THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Tyrosine kinase inhibitor treatment of chronic myeloid leukemia, laboratory and clinical evaluation of therapy related side effects, with particular regard to vascular hematological abnormalities

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The Examination takes place at **Conference Room of Building B, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 12:30 on 02 JUL 2024**

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The PhD Defense takes place at the **Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine**, **University of Debrecen**, **at 14:00 on 02JUL2024**

1. Introduction

Chronic myeloid leukemia (CML) is part of the family of chronic myeloproliferative neoplasms. A fusion gene (*BCR::ABL1*) formed as a result of the reciprocal translocation between the long arms of chromosomes 9 and 22 (Philadelphia chromosome) encodes a protein with unregulated tyrosine kinase activity (BCR::ABL1). This protein with pathologically increased tyrosine kinase activity disrupts the strictly regulated balance of cell differentiation, proliferation, adhesion, and programmed cell death signaling pathways, leading to malignant transformation of *BCR::ABL1* positive cells and forming the clinical picture typical of the disease. Understanding this malignant genetic transformation at molecular level helped to develop specific targeted antitumor therapy, for which inhibition of the abnormal tyrosine kinase activity of the BCR::ABL1 protein was an excellent target. In the late 1990s, Druker and his colleagues developed a molecule known as STI571, later registered as imatinib, which competitively binds to the ATP-binding site of the BCR::ABL1 protein kinase, thus preventing substrate phosphorylation and blocking subsequent signal transduction pathways.

In chronic phase patients, resistant or intolerant to prior use of α-interferon treatment, 90% had a complete hematological response, and 50% had a complete or major cytogenetic response with imatinib treatment at a dose above 300 mg per day. Over the years, the results of controlled clinical trials have confirmed the initial excellent results of tyrosine kinase inhibitor (TKI) treatment surpassing all expectations. Imatinib has very favorable pharmacological properties and has proven to be an oral, highly specific, well-tolerated drug with minimal side effects. During the last two decades, targeted molecular therapy has become a fundamental treatment of malignant hematological diseases. With the development of second (nilotinib, dasatinib, bosutinib) and third (ponatinib) generation tyrosine kinase inhibitors, CML cases that respond poorly to or are intolerant to imatinib have become treatable. However, with second-generation TKI treatments serious side effects can occur at a much higher rate. In connection with nilotinib and ponatinib treatments, cardiovascular events (arterial and venous thromboembolism, acute coronary syndrome, stroke, peripheral vascular disease, hypertension), and with dasatinib treatment, chest fluid accumulation and bleeding (gastrointestinal tract, central nervous system) were observed more frequently.

When CML is diagnosed, more than half of patients are older than 65 years. Accordingly, the occurrence of cardiovascular diseases is also more common. Before starting treatment, it is therefore recommended to carry out detailed cardio-metabolic screening tests, which must be

checked regularly at given times in addition to continuous TKI therapy. Performing screening tests helps to recognize and prevent vascular events leading to ischemic heart disease, ischemic cerebrovascular events, or peripheral arterial occlusive disease, as well as timely recognition and prevention of hypertension. Previous *in vitro* and *ex vivo* studies with dasatinib have confirmed the alteration of primary hemostasis. However, the background of cardiovascular side effects observed with ponatinib treatment is less clear.

"Coated"-platelets (**co**llagen **a**nd **t**hrombin activat**ed**) are sensitive indicators of primary hemostasis. They are formed in connection with double strong agonist (collagen and thrombin) activation, which forms a special subgroup of activated platelets. "Coated"-platelets are a subpopulation of activated platelets with a very significant procoagulant activity. Their common feature is mitochondrial depolarization, persistent cytoplasmic calcium level increase, cell surface expression of phosphatidylserine (PS), and inactivation of fibrinogen (GPIIb/IIIa) receptors. Several functionally active procoagulant proteins - including fibrinogen, factor V, and von Willebrand factor - are on their surface. The overproduction of these procoagulant "coated"-platelets has previously been proven in many thrombotic processes. At the same time, a reduced level of "coated"-platelets can be detected in cases of spontaneous cerebral hemorrhage, lacunar stroke, and severe hemophilia A.

2. Review of the literature

2.1. Chronic myeloid leukemia

Tyrosine kinase inhibitor therapy has brought about a radical change in the treatment of CML. In the 1970s - when only cytotoxic agents were available - the 10-year survival rate was 20%. This increased to 50% with the introduction of allogeneic bone marrow transplantation and α–interferon therapy. A significant change occurred in the last 25 years with tyrosine kinase inhibitor treatment. Today, 10-year survival is nearly 90%; patients' survival is close to that of healthy adults. Currently, for a significant number of patients, TKI treatment can mean continuous, lifelong therapy. The course of the disease can be predicted based on the patient- and disease-specific characteristics recorded at the time of diagnosis (age, spleen size, platelet count, and the proportion of blasts, eosinophilia, and basophilia in the peripheral blood). Several score systems are recommended to determine the prognosis. Among these, the Sokal index is the oldest, and it was also used in most clinical trials. The risk factors of each score system were determined to assess the correlation between response to TKI treatment and survival. Since the majority of patients now die due to causes independent of leukemic progression - while CML

is in long-term and deep molecular remission - new risk assessment score systems have been developed to predict the probability of death related to CML. These are the EUTOS (European Treatment and Outcome Study for CML) and EUTOS long-term survival (ELTS) scores. The ELTS score uses the same simple blood count data, spleen size, and age as the Sokal index. The main difference is in the prognostic value of age, since age affects overall survival less in TKI-treated patients (ELTS score) than in patients treated with conventional chemotherapy (Sokal score).

When applying the currently accepted risk-adapted treatment strategy in the treatment of CML, we always have to choose the therapeutic goal first. In young patients, in the absence of other co-morbidities, the primary goal is achieving fast and deep molecular response (DMR) and treatment-free remission (TFR). For older patients suffering from numerous co-morbidities, survival alone, improvement of quality of life, and prevention of accelerated and blast phases are the goals. In addition to the assessment of the patient's age, general condition, and accompanying diseases, the side effect profile of the chosen TKI is also of paramount importance.

During TKI treatment, based on the latest (Oct 2023) recommendations of the European Leukemia Network (ELN), the hematological, cytogenetic, and molecular responses must be checked at specific times, and based on the results, further actions must be decided (continuation of treatment, modification of therapy, allogeneic stem cell transplantation, etc.). Due to the genetic instability that determines the disease, establishing the cytogenetic status at the time of diagnosis and following it during treatment is important, as fluorescent in situ hybridization (FISH) and PCR tests cannot detect any other chromosomal abnormalities. Certain chromosomal aberrations occur more frequently in CML and affect the outcome of the disease. Quantification of the *BCR::ABL1* fusion gene (reverse transcriptase quantitative PCR method, RT-qPCR) is the basis for monitoring TKI treatment. The standardization of the method (international score, IS) made it possible to compare the results published by individual molecular laboratories and, accordingly, to develop uniform international guidelines. Controlled clinical studies have proven that an *BCR::ABL1* [IS] value below 10% measured in the 3rd month of treatment reliably predicts progression-free survival (PFS). Are values above 1% measured in the 6th month of TKI treatment or above 0.1% measured in the 12th month mean that the treatment is insufficient and in such cases a change of therapy is necessary?

By increasing the sensitivity of RT-qPCR, deep molecular responses can also be evaluated, creating an opportunity to accurately monitor a possible subsequent therapy-free period.

In 30-40% of cases may develop resistance or intolerance to the TKI used during the first-line treatment. The most common cause of resistance is a somatic point mutation of the *BCR::ABL1* fusion gene, which results from the genetic instability of the disease. Currently, more than 100 different mutations are known. Their occurrence is more frequent in the accelerated and blastic phases, and they mean a poor prognosis. The point mutation can create a conformational change of the BCR::ABL1 protein that prevents the specific binding of the TKI molecule to the Abl tyrosine kinase domain (TKD) and can lead to ineffectiveness of the treatment. During the course of the disease, several mutations may develop at the same time, which may affect several *BCR::ABL1* positive cell clones (polyclonal mutation), or develop within the *BCR::ABL1* gene section of a given clone ("compound" mutation). In the case of two or more mutations, the latter is much more common and can cause resistance to several TKIs at the same time. The presence of the T315I mutation causes resistance to all currently available TKI therapies with the exception of ponatinib and asciminib (“gatekeeper” mutation).

TKI resistance can also develop due to inadequate plasma concentrations of the TKI. This is most often caused by insufficient patient compliance and lack of therapy fidelity (adherence). Adherence is mostly influenced by side effects caused by TKI treatment (intolerance). Early, usually hematological side effects (hematological toxicity) can be well controlled by temporarily stopping the TKI, reducing the dose, or switching to another TKI. Prolonged side effects that are not serious (grade 1-2) but have a lasting negative impact on the quality of life represent a significant adherence problem and lead to insufficient therapeutic response as compliance deteriorates. In such cases, it may be necessary to check the TKI plasma level. In case of suspicion of TKI resistance, the accuracy of drug intake and the patient's adherence to therapy must be checked. In case an insufficient response is detected with adequate compliance, it is recommended to change the therapy and perform the *BCR::ABL1* TKD mutation analysis at the same time.

