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Detection of Genetic Mutations in Inherited Glanzmann Thrombasthenia Patients in Hebron-Palestine.

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Detection of Genetic Mutations in Inherited Glanzmann Thrombasthenia Patients in Hebron-Palestine.

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Thesis Approval

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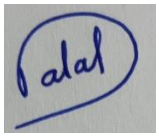
Declaration

I confirm that this thesis, submitted for the master's degree, represents the outcome of our research efforts. The work detailed within this thesis has been conducted following the approval of the research program. All ethical procedures and guidelines have been thoroughly adhered to during the preparation of this thesis.

Signed

Dalal ‘Mohammad Belal’ ‘Mohammad Dawoud’ Al-Hashlamon
Date: 8 /1 /2025



Dedication

Alhamdulillah, first and foremost, I thank Allah for giving me the strength, guidance, and patience to complete this thesis.

To my dear parents, your constant prayers and endless love have been the foundation of my success. You have always believed in me, even when I doubted myself, and for that, I am eternally grateful.

To my beloved husband, your unwavering love, support, and encouragement have been my greatest source of strength throughout this journey. I cannot thank you enough for standing by my side through every challenge and triumph.

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Finally, I am immensely thankful to the family who participated in this research. I sincerely hope that this work contributes to improving their health outcomes.

Abstract

Background:

Glanzmann Thrombasthenia (GT) is a rare autosomal recessive bleeding disorder caused by mutations in genes critical for platelet aggregation, particularly the ITGA2B and/or ITGB3 genes, leading to defective platelet function. This disorder affects platelet function through impaired expression or dysfunction of the αIIbβ3 integrin, which plays a key role in blood aggregation. GT can be classified into three types based on αIIbβ3 integrin expression: Type I, where expression is absent or below 5% of normal levels; Type II, where expression is 5–20% of normal levels; and Type III, where integrin is present but functionally abnormal. Diagnosis involves platelet function tests and sequencing of the ITGA2B and ITGB3 genes. Early diagnosis, management, and genetic counselling are vital for improving patient outcomes and preventing complications. The global prevalence of GT is approximately 1 in 1,000,000 individuals. However, in regions with high rates of consanguinity, this prevalence can rise to as high as 1 in 200,000, or even greater.

Purpose:

This study aims to validate the diagnosis of a clinically suspected GT patient, explore the feasibility of cascade screening for the genetic disorder, and identify pathogenic variants associated with GT within the Palestinian population.

Methods:

Patients meeting the inclusion criteria were selected based on the following criteria: (1) A lifelong bleeding tendency characterized by a normal platelet count, prolonged bleeding time, normal PT, and normal APTT. (2) Primary symptoms included mucocutaneous bleeding, frequent nosebleeds (epistaxis) and easy bruising. (3) Family history of GT, including being an immediate family member of a diagnosed GT patient. The proband's whole blood sample was analysed using WES. Based on the WES findings, specific primers were designed, and RT-PCR was performed. Subsequently, first-degree relatives were screened for the identified variant.

Results:

WES revealed that the proband has a gain variant of uncertain significance (VUS) in the ITGA2B gene: NM_000419.5. This variant involves a duplication spanning exons from 3 to 12, causing an alteration in the reading frame. Notably, this duplication has not been reported in the ClinVar database, suggesting it may be specific to the local population. RT-PCR analysis of the proband's first-degree relatives identified two additional affected family members and six carriers of the variant. However, the results for the proband's parents remain inconclusive, warranting further investigation. A pedigree was constructed to map the inheritance pattern within the family.

Conclusion:

GT remains underdiagnosed and undertreated within our population. Our study highlights the potential of WES as a powerful tool for uncovering the molecular basis of GT, suggesting its integration into diagnostic practices could significantly improve both the understanding and management of this condition. Additionally, the duplication mutation identified in our research, spanning multiple exons, may be unique to our population, as it has not been previously reported in genetic databases. However, further research is needed to confirm its pathogenic significance and explore its broader implications for genetic screening.

Keywords: Glanzmann Thrombasthenia (GT), platelets, WES, ITGA2B.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviation

Acronym	Full Form
GT	Glanzmann Thrombasthenia
GP	Glycoprotein
VWF	Von Willebrand Factor
Fg	Fibrinogen
ET	Essential Thrombocythemia
ADP	Adenine di-phosphate
ISTH	International Society on Thrombosis and Haemostasis
IPFDs	Inherited Platelet Function Disorders
CBC	Complete Blood Count
PT	Prothrombin Time
APTT	Activated Partial Thromboplastin Time
LTA	Light Transmission Aggregometry
rVIIa	Recombinant Activated Factor VII
BMT	Bone Marrow Transplantation
HLA	Human Leukocyte Antigen
HGMD	Human Gene Mutation Database
ER	Endoplasmic Reticulum

Acronym	Full Form
EGF	Epidermal Growth Factor
PGI2	Prostacyclin
EDTA	Ethylenediaminetetraacetic Acid
TIBC	Total Iron Binding Capacity
BUN	Blood Urea Nitrogen
AST	Aspartate Aminotransferase
ALT	Alanine Transaminase
WES	Whole Exome Sequencing
CNVs	Copy Number Variants
RBCs	Red Blood Cells
RT-PCR	Real time-Polymerase chain reaction
PCR	Polymerase Chain Reaction
TAE	Tris-Acetate-EDTA
IVF	In Vitro Fertilization
VUS	Variant of Uncertain Significance
Bp	Base Pairs
NGS	Next Generation Sequencing
PGD	Prenatal Genetic Diagnosis

Acronym	Full Form
SNVs	Single Nucleotide Variants

Chapter One

Introduction

1.1 Background

Glanzmann Thrombasthenia (GT) is a rare autosomal recessive bleeding disorder caused by pathogenic variants in genes essential for platelet aggregation, resulting in impaired platelet function and disrupted hemostasis (Owaidah et al., 2019).

Platelets are blood components derived from bone marrow through the fragmentation of megakaryocyte cytoplasm. They play crucial roles in hemostasis, thrombosis, and inflammation. Platelets contribute to hemostasis by forming blood clots at sites of injury or bleeding through three main stages: adhesion, activation, and aggregation. Additionally, platelets feature a family of transmembrane heterodimeric glycoprotein receptors known as integrins, which consist of two distinct, non-covalently interacting α and β subunits. Among these, the αIIbβ3 integrin is the most prevalent (Repsold & Joubert, 2021).

Integrin αIIbβ3, also known as the platelet fibrinogen receptor, is expressed at around 80,000 copies on the surface of wild-type platelets. (van den Kerkhof et al., 2021) Abnormalities in integrin αIIbβ3 can disrupt blood hemostasis and result in bleeding disorders (A. T. Nurden, Pillois, et al., 2012).

Mutations linked to GT are highly diverse and can include nonsense, missense, and splice site variants, as well as small deletions, insertions, indels, or duplications in the ITGA2B and

ITGB3 genes. Among these, nonsense, missense, and splice site variants are the most frequently observed pathogenic mutations (Botero et al., 2020).

A qualitative or quantitative defect in the platelet glycoprotein (integrin αIIbβ3) as a consequence of genetic mutation in ITGA2B and/or ITGB3 genes causes the inherited disease of platelet dysfunction which called Glanzmann Thrombasthenia (A. T. Nurden, 2006).

GT is clinically classified into three types based on αIIbβ3 integrin expression at the platelet membrane. In Type I GT, the expression of integrin αIIbβ3 is either absent or at a level less than 5% of the wild-type amount, and this type is the most common among GT patients. In Type II GT, the expression of αIIbβ3 integrin is approximately 5-20% of normal levels. In Type III 'variant' GT, αIIbβ3 integrin is present in sufficient quantities ranging from 25–100%, but is qualitatively dysfunctional (A. T. Nurden et al., 2015).

Besides being an inherited platelet disorder, GT can also occur as an acquired condition where normally expressed αIIbβ3 integrins are inhibited. This acquired form of the disorder is known as acquired Glanzmann thrombasthenia. It is essential to distinguish between inherited and acquired GT. Typically, symptoms of inherited GT begin to appear a few days after birth, such as skin bruising, are observed across family lines, and are confirmed by mutations in the ITGA2B and/or ITGB3 genes (Sharma et al., 2011).

Studies on genotype-phenotype correlations in GT patients have shown that those with mutations in ITGA2B or ITGB3 are indistinguishable in terms of phenotype or bleeding severity. Most families have unique mutations, and even when family members share the same mutation, variability in clinical outcomes is observed. The recurrence of certain mutations suggests the presence of mutational hotspots (A. T. Nurden, Pillois, et al., 2012; A. T. Nurden et al., 2015).

Following the clinical diagnosis, various screening and confirmatory laboratory tests should be conducted, including platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT), bleeding time, light transmission aggregometry (LTA), and flow cytometry. To confirm the diagnosis, genetic sequencing of ITGA2B and ITGB3 should be performed to detect the specific mutations involved (Ahammad et al., 2020).

Managing GT patients is crucial to prevent severe bleeding and other complications. Platelet transfusion is considered the gold standard for hemostatic management of GT patients. However, recombinant activated factor VII (rFVIIa) is recommended for use in cases where

patients have platelet antibodies or are refractory to platelet transfusions. Additionally, antifibrinolytics, packed RBC transfusions, and hormonal therapy for adolescent females with GT may be used as part of the treatment (Poon et al., 2016).

Early diagnosis and management can improve the prognosis for GT patients and help reduce the risk of life-threatening complications (Sebastiano et al., 2010). Additionally, genetic counseling is crucial for families with members affected by GT.

1.2 Problem statement

In Palestine, Glanzmann Thrombasthenia (GT) poses a significant yet frequently overlooked public health challenge. Contributing factors include unclear prevalence of genetic variants, limited access to specialized care, insufficient awareness, and the absence of systematic screening. These issues lead to underdiagnosis and suboptimal management of this genetic disorder. Many patients with unexplained bleeding symptoms admitted to Palestinian hospitals often exhibit reduced platelet aggregation and have a family history of bleeding disorders. This results in a considerable burden on both the affected individuals and their families.

Addressing this challenge underscores the urgent need for improved screening and diagnostic protocols, enhanced access to specialized medical care, and greater awareness among both the public and healthcare providers. Such measures are essential to effectively manage GT and reduce its impact on the Palestinian population.

1.3 Study Aim

Aim: To confirm the diagnosis of the clinically diagnosed patient with Glanzmann Thrombasthenia.

1.4 Study Objectives

- To investigate the correlation between genotype and phenotype within the affected families
- Explore the feasibility of cascade screening for the genetic disorder

Chapter Two

Literature Review

2.1 Platelets

Platelets also known as thrombocytes, are the smallest type of blood cells. The average normal platelets counts in healthy individual is between 150×10^9 and 350×10^9 cell/L. Because platelets lack a nucleus, their lifespan is limited to 7 to 10 days after they are formed and released from the megakaryocyte (Gremmel et al., 2016; Josefsson et al., 2020).

Platelets structure consists of a plasma membrane encasing a cytoplasm that contains granules rich in bioactive molecules, such as clotting factors, enzymes, and signalling proteins. The cytoplasm is supported by a network of microtubules and actin filaments, which helps maintain the platelet's shape and support its functions. The outer membrane comprises glycoproteins, including GPIb and GPIIb/IIIa, essential for platelet adhesion and aggregation during processes like hemostasis and thrombosis. This specialized structure enables platelets to rapidly respond to vascular injury and play a key role in clot formation (Rondina & Zimmerman, 2019; Tomaiuolo et al., 2017).

In addition to their well-known roles in thrombosis and hemostasis, (Davì & Patrono, 2007) platelets are increasingly identified as key contributors to various other pathophysiological processes, including inflammation, atherogenesis, tumors growth and metastasis (Gay & Felding-Habermann, 2011), and antimicrobial host defense (Engelmann & Massberg, 2013). Platelet aggregation at improper sites can cause thrombosis and vessel blockage, which can also occur in the placenta and potentially causing miscarriage (Tsikouras et al., 2019).

2.1.1 Role of Platelets in Thrombosis and Hemostasis

The ability of activated platelets to adhere to an injured vessel wall and form aggregates was first documented in the 19th century (Brewer, 2006). Platelets are essential for vascular repair

and hemostasis. Their main function is to gather at sites of blood vessel damage and trigger clotting to prevent blood loss. In their resting state within the bloodstream, platelets are smooth and disc-shaped. When the vessel wall is damaged, exposing collagen and other subendothelial matrix proteins, platelets become activated and undergo a change in shape (Semple et al., 2011).

