Tyrosine kinase inhibitors for cancer treatment

Tyrosine kinase inhibitors for cancer treatment: Effects on platelets

Bibian M.E. Tullemans

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Tyrosine kinase inhibitors for cancer treatment: Effects on platelets

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Promotor
Prof. Dr. J.W.M. Heemskerk

Copromotor
Dr. M.J.E. Kuijpers
Dr. M.J.B. Aarts

Beoordelingscommissie
Prof. Dr. H. ten Cate (Voorzitter)
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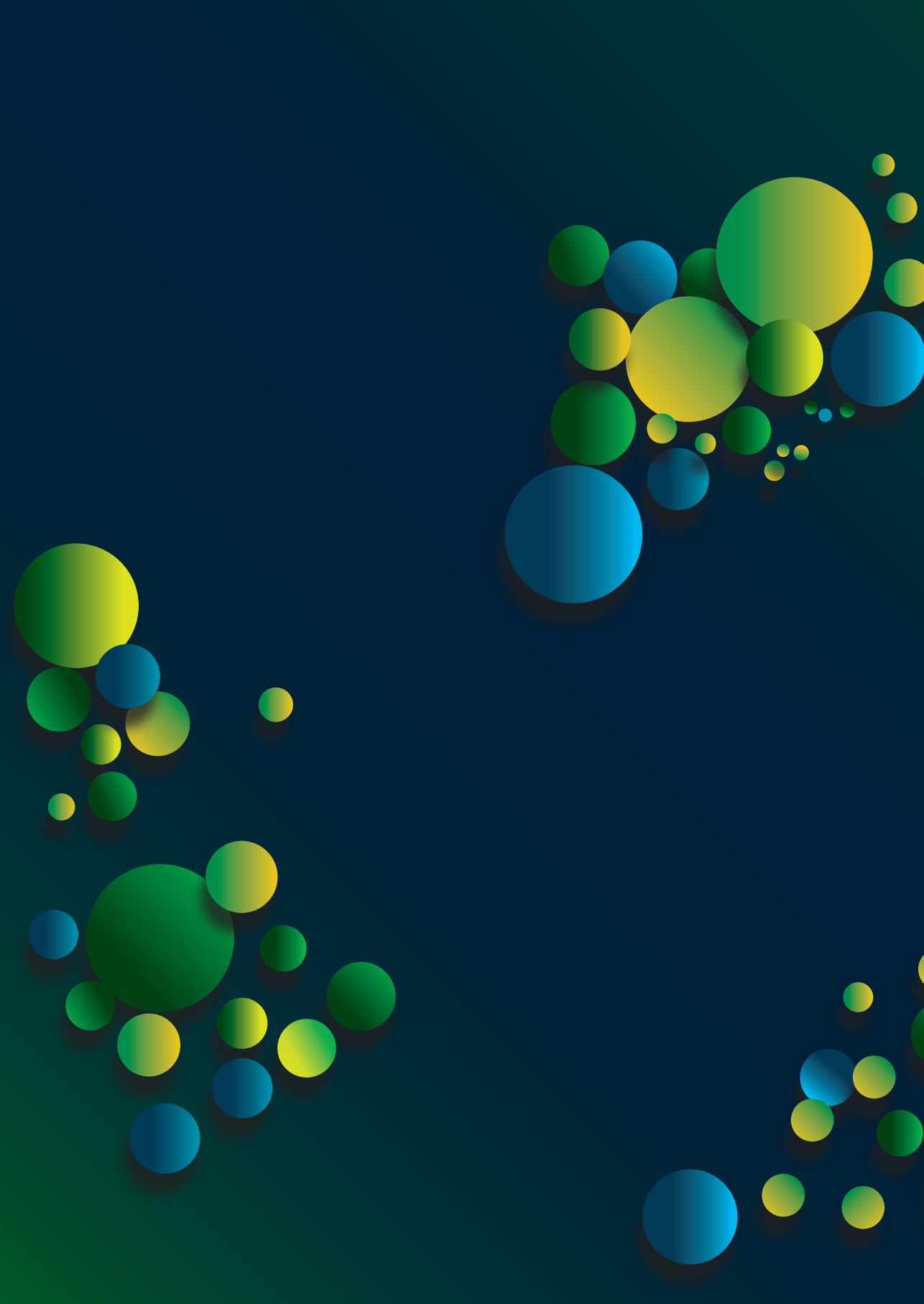