Currently, the National Health Insurance Fund (NEAK) supports three TKIs (imatinib, nilotinib, and dasatinib) in the first line of therapy in Hungary. While imatinib treatment used in the first line improves overall survival (similar to second-generation TKI treatment) with the fewest side effects, second-generation TKI therapy used in the first line can make treatment-

free survival available to patients through a faster and deeper molecular response, also the principle possibility of complete recovery.

In the case of resistance developing in connection with TKI treatments used in the first line, or in the case of side effects (intolerance) that negatively affect the patient's quality of life in the long term, it is necessary to modify the therapy. In the case of primary or secondary resistance, a bone marrow and cytogenetic examination is always necessary to recognize possible clonal evolution (acquired chromosomal aberrations) and to accurately determine the phase of the disease (acceleration? blastic transformation?). Currently, NEAK supports three second-generation TKI treatments (nilotinib, dasatinib, bosutinib) as a second line in Hungary. At the same time, the evidence of second-generation TKI treatment falls short of the experience gained in connection with imatinib treatment, and the side effects caused by second-generation TKIs - which can affect many organ systems - are also much more common.

In the case of resistance to TKI treatment, a mutational analysis of the *BCR::ABL1* gene is also recommended. In the case of a T315I mutation, treatment with ponatinib asciminib or allogeneic stem cell transplantation (allo-SCT) is required.

Allo-SCT is recommended in cases of intolerance or resistance to several TKIs experienced in the first chronic phase of a small number of patients, chromosomal abnormalities that indicate a poor prognosis and T315I mutation detected at diagnosis or occurring during TKI treatment. In cases of intolerance or resistance to second-generation TKI treatment used in the first or second line, the possibility of allo-SCT should also be considered in time. In such cases (line 3 or 4), the alternative TKI treatment is not always able to maintain the appropriate cytogenetic and molecular response. In this case, the chosen second-generation TKI treatment can be supplemented with α-interferon, and ponatinib or, if possible, another TKI treatment in the investigational phase (e.g. vodobatinib) should be considered, and the patient must be prepared for allogeneic stem cell transplantation. In the case of abnormalities indicating acceleration detected at diagnosis, the patient should be considered high risk, and in case of insufficient therapeutic response, allo-SCT is recommended.

Achieving an early and deep molecular response improves both disease-free and overall survival. However, in order to achieve all of this, continuous and accurate molecular biological control of TKI treatment, standardized application of these laboratory methods (within and

between individual laboratories), careful consideration of the achieved therapeutic responses based on uniform guidelines, and, if necessary, appropriate modification of the treatment are necessary. With accurate molecular monitoring of the response to treatment, an increasing proportion of patients can achieve a sufficiently deep molecular response and gain hope for long-term drug- and disease-free survival. In the case of at least 5 years of continuous TKI treatment and a sufficiently deep (MR4.5 or MR5) and long-lasting (at least 2 years) molecular response, if a change of therapy was previously only necessary due to TKI intolerance, suspension of TKI treatment can be attempted. In the case of imatinib, 40% of patients, and 50% of cases of second-generation TKIs, remain in long-term deep molecular remission even without treatment. During the first 6 months of the treatment-free period, close monitoring of the molecular response (performed at least every 4 weeks) is justified. TKI therapy should be restarted if the molecular response is worse than MMR (MMR3).

2.2. Procoagulant, "coated"-platelets

The procoagulant activity of platelets is manifested in several factors. Negatively charged phospholipids (phosphatidylserine, PS) are placed on the surface of activated platelets, which promote the formation of enzyme complexes that are essential for the effective functioning of the coagulation cascade (prothrombinase and tenase). The coagulation cascade consists of a series of chain reactions during which active enzymes are formed from inactive proenzymes and precursor proteins. By amplifying each other's effect and organizing into macromolecular complexes (prothrombinase and tenase complexes), the resulting enzymes strengthen the humoral (coagulation) processes of blood coagulation in addition to platelet aggregation. The prothrombinase complex consists of factor X, a cofactor protein (factor V), the substrate of the enzyme (prothrombin), the anionic phospholipid surface (PS) of the platelet cell membrane, and calcium, which is necessary for the binding of all of these. The advantage of the formation of complexes is that thrombin generation can take place significantly more efficiently on the surface of activated platelets, which are thus localized directly to the site of vessel wall injury.

These events are key to effectively preventing blood loss. At the same time, the overturning of the precise regulation of the process can cause vessel occlusion, which leads to ischemia and infarction of vital organs. Arterial thrombosis is one of the most significant clinical deaths caused by the rupture or erosion of atherosclerotic plaques, which leads to platelet adhesion, aggregation, and thrombus formation in coronary and cerebral arteries causing myocardial infarction and stroke. The study of the complex hemostatic processes underlying the

development of arterial thrombosis is important for understanding the pathophysiology of ischemic cardiovascular diseases.

During their activation, platelets can enter an activated state in different ways, thereby creating two subclasses with heterogeneous cell surface properties and different functions. The two subclasses are aggregating and procoagulant platelets. The subgroup of procoagulant platelets is characterized by a permanent rise in the intracellular calcium level after their activation. As a result, PS exposure on the outer surface of the platelet membrane increases. This leads to an increase in the activity of the tenase and prothrombinase complexes, which promote thrombin and fibrin formation. During the formation of the primary blood clot, the procoagulant platelets come to the surface of the thrombus, so they ensure the formation of a sufficient amount of thrombin and fibrin in the most sensitive place (vessel wall damage).

A subgroup of procoagulant platelets is the "coated"-platelets, which are created by the simultaneous activation of two different, strong agonists (e.g. thrombin and collagen). They are characterized by a large amount of PS on their surface and the ability to irreversibly bind procoagulant proteins released from α-granules, as a result of which they have increased hemostatic activity. The creation of procoagulant platelets occurs mainly through signaling pathways via the GPVI receptor. GPIIb/IIIa is mostly inactive on PS-expressing platelets. On the other hand, the aggregating platelet population is characterized by the fact that they hardly show PS expression. The increase in intracellular calcium level that occurs after their activation subsides quickly, and the GPIIb/IIIa receptor is present in an active conformation on their surface, which binds fibrinogen with high affinity, which is responsible for platelet aggregation.

The flow cytometry test is an easily accessible, simple, and fast hematological diagnostic tool. It is an excellent methodology for quantifying the production capacity or lack of procoagulant platelets, as well as for the analysis of different platelet subpopulations from the point of view of phenotype. Procoagulant activity can also be assessed by other assays, such as *ex vivo* platelet activation-dependent thrombin generation and dynamic flow chamber assays. However, these latter techniques are still in the experimental stage, and their diagnostic applicability requires further standardization studies.

Numerous studies have confirmed the increased formation of "coated"-platelets in patients with a prothrombotic state. The average level of procoagulant platelets was found to be higher in

stroke and TIA compared to the healthy control group. According to the studies, a higher "coated"-platelet level measured at the time of stroke or TIA has prognostic significance in terms of the frequency of recurrence of cerebral circulation disorder. In the case of coronary stenosis, patients belonging to the group characterized by the highest procoagulant platelet level (>50%) have a high risk of developing early ischemic events. Similarly, in patients with asymptomatic carotid artery stenosis, high levels of "coated"-platelets (45%) predicted the risk of stroke or TIA. Furthermore, patients with higher procoagulant platelet levels (42.6%) had more frequent recurrent ischemic events after lacunar stroke. Elevated levels of procoagulant platelets have also been observed in coronary artery disease and heart failure. The platelet potential determination of "coated"-platelets performed in connection with acute vascular events can also help in predicting the more serious course of the disease. In the case of subarachnoid hemorrhage caused by cerebral aneurysm rupture, an increase in procoagulant platelet formation was associated with the development of delayed cerebral ischemia and a significant deterioration of cognitive functions. A higher average level of "coated"-platelets was found in smokers compared to non-smokers. Changes in procoagulant platelet levels were investigated in smoking patients with stroke, some of whom quit smoking after stroke. In their case, a decrease in the degree of formation of "coated"-platelets was observed.

When examining patients with spontaneous intracerebral hemorrhage, a significantly lower procoagulant platelet level was found compared to healthy controls. Examining a similar group of patients, more severe and extensive bleeding developed in those patients with the lowest "coated"-platelet production. Another study found that the outcome of bleeding events and associated death within 30 days was worst in patients with "coated"-platelet levels below 27%. Similarly, patients with subarachnoid hemorrhage had the highest one-month mortality with less than 36.7% "coated"-platelet formation. In essential thrombocythemia, significantly lower "coated"-platelet levels were observed in patients who did not receive hydroxyurea treatment. The production of procoagulant platelets in patients receiving hydroxyurea was almost identical to that of the control group, regardless of whether the patients had a previous thromboembolic event.

3. Objectives

The aim of our work is the detailed processing of patient- and disease-specific data collected during TKI treatment of CML patients cared for at the University of Debrecen, Clinical Center, Clinic of Internal Medicine, Department of Hematology, the evaluation of the results, and the

vascular hematological background of the side effects observed in connection with the given TKI treatment *in vitro* and *ex vivo* testing as follows:

I.
Accurate assessment and organization of comorbidities and possible side effects before starting TKI treatment and during treatment. Evaluation of undesirable side effects and risk-adapted modification of the applied TKI treatment, and prospective processing of the results achieved in this way.

II.
Detailed cardio-metabolic screening of patients to prevent cardiovascular side effects of TKI treatment.

III.
In vitro and *ex vivo* study of primary hemostasis for laboratory characterization of vascular hematological events induced by dasatinib and ponatinib treatment.