Activated platelets adhere to the injury site and attract more platelets, creating a hemostatic plug. The two main surface receptors on platelets, glycoprotein (GP) IIbIIIa (αIIbβ3 integrin) and GPIb, are vital for platelet adhesion and aggregation (Scridon, 2022). The GPIb complex plays a critical role in platelet tethering and adhesion to the damaged vessel wall by binding primarily to immobilized von Willebrand factor (VWF) (Quach & Li, 2020). The interaction between GPIb and VWF transmits intracellular signals to platelets, inducing a conformational change in the αIIbβ3 integrin on the platelet surface which subsequently binds fibrinogen (Fg). Fibrinogen cross-links adjacent platelets, resulting in platelet aggregation and the formation of a platelet plug (Litvinov et al., 2016; Xiao et al., 2004). as shown in Figure 1. However, inappropriate platelet aggregation can lead to severe conditions such as heart attacks or strokes (Bendas & Schlesinger, 2022).

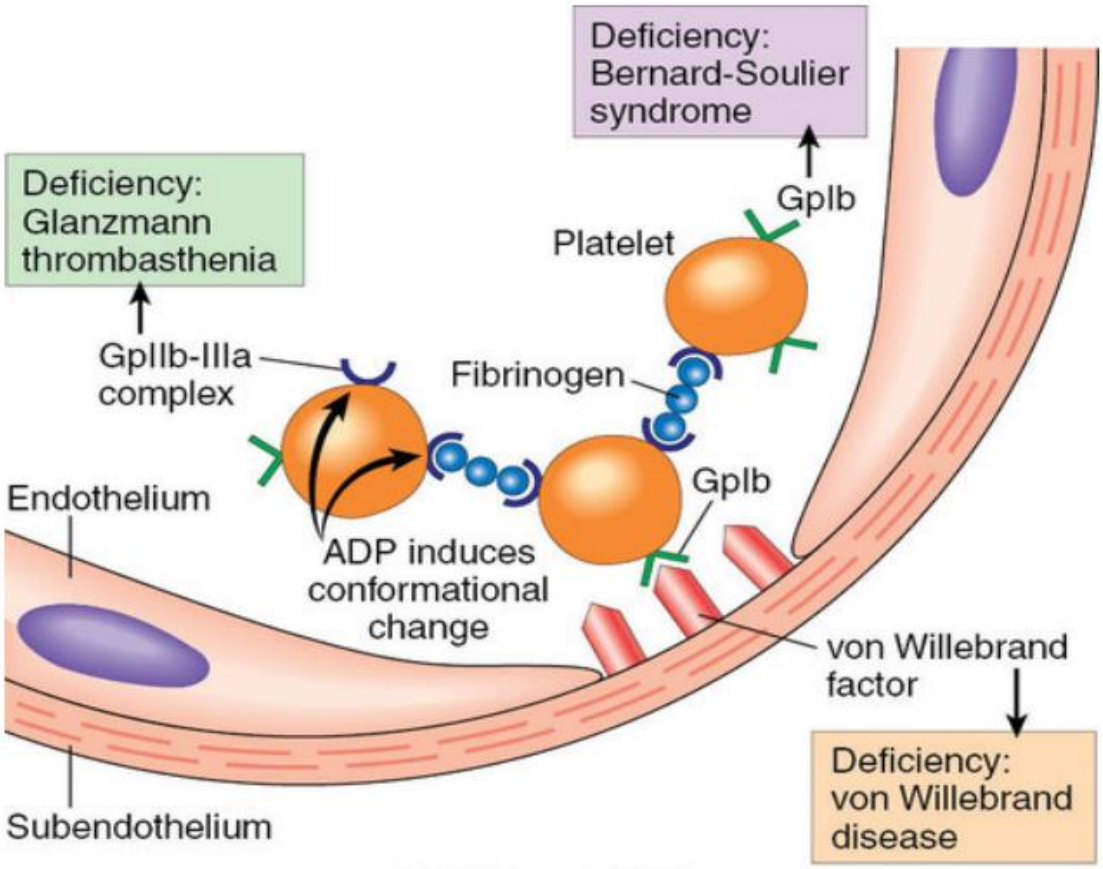

Figure 1: Platelet adhesion and aggregation at sites of vascular injury occur through a coordinated process. The interaction between GPIb and von Willebrand factor (VWF) initiates intracellular signaling within platelets, inducing a conformational change in the αIIbβ3 integrin on the platelet surface. This conformational change enables the integrin to bind fibrinogen, facilitating platelet aggregation. The accompanying figure illustrates the diseases associated with deficiencies in each receptor involved in this process. (Anon 2020).

2.2 Inherited platelet bleeding disorders

Inherited platelet bleeding disorders are generally categorized into two types: qualitative and quantitative defects. Quantitative defects refer to abnormalities in platelet count, where an increase in platelet numbers is known as thrombocytosis, and a decrease is referred to as thrombocytopenia. Qualitative defects are characterized by abnormal platelet function, which disrupts their normal physiological roles. Some platelet disorders may exhibit characteristics of both qualitative and quantitative defects for example Essential Thrombocythemia (ET). Generally, inherited platelets bleeding disorders lead to a range of bleeding symptoms, including mucocutaneous bleeding, epistaxis, easy bruising, menorrhagia, and bleeding following trauma or surgery (Kvernberg et al., 2021; A. Nurden & Nurden, 2011). In this project, the focus will be on Glanzmann thrombasthenia, as this disorder is the subject of this project.

2.3 Glanzmann Thrombasthenia

2.3.1 Overview

Glanzmann thrombasthenia (GT) is a rare autosomal recessive bleeding disorder of platelets, marked by a complete absence, partial deficiency, or functional impairment of the platelet membrane integrin receptor αIIbβ3 (glycoprotein IIb/IIIa). These defects lead to impaired or reduced platelet aggregation (A. T. Nurden et al., 2011). This integrin facilitates platelet aggregation in response to blood vessel injury by the binding of fibrinogen, VWF, and other ligands. When αIIbβ3 binds to these soluble adhesive proteins, it cross-links adjacent platelets, leading to the formation of platelet aggregates (Varga-Szabo et al., 2008).

The worldwide prevalence of GT is estimated to be about 1 in 1,000,000 people in the general population. However, the communities with high rates of consanguinity marriages, such as among Palestine, Jordan, French Gypsy, Manouche, southern Indian populations, and Ashkenazi and Iraqi Jews, the prevalence can be as high as 1 in 200,000 or even greater. GT has been observed in these populations with similar levels of consanguinity (Essawi, 2019; Krause & Graham, 2023; A. T. Nurden et al., 2011).

GT is classified into three types, based on the levels of αIIbβ3 expressed on the platelet surface: Type I, type II, and type III "Variant type". Type I GT is characterized by a complete absence or an expression level of αIIbβ3 integrin below 5% of normal, meanwhile type II GT features αIIbβ3 levels between 5% and 20% of the normal amount. The Variant Type is characterized by αIIbβ3 levels exceeding 20% of the normal level, but with reduced functional activity of the receptor (Botero et al., 2020; Krause & Graham, 2023; Mathews et al., 2021).

2.3.2 Historical background of GT

The first case of Glanzmann thrombasthenia was discovered in 1918 by pediatrician Eduard Glanzmann. He examined a seven-year-old girl in Switzerland who had a history of prolonged bleeding and whose blood failed to clot properly. Glanzmann termed this condition "hereditary hemorrhagic thrombasthenia" (Bellucci & Caen, 2002; Jin et al., 1996; Stevens & Meyer, 2002). Several decades later, in 1966, Caen and Cousin made a significant breakthrough in the physiological analysis of GT. They demonstrated that platelets from GT patients failed to aggregate in response to ADP(Adenine di-phosphate), thrombin, adrenaline, and collagen. Four years later, Caen identified 15 patients whose platelets exhibited reduced or absent aggregation and variable clot retraction. This distinction prompted Caen and his team to classify GT cases

into two groups: Type I and Type II (Caen et al., 1966). Type 3 GT was later identified in 1990 by Nurden and colleagues (George et al., 1990).

2.3.3 Clinical manifestations of GT

The bleeding phenotype is multifactorial, so the symptoms are variable. The majority of heterozygous carriers are asymptomatic, while homozygous or compound heterozygous patients manifest a diversity of clinical phenotypes, starting from severe mucocutaneous bleeding and skin bruising that seem to appear at ages earlier than other symptoms, with a mean age of diagnosis 1-year-old (Kannan & Saxena, 2009; Krause & Graham, 2023). Males with GT are supposed to be diagnosed as a consequence of post-circumcision haemorrhage, an additional common cause of bleeding during childhood is loss of primary teeth, other bleeding symptoms include purpura, epistaxis, gingival bleeding, menorrhagia, bleeding after dental procedures or surgeries (Bellucci & Caen, 2002; Solano et al., 2022). A common symptom in adolescent female patients with GT is heavy menstrual bleeding, this problematic menstrual bleeding may begin at menarche, and in some cases the bleeding stopped at almost 1 month from the onset of menarche (Kadowaki et al., 2021). The prevalence of heavy menstrual bleeding in GT female patients surpasses 9 per 10 cases leading to multiple hospital admissions and many blood transfusions (King et al., 2020; Lu & Yang, 2018). Other less frequent symptoms that associate with complications include hematuria, hemarthrosis and gastrointestinal hemorrhage. Intracranial haemorrhage and organ bleeding are very rare (Mathews et al., 2021; Solano et al., 2022). Due to the severity of this disorder, most patients are diagnosed in childhood, typically before the age of five. However, some individuals reach adulthood without experiencing severe bleeding episodes. Generally, the severity of bleeding, with the exceptions of menorrhagia and pregnancy-related bleeding, tends to decrease with age (Botero et al., 2020).

2.3.4 Diagnosis of GT

The Platelets Physiology Subcommittee of the International Society on Thrombosis and Haemostasis (ISTH), has provided guidelines for diagnosing GT and other inherited platelet function disorders (IPFDs). According to their protocols, initial laboratory tests should be conducted if the patient presents with clear clinical bleeding abnormalities. These tests may include a complete blood count (CBC), activated partial thromboplastin time (APTT), prothrombin time (PT), bleeding time and evaluations for von Willebrand disease, such as VWF antigen levels, ristocetin cofactor activity, and factor VIII coagulant activity. These tests are typically normal in cases of GT and other IPFDs which help exclude more common causes

of bleeding. If these results are inconclusive, further evaluation may involve platelet function studies or next-generation sequencing. Additional screening may include a blood smear, light transmission aggregometry (LTA), assessment of platelet granule release, and analysis of platelet surface glycoproteins by flow cytometry (Krause & Graham, 2023).

The diagnosis of GT is usually overlooked because it shares many clinical and laboratory features with other acquired platelet disorders. It's crucial to consider GT in the differential diagnosis by closely analyzing the patient's medical history (such as unprovoked bruising, severe bleeding following minor trauma, or bleeding episodes), family history (including possible consanguinity), and clinical presentation (such as examining for purpura and ecchymoses). When diagnosing GT, selecting the appropriate laboratory tests is crucial. A normal platelet count in a routine blood smear does not exclude GT, as patients typically have a normal platelet count. The CBC may appear normal or indicate iron deficiency. PT and PTT are also normal. However, a prolonged bleeding time (over 10 minutes) suggests the need for further investigation (Solh et al., 2015).

Light transmission aggregometry (LTA) is widely recognized as the gold standard for diagnosing platelet function. This method involves monitoring centrifuged platelet-rich plasma samples before and after the introduction of an agonist (such as ADP, collagen, epinephrine, arachidonic acid, ristocetin, thrombin receptor-activating peptide, and thromboxane A2 mimetic). The test evaluates various parameters, including shape change, lag phase, percentage of aggregation, slope of aggregation, and deaggregation. LTA is particularly specific for diagnosing GT, as platelet aggregation fails to occur with any agonist except ristocetin, which preserves the reaction. Despite its specificity, LTA is time-consuming and heavily reliant on skilled personnel, requiring experienced laboratories. Additionally, obtaining platelet-rich plasma can be challenging in patients with thrombocytopenia and in pediatric patients, making the test more difficult under these conditions (Krause & Graham, 2023; Solh et al., 2015).

Flow cytometry is a valuable tool in diagnosing GT, as the condition involves a deficiency or dysfunction of glycoprotein receptors on platelets. This technique measures the density of specific platelet receptors using various monoclonal antibodies, focusing on the deficient αIIbβ3 integrin complex. The integrin components are identified as CD41 (alpha IIb) and CD61 (beta 3), while CD42b is a glycoprotein critical for binding VWF. In flow cytometric analysis, CD41 and CD61 levels are typically significantly reduced or absent, whereas CD42b levels remain normal. This pattern, combined with the detection of deficient or non-functional αIIbβ3,

is indicative of a GT diagnosis. However, it is important to recognize that certain cases with defective integrins may still exhibit normal expression levels (George et al., 1990; Gresele & Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015; Iqbal et al., 2016).

