DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Hemostatic alterations in multiple myeloma and in monoclonal gammopathy of undetermined significance

by **Harriet Ghansah**

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LIST OF ABBREVIATIONS AND ACRONYMS

aa	Amino acid
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
APC	Activated protein C
APCR	Activated protein C resistance
APTT	Activated partial thromboplastin time
ASCT	Autologous stem cell transplantation
AUC	Area under the curve
BCA	Bicinchoninic acid
BM	Bone marrow
BSGC	Buffered saline glucose citrate
BTZ	Bortezomib
CAT	Calibrated automated thrombography
CD	Cluster of differentiation
CLA	Clot lysis assay
CLT	Clot lysis time
CRAB	Hypercalcemia, renal failure, anemia, and bone disease
CRP	C-reactive protein
CT	Clotting time
CTI	Corn trypsin inhibitor
DEX	Dexamethasone
DiOC6(3)	3,3′-dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPP	Endogenous plasmin potential
ETP	Endogenous thrombin potential
FDPs	Fibrin degradation products
FITC	Fluorescein Isothiocyanate
FIX	Factor IX
FLC	Free light chain
FM	Fibrin monomer
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
FXIII-A	A subunit of FXIII
FXIII-A_2B_2	Tetrameric structure of FXIII
FXIII-B	B subunit of FXIII
GFP	Gel-filtered platelets
GP	Glycoprotein
HC	Healthy control
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSA	Human serum albumin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgLκ	Immunoglobulin light-chain kappa
IgLλ	Immunoglobulin light-chain lambda
IgM	Immunoglobulin M
IL	Interleukin
ImiD	Immunomodulatory drug
IMWG	International Myeloma Working Group
INR	International normalized ratio
ISI	International sensitivity index
LDH	Lactate dehydrogenase
LEN	Lenalidomide
LT	Lysis time
MCF	Maximum clot firmness
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
PAI-1	Plasminogen activator inhibitor-1
PAP	Plasmin-alpha-2-antiplasmin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Protein C
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PG	Plasmin generation
PL	Phospholipid
PLG	Plasminogen
PMP	Platelet microparticle
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
RISS	Revised International Staging System
RT	Room temperature
rTF	Recombinant tissue factor
rtPA	Recombinant tissue plasminogen activator
Sβ2M	Serum beta-2-microglobulin
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin
TF	Tissue factor

TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
THAL	Thalidomide
TM	Thrombomodulin
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
TRAP	Thrombin receptor-activating peptide
TT	Thrombin time
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
vWF	von Willebrand factor
vWFAct	von Willebrand factor activity
vWFAg	von Willebrand factor antigen
α-granule	Alpha granule
α2-MG	Alpha-2-macroglobulin
α2-PI	Alpha-2-plasmin inhibitor
δ-granule	Dense granule
Δψm	Mitochondrial inner membrane potential

1. INTRODUCTION

Multiple myeloma (MM) is a clonal plasma cell neoplasm [1] that makes up about 1% of global cancers [2, 3] and 10% of all hematological malignancies [4, 5]. The median age at diagnosis is 70 years [1]. Monoclonal gammopathy of undetermined significance (MGUS), a premalignant plasma cell disorder, nearly always precedes MM [6, 7]. The annual risk of MGUS progressing to MM or a related disorder is about 1% [3, 8]. MGUS gets more common with age. It affects 3.2% of people over 50 and 5.3% of people over 70 years [9].

The diagnostic criteria for MGUS include < 10% bone marrow (BM) plasma cells, < 30 g/L serum monoclonal protein concentration, and the absence of CRAB features (hypercalcemia, renal failure, anemia, and bone disease). In contrast, MM is characterized by a 10% or higher BM plasma cell ratio or biopsy-confirmed bony or extramedullary plasmacytoma, in addition to one or more CRAB features or biomarkers of malignancy. The biomarkers of malignancy include a BM plasma cell count of $\geq 60\%$, a serum free light chain (FLC) ratio of 100 or more, and the presence of multiple focal lesions as detected by magnetic resonance imaging [10].

Diverse hemostatic abnormalities have been reported in MM and MGUS, which confer a high thromboembolic and hemorrhagic risk [11–13]. Venous thromboembolism (VTE) is common in patients with MM [4, 14–19] and MGUS [20–23]. While MGUS patients are asymptomatic and do not require treatment, it has been discovered that the choice of therapy has a significant influence on the risk of VTE in MM patients. VTE occurs in 1 – 2% of myeloma patients receiving standard treatment with melphalan and prednisone. Immunomodulatory drug (IMiD) monotherapy also increases VTE risk by 3 – 4% [24] and up to 26% when combined with corticosteroids [19]. Nonetheless, treatment with bortezomib (BTZ), a proteasome inhibitor, has significantly improved the survival rate of patients with

MM and decreased the risk of VTE to < 2% when used alone or in combination therapy [25–27].

Thrombosis in MM patients has also been linked to the production of paraproteins [18], which may cause increased blood viscosity, impaired fibrinolysis [28, 29], decreased protein S activity [30], the development of procoagulant autoantibodies such as lupus anticoagulant [31], and endothelial damage [32]. Elevated levels of fibrinogen, von Willebrand factor (vWF), coagulation factor VIII (FVIII) [13, 30, 33–35], and proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and C-reactive protein (CRP) [36] may also increase the risk of VTE in myeloma patients. Acquired activated protein C resistance (APCR) is said to be common in people with MM and has been linked to a higher risk of thrombosis [37, 38].

The pathophysiology of VTE in MGUS is unclear. Nonetheless, the increased secretion of IL-6 and TNF-α may play a role [20], as these cytokines can trigger tissue factor (TF) expression, increase FVIII, fibrinogen, and vWF transcription, and decrease protein S activity [20, 39].

Bleeding in MM and MGUS patients usually manifests as purpura or epistaxis [40]. In MM, this could be caused by several pathophysiologic mechanisms that involve paraproteins, such as the direct inhibition of fibrin monomer polymerization, impaired platelet adhesion or aggregation, qualitative platelet dysfunction, monoclonal thrombin inhibitor, thrombocytopenia, and acquired von Willebrand syndrome [11, 41–43]. Acquired von Willebrand syndrome is more common in MGUS than other lymphoproliferative disorders [44], possibly because paraproteins bind to vWF, accelerating clearance and lowering circulating levels [45, 46]. Paraproteins have also been shown to inhibit vWF, FVIII [40], and

factor XIII (FXIII), one of the most important regulators of clot firmness and stability, preventing its action on fibrin [47].

FXIII deficiency is rare and can either be inherited or acquired. Congenital FXIII deficiency is caused by defects in both the FXIII-A and FXIII-B genes whereas acquired FXIII deficiency is caused by autoantibodies against a FXIII subunit or decreased synthesis as a result of BM or liver dysfunction, FXIII consumption, or dilution coagulopathy [48]. While acquired FXIII deficiency may cause severe bleeding symptoms [49], its frequency and etiology in MM and MGUS patients remain poorly understood.

2. LITERATURE REVIEW

2.1. Overview of hemostasis

Normal hemostasis is the physiological mechanism that stops bleeding after an injury. It involves a delicate balance of procoagulant and anticoagulant mechanisms to prevent abnormal clotting or bleeding. Hemostasis involves the vessel wall, platelets, coagulation proteins, coagulation inhibitors, and the fibrinolytic system [50].

2.1.1. The vessel wall

The vessel wall and its inner endothelial lining are essential for maintaining the patent vasculature. The endothelium contains thromboregulatory agents such as prostacyclin, nitric oxide [51], and CD39 [52], which work together to prevent thrombus formation. The subendothelial matrix contains collagen, while the smooth muscle and adventitial layers of the vessel wall contain TF. Together, these components form a hemostatic barrier to preserve the high-pressure circulation [53] (Figure 1).

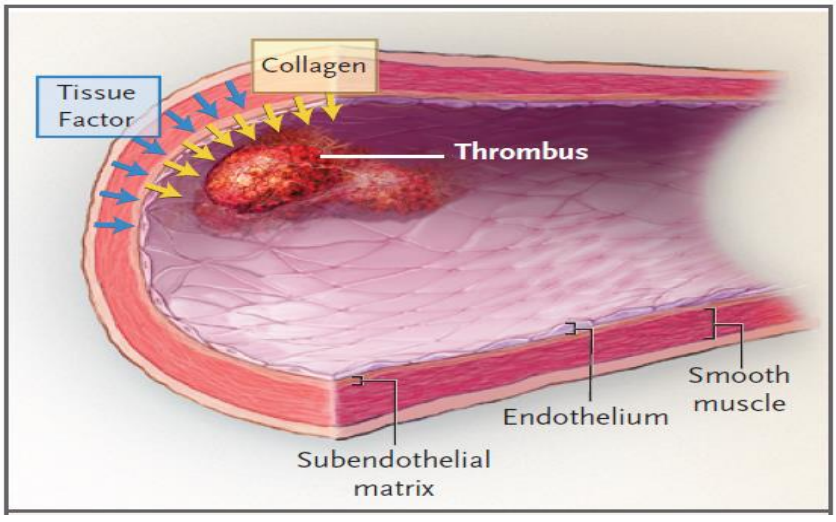

Figure 1. Response to vascular injury. Upon endothelial or vessel wall damage, collagen and tissue factor are exposed to blood, initiating the formation of a thrombus. The yellow and blue arrows represent collagen and tissue factor respectively. Adapted from [53].

2.1.2. Platelets

2.1.2.1. Structure

Platelets measure 2 – 3 μm in diameter [54]. Although anuclear, platelets retain a pool of messenger ribonucleic acid that was transferred from the megakaryocyte during thrombopoiesis, which allows them to synthesize proteins de novo [55]. The platelet membrane is made up of a typical phospholipid (PL) bilayer. The inner, cytoplasmic layer is mostly made up of anionic or polar PLs, namely phosphatidylserine (PS), phosphatidylinositol, and phosphatidylethanolamine, whereas the outer layer consists of neutral PLs, such as phosphatidylcholine and sphingomyelin [56]. The membrane invaginates into the interior of the platelet, forming an open canalicular system. This serves as a passage for the transport of materials into and out of the cell and the release of storage granule contents [57]. Platelets have a cytoskeleton that is mostly made up of actin, spectrin, tubulin, and filamin. Resting platelets are discoid, and their shape is maintained by a circumferential bundle of microtubules (alpha and beta tubulin subunits) located beneath the plasma membrane [58].

Platelets express several transmembrane receptors, including glycoprotein IIb/IIIa (GPIIb/IIIa), which is also designated as $\alpha_{IIb}\beta_3$ for binding fibrinogen and vWF, the GPIb/IX/V complex, which facilitates interaction with vWF, purinergic $P2Y_1$ and $P2Y_{12}$ receptors for adenosine diphosphate (ADP), protease-activated receptor-1 and 4 for thrombin, TPα and TPβ receptors for thromboxane A_2 (TxA_2), and the GPVI receptor for binding to collagen.

Three types of storage granules are found in platelets, namely, alpha granules (α-granules), dense granules (δ-granules), and lysosomes [59]. The α-granules (50 – 80 per platelet) store and secrete adhesive proteins that, through platelet-platelet interactions, mediate the growth and stability of thrombi [60]. They include fibrinogen, vWF, platelet factor 4, P-selectin, CD40 ligand (CD154), platelet-derived growth factor (PDGF), platelet endothelial cell adhesion molecule (CD31), thrombospondin, β-thromboglobulin, factor V

(FV), which constitutes ~20% of the total FV in blood, and GPIIb/IIIa [61]. The δ-granules (2 – 7 per platelet) contain adenosine triphosphate (ATP), ADP, serotonin, polyphosphate, histamine, pyrophosphate, and calcium, which mediate platelet aggregation [62, 63]. Platelet lysosomes contain acid hydrolases, including beta-galactosidase, hexosaminidase, cathepsins, acid phosphatase, beta-glucuronidase, and arylsulfatase. They also express CD63 and lysosome-associated membrane glycoproteins 1/2 [64]. The dense tubular system sequesters calcium ions and contains platelet-activating enzymes like thromboxane synthetase, phospholipase A2, and cyclooxygenase [56]. Platelet mitochondria provide the energy required for normal platelet function [65].

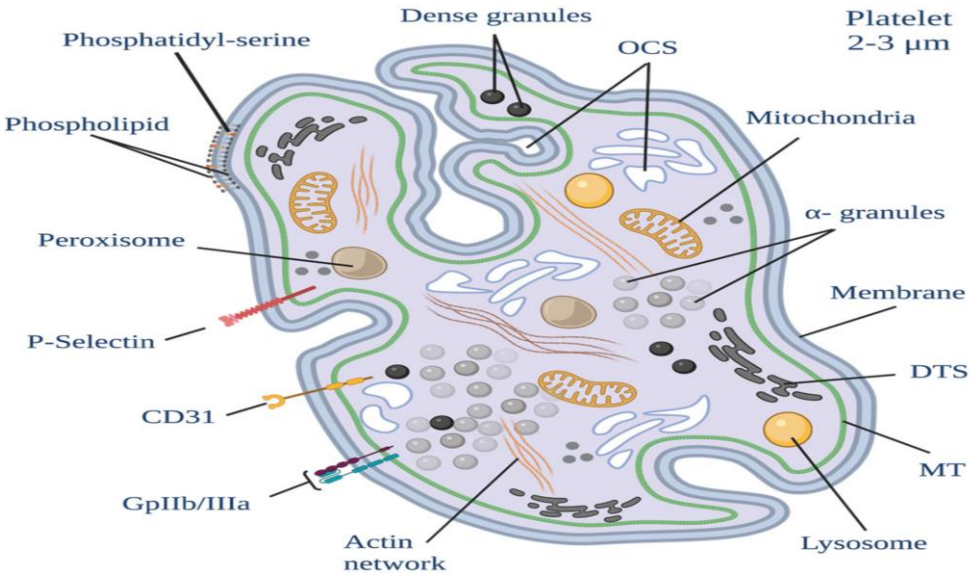

Figure 2. Platelet morphology. Platelets contain alpha granules, dense granules, and lysosomes. The plasma membrane invaginates to form the open canalicular system. The abbreviations shown on the figure are explained as follows: OCS, open canalicular system; α-granules, alpha granules; DTS, dense tubular system; MT, microtubule. Adapted from [54].

2.1.2.2. The role of platelets in primary hemostasis

Upon endothelial or vessel wall damage, collagen and TF are exposed to blood. The exposed collagen causes platelet accumulation and activation, whereas TF triggers blood coagulation [53]. Primary hemostasis begins with platelet adhesion. This process is initiated through the interaction of GPIb/IX/V with its primary ligand, vWF [66, 67]. vWF is

synthesized by endothelial cells; it is either constitutively released into plasma or stored in specialized organelles called Weibel-Palade bodies for on-demand release in response to hemostatic processes [68, 69]. The majority of vWF released by endothelial cells circulates in plasma, while a small amount anchors to the endothelial cell surface. Normally, plasma vWF does not bind to GPIb/IX/V. However, at high shear rate, vWF immobilized on exposed collagen is required to capture platelets [70]. vWF exists in a globular form under physiological shear conditions, concealing binding domains for GPIb (of the GPIb/IX/V complex). Nevertheless, when the shear rate exceeds 500 – 800/sec, vWF undergoes a conformational change where it unfurls into long multimeric strings, exposing its A1 and A3 domain binding sites for GPIb and collagen, respectively [68, 71]. Upon binding to vWF, GPIb/IX/V undergoes structural alteration, initiating intracellular signaling and platelet activation via outside-in signaling. This process eventually causes the activation of GPIIb/IIIa and GPIa/IIa on the platelet surface [66]. GPIa/IIa directly binds to collagen, whereas GPIIb/IIIa binds to vWF, which is linked to collagen [72]. Fibronectin and vitronectin, among other extracellular proteins, contain distinct amino acid (aa) residues that may also serve as direct binding sites for GPIIb/IIIa [73–75]. At low shear rate, the interaction between vWF and GPIb/IX/V may not be required for the initial slowing of platelet velocity. Instead, platelet adhesion can often be achieved through integrin-mediated binding alone [66, 70]. When platelets adhere to the subendothelial matrix proteins, they become activated, and intracellular calcium levels increase. GPVI binding enhances collagen-induced platelet activation. The platelet surface area increases as they change from disc-shaped to elongated cells with extensions of their cytoplasm.

The contents of the platelet α- and δ-granules are released into the extracellular fluid through exocytosis [76]. When ADP binds to the $P2Y_1$ receptor, it triggers various physiological responses in platelets, including shape change, procoagulant activity, generation

of TxA_2, adhesion to immobilized fibrinogen, platelet aggregation, and the formation of thrombi. $P2Y_{12}$ functions similarly to $P2Y_1$, but it also strongly enhances collagen and TxA_2-mediated platelet activation [77]. Polyphosphate may accelerate thrombin activation of coagulation factor XI (FXI), which may help to promote secondary hemostasis [78]. During platelet activation, the release of fibrinogen and vWF from the α-granules may help in cross-linking of the activation-dependent open conformation of GPIIb/IIIa, promoting stable platelet plug formation.

Following injury, numerous chemokines and growth factors are released, including platelet factor 4, vascular endothelial growth factor (VEGF), and PDGF-β, which also aid in the restoration of vascular integrity [79]. Additionally, P-selectin binds to the P-selectin glycoprotein ligand-1 receptor on leukocytes and endothelial cells. As a result, platelets become strongly bound to the endothelium, and leukocytes can be incorporated into thrombus formation [80].

Platelet activation also causes PS to be translocated from the interior to the exterior of the platelet membrane leaflet, where it serves as a charged surface, facilitating the assembly of the intrinsic tenase (VIIIa and IXa) and prothrombinase (Va and Xa) complexes to generate thrombin and thus fibrin [81, 82]. Activated platelets shed PS-enriched microparticles from the surface of their membranes, increasing the platelet surface area for fibrin formation [83, 84]. ADP or TxA_2 released by activated platelets or thrombin formed from activated coagulation factors facilitate the recruitment of more platelets to the site of vessel injury. Furthermore, GPIIb/IIIa undergoes a conformational change on the external surface of the platelet, allowing fibrinogen to bind adjacent platelets to form platelet aggregates/platelet plug.

2.1.3. Coagulation proteins

Coagulation proteins are circulating plasma proteins that are essential components of the coagulation cascade, a sequential process that results in fibrin formation. They are

synthesized by liver hepatocytes, except for TF (earlier designated as factor III), calcium (formerly called factor IV), and FVIII [85]. FVIII is principally produced by hepatic sinusoidal endothelial cells and lymphatic tissue [86]. TF is expressed constitutively in various cells, including smooth muscle cells, fibroblasts, pericytes, and astroglia cells [85–89]. Most of the coagulation proteins circulate in the bloodstream as zymogens that are activated into serine proteases, which serve as catalysts to cleave subsequent zymogens into additional serine proteases [85].

The coagulation cascade is traditionally divided into three distinct pathways: the contact (intrinsic) pathway, which involves factors XII (FXII, Hageman factor), XI (FXI, plasma thromboplastin antecedent), IX (FIX, antihemophilic factor B/Christmas factor), and VIII (FVIII, antihemophilic factor A), the TF (extrinsic) pathway, which involves factor VII (FVII, proconvertin) and TF, and the common pathway, which involves factors X (FX, Stuart-Prower factor), V (FV, proaccelerin/labile factor), II (prothrombin), I (fibrinogen), and XIII (FXIII, fibrin-stabilizing factor). Factor VI does not exist anymore, and high molecular weight kininogen and prekallikrein, which were the more recently discovered coagulation factors, have not been designated with Roman numerals.

2.1.3.1. Factor XIII (FXIII)

FXIII is a large tetrameric molecule that circulates in plasma with a molecular weight of 320 kDa. It is composed of a dimer of the A subunit (FXIII-A_2, 83 kDa) and a dimer of the B subunit (FXIII-B_2, 80 kDa) [90]. The normal plasma concentration of the A_2B_2 heterotetramer is 14 – 28 mg/L [91], with a biological half-life of 9 – 12 days [92]. Physiologically, all of the FXIII-A in plasma is in a complex, whereas FXIII-B is in excess, with about 50% circulating as a free, uncomplexed protein [92]. Monocytes, monocyte-derived macrophages, and platelets also contain FXIII. The B subunits are absent in cellular FXIII [93].

Humans carry the FXIII-A gene on chromosome 6 (6p24 – 25) [94]. It is made up of 14 introns and 15 exons, spanning over 160 kb of DNA [95]. The main site for the synthesis of the A subunit appears to be the BM cells, but hepatocytes may also play a role [96]. FXIII-A has five separate domains: an activation peptide domain (1 – 37 aa residues), a β-sandwich domain (38 – 183 aa residues), a central core domain (184 – 513 aa residues), a β-barrel 1 domain (514 – 628 aa residues), and a β-barrel 2 domain (629 – 731 aa residues) [92, 97]. The catalytic FXIII-A_2 subunit has transglutaminase activity, which covalently crosslinks fibrin polymers, conferring resistance to premature fibrinolysis [97].

Chromosome 1q31 – 32.1 has been identified as the location of the B subunit gene [98], which spans 28 kb of DNA and has 12 exons and 11 introns [99]. Plasma FXIII-B is synthesized by the liver [100]. FXIII-B contains ten sushi domains. Each domain contains ~60 aa residues. The sushi domain folds into a small, dense hydrophobic core surrounded by six β-strands. The strands are held together by two disulfide bridges on either end of the domain [90]. The domains appear to contribute to the binding of the protein to other proteins [93]. The FXIII-B subunits function as carriers, protecting the circulating FXIII-A subunits from proteolysis or systemic clearance [97].

FXIII is activated when thrombin cleaves the activation peptide from the amino terminus of the plasma FXIII-A chains, followed by calcium-dependent dissociation of the B chains [101].

2.1.3.2. The role of coagulation factors in secondary hemostasis

TF forms a 1:1 complex with FVII, which is activated to FVIIa. The TF/FVIIa complex initiates the coagulation cascade by activating FX or FIX. Once the cascade begins, the activation of FX is quickly shut down by tissue factor pathway inhibitor (TFPI). FIXa binds to its cofactor, FVIIIa, on a PL membrane to form the tenase complex, which activates FX. FXa

initiates the common pathway for thrombin generation (TG). It binds to its cofactor, FVa, and Ca^{2+} on a PL membrane to form the prothrombinase complex, which converts prothrombin to thrombin. Thrombin exerts a positive feedback effect on FXI, causing more thrombin to be generated via FIXa and FVIIIa activation of FX [102]. The intrinsic pathway is activated by contact activation via exposed collagen or by autoactivation of FXII when it comes into contact with an artificial surface like ellagic acid or glass. FXIIa activates prekallikrein to kallikrein and FXI to FXIa, which is mediated by high molecular weight kininogen, its cofactor. FXIa again propagates the formation of the tenase complex with subsequent TG. The activation of FV and FVIII by thrombin results in the amplification and propagation of the coagulation cascade. Thrombin subsequently converts fibrinogen to fibrin monomers (FMs). The FMs polymerize to form a fibrin polymer mesh, resulting in a cross-linked fibrin clot. This reaction is catalyzed by activated FXIII (FXIIIa) and results in the formation of a stabilized clot [102]. Furthermore, FXIIIa can covalently cross-link a variety of other proteins, including alpha-2-antiplasmin (α2-antiplasmin), into the fibrin clot, increasing resistance to fibrinolysis.