Chapter 1

General Introduction

Platelets are known to play a crucial role in haemostasis[1-3]. Upon damage to the vessel wall, platelets become activated via different receptors and adhere to the site of injury. Subsequently, a platelet plug is formed, which is stabilised by the formation of fibrin, preventing further blood loss. Unbalanced haemostasis by impaired platelet function or increased platelet reactivity contributes to pathological conditions that involve bleeding or thrombosis, respectively. Yet, platelets are also described to be involved in tumour angiogenesis and cancer progression[4, 5]. In this chapter, a general background is provided of the different receptors that mediate platelet activation and the involvement of tyrosine kinases in this process. Furthermore, a brief overview is given on the role of platelets in malignant disease and the development of tyrosine kinase inhibitors as targeted therapeutic drugs.

Involvement of tyrosine kinases in platelet signalling

For decades, the involvement of platelet function in haemostasis has been thoroughly investigated, which resulted in the knowledge we have today[1-3]. The process of haemostasis is initiated by vessel wall disruption and the exposure of thrombogenic components in the underlying matrix. Platelets can interact with these immobilised and other soluble ligands with a variety of receptors that induce shape change and adhesion to the vessel wall. Ensuing platelet activation can be triggered by different receptors interacting with ligands exposed upon injury. In the following sub-sections, a short description is provided of the major signalling pathways of platelet activation and the tyrosine kinases involved in these processes.

GPIb-IX-V signalling

Tethering of platelets along the vessel wall is initiated by collagen-bound von Willebrand Factor (vWF) through the glycoprotein (GP)Ib-IX-V complex (Figure 1.1). This shear-dependent interaction causes platelet rolling, which enables subsequent firm adhesion to the site of injury via collagen[6]. The interaction between vWF and the GPIbα subunit in the GPIb-IX-V complex leads to weak downstream signalling events. The adapter protein 14-3-3ζ supports downstream signalling and the phosphorylation of Src family kinases (SFK) Src and Lyn[7, 8]. In turn, phosphatidylinositol 3-kinase (PI3K) is activated leading to activation of integrins, stabilised vWF binding, granule secretion and adhesion to the surface. In addition, GPIb-IX-V signalling can also be associated with Fc receptor γ-chain (FcRγ) or FcRγIIa induced activation through the immunoreceptor tyrosine-based activation motif (ITAM) in this receptor[8]. Phosphorylation of the FcRγ (or FcRγIIa) leads to the binding of Syk and induces a cascade of signals comparable to the GPVI receptor (described in the following paragraph). In general, GPIb-IX-V initiates only a limited activation signal in platelets, resulting in transient Ca^{2+} oscillations[6], which mainly helps to induce and amplify platelet activation through other receptors[7, 9].

GPVI signalling

As platelets roll along the damaged vessel wall, GPVI is able to interact with collagen (Figure 1.1). Although GPVI is most efficiently activated by collagen (type I or III), also other ligands have been described to activate this receptor, including physiological (laminin, fibronectin, vitronectin and fibrin(ogen)), synthetic ligands (collagen-related peptide, CRP) or the snake venom toxin convulxin[10]. GPVI is a member of the immunoglobulin superfamily and is associated with the dimeric immunoreceptor tyrosine-based activation motif (ITAM)-containing FcRγ receptor[8, 11, 12]. Activation of GPVI leads to the phosphorylation of the SFKs Fyn and Lyn, which in turn induces clustering of GPVI and phosphorylation of the ITAM motif[1, 8]. This leads to the binding and activation of Syk that will initiate the formation of a complex involving linker for activated T-cells (LAT), Tec family kinases (Btk, Tec), PI3K, phospholipase C-γ2 (PLCγ2) and several other proteins[1, 2, 13]. Through a cascade of signals, calcium increases in the cytosol, promoting processes of platelet activation. As stimulation of GPVI induces strong platelet activation with high sustained calcium elevation, it can promote integrin activation, granule secretion, thromboxane production and procoagulant activity (exposure of phosphatidylserine (PS)), resulting in platelet adhesion, aggregation, and thrombus formation[2, 14].

