SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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

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1. INTRODUCTION

Multiple myeloma (MM) is a clonal plasma cell neoplasm that makes up about 1% of global cancers and 10% of all hematological malignancies. The median age at diagnosis is 70 years. Monoclonal gammopathy of undetermined significance (MGUS), a premalignant plasma cell disorder, nearly always precedes MM. The annual risk of MGUS progressing to MM or a related disorder is about 1%. MGUS is quite prevalent, affecting 3.2% of people over 50 and 5.3% of people over 70 years.

The diagnostic criteria for MGUS include < 10% bone marrow 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 bone marrow 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 bone marrow plasma cell count of $\geq 60\%$, a serum free light chain ratio of 100 or more, and the presence of multiple focal lesions as detected by magnetic resonance imaging.

1.1. Venous thromboembolism (VTE): Risk in MM and MGUS

VTE is common in patients with MM and MGUS. 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 MM patients receiving standard treatment with melphalan and prednisone. Immunomodulatory drug monotherapy also increases VTE risk by 3 – 4% and up to 26% when combined with corticosteroids. 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.

Thrombosis in MM patients has also been linked to the production of paraproteins, which may cause increased blood viscosity, impaired fibrinolysis, decreased protein S activity, the development of procoagulant autoantibodies such as lupus anticoagulant, and endothelial damage. Elevated levels of fibrinogen, von Willebrand factor (vWF), factor VIII (FVIII), and proinflammatory cytokines such as interleukin-6, tumor necrosis factor alpha, and C-reactive protein may also increase the risk of VTE in myeloma patients. Acquired activated protein C (APC) resistance is said to be common in people with MM and has been linked to a higher risk of thrombosis.

The pathophysiology of VTE in MGUS is unclear. Nonetheless, the increased secretion of interleukin-6 and tumor necrosis factor alpha may play a role, as these cytokines can trigger tissue factor expression, increase FVIII, fibrinogen, and vWF transcription, and decrease protein S activity.

1.2. The proteasome and proteasome inhibitors

The proteasome is a large protein complex found in eukaryotic nuclei and cytoplasm. Its main function is to degrade damaged, unfolded, or misfolded proteins within the cell.

Platelets have an active proteasome system, just like nucleated cells, and express the caspase-, trypsin-, and chymotrypsin-like activities of the proteasome. The function of the proteasome in protein degradation in platelets is poorly understood. However, it is known that the proteasome regulates the production and lifespan of platelets.

Proteasome inhibitors cause the accumulation of proteins that would normally be degraded within the cell, resulting in cell death. 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. BTZ binds reversibly to the proteasome β5 subunit. At higher concentrations, it

binds to the β1 and β2 subunits. The β1, β2, and β5 subunits have caspase-like, trypsin-like, and chymotrypsin-like activities, respectively.

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

1.3. 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). The normal plasma concentration of the A_2B_2 heterotetramer is 14 – 28 mg/L, with a biological half-life of 9 – 12 days. 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. Monocytes, monocyte-derived macrophages, and platelets also contain FXIII. The B subunits are absent in cellular FXIII. The main site for the synthesis of the A subunit appears to be the bone marrow cells, but hepatocytes may also play a role. Plasma FXIII-B is synthesized by the liver. The catalytic FXIII-A_2 subunit has transglutaminase activity, which covalently crosslinks fibrin polymers, conferring resistance to premature fibrinolysis. The FXIII-B subunits function as carriers, protecting the circulating FXIII-A subunits from proteolysis or systemic clearance.

1.4. 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. 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. Acquired FXIII deficiency may be caused by autoantibodies against a FXIII subunit, decreased synthesis of a FXIII subunit resulting from impaired bone marrow function or liver disease, FXIII consumption, or dilution coagulopathy. 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. The frequency and etiology of acquired FXIII deficiency in MM and MGUS patients remain poorly understood.

2. AIMS AND OBJECTIVES

2.1. 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.

2.2. 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 thrombin generation (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 phosphatidylserine (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.