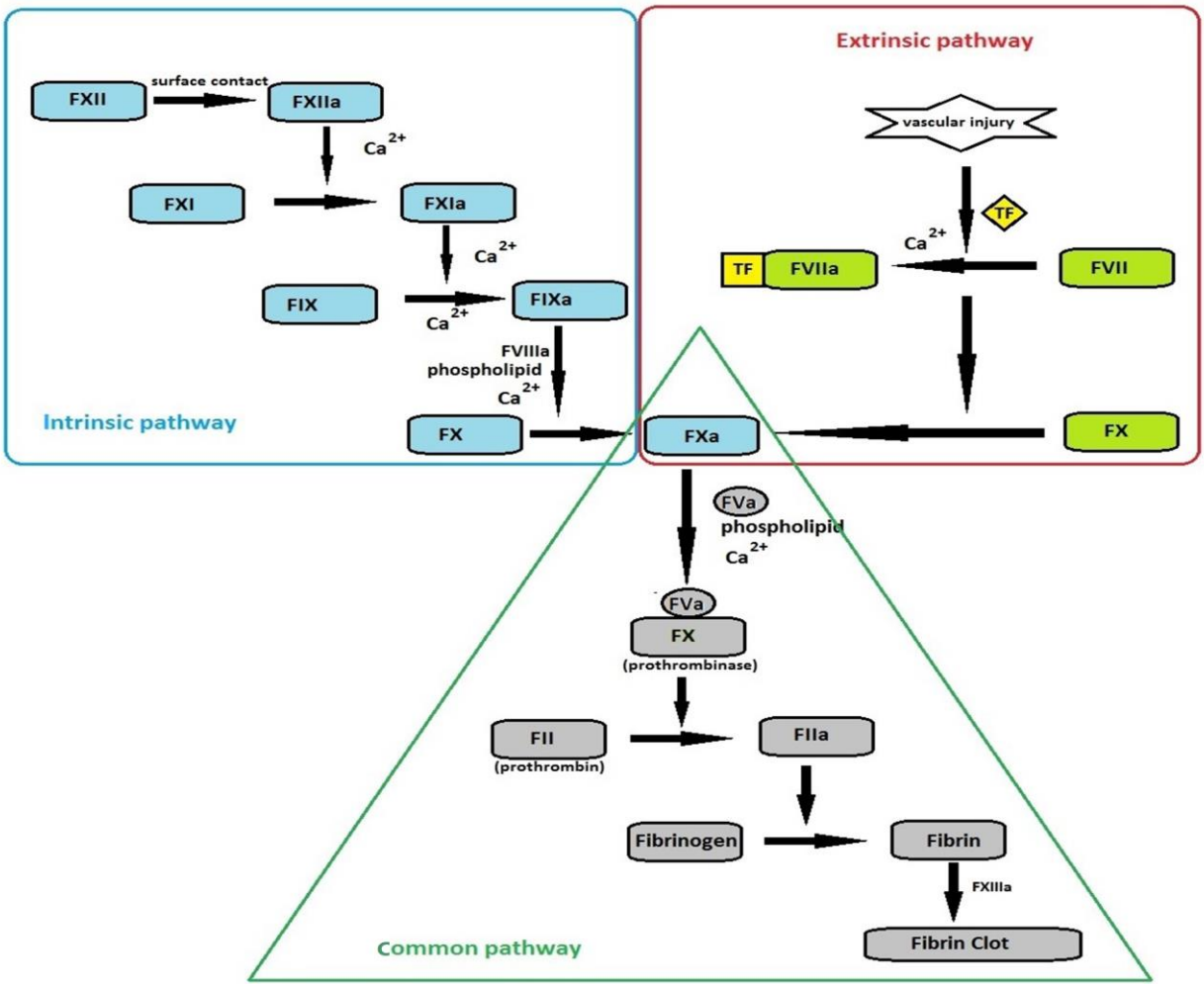

Figure 3. A summary of the blood coagulation pathway. Coagulation is initiated by surface contact or vascular injury, which occurs via the intrinsic and extrinsic pathways, respectively. Both pathways result in factor X activation, which is where the so-called common pathway begins. A fibrin clot is formed when fibrinogen is converted into fibrin. Adapted from [103].

2.1.4. Coagulation inhibitors

The main naturally occurring coagulation inhibitors include antithrombin, protein C (PC), and protein S. Antithrombin inhibits the majority of procoagulant proteases in the coagulation cascade (including FIXa and FXIa), but its main substrates are thrombin and FXa. It binds to thrombin in a 1:1 ratio, producing a thrombin-antithrombin (TAT) complex. This complex inactivates the enzymatic activity of thrombin and causes its elimination from the circulation.

Thirty to 40% of the protein S in human plasma exists as free protein, with the remaining fraction being bound to C4b-binding protein. Only free protein S has anticoagulant properties [104]. In order for PC to have anticoagulant properties, the zymogen must first be activated. While it is true that thrombin has the ability to activate PC, the rate at which this activation occurs under physiological conditions is insufficient to elicit a substantial anticoagulant effect. Thrombomodulin (TM) on endothelial cell surfaces is required for rapid activation of PC. Thrombin binds to TM in a reversible manner and significantly speeds up PC activation by several thousand-fold [105]. Thrombin loses its procoagulant activity after binding to TM. This is because TM, by occupying exosite I of thrombin, prevents thrombin from interacting with other thrombin-binding proteins [106]. The activation of PC by the thrombin-TM complex increases when PC binds to the endothelial protein C receptor [107]. The anticoagulant property of activated PC (APC) is enhanced by two cofactors, namely protein S and the intact form of coagulation FV. While protein S alone is capable of inactivating FVa, protein S and FV act synergistically to inactivate FVIIIa [108].

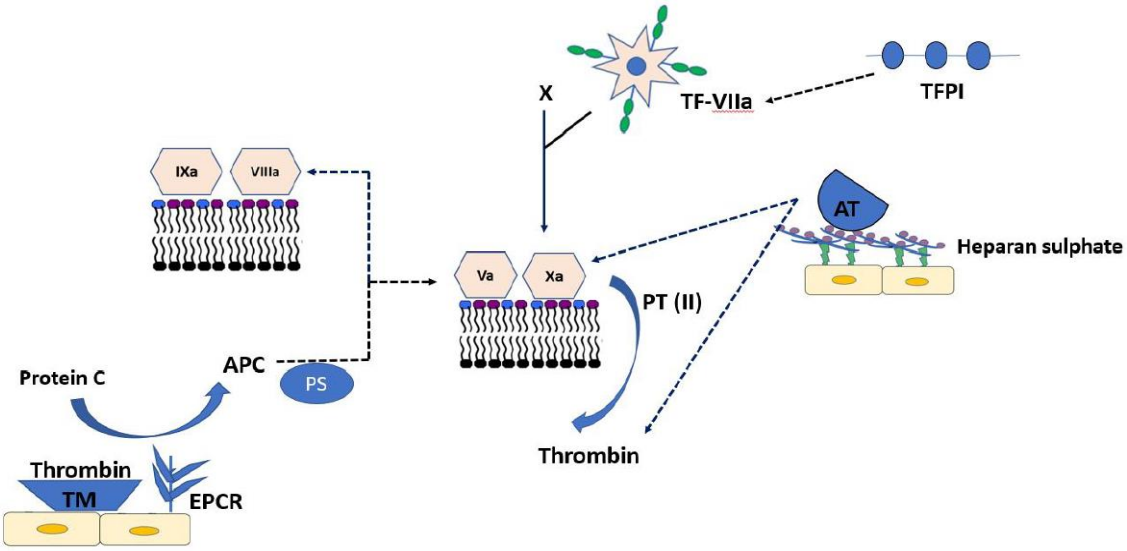

Figure 4. Inhibitors of blood coagulation. Tissue factor pathway inhibitor (TFPI) inhibits the activity of the TF/VIIa complex. Antithrombin (AT) inhibits thrombin and FXa, as well as FIXa and FXIa. Thrombin binding to thrombomodulin (TM) on the surface of endothelial cells causes protein C to be activated, and this is augmented by the endothelial protein C receptor (EPCR). The cofactor activities of FVIIIa and FVa are inhibited by activated protein C (APC) and its co-factor, protein S. Adapted from [109].

2.1.5. The fibrinolytic system

Fibrinolysis is the enzymatic breakdown of the fibrin clot that forms after a vascular injury. The central element in the fibrinolytic system is plasmin. Plasminogen (PLG), the precursor of plasmin, is primarily produced by the liver [110]. The human PLG gene is found on chromosome 6 (6q26 – 27), and spans ~52.5 kb of DNA. It is made up of 18 introns and 19 exons [111]. PLG is a 90 kDa single-chain proenzyme with a blood concentration of ~180 µg/mL (2 µM) and a biological half-life of ~2 days [112]. It is made up of 791 aa that are connected to one another by 24 disulfide bridges, 16 of which give rise to 5 homologous triple loop structures known as kringles [113]. Two enzymes, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), are responsible for the conversion of PLG to its active form, plasmin. During this conversion, the Arg560-Val561 peptide bond of PLG is hydrolyzed, resulting in two chains that are covalently linked by a disulfide bond [114]. tPA is produced and released by vascular endothelial cells, whereas uPA is produced by the urinary epithelium, as well as monocytes and macrophages. tPA and uPA only last for 4 – 8 minutes in the circulation due to the high levels of plasminogen activator inhibitor-1 (PAI-1) [115].

Fibrin, the primary substrate for plasmin, modulates its degradation by binding PLG and tPA on its surface, localizing and increasing plasmin production. In the absence of fibrin, tPA is only a weak PLG activator. Nonetheless, when fibrin is present, it significantly enhances the catalytic efficiency of tPA for PLG activation by at least two orders of magnitude. Following the formation of plasmin, plasmin initiates the cleavage of fibrin into smaller, soluble fragments known as fibrin degradation products (FDPs), while simultaneously exposing carboxy-terminal lysine residues [116]. Multiple FDPs are released, including fibrinopeptide B and D-dimers.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a key regulator of the fibrinolytic system. It is synthesized in the liver and circulates in the blood as a zymogen. TAFI becomes

activated when it comes into contact with thrombin. Activated TAFI (TAFIa) removes carboxy-terminal lysine residues from partially degraded fibrin, thus reducing the binding sites for PLG and plasmin. This, in turn, stabilizes the fibrin thrombi [116]. PAI-1 and alpha-2-plasmin inhibitor (α2-PI) are other regulators of fibrin dissolution. α2-PI rapidly inactivates free plasmin in two steps. The first is a reversible reaction that occurs between the lysine-binding sites of kringle 1 in the heavy chain of plasmin and the carboxyl terminal end of α2-PI. The second step involves the formation of a covalent bond between the active site of plasmin and the reactive region of α2-PI. A stable complex, plasmin-alpha-2-antiplasmin (PAP), is then formed, which serves as a marker for plasmin generation (PG) and, thus, fibrinolysis [117]. PAI-1 is synthesised by a variety of cells, including monocytes and macrophages, vascular endothelial cells, and platelets [118]. It is the primary physiological inhibitor of tPA and uPA that prevents them from converting PLG to plasmin [119].

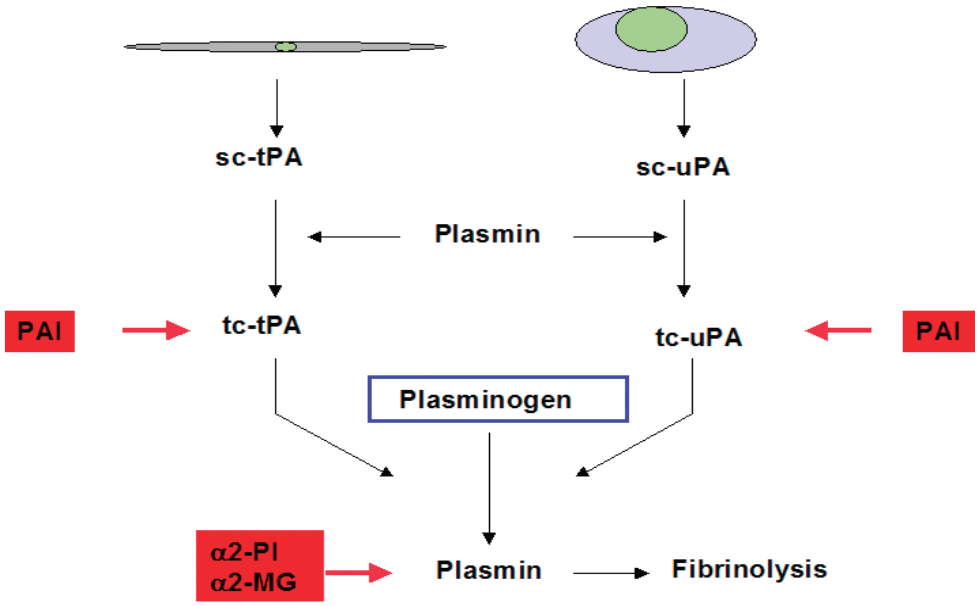

Figure 5. Overview of the fibrinolytic system. Tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) converts plasminogen into plasmin. Plasminogen activator inhibitor (PAI) inhibits tPA and uPA. Plasmin is primarily inhibited by alpha-2-plasmin inhibitor (α2-PI) and, to a smaller extent, by alpha-2-macroglobulin (α2-MG). Following plasmin formation, plasmin converts single-chain tPA (sc-tPA) and single chain uPA (sc-uPA) to their respective two-chain forms, tc-tPA and tc-uPA. Thereafter, plasmin is rapidly inhibited unless it continues to bind to fibrin or to its cell surface receptors. The red boxes represent inhibitors. Adapted from [116].

2.2. *In vitro* coagulation

2.2.1. Standard coagulation (screening) tests

The most commonly used screening tests for investigating coagulation abnormalities are the prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) [120]. PT was introduced by Armand Quick for evaluating prothrombin levels in plasma by adding TF (rabbit brain extract) [121]. The concentration of TF that was used to initiate *in vitro* coagulation in the PT assay was presumably much higher than in the *in vivo* conditions, resulting in rapid TG and its feedback activation of FV. As a result, PT was modified by Langdell and colleagues by introducing "partial thromboplastin." This substance was derived from crude thromboplastin through the processes of ultracentrifugation and dilution, resulting in a PL with a reduced TF concentration [122]. Unlike the “complete” thromboplastin used in the PT assay, this “partial” thromboplastin could distinguish between normal and hemophilic plasma and could still be used in a rapid one-stage assay. The partial thromboplastin time test was also considered for quantifying the amount of FVIII in a given sample [123]. To improve the reliability of the partial thromboplastin time test, a new assay called APTT was introduced. This assay included a contact activator such as kaolin, ellagic acid, or celite [122]. The TT test measures fibrinogen to fibrin conversion by adding exogenous thrombin to platelet-poor plasma (PPP).

There are numerous preparations of thromboplastin reagents, each of which can produce different PT results even when using the same plasma. As a result, to standardize the PT assay, thromboplastin reagents are calibrated against a standard reference provided by the World Health Organisation and assigned an international sensitivity index (ISI). Thereafter, the ISI of each thromboplastin reagent is used to determine the international normalized ratio (INR) using the formula:

$$INR = \left(\frac{PT_{\text{test}}}{PT_{\text{normal}}}\right)^{ISI}$$

The PT test detects deficiencies in coagulation proteins involved in the extrinsic and common blood coagulation pathways and is thus affected by plasma levels of FVII, FX, FV, prothrombin, and fibrinogen. Warfarin and other vitamin K antagonists are regularly monitored for their anticoagulant effects and dose adjustments using PT/INR. APTT, on the other hand, detects deficiencies in coagulation proteins involved in the intrinsic and common pathways and is thus affected by plasma levels of FXII, FXI, FIX, FVIII, FX, FV, prothrombin, and fibrinogen. It is used to monitor treatment with anticoagulants such as unfractionated heparin and direct thrombin inhibitors [124]. Neither the PT nor APTT tests are affected by FXIII deficiency. Heparin prolongs the APTT test, but in therapeutic concentrations, has no effect on the PT test because commercial thromboplastins are combined with exogenous heparin inhibitors [120]. The TT test is used to evaluate fibrinogen abnormalities and to detect inhibitors against thrombin or fibrin. It is useful in the investigation of liver disease and disseminated intravascular coagulation. The test is thus influenced by fibrinogen concentration and/or activity, as well as the presence of heparin and FDPs. The TT test, like PT and APTT, is normal in FXIII deficiency. A prolongation of the PT or APTT test only indicates a deficiency of one or more factors within the relevant coagulation pathways. As a result, additional specific coagulation tests must be performed to identify the underlying cause. Both PT and APTT are also not sensitive enough and thus occur within normal ranges even in clinically significant bleeding disorders such as FXIII deficiency, mild von Willebrand disease, and α2-antiplasmin deficiency [125].

Additionally, these tests provide no information about platelet interactions with coagulation factors *in vivo*. Because activated platelets can locally accumulate coagulation factors, the severity of bleeding during a prolonged PT/APTT test may differ depending on the

platelet count and/or function. Furthermore, because both tests are terminated prior to fibrin polymerization by FXIIIa, it is impossible to determine the overall stability of thrombus formation using PT/APTT tests [122].

2.2.2. Coagulation factor assays

A coagulation factor assay is used to investigate the cause of an unexpectedly prolonged PT or APTT. It is used to detect specific coagulation factor deficiencies or inhibitors. In a factor assay, the plasma being tested is mixed with a control plasma that is deficient in the specific clotting factor under investigation, and the clotting time (CT) is measured. The degree of correction of the CT correlates with the coagulation factor activity in the test plasma. Factor assays are calibrated against dilutions of a normal reference plasma, and the results are reported as a percentage of activity [126]. Factor assays are both specific (they only measure the clotting factor of interest) and sensitive (they can detect low levels of the factor being measured).

2.3. The thrombin generation assay

Traditionally, TG was studied using PT and APTT tests. These tests, however, do not completely reflect the TG that occurs *in vivo*. To begin, plasma tends to clot when < 5% of the total thrombin potential has been generated. As a result, these tests fail to account for > 95% of the thrombin that is produced. Furthermore, the anticoagulant proteins found in plasma are unable to exert their full anticoagulant activity due to insufficient activation. This is especially true for PC, which requires TM on the endothelial cell surface for activation. The plasma and reagents used to perform PT or APTT tests, however, do not contain adequate amounts of TM. The PT and APTT tests can detect a deficiency in one or multiple procoagulant factors, but they cannot determine whether or not this deficiency is counterbalanced by a concomitant anticoagulant factor deficiency [127]. Furthermore, coagulation factor assays can detect specific factor deficiencies, but these may not always agree with the clinical phenotype [128].

The TG assay was developed to evaluate the endogenous capacity of the overall hemostatic potential. It may reveal a bleeding or thrombotic risk [129]. Attempts to measure TG began in 1953 [130, 131], when coagulation was initiated by supplementing plasma or whole blood with TF or cephalin as a source of PL combined with calcium chloride. TG was measured by subsampling the clotting mixture periodically into a fibrinogen-containing tube. The obtained CTs were subsequently utilized to generate a calibration curve, from which the activity of thrombin was derived. This method, however, was deemed time-consuming and subject to high variability, making it unsuitable for use in clinical practice. In 1986, Hemker and colleagues modified this method. They replaced fibrinogen with a synthetic chromogenic substrate that is specific for thrombin. They also used snake venom to defibrinate plasma before measurement and used a computer programme to calculate the parameters that were derived from the TG curve, also known as a thrombogram. In 1993, the same group improved the assay by introducing another substrate (methylmalonyl-methylanalyl-arginyl-pNA) with slow thrombin reactivity [132]. This modification eliminated the time-consuming subsampling method [127]. Later, the chromogenic substrate was replaced with a fluorogenic substrate to avoid the interference caused by the turbidity of the clotting plasma, which required defibrination before testing [127].

TG assays, in general, employ TF to mimic vessel wall damage, and they also require calcium ions. Procoagulant PLs, basically PS (mostly 4 μM), are required in PPP for optimal TF activity and completion of the clotting process [133]. The concentration of TF added to PPP depends on the intended use of the test. For instance, when ≥ 10 pM TF is added, the reaction occurs very quickly with reduced sensitivity to intrinsic pathway coagulation factors. At 2 – 5 pM TF concentrations, however, the reaction is more sensitive to FVIII, FIX, and FXI deficiencies [134]. On the other hand, when platelet-rich plasma (PRP) is used, the platelets take on the role of PLs as an amplifying surface [132].

2.3.1. The calibrated automated thrombography (CAT) method

The CAT method was introduced by Hemker and colleagues for quantifying TG in PPP or PRP [135]. In addition to TF, PL, calcium, and a fluorogenic substrate, this method makes use of a thrombin calibrator, which contains a known concentration of thrombin-like enzyme linked to alpha-2-macroglobulin (α2-MG). This thrombin-like enzyme only reacts with the fluorogenic substrate and is not inhibited by the plasma components. Furthermore, because the colour of the plasma may affect the accuracy of the results, the calibrator is measured continuously for each individual sample [129]. In the TG test, TF and PL are added to plasma in a 96-well plate to activate coagulation. In a subsequent step, a solution containing the fluorogenic substrate (Z-Gly-Gly-Arg-7-amino-4-methylcoumarin) and calcium chloride is added to the reaction mixture. Once thrombin is formed, it proceeds to cleave the fluorogenic substrate, releasing a fluorophore whose fluorescence intensity over time is proportional to the concentration of thrombin generated [135]. The CAT method uses a fluorogenic substrate that is thrombin sensitive. Not only thrombin in its free form can cleave this substrate, but also thrombin bound to α2-MG. This could result in increased TG values. To resolve this problem, the method employs an algorithm that annuls the activity of thrombin linked to α2-MG [135].

The parameters of a thrombogram are the lag time, peak thrombin, endogenous thrombin potential (ETP), time to peak, and StartTail (Figure 6).

