280,6, std: 732). When patients with zero RBC adhesion were not included, the minimum adherent RBC number was 322 and the maximum adherent RBC number was 2366 (mean: 1333, std: 1160).

When the data obtained for each microchannel, including patients with and without RBC adhesion, were compared, there were no significant differences. However, it was observed that there was a slight increase for RBC, hemoglobin, hematocrit, RDW, WBC, platelet, NRBC and ferritin values in the patient group with RBC adhesion compared to patient group without RBC adhesion ones for each microchannel functionalized with 40 μM heme activated HUVECs, fibronectin, and laminin. There was a slight decrease for LDH value in the patient group with RBC adhesion compared to patient group without RBC adhesion ones for each microchannel functionalized with 40 μM heme activated HUVECs, fibronectin, and laminin.

Then, correlation analysis was performed between adherent RBC number and each parameter of patient groups, with and without RBC adhesion. For each analysis, the Spearman r value and its p value were listed in Table 3.8. Significant positive correlations existed between the hemoglobin (r = 0,4751*), white blood cells (WBCs) (r = 0,7255***), platelet (r = 0,6991***) values, and the adherent RBC number of the patient group without RBC adhesion ones (Figure 3.9) for microchannel functionalized with fibronectin.

It was observed that microchannel functionalized with HUVECs and fibronectin had more adherent RBC number than microchannel functionalized with laminin. Therefore, for Forskolin and SQ22536 studies, it was decided to continue with microchannel functionalized with HUVECs and fibronectin.

Table 3.7 Descriptive statistics for beta thalassemia major patients (with and without RBC adhesion) for microchannels functionalized with 40 μM heme activated HUVECs, fibronectin, and laminin.

min-max mean ± std	Patients studied for HUVEC channel (n=22)	Patients having RBC adhesion for HUVEC channel (n=15)	Patients studied for fibronectin channel (n=19)	Patients having RBC adhesion for fibronectin channel (n=11)	Patients studied for laminin channel (n=19)	Patients having RBC adhesion for laminin channel (n=4)
Adherent RBC number	0 – 6996 1130 ±1697	222 – 6996 1657 ± 1839	0 – 30309 3829 ±7542	181 – 30309 6613 ± 9062	0 – 2366 280,6 ± 732	322 – 2366 1333 ± 1160
Age	8 – 42 22,9 ± 12,2	8 – 40 22,3 ± 11,9	8 – 42 22,9 ± 12,6	8 – 42 24,6 ± 13	8 – 42 22,9 ± 12,6	9 – 42 32 ± 15,5
Transfusion time (years)	5 – 41 21 ± 12,5	5 – 39 20,6 ± 11,9	5 – 41 20,7 ± 13	5 – 41 21,9 ± 13,3	5 – 41 20,7 ± 13	9 – 41 29,2 ± 14,7
RBC (4,44-5,06x10^6/μl)	2,2 – 5,2 3,4 ± 0,7	2,2 – 5,2 3,4 ± 0,7	2,2 – 5,2 3,4 ± 0,7	2,9 – 5,2 3,7 ± 0,6	2,2 – 5,2 3,4 ± 0,7	3,2 – 4,1 3,6 ± 0,4
Hemoglobin (13,5-16,9 g/dl)	6,1 – 10,7 8,6 ± 1,2	6,1 – 10 8,6 ± 1,2	6,1 – 10,7 8,6 ± 1,2	7,9 – 10,7 9,3 ± 0,9	6,1 – 10,7 8,6 ± 1,2	8,6 – 10,7 9,4 ± 0,9
Hematocrit (35-45%)	17,4 – 34,2 26,3 ± 4,6	17,4 – 34,2 26,3 ± 4,7	17,4 – 34,2 26,4 ± 4,6	22,3 – 24,2 28,8 ± 3,5	17,4 – 34,2 26,4 ± 4,6	26,2 – 33,5 29,3 ± 3,1
MCV (76-90 fl)	65,3 – 85,2 78,7 ± 5,2	65,3 – 85,2 78,8 ± 5,3	65,3 – 84,1 78,2 ± 5,2	65,3 – 84,1 78 ± 5,6	65,3 – 84,1 78,2 ± 5,2	75,6 – 82,6 80,5 ± 3,3
MCH (23-31 pg)	19,1 – 29,2 25,9 ± 2,6	19,1 – 27,9 25,9 ± 2,5	19,1 – 29,2 25,8 ± 2,7	19,1 – 27,4 25,3 ± 2,6	19,1 – 29,2 25,8 ± 2,7	23,7 – 27,1 25,8 ± 1,5
MCHC (33-36 g/dl)	29,2 – 35,6 32,9 ± 1,8	29,2 – 35,4 32,9 ± 1,7	29,2 – 35,6 32,9 ± 1,9	29,2 – 35,4 32,3 ± 1,7	29,2 – 35,6 32,9 ± 1,9	31,3 – 32,8 32,1 ± 0,6
RDW (11-15%)	12,2 – 30,5 19,1 ± 5,7	12,2 – 30,5 19,1 ± 5,9	12,2 – 30,5 19,3 ± 5,9	12,7 – 30,5 21 ± 5,5	12,2 – 30,5 19,3 ± 5,9	12,7 – 30,5 20,5 ± 7,5
WBC (4,1-11,2x10^9/l)	3,9 – 19,9 9,4 ± 3,8	4,1 – 19,9 10,3 ± 4,1	3,9 – 19,9 9 ± 3,8	7,91 – 19,9 11,1 ± 3,6	3,9 – 19,9 9 ± 3,8	7,9 – 13,8 11 ± 3,3
Platelet (150-400x10^9/l)	114 – 1118 428,7 ± 241	114 – 1118 468,4 ± 264	114 – 807 407,2 ± 195	324 – 807 528,9 ± 157	114 – 807 407,2 ± 195	324 – 807 581 ± 224
NRBC (0.001-0.015)	0,01 - 11,75 1,8 ± 3,7	0,01 - 11,75 1,9 ± 4	0,01 - 11,75 2 ± 3,9	0,01 - 11,75 3,3 ± 4,8	0,01 - 11,75 2 ± 3,9	0,02 - 11,75 3,8 ± 5,3
LDH (150-300 U/l)	n = 15 142 – 380 224 ± 74,8	n = 10 172 – 377 212,5 ± 64,3	n = 13 142 – 380 228,6 ± 79	n = 8 142 – 377 217,9 ± 81,1	n = 13 142 – 380 228,6 ± 79	n = 4 178 – 310 216,3 ± 63
Albumin (2,9-4,5 g/dl)	n = 16 3,9 – 5,1 4,6 ± 0,3	n = 11 4,3 – 5,1 4,6 ± 0,2	n = 14 3,9 – 5,1 4,6 ± 0,3	n = 9 3,9 – 5,1 4,6 ± 0,4	n = 14 3,9 – 5,1 4,6 ± 0,3	n = 4 3,9 - 4,6 4,4 ± 0,3
Total Bilirubin (0,2-1,2 mg/dl)	n = 13 0.5 – 3,8 2,1 ± 1,1	n = 8 1 - 3,8 2 ± 1	n = 12 0,5 – 3,8 2 ± 1	n = 7 0,5 – 3,8 2 ± 1,2	n = 12 0,5 – 3,8 2 ± 1	n = 3 0,5 – 2,9 1,5 ± 1,2
Ferritin (30-150 ng/ml)	n = 12 173 – 4400 1470 ±1136	n = 8 513 – 4400 1759 ± 1211	n = 11 173 – 2186 1204 ± 696	n = 7 513 – 2042 1361 ± 588	n = 11 173 – 2186 1204 ± 696	n = 2 1440 – 1801 1621 ± 255

Table 3.8 Correlation analysis results as the Spearman r value and its p value for each parameter (hematological and biochemical) of patients with adherent RBC number of patient groups with and without RBC adhesion.

Spearman r (p value)	Patients studied for HUVEC channel (n=22)	Patients having RBC adhesion for HUVEC channel (n=15)	Patients studied for fibronectin channel (n=19)	Patients having RBC adhesion for fibronectin channel (n=11)	Patients studied for laminin channel (n=19)	Patients having RBC adhesion for laminin channel (n=4)
Age	0,01612 (0,9433)	0,1773 (0,5244)	-0,04898 (0,8422)	-0,5793 (0,0656)	0,3002 (0,2118)	-0,8000 (0,3333)
Transfusion time (years)	0,03905 (0,8630)	0,1556 (0,5770)	-0,03605 (0,8835)	-0,4636 (0,1546)	0,3079 (0,1998)	-0,8000 (0,3333)
RBC	-0,06428 (0,7762)	-0,09115 (0,7458)	0,4156 (0,0768)	-0,1909 (0,5765)	0,2041 (0,4018)	-0,8000 (0,3333)
Hemoglobin	-0,02067 (0,9272)	-0,1269 (0,6503)	**0,4751*** (0,0398)	-0,2916 (0,3812)	0,2682 (0,2669)	-0,8000 (0,3333)
Hematocrit	-0,0005741 (0,9980)	-0,08579 (0,7602)	0,4420 (0,0581)	-0,3091 (0,3560)	0,2976 (0,2159)	-0,8000 (0,3333)
MCV	0,001148 (0,9960)	0,005362 (0,9874)	0,04470 (0,8558)	-0,08200 (0,8114)	0,3151 (0,1888)	0,4000 (0,7500)
MCH	0,07830 (0,7291)	0,1076 (0,7011)	-0,2836 (0,2393)	0,05467 (0,8754)	-0,1516 (0,5356)	0,4000 (0,7500)
MCHC	-0,08729 (0,6993)	-0,1893 (0,4983)	-0,3704 (0,1186)	0,02278 (0,9517)	-0,2634 (0,2759)	0,4000 (0,7500)
RDW	0,1092 (0,6286)	0,1664 (0,5507)	0,2611 (0,2802)	-0,3909 (0,2366)	0,04189 (0,8648)	-0,4000 (0,7500)
WBC	0,3139 (0,1549)	0,02143 (0,9438)	**0,7255***** (0,0004)	-0,1818 (0,5950)	0,3443 (0,1488)	-0,2000 (0,9167)
Platelet	0,1876 (0,4031)	-0,1929 (0,4901)	**0,6991***** (0,0009)	-0,2545 (0,4511)	0,4378 (0,0608)	0,2000 (0,9167)
NRBC	0,03618 (0,8730)	0,007149 (0,9819)	0,1853 (0,4477)	-0,2005 (0,5517)	0,2475 (0,3069)	-0,4000 (0,7500)
LDH	n = 15 -0,1201 (0,6674)	n = 10 0,2000 (0,5837)	n = 13 -0,3052 (0,3096)	n = 8 0,02381 (0,9768)	n = 13 -0,08724 (0,7840)	n = 4 -0,4000 (0,7500)
Albumin	n = 16 -0,07165 (0,7932)	n = 11 0,1000 (0,7759)	n = 14 0,1551 (0,5958)	n = 9 0,3167 (0,4101)	n = 14 -0,4873 (0,0791)	n = 4 0,2000 (0,9167)
Total Bilirubin	n = 13 0,1356 (0,6594)	n = 8 0,6190 (0,1150)	n = 12 0,1523 (0,6381)	n = 7 0,3929 (0,3956)	n = 12 -0,2845 (0,3742)	n = 3 0,5000 (>0,9999)
Ferritin	n = 12 0,4591 (0,1341)	n = 8 0,4286 (0,2992)	n = 11 0,3164 (0,3396)	n = 7 0,3929 (0,3956)	n = 11 0,3101 (0,3818)	n = 2 Too few pairs