3. METHODS

3.1. 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. The study included 81 subjects: 17 newly diagnosed, untreated MM patients (median age, 68 years, females, 10), 34 MGUS patients (median age, 67 years, females, 19), and 30 age-, sex- and blood group-matched healthy control volunteers (median age, 65 years, females, 23). The non-O blood group distribution in MM, MGUS, and healthy controls was 11 (78.6%), 29 (85.3%), and 20 (66.7%) respectively. The patients were recruited from 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 International Myeloma Working Group criteria. There were 10 (59%) MM patients with IgG paraprotein, 1 (6%) with IgA, 1 (6%) with IgD, and 5 (29%) with light chain disease, 12 of whom had kappa and 5 lambda light chains. Twenty-two (65%) of the MGUS patients had IgG paraprotein, 5 (15%) had IgA, and 7 (20%) had IgM. Of them, 18 had kappa, while 16 had lambda light chain. The frequency of the International Staging System in MM patients was 4 (23%), 2 (12%), and 11 (65%) for stages I, II, and III respectively. The following were the exclusion criteria for both patients and controls: 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, history of other malignancies, exposure to anticoagulant or antiplatelet medications (within the month prior to recruitment), major surgery or trauma within the past month, thrombocytopenia

(platelet count < 100 x 10^9/L), hypofibrinogenemia (fibrinogen concentration < 1.5 g/L), increased cardiovascular risk, acute illnesses, and end-stage renal failure. Moderate hypertension, however, was not considered an exclusion criterion. 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.

3.2. Blood sampling and processing

All study subjects had their peripheral venous blood drawn into EDTA, sodium citrate, or serum separator tubes (Becton Dickinson, San Jose, CA, USA) and processed without delay. Platelet-poor plasma (PPP) was obtained through centrifugation at 1500g for 15 min at room temperature (RT). Platelet-rich plasma (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 thrombin and plasmin generation assays were obtained through a subsequent centrifugation of PPP at 10,000g, 10 min, RT, to remove all residual platelets. For clot lysis assay 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. C-reactive protein, total protein, total calcium, and lactate dehydrogenase concentration were measured in frozen sera.

3.3. Measurement of hematological, biochemical, and hemostasis parameters

Complete blood count was measured from EDTA-anticoagulated blood using an ADVIA-2120i automated hematology analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). The Cobas 6000 chemistry analyzer (Roche Diagnostics, Mannheim, Germany) was used to measure C-reactive protein, total protein, total calcium, and lactate dehydrogenase

concentration. Hemostasis tests were performed using products from Siemens Healthcare Diagnostics on a BCS-XP coagulometer (Siemens Healthcare Diagnostics, Marburg, Germany) unless otherwise specified. Coagulation screening tests [prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT)] were performed using a human recombinant thromboplastin reagent (Dade Innovin), APTT reagent, and BC thrombin reagent respectively. Fibrinogen concentration was measured by the standard method of Clauss. 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. Factor II and factor V 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 vWF antigen (vWFAg) and vWF activity (vWFAct) were measured by the Innovance vWFAg/Act assay. Plasma FXIII activity (Technochrome FXIII, Technochlone, Austria) 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 healthy controls. A sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine FXIII-A_2B_2 antigen levels as well as the total FXIII-B subunits. 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 fibrin monomer (Diagnostica Stago, Asnieres, France) was measured using the Liatest fibrin monomer assay. Plasmin-α2-antiplasmin (PAP) complex (Technoclone, Vienna, Austria) and thrombin-antithrombin (TAT) complex levels were determined using commercially available ELISA kits. Quantitative D-dimer levels were measured using a particle-enhanced, immuno-turbidimetric assay. α2-plasmin inhibitor (α2-PI) activity and plasminogen activity were determined by commercially available methods. APC was determined by an APTT-based assay with a pre-dilution in excess of factor V-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.

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

PBMCs were separated on Histopaque®-1077 (Ficoll) (Sigma-Aldrich, St. Louis, MO, USA). B cells were isolated from PBMCs by magnetic separation (negative isolation, Dynabeads™ Untouched™ Human B Cells Kit, Life Technologies, AS, Oslo) 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 (Sigma-Aldrich, St. Louis, MO, USA) for flow cytometric analysis.

3.5. Isolation of platelets by gel-filtration chromatography

Citrated whole blood from healthy volunteers was diluted with equal volumes of buffered saline glucose citrate (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. The PRP was layered onto a Sepharose CL-2B column (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with buffered saline glucose citrate. 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. The eluates were collected into Eppendorf tubes, and platelet counts were

measured for each fraction. Platelets devoid of plasma proteins as determined by the Pierce bicinchoninic acid protein assay were subsequently used in experiments.

3.6. Flow cytometric measurement of cellular activation and apoptotic markers in BTZ-pretreated samples

B cells were resuspended in RPMI-1640 and pipetted into the wells of a 24-well multidish (Thermo Fisher Scientific, Rochester, NY). The cells were treated with BTZ (Selleckchem, Munich, Germany) at final concentrations of 26 nM, 260 nM, and 2.6 µM and incubated at 37°C for 24 hours. Dimethyl sulfoxide (DMSO 0.2%; Sigma-Aldrich, St. Louis, MO, USA) served as a negative control. The BTZ concentrations used in the experiment have been described as steady-state concentrations in patients receiving BTZ treatment. Cell viability was assessed by trypan blue exclusion and PS expression was measured by annexin V binding using an FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA).