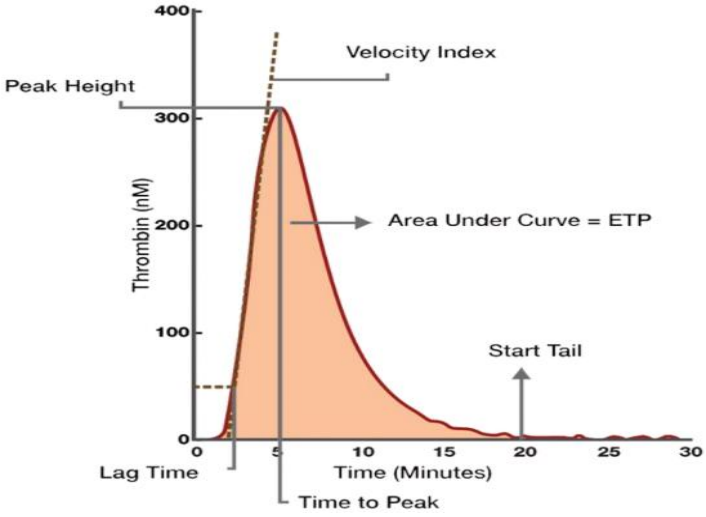

Figure 6. A typical thrombogram and its parameters. Adapted from [136].

Definition of terminologies

(i) Lag time (min): the time until TG begins. It is comparable to the CT in a standard coagulation assay.

(ii) Peak height/peak thrombin (nM): the highest concentration of thrombin generated.

(iii) ETP (nMxmin): the area under the curve(AUC)/the total amount of thrombin formed.

(iv) Time to peak (min): the time until the peak thrombin.

(v) StartTail (min): the time until the endpoint of TG.

(vi) Velocity index [peak thrombin / (time to peak – lag time)], (nM/min): the slope of the curve between lag time and time to peak [127].

Interpretation of TG results

A prolonged lag time with lower ETP and peak thrombin values indicates a decrease in TG potential, which is suggestive of hypocoagulability. Increased TG, on the other hand, is associated with hypercoagulability and is characterized by a shorter lag time and higher ETP and peak thrombin values [137]. In recent years, the use of corn trypsin inhibitor (CTI) in TG assays has become a topic of discussion [138–142]. CTI is added to blood collection tubes prior

to a TG test to inhibit the contact activation pathway via FXIIa inhibition [138, 143]. However, it has been reported that CTI may only be required when using < 1 pM TF in TG tests [140].

2.3.2. Potential applications of the TG assay

The TG assay has been used to monitor the treatment of patients taking oral anticoagulants like rivaroxaban [144] and to assess the efficacy of using prothrombin complex concentrate to reverse its anticoagulant activity [145]. It has also been used to evaluate bleeding risk in patients with congenital deficiencies of FVII, FXII, FXI, FX, FV, and FII [146]. The assay has also proven useful in assessing hypercoagulability in patients carrying the prothrombin G20210A [147] and FV Leiden [148] mutations, as well as in those with antithrombin [149] or protein S deficiencies [150]. In addition, it has shown effectiveness in predicting VTE risk in patients [151–153]. The TG assay has allowed for the estimation of bleeding risk in hemophilia patients who are treated with either FVIII or FVIIa, in addition to evaluating their cardiovascular risk [154, 155]. Segers and colleagues have also discovered that this assay is sensitive to genetic variation in hemostasis-related genes, making it a potentially useful tool for identifying novel genetic risk factors for VTE [156]. Other researchers have found a strong correlation between both ETP and peak thrombin levels and postoperative bleeding tendencies in cardiac surgery patients [157].

2.3.3. Limitations of the TG assay

The scarcity of established reference values for specific TG assay conditions, for instance, the source of TF and its concentration or the use of CTI, makes its clinical application challenging. Ideally, these reference values need to be established in accordance with the adopted protocol for each center and would necessitate similar pre-analytical and analytical conditions for both patient and control test samples. These actions will undoubtedly help to reduce intra- and inter-assay inaccuracies. The duration of the test and the plasma preparation

render it unsuitable for emergency situations [132]. The use of whole blood samples to better replicate *in vivo* conditions must be adopted. Another limitation of the TG assay is its insensitivity to endothelial changes. Endothelial-derived components could be included in the assay to increase its predictive value for the comprehensive evaluation of hemostasis [129].

2.4. Normal plasma cell development

Plasma cells are terminally differentiated cells derived from B cells. Early (pro) B cells arise from hemopoietic stem cells in the BM and undergo rearrangement of their immunoglobulin heavy-chain gene segments. This gene is ~2 Mb in size with four main domains, namely, variable domain, VH (composed of over 100 DNA segments), diversity domain, DH (27 DNA segments), joining domain, JH (6 DNA segments), and constant domain, CH (9 DNA segments). The first rearrangements combine one DH segment with one JH segment via DNA deletions. If this rearrangement is in frame, the pro-B cell continues to differentiate by combining the DH-JH segment with a VH segment and subsequently develops into a precursor (pre) B cell. The regulation of this process involves the action of recombination-activating genes, which recognize specific DNA motifs within the DH, JH, and VH segments. The pre-B cell next rearranges the immunoglobulin light-chain kappa (IgLκ) and immunoglobulin light-chain lambda (IgLλ) genes. This rearrangement begins with the IgLκ gene, which develops into an immunoglobulin M (IgM) kappa-expressing mature B cell. If this rearrangement is unsuccessful, the B cell goes ahead to rearrange the IgLλ gene, which results in an IgM lambda-expressing cell. This phenomenon occurs stochastically, and, in an antigen-independent manner, which explains why there are twice as many kappa producing cells as lambda producing cells. The mature B cells continue their development in the secondary lymphoid organs. Here, molecular rearrangement occurs by somatic hypermutation and in an antigen-dependent manner (in cooperation with T-cells and dendritic cells). Subsequently, stochastic mutations are produced within the immunoglobulin heavy chain DH-

JH-VH segment with the help of an activation-induced deaminase enzyme. Only B cells that produce antibodies that are more specific for the presented antigens will survive; the rest undergo apoptosis.

The final rearrangement occurs by class switch recombination, which also occurs in secondary lymphoid organs and involves the recombination of switch regions with the deletion of interswitch region DNA. This process causes the mature B cell to express a different type of immunoglobulin: immunoglobulin G (IgG), A (IgA), or E (IgE). At last, the mature B cell differentiates into either a memory B cell or a long-lived plasma cell [158] that secretes high-affinity antibody. The plasma cells home to the BM, where they live for months to years [159, 160]. Once BM stromal cells come into contact with plasma cells, they secrete IL-6 [161], which, together with B cell activating factor [162], IL-5, stromal cell-derived factor 1, TNF, and CD44 ligands [163], ensure the survival of the plasma cells.

2.5. Plasma cell development in MGUS and MM

The progression of normal plasma cells to malignant cells involves several oncogenic events, including translocations that involve the immunoglobulin heavy chain locus: t(11;14), t(4;14), t(14;16), and t(6;14), *cyclin D* gene dysregulation, *NRAS* and *KRAS* activating mutations, and nuclear factor kappa B pathway activation. Besides these oncogenic events, the malignant cells are highly dependent on the BM microenvironment [164]. In MM, the malignant plasma cells are localized to the BM in close contact with stromal cells. These are long-lived plasma cells characterized by a markedly low labelling index of 1 – 2% [165]. The immunoglobulin gene sequences in myeloma plasma cells are somatically hypermutated and remain constant throughout the disease [166, 167].

The complications associated with the disease arise as a result of cytokine secretion by the myeloma and stromal cells and interaction through adhesion molecules. Consequently, both stromal cells and osteoclasts are activated, which further supports the growth and survival of

the myeloma cells [168]. The malignant plasma cells produce monoclonal immunoglobulins, also known as monoclonal proteins or paraproteins. In 60% of cases, IgG is the predominant immunoglobulin, followed by IgA (20%), IgD (2%), and IgE (< 0.1%). On rare occasions, a single patient could have more than one monoclonal antibody. In 18% of cases, the paraprotein may be composed of only the light chain of the antibody [169].

2.6. Diagnostic criteria for MM and MGUS

MM was formerly diagnosed with proof of end-organ damage associated with CRAB features. The features of CRAB encompass hypercalcemia, renal insufficiency, anemia, and bone disease. Hypercalcemia is defined as a serum calcium level > 11.5 mg/dL, or 2.88 mmol/L. Renal insufficiency is indicated by a serum creatinine level exceeding 2 mg/dL or 177 μmol/L. Anemia is characterized by a hemoglobin level below 100 g/L or more than 20 g/L below the lower limit of normal. Bone disease manifests as lytic lesions, severe osteopenia, or pathologic fracture [1, 170]. Hypercalcemia is caused by bone destruction and is not very common in myeloma patients [171]. Renal impairment is primarily caused by direct damage to the renal tubules as a result of protein overload, dehydration, hypercalcemia, and nephrotoxic drugs. It affects 20 – 40% of newly diagnosed MM patients [1]. The development of anemia in MM can be attributed to various factors. These include the infiltration of the BM by immature plasma cells, a decrease in the number of erythroid precursors, deficiency of erythropoietin due to renal dysfunction, and impaired iron utilization resulting from elevated production of hepcidin caused by the chronic inflammation commonly observed in myeloma patients [172, 173]. Anemia is prevalent and occurs in approximately 73% of patients upon initial diagnosis and is frequently accompanied by typical symptoms such as dyspnea, weakness, fatigue, and dizziness [171]. In MM, the malignant plasma cells secrete osteoclast-activating factors like TNF-α, IL-1, and IL-6, which promote bone destruction and inhibit osteoblast activity. Osteolytic bone disease causes pathologic fractures and excruciating bone pain, commonly in

the central skeleton. Bone pain may also arise from the presence of expanding plasma cell tumors within the bone, manifesting as a soft tissue mass. Nearly 80% of newly diagnosed MM patients develop bone lesions [171].

The diagnosis of MM based on CRAB features was meant to prevent MGUS and smoldering MM patients from undergoing chemotherapy. Nonetheless, due to significant therapeutic advancements and the discovery of biomarkers capable of differentiating MM from its premalignant stages, the disease definition for MM had to be revised [174–176] by the International Myeloma Working Group (IMWG) in 2014. Together with the CRAB features, the revised criteria allowed the use of specific biomarkers to define MM and advanced imaging techniques to diagnose myeloma bone disease.

In summary, MM is diagnosed based on a BM plasma cell ratio of 10% or more or the presence of biopsy-confirmed bony or extramedullary plasmacytoma. It also requires evidence of any one or more of the CRAB features or biomarkers of malignancy. The individual biomarkers include (i) a BM plasma cell ratio of 60% or more, (ii) a serum FLC ratio of 100 or greater, provided the involved FLC concentration is ≥ 100 mg/L, and (iii) multiple focal lesions on magnetic resonance imaging. MGUS, on the other hand, is characterized by a serum monoclonal protein concentration of < 30 g/L, < 10% BM plasma cells, and no CRAB features [10]. True non-secretory MM requires 30% monoclonal BM plasma cells or a biopsy-confirmed plasmacytoma for diagnosis [177].

2.7. The Revised International Staging System (RISS) for MM

The RISS merges the International Staging System, which is based on serum beta-2 microglobulin (Sβ2M) and serum albumin levels, with disease biology factors like high-risk cytogenetic abnormalities or increased lactate dehydrogenase (LDH) concentration to form a combined prognostic index for clinical management and comparison of data from clinical trials

[178]. It was developed from 11 international trials of 4,445 MM patients at diagnosis. According to this study, myeloma patients diagnosed at stages I, II, and III have 5-year survival rates of 82%, 62%, and 40%, respectively [179]. Stage I is defined by a serum albumin concentration of ≥ 35 g/L, Sβ2M < 3.5 mg/L, no high-risk cytogenetics, and a normal LDH concentration. Stage II is defined as Sβ2M of < 3.5 mg/L, serum albumin concentration < 35 g/L, or Sβ2M 3.5 to < 5.5 mg/L regardless of the serum albumin level. Stage III is defined by Sβ2M levels of > 5.5 mg/L and high-risk cytogenetics [t(4;14), t(14;16), or del(17p)] or elevated LDH [1, 179].

2.8. Treatment of MM

In contrast to MGUS, which does not require treatment, treatment of MM typically consists of various drug combinations such as proteasome inhibitors, IMiDs, corticosteroids, histone deacetylase inhibitors, monoclonal antibodies, nuclear export inhibitors, and high-dose chemotherapy rescued by autologous stem cell transplantation (ASCT) [180]. These drugs work in different ways, but the common goal is to control and destroy the myeloma cells. Prednisone, a corticosteroid, and melphalan, an alkylating agent, have been the gold standard of treatment since the 1960s [181]. Later, high-dose chemotherapy with melphalan rescued by ASCT was introduced [182].

Newer drugs, including the proteasome inhibitor BTZ, thalidomide (THAL), and lenalidomide (LEN), were also incorporated into myeloma treatment [183], which improved patient survival rates. Next-generation IMiDs and proteasome inhibitors, such as carfilzomib and ixazomib, have been developed and approved for treating relapsed or refractory cases. Later, regimens with combinations of monoclonal antibodies and histone deacetylase inhibitors were approved [184]. Currently, MM patients are given induction therapy, which typically consists of a triple combination of BTZ, dexamethasone (DEX), and LEN. Alternative regimens may be chosen based on the suitability of patients for ASCT [180].

2.9. The proteasome, a multicatalytic enzyme

The proteasome is a large protein complex found in eukaryotic nuclei and cytoplasm. It degrades damaged, unfolded, or misfolded proteins [185] and regulates numerous cellular pathways, including apoptosis, cell growth and proliferation, DNA repair, and immune responses [186, 187]. The 26S proteasome is the most studied proteasome complex. It consists of a 20S catalytic core with one or two 19S regulatory subunits attached to either end. The 20S core is barrel-shaped and contains four heptameric rings that are organized into two outer α-rings (α 1-7) and two inner β-rings (β 1-7) [188–191] (Figure 7). The α-rings regulate the entry of proteins (the substrate) into the 20S core and their interactions with the regulatory subunits. The β-rings form the proteolytic core, where the β1, β2, and β5 subunits correspond to the caspase-like, trypsin-like, and chymotrypsin-like activities, respectively [189, 192].

Ubiquitin covalently binds proteins for enzyme-mediated degradation. It is first activated by a ubiquitin-activating enzyme (E1) and then transferred to a ubiquitin-conjugating enzyme (E2). An E3 ligase subsequently binds ubiquitin to the lysine residue of the substrate. To form a polyubiquitin chain, additional activated ubiquitins are bound to internal lysine residues within the already attached ubiquitin. The polyubiquitin chain transports the substrate to the proteasome, where it is degraded by proteolysis [193–196].

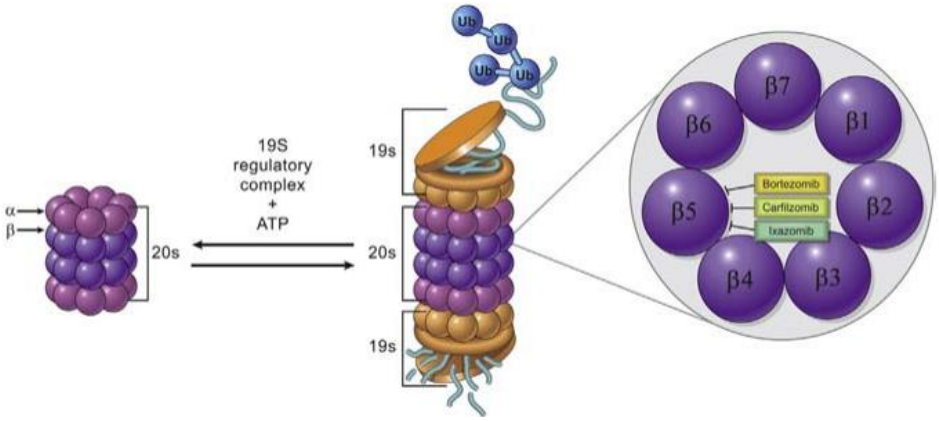

Figure 7. Structure of the proteasome. The 26S proteasome complex is formed by the binding of the 20S catalytic core to the 19S regulatory subunits. Ubiquitin-tagged proteins bind to the 19S subunit and are degraded at the proteolytic β subunit. Proteasome inhibitors mainly inhibit the β5 subunit. Adapted from [197].

2.10. The platelet proteasome

Platelets have an active proteasome system, just like nucleated cells [198, 199]. Yukawa et al. [200] first purified the platelet proteasome with a chromatography column in 1991. They discovered several subunits with trypsin- and chymotrypsin-like activities. In 1993, the same group purified an endogenous activator of the 20S proteasome from human platelets and investigated its effect on three peptidase activities of the proteasome [201]. Later, Ostrowska et al. studied the chymotryptic-like activity of the 20S proteasome [202]. Many years later, a global proteomic analysis identified the caspase-, trypsin-, and chymotrypsin-like activities of the platelet proteasome [203].

Unlike nucleated cells, the function of the proteasome in protein degradation in platelets is poorly understood [198]. However, it is known that the proteasome regulates the production [204] and lifespan [205] of platelets. This is supported by the fact that proteasome inhibition results in a 50% reduction in platelet half-life and subsequently platelet count [198], as well as increased PS exposure, upregulation of pro-apoptotic BAX protein, and a decrease in mitochondrial transmembrane potential [205, 206].

2.11. Proteasome inhibitors

Proteasome inhibitors, as the name implies, cause the accumulation of proteins that would normally be degraded within the cell, resulting in cell death [207, 208]. In 2003, BTZ (Velcade) became the first proteasome inhibitor to be approved for the treatment of both newly diagnosed and relapsed or refractory MM. Carfilzomib (Kyprolis) and ixazomib (Ninlaro) followed in 2012 and 2015, respectively [25, 209].

BTZ is a boronic acid dipeptide with the chemical formula C19H25BN4O4 and the IUPAC name [3-methyl-1-(3-phenyl-2-pyrazinylcarbonylaminopropanoyl)amino-butyl] boronic acid [210]. It binds reversibly to the β5 as well as the β1 and β2 subunits of the proteasome at higher concentrations of the drug [211]. BTZ is also clinically effective in the management of Waldenstrom macroglobulinemia, peripheral T-cell lymphoma, and light-chain amyloidosis when used alone or in combination therapy [212]. Ongoing clinical trials are also evaluating its efficacy in the management of other hematological malignancies, including acute myeloid and lymphoid leukemias, indolent B-cell non-Hodgkin lymphoma, and diffuse large B-cell lymphoma [212].

BTZ inhibits pro-apoptotic protein degradation. It also inhibits IκB (inhibitor of kappa B) degradation, suppresses the nuclear factor kappa B signalling pathway, and prevents the activation of many anti-apoptotic genes that are involved in myeloma progression. Furthermore, BTZ increases the expression of NOXA, a pro-apoptotic protein of the B-cell lymphoma 2 (Bcl-2) protein family that interacts with the anti-apoptotic proteins of the same family to cause myeloma cell death [210].

BTZ has been associated with thrombocytopenia. The mechanism underlying BTZ-induced thrombocytopenia is primarily thought to be related to the inhibition of megakaryocyte proplatelet formation [204, 213] and is not associated with BM injury or decreased thrombopoietin synthesis [214]. The inhibition of megakaryocyte proplatelet formation is

caused by increased levels of activated small GTPase Rho, which is a negative regulator of platelet formation [204, 213]. BTZ also inhibits the human platelet proteasome dose-dependently and affects platelet responsiveness and signalling [215–217].

2.12. Venous thromboembolism: Risk in MM

VTE occurs more frequently in MM patients than in the general population [4, 14–17]. The risk of VTE is higher at diagnosis than after relapse [18]. The pathophysiology of VTE in MM is unclear. Nonetheless, this has been linked to patient-related factors (prolonged immobilization, advanced age, surgery, concomitant infections, hormone therapy, thrombotic history), disease-specific mechanisms, and antimyeloma therapy [24, 218].

In myeloma patients, paraproteins have been linked to thrombotic episodes, potentially causing increased blood viscosity and high levels of immunoglobulins. These factors may impair fibrinolysis, leading to the formation of large clots that occlude blood vessels [28, 29, 43, 219]. They may also act as autoantibodies with procoagulant properties. Lupus anticoagulant, antiprothrombin, antiphospholipid, and anti-protein S antibodies, for example, have been found in MM patients [31, 219, 220]. Paraproteins can also induce endothelial damage [32] and decrease protein S activity [30]. Deitcher et al. reported a severe acquired protein S deficiency in a myeloma patient due to IgG paraprotein binding with free protein S [221]. Furthermore, elevated levels of proinflammatory cytokines like IL-6, TNF-α, and VEGF mediate interactions between plasma cells, BM stromal cells, and endothelial cells, leading to an increase in fibrinogen, vWF, FVIII, and D-dimer levels [30, 34, 37]. Notably, these changes that were already present prior to treatment tend to increase as treatment progresses but decrease after high-dose melphalan and ASCT [35]. Acquired APCR is a significant independent risk factor for VTE in MM patients. According to Zangari et al., 23% of myeloma patients who did not have the FV Leiden mutation at diagnosis had APCR [222].

VTE risk in myeloma patients has also been mainly associated with IMiD treatment [33]. In patients receiving THAL alone, the risk of VTE is < 2% [223]. THAL, DEX, and their combination increase VTE risk by 2.6-, 2.8-, and 8-fold, respectively [224]. In MM patients at diagnosis, THAL and DEX combined treatment have also been shown to increase the incidence of VTE to 12 – 26% [19, 225, 226] and to 2 – 8% in those with relapsed or refractory diseases [227, 228]. The fact that the frequency of VTE in myeloma patients is low when THAL is used alone but significantly increases when used in combination therapy may be due to drug-drug interactions and their combined effect on malignant cells or the vascular endothelium [229]. Similarly, when LEN, a second-generation IMiD, is used alone to treat relapsed or refractory myeloma, the risk of VTE rises by 3 – 5% [230, 231]. However, when combined with high-dose DEX without thromboprophylaxis, the VTE rate in newly diagnosed MM patients rises to 26 – 75% [232, 233] and to 11 – 15% in relapsed or refractory cases [234, 235].

Erythropoietin stimulating agents have also been found to be an independent risk factor for VTE in myeloma patients, increasing the incidence from 1 – 7% in patients receiving high-dose DEX alone and from 5 – 23% in those receiving LEN plus high-dose DEX [33]. Without thromboprophylaxis, proteasome inhibitor treatment with BTZ is associated with a low incidence of thrombotic complications [236, 237]. The underlying mechanism is not well understood. Nonetheless, inhibition of platelet aggregation [238] and upregulation of TM have both been proposed as possible mechanisms [239]. In myeloma patients with acquired APCR, the risk of VTE increases by 50% with THAL treatment [222]. Furthermore, IMiDs have been linked to abnormal TF expression, increased VEGF expression, decreased thrombospondin, and cause a cytokine-mediated APCR. THAL, in particular, has been shown to increase FVIII and vWF levels, as well as platelet aggregation [240].