4. Patients and methods

At the University of Debrecen, Clinical Center, Clinic of Internal Medicine, Department of Hematology, between January 1, 2010, and May 31, 2020, we evaluated the data of 120 patients receiving TKI treatment for chronic phase CML, with particular regard to comorbidities already present at the time of diagnosis, cardiovascular risk and for complications occurring with TKI treatments.

During the primary hemostasis *in vitro* and *ex vivo* tests, citrate-anticoagulated samples from volunteers, healthy donors, and patients receiving various TKI treatments were examined with classic platelet aggregation assays (PFA-100, thrombin generation, collagen-induced aggregometry, ATP secretion). In addition, after the activation of gel-filtered platelets (GFP) with a double agonist (convulxin + thrombin), the formation of "coated"-platelets was determined by flow cytometry with or without TKI treatment.

The study plan and patient documents were approved by the local ethics committee of the University of Debrecen based on RKEB/IKEB decision No. 4875–2017.

For platelet activation and aggregation studies, we used gel-filtered platelet (GFP) samples from healthy volunteers (n=11) (*in vitro* studies) and patients receiving dasatinib, ponatinib, or nilotinib therapy (*ex vivo* studies) for chronic phase CML (n=20) or Philadelphia chromosome-positive acute lymphoid leukemia (n=1). Antiplatelet treatment (if any) was suspended 7 days before the study, and since diabetes mellitus can also affect the level of "coated"-platelets, diabetic patients were excluded from the study. Patients receiving TKI treatment had been under continuous TKI treatment for at least 4 months at the time of sampling.

For the tests, whole blood was drawn from the elbow vein into a tube containing 0.109 mol/L (3.2%) sodium citrate (Becton Dickinson, San Jose, CA). During the preparation of platelet-rich plasma (PRP), the sample was centrifuged at 170 g for 10 minutes at 20 °C. When preparing platelet-poor plasma (PPP), the blood was centrifuged at 1500 g for 15 minutes at 20 °C.

4.1. Laboratory examination of platelet function

Platelet function was screened with a PFA-100 device (Siemens, Deerfield, IL, USA) using collagen/epinephrine (C/Epi) and collagen/ADP (C/ADP) cartridges by determining the closure time (CT). Platelet aggregation and secretion induced by 1 μg/ml fibrillar collagen (Takeda, Linz, Austria) or 500 μg/ml AA (Labexpert Ltd., Debrecen, Hungary) were investigated using a Chrono-Log 700 lumi-aggregometer (Chrono) (Log Corp., Havertown, PA, USA). The platelet count in the platelet-rich plasma was adjusted to 250×10^9/L using the platelet-poor plasma. The aggregation process was followed for 8 minutes and the results were expressed as a percentage of the maximum change in light transmission (ΔTmax %). The amount of ATP released during platelet activation was determined by the bioluminescence method using the luciferin-luciferase reagent (Biotherma AB, Handen, Sweden). Maximal ATP secretion was expressed as μmol ATP/10^{11} platelet count.

4.2. Preparation of platelet-rich plasma and planning of *in vitro* and *ex vivo* studies

After the centrifugation described above, the number of PRP platelets was adjusted to 250 x 10^9/L by adding platelet-poor plasma. PRP from healthy volunteers was used to study the effect of ponatinib *in vitro*. The following PRP samples were prepared:

- not pretreated, activated with collagen or ADP or convulxin
- pretreated with ponatinib, activated with collagen or ADP or convulxin

For pretreatment, ponatinib was used in final concentrations of 75, 150 and 1000 nM (10 min, 37 ºC). 1 μg/ml collagen or 5 μM ADP or 125 ng/ml convulxin were used to activate pretreated platelets.

During *ex vivo* studies, PRPs prepared from blood samples of patients receiving dasatinib, ponatinib or nilotinib treatment were activated using 1 μg/ml collagen or 5 μM ADP or 500 μg/ml arachidonic acid or 125 ng/ml convulxin.

4.3. Production of gelfiltered platelets

Gel filtration of PRP samples was performed on a Sepharose CL-2B column (Sigma-Aldrich, Saint Louis, MO, USA), and purified platelets were adjusted to a cell concentration of 40 x 10^9/L in BSGC.

4.4. Flow cytometric analysis

All flow cytometry analyzes were performed on a BD FACS-Canto II (Becton Dickinson, Mansfield, MA, USA) and data were analyzed using BD FACSDiva software (version 6.1.3).

Evaluation of "Coated"-platelets

Platelet activation was evaluated in a total volume of 100 μl, which contained Mix buffer (79 μl), 40x10^9/l GFP (10 μl), 1 μl biotinylated fibrinogen (100 μg/ml; Sigma-Aldrich) and 10 μl agonist. The final concentration of convulxin was 125 ng/ml (Pentapharm, Basel, Switzerland), and the concentration of thrombin was 0.5 U/ml (bovine thrombin; Sigma-Aldrich). Mix buffer was prepared from the following components: 10 mM HEPES, 1 mg/ml BSA, 2.5 mM $CaCl_2$, 1.25 mM $MgCl_2$ and 150 mM NaCl. The mixture was incubated for 10 minutes at 37°C, then the reaction was stopped with 200 μl of 1% (w/v) paraformaldehyde and fixed for 20 minutes at room temperature. After fixation, 3.5 ml of PBS containing 1 mg/ml BSA (PBS/BSA) was added and the sample was centrifuged at 1500 x g for 15 min. The pellet was resuspended in PBS/BSA labeled with 200 μl anti-CD41a-PECy and Streptavidin-PE, incubated for 20 minutes at room temperature in the dark. After washing with PBS, centrifuged again as mentioned above, and finally was resuspended in PBS. The proportion of "coated"-platelets was determined as percentage (%).

Detection of the active conformation of αIIbβ3 integrin

The active conformation of integrin αIIbβ3 was determined by using unactivated samples, or samples activated with 20 μM thrombin receptor activating peptide (TRAP) or 25 ng/ml

convulxin (GPVI agonist). Fluorescein isothiocyanate (FITC)-conjugated PAC1 and phycoerythrin-cyanin 5 (PECy5)-conjugated CD41a antibodies connection were determinated by flow cytometry.

Examination of platelet-monocyte heterotypic aggregates

To determine platelet-monocyte aggregates, whole blood from healthy donors (n=6) were incubated with 75, 150, and 1000 nM ponatinib or vehicle (0.2% DMSO) for 10 min at 37°C, and then the samples were activated with or without convulxin and then incubated with CD14 phycoerythrin (PE) and CD42a FITC. The red blood cells were lysed, the samples were washed twice with phosphate-buffered saline (PBS), and then fixed with paraformaldehyde. The percentage of platelet-monocyte aggregates was determined by fluorescein isothiocyanate (FITC)-conjugated CD42a binding. In each case, the results were compared with "non-immune" immunoglobulin G (IgG) control samples serving as isotype controls.

4.5. Applied statistical methods

GraphPad Prism version 6.01 was used for statistical analyses. The distribution of the data was evaluated using the Kolmogorov-Smirnov test. The statistical significance of the differences between the *in vitro* experimental groups was analyzed by one-factor ANOVA in the case of Gaussian distribution and by the Kruskall-Wallis test in the case of non-Gaussian distribution. The statistical significance of the differences between the results of CML patients before drug administration (0 hours) and 1 and 4 hours after drug administration was analyzed using the paired Student's t-test in the case of a Gaussian distribution, and the Wilcoxon rank test in the case of a non-Gaussian distribution. Differences were considered significant if p-values were below 0.05. Survival of CML patients receiving TKI treatment was determined using the Kalpan-Meier estimate.

5. Results

5.1. Clinical examination of the CML patient group

Between January 1, 2010, and May 31, 2020, we evaluated the data of 120 patients receiving TKI treatment for chronic phase CML (previously diagnosed and under treatment, or newly diagnosed patients during this period). The average age at diagnosis was 49 years (15–82 years), and the average follow-up time from diagnosis was 110 months (3–353 months). The total duration of TKI treatment was 14 303 months. A significant number of patients (78.3%)

received imatinib treatment in the first line. In addition to the first-line imatinib treatment, intolerance or resistance developed in 34.2%.

Accelerated or blast phase occurred in 3 cases in addition to the second and third-generation TKI treatment used in the second or multiple lines. All three cases developed in the presence of the T315I mutation and were already under treatment with ponatinib, and had a fatal outcome. Allogeneic stem cell transplantation could not be performed in the case of 2 patients due to old age and the case of 1 patient due to the lack of a suitable donor. In the case of one patient, due to TKI resistance (confirmed, multiple *BCR::ABL1* mutation) and intolerance (severe and prolonged hematological toxicity), asciminib therapy was started after a total of five types of TKI treatment, with which a sufficiently deep molecular response was developed, but in the end, due to additional chromosomal abnormalities, the HLA matched sibling donor allogeneic stem cell transplantation was performed.

In the examined period, the mortality associated with CML was 1%. PFS was 81% and overall survival was 87%. The reason for switching from first-line imatinib treatment to second-generation TKI was intolerance in 68% and resistance in 32% (primary resistance 5%). In the examined period, we reviewed in detail the side effects occurring during TKI therapy, with particular attention to cardiometabolic abnormalities. We evaluated the need for therapy modifications due to unwanted side effects, as well as the profession-specific clinical practice of treating complications.