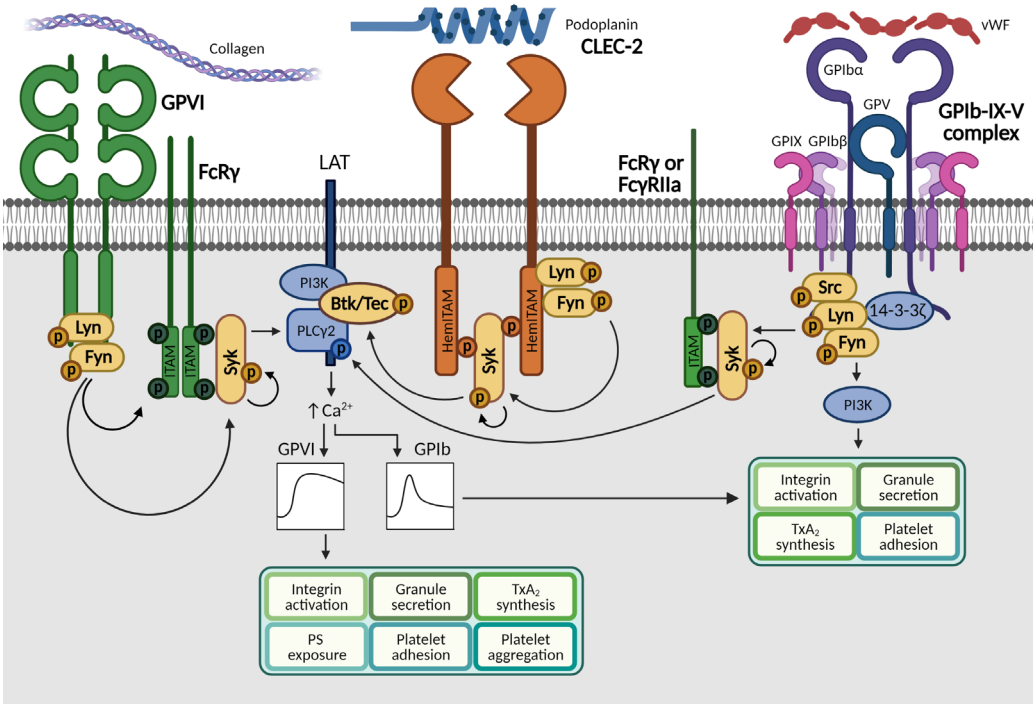

Figure 1.1. Schematic overview of signalling cascades upon activation via collagen receptor GPVI, podoplanin receptor CLEC-2 and von Willebrand factor (vWF) receptor GPIb-IX-V. Shown are the most potent agonists for each receptor. For abbreviations: see text. Modified after references [1, 2, 7, 8, 12, 13].

CLEC-2 signalling

A less well studied receptor that mediates platelet activation is C-type lectin-like type II membrane glycoprotein (CLEC-2) (Figure 1.1). CLEC-2 is activated through its natural ligand podoplanin or the snake venom toxin rhodocytin[15, 16]. The CLEC-2 receptor contains a single hemi-immunoreceptor tyrosine-based activation motif (hemITAM), and its signalling involves many molecules that are also present in the GPVI pathway. Upon activation the hemITAM motif becomes phosphorylated through Fyn and Lyn (and possibly Btk or Tec)[1, 13, 17]. This enables dimerization of CLEC-2 and the binding of Syk to the hemITAM motif, leading to the downstream signalling through the LAT complex, inducing an increase of intracellular calcium and resulting in platelet activation[1, 2]. Although GPVI and CLEC-2 signalling are in many ways alike, CLEC-2 signalling appears to be more reliant on Syk, whereas GPVI signalling is more SFK dependent[18].