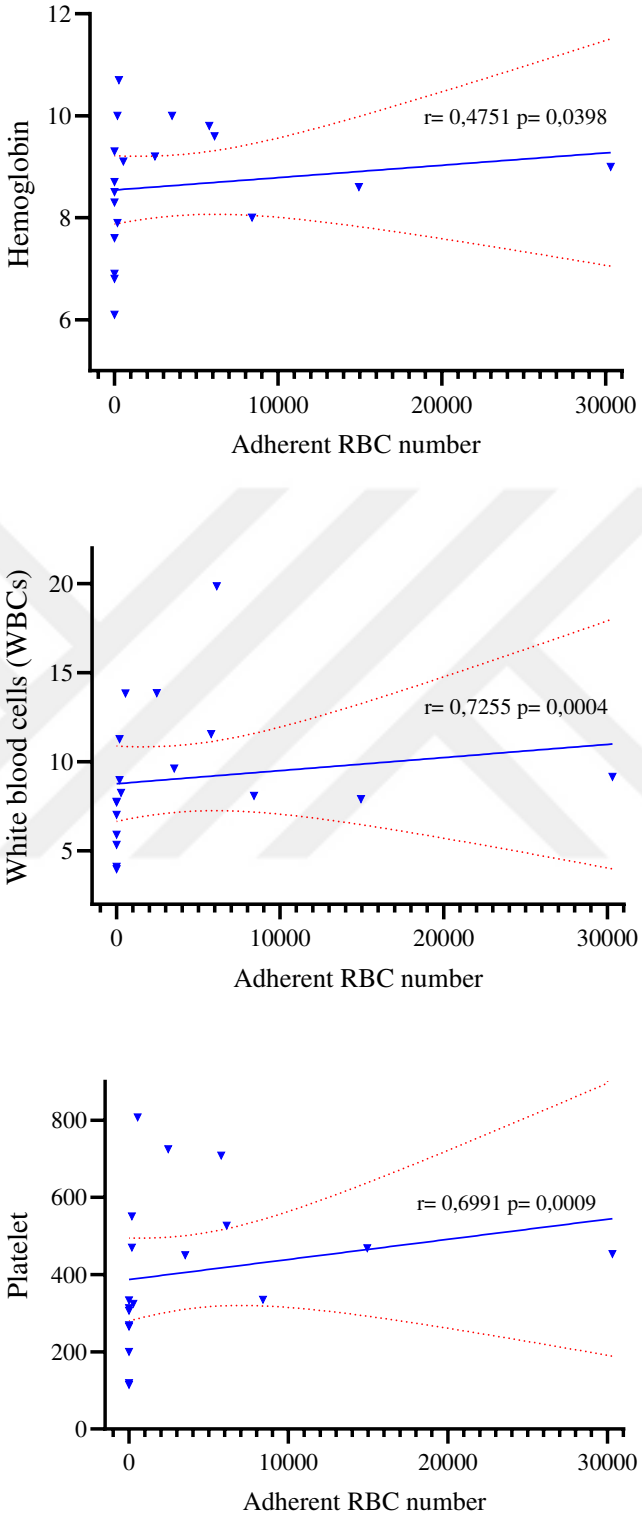


Figure 3.9 Significant positive correlations between the hemoglobin (r =0,4751, p =0,0398), white blood cells (WBCs) (r =0,7255, p =0,0004), platelet (r =0,6991, p =0,0009) values, and the adherent RBC number of the patient group including zero RBC adhesion ones.

3.6 Study population for Forskolin and SQ22536 studies

Four female and six male beta thalassemia major patients between the ages of 8 and 40 (mean: 21,22) (Table 3.9) were included in the study with Forskolin and SQ22536. These patients were chosen according to the previous adhesion study that showed adherent RBCs in microchannel functionalized with 40 μM heme activated HUVECs, and fibronectin. Their first transfusion year, frequency of transfusion, whether a splenectomy has been performed, and the active ingredients of the drugs that patients use were recorded. Splenectomy was performed for three patients. It has been observed that most of the drugs used by patients are iron chelators such as DFX and DFP.

For the study with Forskolin and SQ22536, beta thalassemia major patients' hematological (RBC count, Hb amount, Hct, MCV, MCH, MCHC (RBC's hemoglobin amount in relation to its size), RDW (the size and volume variation of RBCs), WBC, Plt, and NRBC count), and biochemical parameters (LDH, albumin, Total bilirubin, ferritin) are listed in Table 3.10 and Table 3.11 respectively.

Table 3.9 Clinical phenotype of beta thalassemia major patients included in the study with Forskolin and SQ22536.

	Birth Year	**Sex**	**First Transfusion Year**	**Frequency of Transfusion**	**Splenectomy (Yes/No)**	**Active ingredients of the drugs**
Patient 1	1999	M	1999	Once in 3 weeks	YES - 2011	DFX
Patient 2	2016	M	2019	Once in 3 weeks	NO	DFX, Dvit, Bvit
Patient 3	1993	F	1995	Once in 3 weeks	NO	DFX, Folic Acid, Bvit
Patient 4	1984	M	1985	Once in a month	YES - 2000	DFX, Zinc, Folic Acid, Bvit, Dvit, Prednisolone
Patient 5	2009	F	2011	Once in 3 weeks	NO	DFP, Folic Acid, Zinc
Patient 6	1987	M	1987	Once in 3 weeks	YES - 2006	DFX
Patient 7	2006	M	2006	Once in a month	NO	DFX, Folic Acid, Evit, Dvit
Patient 8	1990	M	1990	Once in 2 weeks	NO	DFX
Patient 9	2012	F	2016	Once in a month	NO	DFX
Patient 10	2015	F	2015	Once in 3 weeks	NO	DFX

Table 3.10 Beta thalassemia major patients' hematological parameters for the study with Forskolin and SQ22536.

	RBC	Hb	Hct	MCV	MCH	MCHC	RDW	WBC	Plt	NRBC
Patient 1	3,30^{-}	9,10^{-}	25,40^{-}	77,90	27,90	35,80	15,80^{+}	15,65^{+}	1169^{+}	3,17^{+}
Patient 2	3,20^{-}	9,00^{-}	25,20^{-}	79,70	28,50	35,70	14,70	7,34	558^{+}	0,01
Patient 3	3,50^{-}	9,70^{-}	27,40^{-}	77,80	27,60	35,40	19,80^{+}	10,57	205	0,01
Patient 4	3,80^{-}	9,30^{-}	28,60^{-}	75,50^{-}	24,50	32,50^{-}	31,20^{+}	15,64^{+}	643^{+}	28,88^{+}
Patient 5	3,40^{-}	9,00^{-}	25,60^{-}	75,70^{-}	26,60	35,20	17,60^{+}	4,58	203	0,09^{+}
Patient 6	3,50^{-}	10,10^{-}	27,80^{-}	80,10	29,10	36,30^{+}	17,10^{+}	20,45^{+}	605^{+}	2,4^{+}
Patient 7	3,70^{-}	8,40^{-}	25,00^{-}	67,20^{-}	22,60^{-}	33,60	27,90^{+}	6,38	235	0,45^{+}
Patient 8	1,90^{-}	5,20^{-}	14,50^{-}	76,70	27,50	35,90	14,20	4,25	55^{-}	0,12^{+}
Patient 9	4,58	8,90^{-}	28,40^{-}	62,00^{-}	19,40^{-}	31,30^{-}	26,60^{+}	4,68	143^{-}	0,25^{+}
Patient 10	3,54^{-}	9,90^{-}	27,40^{-}	77,40	28,00^{-}	36,10^{+}	12,50	6,54	400	0,01

RBC: Red blood cell (4,44-5,06x10^{6}/µl), Hb: Hemoglobin (13,5-16,9 g/dl), Hct: Hematocrit (35-45%), MCV: Mean corpuscular volume (76-90 fl), MCH: Mean corpuscular hemoglobin (23-31 pg), MCHC: Mean corpuscular hemoglobin concentration (33-36 g/dl), RDW: Red cell distribution width (11-15%), WBC: White blood cell (4,1-11,2x10^{9}/l), Plt: Platelet count (150-400x10^{9}/l), NRBC: Nucleated red blood cell (0.001-0.015).
($^{-}$ below average, $^{+}$ above average)

Table 3.11 Beta thalassemia major patients' biochemical parameters for the study with Forskolin and SQ22536.

	LDH	Albumin	Total Bilirubin	Ferritin
Patient 1	126^{-}	--	--	779^{+}
Patient 2	163	4,04	1,08	1792^{+}
Patient 3	135^{-}	4,51^{+}	1,07	--
Patient 4	276	4,98^{+}	2,99	1004^{+}
Patient 5	198	4,70^{+}	1,56^{+}	--
Patient 6	--	--	--	--
Patient 7	557^{+}	--	3,32^{+}	--
Patient 8	--	--	--	--
Patient 9	386^{+}	4,57^{+}	2,07^{+}	420^{+}
Patient 10	193	4,65^{+}	--	1899^{+}

LDH: Lactate Dehydrogenase (150-300 U/l), Albumin (2,9-4,5 g/dl), Total Bilirubin (0,2-1,2 mg/dl), Ferritin (30-150 ng/ml).
($^{-}$ below average, $^{+}$ above average)

3.7 Determination of the adherent RBC numbers for microchannels functionalized with endothelial cells and fibronectin after using Forskolin and SQ

After blood sample collection, whole blood samples incubated with Forskolin and SQ22536 perfused into the microchannels functionalized with 40 μM Heme activated HUVECs and fibronectin, and the microchannels were rinsed with HEPES-buffered basal cell culture medium. Image analysis for RBCs adhesion into the microchannels was made by scanning 32 mm^2 field of view in the middle of the microchannel under 10x objectives using an inverted microscope.

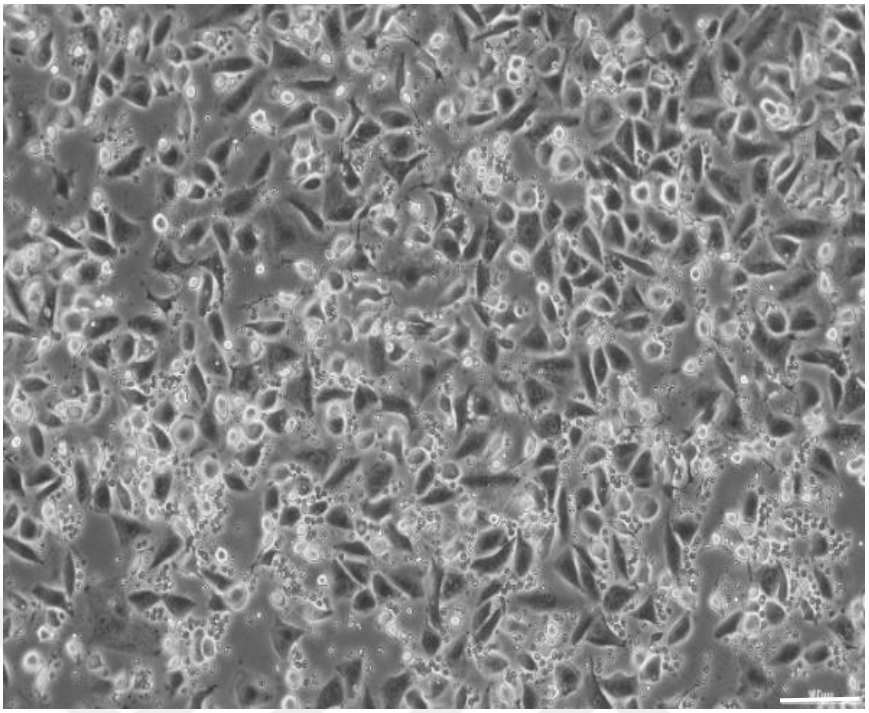
Representative images of adherent RBCs after using Forskolin and SQ22536 for microchannels functionalized with 40 μM heme activated HUVECs (Figure 3.10), and fibronectin (Figure 3.11, Figure 3.12) was recorded.