2.13. Venous thromboembolism: Risk in MGUS

VTE risk in MGUS is well established. A single-center study of 310 MGUS patients found that 19 (6.1%) of them developed VTE during a follow-up period of 44 months [21]. VTE risk was associated with a high paraprotein concentration at the time of diagnosis. Another study looked at 174 MGUS patients and found VTE in 13 (7.5%) of them after a median follow-up period of 8 years [22]. In the univariate analysis, it was found that having a personal or familial history of VTE was a major risk factor [22]. VTE also correlated with low serum albumin levels, high leukocyte count, and immobility. Cohen and Sarid conducted a comparative analysis of VTE in 166 MGUS patients and healthy controls (HCs) and found that MGUS patients had a 38% higher risk of VTE [241]. Muslimani and colleagues also found VTE in 8% of patients diagnosed with MGUS, representing a 23-fold higher risk than the general population [20]. A study involving over four million military veterans in the United States also discovered a three-fold increase in the risk of deep vein thrombosis among MGUS patients [14], while a Swedish study found a 2.1-fold increase in the risk of VTE [242].

The pathophysiology of VTE in MGUS is unclear. Nevertheless, increased TNF-α and IL-6 secretion can stimulate TF expression, increase transcription of fibrinogen, FVIII, and vWF, and decrease protein S activity [20, 39].

2.14. The hypercoagulable state in MM and MGUS: The utilization of the TG assay

The TG assay has been widely used as a functional assay to assess prothrombotic risk in MM and MGUS patients. Several studies have found significant differences in TG assay parameters between newly diagnosed MM or MGUS patients and HC volunteers. This could be explained by variations in the amount of TF used in the test, the inclusion of anticoagulants like APC or TM in the test, and the inhibition of contact activation with CTI. Tiong et al. found a shorter lag time and time to peak, increased velocity index, and increased resistance to TM inhibition in myeloma patients using 1 pM or 5 pM TF, with or without CTI, whereas MGUS

subjects were comparable to HCs in all TG assay parameters studied [218]. Crowley et al. found that with 1 pM TF, MM and MGUS patients had significantly higher peak thrombin levels than healthy volunteers, whereas lag time, time to peak, and ETP were comparable across groups. When APC was included in the TG test, ETP in control plasma was significantly lower than in MM or MGUS plasma [243]. Another study that used 1 pM TF in the TG test found that patients with MM and MGUS had higher ETP and peak thrombin, as well as shorter lag time and time to peak, when compared to control subjects [244]. Furthermore, TG measured with 5 pM TF revealed that myeloma patients had a significantly shorter time to peak and increased velocity index than control subjects, although the lag time, ETP, and peak thrombin values did not differ between groups. However, consistent with a study by Leiba et al. [245], 1 pM TF resulted in no differences in TG assay parameters between patients and controls [246].

2.15. Fibrinolytic activity in patients with MM

There has been little research into the fibrinolytic activity of patients with MM. According to one study, myeloma patients have lower fibrinolytic activity, which is primarily attributable to increased PAI-1 activity. The increase in PAI-1 activity has been linked to elevated IL-6 levels [247]. The fibrinolytic capacity, as determined by the clot lysis time (CLT), did not differ between MM patients and HCs at the time of diagnosis. However, during treatment induction with THAL or vincristine-based combination chemotherapy, fibrinolysis was significantly impaired in myeloma patients, which may help to explain the increased thrombotic risk observed during the first few months of treatment [248]. Another study found that patients with MM have altered plasma fibrin clot structure and function, with clot formation occurring more slowly and with a decreased ability to lyse. This mechanism has been linked to increased TG potential during clot formation, which modifies the coagulant properties of fibrinogen and perhaps other proteins involved in fibrin formation and/or degradation, resulting in more compact clots composed of thin fibers [32]. IgG purified from

myeloma samples has also been shown to inhibit fibrin polymerization, resulting in clots with thinner fibrin fibers that are more resistant to fibrinolysis [29].

2.16. FXIII deficiency

FXIII deficiency is rare. It affects one in every 1 – 2 million people in Europe and North-America and accounts for 6% of all rare bleeding disorders [249]. It is inherited as an autosomal recessive trait. People with severe deficiencies are classified as either homozygotes or compound heterozygotes. Congenital FXIII deficiency can be caused by defects in both the FXIII-A and FXIII-B genes [48]. There are two types: type I and type II. Type I deficiency is a quantitative defect caused by decreased synthesis of the protein, while type II is a qualitative defect characterized by a normal or near-normal concentration of functionally defective FXIII-A antigen [250]. A severe inherited deficiency in FXIII causes abnormal wound healing, excessive bleeding, and recurrent spontaneous miscarriages in women. About 80% of cases are associated with delayed umbilical cord bleeding [48]. Intracranial bleeding occurs in approximately 30% of patients. Other symptoms include intramuscular and subcutaneous hematomas, ecchymoses, bleeding from the mouth and gingiva, and prolonged bleeding after trauma [92].

Acquired FXIII deficiency may be caused by autoantibodies against a FXIII subunit, decreased synthesis of a FXIII subunit resulting from impaired BM function or liver disease, FXIII consumption, or dilution coagulopathy [48]. Autoantibodies against FXIII subunits can either be neutralizing or non-neutralizing. Neutralizing autoantibodies affect the activity of FXIIIa or its activation. The non-neutralizing type forms an immune complex with FXIII subunits, which is subsequently cleared from the plasma by the reticuloendothelial system [48]. Acquired FXIII deficiency, characterized by significantly reduced plasma FXIII levels, has been associated with pulmonary embolism, leukemia, Crohn disease, myelodysplastic syndrome, disseminated intravascular coagulation, Henoch-Schönlein purpura, ulcerative

colitis, liver cirrhosis, sepsis, major surgery, and stroke [250]. The frequency and etiology of acquired FXIII deficiency in MM and MGUS patients remain poorly understood.

3. AIMS AND OBJECTIVES

3.1. Aims of study

Project 1. We aimed to investigate the effect of BTZ on platelet function.

Project 2. We aimed to study hypercoagulable changes and fibrinolytic alterations in newly diagnosed MM and MGUS patients.

3.2. Detailed objectives

Project 1. Effect of BTZ on platelet function

In this project, we hypothesized that inhibition of human platelet proteasome activity by BTZ results in a procoagulant platelet phenotype with subsequent TG. Our objectives were:

1. To develop an *in vitro* experimental approach to optimally study the effect of BTZ on platelet function.
2. To investigate the effect of BTZ on platelet activation.
3. To determine whether BTZ-induced platelet PS expression causes subsequent TG.

Project 2. Hypercoagulable changes and fibrinolytic alterations in MM and MGUS

Our objectives were:

1. To analyze plasma procoagulant and anticoagulant factors in newly diagnosed MM and MGUS patients.
2. To investigate hypercoagulable changes and fibrinolytic alterations in patients with MM and MGUS.
3. To investigate the potential role of APC in thrombotic risk in MM and MGUS patients.
4. To determine the frequency and etiology of acquired FXIII deficiency and several parameters of fibrinolysis in MM and MGUS patients.

4. MATERIALS AND METHODS

4.1. Materials

BTZ (Velcade) was obtained from Selleckchem (Munich, Germany), solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), and stored in small aliquots at –20^{o}C until needed. Bovine thrombin, thrombin receptor-activating peptide (TRAP), 3,3′-dihexyloxacarbocyanine iodide [DiOC6(3)], human serum albumin (HSA), Sepharose CL-2B column, Histopaque®-1077 (Ficoll), and RPMI-1640 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sigma-Aldrich (St. Louis, MO, USA) also supplied the salts and powder that were used in the preparation of phosphate buffered saline (PBS), buffered saline glucose citrate (BSGC), paraformaldehyde fixative, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Dynabeads™ Untouched™ Human B Cells Kit was purchased from Life Technologies (AS, Oslo). The 24-well multidish was obtained from Thermo Fisher Scientific (Rochester, NY). Anti-CD62P-PE, anti-CD42a-FITC, and mouse IgG1-PE antibodies, as well as Annexin V-FITC and Annexin V-binding buffer were obtained from Becton Dickinson (San Jose, CA, USA). Ethylene diamine tetra-acetic acid (EDTA), 3.2% (0.105 M) sodium citrate, and serum separator tubes were also obtained from Becton Dickinson (San Jose, CA, USA). Anti-CD41-PE antibody was purchased from DAKO (Glostrup, Denmark). The size calibration beads (Megamix-Plus FSC) were purchased from Biocytex (Marseille, France). The reagents or kits for measuring PT (human recombinant thromboplastin reagent, Dade Innovin), APTT, TT, FII activity, FVIII activity, fibrinogen, protein S activity, vWF antigen (vWFAg), vWF activity (vWFAct), TAT complex, D-dimer, α2-PI activity, PLG activity, as well as FV-deficient plasma were all obtained from Siemens Healthcare Diagnostics Products (GmbH, Marburg, Germany). The FM kit was purchased from Diagnostica Stago (Asnieres, France). Technozym PAP complex enzyme-linked immunosorbent assay (ELISA) kit (Technoclone, Vienna, Austria) was used for the

measurement of PAP complex. APC was purchased from CellSystems (Troisdorf, Germany). Plasma FXIII activity was measured using commercially available reagents (Technochrome FXIII, Technochlone, Austria). The reagents, calibrator, Fluo-Buffer, and fluorogenic substrate used in the TG and PG assays were obtained from Synapse Research Institute, Maastricht, The Netherlands. Human TF (Innovin, Siemens, Marburg, Germany) and recombinant tissue plasminogen activator (rtPA) (Alteplase, Boehringer Ingelheim, Ingelheim, Germany) were obtained as indicated. The 96-well black and transparent microplates were purchased from Greiner Bio-One North America Inc., Monroe, MI, USA, and Greiner Bio-One Inc. GmbH, Kremsmünster, Austria, respectively.

4.2. Study population

Project 1. Informed consent was obtained from a cohort of 41 healthy volunteers who had not taken any medications that could potentially affect platelet function in the two weeks preceding the blood sampling procedure. The study received ethical approval from the Ethics Committee of the University of Debrecen, with the approval number RKEB/IKEB 4875-2017.

Project 2. Individuals who had recently been diagnosed with MM or MGUS were recruited from two medical centers in Hungary: The Department of Internal Medicine at the University of Debrecen and the Department of Hematology at the Jósa András Teaching Hospital in Nyíregyháza. The diagnoses of MM and MGUS were established using the IMWG criteria [10]. The MM patients were grouped into stages I, II, and III using the International Staging System for MM [251]. The following were the exclusion criteria for both patient groups: a recent history of arterial or VTE or a hemorrhagic disorder (within the last 6 months before recruitment), a known history of thrombophilia, antiphospholipid syndrome, liver dysfunction, any previous malignancy except for MM or MGUS, exposure to anticoagulant or antiplatelet medications (within the month prior to recruitment), major surgery or trauma within the past month, thrombocytopenia (platelet count $< 100 \times 10^9$/L), hypofibrinogenemia

(fibrinogen concentration < 1.5 g/L), and end-stage renal failure. The control group consisted of healthy blood donors who were matched with the patients for age, sex, and blood group. Control subjects with known hemorrhagic or thrombotic disorders, such as thrombocytopenia or hypofibrinogenemia, malignancy, autoimmune disorders, including antiphospholipid syndrome, and chronic kidney or liver disease, were excluded. Those who were taking anticoagulants, had an increased risk of cardiovascular events, or had recently experienced acute illnesses, major surgery, or trauma within the past month were also excluded from the study. Moderate hypertension, however, was not considered an exclusion criterion. The patients in the MM and MGUS groups were comparable in terms of thrombotic risk factors (e.g., cardiovascular risk factors). Table 1 shows the demographic and clinical data of the study population. Serum protein electrophoresis and immunofixation were performed on all samples from MM and MGUS patients. The gamma (γ) or beta (β) globulin region revealed homogeneous paraprotein fractions, which were later identified as IgM, IgA, IgD, or IgG kappa or lambda. None of the control samples showed monoclonal proteins on serum protein electrophoresis. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Debrecen (approval number: DE RKEB/IKEB 5906-2021). Before recruitment, all study participants provided voluntary written informed consent.

Table 1. Demographic and clinical data of the study population

	HC (n = 30)	MGUS (n = 34)	MM (n = 17)
Age, yr	65 (61 – 71)	67 (65 – 73)	68 (63 – 76)
Female, n (%)	23 (76.7)	19 (55.9)	10 (71.4)
ABO blood group (non-O), n (%)	20 (66.7)	29 (85.3)	11 (78.6)
Subtype, n (%)	N/A		
IgG		22 (65)	10 (59)
IgA		5 (15)	1 (6)
IgD		0	1 (6)
IgM		7 (20)	0
Light chain disease		0	5 (29)
Kappa		18 (53)	12 (71)
Lambda		16 (47)	5 (29)
ISS stage, n (%)	N/A	N/A	
I			4 (23)
II			2 (12)
III			11 (65)

Median (interquartile range) or frequency (%) are shown. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; ISS, International Staging System; N/A, not applicable.

4.3. Blood sampling and processing

All study subjects had their peripheral venous blood drawn into EDTA, sodium citrate, or serum separator tubes and processed immediately. PPP was obtained through centrifugation at 1500g for 15 min at room temperature (RT). PRP was prepared by centrifugation at 170g, 15 min, RT, and the platelet counts were determined using a Sysmex XP-300 hematology analyzer (Sysmex, Kobe, Japan) and adjusted to 250 x 10^9/L with PPP. Plasma samples for TG and PG assays were obtained through a subsequent centrifugation of PPP at 10,000g, 10 min, RT, to remove all residual platelets. For clot lysis assay (CLA) and functional hemostasis assays, plasma samples were obtained by a subsequent centrifugation of PPP at 1500g, 15 min,

RT. The PPP samples were stored in aliquots at –70◦C until use. Total protein, CRP, LDH, and total calcium were measured in frozen sera.

4.4. Isolation of B cells from peripheral blood mononuclear cells (PBMCs)

PBMCs were separated on Histopaque®-1077 (Ficoll). B cells were isolated from PBMCs by magnetic separation (negative isolation, Dynabeads™ Untouched™ Human B Cells Kit) using beads in combination with an antibody mix [(contains biotinylated mouse IgG antibodies for CD2, CD14, CD16 (specific for CD16a and CD16b), CD36, CD43, and CD235a (Glycophorin-A)] to remove all other cells from the sample, leaving the B cells in suspension. This technique resulted in 97% cell viability. The cell count was adjusted to 2 x 10^6/mL in RPMI-1640 for flow cytometric analysis.

4.5. Isolation of platelets by gel-filtration chromatography

Citrated whole blood from healthy volunteers was diluted with equal volumes of BSGC (129 mM NaCl, 1.6 mM KH_2PO_4, 14 mM sodium citrate, 11 mM glucose, and 10 mM NaH_2PO_4; pH 7.3) in plastic tubes and centrifuged immediately at 170g, 15 min, RT to obtain PRP. Gel-filtration was performed as previously described [252]. Briefly, PRP was layered onto a Sepharose CL-2B column equilibrated with BSGC. The column was packed with beads which allowed plasma solutes to enter the gel of the matrix, while platelets, by virtue of their size, were only restricted to the spaces between the beads. The platelets therefore moved faster through the column and were eluted ahead of all the other plasma components (Figure 8). The eluates were collected into Eppendorf tubes, and platelet counts were measured for each fraction. Platelets devoid of plasma proteins; fractions 7 – 11 (Figure 9) as determined by the Pierce bicinchoninic acid (BCA) protein assay were subsequently used in experiments.

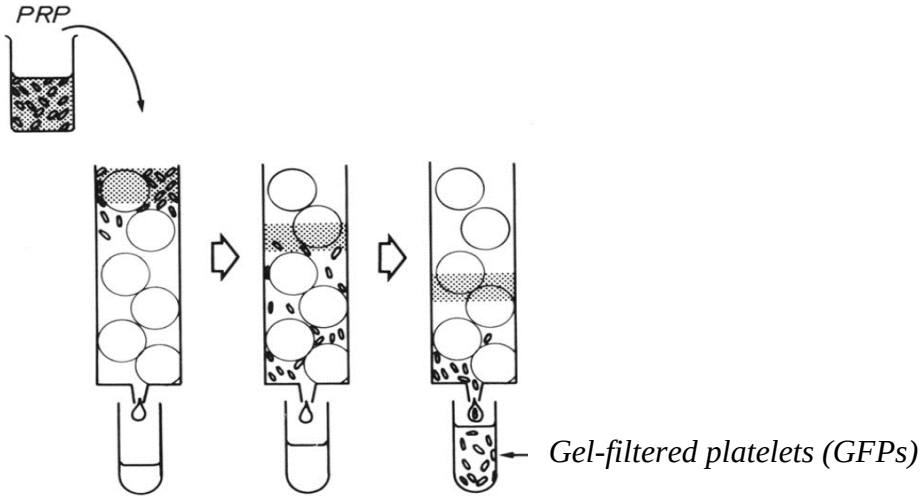

Figure 8. Illustration of platelet gel-filtration chromatography. The large circles, tiny dots, and elliptical shapes, respectively, represent the beads of the Sepharose CL-2B column, plasma solutes, and platelets. Adapted from [252].

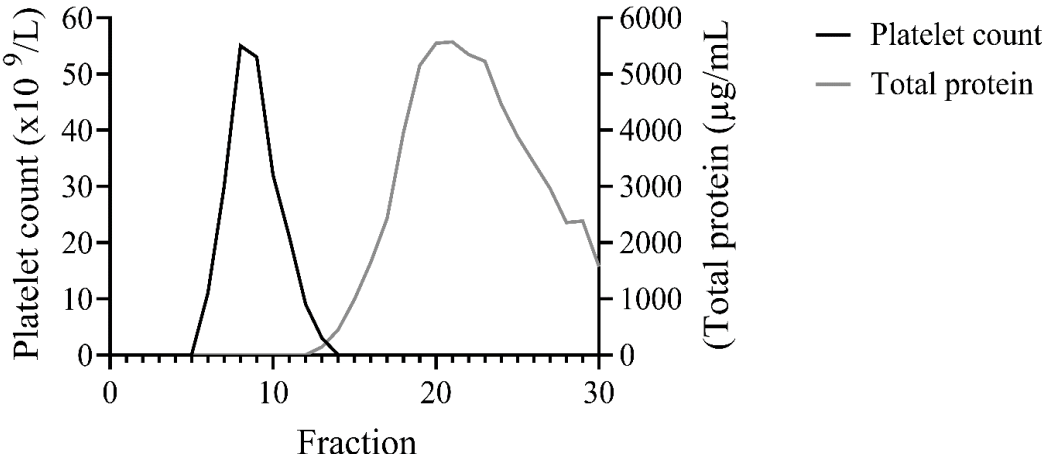

Figure 9. A graph showing the eluted platelet fractions. Gel-filtration chromatography was used to isolate thirty platelet fractions. The platelet count of each fraction was first measured and then centrifuged at 1500g, 15 min, RT to sediment the cells. The Pierce BCA protein assay was used to detect the presence of proteins in the supernatants, and a microplate reader (LabSystems MULTISKAN MS microplate reader) was used to measure the total protein concentrations. Platelet fractions totally devoid of proteins (fractions 7 – 11) were used in all experiments.

4.6. Measurement of hematological, hemostasis, and biochemical parameters

Complete blood count was measured from EDTA-anticoagulated blood using an ADVIA-2120i automated hematology analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). PT, APTT, and TT tests were performed on a BCS-XP coagulometer (Siemens Healthcare Diagnostics, Marburg, Germany). The Cobas 6000 chemistry analyzer (Roche Diagnostics, Mannheim, Germany) was used to measure CRP, LDH, total protein, and total calcium levels. Fibrinogen levels were measured by the standard method of Clauss (reference range, 1.5 – 4.0 g/L). Protein S activity was measured using a clotting assay which is based on plasma protein S cofactor activity, which enhances the anticoagulant function of APC. FII and FV activities were measured by a PT-based clotting factor assay using the appropriate factor-deficient plasma. FVIII activity was measured by a chromogenic assay, while vWFAg and vWFAct were measured by the Innovance vWFAg/Act assay. Plasma FXIII activity was measured by an ammonia release assay. Where FXIII activity was below the lower limit of normal (69%), the assay was repeated to determine whether an inhibitor was present. This was done by performing a 1:1 mixing study of the plasma from the patient and HCs. A sandwich ELISA was used to determine FXIII-A_2B_2 antigen levels [253] as well as the total FXIII-B subunits [254]. Free FXIII-B subunit levels were calculated based on FXIII-A_2B_2 and total FXIII-B subunit levels using the following formula: free FXIII-B = (total FXIII-B) – [FXIII-A_2B_2 x (0.49)]. Quantitative FM was measured using the Liatest FM assay. PAP and TAT complex levels were determined using commercially available ELISA kits. Quantitative D-dimer levels were measured using a particle-enhanced, immuno-turbidimetric assay. α2-PI activity and PLG activity were determined by commercially available methods. APC was determined by an APTT-based assay with a pre-dilution in excess of FV-deficient plasma. The ratio between the APTT with and without APC was calculated (APC sensitivity ratio, APC-sr). The cut-off value for normal APC-sr was established according to the manufacturer's

recommendation. Patients with APC-sr values below 2.0 were classified as APC-resistant.

Prolonged PT, APTT, and TT were defined as > 12.5 s, > 37.7 s, and > 25 s, respectively. The activity ranges for FII and FV were set at 70 – 120%, and for FVIII, 60 – 150%. Abnormal protein S activity was defined as values below the reference range of 60 – 130%, whereas vWFAg and vWFAct were defined as 50 – 160% and 61 – 179% respectively. The cut off values for CRP were < 4.6 mg/L for females and < 5.2 mg/L for males.

4.7. Flow cytometry

4.7.1. PS expression in magnetically isolated B cells

B cells were resuspended in RPMI-1640 and pipetted into the wells of a 24-well multidish. The cells were treated with BTZ at final concentrations of 26 nM, 260 nM, and 2.6 µM and incubated at 37ºC for 24 h. 0.2% DMSO served as a negative control. Cell viability was assessed by trypan blue exclusion. The cells were washed and stained with annexin V FITC for 15 min, RT, in the dark, and immediately resuspended in annexin V binding buffer for flow cytometric analysis.