5.1.1. Hematological toxicity

The most common abnormalities in the blood count (anemia, neutropenia, or thrombocytopenia) were observed with dasatinib treatment, and grade 3-4 thrombocytopenia occurred most frequently (69%) during dasatinib therapy. By reducing or temporarily omitting the dose of dasatinib, and then reintroducing the drug at a reduced dose (1x50 mg daily) after the cytopenia resolved, no change of therapy was necessary in any case. Since the blood count was permanently stable in all cases with the reduced dose of dasatinib, the daily dose of 100 mg of dasatinib could be returned. Even with bosutinib treatment, hematological toxicity was frequently observed. In two cases, a change of therapy was necessary due to prolonged grade 3 neutropenia. With treatment with nilotinib 2x300 mg per day, 35% of patients developed serious (grade 3-4) hematological side effects, and in two of these cases, neutropenia was very long-lasting (3 and 7 months). Neutropenia observed with imatinib treatment occurred more

often in connection with increased doses (600 mg or 800 mg per day). With the standard (400 mg daily) dose of imatinib, we observed the fewest hematological side effects. Due to the small number of patients receiving ponatinib treatment (n=9), the observed toxicity was not representative. In one case, grade 2 thrombocytopenia was observed at a dose of 1x45 mg ponatinib per day.

5.1.2. Cardiovascular abnormalities

Based on the risk classification defined by the European Society of Cardiology, we started imatinib treatment in all patients with high and moderate cardiovascular risk. We did not experience any cardiovascular complications with first-line imatinib treatment (n = 94). In the absence of low cardiovascular risk, young age (≤60 years), high ELTS score, and other comorbidities (diabetes mellitus, COPD, hypertension, peripheral arterial vascular disease, etc.), we started second-generation TKI (dasatinib or nilotinib) therapy. No cardiovascular complications were observed with dasatinib treatment (n = 6). In the case of nilotinib therapy (n = 17), stroke occurred in 2 patients, peripheral arterial occlusive disease in 3 patients, and hypertension in 5 patients. In the cases of stroke and vasoconstriction, the treatment was continued with imatinib (n = 3), or due to the deep molecular response achieved, the TKI treatment was successfully discontinued permanently (TFR, n = 2). In case of hypertension detected during the treatment (n=5), the patient's therapy was switched to bosutinib (n=3) or dasatinib (n=2), and appropriate antihypertensive treatment was started. QTc-time prolongation or cardiac ultrasound abnormalities suggestive of heart failure were not detected in any patient. We observed three cases of vascular complications (one stroke, one occlusive arterial disease and one deep vein thrombosis) with 1x45 mg ponatinib treatment per day (n = 9) in the case of T315I-mutation or previous imatinib and at least two second-generation TKI treatments in multiple therapeutic lines. All of the patients were previously treated with nilotinib. We did not experience any cardiovascular complications during second or third-line bosutinib therapy.

5.1.3. Pulmonary events

In addition to high-dose (1×600 mg daily) imatinib treatment (n=3), a non-serious (grade 2) pleural effusion developed in one case, which became reversible after temporary discontinuation of imatinib therapy and administration of diuretics. A non-serious (grade 2) pleural effusion was observed in one case with the first-line treatment of 1×100 mg dasatinib daily (n = 6). After the temporary withdrawal of dasatinib and administration of diuretics, the dasatinib was reduced (1×70 mg per day), and later the full dose could be safely returned.

During second-line dasatinib therapy (n = 40), mild (grade 1-2) pleural effusions occurred in four cases and severe (grade 3) in two cases. A change of therapy (bosutinib) was necessary due to severe hydrothorax.

5.1.4. Metabolic abnormalities

Type 2 diabetes was known in 17 cases when the diagnosis of CML was established. In the vast majority of these patients (n=15), imatinib treatment was started, and the blood glucose balance remained balanced even with imatinib treatment. Nilotinib 2×300 mg per day therapy was started in two cases, the blood glucose level did not change, the previous antidiabetic treatment did not need to be modified, but in both cases, during the treatment, increased lipid values were observed, and statin therapy became necessary. In the case of nilotinib therapy (n=17) used in the first line (2×300 mg per day), reduced glucose tolerance developed in three patients. In two cases, we switched to dasatinib, in one case nilotinib treatment could be continued permanently with diet and lifestyle changes. This patient achieved DMR, and nilotinib became definitively discontinued (TFR). In the second line, in addition to nilotinib therapy 2×400 mg per day (n=24), diabetes requiring antidiabetic treatment (metformin) was observed twice. After stopping nilotinib, metformin could be stopped in addition to dasatinib treatment.

5.1.5. Gastroenterological complications

The most frequent gastroenterological complication (diarrhea) occurred with bosutinib treatment (n=12), which was mild (grade 1-2) in the majority of cases (n=10), and occurred 2-4 days after the start of treatment. With the temporary suspension of bosutinib treatment, symptomatic therapy (loperamide), and then a gradual dose structure from a low dose (1×100 mg per day), the symptoms of diarrhea were well controlled. Two elderly patients developed DMR with 1×200 mg bosutinib therapy per day, and no further dose increase was performed on them. Two patients developed severe (grade 3) diarrhea, in their case bosutinib had to be discontinued permanently, and the therapy needed to be modified.

Mild (grade 1 or 2) diarrhea was observed in 3 cases in connection with nilotinib treatment (n=17) given in the first line, 2×300 mg daily. Symptoms resolved following temporary discontinuation of nilotinib and did not recur after reinitiation.

Our patients were also screened for hepatitis serology, which proved to be negative for hepatitis B and C viruses in all cases. In addition to the TKI treatment, no major liver function abnormalities were detected, so no therapy modification was necessary.

During treatment with 1x100 mg dasatinib per day, severe gastrointestinal bleeding requiring transfusion occurred twice in our patients. Colonoscopic examination confirmed hemorrhagic colitis in both cases. During the examination of primary hemostasis, in addition to the inhibition of platelet aggregation, a reduced formation of "coated"-platelets was also detected. The latter is a sensitive indicator of the inhibitory effect of dasatinib on primary hemostasis. We stopped dasatinib and switched to imatinib treatment. No further gastrointestinal bleeding was observed.

5.1.6. Conception and pregnancy

With the intention of family planning, in the case of a young female patient with a molecular response of MR4.5 depth (*BCR::ABL1* [IS]: 0.0032%), nilotinib treatment was temporarily suspended several times for the period of *in vitro* fertilization, but conception did not occur even after several attempts. Due to the gradual loss of DMR, nilotinib treatment was started again, after which the *BCR::ABL1* [IS] value decreased to the previous, sufficiently low level within 6 months (*BCR::ABL1* [IS]: 0.002%). A young male patient of ours who was treated with dasatinib and later with ponatinib conceived a child during both TKI therapies and then gave birth to healthy children.

5.2. Examination of primary hemostasis during TKI treatment

5.2.1. Investigation of the effect of dasatinib on primary hemostasis

Based on the previous observations that dasatinib inhibits platelet aggregation and that reduced "coated"-platelet formation is a sensitive indicator of bleeding, platelet aggregation and "coated"-platelet function tests were performed. For a detailed analysis of the *in vitro* and *ex vivo* effects of dasatinib on primary hemostasis, we examined the platelet functions of healthy volunteers and CML patients treated with dasatinib and (as a negative control) nilotinib (results of the *in vitro* studies have been presented previously by the co-author).

The formation of "coated"-platelets in gel-filtered platelets and the aggregation processes were investigated with the PFA-100 platelet function test (whole blood) and platelet aggregometry (PRP). We hypothesized that the formation of "coated"-platelets can be a sufficiently sensitive and effective tool for the detection of hemorrhagic conditions associated with platelet activation abnormalities occurring during dasatinib treatment.

Platelet aggregation and ATP release in CML patients treated with dasatinib or nilotinib were examined immediately before (0 h) and 1 and 4 h after drug administration. A significant

decrease in maximal aggregation was observed after 1 hour for both tested platelet agonists (AA: $p \leq 0.05$, collagen: $p \leq 0.01$). Aggregation curves normalized 4 hours after taking the drug. Similar changes were observed when examining the release of ATP. There was no change in the nilotinib group.

The PFA-100 measurements were also performed before the administration of the given TKI (0 hours), and 1 and 4 hours afterwards. In the samples from CML patients treated with dasatinib, during the PFA-100 tests, we observed a significant prolongation of the C/Epi closing time 1 hour after administration compared to the baseline results ($p \leq 0.05$). Closing times were nearly normalized 4 hours after dasatinib administration. In the group treated with nilotinib, we did not notice any changes here either.

CD41a-positive events were evaluated using a dot plot recorded using a flow cytometer, and "coated"-platelets were identified by detecting bound fibrinogen. Similar to the *in vitro* studies, in *ex vivo* samples from CML patients treated with dasatinib, we observed a significant decrease in the formation of "coated"-platelets 1 hour after taking the drug. This effect was still detectable 4 hours after drug exposure in the dasatinib group. No changes were observed in the group treated with nilotinib.

5.2.2. Investigation of the effect of ponatinib on primary hemostasis

To further analyze in detail the controversial effects of ponatinib on platelet function, in our experiments, we examined the *in vitro* effect of ponatinib on collagen-induced platelet aggregation, ATP secretion, and "coated"-platelet formation. Furthermore, we evaluated the effect of ponatinib on collagen-induced platelet aggregation based on *ex vivo* samples. Platelet activation occurs through several agonist-specific platelet receptors. During the process of primary hemostasis, platelet adhesion to extracellular matrix proteins is followed by platelet activation and aggregation. During these processes, ADP is released from the dense granules of the platelet, which promotes the formation of a thrombus by causing further platelet activation.