Adherent RBC numbers for microchannels functionalized with 40 μM heme activated HUVECs (n=9) and fibronectin (n=8) are determined and listed in Table 3.12 and Table 3.13 respectively, for beta thalassemia major patients after using vehicle, Forskolin and SQ22536.

For Forskolin and SQ22536 studies, 9 of 10 patients had adherent RBCs in the microchannel functionalized with 40 μM heme activated HUVEC, while 8 of 10 patients had adherent RBCs in the microchannel functionalized with fibronectin. The chemical effect of Forskolin and SQ22536 was also examined based on the vehicle (PBS and DMSO) used in their preparation.

Descriptive statistics were made for beta thalassemia major patients. Data were listed as minimum and maximum values, with their means and standard deviations (std) in Table 3.14.

A



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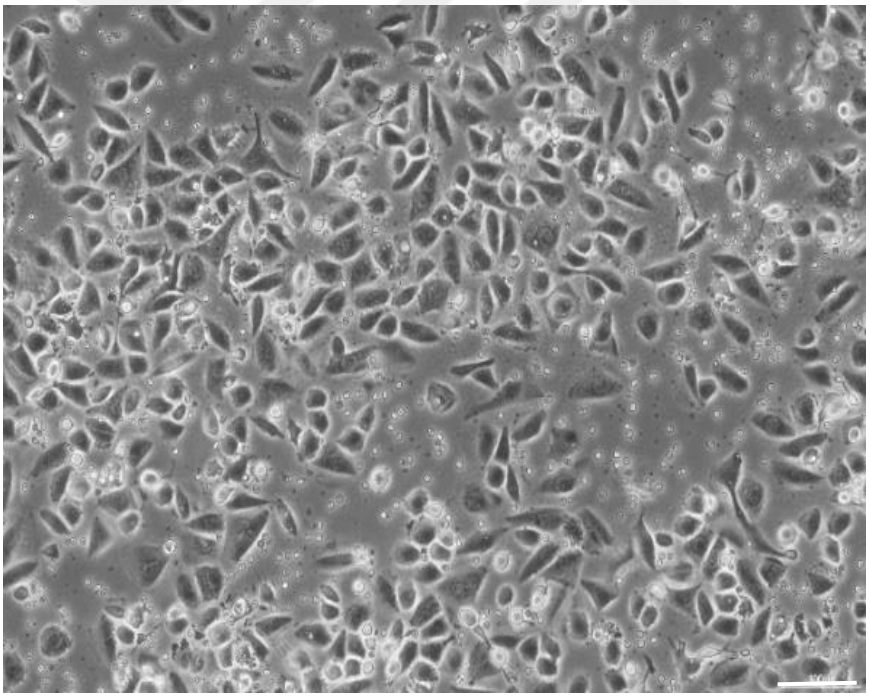

Figure 3.10 Representative images of adherent RBCs after using Forskolin (A) and SQ22536 (B) for microchannel functionalized with 40 μM heme activated HUVECs (Scale bar=100 μm).

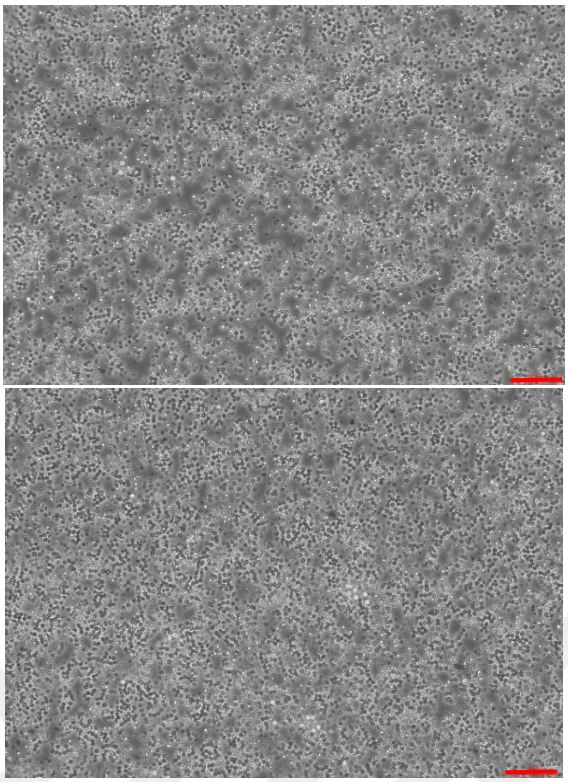

Figure 3.11 Representative images of adherent RBCs after using Forskolin for microchannel functionalized with fibronectin (Scale bar=100 μm).

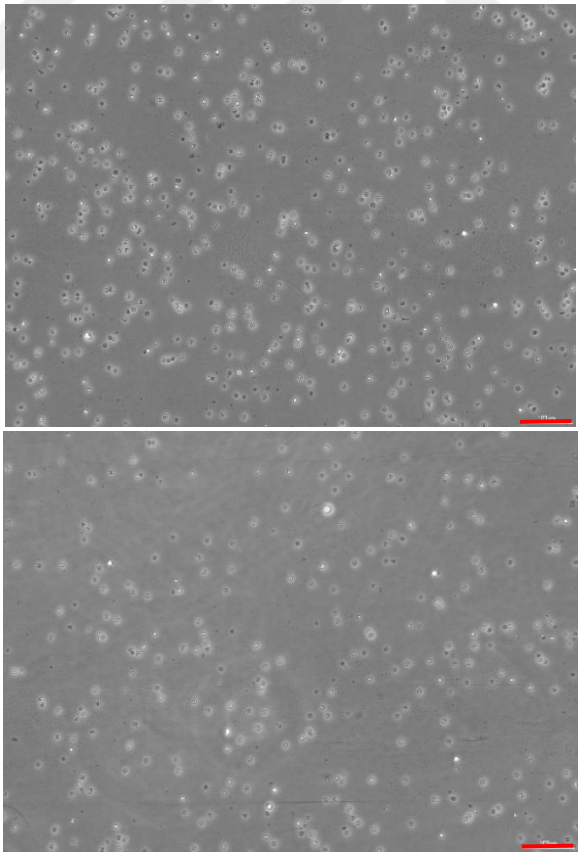

Figure 3.12 Representative images of adherent RBCs after using SQ22536 for microchannel functionalized with fibronectin (Scale bar=100 μm).

Table 3.12 Beta thalassemia major patients' adherent RBC numbers for microchannels functionalized with 40 μM heme activated HUVECs after using vehicle, Forskolin and SQ22536.

	Adherent RBC Number (Control)	**Adherent RBC Number (Vehicle)**	**Adherent RBC Number (Forskolin)**	**Adherent RBC Number (SQ22536)**
Patient 1	3826	--	19124	39986
Patient 2	2259	3975	16869	40987
Patient 3	915	--	58092	68704
Patient 4	1340	4126	1006	41126
Patient 5	1114	--	12065	27752
Patient 6	0	--	--	--
Patient 7	2600	4375	17259	41256
Patient 8	4750	--	20140	11789
Patient 9	1347	494	2653	53326
Patient 10	15599	8056	8398	43700

Table 3.13 Beta thalassemia major patients' adherent RBC numbers for microchannels functionalized with fibronectin after using vehicle, Forskolin and SQ22536.

	Adherent RBC Number (Control)	**Adherent RBC Number (Vehicle)**	**Adherent RBC Number (Forskolin)**	**Adherent RBC Number (SQ22536)**
Patient 1	3227	--	33459	43567
Patient 2	5874	2124	73672	938
Patient 3	10591	--	123196	27787
Patient 4	4398	--	56493	115672
Patient 5	0	--	--	--
Patient 6	2520	1634	18468	10247
Patient 7	4075	2524	2565	1814
Patient 8	0	--	--	--
Patient 9	2447	969	6479	3800
Patient 10	2071	4370	24446	42712

For the microchannels functionalized with 40 μM heme activated HUVECs, the control microchannel that untreated blood perfused had minimum 915 adherent RBCs and maximum 15599 adherent RBCs (mean: 3750, std: 4630). When the chemical effect was examined with vehicle that applied to the blood, there was a slight increase of adherent RBCs in comparison with the untreated blood (control).

When Forskolin and SQ22536 applied to the blood, adherent RBC number was increased. For Forskolin, the minimum adherent RBC number was 1006 and the maximum adherent number was 58092 (mean: 17290, std: 16816). For SQ22536, the minimum adherent RBC number was 11789 and the maximum adherent number was 68704 (mean: 40958, std: 15639). There was a significant increase for adherent RBC number after applying SQ22536 to the blood compared to control ($p<0,001$) and vehicle ($p = 0.013$) as seen Figure 3.13.

For the microchannels functionalized with fibronectin, the control microchannel that untreated blood perfused had minimum 2071 adherent RBCs and maximum 10591 adherent RBCs (mean: 4400, std: 2797). When the chemical effect was examined with vehicle that applied to the blood, there was a slight decrease of adherent RBCs in comparison with the control blood.

When Forskolin and SQ22536 applied to the blood, adherent RBC number was increased. For Forskolin, the minimum adherent RBC number was 2565 and the maximum adherent number was 123196 (mean: 42347, std: 40632). For SQ22536, the minimum adherent RBC number was 938 and the maximum adherent number was 115672 (mean: 30817, std: 38546). There was a significant increase for adherent RBC number after applying Forskolin to the blood compared to vehicle ($p = 0,0135$) as seen Figure 3.14.