4.7.2. Platelet PS expression and platelet microparticle (PMP) determination

Platelet PS expression was measured by annexin V-binding. Human PRP and GFPs were incubated with BTZ at different concentrations (26 nM, 260 nM, and 2.6 µM), thrombin (1 U/mL) or TRAP (40 µM), and DMSO (0.2%) for 15 and 60 min at 37ºC. In separate experiments, GFPs were supplemented with HSA (10, 20, and 40 mg/mL) at RT before incubation with BTZ at 37ºC for 60 min. In all experiments, 5 µL of pretreated sample was stained with 5 µL each of annexin V-FITC and anti-CD41-PE antibody in 35 µL of annexin V-binding buffer (2.5 mM calcium chloride). The samples were incubated for 15 min at RT in the dark, diluted to 550 µL with annexin V-binding buffer, and analyzed immediately on an FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA). Ten thousand platelets were

acquired per sample and analyzed with the Kaluza software (Beckman Coulter, Brea, CA, USA). The PMP gate was set using size (0.3 – 1 μm) calibration beads (Megamix-Plus FSC) and PMP analysis was performed from tubes of PS-stained GFP samples that had been incubated with BTZ for 60 min.

4.7.3. Platelet P-Selectin expression

At 37°C, human GFPs were treated with BTZ (26 nM, 260 nM, and 2.6 μM), thrombin (1 U/mL), and DMSO (0.2%) for 15 and 60 min. In separate experiments, GFPs were supplemented with 40 mg/mL HSA prior to incubation with BTZ for 15 min. The samples were fixed with 1% paraformaldehyde (pH 7.4) for 1 h at RT in the dark, followed by a 15-min wash with PBS at 2500g. Forty microliters of washed resuspended platelets were stained with 5 μL each of anti-CD42a-FITC and anti-CD62P-PE antibodies. After two more washes, the samples were resuspended in PBS for flow cytometry. Ten thousand platelets were collected per sample and analyzed with Kaluza software.

4.7.4. Platelet mitochondrial inner membrane potential (Δψm) depolarization

Five microliters of GFPs treated with BTZ (26 nM, 260 nM, and 2.6 μM), thrombin (1 U/mL), or DMSO (0.2%) for 15 or 60 min were diluted 1:20 with BSGC and stained with 20 μL of 1.5 μM DiOC6(3) dye for 20 min at RT in the dark. The samples were diluted to 560 μL with BSGC, and platelets were acquired based on forward scatter (FSC) and side scatter (SSC) properties. The fluorescence of DiOC6(3)-stained platelets was analyzed on the SSC-FL1 dot plot. The percentage of depolarized platelets was determined as a decrease in fluorescence of the DiOC6(3)-stained platelets.

4.8. Thrombin Generation Assay

TG was assessed by the CAT method (Fluoroskan Ascent FL fluorimeter, Thrombinoscope BV, Maastricht, The Netherlands). In project 1, GFPs were treated with BTZ (26 nM, 260 nM, and 2.6 μM), TRAP (40 μM), and DMSO (0.2%) at 37°C for 60 min. The pretreated platelets were resuspended in autologous PPP to a final concentration of 20 x 10^9/L. TG was measured with PRP reagent [1 pM recombinant TF (rTF)]. In project 2, TG was measured in PPP using PPP low reagent (1 pM rTF, 4 μM PL). In both projects, 20 μL of reagent or calibrator containing thrombin-α2-MG complex was pipetted into a 96-well black plate. Eighty microliters of samples were added and incubated at 37°C for 10 min. TG was initiated by automatic dispensation of 20 μL of FluCa solution [Fluo-Buffer (100 mmol/L calcium chloride)] and fluorogenic substrate (*Z*-Gly-Gly-Arg- AMC) into each well (final calcium chloride concentration, 16.67 mmol/L). In project 2, to achieve a 10 nM APC final concentration in the total reaction volume of 120 μL, 7 μL of PPP was removed and replaced with 7 μL of 170 nM stock APC solution without further incubation. The following TG parameters were computed using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands): lag time, peak thrombin, time to peak, StartTail, and ETP. All measurements were performed in triplicate.

4.9. Plasmin generation assay

PG was assessed in plasma by the CAT method (Fluoroskan Ascent FL fluorimeter, Thrombinoscope BV, Maastricht, The Netherlands) as published earlier, with some modifications [255]. Briefly, 70 μL of reagent containing 5 pM TF, 4 μM PL, and 1.25 μg/mL tPA or calibrator was pipetted into a 96-well black plate. Thirty microliters of freshly thawed PPP was added and incubated at 37°C for 10 min. PG was started by the automatic dispensation of 20 μL of a solution containing plasmin substrate (Boc-Glu-Lys-Lys-AMC) and calcium chloride (final concentrations, 500 μM and 16.7 mM respectively). The following PG

parameters were evaluated using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands): lag time (the time until plasmin generation begins), peak plasmin (the highest concentration of plasmin generated), time to peak (the time until the peak plasmin), and endogenous plasmin potential (the area under the curve). All measurements were carried out in triplicate.

4.10. *In vitro* clot lysis assay

CLA was performed as previously described [256]. Briefly, plasma samples were thawed in a water bath at 37°C. In the wells of a 96-well microtiter plate, a clot induction and lysis mix was prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, pH 7.4), where citrated plasma was mixed with 1000-fold diluted human TF and 100 ng/mL rtPA. The plasma was 1.2 times diluted with buffer. Clotting and subsequent lysis were induced by automatically pipetting HEPES buffer containing calcium chloride (21 mM) into each sample well. All concentrations are provided as final concentrations in a 100 μL well volume. Turbidity was monitored at 340 nm every minute for 300 min at 37°C using a TECAN Infinite m200 microplate reader (TECAN Trading AG, Mannedorf, Switzerland). Samples were measured in quadruplicate. The Shiny App Software tool was used to analyze curves [257]. Clot formation and lysis were defined using the following variables calculated from the turbidimetric curves: maximum absorbance, time to maximum absorbance, various CLT points: 10%CLT, 50%CLT, 90%CLT, and CLA AUC. CLTs were defined as the time from the 10%, 50%, or 90% point, from clear to maximum turbidity, to the 10%, 50%, or 90% point in the transition from maximum turbidity to the final baseline turbidity, respectively.

4.11. Viscoelastometry

The ClotPro point-of-care device (Enicor GmbH, Munich, Germany) and its consumables were used for viscoelastometric tests. Briefly, clot formation is initiated by electronically pipetting 340 µL of citrated whole blood into an 'active tip' filled with activator reagents [258, 259]. The sample is mixed with the reagent during pipetting and immediately transferred into a cylinder-shaped cup containing a pin. An elastic element causes the cup to rotate in both clockwise and anticlockwise directions during the measurement, while the pin remains in place. As a clot forms, the analyzer records the restriction of movement in the cup. The data is used to generate thromboelastographic amplitude values that are plotted against time. The measurements were performed at 37°C for 40 min and automatically terminated by the software. Quality control measurements were routinely performed as recommended by the manufacturer. The tPA-assay is a TF-activated coagulation assay that also contains rtPA (650 ng/mL). The following ClotPro parameters were evaluated: CT (s); the time from the start of the test until a clot amplitude of 2 mm is reached], maximum clot firmness [MCF (mm); the maximum amplitude reached during the test], and lysis time [LT (s); the time from CT until 50% of fibrinolysis is detected].

4.12. Statistical Analysis

GraphPad Prism (GraphPad Prism Inc., La Jolla, CA, USA) was used to analyze the data. The test for normality was performed by the Kolmogorov-Smirnov test. Differences between categorical variables were assessed by the $\chi 2$ test and expressed as the frequency (%) of subjects in each category. Continuous variables were expressed as mean ± SD or median and interquartile range. Ordinary one-way Analysis of Variance (ANOVA), followed by Dunnett's or Tukey's post hoc test or Kruskal-Wallis with Dunn's post hoc test, was used to compare differences between groups with Gaussian and non-Gaussian distributions, respectively. Student's *t*-test for paired data or Wilcoxon matched pairs signed rank test was

used to compare differences in TG with and without APC among individual groups depending on their distributions. Pearson's or Spearman's correlation coefficient was used to determine the strength of correlation between continuous variables. For all comparisons, a p-value < 0.05 was considered statistically significant.

5. RESULTS

This chapter presents the results of both Project 1 and Project 2.

Project 1. Effect of BTZ on platelet function

5.1. BTZ induces B cell apoptosis

In preliminary experiments, we confirmed the apoptosis-inducing effect of BTZ on peripheral blood B cells of a healthy donor. Consistent with the results of a previous study [260], we observed an increase in PS exposure in all BTZ-pretreated samples compared to DMSO (Figure 10).

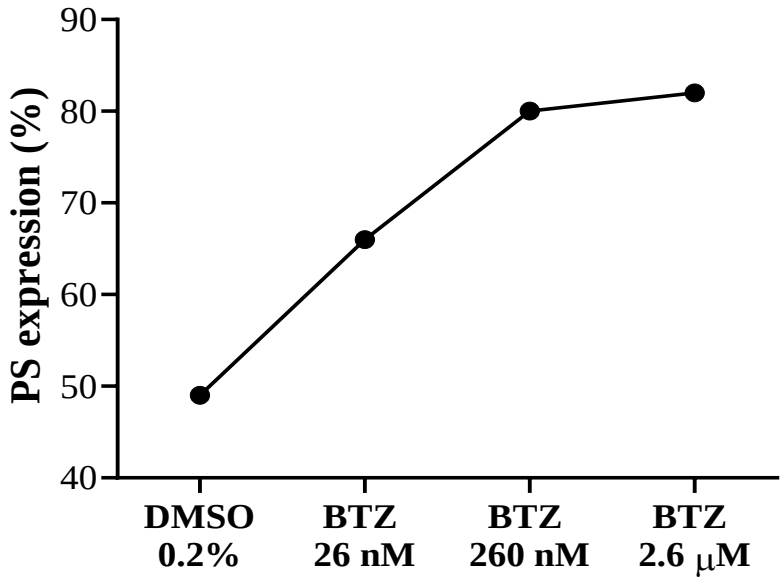

Figure 10. PS expression in BTZ-pretreated B cells. PS, phosphatidylserine; DMSO, dimethyl sulfoxide; BTZ, bortezomib, (*n* = 1).

5.2. Platelet activation and apoptosis studies

To determine whether BTZ causes platelet activation, we first measured PS exposure in human PRP pretreated with BTZ (26 nM, 260 nM, and 2.6 μM), TRAP (40 μM) as a positive control, and DMSO (0.2%) as a negative control. The BTZ concentrations used in the experiment have been described as steady-state concentrations in patients receiving BTZ

treatment [261]. Following a 60-min treatment, BTZ resulted in nonsignificant PS exposure in PRP, while significantly increased PS expression was observed in TRAP-stimulated platelets (Figure 11).

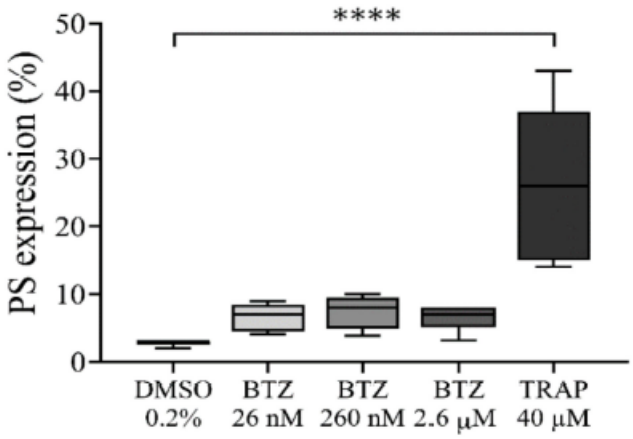

Figure 11. PS expression in BTZ-pretreated platelet-rich plasma. ****$p < 0.0001$ ($n = 5$) compared to the DMSO group. PS, phosphatidylserine; DMSO, dimethyl sulfoxide; BTZ, bortezomib; TRAP, thrombin receptor-activating peptide.

As 83% of BTZ binds to human plasma proteins at therapeutic concentrations [262, 263], we postulated that plasma proteins could neutralize its direct effect on platelets in an *in vitro* setting. The next step was to investigate its potential effect on plasma-free platelets. Earlier experiments utilizing washed platelets and GFPs demonstrated that gel-filtration is a simple method of platelet preparation that yields significantly reduced levels of 'artificial' P-selectin expression. We were able to confirm that the GFPs used in this study were completely free of plasma proteins by measuring the protein concentration of all eluates using the Pierce BCA protein assay.

Like the PRP, the GFPs were treated with increasing concentrations of BTZ. Thrombin served as the positive control. The dot plots in Figure 12A show the effect of BTZ and thrombin on platelet PS exposure. PS exposure increased significantly in thrombin-treated platelets at 15 and 60 min. Similarly, BTZ pretreatment increased PS exposure dose-dependently at 15 min

and resulted in a double increase in PS at 60 min (Figure 12B). These findings justified the stimulatory effect of BTZ on isolated platelets. As a result, GFPs were used in subsequent experiments.

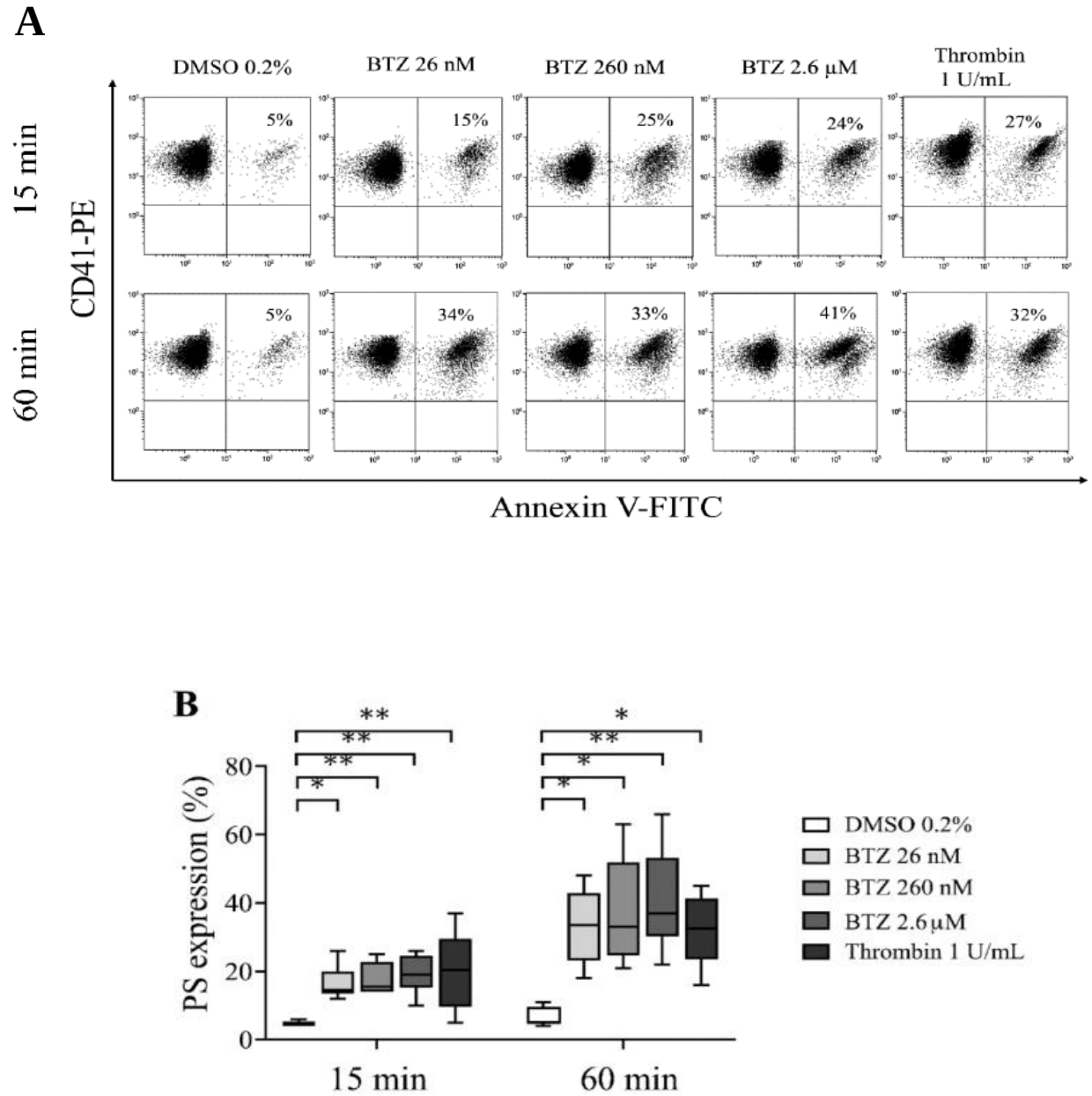

Figure 12. PS expression in BTZ-preincubated gel-filtered platelets. Panel A is a representative dot plot of the percentage PS expression based on CD41 and annexin V double positivity (upper right quadrant). Panel B shows the percentage of PS expression in the BTZ-treated groups compared to DMSO. *$p < 0.05$ and **$p < 0.01$ ($n = 6$) compared to the DMSO group. PS, phosphatidylserine; DMSO, dimethyl sulfoxide; BTZ, bortezomib.

To verify the possible effect of BTZ on platelet activation, we examined platelet *a*-granule secretion by measuring surface P-selectin expression. Both BTZ and thrombin showed a significant increase in P-selectin expression after 15 min of incubation; by 60 min, BTZ showed a dose-dependent increase in these values (Figure 13).

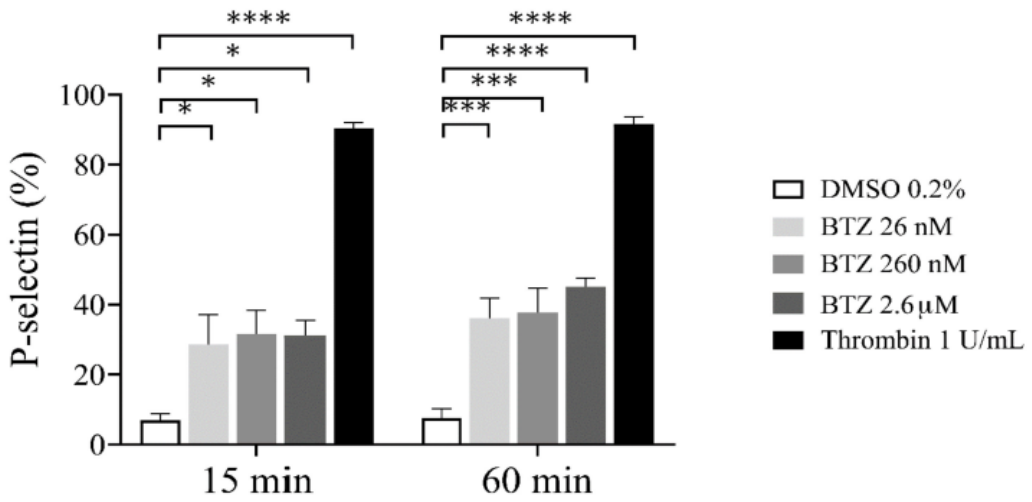

Figure 13. P-selectin expression in BTZ-preincubated gel-filtered platelets. *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ ($n = 5$) compared to the DMSO group. DMSO, dimethyl sulfoxide; BTZ, bortezomib.

Next, we investigated the effect of BTZ on platelet lifespan by measuring the platelet Δψm depolarization. Changes in the Δψm were monitored using DiOC6(3) dye. Depolarization of the Δψm was quantified as an increase in the percentage of depolarized cells and a decrease in the fluorescence of DiOC6(3)-stained platelets. Figure 14A shows the percentage of depolarized cells in one of twelve experiments. At the specified times, the percentage of depolarized cells in BTZ-treated platelets was significantly higher as was the percentage in thrombin-treated platelets (Figure 14B). This was accompanied by a decrease in the median fluorescence intensity (Figure 14C).

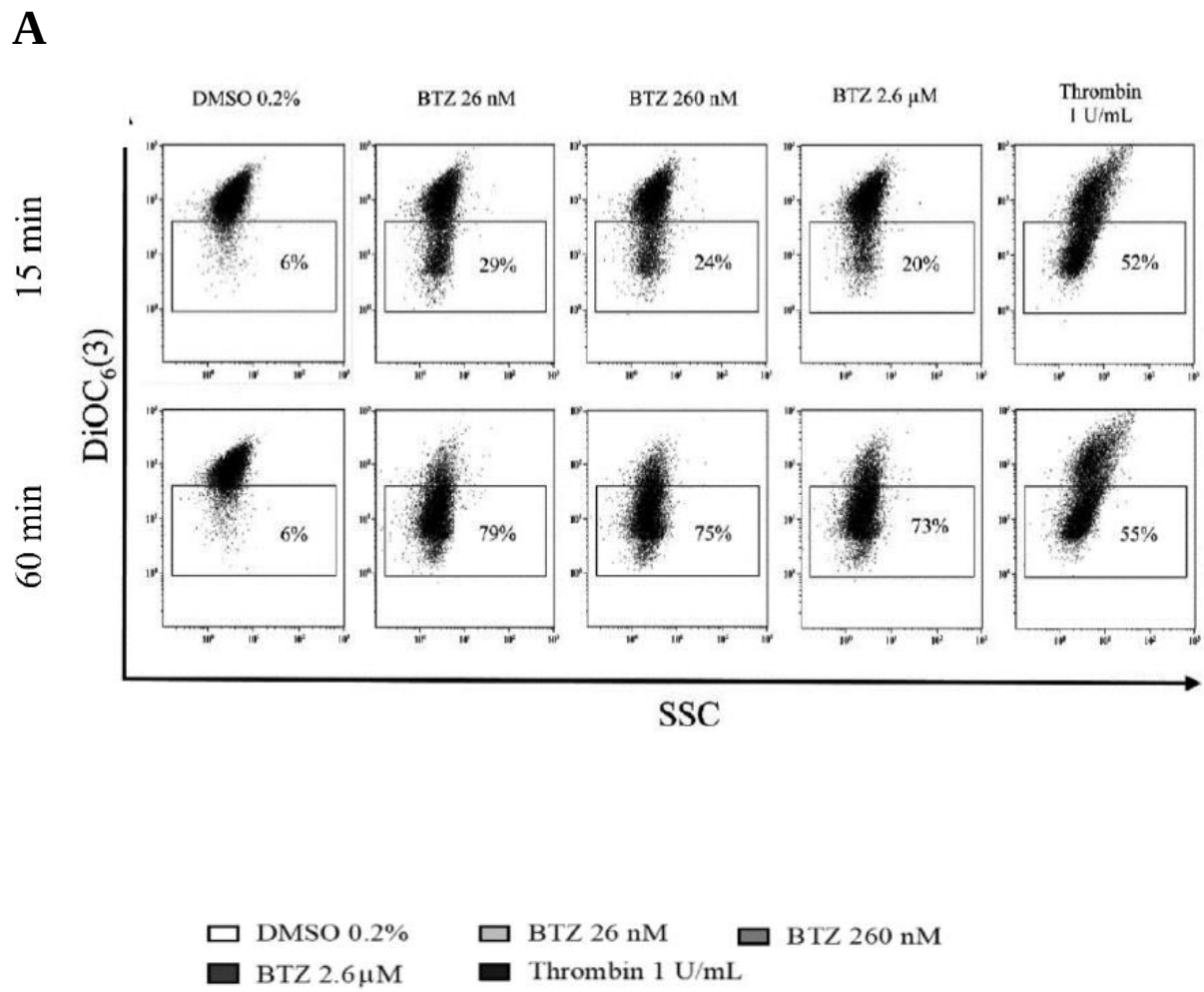

Figure 14. BTZ induces Δψm depolarization of gel-filtered platelets. Panel A is a representative dot plot of the percentage of depolarized cells in one of twelve experiments. Depolarization of the Δψm was quantified as an increase in the percentage of depolarized cells (B) and a decrease in the fluorescence of DiOC6(3)-stained platelets (C). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ ($n = 12$) compared to the DMSO group. DMSO, dimethyl sulfoxide; BTZ, bortezomib; SSC, side scatter.