The ISTH guidelines recommend molecular genetic testing for cases where a diagnosis remains unclear after standard laboratory studies. The most comprehensive method for diagnosing GT is through mutation analysis. This involves sequencing the genomic DNA of the 45 exons that make up the αIIbβ3 complex, along with the splice sites of the ITGB3 and ITGA2B genes. In general, diagnosing GT is characterized by a normal platelet count and prolonged bleeding time. Platelets fail to aggregate under the conditions used in LTA, a finding uniquely indicative of GT (Gresele & Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

2.3.5 Managements of GT

Despite the variability in bleeding frequency and severity among patients with GT, platelet transfusions are the standard treatment for most cases. However, repeated transfusions can increase the risk of alloimmunization against αIIbβ3, leading to platelet refractoriness. Other treatment options include compression, fibrin glue, and gauze soaked in tranexamic acid. Epistaxis can be managed with compression, nasal packing, fibrin sealants, cauterization, and tranexamic acid. Menorrhagia is typically controlled with high doses of progesterone and ongoing use of oral contraceptives. Severe bleeding from trauma and gastrointestinal bleeding often requires blood component transfusions (A. T. Nurden, Freson, et al., 2012; Poon et al., 2015).

Recombinant activated factor VII (rVIIa) is frequently utilized to treat both inherited and acquired platelet disorders. For patients with GT, rVIIa has been applied to manage bleeding episodes with varying degrees of success. It is especially beneficial for those who have developed alloimmunization against αIIbβ3 and have platelet refractoriness (A. T. Nurden, Freson, et al., 2012; Poon et al., 2016).

Furthermore, bone marrow transplantation (BMT) has been established as a curative option for several GT patients, particularly in cases of recurrent severe bleeding and platelet refractoriness caused by anti-αIIbβ3 antibodies. In such situations, allogenic BMT remains the only therapeutic option that has been successfully reported (Fiore et al., 2018; Wiegering et al., 2013). Another promising therapeutic option that is still in the experimental stages is gene therapy, by trials to increase αIIbβ3 expression in GT patients (Mathews et al., 2021; Sullivan et al., 2014).

2.3.6 Prognosis of GT

GT is a serious bleeding disorder where life-threatening or fatal bleeding can occur spontaneously or in response to invasive procedures, severe trauma, or childbirth. The wide range of mutations causing GT leads to varying clinical presentations, differing in severity (Lee & Poon, 2018; Poon et al., 2016). With proper preparation and coordination by the healthcare team, patients can manage the condition effectively, as demonstrated by a 52-year-old man with GT who successfully underwent open aortic valve replacement (Sheikh et al., 2014). Generally, bleeding episodes in GT are less life-threatening compared to those seen in inherited coagulation disorders (Iqbal et al., 2016).

2.3.7 Complication of GT

Patients with GT who experience chronic mild bleeding may develop iron deficiency anemia, although most complications associated with GT stem from its treatment rather than the disease itself (Lee & Poon, 2018).

Approximately 17% of GT patients receiving leukocyte-reduced platelet transfusions develop anti-HLA (Human leukocyte antigen) antibodies, and this percentage increases significantly if non-leukocyte-reduced platelets are used. Among those who develop anti-HLA antibodies, about half become refractory to future platelet transfusions (Lee & Poon, 2018; Poon et al., 2016). Additionally, patients with specific mutations may produce antibodies against the alpha IIb beta 3 integrin surface antigens. GT patients can also develop anti-platelet antibodies, which can cross the placenta during pregnancy. Studies of pregnant women with GT and anti-platelet antibodies have shown a range of fetal effects, from thrombocytopenia to neonatal death due to intracranial haemorrhage (Barg et al., 2018).

2.3.8 Molecular basis of GT

2.3.8.1 ITGA2B and ITGB3 genes

GT is a platelet aggregation disorder caused by quantitative or qualitative defects in the integrins αIIb and β3. These integrins are encoded by the ITGA2B and ITGB3 genes.(Botero et al., 2020) The ITGA2B and ITGB3 genes are located on chromosome 17, at positions 17q21.31 and 17q21.32, respectively. The ITGA2B gene spans 65 kbp and consists of 30 exons, with 218 mutations documented in the Human Gene Mutation Database (HGMD) causing GT. Whereas, the ITGB3 gene spans 17 kbp and contains 15 exons, with 143 recorded mutations (Mathews et al., 2021). A greater percentage of pathogenic variants are found in ITGA2B, likely due to its larger size, consisting of 30 exons, compared to ITGB3, which has 15 exons. The clinical phenotypes associated with mutations in either gene are indistinguishable (A. T. Nurden et al., 2011).

Pathogenic nonsense, missense, and splice site variants are frequently found in these genes, while large deletions and duplications are rare but have been documented (A. T. Nurden & Pillois, 2018). Pathogenic missense variants can disrupt biosynthesis subunit in megakaryocytes, obstruct the transport of pro-αIIbβ3 complexes from the endoplasmic reticulum (ER) to the Golgi apparatus, or prevent the export of mature complexes to the cell surface. Many of these variants impact the β-propeller region of αIIb and the epidermal growth factor domains of β3 (Richards et al., 2015). Figure 2 shows the schematic representation of the ITGA2B gene, which illustrates a wide spectrum of mutations that cause GT (A. T. Nurden, Pillois, et al., 2012). Figure 3 present the schematic representation of the ITGB3 gene, which shows a wide spectrum of mutations that lead to GT. This data was compiled from a comprehensive literature survey and consultations with the GT database. Missense mutations causing GT are widely distributed across both genes, whereas variant forms are more likely associated with ITGB3 gene defects (A. T. Nurden, Pillois, et al., 2012).

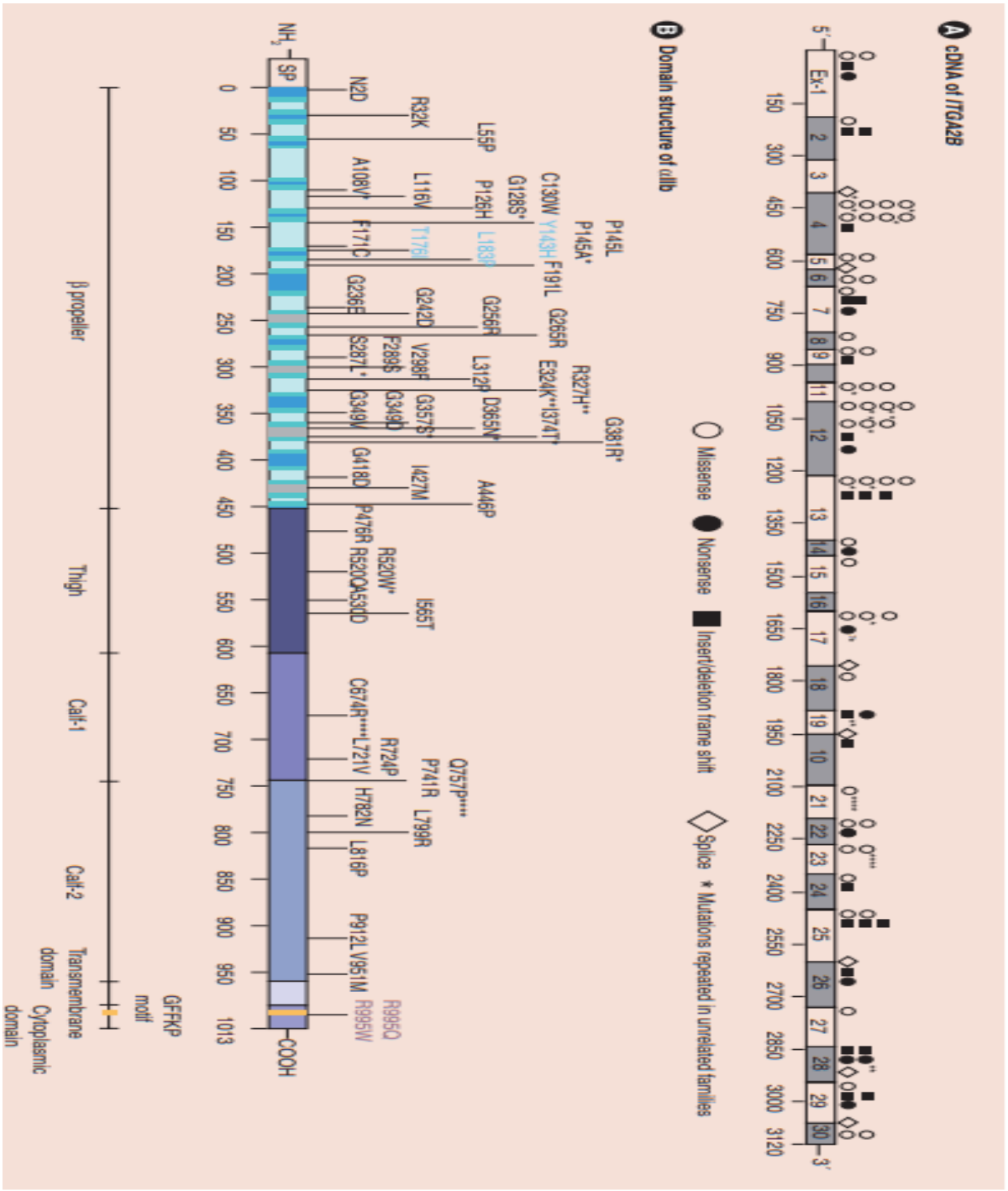

Figure 2: The schematic representation of the ITGA2B gene highlights a diverse array of mutations associated with GT. In panel (A), the mutations are depicted using symbols corresponding to their type and are distributed throughout the gene. Panel (B) provides a detailed view of missense mutations, identified by their single-letter amino acid codes, aligned with the structural domains of the subunit. Mutations that primarily impair αIIbβ3 expression are shown in black, while those in blue represent variant forms with nonfunctional αIIbβ3. Mutations depicted in mauve are linked to macrothrombocytopenia. Asterisks denote specific points of interest within the gene (A. T. Nurden, Pillois, et al., 2012).