Integrin activation

Upon platelet activation, one of the induced responses is the activation of integrins through inside-out signalling (Figure 1.2A)[1, 19]. Integrins are transmembrane proteins consisting of an α and β chain that exist as a noncovalent heterodimeric complex. Integrins are important for stable adhesion to the vessel wall and platelet aggregate formation[1]. For integrins to become active, a conformational change in this complex is necessary to obtain a high-affinity ligand binding state[2, 20]. In the low-affinity state of integrins, Csk forms a complex with Src and maintains the β-chain in an inactive state. Activation induces dissociation of Csk from the complex which leads to the activation of Src[8, 21]. Subsequently, the actin cytoskeletal proteins talin-1 and kindlin-3 bind to Src and provoke the conformational change that leads to the increase in affinity (Figure 1.2A).

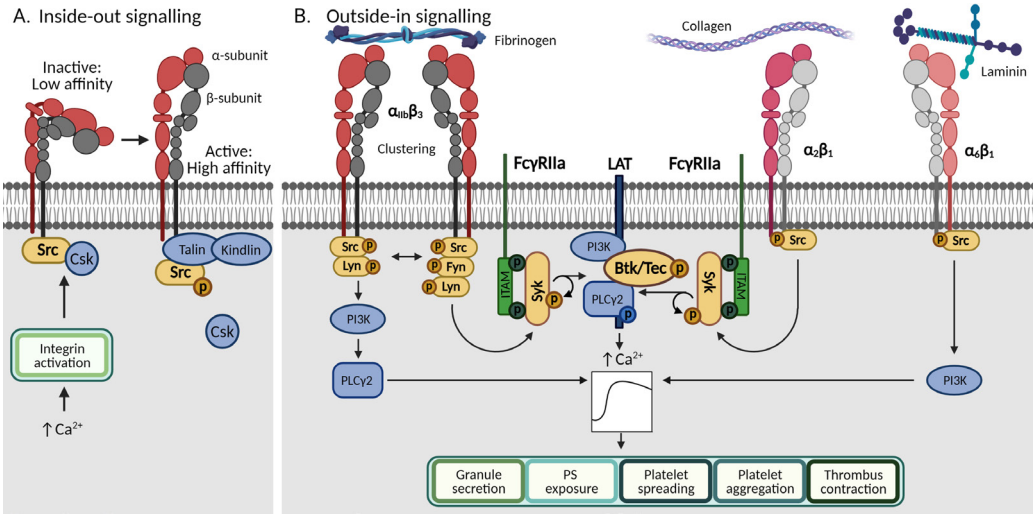

Figure 1.2. Schematic overview of (A) inside-out and (B) outside-in signalling of platelet integrins including $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and $\alpha_6\beta_1$. Shown are the most potent agonists for each receptor. For abbreviations: see text. Modified after references [1, 2, 8, 23, 31].

This allows integrins to interact with several adhesive or soluble compounds[1, 2]. There are several integrins expressed on platelets ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha V\beta_3$ and $\alpha_{IIb}\beta_3$), but for this thesis background only the integrins $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ are relevant.

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is the most abundant (integrin) receptor on platelets with over 80,000 copies on the membrane and additional copies in the α-granules[22, 23]. Several ligands can interact with activated integrin $\alpha_{IIb}\beta_3$, including fibrin, fibrinogen, vWF and fibronectin[21, 23]. Ligand binding to integrin $\alpha_{IIb}\beta_3$ contributes to platelet activation via a process called outside-in signalling that relies on clustering of the receptor (Figure 1.2B)[1, 2]. This activates a cascade of events that involves the activation of the SFKs Src, Lyn and/or Fyn. This can result in direct stimulation of PI3K and PLCγ2 signalling, although this can also occur indirectly via the activation of the ITAM signalling pathway involving Syk[2, 8, 24]. Both pathways result in an increase of intracellular calcium levels and drive processes such as platelet spreading, stable adhesion, aggregation, thrombus contraction and procoagulant activity of platelets[23, 25].