We also measured PS expression in different human platelet samples. PRP was first incubated with BTZ at final concentrations of 26 nM, 260 nM, and 2.6 µM for 60 min at 37°C. DMSO (0.2%) and thrombin receptor-activating peptide (TRAP 40 µM; Sigma-Aldrich, St. Louis, MO, USA) served as negative and positive controls respectively. Subsequently, gel-filtered platelets (GFPs) were incubated with BTZ (at the same concentrations as in PRP) for 15 and 60 min at 37°C. Here, thrombin 1 U/mL (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control. In separate experiments, GFPs were supplemented with human serum albumin (10, 20, and 40 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at RT before incubation with BTZ or thrombin for 15 or 60 min at 37°C. PS expression was measured in both PRP and GFP samples by annexin V binding using an FC 500 flow cytometer. We analyzed the number of annexin V-positive platelet-derived microparticles from tubes of PS-stained GFP samples. The platelet microparticle gate was set using size (0.3 – 1 µm) calibration beads (Megamix-

Plus FSC, Biocytex, Marseille, France). P-selectin expression was measured in BTZ-preincubated GFPs using anti-CD62P-PE antibodies (Becton Dickinson, San Jose, CA, USA). For all PS and P-selectin measurements, ten thousand platelets were acquired per sample and analyzed with the Kaluza software (Beckman Coulter, Brea, CA, USA).

Depolarization of the platelet mitochondrial inner membrane potential ($\Delta\psi m$) was determined in BTZ-preincubated GFPs using 3,3′-dihexyloxacarbocyanine iodide [(DiOC6(3)] dye (Sigma-Aldrich, St. Louis, MO, USA). 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.

3.7. Thrombin Generation Assay

TG was assessed by the calibrated automated thrombography method using Fluoroskan Ascent FL fluorimeter with Thrombinoscope reagents and software (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 1 pM TF (tissue factor). In project 2, TG was measured in PPP using 1 pM TF, 4 μM PL (phospholipid). In both projects, 20 μL of reagent or calibrator containing thrombin-α2-macroglobulin complex was pipetted into a 96-well black plate (Greiner Bio-One North America Inc., Monroe, MI, USA). 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 $CaCl_2$)] and fluorogenic substrate (*Z*-Gly-Gly-Arg- AMC) into each well (final $CaCl_2$ concentration, 16.67 mmol/L). In project 2, to achieve a 10 nM APC (CellSystems, Troisdorf, Germany) 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 assay parameters were computed using the Thrombinoscope software: lag time (the time until TG begins), peak thrombin (the highest concentration of thrombin generated), time to peak (the time until the peak thrombin), and endogenous thrombin potential (ETP; the area under the curve). All measurements were performed in triplicate.

3.8. Plasmin generation assay

Plasmin generation was measured in plasma using the Fluoroskan Ascent FL fluorimeter (Thrombinoscope BV, Maastricht, The Netherlands). Briefly, 70 µL of reagent containing 5 pM TF, 4 µM PL, and 1.25 µg/mL tPA (tissue plasminogen activator) or calibrator was pipetted into a 96-well black plate (Greiner Bio-One North America Inc., Monroe, MI, USA). Thirty microliters of freshly thawed PPP was added and incubated at 37^{o}C for 10 min. Plasmin generation was started by the automatic dispensation of 20 µL of a solution containing plasmin substrate (Boc-Glu-Lys-Lys-AMC) and $CaCl_2$ (final concentrations, 500 µM and 16.7 mM respectively). The following plasmin generation assay 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. The reagents, calibrator, and substrate used in the plasmin generation assay were obtained from Synapse Research Institute (Maastricht, The Netherlands).

3.9 *In vitro* clot lysis assay

Plasma samples were thawed in a water bath at 37°C. In the wells of a 96-well microtiter plate (Greiner Bio-One Inc. GmbH, Kremsmünster, Austria), a clot induction and lysis mix was prepared in HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween20, pH 7.4), where citrated plasma was mixed with 1000-fold diluted human TF ((Innovin, Siemens,

Marburg, Germany) and 100 ng/mL rtPA (Alteplase, Boehringer Ingelheim, Ingelheim, Germany). The plasma was 1.2 times diluted with buffer. Clotting and subsequent lysis were induced by automatically pipetting HEPES buffer containing $CaCl_2$ (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. Clot formation and lysis were defined using the following variables calculated from the turbidimetric curves: maximum absorbance, time to maximum absorbance, various clot lysis time (CLT) points: 10%CLT, 50%CLT, 90%CLT, and clot lysis assay AUC (area under the curve). 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.