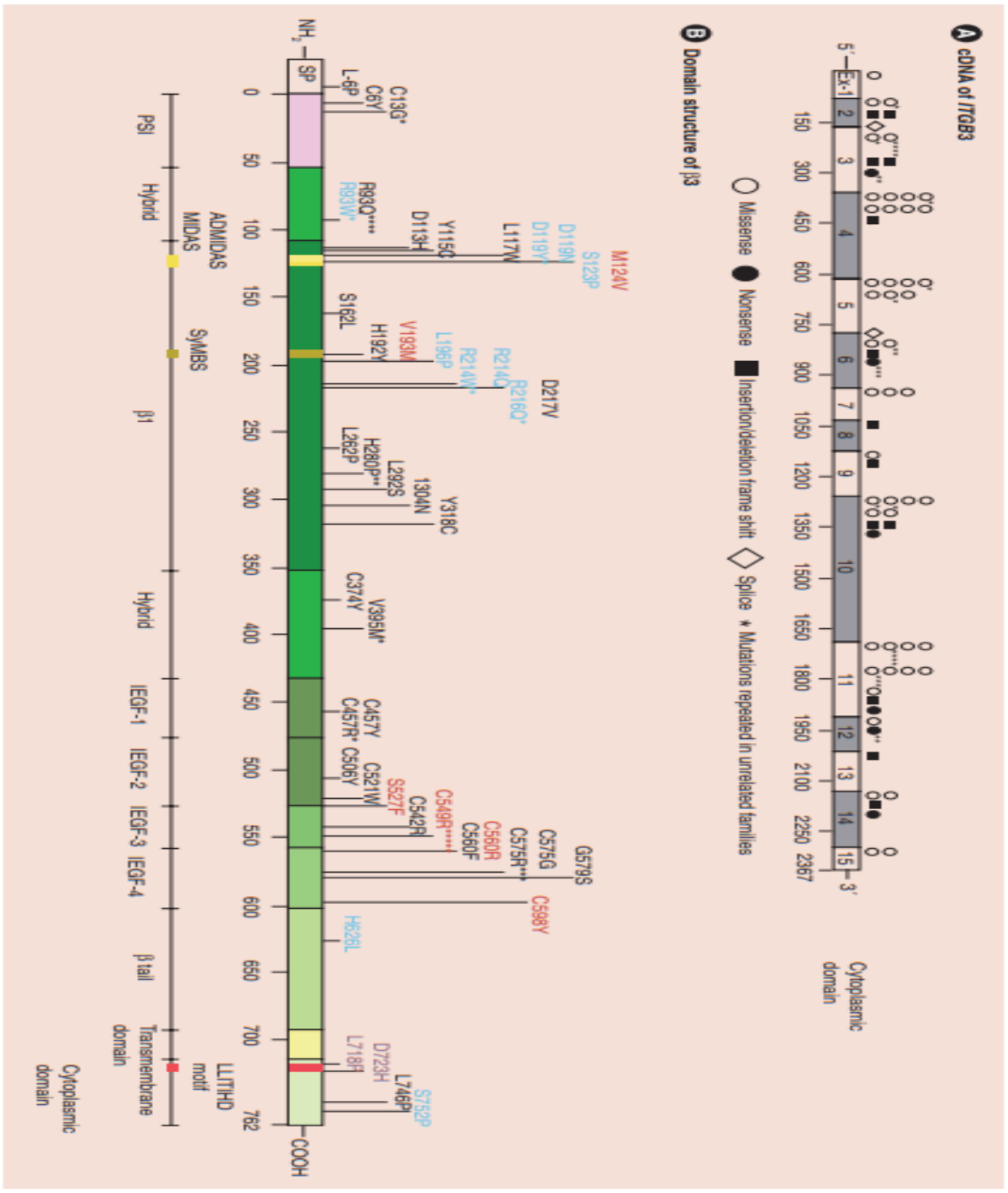

Figure 3: The schematic representation of the ITGB3, showing a broad spectrum of mutations associated with GT. In panel (A), the mutations are represented by symbols corresponding to their type, with their distribution spanning the entire gene. In panel (B), missense mutations are detailed using single-letter amino acid codes and are aligned with the structural domains of each subunit. Mutations shown in black predominantly impair αIIbβ3 expression, while blue mutations indicate variant forms with nonfunctional αIIbβ3. Mutations in red signify substitutions that activate the integrin, and mauve mutations are associated with macrothrombocytopenia. Asterisks highlight the frequency of reported mutations in apparently unrelated families (Alan T Nurden et al. 2012).

2.3.8.2 Platelet receptors αIIbβ3 integrin

The α-subunit of αIIbβ3 is composed of 1008 amino acids and includes a cytoplasmic tail, a transmembrane domain, two calf domains, a thigh domain, and a head (β-propeller domain) which serves as the primary domain for ligand binding (Anderson et al., 2014; Ma et al., 2007). Figure (4) The β-subunit of αIIbβ3 is composed of 772 amino acids and includes a cytoplasmic tail, a transmembrane domain, a membrane-proximal β-tail domain (βTD domain), four epidermal growth factor (EGF) domains, a hybrid domain and a β3A domain (Ma et al., 2007).

The β3 subunit is made up of a single polypeptide chain stabilized by disulfide bonds. Upon activation, it undergoes rearrangement with a disulfide exchange reaction, resulting in the formation of free thiols. This integrin contains multiple binding sites for divalent cations, which are essential for ligand binding. The cytoplasmic domains of both subunits create a binding site for intracellular cytoskeletal molecules. The interaction between the α- and β-subunits is mediated by the β-propeller domain and the β3 domain (Jordan & Gibbins, 2006; Levin et al., 2013). Nitric oxide and prostacyclin (PGI2), made by healthy endothelial cells, prevent the activation of platelets in their resting state (Dorris & Peebles Jr., 2012).

Before platelet activation, αIIbβ3 exists in its resting state with non-active bent conformation, which has a low affinity for ligand binding. At the time of platelet activation, αIIbβ3 exposed to physiological agonists such as ADP, epinephrine, collagen and thrombin, thus the integrin changes its conformation and straightens (to its active form), which in turn causes the regions that are essential for the binding of Fg and other soluble adhesive proteins to be exposed and allows ligand binding. In response, fibrinogen will bind, facilitating the straightening of αIIb and β3 subunits and other mechanisms that eventually enable platelet to spread and fibrin clot stabilization. Any abnormalities in integrin αIIbβ3 will affect blood haemostasis and lead to a bleeding disorder termed as GT (Bye et al., 2016).

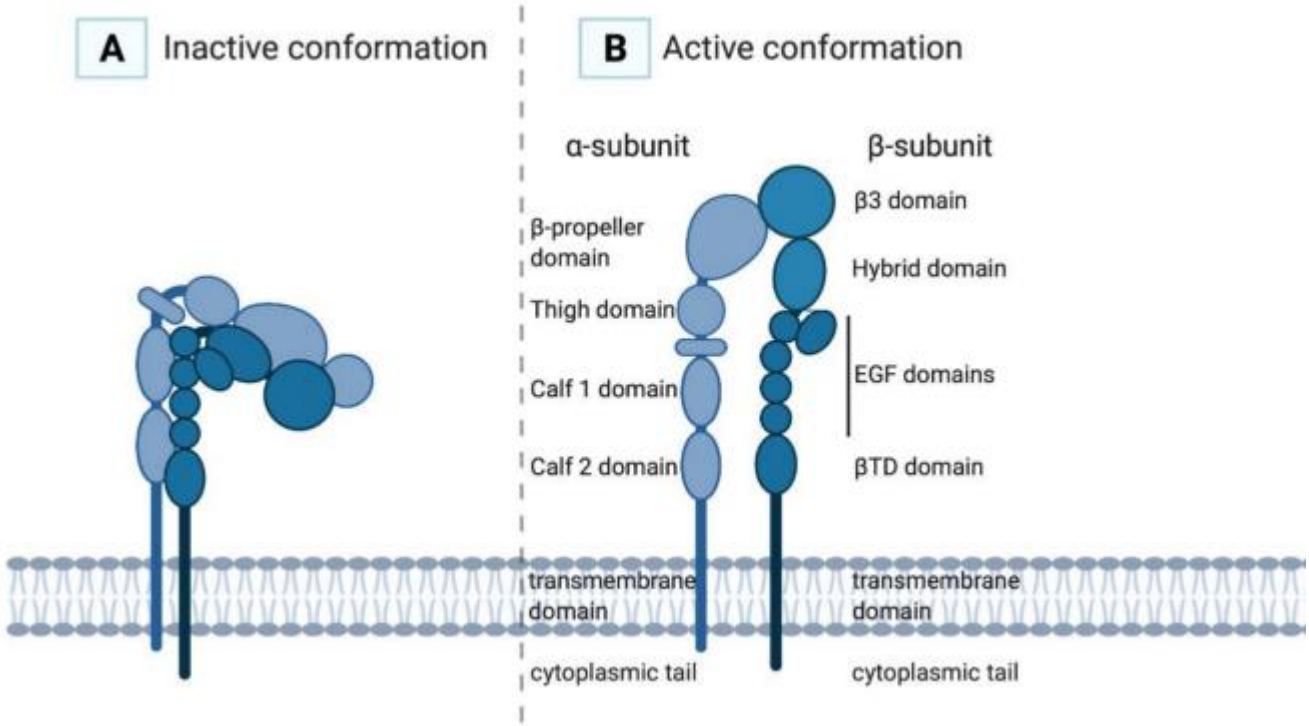

Figure 4: (A) The inactive conformation of the integrin αIIbβ3, its ligand-binding site is not readily accessible to ligands. In contrast, (B) in the active conformation, the ligand-binding site becomes exposed and exhibits a high affinity for its ligands (A. T. Nurden et al., 2013).

The formation of the αIIbβ3 complex in the ER is a prerequisite for its transport to the Golgi apparatus and subsequent incorporation into the platelet membrane. Defects in αIIbβ3 biogenesis can cause the αIIb protein to be retained in the ER or degraded rapidly. Uncomplexed β3 can either bind to αv to form the αvβ3 receptor (vitronectin receptor) or be rapidly degraded. Consequently, defects in ITGA2B and/or ITGB3 can disrupt the normal synthesis of αIIbβ3, leading to insufficient integrin expression on the platelet membrane and resulting in Glanzmann thrombasthenia (A. T. Nurden et al., 2013).

Chapter Three

Materials and Methods

3.1 Subject selection

The Palestinian Ministry of Health and the Al-Quds University ethical committee approved this research. The Ministry of Health permitted us to visit Hebron Governmental Hospital (Princess Alia Hospital) in Hebron, collect samples and data from patients who attend the Haematology department and perform some clinical laboratory tests in the hospital laboratory.

Samples were collected according to some inclusion and exclusion criteria. The inclusion criteria:

1. Patients with lifelong bleeding tendency characterized by normal platelet count, prolonged bleeding time, normal prothrombin time (PT), normal activated partial thromboplastin time (APTT).
2. The primary symptoms include a history of mucocutaneous bleeding, frequent nosebleeds (epistaxis), easy bruising, gum bleeding (gingivorrhagia), and in girls, heavy menstrual bleeding at menarche, menometrorrhagia, and postpartum hemorrhage. Additional symptoms may include gastrointestinal (GI) bleeding, excessive bleeding following invasive procedures, spontaneous bruising, or severe bleeding episodes after minor trauma.
3. Or family history or immediate family member of GT patient.

Patients with another reason of thrombasthenia, thrombocytopenia, or with acquired thrombasthenia caused by autoimmune disorders or medications were excluded.

Four families met our inclusion criteria and agreed to participate in the study. However, due to budgetary constraints, we selected one family to initiate the project. From the selected family thirteen members agreed to participate in our study; each given a code (e.g., GTK01, GTK02,

GTK03, etc). Among this family, three participants met all the inclusion criteria and clinically diagnosed as GT, while the remaining ten were immediate family members. We selected one of the three GT diagnosed patients as our proband based on the severity of her symptoms, the frequency and duration of bleeding episodes, and the longest recorded bleeding time (approximately 16 minutes).

A questionnaire was filled out to get the essential information about the demographic, personal and clinical data for each patient (see appendix 2). Written informed consent was obtained from each participant for blood sample collection, conducting genetic studies, and publishing the research results. (see appendix 1).

Personal and clinical data collected: medical history, age, gender, consanguinity and type of bleeding symptoms, i.e. epistaxis, cutaneous bleeding, gum bleeding, gastrointestinal bleeding, bleeding at circumcision (in males), bleeding at dental extraction, muscle hematoma, hematuria and menorrhagia.

We also selected a control group of twelve individuals from the same Palestinian population to assess gene expression in healthy, normal individuals who have no history of bleeding episodes or family history of inherited bleeding disorders.

We faced challenges in obtaining samples and encouraging participants to take part in our study due to the stigma associated with inherited diseases and the fear of unexpected results. Additionally, another challenge was reaching the patients to collect the blood samples.

3.2 Blood sampling

Peripheral blood was collected from participants in three tubes: Ethylenediaminetetraacetic acid (EDTA), plain, and sodium citrate. The samples were processed on the same day for CBC, biochemistry, and coagulation tests. Additionally, whole blood in an EDTA tube was divided into aliquots and stored at -20°C for DNA extraction. For the control group, blood samples were collected in EDTA tubes.

3.3 Laboratory tests measurements

A bleeding time test was performed for the participant in this study using the Duke method. CBC test (Haemoglobin, haematocrit, Mean Corpuscular Volume, Mean corpuscular haemoglobin and platelet), coagulation tests (PT, APTT, INR and fibrinogen) and some biochemistry tests (ferritin, iron, total iron binding capacity (TIBC), glucose, creatinine, blood urea nitrogen (BUN), uric acid, serum lights and liver profile; Aspartate aminotransferase (AST) and Alanine Transaminase (ALT).

3.4 Whole exome sequencing (WES) test

The WES test was performed in Istishari Arab Hospital, Ramallah, at the molecular and genetics laboratory, for the selected proband. WES was then conducted, which included the analysis of CNVs and the mitochondrial genome. DNA quantification was carried out using qubit v.3, and its quality was confirmed via gel electrophoresis.

For library preparation, the TruSeq Capture Exome Kit (Illumina) was utilized, enabling coverage of 45 Mb of exonic content. This kit's probe set was constructed to enrich 214,405 exons. Subsequently, sequencing was performed on the NextSeq 500, and the reads were aligned to the reference human genome (h19) using the BWA aligner. Prior to variant calling with GATK (Genome Analysis Toolkit), the BAM-formatted mapped reads underwent several preprocessing steps, including the elimination of PCR duplicates, realignment around indels, and base quality recalibration.

The last list of variants was annotated using ANNOVAR with data from various databases, involving minor allele frequency (MAF) information like PopFreqMax and variant effect predictors such as SIFT, PolyPhen-2, and REVEL.

To enhance reliability, variants with low coverage, synonymous changes, those predicted to be benign by SIFT, PolyPhen-2, and REVEL, and those with a MAF > 1% in gnomAD, PopFreqMax, and Palestinian in-house database were filtered out.