In comparison to integrin $\alpha_{IIb}\beta_3$, integrin $\alpha_2\beta_1$ (GPIa/IIa) is less abundantly expressed with only 2,000-4,000 copies per platelet[23]. The ligands that integrin $\alpha_2\beta_1$ can interact with are fibrillar and soluble collagens (Figure 1.2B)[1]. The activation of outside-in signalling of $\alpha_2\beta_1$ is thought to be similar to that of $\alpha_{IIb}\beta_3$ in which activation of Src leads to the activation of the Syk-dependent ITAM signalling[23, 26]. The role of this integrin is believed to be mainly complimentary to GPVI via maintaining and stabilising platelet adhesion[27-29].

The integrin $\alpha_6\beta_1$ is a moderately expressed protein with around 10,000 copies per platelet[22]. Integrin $\alpha_6\beta_1$ serves as a receptor for laminin (Figure 1.2B) and is described to be moderately important in thrombus formation[30]. It is suggested that activation of $\alpha_6\beta_1$ mainly involves the activation of Src and subsequently PI3K, resulting in cytoskeletal rearrangement and platelet spreading[31].

G-Protein Coupled Receptor signalling

Another platelet activation response is the release of soluble autocrine agonists (e.g., ADP and thromboxane A_2 (TxA_2)) at the site of injury. Several so-called secondary mediators, such as ADP, are secreted from the α- and δ-granules upon activation. TxA_2 is formed from arachidonic acid in the platelet membrane via several steps involving cyclooxygenase-1. In addition, thrombin is an important soluble platelet agonist that arises during coagulation. In general, these soluble agonists activate platelets via G protein-coupled receptors (GPCRs). GPCRs are transmembrane proteins with seven domains passing through the cell membrane[32]. Members of this family include the ADP receptors $P2Y_1$ and $P2Y_{12}$, the TxA_2 receptor (TP) and the receptors for thrombin, protease-activated receptor (PAR)1 and 4[27]. Tyrosine kinases are not considered to be involved in the downstream signalling of these receptors, with the exception of the $P2Y_{12}$ receptor (Figure 1.3). The $P2Y_1$, TP and PAR receptors all rely on downstream signalling via the G-proteins G12/13α

and/or Gqα. Activation via G12/13α leads to activation of Rho-associated protein kinase (ROCK) resulting in cytoskeletal rearrangement and shape change in platelets[33]. On the other hand, Gqα-induced signalling activates phospholipase Cβ (PLCβ) followed by intracellular calcium rises resulting in strong platelet responses such as integrin activation and granule secretion[33]. Only $P2Y_{12}$ is aberrant in its signalling as compared to the other GPCRs, as it is related to the G-protein Giα[34]. Stimulation of $P2Y_{12}$ induces a cascade involving the activation of tyrosine kinases (Fyn and Lyn) and subsequently PI3K and Akt. Eventually, this results in integrin activation and aggregation of platelets. In addition, stimulation of Giα reduces cAMP, which inhibits platelet activation[35].

TAM signalling

Another soluble ligand is Gas6, a plasma protein that is increased in response to inflammation, survival, growth arrest and other biological processes[27]. Furthermore, Gas6 is present in the α-granules of platelets and released during activation[36]. Gas6 is the ligand for the tyrosine kinase receptors Tyro3, Axl and Mer (TAM), which are important in clearance of apoptotic cells and resolution of inflammation[37, 38]. In addition, a role of TAM in platelet aggregation and clot formation is reported[38, 39]. It is believed that the activation of TAM synergizes with $P2Y_{12}$ signalling, thereby persisting $\alpha_{IIb}\beta_3$ activation and platelet aggregation, a pathway that is dependent on the phosphorylation of Akt through PI3K[38]. Although not much is known with regard to the involvement of tyrosine kinases downstream of TAM receptors in platelets, TAM receptors are tyrosine kinases itself and are described to signal via Src and/or Fyn resulting in downstream signalling of PI3K, PLCγ and ERK in other cells such as fibroblasts[40-43]. This might point towards a possible involvement of (one of) these kinases in this pathway.