3.10. 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. 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. The tPA-assay is a tissue factor-activated coagulation assay that also contains rtPA (650 ng/mL). The following ClotPro parameters were evaluated: clotting time (the time from the start of the test until a clot amplitude of 2 mm is

reached), maximum clot firmness **(**the maximum amplitude reached during the test), and lysis time (the time from clotting time until 50% of fibrinolysis is detected).

3.11. 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 median and interquartile range. Ordinary one-way Analysis of Variance, 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.

4. RESULTS

Project 1. Effect of BTZ on platelet function

4.1. 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, we observed an increase in PS exposure in all BTZ-treated samples compared to DMSO.

4.2. Platelet activation and apoptosis studies

To determine whether BTZ causes platelet activation, we first measured PS exposure in human PRP pretreated with BTZ. Following a 60-min treatment, BTZ resulted in nonsignificant PS exposure in PRP, while significantly increased PS expression was observed in TRAP-stimulated platelets. As 83% of BTZ binds to human plasma proteins at therapeutic concentrations, 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. Thus, we treated GFPs with increasing concentrations of BTZ. 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. These findings justified the stimulatory effect of BTZ on isolated platelets. As a result, GFPs were used in subsequent experiments.

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.

Next, we investigated the effect of BTZ on platelet lifespan by measuring the platelet $\Delta\psi m$ depolarization. Changes in the $\Delta\psi m$ were monitored using DiOC6(3). 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. At 15 and 60 min, the percentage of depolarized cells in BTZ-treated platelets was significantly higher as was the percentage in thrombin-treated platelets. This was accompanied by a decrease in the median fluorescence intensity.

We also investigated the possible role of BTZ in inducing PS expression in platelet microparticles. Compared to the control group, all the BTZ-treated groups showed a significantly higher number of annexin V-positive platelet microparticles while TRAP showed only a weak stimulatory effect.

4.3. Functional assays for enhanced PS expression

In the TG test, peak thrombin was higher, and time to peak was shorter in all the BTZ and TRAP-treated groups compared to the control group. ETP was increased after TRAP pretreatment, unlike BTZ.

4.4. 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 human serum albumin 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 human serum albumin 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. We found no differences in PS expression between thrombin-treated

GFPs with and without albumin. Similarly, albumin had no effect on thrombin-induced P-selectin expression.

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

4.5. Baseline characteristics of the study cohort

Age, sex, and blood group distribution were comparable across groups. Hemoglobin concentration and red blood cell count were lower in MM patients compared to MGUS patients and healthy controls, whereas red cell distribution width was higher. C-reactive protein, total protein, and lactate dehydrogenase concentration were higher in MM and MGUS patients than in controls.

4.6. Hemostatic parameters of the study cohort

The results of coagulation screening tests in MM or MGUS patients and healthy controls were not significantly different. Protein S activity was lower in the patient groups compared to controls. The APC ratio was comparable across groups. FVIII activity, vWFAg, and vWFAct levels were increased in MM and MGUS patients compared to controls.

4.7. Plasma TG and sensitivity to APC inhibition

ETP was significantly increased in MM patients [median: 1595 (IQR: 1293 – 1848) nMxmin in patients vs. 1292 (IQR: 1038 – 1637) nMxmin in controls, $p = 0.0435$]. 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, $p = 0.0024$]. 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, $p = 0.0019$].

In *in vitro* experiments, adding APC to plasma samples from MGUS patients and healthy controls 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, $p = 0.0560$]. Peak thrombin was reduced in healthy controls 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. 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%. APC also significantly prolonged the lag time and time to peak parameters in plasma from healthy controls and MGUS patients but not in MM plasma. The observed phenomena indicate a greater resistance of MM plasma to APC inhibition.

4.8. 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. Similarly, vWFAg correlated significantly with ETP but only moderately with peak thrombin. 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, $p = 0.0032$). C-reactive protein 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 healthy controls but not in MM and MGUS patients.

4.9. FXIII antigen and activity levels of the study cohort

FXIII-A_2B_2 antigen and FXIII activity levels were lower in MM patients compared to MGUS patients and healthy controls. 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 healthy controls (r = 0.6780, 95% CI: 0.3688 – 0.8520, p = 0.0004). There was no difference in total FXIII-B levels between MM or MGUS patients and control subjects. Nonetheless, the ratio of free/total FXIII-B was significantly higher in MM patients than in MGUS and healthy controls.