5.3. Functional assays for enhanced PS expression

Since BTZ increases PS exposure in GFPs, the TG test was performed as a global evaluation of the hemostatic system. The representative TG curves demonstrate the effect of BTZ on thrombin formation *in vitro* (Figure 15A). Compared to the control group, peak thrombin and velocity index were significantly higher in all the BTZ and TRAP-treated groups (Figure 15B,G). ETP was significantly increased after TRAP pretreatment, unlike BTZ (Figure 15C). Although the lag time values were shorter, no significant differences were found between the BTZ-treated groups and the control group (Figure 15D). The time to peak (Figure 15E) and StartTail (Figure 15F) parameters, however, were significantly shorter compared to the control.

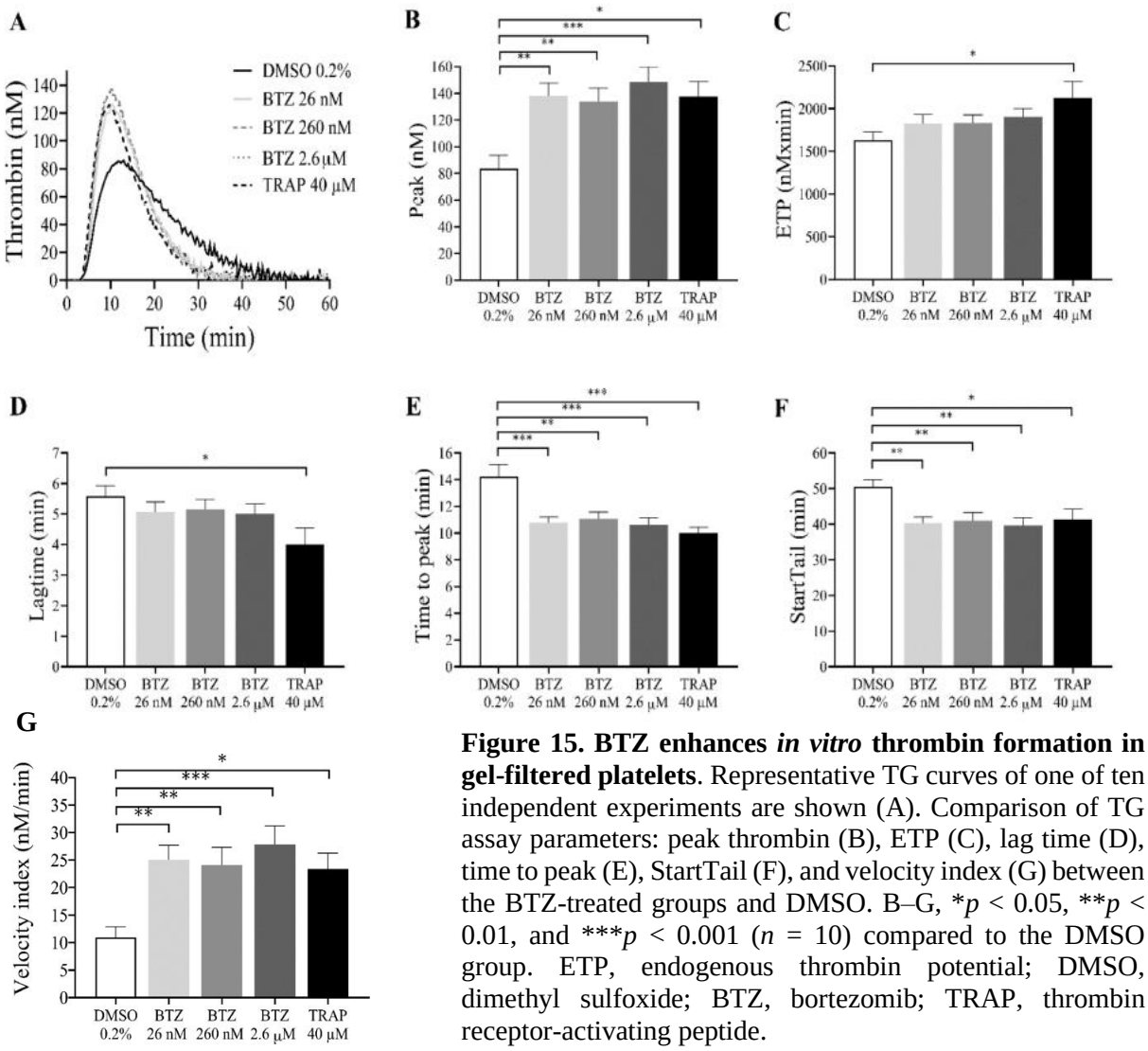

Figure 15. BTZ enhances *in vitro* thrombin formation in gel-filtered platelets. Representative TG curves of one of ten independent experiments are shown (A). Comparison of TG assay parameters: peak thrombin (B), ETP (C), lag time (D), time to peak (E), StartTail (F), and velocity index (G) between the BTZ-treated groups and DMSO. B–G, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ($n = 10$) compared to the DMSO group. ETP, endogenous thrombin potential; DMSO, dimethyl sulfoxide; BTZ, bortezomib; TRAP, thrombin receptor-activating peptide.

Platelets release microparticles through cellular blebbing when they become activated or undergo apoptosis [264]. Platelet-derived microparticles express PS, which provides a surface for the assembly of the prothrombinase complex (FXa/FVa), facilitating thrombin formation. In this regard, we investigated the possible role of BTZ in inducing PS expression in PMPs. Compared to the control group, all the BTZ-treated groups showed a significantly higher number of annexin V-positive PMPs (Figure 16). TRAP showed only a weak stimulatory effect on PMP formation, which is consistent with previous findings [265].

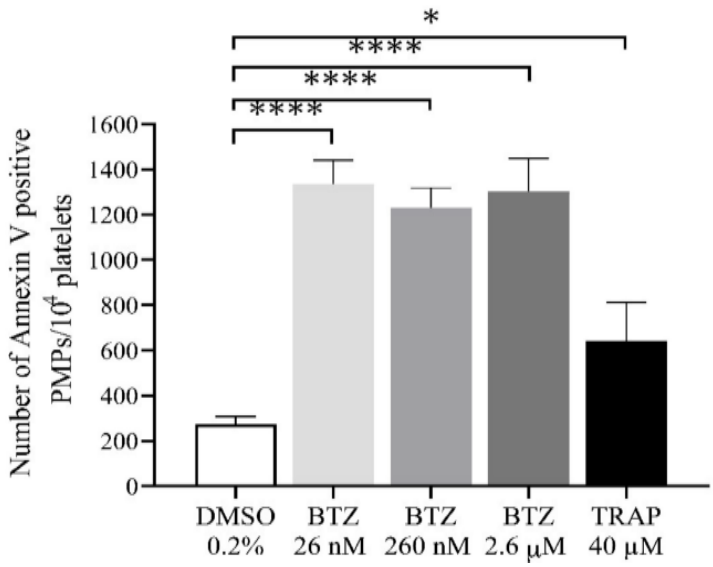

Figure 16. Annexin V-positive PMPs in BTZ-pretreated gel-filtered platelets. *$p < 0.05$ and ****$p < 0.0001$ ($n = 10$) compared to the DMSO group. PMPs, platelet microparticles; DMSO, dimethyl sulfoxide; BTZ, bortezomib; TRAP, thrombin receptor-activating peptide.

5.4. Plasma proteins neutralize the effect of BTZ on human platelets *in vitro*

To demonstrate that plasma proteins inhibit the effect of BTZ on platelets *in vitro*, GFPs were either supplemented with HSA or buffer without further incubation before subsequent treatment with BTZ and thrombin. Albumin was used in this series of experiments because it is the most abundant plasma protein. To mimic the experiment on PS expression in BTZ-pretreated PRP, GFPs with and without HSA supplementation were treated with BTZ for 60 min, as BTZ did not significantly induce PS expression in PRP after this time point.

PS expression was increased in the absence of albumin supplementation, as expected. P-selectin expression was similarly increased following a 15-min BTZ pretreatment in the absence of albumin. However, these effects were attenuated in the presence of albumin (Figure 17A,B). Thrombin 1 U/mL was used as a positive control in these measurements. We found no differences in PS expression between GFPs with and without albumin. Similarly, albumin had no effect on thrombin-induced P-selectin expression (Table 2).

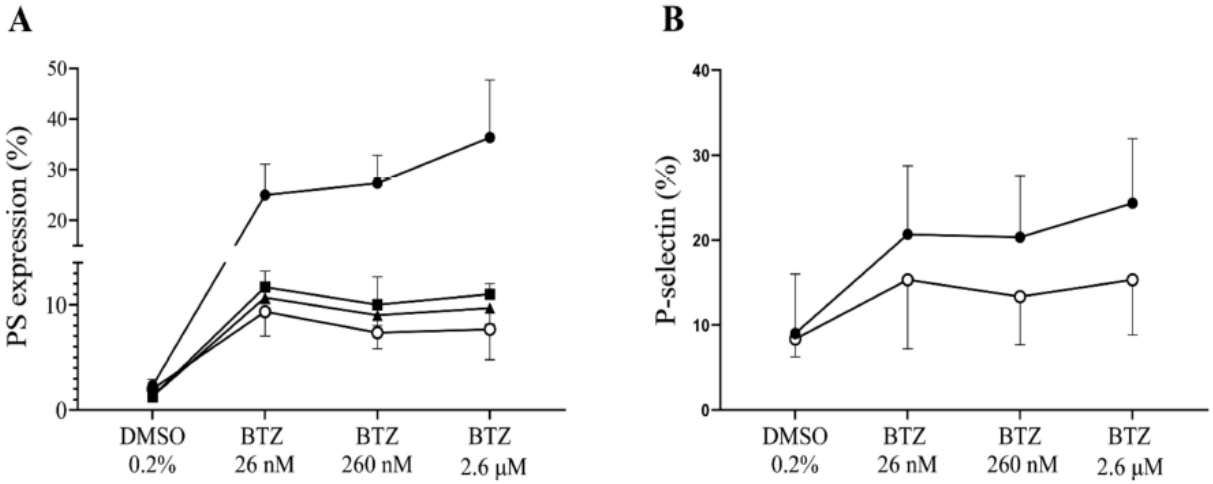

Figure 17. Albumin neutralizes the effect of BTZ on gel-filtered platelets *in vitro*. PS expression (A) and P-selectin expression (B) in BTZ-treated platelets without albumin are represented by lines with solid circle. Lines with squares, triangles, and open circles represent albumin concentrations of 10, 20, and 40 mg/mL, respectively (n = 3). PS, phosphatidylserine; DMSO, dimethyl sulfoxide; BTZ, bortezomib.

Table 2. Thrombin-induced PS and P-selectin expression in gel-filtered platelets with and without albumin supplementation

Buffer/HSA	PS expression (%)	P-selectin (%)
Buffer	31 ± 5.7	94.7 ± 2.5
10 mg/mL	32 ± 7.6	-
20 mg/mL	33 ± 6.6	-
40 mg/mL	31 ± 7.5	95 ± 2.6

HSA, human serum albumin; PS, phosphatidylserine.

Project 2. Hypercoagulable changes and fibrinolytic alterations in MM and MGUS

5.5. Baseline characteristics of the study cohort

Table 3 shows the baseline characteristics of the study population. Age, sex, and blood group distribution were comparable across groups. Hemoglobin concentration and red blood cell count were significantly lower in MM patients compared to MGUS patients and HCs, whereas red cell distribution width was higher. Total protein, CRP, and LDH concentration were significantly higher in MM and MGUS patients than in controls.

Table 3. Baseline characteristics of the study cohort

Parameters	HC (n = 30)	MGUS (n = 34)	MM (n = 14)	p-value
Age, yr	65 (61 – 71)	67 (65 – 73)	68 (63 – 76)	0.0914
Female, n (%)	23 (76.7)	19 (55.9)	10 (71.4)	0.1947
ABO blood group (non-O), n (%)	20 (66.7)	29 (85.3)	11 (78.6)	0.2079
Hb, g/L	141.1 ± 11.5	134.5 ± 17.1	102.8 ± 17.7	< 0.0001 *< 0.0001, #< 0.0001
RBC, $x10^{12}$/L	4.7 (4.5 – 5.1)	4.4 (4.1 – 4.9)	3.1 (2.8 – 4.0)	< 0.0001 *< 0.0001, #0.0003
MCV, fL	89.6 (86.2 – 93.0)	91.1 (88.1 – 93.5)	92.2 (89.9 – 96.3)	0.0553 *0.0484
RDW, %	13.6 (13.3 – 14.0)	14.0 (13.2 – 14.6)	15.8 (13.9 – 18.6)	0.0043 *0.0029, #0.0433
PLT, $x10^{9}$/L	245.6 ± 56.6	233.0 ± 68.1	209.9 ± 72.4	0.2537
MPV, fL	8.3 ± 1.1	9.2 ± 1.3	8.9 ± 1.1	0.0093 +0.0067
WBC, $x10^{9}$/L	6.4 (5.3 – 7.8)	6.0 (4.6 – 8.0)	7.3 (6.0 – 8.2)	0.2176
CRP, mg/L	1.6 (0.8 – 2.8)	4.8 (1.4 – 6.3)	5.7 (2.0 – 17.0)	0.0043 +0.0205, *0.0133
LDH, U/L	111.5 (79.5 – 133.3)	188.0 (161.0 – 209.0)	158.5 (137.8 – 197.3)	< 0.0001 +< 0.0001, *0.00065
Total protein, g/L	70.6 ± 10.2	76.5 ± 7.0	101.6 ± 28.2	< 0.0001 *< 0.0001, #< 0.0001
Total calcium, mmol/L	2.4 ± 0.3	2.4 ± 0.1	2.5 ± 0.3	0.2990

Mean ± SD, median (interquartile range), or frequency (%) are shown. p-values without annotation represent the overall ANOVA or Kruskal-Wallis test results for the indicated parameters, while p-values with annotation show the outcome of the post-hoc analysis between two groups only where significant: comparison between +HC vs. MGUS, *HC vs. MM, #MGUS vs. MM. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; Hb, hemoglobin; RBC, red blood cell; MCV, mean cell volume; RDW, red cell distribution width; PLT, platelet; MPV, mean platelet volume; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase.

5.6. Hemostatic parameters of the study cohort

5.6.1. Coagulation profile

The results of coagulation screening tests in patients with MM or MGUS and those of HCs were not significantly different. The median fibrinogen levels did not differ between groups. Patients with MM and MGUS had statistically lower protein S activity but higher FV activity when compared to HCs. Protein S activity was reduced in 6 (43%) of the MM patients, 1 (3%) of the MGUS patients, but not in any of the HCs. The APC ratio was comparable across groups (Table 4).

Table 4. Coagulation profile of the study population

	HC (n = 30)	MGUS (n = 34)	MM (n = 14)	p-value
PT, s	8.4 (7.9 – 8.7)	8.4 (8.0 – 8.7)	9.0 (7.9 – 10.1)	0.2283
INR	0.93 (0.89 – 0.96)	0.96 (0.91 – 0.98)	1.00 (0.88 – 1.12)	0.0846
APTT, s	27.5 ± 2.5	29.4 ± 3.9	28.6 ± 4.8	0.1307
TT, s	17.9 (17.2 – 18.7)	17.6 (16.9 – 18.6)	18.5 (17.1 – 21.7)	0.2553
Fibrinogen, g/L	3.7 (3.3 – 4.1)	3.7 (3.1 – 4.2)	3.8 (3.3 – 4.5)	0.6511
FII activity, %	121.1 ± 19.3	111.1 ± 17.3	104.1 ± 21.4	0.0144 *0.0175
FV activity, %	91.7 ± 39.2	123.9 ± 31.9	118.6 ± 34.9	0.0016 +0.0015
Protein S activity, %	111.1 ± 22.2	94.8 ± 21.5	76.7 ± 41.6	0.0005 +0.0407, *0.0004
APC ratio	2.73 ± 0.35	2.88 ± 0.44	2.76 ± 0.26	0.2919

Mean ± SD or median (interquartile range) are shown. p-values without annotation represent the overall ANOVA or Kruskal-Wallis test results for the indicated parameters, while those with annotation show the outcome of the post-hoc analysis between two groups only where significant: comparison between +HC vs. MGUS, *HC vs. MM. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PT, prothrombin time; INR, international normalized ratio; APTT, activated partial thromboplastin time; FII, factor II; FV, factor V; APC, activated protein C.

5.6.2. FVIII and vWF levels

FVIII activity, vWFAg, and vWFAct levels were significantly increased in MM patients compared to HCs (Figure 18A,B,C). MGUS patients also had elevated FVIII activity and vWFAg levels compared to HCs (Figure 18A,B). FVIII activity was higher than normal in 71% of MM patients, 46% of MGUS patients, and 17% of HCs. vWFAg levels were higher than normal in 75% of MM patients, 64% of MGUS patients, and 28% of HCs. For vWFAct, 67% of MM, 46% of MGUS, and 8% of HCs had values that were higher than the upper limit of normal.

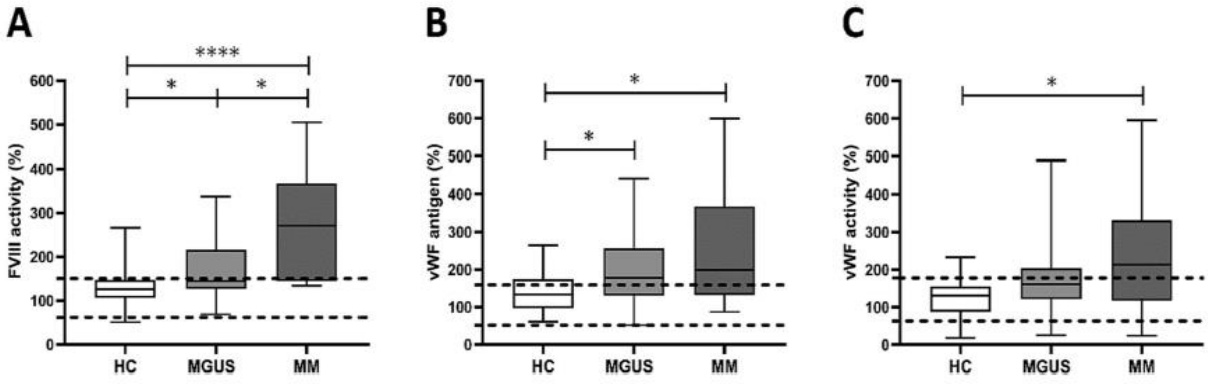

Figure 18. FVIII and vWF levels are elevated in MM and MGUS patients compared to HCs. The horizontal dashed lines indicate the reference ranges for FVIII activity (A), 60 – 150%, vWF antigen (B), 50 – 160%, and vWF activity (C), 61 – 179%. *$p < 0.05$ and ****$p < 0.0001$ (A – C). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; FVIII, factor VIII; vWF, von Willebrand factor.

5.7. Plasma TG and sensitivity to APC inhibition

Untreated MM and MGUS patients had higher levels of TG than HCs (Figure 19A – F). ETP was significantly increased in MM patients [median: 1595 (IQR: 1293 – 1848) nMxmin in patients vs. 1292 (1038 – 1637) nMxmin in controls, $p = 0.0435$] (Figure 19B). Peak thrombin was 70% higher in MM patients compared to controls [291.3 (229.8 – 342.1) nM in patients vs. 171.4 (123.2 – 244.4) nM in controls, $p < 0.0001$], while MGUS patients had a 46% higher peak thrombin [249.6 (180.1 – 305.4) in patients vs. 171.4 (123.2 – 244.4)

nM in controls, $p = 0.0024$] (Figure 19C). The time to peak in MM patients was significantly shorter [9.4 (8.3 – 11) min in patients vs. 11.5 (10.6 – 12.8) min in controls, $p = 0.0019$] (Figure 19D). The StartTail parameter was also shorter in the patient groups compared to controls (Figure 19E). The velocity index was more than twice as high in MM patients [101.9 (74.2 – 125) nM/min in patients vs. 40.8 (26.5 – 64.1) nM/min in controls, $p < 0.0001$] and 84% higher in MGUS patients [75.2 (47.6 – 104.6) nM/min in patients vs. 40.8 (26.5 – 64.1) nM/min in controls, $p = 0.0003$] (Figure 19F).

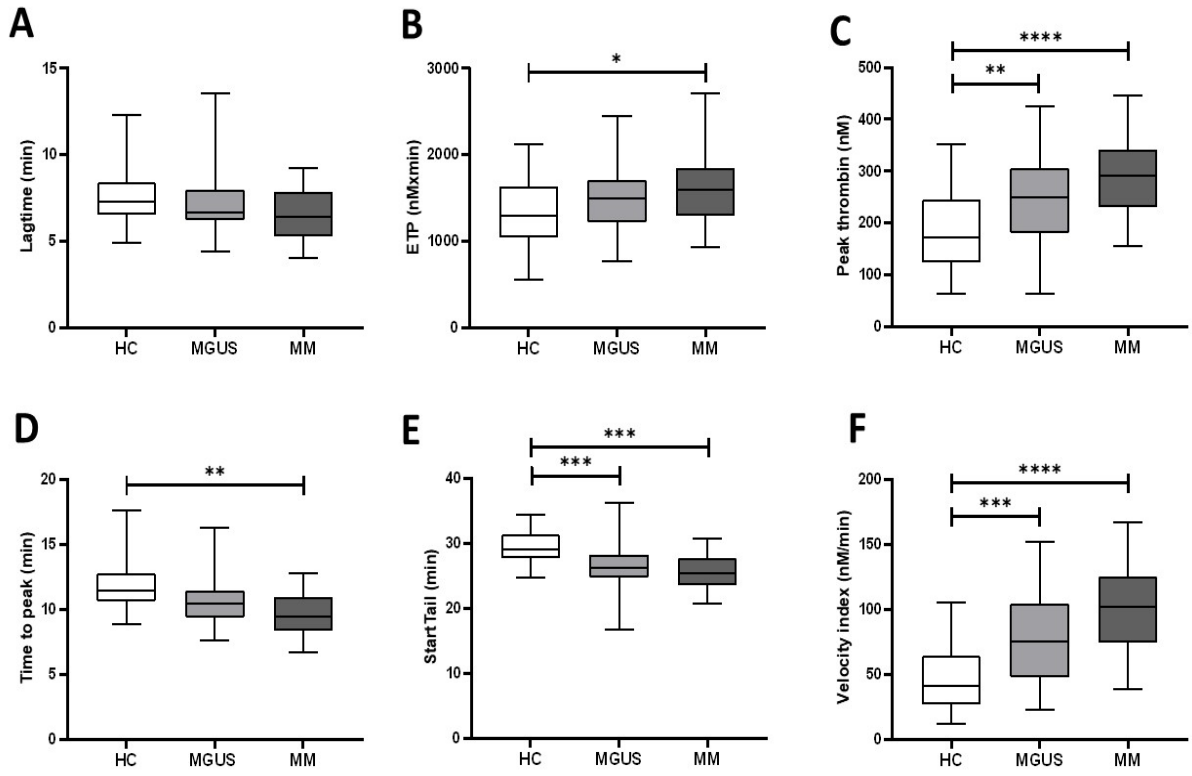

Figure 19. TG is increased in MM and MGUS patients compared to HCs. Comparison of TG assay parameters: lagtime (A), ETP (B), peak thrombin (C), time to peak (D), StartTail (E), and velocity index (F) between the patient and control groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (B – F). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; ETP, endogenous thrombin potential.