3.5 Primers design and Housekeeping Genes

Depending on WES result, primers for the segregation analysis of the ITGA2B gene were designed using Primer3 software. Four pairs of primers were used to amplify the ITGA2B gene. The first pair was designed for exon 7, and the second pair for exon 9. Both of these pairs included intronic regions in their sequences. Additionally, two more pairs of primers were designed specifically for exonic regions, excluding introns. The third pair targeted exon 4, while the fourth pair targeted exon 7. The sequences of all primers used are listed in Table1.

Several housekeeping genes, including RPPH1, ABL, and ZNF80, were used in this study. The sequences of these housekeeping genes are provided in Table 1.

Table 1: Forward and reverse primers utilized for the amplification of the ITGA2B gene and the housekeeping genes used in this study.

Exon/ housekeeping gene	Sequence	
Exon7	Exon7-F	GACACGTGCCACAAAAGGAT
	Exon7-R	TCTTAGGTACGTGCCCATCC
	Amplifying a 166 bp	
Exon9	Exon9-F	AGAAAGGGTGGTTTGGTGGA
	Exon9-R	GCTCTCCCTATCGCCAGAAT
	Amplifying a 170 bp	
Exon4	Exon4-F	GCTGAGAAGACGCCCGTA
	Exon4-R	CGTAAATGCGGCTCAGGG
	Amplifying a 103 bp	
Exon7	Exon7-F	CCAGTTGCGGATATTTTCTCGA
	Exon7-R	CCGTCGAAGTACTCTGGGTT
	Amplifying a 104 bp	
RPPH1	RPPH1-F	GTAAAACGACGGCCAGTCCGCGATATTGAGCTCCGAA
	RPPH1-R	AACAGCTATGACCATGGGTACCTCACCTCAGCCATT
ABL	ABL1_F	TCTGAGGTCTGCTGCAAAGG
	ABL1_R	GGGGACATGCCATAGGTAGC
ZNF80	ZNF80_F	CTGTGACCTGCAGCTCATCCT
	ZNF80_R	TAAGTTCTCTGACGTTGACTGATGTG

3.6 DNA extraction

Genomic DNA was extracted from EDTA whole blood samples of 13 participants and 12 controls using the Genomic DNA Mini Kit (Blood/Cultured Cell), following the manufacturer's instructions (Epicenter). The concentration and purity of the extracted DNA were assessed using a Nanodrop spectrophotometer, with a DNA purity value of >1.7.

3.7 Real-time Polymerase chain reaction (RT-PCR) test

For our participants and control group duplication events were validated by quantitative real-time PCR on genomic DNA, using primers targeting sequences within the suspected copy number variation (CNV) region. Amplification was normalized against three reference genes: ZNF80, RPPH, and ABL. Each qPCR reaction included 10 ng of genomic DNA, 200 nM of each primer, and 10 µl of Fast SYBR Green master mix (Life Technologies, Grand Island, NY). The qPCR amplification was performed on a BioRad system following a standard thermocycling protocol: an initial denaturation at 95°C for 3 minutes, 40 cycles of 95°C for 20 seconds, and 60°C for 1 minute, followed by a melting curve analysis. Each sample was run in triplicate. Variations in genomic sequence abundance were determined using the $2-\Delta\Delta CT$ method, with error estimation performed through standard error propagation techniques. The results were confirmed at Isteshari Hospital laboratory.

3.8 Analysis of RT-PCR data

The patient samples, control samples, and housekeeping genes were coded and entered into Excel program for analysis. This process involved calculating the copy number variant (CNV) based on the following steps: determining the ct (Cycle threshold) value, calculating the ct mean, Δct, ΔΔct, and finally, the fold change.

Calculation done by using these equations:

1. Δct = ct value of target gene – ct value of endogenous control gene.
2. ΔΔct = Δct value of sampels – average Δct of controls
3. Fold chain (FC) = 2 ^ - ΔΔct
4. CNV = 2 FC (Sicko et al., 2022; Zampaglione et al., 2020)

Chapter Four

Results

4.1 GT Proband Medical History

GTK04 is a 16-year-old female who first presented with unusual skin bruising at one month of age. As her first year progressed, she experienced increasing bruising, along with episodes of epistaxis and mucocutaneous bleeding. Throughout her childhood, she also developed throat bleeding following throat inflammation, gastrointestinal bleeding, and excessive bleeding from minor wounds and dental procedures. The latter required blood transfusions, including packed RBCs and platelets, due to her hemoglobin level dropping to 6 mg/dL.

During puberty, she experienced severe bleeding following menarche, necessitating hospital admission and transfusions of packed RBCs and platelets. Subsequently, nearly every menstrual cycle was marked by severe bleeding, leading to multiple hospitalizations and blood transfusions. At age 14, she developed an ovarian cyst that ruptured within her uterus, causing severe bleeding and a hemoglobin drop to 5 mg/dL. Many units of packed RBCs and platelets were transfused to stabilize her condition and control the bleeding.

A review of the family history showed that the patient's sister experiences similar but less severe symptoms, while one of the patient's nephews exhibit comparable symptoms as well (All first-degree relatives with similar symptoms were included in our study). Notably, the patient's parents are first cousins, confirming the presence of consanguinity. This patient met all of our inclusion criteria and was chosen as the proband for our study.

4.2 Identification of the variant through WES

With the help of the analysis of WES data, which included CNVs and the mitochondrial genome, we identified duplication of uncertain significance (VUS) in the ITGA2B gene:

NM_000419.5. This finding supports our clinical diagnosis of suspected autosomal recessive GT. No other variants with clinical significance related to the GT phenotype were identified.

4.3 Interpretation of the ITGA2B variant

The duplication in ITGA2B spanning exons from 3 to 12, identified through WES and confirmed by RT-PCR. This duplication is suspected to be the cause of GT in our patients and appears to be a novel mutation, as it has not been previously documented. However, further studies are required to confirm its pathogenicity in the future.

4.4 Laboratory test results

Some of the laboratory test results for the proband and her family members are presented in Table 3.

The laboratory results reveal that the proband (GTK04) and her sister (GTK03) share similar abnormalities, including prolonged bleeding time (>10 minutes), normal platelet count, PT and APTT. The proband's parents (GTK01 and GTK02) exhibit normal bleeding times, PT and platelet count, with elevated APTT levels. One nephew (GTK05) shows prolonged bleeding time, elevated APTT, and slightly elevated platelet count. Another nephew (GTK06) also has prolonged bleeding time and elevated APTT, with normal platelet count and PT.

Other family members (GTK07–GTK13) generally have normal bleeding times, PT, and platelet count, with some variations in APTT, particularly elevated levels in GTK08 and GTK10, warranting further investigation. Haemoglobin levels show mild variation across the family, with some members exhibiting low levels.

Table 2: Summary of selected lab test results for our study subjects.

Test / Code	*Bleeding Time*	*Haemoglobin (Current)*	*Platelet*	*PT*	*APTT*	*INR*
GTK04	>10	10.5	344	14.6	32	1.13
GTK01	2	9.5	274	12.7	46.1	0.98
GTK02	3	14.6	239	13.4	40.5	1.03
GTK03	>10	9.6	272	11.2	34.7	0.78
GTK05	>10	9.9	438	15.3	53.8	1.18
GTK06	>10	8.5	327	15	41.3	1.17
GTK07	1	13.3	412	13.8	40.1	1.06
GTK08	3	12.9	225	13.0	38.9	1.00
GTK09	2	15.2	200	12.6	34.6	0.97
GTK10	3	15.5	193	12.6	42.3	0.97
GTK11	2	12.4	296	13.8	36	1.06
GTK12	1	12.5	211	13.7	37.3	1.06
GTK013	2	13.9	273	16	31.4	1.24

4.5 Family pedigree

A family pedigree for GT participants was created based on the clinical diagnosis and the symptoms reported by each participant. It includes the selected family members who took part in our study. The pedigree shows three patients: K3, K4, and K6. Additionally, it includes eight carriers: K1, K2, K7, K8, K10, K11, K12, and K13, and two normal individuals: K5 and K9. The other family members declined to participate in our study. This pedigree is presented in Figure 5.

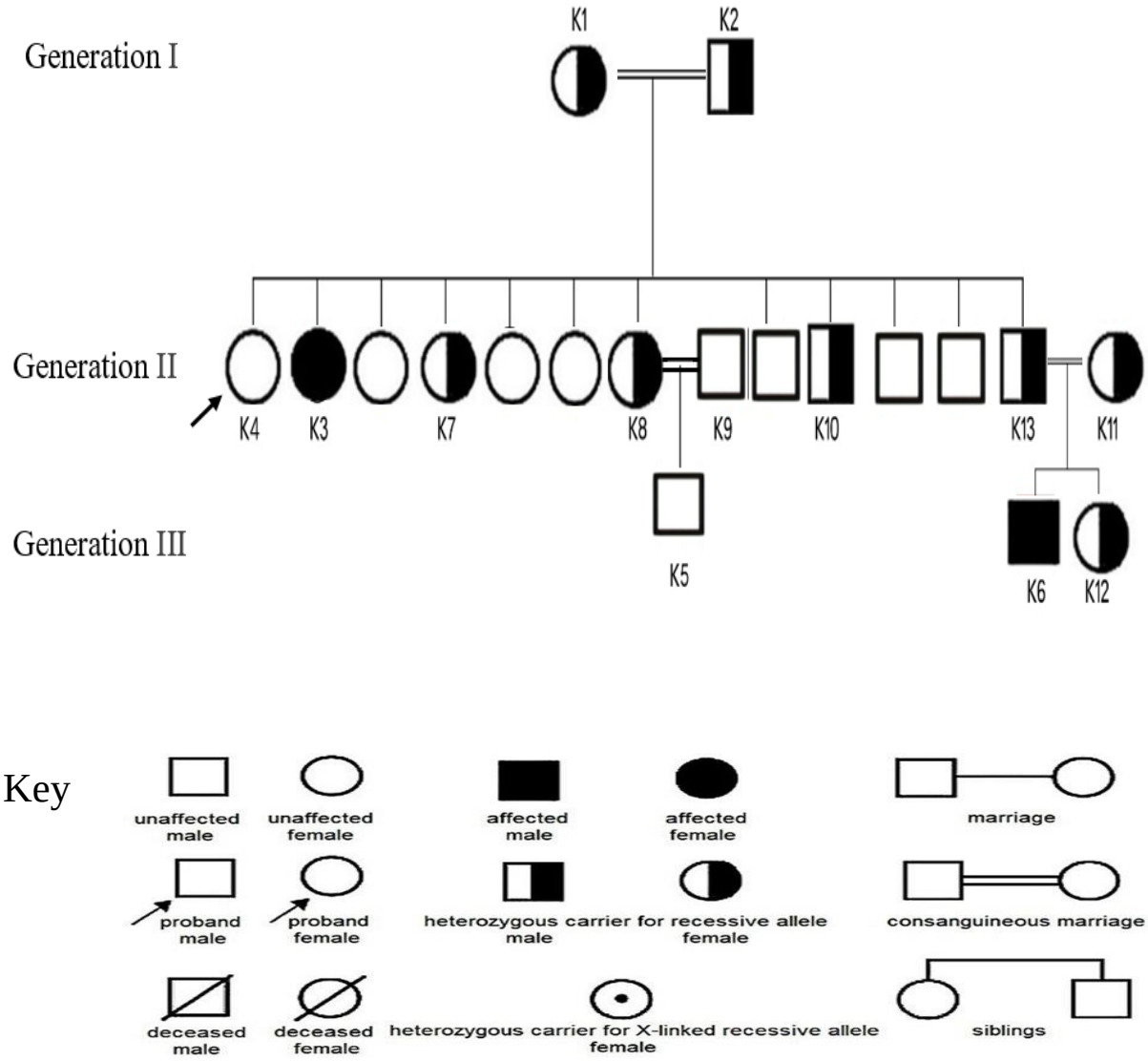

Figure 5: The pedigree of the participating family members is shown in the diagram, which categorizes the participants into patients, carriers, and normal individuals.

4.6 RT-PCR results

Following the WES findings, RT-PCR was performed using various primers and housekeeping genes. CNV was calculated using the equations detailed in the previous chapter, and the results are presented in the following figures.

Figure 6 illustrates the CNV of Exon-9 relative to the housekeeping gene RPPH for both control samples (NC10, NC11, and C1–C10) and participant samples (K1–K13). In the control group, CNV values remain consistent, clustering around one. However, in the participant group, CNV values vary widely. Notable increases are observed in K3 and K4, suggesting significant

amplification in these individuals. K6 also shows elevated CNV, although less pronounced than K3 and K4. Moderate CNV increases are observed in participants K9, K10, K11, and K13. Interestingly, the proband's parents (K1 and K2) exhibit CNV values close to 1, similar to the control group, which appears inconsistent with expectations and warrants further investigation.