4.10. 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. Quantitative fibrin monomer was significantly increased in patients with MM [median: 4.5 (IQR: 3.3 – 6.1) µg/mL, p = 0.0009] and MGUS [median: 3.7 (IQR: 2.7 – 5.0) µg/mL, p = 0.0246] compared to controls [median: 2.5 (IQR: 2.3 – 3.1) µg/mL]. MM 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]. 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]. 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].

4.11. 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. ClotPro-tPA lysis time was significantly prolonged in MM patients

compared to controls (p = 0.0427), while the maximum clot firmness was higher (p = 0.0106). There were no significant differences in plasmin generation or clot lysis assay parameters between patients and controls. FXIII activity levels showed a significant positive correlation with ClotPro-tPA lysis time (r = 0.5136, 95% CI: 0.1286 – 0.7640, p = 0.0122) and peak plasmin (r = 0.4595, 95% CI: 0.0583 – 0.7329, p = 0.0274) in healthy controls. Similarly, FXIII A_2B_2 levels showed a significant positive correlation with ClotPro-tPA lysis time (r = 0.4591, 95% CI: 0.1113 – 0.7067, p = 0.0122) in healthy controls. Such associations were abolished in patients with MM and MGUS.

5. DISCUSSION

The antiproliferative and antitumor properties of BTZ make it extremely effective in a variety of hematological malignancies. Notably, it has significantly improved the management of newly diagnosed, refractory, or relapsed MM.

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.

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 parameter was significantly shorter in all BTZ-pretreated groups, the peak thrombin concentration was significantly higher, but there was no significant difference in 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 platelet microparticles. In our study, the BTZ-preincubated samples showed much higher platelet microparticles than the TRAP samples. As a result, it is highly probable that the higher platelet microparticle 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 human serum albumin 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 bone marrow 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 since thrombosis is frequent in patients with MM and has been linked to proteasome inhibitor treatment in some cases.

In our other study, we found that TG quantity parameters in newly diagnosed, untreated MM patients were significantly higher than in a healthy control group, similar to MGUS patients. We also found elevated FVIII and vWF levels in both patient groups. These findings are in line with previous researches linking these phenomena 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 bone marrow neovascularization. Furthermore, it has been discovered that bone marrow angiogenesis increases as plasma cell dyscrasia progresses from the precursor MGUS stage to advanced MM, implying a potential link between angiogenesis and disease progression. 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 C-reactive protein levels of all our study subjects and

determined their correlations with ETP and peak thrombin values. In healthy control subjects, C-reactive protein 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 APC to plasma from MM patients resulted in only a minimal reduction of 1.6% in ETP and 5% in peak thrombin, whereas a previous study found 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 acquired APC resistance as determined by an APTT-based resistance test. Contrary to previous reports, we found a significant reduction in protein S activity in MM and MGUS patients compared to control subjects, 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 healthy controls 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 similar between groups, indicating that the decreased FXIII levels found in MM 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 healthy controls. However, in two of these patients, FXIII-A_2B_2 antigen levels were below the normal

range. Mixing studies did not reveal the presence of an FXIII inhibitor in any of the cases where FXIII activity was low. Based on the results of the mixing studies and total FXIII-B levels, an immune-mediated inhibitory mechanism was ruled out. The significance of these findings in MGUS patients 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 and critically ill patients. Our results are consistent with other studies that show acquired FXIII deficiency and hyperfibrinolysis in newly diagnosed pediatric hematological malignancies.

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 plasmin generation assay and clot lysis assay in our patient cohort did not differ from healthy controls, 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 between MM and MGUS patients and healthy controls. MM patients had significantly increased clot firmness, indicating a prothrombotic state. The lysis time 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. 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. In this study, both MM and MGUS patients had elevated levels of fibrin monomer, TAT complex, D-dimer, and PAP complex, indicating marked hypercoagulability and secondary hyperfibrinolysis.

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.

6. 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.

7. NEW SCIENTIFIC FINDINGS

Project 1. Effect of bortezomib on platelet function

- Bortezomib induces platelet activation and apoptotic processes in human gel-filtered 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 multiple myeloma patients at the time of diagnosis due to increased activation and consumption.

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

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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.
   DOI: http://dx.doi.org/10.1016/j.thromres.2023.12.004
   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.
   DOI: http://dx.doi.org/10.1016/j.thromres.2023.01.010
   IF: 7.5 (2022)
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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