Table 3.14 Descriptive statistics for beta thalassemia major patients for microchannels functionalized with 40 μM heme activated HUVECs and fibronectin after using vehicle, Forskolin and SQ22536.

min-max mean ± std	**Patients studied for HUVEC channel (n=9)**	**Patients studied for fibronectin channel (n=8)**
Adherent RBC number Control	915 – 15599 3750 ± 4630	2071 – 10591 4400 ± 2797
Adherent RBC number Vehicle	n = 5 494 – 8056 4205 ± 2678	n = 5 969 – 4370 2324 ± 1282
Adherent RBC number Forskolin	1006 – 58092 17290 ± 16816	2565 – 123196 42347 ± 40632
Adherent RBC number SQ22536	11789 – 68704 40958 ± 15639	938 – 115672 30817 ± 38546
Age	8 – 40 21,22 ± 11,61	8 – 40 22,38 ± 12,61
Transfusion time (years)	5 – 39 20 ± 12,28	5 – 39 21,25 ± 13,30
RBC (4,44-5,06x10^6/μl)	1,90 – 4,58 3,43 ± 0,70	3,20 – 4,58 3,64 ± 0,42
Hemoglobin (13,5-16,9 g/dl)	5,20 – 9,90 8,72 ± 1,39	8,40 – 10,10 9,30 ± 0,56
Hematocrit (35-45%)	14,50 – 28,60 25,28 ± 4,27	25,00 – 28,60 26,90 ± 1,47
MCV (76-90 fl)	62,00 – 79,70 74,43 ± 5,86	62,00 – 80,10 74,70 ± 6,54
MCH (23-31 pg)	19,40 – 28,50 25,84 ± 3,08	19,40 - 29,10 25,95 ± 3,45
MCHC (33-36 g/dl)	31,30 – 36,10 34,61 ± 1,72	31,30 – 36,30 34,59 ± 1,88
RDW (11-15%)	12,50 - 31,20 20,03 ± 6,82	12,50 - 31,20 20,70 ± 6,94
WBC (4,1-11,2x10^9/l)	4,25 - 15,65 8,40 ± 4,52	4,68 - 20,45 10,91 ± 5,69
Platelet (150-400x10^9/l)	55 – 1169 401,20 ± 347,20	143 – 1169 494,80 ± 333,10
NRBC (0.001-0.015)	0,01 - 28,88 3,66 ± 9,51	0,01 - 28,88 4,39 ± 9,96
LDH (150-300 U/l)	n = 8 126 – 557 254,30 ± 149,00	n = 7 126 – 557 262,30 ± 159,10
Albumin (2,9-4,5 g/dl)	n = 6 4,04 – 4,98 4,57 ± 0,30	n = 5 4,04 – 4,98 4,55 ± 0,33
Total Bilirubin (0,2-1,2 mg/dl)	n = 6 1,07 - 3,32 2,01 ± 0,96	n = 5 1,07 - 3,32 2,10 ± 1,04
Ferritin (30-150 ng/ml)	n = 5 420 – 1899 1179,00 ± 644,40	n = 5 420 – 1899 1179,00 ± 644,40

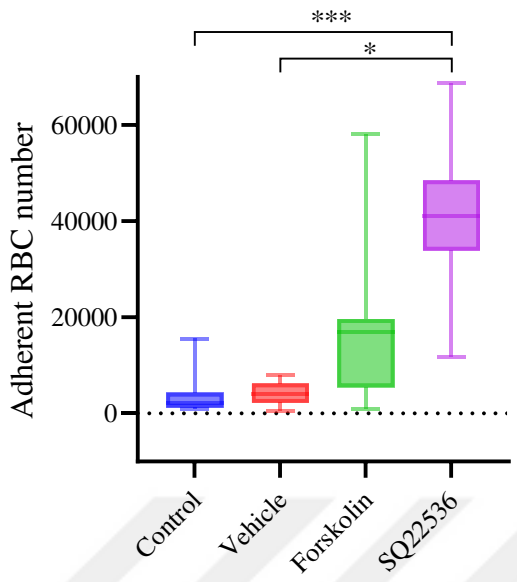

Figure 3.13 The comparison of adherent RBC number for microchannels functionalized with 40 μM heme activated HUVECs after perfusion of untreated blood, treated blood with vehicle, Forskolin, and SQ22536 separately (*** p<0,001, * p<0.05, Kruskal-Wallis test).

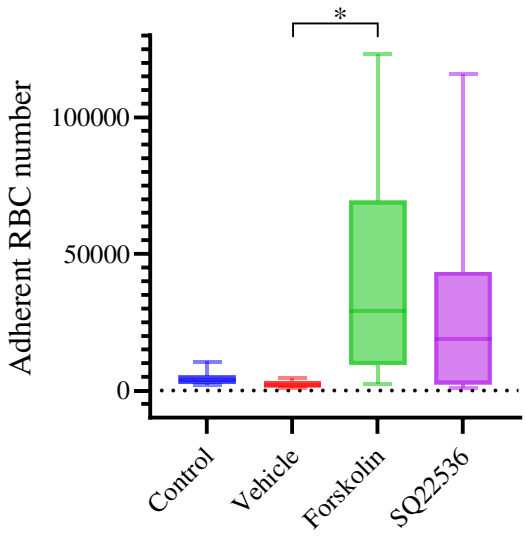

Figure 3.14 The comparison of adherent RBC number for microchannels functionalized with fibronectin after perfusion of untreated blood, treated blood with vehicle, Forskolin, and SQ22536 separately (* p<0,05, Kruskal-Wallis test).

Then, correlation analysis was performed between adherent RBC number for untreated blood and each parameter of patient groups. For each analysis, the Spearman r value and its p value were listed in Table 3.15. There were no significant correlations between adherent RBC number for untreated blood and each parameter of patient groups.

Table 3.15 Correlation analysis results as the Spearman r value and its p value for each parameter (hematological and biochemical) of patients with adherent RBC number in control microchannels functionalized with 40 μM heme activated HUVECs and fibronectin.

Spearman r (P value)	**Adherent RBC number for HUVEC channel (n=9) (Control)**	**Adherent RBC number for fibronectin channel (n=8) (Control)**
Age	-0,2000 (0,6134)	0,2381 (0,5821)
Transfusion time (years)	-0,1167 (0,7756)	0,2143 (0,6191)
RBC	-0,2833 (0,4630)	-0,3713 (0,3651)
Hemoglobin	-0,1590 (0,6835)	-0,1667 (0,7033)
Hematocrit	-0,4686 (0,2048)	-0,2515 (0,5487)
MCV	0,1667 (0,6777)	0,2143 (0,6191)
MCH	0,3333 (0,3853)	-0,02381 (0,9768)
MCHC	0,6667 (0,0589)	-0,2619 (0,5364)
RDW	-0,6333 (0,0760)	0,2857 (0,5008)
WBC	-0,1333 (0,7435)	0,2143 (0,6191)
Platelet	0,1167 (0,7756)	0,04762 (0,9349)
NRBC	0,03390 (0,9461)	-0,07319 (0,8872)
LDH	n = 8 -0,04762 (0,9349)	n = 7 -0,2857 0,5560
Albumin	n = 6 -0,1429 (0,8028)	n = 5 -0,6000 (0,3500)
Total Bilirubin	n = 6 0,6000 (0,2417)	n = 5 -0,7000 (0,2333)
Ferritin	n = 5 0,5000 (0,4500)	n = 5 0,000 (>0,9999)

3.8 The effect of shear stress on RBC deformability for beta thalassemia major patients

After deformability measurements, it was observed that RBC deformability was slightly decreased by continuous 5 Pa SS application at high shear stress (>5.15 Pa) levels for untreated patient blood samples. When deformability changes were examined patient by patient, it was observed that the ones that have decreased deformability (Patient 3 and Patient 6) had splenectomy different than others (Figure 3.15).

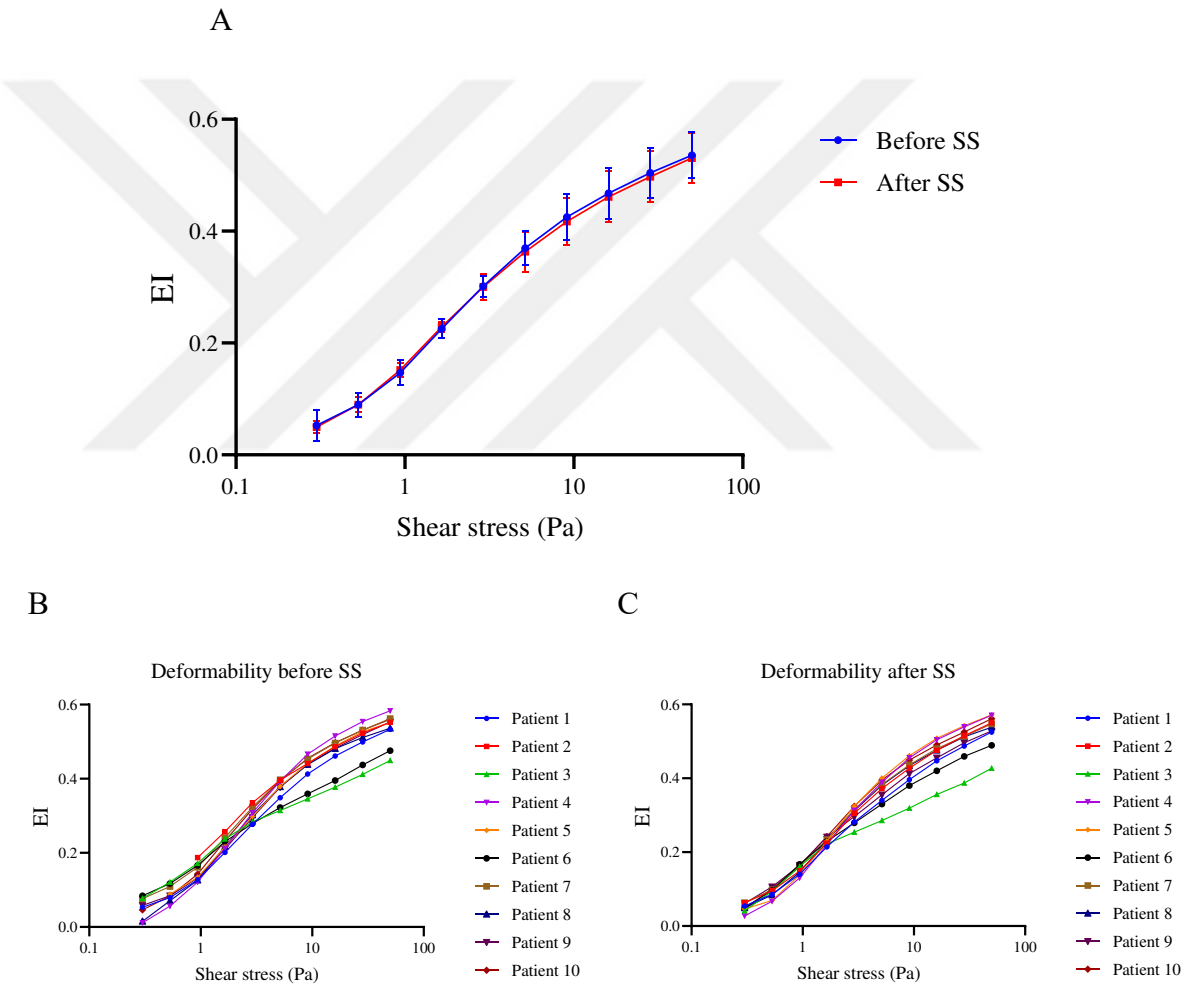

Figure 3.15 RBC deformability curves before and after SS application for beta thalassemia major patients. A. EI changes before and after shear stress (n=10). B. RBC deformability curves before SS application for each beta thalassemia major patient (n=10). C. RBC deformability curves after SS application for each beta thalassemia major patient (n=10). (Two-way ANOVA).