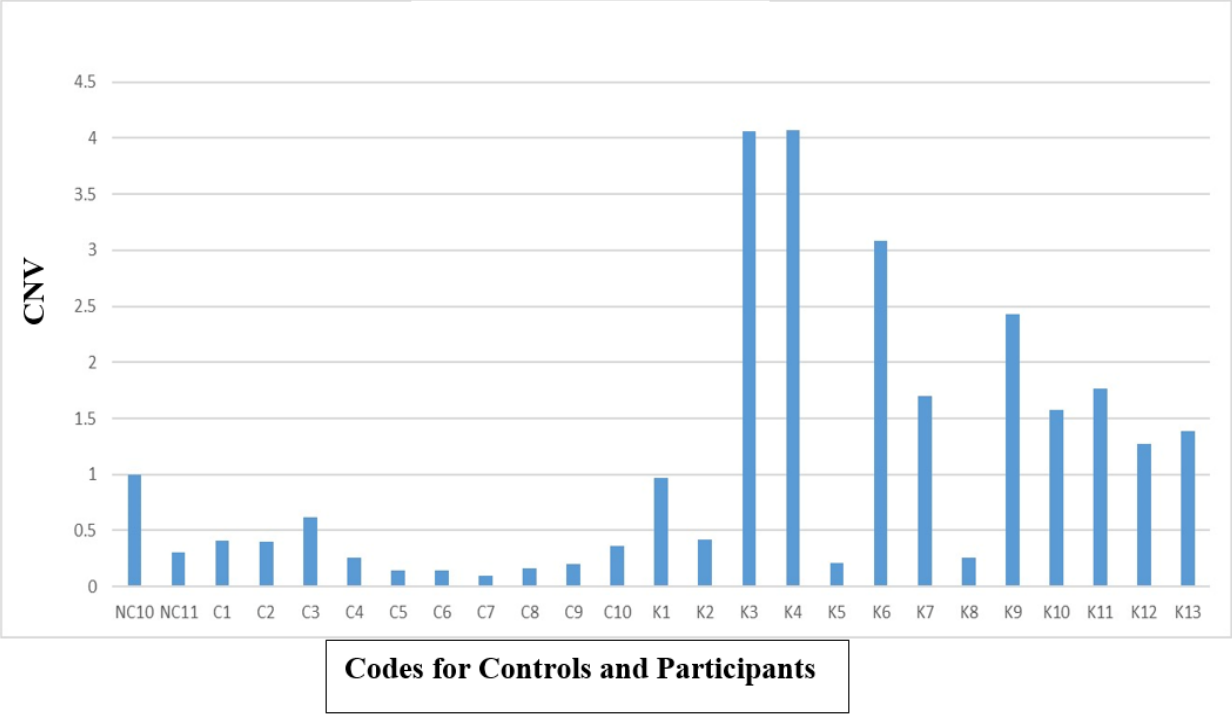

Figure 6: CNV for the 12 control samples and 13 subjects.

We then proceeded with our work using two pairs of primers, which include intronic regions in their sequences, targeting exons 7 and 9, with ZNF80 serving as the housekeeping gene. As illustrated in Figures 7 and 8, the CNV values typically ranged from 0.5 to 2. Notably, our proband K4 showed CNV values of 1.8 and 2, which we found to be unsatisfactory.

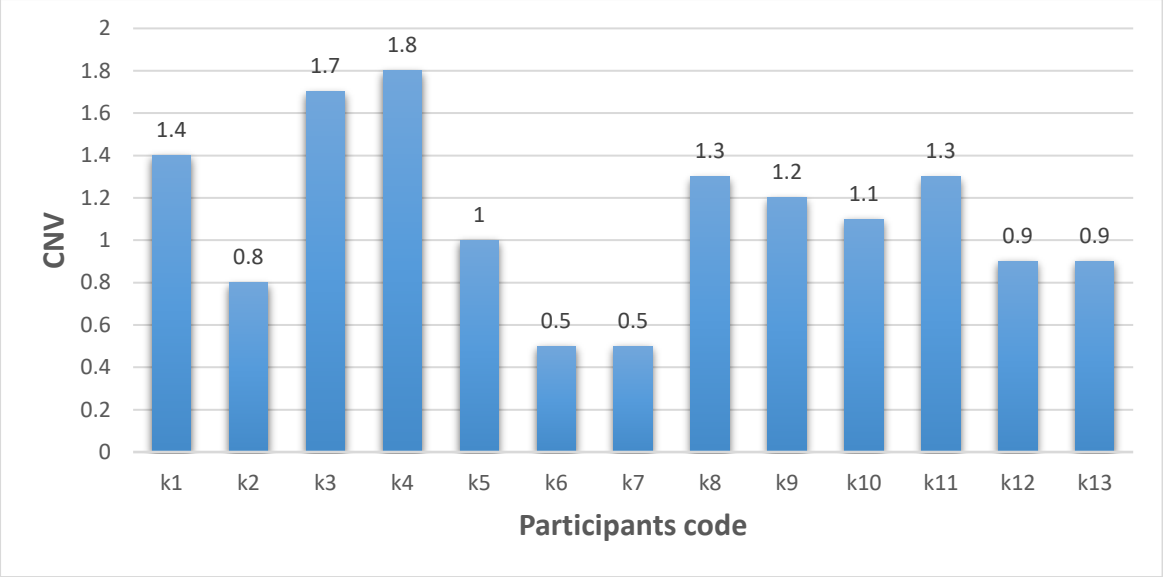

Figure 7: The figure illustrates the CNV of our participants using primers targeting exon 7, with ZNF80 as the housekeeping gene.

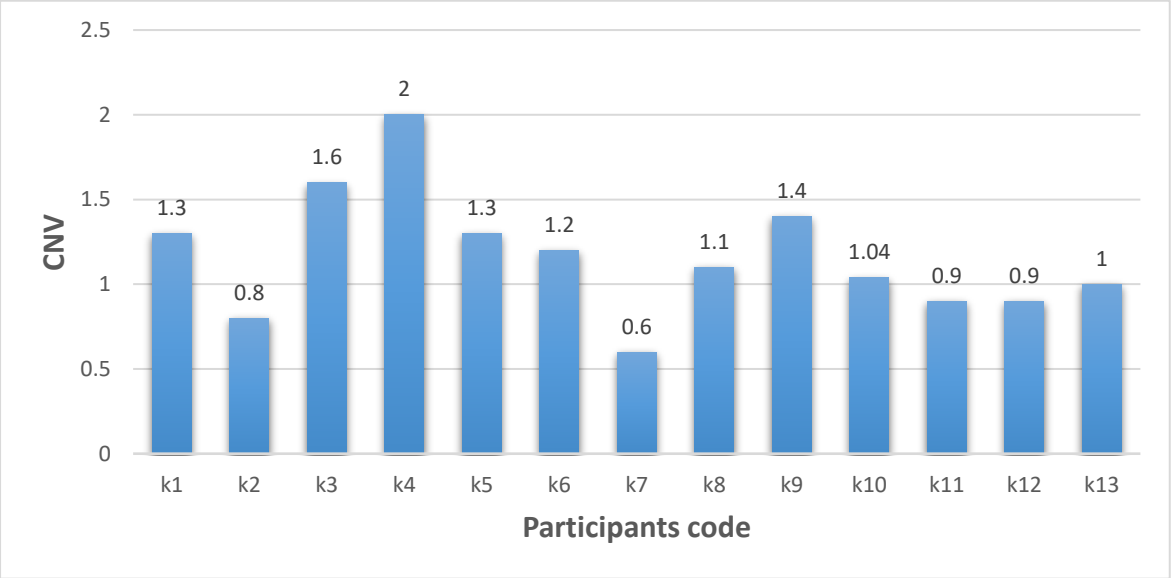

Figure 8: The figure depicts the CNV of our participants using primers targeting exon 9, with ZNF80 serving as the housekeeping gene.

Figures 9 and 10 shows primers targeting the exonic regions, excluding the intronic sequences, specifically targeting exons 4 and 7, with ZNF80 serving as the housekeeping gene. Both figures present similar CNV results, showing a high CNV for K4, K3, and K6, who are considered GT patients. Additionally, the probands' parents (K1 and K2) exhibit CNV values below 1. The CNV for the other participants ranges between carrier and normal levels.

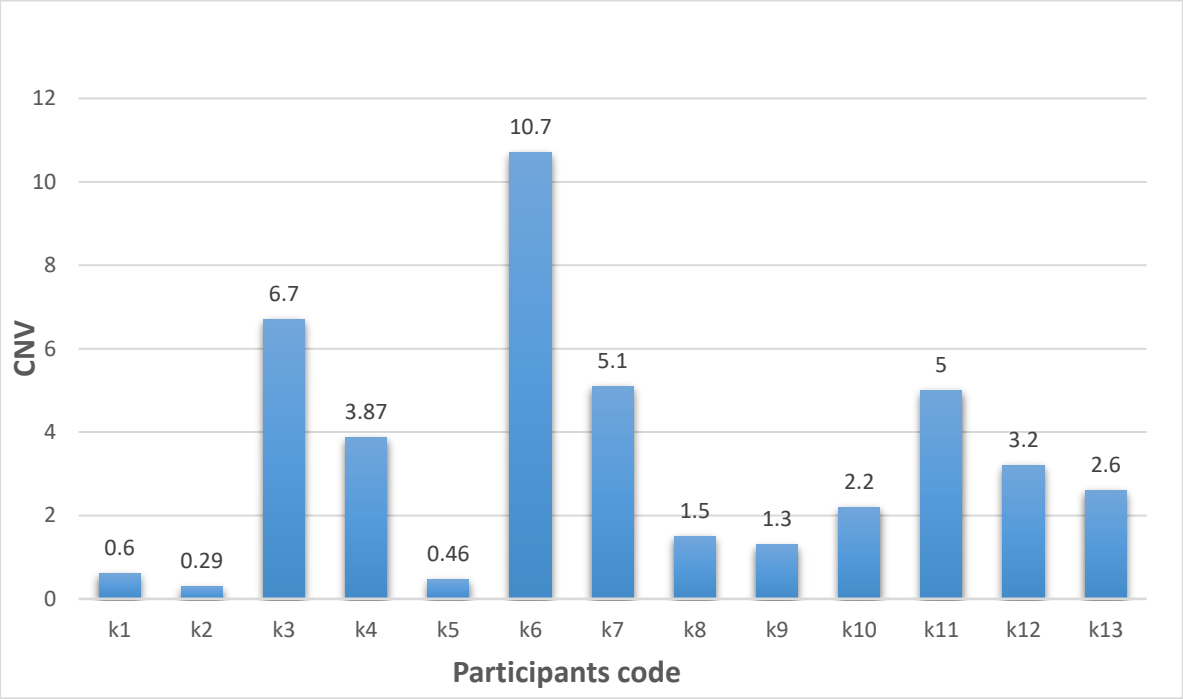

Figure 9: The figure displays the CNV of our participants, using primers specific to exon 4, with ZNF80 as the housekeeping gene.

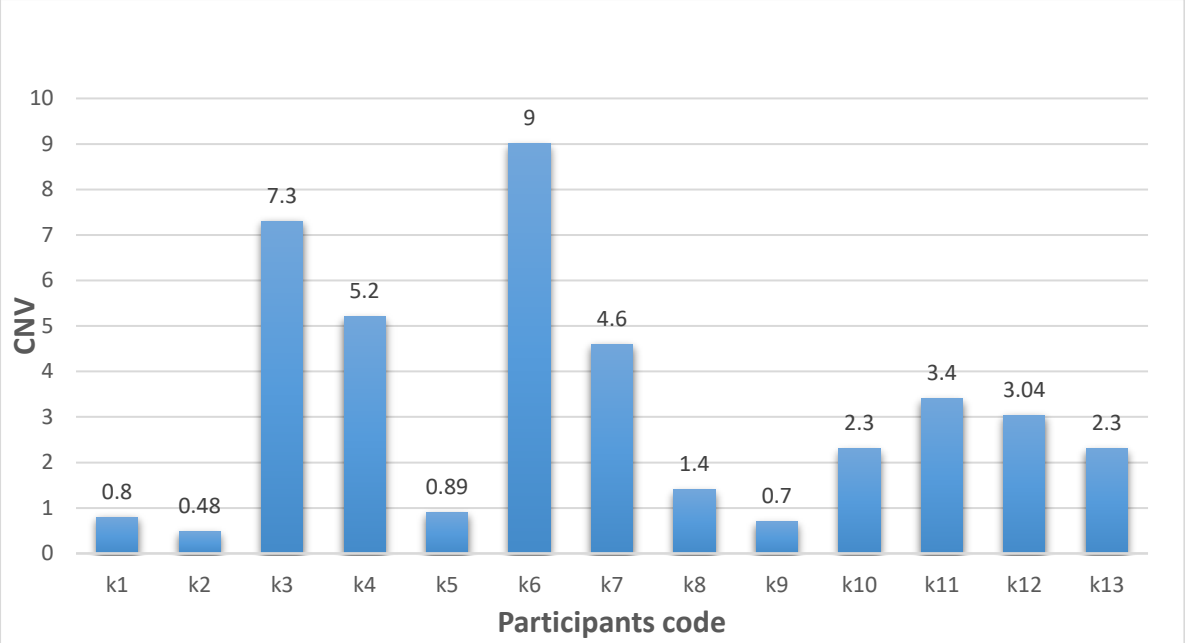

Figure 10: The figure shows the CNV of our participants, determined using primers specific to exon 7, with ZNF80 serving as the housekeeping gene.