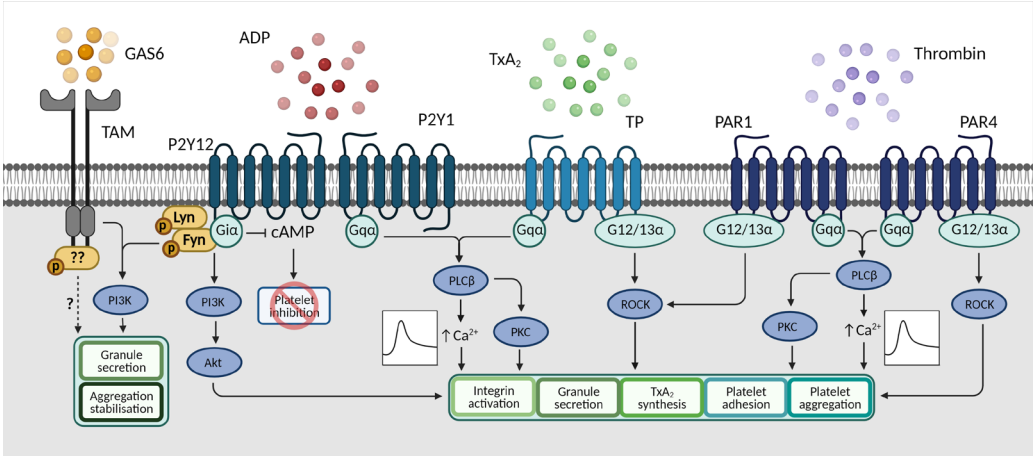

Figure 1.3. Schematic overview of signalling cascades upon platelet activation of the Gas6 receptors Tyro-3, Axl and Mer (TAM) and G-protein coupled receptors (GPCR), i.e. ADP receptors $P2Y_{12}$ and $P2Y_1$, thromboxane A_2 (TxA_2) receptor TP and thrombin receptors protease-activated receptors (PAR)1 and 4. Shown are the most potent agonists for each receptor. For abbreviations: see text. Modified after references [2, 8, 33-35, 38].

Roles of platelets in malignant disease

Platelets are not only important in haemostasis to prevent excessive bleeding or thrombosis, they are also involved in malignant diseases and inflammation[44, 45]. It is known that cancer patients are at risk for thrombosis[46, 47], as aberrations in coagulation and haemostasis were observed in these patients more than 150 years ago[48]. Nowadays, the interactions between platelets and cancer have been more clearly described[49]. Solid tumours are known to induce thrombocytosis, an elevated platelet count >400x10^9/L, but tumour cells are also able to induce platelet activation[4, 45]. Platelets (and their function) are suggested to be important in the growth of tumours and formation of metastasis via angiogenesis. Platelets contain many anti- and proangiogenic molecules in their granules which are secreted upon their activation. Platelets are also described as main transporters of vascular endothelial growth factor (VEGF), which is an important stimulus of angiogenesis[50]. Furthermore, platelets are able to sequester all kinds of plasma proteins into their granules through active endocytosis[51]. In order for platelets to release their granule content close to the tumour site, platelet activation is necessary. Tumour cells are known to express or secrete several proteins that are platelet-activating, such as thrombin, ADP and podoplanin[52]. Platelet activation through PAR1 and $P2Y_{12}$ is proposed to mainly induce the secretion of VEGF[45]. Other than VEGF, platelets are also able to release other pro-angiogenic growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) from specific α-granules. Release of these factors can promote angiogenesis, tumour neovascularization and growth[53, 54]. Besides stimulating angiogenesis and tumour growth, platelets can also be involved in tumour cell survival and metastasis[52]. Platelets express various adhesion molecules on their surfaces (integrins, selectins, etc.), allowing them to bind to tumour cells after entering into the blood vessels. This results in shielding and protection of the tumour cells from the immune system[55, 56], as well as the high shear forces in the blood stream[57, 58]. In addition, platelets in these so-called platelet-tumour aggregates can also help in arrest and stabilisation of tumour cells to the endothelial wall of a distant blood vessel, hence further facilitating metastasis[59]. Altogether, this shows the involvement of platelets in tumour progression and metastasis.