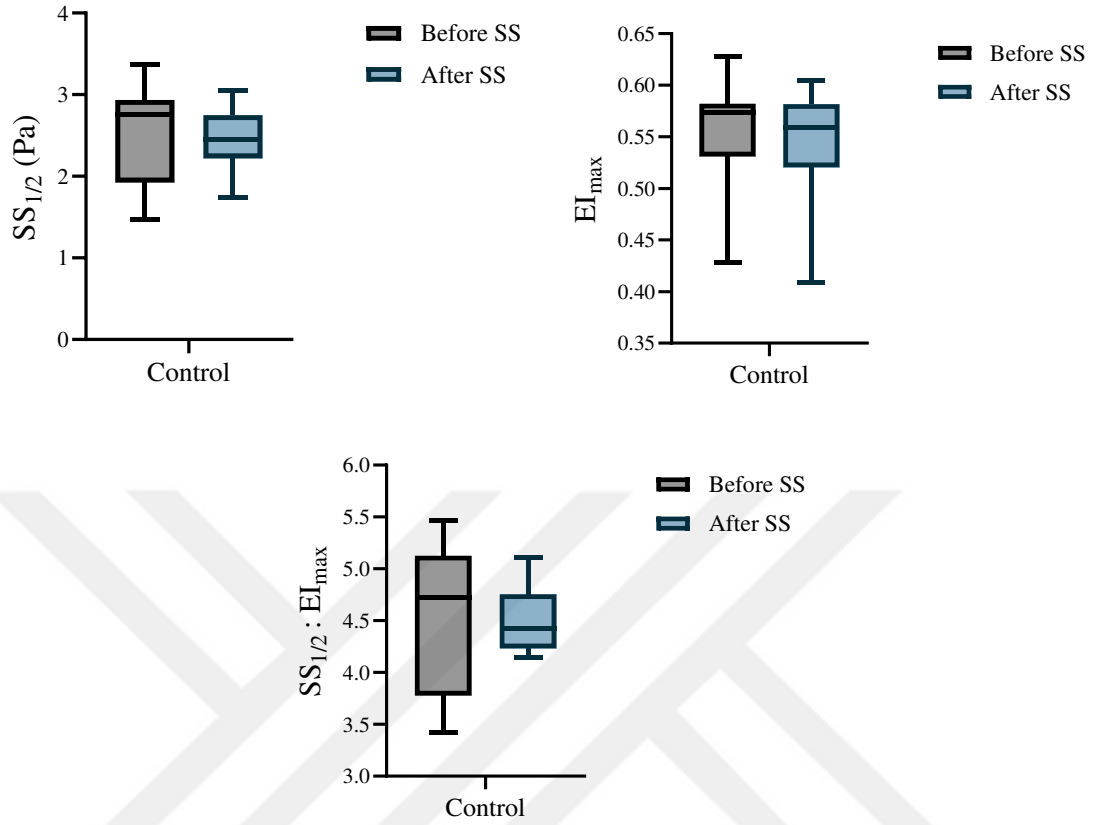

Figure 3.16 RBC deformability parameters EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} for untreated blood of beta thalassemia patients before and after SS application (n=10, Two-way ANOVA).

When EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were examined before and after shear stress for beta thalassemia patients, there were not any significant changes (Figure 3.16).

3.9 The effect of Forskolin on RBC deformability for beta thalassemia major patients

The application of Forskolin with shear stress impaired the deformability compared to the vehicle. At low shear stress (<5.15 Pa), it was observed that RBC deformability was slightly increased after SS both for vehicle and Forskolin treated blood. At high shear stress (>9.09 Pa), it was observed that RBC deformability was slightly decreased after SS both for vehicle and Forskolin treated blood. There were not any significant differences between vehicle and Forskolin treated blood before and after SS (Figure 3.17).

EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were not altered by Forskolin treatment compared to the vehicle before and after SS application. When Forskolin was applied, although EI_{max} were not changed significantly before and after SS application, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were significantly decreased after continuous SS ($p < 0,05$, Figure 3.18).

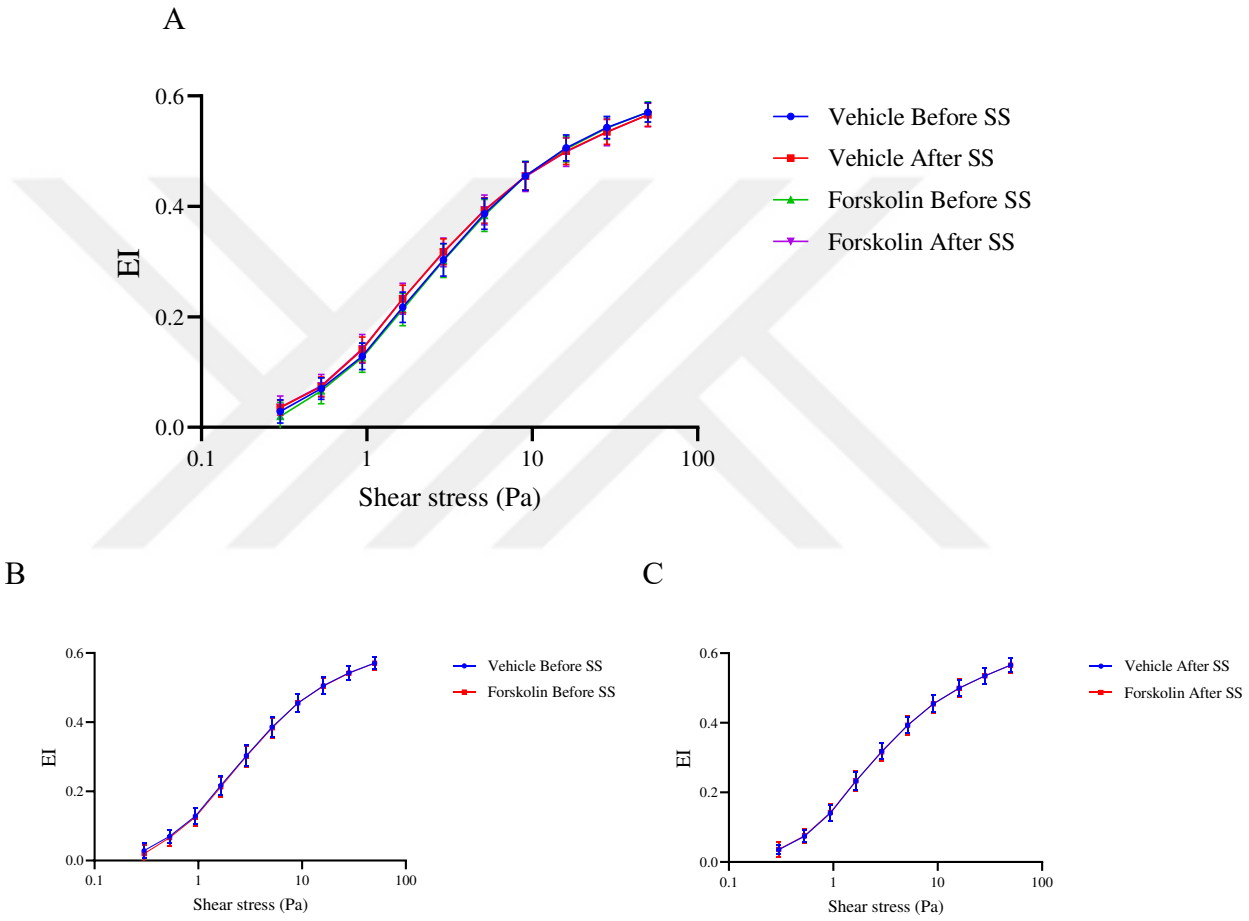

Figure 3.17 RBC deformability curves in Forskolin groups before and after SS application (n=10). A. EI changes between before and after 5 Pa SS in Forskolin and vehicle (n=10). B. EI changes before 5 Pa SS between Forskolin and the vehicle (n=10). C. EI changes after 5 Pa SS between Forskolin and the vehicle (n=10). (Two-way ANOVA).

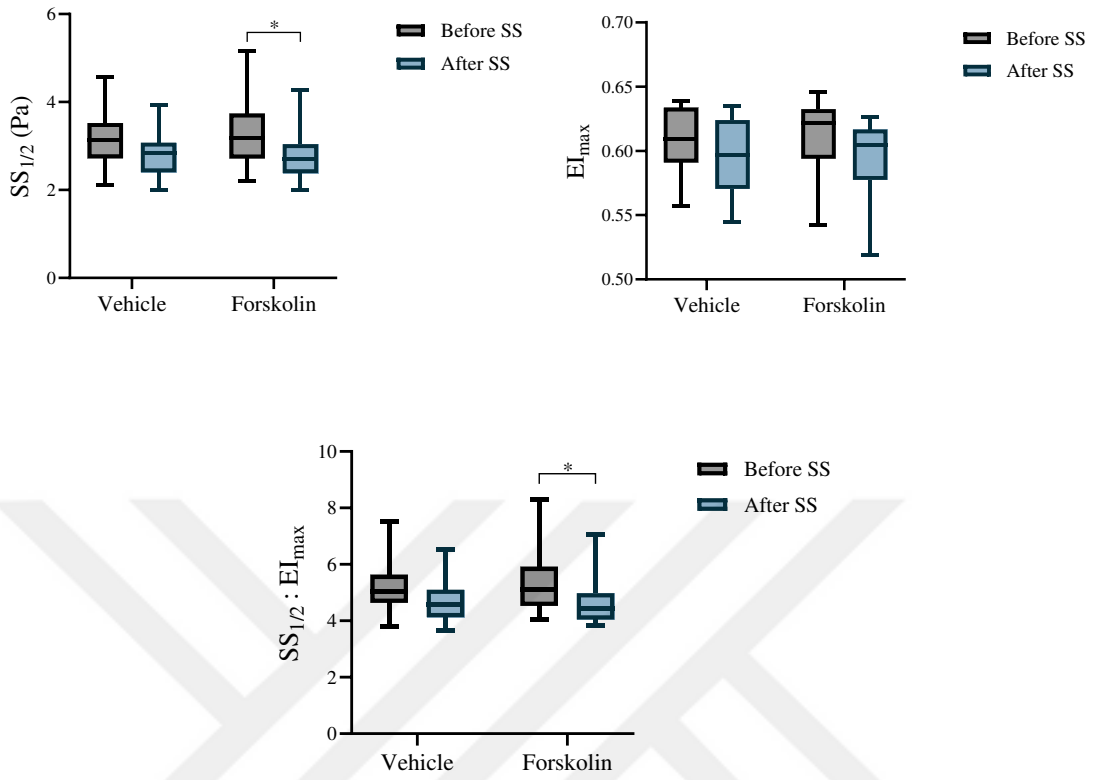

Figure 3.18 RBC deformability parameters EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} in Forskolin groups before and after SS application (* $p<0,05$, n=10, Two-way ANOVA).

3.10 The effect of SQ22536 on RBC deformability for beta thalassemia major patients

The application of SQ22536 with shear stress impaired the deformability compared to the vehicle. At low shear stress (<5.15 Pa), it was observed that RBC deformability was slightly increased after SS both for vehicle and SQ22536 treated blood. At high shear stress (>9.09 Pa), it was observed that RBC deformability was slightly decreased after SS both for vehicle and SQ22536 treated blood. There were not any significant differences between vehicle and SQ22536 treated blood before and after SS (Figure 3.19).

EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were not altered by SQ22536 treatment compared to the vehicle before and after SS application. When vehicle was applied, although EI_{max} were not changed significantly before and after SS application in the vehicle, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were significantly decreased after SS application ($p < 0,05$, Figure 3.20).