Chapter Five

Discussion

GT is an inherited condition that runs in families. If both parents are carriers, there is a 25% chance that the disease will be passed on to any of their children. As a serious bleeding disorder, it is crucial to closely monitor any patient with a diagnosis of GT and to screen their family members for the condition. Early diagnosis and timely intervention are essential to managing the disease effectively. This approach aligns with a crucial health goal of alleviating the burden of bleeding disorders and enhancing patient outcomes, thereby significantly improving the quality of life for individuals with GT (Mathews et al., 2021; Poon & Safdari, 2023).

To explore the genetic basis of GT, four families agreed to participate in our study. However, due to limited budget, only one family, including the proband (GTK04), was selected for analysis. The proband born to consanguineous parents, showed early signs of GT, such as excessive bleeding and bruising. Her condition worsened during adolescence, particularly during menstruation, with severe bleeding episodes requiring frequent hospitalizations and blood and platelets transfusions. A ruptured ovarian cyst later caused critical bleeding, emphasizing the severity of her condition.

We then decided to conduct further investigations within the proband's family, beginning with initial laboratory tests on the family members. The laboratory results reveal several interesting patterns and inconsistencies within the proband's family. The proband's sister (GTK03) exhibits similar symptoms, though with less severe bleeding episodes and fewer hospital admissions. Both the proband and her sister display prolonged bleeding times (>10 minutes), normal platelet counts, and normal PT, which are characteristic of GT. The elevated APTT levels in the proband's parents (GTK01 and GTK02) may suggest a possible underlying issue

that requires future investigation, although they do not show bleeding abnormalities. This could indicate that they are carriers of the condition or may be affected by another undiagnosed factor influencing coagulation.

In the case of the proband's nephews, GTK05 and GTK06, both demonstrate prolonged bleeding times and elevated APTT levels. However, GTK05 has a slightly elevated platelet count and was diagnosed with an inherited bleeding disorder other than GT, indicating the possibility of a separate genetic issue. GTK06 presents symptoms consistent with GT, initially manifesting as post-circumcision hemorrhage, skin bruising, mucocutaneous bleeding, and bleeding after minor injuries. He exhibits prolonged bleeding time, normal platelet count, and PT, along with elevated APTT. Further investigation is needed to determine whether additional abnormalities are present alongside GT. The remaining family members (GTK07–GTK13) generally show normal results, with some variations in APTT, particularly elevated levels in GTK08 and GTK10. These variations may require additional testing to rule out other clotting disorders or to confirm whether they are asymptomatic carriers of a genetic mutation. Mild variations in hemoglobin levels across the family members, with some exhibiting lower values, may be indicative of other undiagnosed hematologic issues or may be unrelated to the bleeding disorders. Overall, the results largely support the presence of GT in the proband, her sister GTK03, and her nephew GTK06. They also highlight the complexity of inherited bleeding disorders, with multiple family members showing varied coagulation profiles. Further genetic testing and investigations are necessary to better understand the genetic background and to rule out other potential bleeding disorders within this family.

The next step involved performing WES for the proband to identify the causative mutation affecting our proband and her family. WES is a fast and powerful tool for analysing genetic information, making it especially useful for investigating complex genetic disorders (Choi et al., 2009). In the study of rare disorders, WES is a valuable tool for minimizing errors when detecting mutations in hotspot regions. For Mendelian disorders, Next Generation Sequencing (NGS) is particularly useful for understanding disease pathogenesis and identifying novel gene mutations linked to the condition. Additionally, NGS is highly effective in investigating genetic illnesses with recessive inheritance patterns, as it allows for the rapid identification of homozygous harmful mutations by comparing the coding sequences of affected individuals to those of unaffected ones (Bamshad et al., 2011).

Through the analysis of WES data, we identified a variant of uncertain significance (VUS) in the ITGA2B gene. Specifically, a duplication spanning from exon 3 to exon 12 was detected in our proband. While 26 duplication variants in this gene are listed in the ClinVar database, all are base pair (bp) duplications ranging from 1 to 18 bp. In contrast, our proband's duplication measures 2.5 kb (approximately 2500 bp). None of the reported variants involves full exon or multi-exon duplications. Therefore, the variant identified in our proband is novel and has not been reported or submitted to the ClinVar database or other databases. As a result, the clinical significance and functional characterization of this variant have not been previously described.

After obtaining the results from the WES test, primers were designed to continue the investigation of the proband's family using the RT-PCR technique. To ensure the reproducibility of the tests, it is advisable to design two pairs of primers for any quantitative PCR analysis.

When employing RT-PCR, implementing a normalization strategy is crucial for ensuring the reliability of the data. A common method involves comparing the expression of the target gene with that of an endogenous control or reference gene within the same sample. Housekeeping genes, which are essential for fundamental cellular functions and are consistently expressed across various physiological and experimental conditions, are frequently utilized as reference genes. Consequently, it is vital to assess the stability of the selected normalizing gene in the cells being studied to ensure accurate analysis of the target gene's expression (Gong et al., 2016; Panina et al., 2018; Piazza et al., 2017).

In this study, the housekeeping genes RPPH1, ABL, and ZNF80 were utilized. ZNF80 and RPPH1 provided better results than ABL, likely due to their more stable and consistent expression across different samples, making them more reliable for normalization. In contrast, ABL may exhibit variable expression levels under certain experimental conditions, resulting in less reliable outcomes.

Following that, we continue our study by analyzing the ITGA2B gene in a control group of healthy individuals from the same Palestinian population. The control group was selected for better understanding the genetic background of this population, alongside the family members involved in the study. As shown in Figure 6, the CNV values in the control group remain consistent, clustering around one, indicating no significant variation or duplication in the ITGA2B gene. This suggests that the control group demonstrates normal genomic integrity for this region.

In contrast, the participant group shows a wide range of CNV values. Notable increases in CNV are observed in K3 and K4, suggesting significant amplification in these individuals, which may indicate a genetic alteration linked to their condition. K6 also displays elevated CNV, though the increase is less than in K3 and K4, pointing to amplification. Moderate increases in CNV are seen in participants K9, K10, K11, and K13, which could suggest a variation in this gene region.

Interestingly, the proband's parents (K1 and K2) show CNV values near one, similar to the normal control group, which appears inconsistent with expectations. Given that the proband exhibits amplified CNV, it is unusual that the parents who are presumed carriers, do not show a similar amplification. This discrepancy warrants future investigation to understand the inheritance pattern and to explore whether other genetic or epigenetic factors could be influencing the CNV in these individuals.

Afterward, we employed two pairs of primers that incorporated intronic regions in their sequences (one designed for exon 7 and the other for exon 9) along with the three housekeeping genes mentioned earlier. However, the results we obtained were unsatisfactory due to inconsistencies in the data, with CNVs among all participants being similar. Notably, the suspected patient (GTK04) exhibited CNVs of less than three, as illustrated in Figures 7 and 8. The unsatisfactory results could stem from several factors, including potential technical issues, primer efficiency, or inaccuracies in the quantification process. The inclusion of intronic regions in the primers may also have contributed to variability, as these regions can exhibit different levels of amplification compared to exonic regions.

These results raised new questions, leading us to suspect that the issue might lie with the primers, particularly due to their inclusion of intronic regions. Consequently, we designed two additional pairs of primers specifically targeting exonic regions while excluding introns. The third pair was targeted exon 4, and the fourth pair focused on exon 7. Additionally, the three housekeeping genes previously mentioned were incorporated into the analysis. Utilizing these primers yielded improved results. As shown in Figures 9 and 10, our proband (GTK04) exhibited CNVs of 3.87 and 5.2 respectively; this validated the accuracy of the work and established the proband as a reliable positive control. Additionally, the proband's sister, GTK03, who shares similar symptoms, also displayed elevated CNVs indicative of a gene duplication. Furthermore, the proband's nephew, GTK06, who exhibits significant symptoms consistent with GT, can also be classified as a patient.

However, these results remain questionable. Since GT is an autosomal recessive disorder, the proband's parents (GTK01 and GTK02) should be carriers with CNVs of at least 1.5. Surprisingly, our findings showed their CNVs to be within the normal range. To resolve this issue, several steps were taken to address the issue. First, it was confirmed that they are indeed the biological parents of the proband. Then, new samples were collected to rule out any errors during sample collection or labelling and repeated the tests using the three housekeeping genes. Despite these efforts, the results for the parents still appeared normal, which suggests that further investigation is needed.

To clarify the genetic status of the proband's parents, we recommend performing WES for both to confirm the presence or absence of the mutation. This will enable more accurate interpretation and lead to more precise conclusions regarding this family's genetic background.

While GTK06 is confirmed as a patient, his parents GTK11 and GTK13, and his sister, GTK12, exhibit CNV values that fall within a range suggestive of carrier status or potentially resembling that of a patient, but they don’t have any bleeding abnormalities or symptoms related to GT. Additionally, the proband's other sister, GTK07, appears unaffected, with no history of bleeding episodes or GT symptoms. However, her CNV values also fall within a range that could indicate carrier status or mimic those of a patient. This underscores a limitation of our technique, as it cannot reliably differentiate between heterozygous and homozygous carriers. In addition, the proband's brother, GTK10, appears to be a carrier based on his CNV results.

Most families present with distinct mutations, and even when the same genetic mutation is shared among family members, there is considerable variability in clinical manifestations. The recurrence of specific mutations may indicate the presence of mutational hotspots (A. T. Nurden et al., 2015; A. T. Nurden, Pillois, et al., 2012).

The mutation in our study is a copy number variant (CNV)**,** which presents distinct challenges in analysis due to its complexity and inherent variability. CNVs involve large-scale changes in the genome, such as deletions or duplications of segments of DNA, and can affect multiple genes or regulatory regions, complicating the identification of functional consequences. Unlike single nucleotide variants (SNVs), CNVs can lead to changes in gene dosage, potentially resulting in over-expression or under-expression of the affected genes, and these effects might not be easily detected through traditional methods such as PCR or sequencing alone. In this context, in silico prediction programs could provide valuable insights into the potential impact

of the CNV on protein function. Tools like PolyPhen-2, and CADD are primarily designed for single nucleotide changes, but some have been extended to evaluate the effects of structural variants, including CNVs. These programs can help predict how the duplication or deletion of exonic or regulatory regions might influence the expression levels of the affected genes or alter the structure and function of the resulting protein. Furthermore, computational tools that assess gene dosage effects or disruption of regulatory elements can complement experimental findings by offering predictions on how changes in gene copy number might contribute to the disease phenotype.

CNVs can disrupt gene function by affecting not only coding regions but also regulatory elements, their pathogenicity might result from both loss-of-function and gain-of-function mechanisms. By integrating in silico predictions with experimental data, such as RT-PCR**,** CNV detection assays**,** and functional validation**,** a more comprehensive understanding of the CNV's potential role in disease can be achieved. Therefore, using a combination of experimental techniques and computational predictions will enhance the ability to interpret the findings and provide a clearer understanding of the biological consequences of the CNV in question.(Gažiová et al., 2022; Sládeček et al., 2023)

To confirm the pathogenicity of the variant, future studies should include a combination of functional, genetic, and clinical approaches. In vitro experiments and gene editing technologies, such as CRISPR-Cas9, can help assess the variant's impact on protein function and cellular behaviour. Population studies should focus on segregation patterns in families and the variant’s frequency in diverse populations. In silico predictions can be refined with structural modelling and pathogenicity scoring tools. Additionally, analysing clinical outcomes in individuals with the variant can help establish a direct link to disease, while follow up studies and monitoring carriers over time will provide valuable information on disease progression. Together, these studies will validate the variant’s role in disease and enhance our understanding of its pathogenic mechanisms.