In *in vitro* experiments, adding APC to plasma samples from MGUS patients and HCs attenuated TG in both groups. In the presence of 10 nM APC, ETP was significantly reduced in both control [1292 (1038 – 1637) nMxmin vs. 648.2 (348.9 – 1202) nMxmin, $p < 0.0001$] and MGUS plasmas [1489 (1226 – 1711) nMxmin vs. 1078 (671 – 1266) nMxmin, $p < 0.0001$] in contrast to MM plasma [1595 (1293 – 1848) nMxmin vs. 1569 (1205 – 1733) nMxmin, $p = 0.0560$], where the difference was only 1.6% (Figure 20A). The observed phenomena indicate a greater resistance of MM plasma to APC inhibition as compared to plasmas from MGUS patients and HCs. Like ETP, peak thrombin was reduced in HCs from 171.4 (123.2 – 244.4) nM to 97.2 (51.3 – 190.3) nM, $p < 0.0001$, representing a 43% reduction, and in MGUS patients from 249.6 (180.1 – 305.4) nM to 170.7 (112.6 – 233.0) nM, $p < 0.0001$, representing a 32% reduction (Figure 20B). However, the difference between TG without APC [291.3 (229.8 – 342.1) nM] and TG with APC [277.3 (228.1 – 329.4) nM], $p = 0.4952$ in MM plasma was only 5% (Figure 20B). This further explains the increased sensitivity of HC and MGUS plasmas to the anticoagulant effect of APC. APC also significantly prolonged the lag time in plasma from HC subjects [7.3 (6.5 – 8.4) min vs. 8.0 (7.1 – 9.5) min, $p < 0.0001$] and MGUS patients [6.7 (6.2 – 8.0) min vs.7.2 (6.7 – 8.9) min, $p < 0.0001$], but not in plasma from MM patients [6.4 (5.3 – 7.9) min vs. 6.5 (5.6 – 8.1) min, $p = 0.4765$] (Figure 20C). Similarly, the time to peak parameter was significantly prolonged in HC and MGUS plasmas but not in MM plasma (Figure 20D).

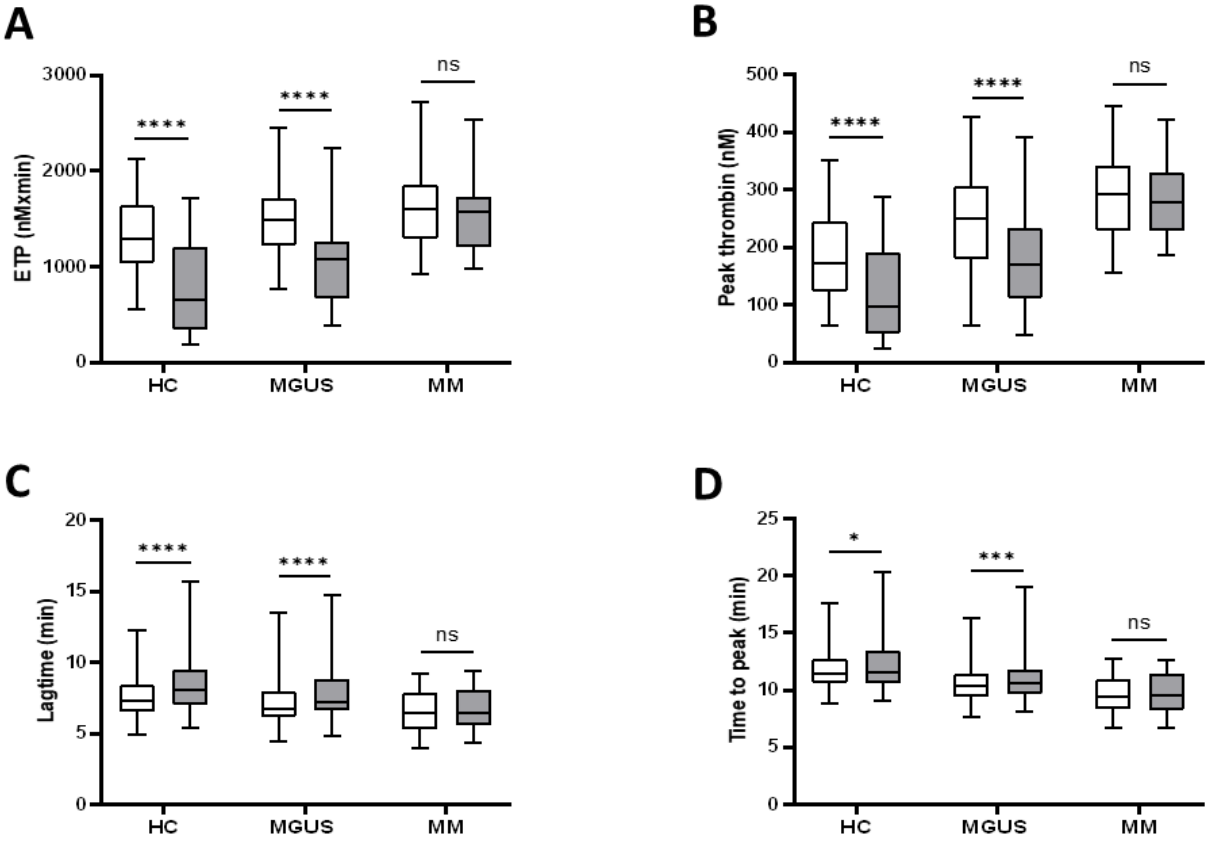

Figure 20. The sensitivity of MM plasma to the anticoagulant effect of APC is reduced. Comparison of TG assay parameters: ETP (A), peak thrombin (B), lagtime (C), and time to peak (D) with and without APC. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, and ns-nonsignificant (white bars: TG without APC; grey bars: TG with APC). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; ETP, endogenous thrombin potential.

5.8. Correlations between hemostasis or biochemical parameters and TG quantity parameters in patients and healthy controls

In MGUS patients, ETP and peak thrombin showed a significant positive correlation with FVIII activity (Figure 21A,B). Similarly, vWFAg correlated significantly with ETP (Figure 21C) but only moderately with peak thrombin (Figure 21D). The strongest correlations were found between vWFAct and ETP ($r = 0.5591$, 95% CI: 0.2627 – 0.7590, $p = 0.0006$) and peak thrombin ($r = 0.4915$, 95% CI: 0.1739 – 0.7165, $p = 0.0032$) (Figure 21E,F). FVIII activity and vWF levels were found to be positively correlated in all groups, as expected ($p < 0.05$ in all groups). Furthermore, CRP levels also correlated with ETP ($r = 0.6408$, 95% CI: 0.3416 –

0.8220, $p = 0.0002$) and peak thrombin ($r = 0.6925$, 95% CI: 0.4212 – 0.8500, $p < 0.0001$) in HC subjects but not in MM and MGUS patients.

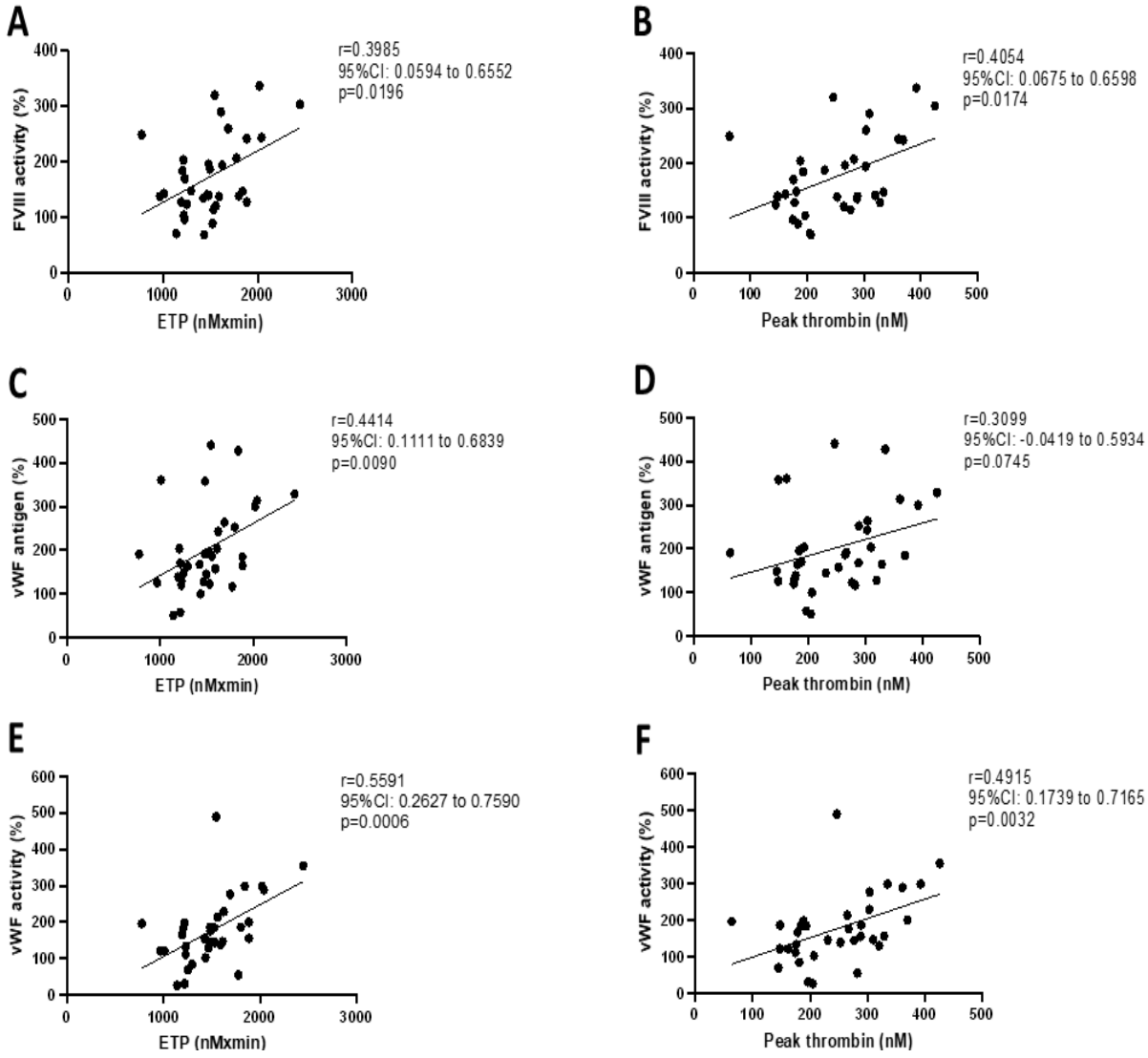

Figure 21. Correlations between hemostasis and TG quantity parameters in MGUS patients. Spearman's correlations between FVIII activity and ETP (A), peak thrombin (B), vWF antigen and ETP (C), peak thrombin (D) and vWF activity and ETP (E), peak thrombin (F) are shown. FVIII, factor VIII; vWF, von Willebrand factor; ETP, endogenous thrombin potential; CI, confidence interval.

5.9. FXIII antigen and activity levels of the study cohort

FXIII-A_2B_2 antigen and FXIII activity levels were significantly lower in MM patients compared to MGUS patients and HCs (Figure 22A,B). In 38% (6 out of 16) of MM patients, FXIII-A_2B_2 levels were below the lower reference limit. There was a strong positive correlation

between FXIII-A_2B_2 and FXIII activity in all the groups studied: MM (r = 0.6061, 95% CI: 0.0827 – 0.8674, p = 0.0281), MGUS (r = 0.8200, 95% CI: 0.6226 – 0.9193, p < 0.0001), and HC (r = 0.6780, 95% CI: 0.3688 – 0.8520, p = 0.0004). FXIII activity and FXIII-A_2B_2 levels were slightly below the reference range in two MGUS patients. Mixing studies did not reveal the presence of an FXIII inhibitor in any of the cases where FXIII activity was low. There was no difference in total FXIII-B levels between HCs and patients with MM or MGUS (Figure 22C). Based on the results of the mixing studies and FXIII-B levels, an immune-mediated inhibitory mechanism was ruled out. As shown in Figure 22D, free/total FXIII-B was significantly higher in MM patients than in MGUS patients and HCs, suggesting that the underlying pathomechanism of low FXIII levels in MM patients is most likely due to increased FXIII activation and consumption.

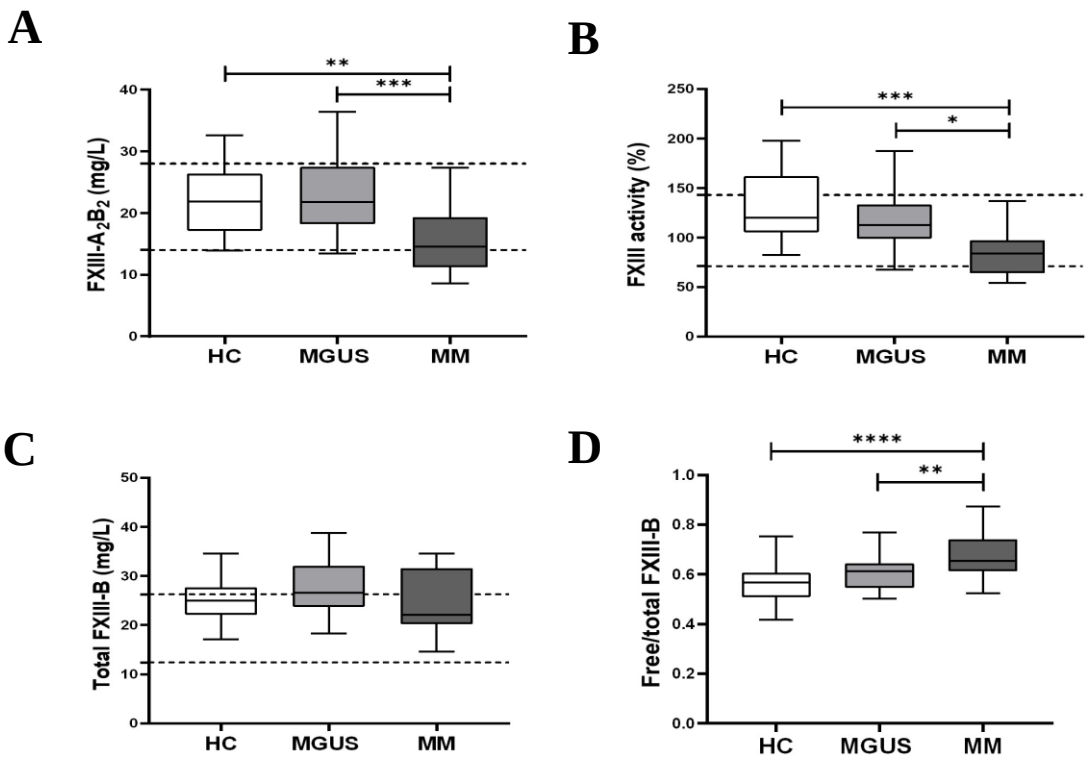

Figure 22. Low FXIII levels in patients with MM. The horizontal dashed lines indicate the reference ranges for FXIII-A_2B_2 (A), 14 – 28 mg/L, FXIII activity (B), 69 – 143%, and total FXIII-B (C), 12.4 – 26.3 mg/L. The ratio of free/total FXIII-B is shown in panel D. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 (A,B,D). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; FXIII-A_2B_2, factor XIII antigen; FXIII-B, factor XIII-B.

5.10. Prothrombotic and fibrinolytic markers of the study population

To better understand the pathomechanism underlying the decreased FXIII levels found in MM patients, we evaluated additional prothrombotic as well as fibrinolytic markers in our study cohorts. Fibrinogen levels were comparable across groups (Figure 23A). Quantitative FM was significantly increased in patients with MM [median: 4.5 (IQR: 3.3 – 6.1) µg/mL, p = 0.0009] and MGUS [3.7 (2.7 – 5.0) µg/mL, p = 0.0246] compared to HCs [2.5 (2.3 – 3.1) µg/mL] (Figure 23B). Myeloma patients had significantly higher TAT complex levels than controls [6.6 (4.8 – 10.0) µg/L vs. 3.3 (2.8 – 5.1) µg/L, p = 0.0004] (Figure 23C), suggestive of hypercoagulability and increased TG.

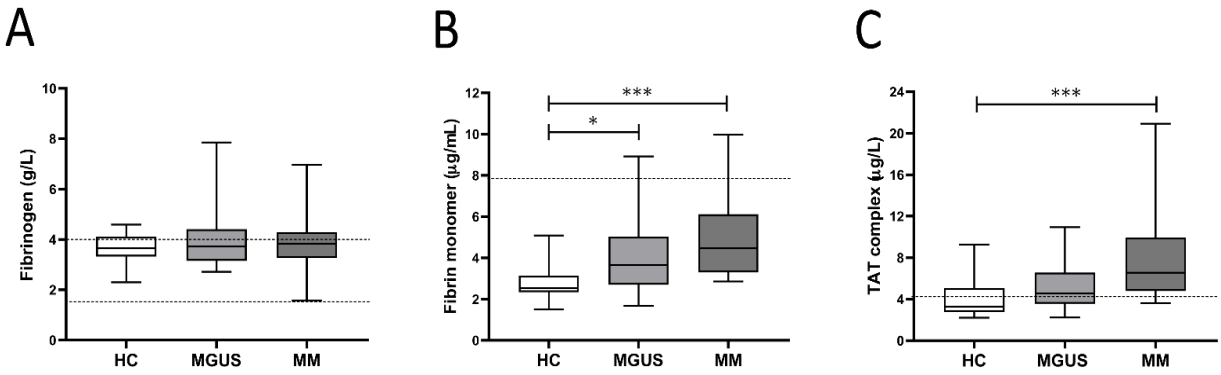

Figure 23. Fibrinogen, quantitative fibrin monomer, and TAT complex levels in HCs, MGUS, and MM patients. The horizontal dashed lines indicate the reference ranges or cut off values for fibrinogen (A), 1.5 – 4.0 g/L, FM (B), < 7.8 µg/mL, and TAT complex (C), < 4.2 µg/L. *p < 0.05 and ***p < 0.001 (B,C). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; TAT, thrombin-antithrombin.

The median D-dimer levels were higher in MM [1.4 (0.7 – 2.5) µg/mL, p = 0.0002] and MGUS [0.7 (0.5 – 1.2) µg/mL, p = 0.0095] patients than in control subjects [0.4 (0.3 – 0.8) µg/mL] (Figure 24A). This increase was associated with a significant elevation in PAP complex levels in patients [485.2 (196.3 – 689.0) ng/mL, p < 0.0001 for MM and 320.9 (199.9 – 588.0) ng/mL, p < 0.0001 for MGUS] compared to controls [153.0 (124.2 – 217.8) ng/mL]

(Figure 24B), implying enhanced fibrinolysis in both patient groups. PLG activity (Figure 24C) and α2-PI activity (Figure 24D) did not differ between patients and controls.

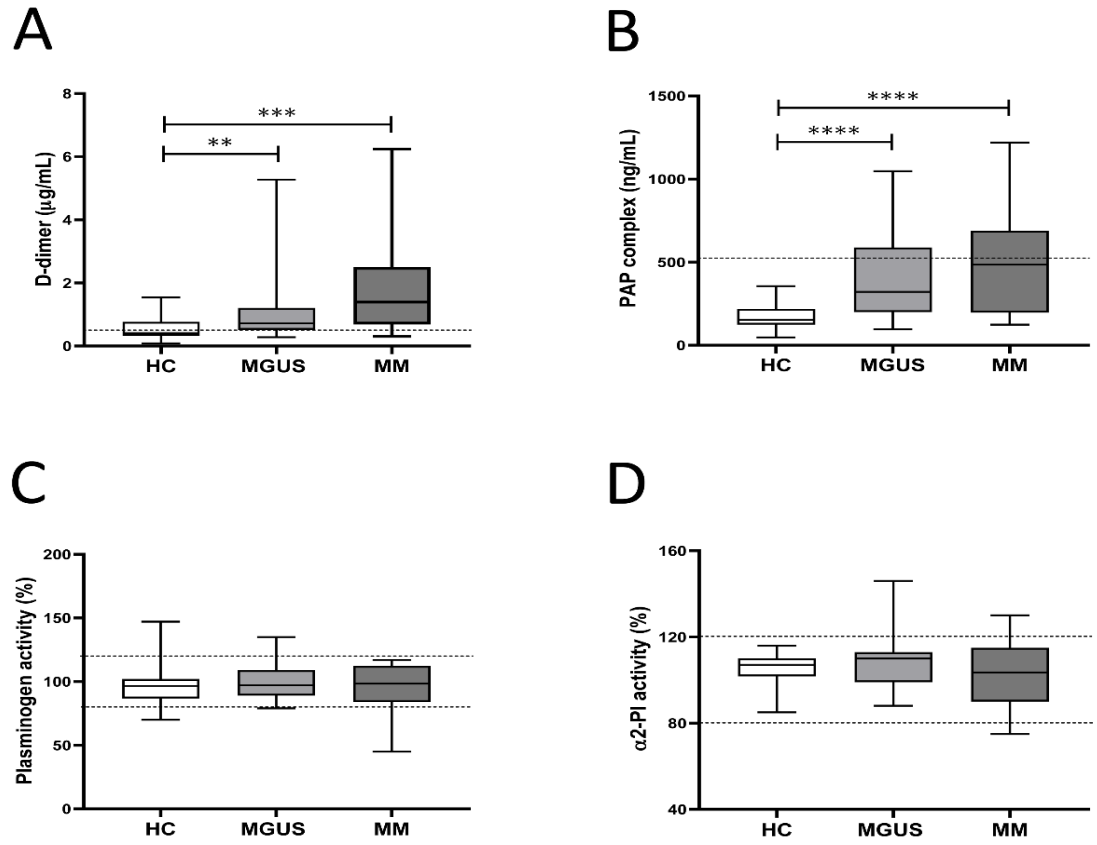

Figure 24. D-dimer (A), PAP complex (B), plasminogen activity (C), and α2-PI activity (D) in HCs, MGUS, and MM patients. The horizontal dashed lines indicate the reference ranges or cut off values for D-dimer (A), < 0.5 µg/mL, PAP complex (B), < 514 ng/mL, plasminogen activity (C), 80 – 120%, and α2-PI activity (D), 80 – 120%. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ (A,B). HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PAP, plasmin-*α2*-antiplasmin*;* α2-PI, α2-plasmin inhibitor.