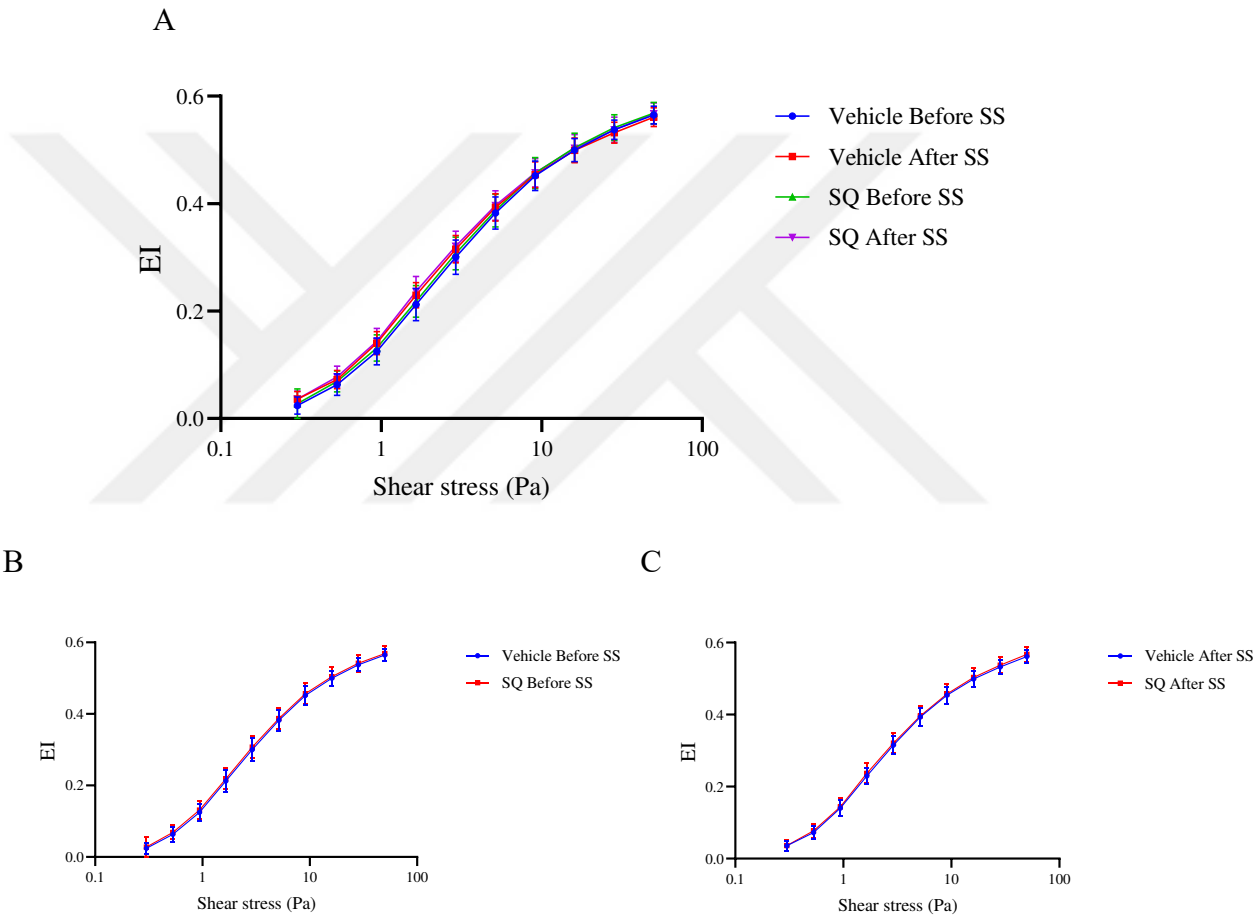

Figure 3.19 RBC deformability curves in SQ22536 groups before and after SS application (n=10). A. EI changes between before and after 5 Pa SS in SQ22536 and vehicle (n=10). B. EI changes before 5 Pa SS between SQ22536 and the vehicle (n=10). C. EI changes after 5 Pa SS between SQ22536 and the vehicle (n=10). (Two-way ANOVA).

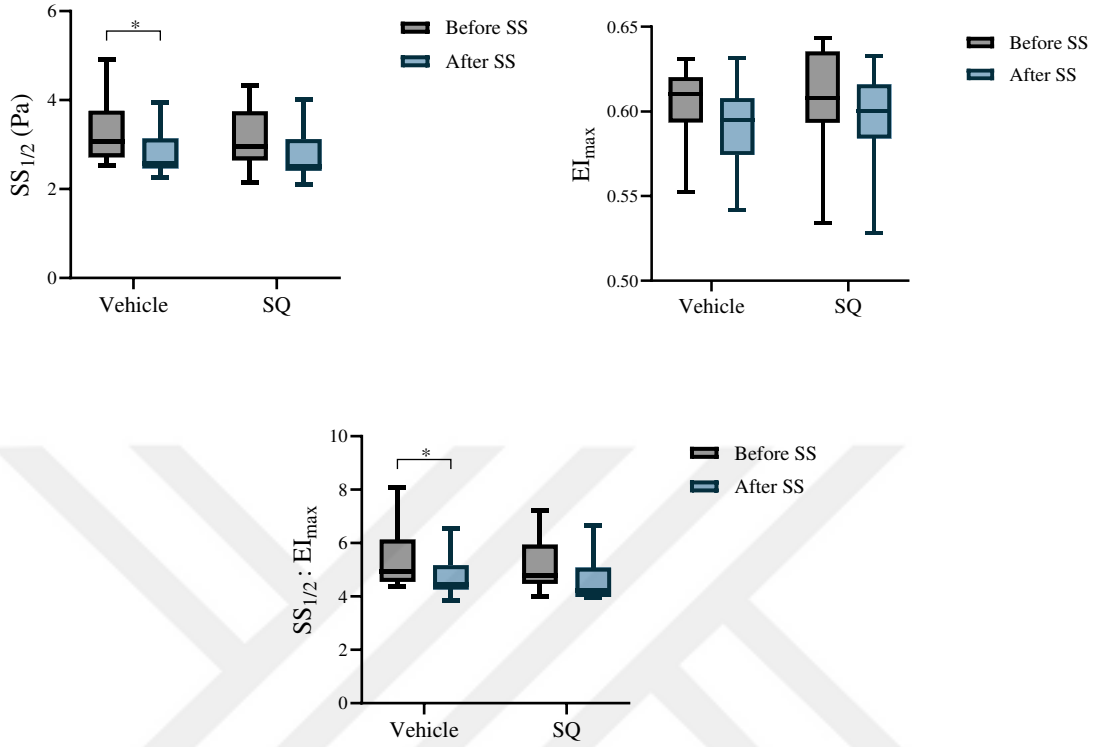

Figure 3.20 RBC deformability parameters EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} in SQ22536 groups before and after SS application (* $p<0,05$, n=10, Two-way ANOVA).

3.11 Determination of the Plasma Hemoglobin

After hemoglobin standards were prepared as 10 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0,5 mg/ml, and 0 mg /ml (only PBS), and their absorbance values measured at 540 nm, the calibration curve was plotted with absorbance values versus hemoglobin concentration (mg/ml) as seen Figure 3.21. The equation of curve was determined as y=0,0623x-0,0023.

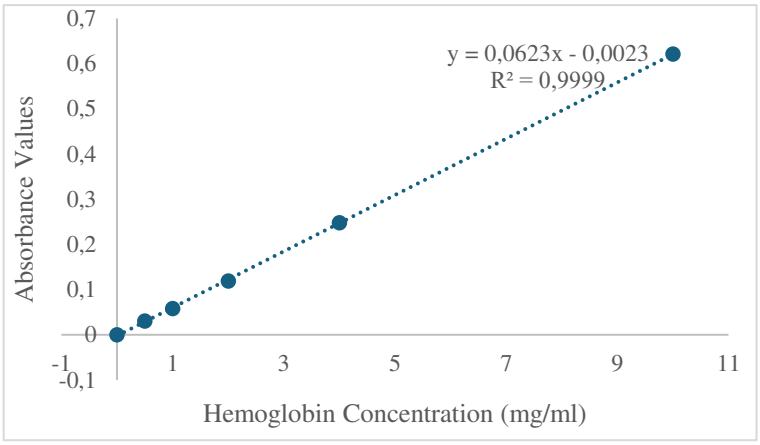

Figure 3.21 The calibration curve with absorbance values versus hemoglobin concentration (mg/ml). The equation of curve was y=0,0623x-0,0023 with R^2 =0,9999.

After the curve was obtained, the absorbance values of each healthy donor' and patient' plasma sample was measured at 540 nm. Finally, the total hemoglobin concentration (mg/ml) of plasma sample of each healthy donor and patient was determined directly from the calibration curve. The minimum plasma hemoglobin concentration that healthy donor had was 1,22 and the maximum plasma hemoglobin concentration that healthy donor had was 1,59 (mean: 1,36).

The hemoglobin concentration of plasma sample for beta thalassemia intermedia patients was listed in Table 3.5. For beta thalassemia major patients studied for determining adhesion in microchannels functionalized with 40 μM heme activated HUVECs, fibronectin and laminin, their hemoglobin concentration of plasma sample were listed in Table 3.16.

The minimum plasma hemoglobin concentration that patient had was 2,13 and the maximum plasma hemoglobin concentration that patient had was 6,08 (mean: 3,14, std: 1,06) for patients studied in microchannel functionalized with 40 μM heme activated HUVECs. The minimum plasma hemoglobin concentration that patient had was 2,13 and the maximum plasma hemoglobin concentration that patient had was 5,63 (mean: 2,90, std: 0,84) for patients studied in microchannel functionalized with fibronectin and laminin.

Table 3.16 Plasma hemoglobin concentration of beta thalassemia major patients studied for determining adhesion in microchannels functionalized with 40 μM heme activated HUVECs, fibronectin and laminin.

	Plasma Hemoglobin Concentration (mg/ml)
Patient 1	4,27
Patient 2	6,08
Patient 3	3,61
Patient 4	2,49
Patient 5	2,65
Patient 6	2,15
Patient 7	4,28
Patient 8	2,97
Patient 9	2,53
Patient 10	2,13
Patient 11	2,64
Patient 12	2,22
Patient 13	3,20
Patient 14	2,36
Patient 15	2,75
Patient 16	2,61
Patient 17	5,63
Patient 18	2,27
Patient 19	3,38
Patient 20	3,13
Patient 21	2,86
Patient 22	2,85

Then, correlation analysis was performed between plasma hemoglobin concentration and adherent RBC number of patient groups, with and without RBC adhesion. For each analysis, the Spearman r value and its p value were listed in Table 3.17. A significant positive correlation existed between the plasma hemoglobin concentration (r = 0,4263*) and the adherent RBC number of the patient group without RBC adhesion (Figure 3.22) for microchannel functionalized with 40 μM heme activated HUVECs.

Table 3.17 Correlation analysis results as the Spearman r value and its p value for plasma hemoglobin concentration of patients with adherent RBC number of patient groups with and without RBC adhesion.

Spearman r (p value)	**Patients studied for HUVEC channel (n=22)**	**Patients having RBC adhesion for HUVEC channel (n=15)**	**Patients studied for fibronectin channel (n=19)**	**Patients having RBC adhesion for fibronectin channel (n=11)**	**Patients studied for laminin channel (n=19)**	**Patients having RBC adhesion for laminin channel (n=4)**
Plasma Hemoglobin Concentration (mg/ml)	**0,4263*** (0,0479)	0,1107 (0,6952)	-0,06198 (0,8010)	-0,6000 (0,0562)	-0,03689 (0,8808)	-0,2000 (0,9167)

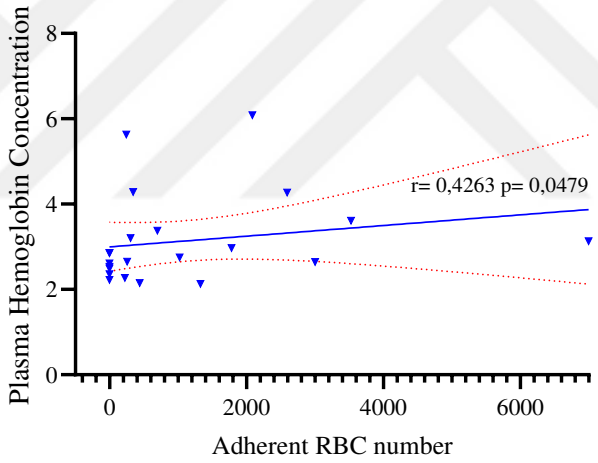

Figure 3.22 Significant correlation between the plasma hemoglobin concentration (r = 0,4263, p = 0,0479) and the adherent RBC number of the patient group without RBC adhesion ones for microchannel functionalized with 40 µM heme activated HUVECs.