Chapter Six

Conclusion, Recommendations, Limitations and Future perspectives

6.1Conclusion and recommendations

Integrating all findings and components comprehensively, GT remains underdiagnosed and undertreated within our population. Our results emphasize the potential of WES as a robust tool for identifying the molecular basis of GT. Integrating WES into diagnostic practices has the potential to greatly enhance both the understanding and management of GT in the future. Additionally, our study offers valuable insights into the clinical presentation and molecular basis of GT cases.

The duplication mutation identified in our research, which spans multiple exons, may be unique to our population since it has not been previously reported or submitted to genetic databases. However, further investigation is essential to confirm its pathogenic role and broader implications.

For GT, we strongly recommend universal screening for individuals with a family history of this condition or other related bleeding disorders. Additionally, genetic counselling should be provided to patients diagnosed with GT or exhibiting signs of inherited bleeding disorders within their families. Early detection and genetic counselling are essential for effective management and can significantly reduce the risk of severe bleeding episodes.

In the case of GT, genetic diagnosis by WES has proven to be the gold standard for confirming the condition. Relying solely on clinical criteria to diagnose GT can lead to underestimating the number of cases. This is because milder or atypical symptoms may go unnoticed, and the overlap of symptoms with other bleeding disorders can result in misdiagnosis. Additionally, GT has a variable phenotype, meaning some individuals may experience very mild or no symptoms at all, making it harder to diagnose based on clinical signs alone. Carriers of the

disorder, who may not show significant symptoms, are also often overlooked. Furthermore, genetic mutations that don't present typical clinical features may be missed without genetic testing. This highlights the importance of genetic testing for accurate diagnosis.

6.2 Limitations

The primary limitations of this study arise from time constraints, limited funding, and the social stigma surrounding inherited diseases. With more time, we could have included additional participants and families from the same region and other regions, which would have allowed us to better generalize the findings to the Hebron population and eventually to the wider Palestinian population. Consistent funding would have provided the opportunity to further investigate the clinical significance of the variant we identified. Additionally, with more resources, we could have conducted WES on other samples suspected of having different variants. Another significant challenge was the stigma attached to inherited diseases within our society, which reduced the sample size, particularly among female participants.

6.3 Future perspectives

We intend to persist with cascade screening for the family members participating in our study to identify as many GT cases as we can. Additionally, we aim to explore other families from various regions in Palestine for this variant and potentially other universally confirmed variants. Our team is organizing community-based lectures to emphasize the importance of screening for genetic diseases, as well as the value of genetic counselling, particularly in cases of consanguinity. We seek to change the public perception of genetic diseases, moving away from the stigma associated with them.

Ultimately, we envision the potential application of in vitro fertilization (IVF) combined with prenatal genetic diagnosis (PGD) to identify and select genetically normal embryos for implantation into the mother’s uterus. This approach offers carriers or patients the opportunity to have healthy, genetically normal offspring.

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Appendixes

Appendix (1): Consent form.

Al-Quds University
Faculty of Medicine
Abu-Dios, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

Consent Form for Genetic Tests

موافقة على إجراء فحوصات جينية

اسم المريض: _____

رقم الهوية: _____ تاريخ الميلاد: ____/__/__

رقم الهاتف: _____ الجنس: انثى / ذكر

العنوان: _____

✓ تمت الموافقة على اجراء الفحوصات التشخصية والجينية بواسطة:

☐ شخص بالغ

☐ أحد الوالدين او ولي أمر الاسم:

_____

✓ موافقة المريض:

أوافق انا _____ على اجراء الفحوصات التي تمت مناقشتها مع _____ لي.

توقيع المريض _____ التاريخ ____/__/__

أو

✓ موافقة ولي أمر المريض:

أوافق انا _____ على اجراء الفحوصات التي تمت مناقشتها مع _____ (الرجاء ذكر صلة القرابة)_____

توقيع ولي الأمر _____ التاريخ ___/__/__

✓ إذن بنشر نتائج الفحوصات الجينية:

أسمح بنشر نتائج فحوصاتي/ فحوصات طفلي دون ذكر الاسم وذلك لأهداف البحث العلمي. نعم/ لا

أنا دلال الهشلمون ناقشت مع (المريض/ والده / ولي امره) السبب المقترح لإجراء الفحوصات الجينية منها والتشخيصية. وتم إطلاع (المريض/ والده / ولي امره) على طبيعة العمل والنتائج المحتملة والقيود والمخاطر للاختبار الجيني المقترح.

التوقيع:

_____

Appendix (2): Questionnaire

Graduate Studies
Faculty of Medicine
Biochemistry and Molecular Biology
Master Program

جامعة القدس
Al-Quds University

Questionnaire

استبيان لدراسة بعنوان: الكشف عن المتغيرات الجينية لمرض (وهن صفيحات لغلانزمان) Glanzmann Thrombasthenia الوراثي لدى مرضى من مدينة الخليل.

نحن مجموعة بحثية من جامعة القدس ضمن برنامج ماجستير في الكيمياء الحيوية والاحياء الجزيئية، نقوم بهذا البحث ليتم نشره للتخرج ولنيل شهادة الماجستير.

هذه الدراسة تهدف الى الكشف عن المتغيرات الجينية عند المرضى الذين يعانون من مشاكل في تجمع الصفائح الدموية الوراثي . بمشاركتك في هذه الدراسة ف انت توافق على استعمالنا لسجلك المرضي ونتائج الفحوصات والدارسة لأغراض البحث العلمي فقط، علما ان معلوماتك الشخصية لن يتم الكشف عنها وسيتم التعامل معها ب سرية تامة.

من فضلك هل يمكنك الاجابة على هذه الاسئلة؟ يمكنك الاستفسار عن اي نقطة غير واضحة.

❖ *المعلومات الشخصية*

1. الاسم الرباعي _____
2. رقم الهوية _____
3. العنوان السكن _____
4. تاريخ الميلاد _____
5. الجنس ذكر / انثى
6. الحالة الاجتماعية _____

أعزب، هل والديك أقارب؟ درجة القرابة _____

متزوج، هل زوجك/ زوجتك من اقاربك؟ درجة القرابة _____

❖ *المعلومات الطبية*

الرجاء الإجابة على الأسئلة التالية مع ذكر التدخل الطبي او الشخصي لوقف العرض المذكور ان وجد

1. هل تعاني من كدمات غير مبررة على الجلد؟ ◯ نعم ◯ لا
   من متى بدأت بالظهور؟
   التدخل الطبي او الشخصي (ضغط على موضع النزيف، استخدام علاج معين)
   _____

2. هل تعاني من الرعاف (نزيف الانف)؟ ◯ نعم ◯ لا

فترة تكرر الرعاف؟

---

3. في الطفولة عند تبديل الاسنان اللبنية هل تعرضت لنزيف؟ ◯ نعم ◯ لا

مدة النزيف؟

---

4. هل عانيت من النزيف من الجروح الخفيفة؟ ◯ نعم ◯ لا
مدة النزيف؟

---

5. هل عانيت من نزيف من اللثة؟ ◯ نعم ◯ لا

مدة النزيف؟

---

6. هل عانيت من نزيف بعد اي اجراء طبي بالأسنان؟ ◯ نعم ◯ لا

مدة النزيف؟

---

7. هل عانيت من نزيف بعد عمليه جراحيه؟ ◯ نعم ◯ لا

مدة النزيف؟

---

8. للذكور، هل عانيت من نزيف بعد الطهور؟ ◯ نعم ◯ لا

مدة النزيف؟

---

9. للإناث، هل عانيت من نزيف أثناء ال دورة الشهرية؟ تحديدا ب اول دورة شهرية؟ ◯ نعم ◯ لا

مدة النزيف؟

_____

10. هل عانيت من نزيف اثناء الحمل او الولادة او ما بعد الولادة؟ ◯ نعم ◯ لا

مدة النزيف؟

_____

11. هل عانيت من نزيف بالمفاصل، الجهاز الهضمي او نزيف بأحد أعضاء الجسم؟ ◯ نعم ◯ لا

مدة النزيف؟

_____

12. هل تتابع علاجك عند اخصائي أمراض دم؟ ◯ نعم ◯ لا

13. هل تستخدم ادوية دورية؟

اذكر اسم الدواء _____

14. هل سبق وتم نقل اليك أحد مكونات الدم؟ (خلايا دم حمراء، بلازما او صفائح) ◯ نعم ◯ لا

كم مرة؟_____

سبب نقل الدم؟_____

15. هل سبق وقمت بإجراء فحص جينات؟ ◯ نعم ◯ لا

16. هل هناك فرد من افراد عائلتك لديهم مرض وهن صفيحات لغلانزمان؟ ◯ نعم ◯ لا

صلة القرابة_____

التاريخ	توقيع الباحث	توقيع المريض أو احد والديه
_____	_____	_____

شكرا لتعاونكم

Appendix (3): Ethical approval

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
عمادة كلية الطب
ابوديس/القدس

Research Ethics subcommittee of Faculty of medicine

Letter of Ethical approval

Date14/6/2023

Ref#: R3-14-23

Dear Applicants: Dr. Kifaya Suleiman and Miss. Dalal Al-Hashlamoon
Biochemistry and Molecular Biology master program
The Research Ethics subcommittee of faculty of medicine has recently reviewed your proposal entitled "Detection of Genetic Mutations in Inherited Glanzmann Thrombasthenia in Hebron-Palestine." Your proposal is deemed to meet the requirements of research ethics subcommittee at Al-Quds University.

Note: This letter can be used to apply for the central Al-Quds University research ethics committee if needed

Best of luck,

Dr. Suheir Ereqat
Head of research ethics subcommittee
Biochemistry and Molecular Biology master program
Faculty of Medicine-Al-Quds University

P.O. Box 20002
Tell: 02-2799203
Fax: 02-2796110

ص . ب 20002
هاتف: 022799203
فاكس022796110

الكشف عن المتغيرات الجينية المسببة لمرض وهن الصفيحات غلانزمان في منطقة الخليل، فلسطين

إعداد: دلال "محمد بلال" "محمد داوود" الهشلمون

إشراف: د. كفاية عزمي

الملخص بالعربية

مرض وهن الصفيحات غلانزمان (GT) هو اضطراب نزيف نادر وراثي متنحي يحدث بسبب طفرات في الجينات ITGA2B و ITGB3 ، التي تُعد ضرورية في تجميع الصفائح الدموية. تؤدي هذه الطفرات إلى خلل في وظيفة الصفائح بسبب تعطل او خلل في وظيفة المستقبل αIIbβ3، الذي يلعب دورًا رئيسيًا في عملية تجلط الدم. يتم تصنيف GT إلى ثلاثة أنواع حسب وظيفة وعدد المستقبلαIIbβ3 : النوع الأول (غياب المستقبل أو أقل من 5% من المستويات الطبيعية)، النوع الثاني (5-20% من المستويات الطبيعية)، والنوع الثالث (عدد المستقبل طبيعي ولكن وظيفة غير طبيعية). يتم التشخيص عن طريق اختبارات جينية، اختبار وظيفة الصفائح، فحص للجينات ITGA2B و ITGB3. يعتبر التشخيص المبكر والاستشارة الجينية أساسيين لتحسين النتائج والوقاية من المضاعفات. تنتشر GT على مستوى العالم بمعدل 1 من كل مليون شخص، لكن في المناطق ذات معدلات زواج الأقارب المرتفعة قد يصل الانتشار إلى 1 من كل 200,000.

هدفت هذه الدراسة إلى تأكيد تشخيص مرضى مشتبه في إصابتهم بـGT ، ودراسة إمكانيات الفحص التسلسلي للأسر، وتحديد الطفرات المسببة لـ GT في السكان الفلسطينيين. تم اختيار المرضى بناءً على معايير محددة مثل الميل للنزيف المستمر، وعدد الصفائح الدموية الطبيعي، وتاريخ عائلي للإصابة بـGT .

أظهرت نتائج التسلسل الكامل للجينوم (WES) وجود طفرة غير مؤكدة الأهمية (VUS) في جين ITGA2B تتضمن تكرارًا يمتد من exon 3 إلى 12. لم يتم الإبلاغ عن هذه الطفرة مسبقًا، مما يشير إلى أنها قد تكون خاصة بالسكان المحليين. أكدت تحليلات RT- PCR وجود الطفرة في أفراد العائلة بينهم مصابين واخرين حاملين لهذه الطفرة ،في حين كانت نتائج والدي المريض غير حاسمة وتتطلب مزيدًا من التحقيق. تؤكد الدراسة على إمكانات WES كأداة تشخيصية لـ GT وتسلط الضوء على الحاجة إلى مزيد من البحث لتأكيد أهمية الطفرة المكتشفة من الناحية المرضية.