In our experiments, we investigated collagen and ADP-induced aggregation in the PRP environment in the presence or absence of ponatinib. The aggregation response to collagen and ATP secretion proved to be concentration-dependent, and this phenomenon became significant at 1000 nM ponatinib concentration for both parameters tested (maximum change in light

transmittance (ΔTmax) and ATP secretion). On the other hand, during ADP (5 μM)-induced aggregation, neither platelet aggregation nor ATP secretion was affected by ponatinib. Activation of integrin αIIbβ3 is one of the most important steps during platelet aggregation. For this reason, we investigated the effect of ponatinib on αIIbβ3 levels activated by PAC1 (a monoclonal antibody capable of binding to the fibrinogen binding site on the activated form of the GPIIb/IIIa receptor). After ponatinib pretreatment, platelets were activated with convulxin, and PAC1 binding was measured. We observed that ponatinib pretreatment dose-dependently decreased the percentage of PAC1-binding platelets in convulxin-activated samples, and these changes were significant at a final ponatinib concentration of 1000 nM in convulxin-activated samples.

During *in vitro* studies, GFP from healthy controls was activated with a mixture of convulxin and thrombin, and the amount of "coated"-platelets was measured by flow cytometry. Ponatinib pretreatment inhibited the formation of "coated"-platelets in a dose-dependent manner. After summarizing the results of six parallel experiments, we concluded that ponatinib pretreatment significantly inhibited the formation of "coated"-platelets even at a therapeutic concentration (150 nM), and the amount of "coated"-platelets showed a further decrease at a final ponatinib concentration of 1000 nM.

It was also previously known that treatment of CML patients with ponatinib had a modest effect on platelet function. To further investigate the clinical significance of these results, we examined samples from five patients suffering from the chronic phase of CML and receiving ponatinib treatment. Blood samples were collected immediately before ponatinib administration (0 h) and 4 h after TKI administration. The daily dose of ponatinib was different between individual patients, so the data could not be evaluated together but were evaluated as individual results. Similar to the *in vitro* studies, collagen-induced platelet aggregation was determined in PRP samples from ponatinib-treated patients. In four patients, a reduced collagen-induced aggregation response was measured in samples taken before intake. In addition, we observed that three patients had a reduced collagen-induced aggregation response 4 h after ponatinib administration. Ponatinib significantly inhibited collagen-induced aggregation in patients receiving ponatinib (post-dose samples) compared to control platelet aggregation ($p=0.003$). It should be noted that aggregation tests were performed in a patient receiving ponatinib treatment 1x30 mg per day before the start of ponatinib therapy and after 2 weeks of ponatinib treatment. The ΔTmax values of collagen-induced aggregations were

observed as follows: 86% before ponatinib treatment, 73% during the treatment period in the pre-dose sample, and 75% in the four-hour post-dose sample.

According to previous literature reports, ponatinib exerts a pro-thrombotic and pro-inflammatory effect in CML patients undergoing TKI treatment. We hypothesized that the analysis of monocyte-platelet aggregates could be a sensitive indicator of these processes. We also examined the effect of ponatinib on the formation of monocyte-platelet heterotypic aggregates. Our results showed that ponatinib has an inhibitory effect on platelet function in both PRP and GFP environments. We evaluated the change in the level of heterotypic aggregates after ponatinib pretreatment in samples not activated and activated with the GPVI receptor agonist (convulxin) using a flow cytometry test method. By pre-treating non-activated platelets with different concentrations of ponatinib, we did not observe any changes in the level of monocyte-platelet heterotype aggregates. After ponatinib pretreatment and convulxin activation, the percentage of monocyte-platelet aggregates increased in a dose-dependent manner, and this effect became significant at 1000 nM ponatinib pretreatment.

6. Discussion

6.1. Side effects caused by TKI treatment

6.1.1. Hematological toxicity

The broad-spectrum BCR::ABL1 inhibition of dasatinib is behind the frequent hematological toxicity observed with dasatinib treatment. By inhibiting the function of other tyrosine kinases (c-KIT, EPH A2, SRC, LYN, PDGFR) that are less specific but important for hematopoiesis, dasatinib therapy often leads to the development of cytopenia. Anemia observed with TKI treatment is a consequence of c-KIT inhibition caused by TKIs. This receptor is essential for normal hematopoiesis and the development of erythroid cells. A possible cause of neutropenia is the nonspecific binding of TKIs to several key kinase targets of the immune system (LCK, LYN, SRC). In all cases, neutropenia resolved quickly by stopping dasatinib or reducing the dose. Similarly, imatinib also inhibits LCK, but based on *in vitro* data, the inhibitory effect of LCK is inferior to that of dasatinib. This is based on the fact that a higher-than-normal serum concentration is required to induce immunomodulating effects. This is consistent with our observation that neutropenia occurred more frequently with higher imatinib doses.

The reason for the development of thrombocytopenia can also be explained by the inhibition of SFK, which is one of the important signaling proteins of megakaryocytes and platelets. SFKs

mediate signals from several platelet surface receptors that influence platelet dissemination and migration into the vascular environment. Therefore, despite an increase in the number of mature megakaryocytes in the bone marrow, aspecific inhibition of SFKs causes thrombocytopenia by inhibiting migration and pro-platelet formation. The more frequent thrombocytopenia experienced in connection with dasatinib and bosutinib treatment may be related to the inhibition of SFK-mediated receptor mechanisms. In connection with dasatinib therapy, grade 3-4 thrombocytopenia was observed in 69% of patients. In such a case, there was no need to change therapy by reducing the dose of dasatinib or temporarily omitting it, and then reintroducing the drug at a reduced dose (1x50 mg per day) after the cytopenia resolved. After the blood count was stable with the reduced dose of dasatinib, 100 mg of dasatinib per day could be returned in all cases.

6.1.2. Cardiovascular side effects

When CML is diagnosed, more than half of patients are older than 65 years. Accordingly, the incidence of cardiovascular diseases is also more frequent, which we also experienced in our patient data. In addition to long-term (up to a decade) treatment, the cardiovascular risk caused by individual TKIs (arterial and venous thromboembolism, acute coronary syndrome, stroke, peripheral vascular disease, hypertension) can vary greatly in frequency and severity. Cardiovascular risk is particularly significant in patients older than 65 years receiving second- or third-generation TKI therapy. Therefore, it is recommended to carry out detailed cardio-metabolic screening tests before starting treatment, which must be checked regularly at given times in addition to continuous TKI therapy.

Imatinib, dasatinib, or bosutinib are preferred for first-line treatment of chronic phase CML in patients at very high risk of cardiovascular disease. In such patients, nilotinib treatment is not recommended, and we can only decide on its use after a thorough consideration of the risk factors, the risk of the disease, and the expected benefits of the treatment. Among our patients, we always started imatinib treatment in case of a high risk recognized based on the performed cardiovascular screening tests. Among these patients, no cardiovascular complications were observed during the study period. In patients with low or moderate cardiovascular risk, either TKI can be chosen. Performing screening tests helps to detect and prevent vascular events leading to ischemic heart disease (IHD), ischemic cerebrovascular events (ICVE), or peripheral arterial occlusive disease (PAOD) in time, as well as hypertension. Most clinical studies

reported an increased cardiovascular risk in patients treated with nilotinib and ponatinib. Cardiovascular screening is of particular importance in these cases.

In a multicenter, randomized clinical trial prior to the registration of nilotinib, 13.4% of patients receiving first-line treatment with nilotinib 2x400 mg daily developed some type of cardiovascular complication (IHD: 8.7%, ICVE 3.2%, PAOD: 2.5%) of which 8.7% were severe. In a single-arm, multicenter, phase 3b clinical trial, cardiovascular complications were experienced in 6% of patients treated with nilotinib 2x300 mg daily in the first line (IHD: 3.4%, ICVE 0.8%, PAOD: 1.9%), of which 3.5% -a was serious. The frequency of cardiovascular side effects in CML patients treated with nilotinib clearly increases with the duration of therapy and age. With a daily dose of 2x300 mg of nilotinib, the occurrence of IHD in each age group is significantly more common in the elderly (>75 years). In our patients who received nilotinib therapy (n = 17), we observed the development of stroke (n = 2), peripheral arterial occlusive disease requiring intervention (n = 3), and hypertension (n = 5). In the cases of stroke and vasoconstriction, the treatment was continued with imatinib administration (n = 3), and due to the achieved DMR, the TKI treatment was successfully permanently suspended in two cases (TFR). Our results observed in connection with nilotinib treatment are consistent with the frequency of cardiovascular complications reported in the international literature. Carrying out the close cardio-metabolic screening tests we have used since 2015 has facilitated the timely recognition of possible side effects and the appropriate profession-specific investigation and treatment.