Finally, the hemoglobin concentration of plasma sample of beta thalassemia major patients studied for Forskolin and SQ22536 studies were listed in Table 3.18. The minimum plasma hemoglobin concentration that patient had was 2,32 and the maximum plasma hemoglobin concentration that patient had was 7,38 (mean: 3,71, std: 1,65) for patients studied in microchannel functionalized with 40 µM heme activated HUVECs.

The minimum plasma hemoglobin concentration that patient had was 2,32 and the maximum plasma hemoglobin concentration that patient had was 7,38 (mean: 3,71, std: 1,69) for patients studied in microchannel functionalized with fibronectin.

Table 3.18 Plasma hemoglobin concentration of beta thalassemia major patients studied for Forskolin and SQ22536 studies.

	Plasma Hemoglobin Concentration (mg/ml)
Patient 1	7,38
Patient 2	2,79
Patient 3	3,09
Patient 4	5,11
Patient 5	2,50
Patient 6	3,21
Patient 7	2,32
Patient 8	4,40
Patient 9	2,87
Patient 10	2,97

Then, correlation analysis was performed between adherent RBC number for untreated blood and the plasma hemoglobin concentration of patient groups. For each analysis, the Spearman r value and its p value were listed in Table 3.19. There were no significant correlations between groups.

Table 3.19 Correlation analysis results as the Spearman r value and its p value for plasma hemoglobin concentration of patients with adherent RBC number in control microchannels functionalized with 40 μM heme activated HUVECs and fibronectin.

Spearman r (P value)	**Adherent RBC number for HUVEC channel (n=9) (Control)**	**Adherent RBC number for fibronectin channel (n=8) (Control)**
Plasma Hemoglobin Concentration (mg/ml)	0,1667 (0,6777)	-0,02381 (0,9768)

Chapter 4

DISCUSSION

In this study, we investigated the RBC adhesion for beta thalassemia patients under flow conditions using microfluidic chip whose microchannels functionalized with 40 μM heme activated HUVECs, fibronectin and laminin. We demonstrated that increased RBC adhesion for beta thalassemia major patients in microchannels functionalized with 40 μM heme activated HUVECs and fibronectin compared to the laminin. There were significant positive correlations between the hemoglobin, WBCs, platelet values, and the adherent RBC number of patient group for microchannel functionalized with fibronectin. Pathway studies conducted with Forskolin and SQ22536 showed that RBC adhesion changed by targeting molecules of AC-cAMP-PKA signaling pathway. In addition, RBC deformability was measured for beta thalassemia major patients. It was observed that RBC deformability was slightly decreased after shear stress at high shear stress levels for untreated patient bloods, and the application of Forskolin and SQ22536 with shear stress impaired the deformability. Lastly, plasma hemoglobin concentration of each patient was determined by Drabkin's Assay and there were higher plasma hemoglobin concentrations in beta thalassemia major patients' plasma samples. A significant positive correlation existed between the plasma hemoglobin concentration and the adherent RBC number of patient group for microchannel functionalized with 40 μM heme activated HUVECs.

Beta thalassemia is an inherited genetic disease that impacts the hematopoietic system, caused by mutations in the beta globin gene. The primary characteristic of beta thalassemia is ineffective erythropoiesis due to the reduced or absent beta globin production, resulting in low or no production of HbA. This deficiency in beta globin leads to an accumulation of free alpha globin chains in the erythroid cells' cytoplasm, affecting the erythroid cells' physiology. These free α-globin chains aggregate and precipitate, causing cytotoxic effects, oxidative stress, and decrease the erythroid cells' survival, which in turn results in high levels of hemolysis (Gambari & Finotti, 2024).

In addition to beta thalassemia, hemolytic diseases, including sickle cell disease, malaria and autoimmune hemolytic anemia, continue to pose significant clinical challenges. These conditions lead to the breakdown of RBCs, releasing hemoglobin and heme into plasma. Extracellular hemes are highly proinflammatory, activating immune cells and ECs, which contributes to the pathogenesis of the disease. As a result, patients experiencing severe hemolytic events may have plasma heme concentrations reaching 20–50 μM (Muller-Eberhard et al., 1968; Roumenina et al., 2016).

This endothelial cells' activation causes the overexpression of adhesion molecules and adhesion of RBCs to the endothelial cells. Various research has been carried out to determine the molecular and physical characteristics involved in this interaction between RBCs and ECs, including SCD, malaria, PV, RVO, diabetic mellitus, hypertension, and stroke (Barshtein et al., 2022; Wautier & Wautier, 2020; Wautier et al., 1981; Wautier & Wautier, 2013; Wautier et al., 2007; Wautier et al., 2011). Patients with beta thalassemia also experience changes in their RBC flow characteristics like adherence to ECs, leading to microcirculatory issues. It was found that there was significantly higher RBC adherence to ECs in BTM, and even greater RBC adherence to ECs in BTI under static conditions (Hovav et al., 1999). Despite these findings, the underlying mechanisms and molecules involved in RBC adhesion in beta thalassemia patients remain not fully understood.

The most studied adhesion molecules on RBCs, for the diseases having adhesion, are ICAM-4, α4β1, Lu/BCAM, CD36, CD47, Neurothelin, Sema7A, and LFA-3 (Aricescu & Jones, 2007; Joneckis et al., 1993; Wautier & Wautier, 2013). Therefore, the major aim

of this study was to elucidate which adhesion molecules mediate the RBC adhesion to ECs for beta thalassemia patients and determine this RBC adhesion under flow conditions unlike the previous study with beta thalassemia patients. For this aim, a microfluidic chip designed for single-use and disposal was used for this study. It is simple and cost-efficient yet allows thorough analysis of biophysical properties at the level of individual cells. To examine the RBC adhesion to different adhesion sites, the microchannels of microfluidic chip was functionalized with HUVECs, fibronectin and laminin (Alapan et al., 2016; Kucukal et al., 2018).

After image analysis in this study, it was observed that the most adherent RBC number was in microchannel functionalized with fibronectin for beta thalassemia major patients. 15 of 22 patients had adherent RBC in microchannel functionalized with 40 μM heme activated HUVECs, and the minimum adherent RBC number was 222 and the maximum adherent RBC number was 6996 (mean: 1657, std: 1839). 11 of 19 patients had adherent RBC in microchannel functionalized with fibronectin, and the minimum adherent RBC number was 181 and the maximum adherent RBC number was 30309 (mean: 6613, std: 9062). 4 of 19 patients had adherent RBC in microchannel functionalized with laminin, and the minimum adherent RBC number was 322 and the maximum adherent RBC number was 2366 (mean: 1333, std: 1160). When the clinical and laboratory findings of patients having the adherent RBC number were examined one by one, there was not any specific indication that causes RBC adhesion for these patients. In addition, there were not any significant differences for patient clinical and laboratory findings between adhesion sites used for functionalization of the microchannel.

Integrins, which are transmembrane proteins, facilitate cell adhesion. RBCs and reticulocytes express one of the most characterized integrin which is α4β1 on the cell surface mediating adhesion to VCAM-1, fibronectin, and thrombospondin (Lou et al., 2021). The expression and activation of α4β1 integrin on RBCs increase under certain conditions, such as inflammation or disease states like SCD, which facilitates the RBC adhesion to VCAM-1 and fibronectin (Gee & Platt, 1995; Kumar et al., 1996). Such a condition may also be present in the beta-thalassemia major patients in this study; there might be an increased expression of α4β1 integrin in their RBCs. In addition to this, an increase in WBC count is the key indicator of inflammation (Foy et al., 2022). The

increased RBC adhesion in microchannel functionalized with fibronectin in this study could also be explained by increased WBC count, since there was a significant positive correlation between WBC count and adherent RBC number in microchannel functionalized with fibronectin (r =0,7255***).

The second objective of this study was to examine the changes in RBC adhesion in microchannels functionalized with 40 μM heme activated HUVECs and fibronectin for beta thalassemia major patients by using a blocker or an activator targeting AC-cAMP-PKA signaling pathway. For this reason, Forskolin was used for the stimulation of AC and SQ22536 was used for the inhibition of AC. The reason of targeting AC was that the AC-cAMP-PKA signaling pathway is particularly significant for researching the mechanisms of RBC deformability adaptive regulation and RBC adhesion to the endothelium (Gauthier et al., 2005; Zennadi et al., 2012). The central component of this pathway is the enzyme AC, which produces cAMP from ATP when the GPCR is stimulated. There is compelling evidence that this pathway operates in RBCs and plays a crucial role in adjusting their micromechanical properties (Semenov et al., 2019). In addition, activation of the AC-cAMP-PKA signaling pathway enhances sickle RBC adherence to the endothelium through the Lu/BCAM adhesion molecule (Gauthier et al., 2005). This abnormal adherence is believed to play a crucial role in the onset and progression of vaso-occlusive crises (Hebbel, 1997). Furthermore, the ERK1/2 signaling molecule is activated by the AC-cAMP-PKA signaling pathway, phosphorylating the ICAM-4 adhesion molecule on RBCs, further enhancing sickle RBC adhesion. Blocking the activities of AC or PKA can reverse this mechanism and reduce sickle RBC adhesion to the endothelium (Goksel et al., 2023; Zennadi et al., 2012). PKA activation is suppressed, and cAMP levels are decreased by AC inhibition. PKA controls ion channel activity and targets many membrane proteins. In the RBC membrane, PKA phosphorylates adducin, dematin, and Protein 4.1, which encourages the spectrin network to dissociate and decreases stability of the membrane (Chen et al., 2013; Cohen & Gascard, 1992; Koshino et al., 2012). Therefore, in this study, AC was stimulated or inhibited to determine the changes of RBC adhesion in microchannel functionalized with 40 μM heme activated HUVECs and fibronectin.

Image analysis of pathway studies showed that there was significantly increase RBC adhesion for beta thalassemia major patients after applying both Forskolin and SQ22536 separately. For microchannel functionalized with 40 μM heme activated HUVECs, after Forskolin was applied, the minimum adherent RBC number was 1006 and the maximum adherent number was 58092 (mean: 17290, std: 16816). After SQ22536 was applied, the minimum adherent RBC number was 11789 and the maximum adherent number was 68704 (mean: 40958, std: 15639). There was a significant increase for adherent RBC number after applying SQ22536 to the blood compared to control ($p<0,001$) and vehicle ($p = 0.013$). For microchannel functionalized with fibronectin, after Forskolin was applied, the minimum adherent RBC number was 2565 and the maximum adherent number was 123196 (mean: 42347, std: 40632). After SQ22536 was applied, the minimum adherent RBC number was 938 and the maximum adherent number was 115672 (mean: 30817, std: 38546). There was a significant increase for adherent RBC number after applying Forskolin to the blood compared to vehicle ($p = 0,0135$).