5.11. Global assay parameters

To determine whether the decrease in FXIII levels in myeloma patients affects the balance of fibrinolysis, global assays were performed, and lysis parameters were correlated with FXIII levels. Table 5 shows the results of Clotpro tPA-assay measurements. The CT and LT parameters were significantly prolonged in myeloma patients compared to HCs, while the

MCF was higher. There were no significant differences in PG assay or CLA parameters between patients and controls (Tables 6 & 7).

Table 5. Comparison of ClotPro tPA-assay parameters among the study groups

Parameters	HC (n = 30)	MGUS (n = 33)	MM (n = 17)	p-value
tPA-assay				
CT (s)	49.0 (43.8 – 57.0)	50.0 (46.3 – 57.5)	56.0 (47.0 – 65.0)	0.0397 *0.0369
MCF (mm)	31.7 ± 6.4	33.8 ± 9.2	39.2 ± 9.0	0.0141 *0.0106
LT (s)	230.0 (211.0 – 246.0)	247.0 (217.5 – 289.0)	247.0 (219.5 – 358.0)	0.0357 *0.0427

Mean ± SD or median (interquartile range) are shown. Comparison between *HC vs. MM. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; tPA, tissue plasminogen activator; CT, clotting time; MCF, maximum clot firmness; LT, lysis time.

Table 6. Plasmin generation assay parameters of the study population

Parameters	HC (n = 30)	MGUS (n = 33)	MM (n = 17)	p-value
Lagtime (min)	2.8 (2.7 – 3.0)	3.0 (2.7 – 3.2)	3.0 (2.7 – 3.3)	0.1801
EPP (nMxmin)	246.0 (237.8 – 277.2)	229.1 (155.2 – 272.4)	239.2 (207.6 – 271.0)	0.1223
Peak plasmin (nM)	49.5 ± 6.7	49.4 ± 11.4	48.7 ± 9.3	0.9530
Time to peak (min)	5.0 (4.9 – 5.2)	5.2 (4.9 – 5.5)	5.2 (5.0 – 5.3)	0.4178

Mean ± SD or median (interquartile range) are shown. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; EPP, endogenous plasmin potential.

Table 7. *In vitro* clot lysis assay parameters of the study population

Parameters	HC (n = 30)	MGUS (n = 33)	MM (n = 15)	p-value
Maximal absorbance	1.4 (1.3 – 1.6)	1.5 (1.3 – 1.6)	1.2 (1.1 – 1.6)	0.0617
Time to maximal absorbance (min)	12.0 (8.0 – 17.3)	10.0 (8.0 – 13.0)	12.0 (9.0 – 16.5)	0.3014
10%CLT (min)	18.0 (14.0 – 30.2)	21.0 (14.5 – 29.0)	24.8 (14.3 – 27.0)	0.8502
50%CLT (min)	37.5 ± 19.4	34.0 ± 11.8	30.5 ± 12.0	0.3393
90%CLT (min)	58.1 ± 18.7	56.5 ± 20.2	46.5 ± 15.8	0.1401
CLA AUC (OD*min)	20.7 (17.0 – 29.0)	24.0 (20.1 – 27.6)	18.2 (15.1 – 23.2)	0.1264

Mean ± SD or median (interquartile range) are shown. HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; CLT, clot lysis time; CLA AUC, clot lysis assay area under the curve.

5.12. Correlations between hemostasis and global assay parameters in patients and healthy controls

FXIII-A_2B_2 and FXIII activity levels showed a significant positive correlation with ClotPro tPA LT and peak plasmin in HCs. Such associations were abolished in patients with MM and MGUS. Similarly, α2-PI activity and PAP complex levels were positively correlated with ClotPro tPA LT and peak plasmin in HCs but not in the patient groups (Table 8).

Table 8. Correlations between hemostasis and global assay parameters in patients and healthy controls

	FXIII activity (%)			FXIII-A_2B_2 (mg/L)			α2-PI activity (%)			PAP complex (ng/mL)		
	HC	MGUS	MM	HC	MGUS	MM	HC	MGUS	MM	HC	MGUS	MM
ClotPro tPA lysis time	*r* = 0.5136 (0.1286 – 0.7640) *p* = 0.0122	*r* = 0.2147 (–0.2167 – 0.5759) *p* = 0.3253	*r* = 0.3282 (–0.2720 – 0.7445) *p* = 0.2736	*r* = 0.4591 (0.1113 – 0.7067) *p* = 0.0122	*r* = 0.1570 (–0.1969 – 0.4747) *p* = 0.3828	*r* = 0.4183 (–0.1136 – 0.7638) *p* = 0.1077	*r* = 0.3737 (0.0154 – 0.6469) *p* = 0.0420	*r* = 0.0961 (–0.2654 – 0.4341) *p* = 0.5945	*r* = 0.3747 (–0.1643 – 0.7414) *p* = 0.1520	*r* = 0.5050 (0.1589 – 0.7406) *p* = 0.0052	*r* = 0.0205 (–0.3345 – 0.3705) *p* = 0.9096	*r* = –0.1502 (–0.6113 – 0.3870) *p* = 0.5760
Peak plasmin	*r* = 0.4595 (0.0583 – 0.7329) *p* = 0.0274	*r* = –0.1319 (–0.5160 – 0.2964) *p* = 0.5486	*r* = 0.3645 (–0.2334 – 0.7624) *p* = 0.2208	*r* = 0.3445 (–0.0251 – 0.6313) *p* = 0.0672	*r* = 0.1655 (–0.1885 – 0.4815) *p* = 0.3572	*r* = 0.3366 (–0.1909 – 0.7133) *p* = 0.2023	*r* = 0.3685 (0.0094 – 0.6433) *p* = 0.0451	*r* = –0.2589 (–0.5603 – 0.1031) *p* = 0.1457	*r* = –0.0722 (–0.5483 – 0.4392) *p* = 0.7903	*r* = 0.4900 (0.1394 – 0.7314) *p* = 0.0070	*r* = 0.1858 (–0.1785 – 0.5053) *p* = 0.3005	*r* = 0.3317 (–0.1963 – 0.7106) *p* = 0.2095

tPA, tissue plasminogen activator; FXIII, factor XIII; FXIII-A_2B_2, factor XIII antigen; α2-PI, α2-plasmin inhibitor; PAP, plasmin-α2-antiplasmin; HC, healthy control; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma. The 95% confidence intervals are provided in brackets.

6. DISCUSSION

BTZ inhibits the proteasomal degradation of various regulatory proteins. Its antiproliferative and antitumor properties make it extremely effective in a variety of hematological malignancies. Notably, it has significantly improved the management of newly diagnosed, refractory, or relapsed MM and mantle cell lymphoma [187, 266–268]. BTZ has a higher therapeutic efficacy than high-dose DEX in the treatment of patients with relapsed MM [269].

In this study, we hypothesized that inhibition of human platelet proteasome activity by BTZ results in a procoagulant platelet phenotype with subsequent TG. In PRP pretreated with BTZ, we observed no significant increase in PS expression. However, there was a significant increase in PS expression in GFPs 15 min after BTZ treatment, which increased further at 60 min. Our findings corroborate those of Nayak et al., who showed that in mice, BTZ doses of 0.1 mg/kg and 0.3 mg/kg significantly increased PS expression in washed platelets [205].

Since PS expression increases in response to platelet activation or apoptosis, we measured the platelet $\Delta\psi m$ depolarization to confirm our findings. The percentage of depolarized cells increased with time, suggesting that BTZ also has an effect on platelet apoptosis. To verify the possible effect of BTZ on platelet activation, we measured surface P-selectin expression. We found markedly elevated P-selectin expression in all the BTZ-pretreated samples.

Our objective was to demonstrate that the increase in extracellular PS could induce subsequent TG. In the TG assay, the time to peak and StartTail parameters were significantly shorter in all BTZ-pretreated groups, the peak thrombin concentration was significantly higher, but there were no significant differences in lag time and ETP. Notably, the BTZ-pretreated groups showed higher peak thrombin values than the positive control (TRAP). This led us to consider that, besides platelets, the enhanced TG in these samples may also be influenced by

PMPs. In our study, the BTZ-preincubated samples showed much higher PMPs than the TRAP samples. As a result, it is highly probable that the higher PMP number also played a role in increasing TG.

The observed difference in PS expression between PRP samples and GFPs could be clarified by supplementing GFPs with HSA prior to BTZ pretreatment. The addition of albumin reduced the effect of BTZ on GFPs and caused minimal PS expression, comparable to PRP, demonstrating that albumin neutralizes the stimulatory effect of BTZ on human platelets *in vitro*. However, it is important to consider that targeted cells in various anatomical sites, including the BM microenvironment, are surrounded by a different protein milieu where BTZ can exert its action more effectively.

Our findings support our hypothesis and may have pathological implications. Hypoalbuminemia is a significant prognostic factor for MM [270]. Previous studies have established a correlation between low serum albumin levels and increased IL-6 levels, a potent growth factor for myeloma cells, reflecting the severity of the disease and cell proliferation [271, 272]. Thrombosis is frequent in myeloma patients [16, 17] and has been linked to proteasome inhibitor therapy in some cases [273]. PS-exposing platelets, red blood cells, and leukocytes have been detected in newly diagnosed MM patients, which may contribute to the thrombotic events observed in a large number of them [274].

Thrombosis in MM has also been associated with IMiD drug treatment, showing an increase of up to 26% [19]. Similarly, thrombosis has been observed in patients with MGUS, who typically do not undergo any treatment for their condition [14, 21–23, 275]. In this study, we found that the levels of TG in newly diagnosed MM patients were significantly higher than in a HC group, similar to MGUS patients. ETP and peak thrombin levels were higher in our patient cohorts, and the lag time and time to peak were shorter when compared to HCs. Our findings agree with those of Nielsen et al. [244]. In a study by Crowley and co., the lag time,

ETP, and time to peak parameters in MM and MGUS patients were not significantly different from controls [243]. The peak thrombin level in their patient cohort was higher than in controls, though less significantly different. While the present study included only untreated myeloma patients, half of the cohort of MM patients in Crowley and co.'s study had refractory MM, which may explain the differences in the results of the two studies. Other studies have reported no significant differences, particularly in ETP and peak thrombin values, between newly diagnosed MM or MGUS patients and control subjects [218, 245, 246]. On the contrary, some studies have found lower levels of TG in newly diagnosed MM patients, where the lag time and time to peak parameters were significantly prolonged and the ETP and peak thrombin values were reduced compared to controls [24, 276]. This hypocoagulable profile has been linked to an increase in the plasma concentration of TFPI and TM [24].

Elevated levels of plasma FVIII and vWF increase the risk of VTE [13, 277–280]. Kamphuisen et al. found that FVIII levels exceeding 150% are associated with a fivefold increase in the risk of thrombosis [278]. In the present study, we found elevated FVIII and vWF levels in MM and MGUS patients. These findings are in line with previous studies [32, 218] linking this phenomenon to hypercoagulability. The underlying cause of the elevated FVIII and vWF remains somewhat unclear. Nonetheless, we found a significant correlation between FVIII and vWF levels in all our studied groups, suggesting endothelial damage. Elevated vWF levels in MM patients have also been associated with increased BM neovascularization [281]. Furthermore, Rajkumar and colleagues discovered that BM angiogenesis increases as plasma cell dyscrasia progresses from the precursor MGUS stage to advanced MM, implying a potential link between angiogenesis and disease progression [282]. This phenomenon may explain the elevated FVIII and vWF levels in MGUS patients. In this study, notably, peak thrombin and ETP were positively correlated with FVIII and vWF levels

in MGUS patients. This key finding may help to explain the hypercoagulable profile of these patients.

In order to exclude the potential influence of acute phase reactions on the elevated FVIII and vWF levels, we measured the CRP levels of all our study subjects and determined their correlations with ETP and peak thrombin values. In HC subjects, CRP correlated positively with ETP and peak thrombin, but not in MM and MGUS patients, suggesting that the increased FVIII and vWF levels in our patient cohort are not due to inflammation. Moreover, since the blood group distribution in our patient and control groups was similar, the elevated vWF levels in our patient cohorts cannot be attributed to differences in blood group distribution.

Thrombosis in cancer patients, including those with MM, has been associated with the APC pathway. In this study, we show that APC does not have an inhibitory effect on TG in MM patients, which may increase their prothrombotic tendencies. The addition of 10 nM APC to plasma from MM patients resulted in only a minimal reduction of 1.6% in ETP and 5% in peak thrombin, whereas Crowley and colleagues [243] observed a much higher reduction of 59% and 65%. Exogenous APC attenuated TG in MGUS patients, confirming that the APC pathway may not be implicated in the observed thrombotic risk in these individuals. The significance of these results lies in the fact that none of our patients or control subjects had APCR as determined by an APTT-based resistance test. Contrary to a previous study [218], we found a significant reduction in protein S activity in MM and MGUS patients compared to HCs, adding to our understanding of the increased thrombotic risk in these patients.

We also evaluated the levels of FXIII among our study participants. FXIII-A_2B_2 antigen and FXIII activity levels were significantly lower in newly diagnosed MM patients than in HC volunteers and MGUS patients. Notably, 38% of myeloma patients had FXIII-A_2B_2 antigen levels below the lower limit of normal. Total FXIII-B levels were not significantly different between our study groups, indicating that the decreased FXIII levels found in myeloma patients

are most likely due to increased consumption of the FXIII-A active subunit as a result of excessive activation of coagulation. FXIII levels were not significantly different between MGUS patients and HCs. However, in 2 (6%) of these patients, FXIII-A_2B_2 antigen levels were below the normal range. The significance of these findings remains to be determined.

Furthermore, D-dimer and PAP-complex levels were elevated in MM patients, indicating increased fibrinolytic turnover. The ratio of the free/total FXIII-B subunit was also increased. Taken together, these findings suggest increased FXIII activation. Since D-dimers are formed from cross-linked fibrin, FXIII is most probably consumed during this process of ongoing coagulation and fibrinolysis activation, resulting in an increase in the ratio of the free/total FXIII-B subunit. A similar phenomenon has been observed in other clinical situations, such as in COVID-19 [283] and critically ill patients [284]. Our results are consistent with other studies that show acquired FXIII deficiency and hyperfibrinolysis in newly diagnosed pediatric hematological malignancies [285].

The clinical implications of such low FXIII levels in MM patients are difficult to predict. A longitudinal study may be needed to investigate the potential pathological bleeding tendencies in MM patients with acquired FXIII deficiency and the potential benefits of FXIII replacement therapy. The results of both PG assay and CLA in our patient cohort did not differ from HCs, probably because of their reduced sensitivity to alterations in the fibrinolytic system. In our study, we performed the first-ever comparison of ClotPro tPA-assay parameters, a method used to assess the viscoelastic properties of whole blood, between MM and MGUS patients and HCs. MM patients had significantly increased clot firmness, indicating a prothrombotic state. The LT was also significantly prolonged. It has been reported that plasma fibrin clot structure and function are altered in myeloma patients at diagnosis; fibrin clot formation occurs at a slower rate and has a decreased lysis capacity [32]. This could explain our results.

The results of the ClotPro tPA-assay parameters, however, showed no correlation with FXIII levels. Again, in both patient groups, there was no correlation between α2-PI, PAP complex, and FXIII levels and the outcomes of global fibrinolytic assays. This suggests that the imbalance in fibrinolysis can be explained by other key players. These findings also imply that the loss of FXIII in myeloma patients is secondary to ongoing fibrinolysis and not the primary cause.

Alterations in fibrinolysis could be a major, albeit frequently overlooked, cause of bleeding in patients. Secondary or 'reactive' hyperfibrinolysis occurs as a result of increased activation of the fibrinolytic system in response to a state of hypercoagulability. In this study, both myeloma and MGUS patients had elevated levels of FM, TAT complex, D-dimer, and PAP complex, indicating marked hypercoagulability and secondary hyperfibrinolysis. The increase in FM levels in myeloma patients is consistent with previous research [286]. This study reports, for the first time, elevated FM and TAT complex levels in MGUS patients. Elevated D-dimer levels in MM and MGUS patients have also been previously reported [37, 287]. In this study, increased D-dimer levels were found in 88% of MM patients, 76% of MGUS patients, and 37% of HCs.

Our study does have limitations. The limited availability of patients for recruitment can be attributed to the rigorous implementation of exclusion criteria during the recruitment process. This needs to be considered when interpreting our findings. Due to the small number of patients, we were unable to determine a link between the results of hemostasis tests and the stage of MM diagnosis. The lack of longitudinal laboratory assays to determine how fibrinolysis markers change over the course of disease is a limitation of our study. The investigation of long-term outcomes in MM and MGUS patients, specifically in relation to the development of bleeding or thromboembolic complications, was not within the purview of our study. The strength of our study, however, lies in its novel study design, which employs an in-

depth panel of hemostasis assays to investigate hypercoagulability and fibrinolysis activation in patients with MM and MGUS.

7. SUMMARY

While bortezomib has shown promise in reducing the risk of venous thromboembolism in multiple myeloma, it has also been associated with thrombocytopenia, resulting in bleeding episodes that require platelet transfusion. Our study adds to our understanding of the effect of bortezomib on platelet function. We found that bortezomib induces platelet activation and apoptosis in human gel-filtered platelets via increased phosphatidylserine and P-selectin expression, mitochondrial inner membrane potential depolarization, platelet microparticle formation, and thrombin generation. Nevertheless, the presence of albumin diminishes these effects.

Our other study found that hypercoagulable changes in patients with newly diagnosed multiple myeloma and monoclonal gammopathy of undetermined significance are associated with elevated levels of factor VIII and von Willebrand factor, increased thrombin generation, thrombin-antithrombin complex, quantitative fibrin monomer, and decreased protein S activity. We also found that, unlike in patients with monoclonal gammopathy of undetermined significance, activated protein C cannot exert its inhibitory effect on thrombin generation in patients with multiple myeloma and that this phenomenon is unrelated to activated protein C resistance. In addition, we found significant correlations between thrombin generation assay quantity parameters and the levels of factor VIII and von Willebrand factor in patients with monoclonal gammopathy of undetermined significance, which may help to explain their hypercoagulable states.

We also observed elevated D-dimer and plasmin-α2-antiplasmin complex levels in patients with multiple myeloma and monoclonal gammopathy of undetermined significance, suggesting secondary hyperfibrinolysis. Furthermore, we provided the first comprehensive evaluation of factor XIII deficiency in patients with multiple myeloma. Factor XIII levels were markedly reduced in multiple myeloma patients, which is most likely due to increased

consumption of the factor XIII-A active subunit as a result of hypercoagulation and ongoing fibrinolysis. A longitudinal study may be needed to investigate the potential pathological bleeding tendencies in multiple myeloma patients with acquired factor XIII deficiency and the potential benefits of factor XIII replacement therapy. Our findings suggest that, while monoclonal gammopathy of undetermined significance is benign, hemostasis alterations are already evident at presentation, raising the need for awareness during monitoring.

8. NEW SCIENTIFIC FINDINGS

Project 1. Effect of bortezomib on platelet function

- Bortezomib induces platelet activation and apoptotic processes in gel-filtered human platelets.
- Bortezomib can also induce subsequent thrombin generation and shorten the time for thrombin formation.
- These effects are mostly eliminated in the presence of plasma albumin.

Project 2. Hypercoagulable changes and fibrinolytic alterations in multiple myeloma and monoclonal gammopathy of undetermined significance

- Hypercoagulability in newly diagnosed patients with multiple myeloma and monoclonal gammopathy of undetermined significance is associated with elevated factor VIII and von Willebrand factor levels, increased thrombin formation, thrombin-antithrombin complex, and fibrin monomers, and decreased protein S activity.
- Factor VIII and von Willebrand factor levels in patients with monoclonal gammopathy of undetermined significance show a correlation with thrombin generation assay quantity parameters.
- Plasma from patients with multiple myeloma is less sensitive to the activated protein C-mediated anticoagulant effect compared to plasma from patients with monoclonal gammopathy of undetermined significance. This difference does not appear to be related to activated protein C resistance.
- Factor XIII levels are low in patients with multiple myeloma at the time of diagnosis due to increased activation and consumption.

- D-dimer and plasmin-α2-antiplasmin complex levels are elevated in patients with multiple myeloma and monoclonal gammopathy of undetermined significance, suggesting secondary hyperfibrinolysis.

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10. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

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Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Ghansah, H.**, Orbán-Kálmándi, R. A., Bekéné Debreceni, I., Katona, É., Rejtő, L., Váróczy, L., Lóczi, L., Laat, B. d., Huskens, D., Kappelmayer, J., Bagoly, Z.: Low factor XIII levels and altered fibrinolysis in patients with multiple myeloma.
   Thromb. Res. 234, 12-20, 2024.
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   IF: 7.5 (2022)
2. **Ghansah, H.**, Bekéné Debreceni, I., Váróczy, L., Rejtő, L., Lóczi, L., Bagoly, Z., Kappelmayer, J.: Patients with multiple myeloma and monoclonal gammopathy of undetermined significance have variably increased thrombin generation and different sensitivity to the anticoagulant effect of activated protein C.
   Thromb. Res. 223, 44-52, 2023.
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3. **Ghansah, H.**, Bekéné Debreceni, I., Fejes, Z., Nagy, B. J., Kappelmayer, J.: The Proteasome Inhibitor Bortezomib Induces Apoptosis and Activation in Gel-Filtered Human Platelets.
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11. KEYWORDS

- Activated protein C
- Bortezomib
- Factor XIII
- Fibrinolysis
- Gel-filtered platelets
- Monoclonal gammopathy of undetermined significance
- Multiple myeloma
- Platelet activation and apoptosis
- Thrombin generation
- Thrombosis

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