In CML patients treated with ponatinib at a dose of 1x45 mg per day, 31% developed some kind of cardiovascular or venous thromboembolic complication (IHD: 16%, ICVE 13%, PAOD: 14%, venous thrombosis: 6%), of which 26% it was severe (grade 3-4). In the case of some patients, several cardiovascular complications may develop at the same time. The frequency of side effects is dose-dependent. Reducing the average daily dose of ponatinib by 15 mg - while achieving and maintaining an adequate clinical response - is associated with a 33% reduction in cardiovascular risk. Currently, the reduced daily dose of ponatinib is 1x30 mg, which can be reduced to 15 mg per day after achieving an adequate molecular response (MR3). In addition to the ineffectiveness of preventive imatinib and two second-generation TKI treatments, or 1×45 mg ponatinib treatment per day (n = 9) used in case of T315I mutation, vascular complications were observed in three cases (one stroke, one arterial occlusive disease and one deep vein thrombosis). All of the patients were previously treated with nilotinib.

During *in vitro* experiments with nilotinib and ponatinib, pro-atherogenic (increased ICAM-1 expression) and anti-angiogenic effects (VEGF receptor inhibition) were demonstrated for both TKIs, which may play a role in the more frequent PAOD observed during treatment. PAOD occurred in 14% of patients treated with ponatinib at a dose of 1x45 mg per day, with a median treatment time of five years, of which 11% were severe. For this reason, ponatinib treatment is contraindicated in the case of vasoconstrictive disease. In cases of mild or moderately severe vasoconstriction, if nilotinib is the only TKI of choice, it can be used with due caution and only with close follow-up (ankle-brachial index, peripheral arterial Doppler examination, blood pressure control, etc.). In such cases, correction of all cardiovascular risk factors is recommended, although there is no clear clinical evidence that this reduces the increased vascular risk caused by ponatinib or nilotinib.

Based on the international literature data, the development of high blood pressure was not observed more often with imatinib, dasatinib, and bosutinib treatment, which was also confirmed by our results.

6.1.3. Pulmonary side effects

Pleural effusion most often developed with dasatinib treatment (first or second line) in our patients (n = 7/51), which in most cases was not serious (grade 1-2). In two cases, severe (grade 3) hydrothorax was observed, which necessitated a change of therapy (bosutinib). According to literature data, chest fluid collection can usually appear a few months after the start of treatment, but it can also develop later - even several years after the start of treatment. According to a statistical analysis conducted on a database of side effects reported in connection with various TKI treatments with a very large number of patients, pleural effusion occurred with an outstanding frequency in patients treated with dasatinib. Among the possible "off-target" molecular signaling pathways, the role of inhibition of the proto-oncogene Lyn belonging to the SRC tyrosine kinase (SRK) family has been suggested. Disruption of this signaling pathway leads to oligoclonal proliferation of large granular lymphocytes (LGLs), which initiate a T-cell-mediated cytotoxic process on endothelial cells. In controlled clinical trials, pleural effusion was observed in 13-39% of imatinib-resistant or intolerant patients with second-line dasatinib treatment. The rate of hydrothorax development was higher in advanced CML and was also related to dasatinib dose. It is important to note, however, that chest effusion experienced with TKI treatment is in most cases a mild, non-serious side effect (grade 1 or 2) with few symptoms,

which resolves by reducing the TKI dose, and dasatinib can be started again. For first-line dasatinib, the median time to onset of chest fluid was 10 months, with 89% occurring within the first 8 weeks of treatment. When used in the second line, the median time for hydrothorax to appear was between 5-11 months, but there were also cases when it appeared 3 years after the start of treatment. It is most often accompanied by symptoms of dry cough, fatigue, chest pain, and dyspnea, in which cases physical and imaging tests (chest X-ray, high-resolution computed tomography) are mandatory.

Although fluid collection occurs less frequently with imatinib treatment, pericardial and pleural fluid collection is more common at doses higher than the standard dose (600 mg or 800 mg per day). Among our patients, in addition to high-dose (1×600 mg daily) imatinib treatment (n=3), a non-serious (grade 2) chest fluid collection developed in one case, which was reversible after temporary discontinuation of imatinib therapy and the administration of a diuretic at a dose of 1×400 mg daily changed.

6.1.4. Metabolic abnormalities

Disruption of carbohydrate and fat metabolism is also a frequently observed side effect during TKI treatment. Hyperglycemia most often develops with nilotinib treatment. In the case of first-line nilotinib treatment, elevated blood sugar values were detected in 50% of patients, of which 7% had severe (grade 3 or 4) hyperglycemia. Severe (grade 3 or 4) hyperglycemia occurred in 12% of patients with imatinib-resistant or intolerant chronic phase CML treated with nilotinib. In the case of an accelerated phase, this rate was 6.7%. Hyperglycemia developed on average one week after starting treatment. Among normoglycemic, non-diabetic patients at the start of nilotinib treatment, diabetes developed in 20.1% during a 3-year follow-up period. In 31% of CML diabetic patients treated with nilotinib, modification of the antidiabetic treatment was necessary. Ketoacidosis did not occur, but 60% developed severe (grade 3 or 4) hyperglycemia.

Similar to the metabolic deviations reported in the international literature, we also observed disturbances in sugar balance and fat metabolism among our patients with TKI treatment. At the time of CML diagnosis, 17 patients had known type 2 diabetes. The majority of these patients (n=15) received imatinib treatment. The blood sugar balance remained balanced throughout the imatinib treatment. In addition to previously known diabetes, nilotinib treatment was started in 2 cases (due to young age and high risk). We drew the attention of the patients to the strict adherence to the diet, so in addition to the previous antidiabetic treatment

(metformin) and diet, the blood sugar balance remained in balance, there was no need to modify the oral antidiabetic treatment, and their molecular response was adequate. However, during the treatment, elevated lipid values appeared in both cases, and statin therapy became necessary. In the case of first-line nilotinib 2×300 mg daily therapy (n=17), reduced glucose tolerance was confirmed in 3 of our patients. In two cases we switched to dasatinib, in one case nilotinib treatment could be continued permanently with diet and lifestyle changes. Later, this patient achieved a deep molecular response and nilotinib was definitively discontinued (TFR). In the second line, with 2×400 mg nilotinib therapy per day (n=24), diabetes requiring diet and metformin treatment was observed on two occasions. After nilotinib was discontinued, in addition to dasatinib treatment, metformin could also be discontinued.

The different, non-specific effects of individual TKIs beyond the inhibition of BCR::ABL1 (c-KIT, PDGFR, SRC) affect the molecular pathways of the regulation of metabolic processes at different points. In the case of imatinib and dasatinib, improvements in blood sugar and lipid profile are observed, in the case of nilotinib, reduced glucose tolerance, diabetes mellitus and

deterioration of fat metabolism parameters is observed. The beneficial effect of imatinib on blood sugar management results in increased insulin secretion by paralyzing pancreatic β-cell apoptosis, and increased peripheral tissue insulin sensitivity by inhibiting the TNF-α pathway and endoplasmic reticulum stress.

The molecular basis for the unfavorable metabolic profile of nilotinib is controversial and less clear. In one case study, decreased insulin secretion was reported during nilotinib therapy, which proved to be reversible after discontinuation of treatment. In another study, changes in blood sugar metabolism were examined in connection with nilotinib therapy in 37 patients, and hyperinsulinemia and insulin resistance were established. Cardiometabolic screening and follow-up of patients before starting TKI treatment and during treatment is extremely important, as timely recognition and treatment of possible metabolic derailment can prevent the development of increased cardiovascular side effects in connection with the therapy.

After starting TKI treatment with known diabetes, stricter control of blood sugar levels and, if necessary, modification of antidiabetic therapy are recommended. In such cases, it is recommended to choose another TKI instead of nilotinib treatment, but properly treated type 2 diabetes mellitus alone does not constitute an absolute contraindication for nilotinib treatment.

Close blood sugar control, glycosylated hemoglobin levels, and consultation with a diabetologist are recommended. Although hyperglycemia (grade 3 or 4) developed in half of the first-line diabetic patients treated with nilotinib during clinical trials, no serious complications (ketoacidosis, hyperosmolar coma, hospitalization) were observed. Almost three-quarters of the patients (74%) did not need to change their antidiabetic treatment, and no cardiovascular disease developed during the follow-up period.

In the case of nilotinib treatment, 22% of patients experienced a mild (grade 1 or 2) increase in cholesterol levels. Three months after nilotinib treatment, the median increase in LDL-cholesterol was 33 mg/dL. Mild (grade 1 or 2) hypertriglyceridemia occurred in 12% of patients receiving ponatinib therapy resistant to prior TKI treatments. In the case of bosutinib and dasatinib treatment, no deviations in the lipid balance were detected. High cholesterol values that develop early (3 months) in connection with nilotinib treatment showed a correlation with the development and severity of PAOD. Monitoring of lipid levels is recommended before starting therapy and during treatment. In the case of persistent hypercholesterolemia (>6.2 mmol/l) with nilotinib therapy, the patient is considered to be at high risk for PAOD, and appropriate statin treatment should be started as soon as possible. During our studies, among our patients receiving TKI treatment, we observed a difference indicating a lipid metabolism disorder during nilotinib treatment. In the case of nilotinib treatment (n=2) started with previously known diabetes, although the blood glucose balance did not deteriorate, elevated lipid values were observed and statin therapy became necessary.