An increase in RBC adhesion after Forskolin application was expected, since it is an activator of AC. This activation increases the cAMP levels in RBCs that resulted in increased adhesiveness of RBC. An increase in RBC adhesion after SQ22536 application was unexpected, since it was an AC inhibitor. Increasing adherent RBC after SQ22536 application explains that RBC adhesion to the HUVECs and fibronectin has different regulators, therefore only inhibiting AC had no inhibitory effect on RBC adhesion. AC, PKA, and PDE are the critical components of AC-cAMP-PKA signaling pathway (Adderley et al., 2011). The elevation in RBC adhesion due to the inhibition of cAMP signaling could be attributed to the interaction between various signaling pathways, including cGMP-dependent mechanisms via PDEs (Sadek et al., 2020; Zaccolo & Movsesian, 2007; Zhao et al., 2016). Intracellular cGMP has the ability to activate extracellular signal-regulated kinase (ERK), a member of the MAPK family (Li et al., 2003). In addition, increased adherent RBC number could also explained by increased adenosine signaling after applying SQ22536, which is an adenosine derivative inhibiting erythroblast development, decreasing gamma globin gene expression, and reducing the production of HbF (Bhanu et al., 2008; Keefer et al., 2006).

The third objective of this study was to measure RBC deformability for beta thalassemia major patients. Deformability measurements were taken before and immediately after the physiological shear stress (5 Pa) application. The enhancement of RBC deformability due to shear forces could notably influence blood flow dynamics, particularly in tissues with blood vessels that have a reduced ability to adjust their diameter. This might act as a compensatory mechanism to maintain sufficient microcirculatory perfusion (Meram et al., 2013). Maintaining shear-induced enhancement of deformability is crucial under physiological conditions. However, this enhancement is disrupted in hematological disorders like SCD and hereditary hemoglobinopathies, as well as in other pathological conditions such as diabetes mellitus, diabetic nephropathy, and non-diabetic hypertension (Alapan et al., 2014; Clark et al., 1983; Tsuda, 2020). In this study, it was also observed that RBC deformability was slightly decreased after SS at high shear stress levels for untreated patient bloods. The primary factors influencing RBC deformability are intracellular viscosity, the surface area-to-volume ratio, the cytoskeleton structure, the cytoplasm's ionic content which affects RBC hydration, and metabolic processes regulating redox state and ATP levels (Diez-Silva et al., 2010; Gallagher, 2013; Ugurel et al., 2022). Since decreased deformability indicates that these cells are more rigid and prone to hemolysis (Horobin et al., 2017; Simmonds & Meiselman, 2016), decreased deformability observed in this study could explain the increased RBC adhesion in beta thalassemia major patients.

When deformability changes were examined patient by patient, it was observed that the ones that have decreased deformability (Patient 3 and Patient 6) had splenectomy different than others. Splenectomy is known to enhance RBC count, Hb levels, and hematocrit, while reducing MCHC and the reticulocytes' percentage (Berrevoets et al., 2021). At the cellular level, RBC size increases post-splenectomy, and microspherocytes are no longer detectable. However, splenectomy has minimal impact on correcting cytoskeletal membrane defects (Reliene et al., 2002). A recent study has indicated that RBC deformability, assessed via osmotic gradient ektacytometry, is not improved following splenectomy (Berrevoets et al., 2021) as seen in this study.

Furthermore, Forskolin and SQ22536 were applied to the blood samples of beta thalassemia major patients to observe changes in RBC deformability. Forskolin and SQ22536 were used to observe the effect of AC-cAMP-PKA signaling pathway on RBC deformability for beta thalassemia major patients.

It was indicated that AC stimulation by Forskolin reduces RBC deformability, likely due to increased cAMP levels. However, some studies have shown that Forskolin enhances the RBC deformability of healthy donors (Muravyov & Tikhomirova, 2013). However, Semenov et al. (2019) demonstrated that Forskolin's effect on RBC deformability depends on both the drug dosage and the shear stress level. It was also observed that Forskolin increased RBC deformability prior to the constant shear stress application but decreased it after the shear stress application. The reduction in RBC deformability caused by Forskolin after shear stress in SCD patients may be due to calcium influx through shear stress-sensing mechanisms, which could activate AC and elevate intracellular cAMP levels (Kaestner et al., 2020). The reduction in RBC deformability by the stimulation of AC was notably observed at high shear stresses, showing that Forskolin's effect depends on shear stress (Goksel et al., 2023). In this study, the application of Forskolin with shear stress impaired the deformability compared to the vehicle. At low shear stress (<5.15 Pa), it was observed that RBC deformability was slightly increased after SS both for vehicle and Forskolin treated blood. At high shear stress (>9.09 Pa), it was observed that RBC deformability was slightly decreased after SS both for vehicle and Forskolin treated blood. There were not any significant differences between vehicle and Forskolin treated blood before and after SS.

The parameter of $SS_{1/2}$ serves as a global index of RBC deformability, while EI_{max} represents the maximum elongation index at infinite shear stress. Although EI_{max} can be influenced by cell shape and membrane properties, it is not affected by cytoplasmic viscosity (Baskurt et al., 2009). A lower $SS_{1/2}$ typically indicates increased RBC deformability, but it may also correlate with a lower EI_{max}. Therefore, the $SS_{1/2}$:EI_{max} ratio should be considered, as it reflects how EI depends on SS regardless of changes in EI_{max} (Baskurt & Meiselman, 2013). In this study, EI_{max}, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were not altered by Forskolin treatment compared to the vehicle before and after SS application for beta thalassemia major patients. After SS application, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values

were significantly decreased in Forskolin treated blood samples ($p < 0,05$), although EI_{max} was not significantly changed. Since EI_{max} remains unchanged here, it implies that the deformability has impaired because of SS. In this study, that was shown that Forskolin's effect depends on shear stress in beta thalassemia major patients as seen SCD patients.

It is also found that the application of SQ22536 impaired the deformability compared to the vehicle before and after SS. At low shear stress (<5.15 Pa), it was observed that RBC deformability was slightly increased after SS both for vehicle and SQ22536 treated blood. At high shear stress (>9.09 Pa), it was observed that RBC deformability was slightly decreased after SS both for vehicle and SQ22536 treated blood. There were not any significant differences between vehicle and SQ22536 treated blood before and after SS. It is shown that the inhibition of AC-cAMP-PKA signaling pathway by SQ22536 significantly enhanced RBC deformability in SCD patients (Ugurel et al., 2019) as seen beta thalassemia major patients at low shear stress in this study. The enhancements in RBC deformability observed in SCD with SQ22536 could be attributed to the activation of adenosine signaling rather than the inhibition of AC. Since adenosine signaling has been demonstrated to promote vasodilation, enhance blood flow, and decrease inflammation, a potential therapeutic role for adenosine molecules in SCD has been explored. Adenosine signaling activity is crucial for the proper function of erythrocyte physiology. Nonetheless, sickling is a result of increased adenosine signaling. It has been demonstrated that adenosine derivatives, such as SQ22536, limit erythroblast development, lower gamma globin gene expression, and lower the synthesis of HbF (Bhanu et al., 2008; Keefer et al., 2006). SQ22536 has greatly increased RBC deformability, although its potential off-target effects on the pathophysiology of SCD cannot be completely ruled out. Adenosine signal transduction exhibits varying impacts on sickle cell physiology through its many receptors (Jackson et al., 1996; Ugurel et al., 2019; Zhang & Xia, 2012). The enhancement of red blood cell deformability observed in beta thalassemia major patients as a result of cAMP signaling inhibition may be related to the interactions among many signaling pathways, such as those involving cGMP-dependent mechanisms through PDEs (Sadek et al., 2020; Zaccolo & Movsesian, 2007; Zhao et al., 2016). Extracellular signal-regulated kinase (ERK), a member of the MAPK family, can be activated by intracellular cGMP (Li et al., 2003). EI_{max}, $SS_{1/2}$ and

$SS_{1/2}$:EI_{max} values were not altered by SQ22536 treatment compared to the vehicle before and after SS application. When vehicle was applied, although EI_{max} were not changed significantly before and after SS application, $SS_{1/2}$ and $SS_{1/2}$:EI_{max} values were significantly decreased after SS ($p < 0,05$).

Finally, plasma hemoglobin concentration was determined by Drabkin's Assay. The minimum plasma hemoglobin concentration that patient had was 2,13 and the maximum plasma hemoglobin concentration that patient had was 6,08 (mean: 3,14, std: 1,06) for patients studied in microchannel functionalized with 40 μM heme activated HUVECs. The minimum plasma hemoglobin concentration that patient had was 2,13 and the maximum plasma hemoglobin concentration that patient had was 5,63 (mean: 2,90, std: 0,84) for patients studied in microchannel functionalized with fibronectin and laminin. It was observed that plasma hemoglobin concentration was increased for beta thalassemia major patients than healthy controls. In addition, a significant positive correlation existed between the plasma hemoglobin concentration ($r = 0,4263^*$) and the adherent RBC number of patient group for microchannel functionalized with 40 μM heme activated HUVECs. It indicates that hemolysis occurred in these patients and hemoglobin leaked into the plasma.

Limitations of this study are a restricted number of patients included for RBC adhesion studies. The fact that RBC adhesion is observed in some patients but not in others remains unexplained within this study, as no significant differences were found among the patients. This suggests the presence of other patient-specific variables and highlights the necessity of conducting these studies with a larger patient cohort to achieve more comprehensive insights. In addition to this, pathway studies should be advanced. By targeting different molecules to activate or inhibit them, the impact of the AC-cAMP-PKA signaling pathway on RBC adhesion can be more thoroughly investigated. Furthermore, RBC adhesion should be examined from the perspective of adhesion molecules present on RBCs. It is crucial to determine whether these adhesion molecules increase in RBCs due to increased adhesion observed in patients. Furthermore, this aspect should be incorporated into pathway studies. Finally, the number of patients in studies involving beta thalassemia intermedia is very limited, and only RBC adhesion to endothelial cells has been examined. The sample size for this patient group should be

increased, and investigations should focus on interactions with other adhesion molecules other than the endothelial cells, such as fibronectin and laminin.

In conclusion, increased RBC adhesion of beta thalassemia major patients was observed in microchannels functionalized with 40 μM heme activated HUVECs and fibronectin. This preliminary study was conducted for the first time to determine which adhesion site is predominantly involved in RBC adhesion in beta thalassemia major patients. Subsequently, studies were conducted on the AC-cAMP-PKA signaling pathway possibly targeting RBC adhesion molecules. For this purpose, AC was activated via Forskolin, and inhibited via SQ22536. It has been determined that both Forskolin and SQ22536 have an increasing effect on RBC adhesion in microchannels functionalized with 40 μM heme activated HUVECs and fibronectin. In addition to this, RBC deformability analysis has also been conducted for beta thalassemia major patients. It was observed that RBC deformability was slightly decreased after SS for untreated patient bloods, and the ones that have decreased deformability had splenectomy different than others. Deformability studies have also been conducted with the Forskolin and SQ22536 application. The application of Forskolin and SQ22536 with shear stress impaired the deformability. Lastly, it was found that plasma hemoglobin concentration was higher in beta thalassemia major patients' plasma samples than healthy controls. For these reasons, this study has contributed to understanding the pathophysiology of beta thalassemia major patients and importance of targeting AC-cAMP-PKA signaling pathway for RBC adhesion by showing Forskolin and SQ22536 effects.

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