6.1.5. Gastrointestinal side effects

Among the gastrointestinal symptoms caused by TKI treatment, diarrhea is one of the most common side effects. In clinical trials, it was mostly observed with bosutinib treatment. In the case of bosutinib 1x500 mg per day, significant (grade 3 or 4) persistent and recurrent diarrhea - occurring in the first month of treatment - was observed in 8-11%. However, it was only very rarely necessary to change TKI due to diarrhea. The severity of symptoms was related to the dose of bosutinib. 67% of patients needed symptomatic treatment with loperamide, atropine, or diphenoxylate due to diarrhea. Among our patients, with 1x500 mg bosutinib treatment per day (n=12), a significant number of cases (n=10) developed mild diarrhea (grade 1-2), which appeared 2-4 weeks after the start of the treatment. In two cases, due to severe (grade 3) diarrhea, bosutinib had to be permanently discontinued, and a modification of the therapy was necessary. In mild cases, with the temporary suspension of bosutinib treatment, symptomatic

therapy (loperamide), and repeated gradual dose increase from a low dose (1×100 mg per day), diarrhea symptoms were well controlled. In two elderly patients - who only tolerated a daily dose of 1×200 mg bosutinib due to diarrhea - persistent MMR developed after 12 months of treatment. According to our experience, at the start of the treatment, a gradual build-up of the therapeutic target dose is recommended, which significantly reduces the frequency and extent of the development of gastrointestinal symptoms. Loperamide is effective in relieving the symptoms of diarrhea.

Due to diarrhea observed in connection with dasatinib therapy, temporary suspension of treatment was necessary in 1-3% of patients, and dose reduction was necessary in 2%. No significant diarrhea was observed with ponatinib treatment. There was no diarrhea among our patients receiving dasatinib and ponatinib treatment. Mild (grade 1 or 2) diarrhea was observed in 3 cases in connection with nilotinib treatment (n=17) given in the first line (2×300 mg daily). Symptoms resolved following temporary discontinuation of nilotinib and did not recur after reinitiation.

Gastrointestinal bleeding may occur with dasatinib treatment, despite a maintained platelet count. Regardless of the phase of the disease, it was detected in 17% of cases and most often appeared in the form of hemorrhagic colitis. Coagulation abnormalities could be verified in 3%, the platelet count was below 100 x 10^9/L in two-thirds of the cases. Thrombocytopenia and the advanced stage of the disease can be considered independent risk factors for the occurrence of gastrointestinal bleeding. No increased tendency to gastrointestinal bleeding was observed in connection with first-line dasatinib or other TKI treatment. In addition to the inhibitory effect of dasatinib on collagen-induced platelet aggregation, its inhibitory effect on the formation of procoagulant platelets considered a much more sensitive indicator of primary hemostasis, plays a role in the development of bleeding.

In case of gastrointestinal bleeding occurring with dasatinib treatment, the source of the bleeding must be confirmed. By evaluating the results of gastroscopic and colonoscopy examinations, it is possible to decide whether to reduce the dose of the drug or switch to another TKI. During treatment with 1x100 mg dasatinib per day, severe gastrointestinal bleeding requiring transfusion occurred twice in our patients. Colonoscopic examination confirmed hemorrhagic colitis in both cases. During the examination of primary hemostasis, in addition to the inhibition of platelet aggregation, a decrease in the formation of "coated"-platelets was also

demonstrated. The latter is a sensitive indicator of the inhibitory effect of dasatinib on primary hemostasis. We stopped dasatinib and switched to imatinib treatment. No further gastrointestinal bleeding was observed.

Based on the results of an extensive meta-analysis, the chance of severe (grade 3 or 4) liver damage is significantly more frequent with any TKI treatment compared to the control arm. Hepatotoxicity associated with TKI treatment leading to fatal liver failure has so far only been described in connection with a few patients receiving treatment with imatinib and ponatinib. During the histological examination, in most cases with a fulminant course, in addition to a non-specific inflammatory infiltrate, massive, viral hepatitis-like necrosis of the liver tissue was noted, which was formed by inflammatory idiosyncratic processes. Liver function abnormalities (increased GOT or GPT levels) can develop with varying frequency and severity but with all TKI treatments. Hepatotoxicity caused by TKI treatment is mild in 25-35% of cases (grade 1 or 2) and involves only a temporary increase in transaminase levels. A severe (grade 3 or 4) form occurred in 2% and most often developed with ponatinib treatment. The increase in transaminase levels usually occurs 2-8 weeks after the start of treatment, but in connection with imatinib treatment it can occur at a later time as well. An increase in GOT or GPT levels was observed a median of 46 days (1-334 days) after the start of ponatinib treatment. Among our patients, in addition to TKI treatment, we did not notice any significant (grade 3-4) liver function abnormalities, so no therapy modification was necessary.

6.1.6. Conception and pregnancy

Men who took imatinib, bosutinib, dasatinib, or nilotinib did not have an increased risk of birth defects in their offspring. In the case of ponatinib therapy, data are incomplete. Changes in sperm quality and morphology may be present at diagnosis and remain unchanged after imatinib treatment. According to current recommendations, men with family planning intentions do not need to stop TKI treatment.

In case of pregnancy during TKI treatment, TKI treatment must be stopped in the first trimester and a fetal ultrasound examination must be performed immediately. The teratogenicity of TKI during organogenesis is due to non-specific, probably PDGFR inhibition. With dasatinib treatment in the second trimester, hydrops fetalis occurs more often, so for safety reasons, all TKIs are contraindicated during pregnancy. Although imatinib has been used safely in the

second and third trimesters, routine use is not recommended based on the limited experience available.

Treatment of CML diagnosed during pregnancy should be started individually. Termination of pregnancy should definitely be considered if the diagnosis is confirmed at advanced, high-risk disease. In the case of a low-risk disease, if the white blood cell count is low, we can wait until delivery to start the treatment under close monitoring. In case of thrombocytosis, the administration of acetylsalicylic acid and/or low molecular weight heparin is recommended. In case of a high white blood cell count, leukapheresis and/or α-IFN can be safely used. Close cooperation with obstetrician colleagues is recommended, and regular fetal ultrasound examinations are important. TKIs are excreted in breast milk in small concentrations, their use during breastfeeding is not recommended.

Treatment can be suspended for the period of family planning for women with a long-lasting deep molecular response suitable for suspension of treatment. Any subsequent TKI treatment depends on the persistence or loss of MMR. Women who lose MMR during TFR and are already pregnant are likely to carry the pregnancy to term without needing to restart treatment. Women who lose MMR and are not yet pregnant should restart TKI treatment. After stopping imatinib, switching to a second-generation TKI should be considered, and if a deep molecular response can be achieved again, another treatment-free period can be tried. Female patients who desire pregnancy without achieving a sustained deep molecular response (often due to older age and/or social pressure) are a great challenge. In such cases, possible solutions include replacing the TKI treatment with α-IFN, or considering alternative methods of conception (surrogacy).

6.2. Primary hemostasis during TKI treatment

6.2.1. *In vitro* and *ex vivo* effects of dasatinib on primary hemostasis

During phase III clinical trials, the incidence of bleeding complications was 9–40% (grade 1–4) with dasatinib. Of these, severe (grade 3-4) bleeding complications occurred in 8%. The bleeding mainly affected the gastrointestinal tract and the central nervous system. Serious bleeding events in patients receiving dasatinib have been reported in several additional case reports. Previous *in vitro* and *ex vivo* studies with dasatinib have confirmed the insufficient functioning of primary hemostasis. The published results confirmed the inhibition of platelet activation, the reduction of the thrombus volume measured on the collagen matrix under flow conditions, the prolonged closing time of C/Epi with the PFA-100 test, and the reduced platelet

aggregation induced by epinephrine and AA. During our studies with dasatinib the platelet functions of patients receiving treatment with dasatinib or nilotinib were checked before (0 hours) and after (1 and 4 hours) taking the TKIs. Nilotinib was used as a negative control, as previous observations showed that nilotinib has no effect on platelet aggregation. Based on our experience, dasatinib treatment resulted in an impairment of platelet activation and aggregation. Nilotinib had absolutely no effect on these processes. These data are consistent with previous observations, despite other investigators using only a 10-min preincubation with dasatinib in *in vitro* experiments. According to our studies, the detection of the reduced "coated"-platelet-forming effect of procoagulant platelets activated with a double agonist proved to be a more sensitive method than PFA-100 and platelet aggregation.

Platelet collagen receptor (GPVI) regulates platelet activation in a tyrosine kinase-dependent manner. SRC family kinases (SFKs) play a key role in platelet activation (LYN, LCK, YES, FYN). Lyn is highly expressed in human platelets and binds to the cytoplasmic domain of GPVI to phosphorylate and activate SYK. Dasatinib is a potent multikinase inhibitor (ABL, c-KIT, EPH A2, PDGF-β, SFK). Although nilotinib is also a second-generation TKI that blocks several tyrosine kinases (ABL, PDGFR, c-KIT, ARG, EPH B4), it does not inhibit SFK. Thrombin exerts its effects through a G-protein-mediated protease-activated receptor-mediated activation of PLCβ, which results in calcium mobilization and PKC activation, as well as increased activation of calcium-calmodulin-dependent kinases, MAP kinases, and phospholipase A2. This latter signaling pathway is not affected by either dasatinib or nilotinib therapy. Since dasatinib has a significant inhibitory effect on the production of "coated"- platelets, while nilotinib does not, we hypothesize that the reduction of "coated"-platelet formation in CML patients receiving dasatinib treatment occurs through the inhibition of SFK kinases associated with the GPVI receptor.

Previous platelet aggregation tests confirmed impairment of platelet activation and aggregation with dasatinib treatment. Studies have proven that dasatinib effectively inhibits platelet signaling and collagen-induced platelet activation processes. However, their data confirmed different platelet aggregation results and a longer-lasting inhibitory effect of collagen on platelet function. The main reasons for these differences may be the different conditions of the experiments. According to their results, in patients with CML (n = 6), the inhibitory effect of dasatinib treatment on platelet aggregation lasted longer than 4 hours. It is important to note that in the previously reported studies, half of the patients received 70 mg twice daily, and the

other half received 100 mg dasatinib once daily. In our experiments, all patients (n = 10) received 100 mg dasatinib daily. Based on pharmacokinetic studies, the half-life of dasatinib is 3-6 hours, the C_{max} concentration is reached within 1 hour after taking the drug, and the effects of the dosage regimen (once or twice a day) have a significant impact on the safety and efficacy of dasatinib. The inhibitory effect of dasatinib therapy on platelet function is thus probably less pronounced after 4 hours.

Gastrointestinal bleeding is a more common adverse event with dasatinib than with other TKIs. Although collagen-induced platelet aggregation is abnormal in all dasatinib-treated patients, only a fraction of patients develop a clinically significant bleeding complication. The reason for this could most likely be another effect of dasatinib, independent of the processes of primary hemostasis. Unlike other TKIs, dasatinib is also known to significantly affect the barrier function of the vascular endothelium. The resulting temporary increase in vascular permeability - which is further enhanced by the decrease in the formation of "coated"-platelets as confirmed by our tests - as well as the reduced platelet aggregation all contribute to the development of hemorrhagic complications.

6.2.2. *In vitro* and *ex vivo* effects of ponatinib on primary hemostasis

Countless laboratory studies have already analyzed the cause of the prothrombotic effect of ponatinib. Studies have shown that ponatinib induces a thrombo-inflammatory response in collagen and $FeCl_3$ injury models. Other authors have confirmed in mouse experiments that pontainib treatment increases P-selectin expression and GPIIb/IIIa receptor-dependent platelet activation. However, the observed activation effect was pronounced only at lower concentrations of agonists (collagen or thrombin). According to pharmacokinetic studies, the half-life of ponatinib is 27-34 hours, and mean ponatinib C_0 values after daily administration of 15, 30, or 45 mg of ponatinib demonstrated plasma concentrations of 26.2 nM, 56.1 nM, and 64.3 nM. In these studies, it was shown that ponatinib reached C_{max} values 4-6 hours after administration, which were 77 nM and 145 nM at 15 mg and 45 mg daily doses, respectively. Therefore, in our experiments, we examined the effect of ponatinib on the changes induced by platelet agonists at clinically relevant therapeutic (75 and 150 nM) and above-therapeutic (1000 nM) concentrations. In our previous experiments, we confirmed the inhibitory effect of procoagulant dasatinib on platelet formation and thrombus retraction. Dasatinib exerts this effect by inhibiting various tyrosine kinase signaling pathways (LCK, LYN, SRC). Since ponatinib is also able to inhibit these kinase pathways, it is assumed that it also causes the

inhibition of platelet aggregation and ATP release through this "off-target" pathway. At the same time, the inhibitory effect on the platelet aggregation response induced by collagen was observed only at a concentration well above the therapeutic level (1000 nM). Our results are consistent with a previous study that used washed platelets. In this study environment, ponatinib inhibited CRP-induced platelet aggregation at concentrations as low as 100 nM. Loren et al found that the addition of ponatinib to washed platelets *in vitro* inhibited GPVI activation pathways, and platelets treated with ponatinib showed little adherence to surfaces coated with fibrinogen or collagen, similar to platelets treated with a specific SRC family kinase inhibitor. Additionally, fibrinogen- or collagen-bound platelets treated with ponatinib showed reduced phosphorylation of specific kinases of the GPVI pathway, similar to SRC family kinase inhibitor-treated platelets.

After ponatinib pretreatment, we detected the activation of GPVI receptors, and in parallel, we observed a decrease in the binding of the monoclonal antibody PAC1 to blood platelets - which is an indicator of the activated state of the GPIIb/IIIa receptor. This effect was significantly greater at 1000 nM ponatinib concentration. Consistent with our observations, Deb et al found that ponatinib could reduce the mean fluorescence intensity of PAC1-binding platelets upon activation of PAR1 and PAR4 receptors at a final concentration of 145 nM. Examining the effect of ponatinib on GPVI receptor activation in whole blood samples, no differences were found.

Based on our previous studies with dasatinib, we hypothesized that the detection of "coated"-platelets with a flow cytometer may be a more sensitive method for identifying the inhibitory effect of ponatinib than classical platelet aggregation tests. During our experiments carried out in the GFP environment, we observed a decrease in the production of "coated"-platelets when the samples were co-activated via the GPVI (convulxin) and PAR1 (thrombin) receptors. This effect was already significant at a ponatinib concentration of 150 nM and was halved at a concentration of 1000 nM. We consider the observed inhibitory effect of ponatinib on "coated"-platelets to be clinically relevant, as we previously found that the flow cytometry method used for their detection is more sensitive than the classical platelet aggregometry test methods.

Treatment with ponatinib is limited to a small proportion of CML patients. It is recommended for patients who have failed other TKI treatments or who have the rare T315I mutation of *BCR::ABL1*. At the time of our studies, a total of 22 patients in Hungary received ponatinib

treatment. Only a limited number of these patients (n=5) had access to evaluate the effect of ponatinib treatment on platelet function. In patients receiving long-term ponatinib treatment, the maximal platelet aggregation (ΔT_{max} value) tested with collagen was already significantly reduced in the samples taken before the next dose (C_0) and remained below the reference range. Thus, both *in vitro* and *ex vivo* results support the inhibitory effect of ponatinib on platelet activation induced by classical platelet agonists through the GPVI receptor.

In vitro studies were performed under controlled conditions on PRP or GFP samples from healthy volunteers not taking ponatinib. In the case of CML patients, several medications taken at the same time can affect the free concentration of ponatinib, which is otherwise highly protein-bound in plasma. Furthermore, other comorbidities of the patients and the effect of ponatinib in plasma may differ significantly compared to the *in vitro* PRP or GFP samples. Based on our tests, we found that the inhibitory effect of ponatinib on platelets is related to the inhibition of Sarcoma family kinases (LYN, FYN, and SRC). In a previous study, the degree of inhibition was found to be similar for dasatinib and ponatinib, which may explain its attenuating effect on platelet activation through the GPVI receptor. Since LYN is constitutively active, the lower ponatinib dose results in an open conformation of p-LYN by blocking only the more sensitive inhibitory site of p-LYN Y507. Studies have also shown that treatment with ponatinib changes the functions of both the blood vessel wall and platelets, making the latter hyperactive. In mice, ponatinib therapy can enhance vWF-mediated platelet adhesion to microvascular endothelium. Platelet adhesion was mediated by binding of the released A1 domain GPIbα to the endothelium or vWF associated with the leukocyte-platelet complex. These processes occur both in the large arteries and in the peripheral microcirculation. The latter can cause ischemic changes in the functioning of individual organs (e.g. ventricular myocardium).

Cardiovascular events in patients taking ponatinib may result from effects on platelets and other cells in the circulation. In our experimental setup, we examined the inhibitory effect of ponatinib on platelets in the context of PRP and GFR. However, the results of monocyte-platelet aggregate formation in whole blood samples indicated a possible activating effect of ponatinib on platelets and/or monocytes. One of the limitations of our study results was the small number of samples from patients treated with ponatinib. Furthermore, the inhibitory effect of ponatinib was verified in a purified experimental environment. In summary, we demonstrated the inhibitory effect of ponatinib on human platelets in in *vitro* experiments and *ex vivo* samples by

using lumi-aggregation studies and the detection of "coated"-platelets by flow cytometry, where the final hemostatic effect largely depends on the measurement conditions.

7. New findings

In the course of our investigations, we analyzed data from 120 patients with CML cared at the University of Debrecen Clinical Center, Clinic of Internal Medicine, Department of Hematology. We evaluated the progression-free and overall survival results through a detailed analysis of patient and disease-specific data. We examined in detail the side effects occurring in connection with the given TKI treatments, the detailed description of which, supplemented by literature data, was published for the first time in Hungary. To further investigate the background of the symptoms suggestive of bleeding in connection with dasatinib treatment, and the increased cardiovascular risk associated with ponatinib therapy, in addition to the classic lumi-aggregation tests, we determined the changes in the formation of "coated"-platelets, which play a key role in primary hemostasis.

We confirmed that a significant decrease in maximum aggregation occurs 1 hour after taking the drug in *ex vivo* samples from CML patients. Aggregation curves normalized 4 hours after dosing.

We also found that ponatinib pretreatment inhibits the formation of "coated"-platelets in a dose-dependent manner, as well as significantly inhibits collagen-induced aggregation in the post-dose samples of patients taking 1x30 mg ponatinib per day compared to the aggregation of control platelets. Our results also confirmed that ponatinib has an inhibitory effect on platelet activation induced by GPVI agonists.

Based on all of this, we proved for the first time that the "coated"-platelet determination is a more sensitive indicator of primary hemostasis abnormalities caused by dasatinib than classical aggregation tests and that the increased cardiovascular events detected in connection with ponatinib treatment are other than the primary hemostasis abnormalities (plasma concentration, endothelial dysfunction) may develop as a result of the influence of factors.

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9. Publications

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A PhD értekezés alapjául szolgáló közlemények

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