Off-target effects of tyrosine kinase inhibitors

As more knowledge is gained on cancer progression and the underlying mechanisms, new types of therapeutic drugs have been developed to interfere with these processes, including angiogenesis. In the last decades, several types of tyrosine kinase inhibitors (TKIs) have been developed to target (multiple) receptor and/or downstream tyrosine kinases which are important in signalling pathways that mediate tumour progression. As these TKIs can be orally administered and are overall well tolerated in patients, increasing numbers are being developed and several have been approved for clinical use in several types of cancer[27]. However, given that TKIs inhibit important tyrosine kinases in cancer cells, due to a high tyrosine kinase expression in other cells

such as platelets, “off-target” effects can be expected. Hence, for several TKIs (severe) side effects have been reported, while also cancers can become resistant toward these drugs.

Aims and outlines of this thesis

This thesis has as overall aim to provide better insight into the off-target effects of tyrosine kinase inhibitors (TKIs) used for cancer treatment on platelet functions. The general introduction in **this Chapter** provides background information on key pathways of platelet activation and the involvement of most relevant tyrosine kinases in these pathways. **Chapter 2** reviews nearly 40 multi-target TKIs that are currently used for treatment of different types of cancer, to prolong progression-free survival. This chapter describes how several TKIs can inhibit activation mechanisms in platelets, and which are the clinical consequences of these antiplatelet effects. In **Chapter 3**, we investigated effects of the antiangiogenic compound pazopanib on platelet function *in vitro* and in blood of advanced renal cell carcinoma (RCC) patients during treatment. Patients treated with pazopanib are reported to have an increased mild bleeding risk. Given the well-established role of protein tyrosine kinases in platelet activation, we hypothesized that the increased bleeding risk could be due to an off-target effect by pazopanib on platelet tyrosine kinases. The focus of **Chapter 4** is sunitinib, another multi-target TKI used for the treatment of advanced RCC. Sunitinib was already described to affect collagen-induced activation under non-coagulating conditions. We investigated the effects of sunitinib on thrombus formation induced by other tyrosine kinase-dependent receptors, as well as the effects under coagulating conditions in a whole blood microfluidics system. Since cancer patients often experience cardiovascular diseases as a co-morbidity, they are often on a combined therapy of sunitinib with antiplatelet or anticoagulant drugs. As both are associated with an increased bleeding risk, we examined the synergistic effects of sunitinib with aspirin, as a common antiplatelet drug, on thrombus and fibrin formation. In **Chapter 5** we describe a patient study with sunitinib-treated RCC patients. Here we explored the effects of sunitinib-treatment on quantitative and qualitative platelet traits in relation to the sunitinib levels in plasma or serum, and also assessed the occurrence of bleeding to evaluate the haemostatic consequences and clinical relevance.

Several types of TKIs have also been reported to increase the bleeding risk, often lacking a clear explanation. It is of importance to investigate possible underlying antiplatelet mechanisms, to come to better-informed treatment decisions, especially when prescribed in combination with antiplatelet or anticoagulant drugs. Therefore, in **Chapter 6**, we investigated the effect of three Btk inhibitors (acalabrutinib, ibrutinib and MK-1026) on platelet function pathways *in vitro* and in patients. Furthermore, we assessed the off-target effects in Btk knock-out mice.

In the literature, the effects of other TKIs on platelets have only partially been assessed. The reported effects of several TKIs on the GPVI pathway in platelets make those TKIs, at a lower dose, interesting as possible antiplatelet drugs, since GPVI inhibition is reported not to be associated

with an increased bleeding tendency. On the other hand, effects on other platelet signalling pathways remain largely unknown, which may possibly explain the reported increased risk of bleeding. To explore whether TKIs are of potential interest as antiplatelet drugs, we systematically investigated the effects of eight clinically used TKIs on (physiological) platelet function in whole blood under flow and isolated platelets using different agonists in **Chapter 7**. In the last chapter of this thesis, **Chapter 8**, the most important findings of this thesis are highlighted and brought in context with the current literature.

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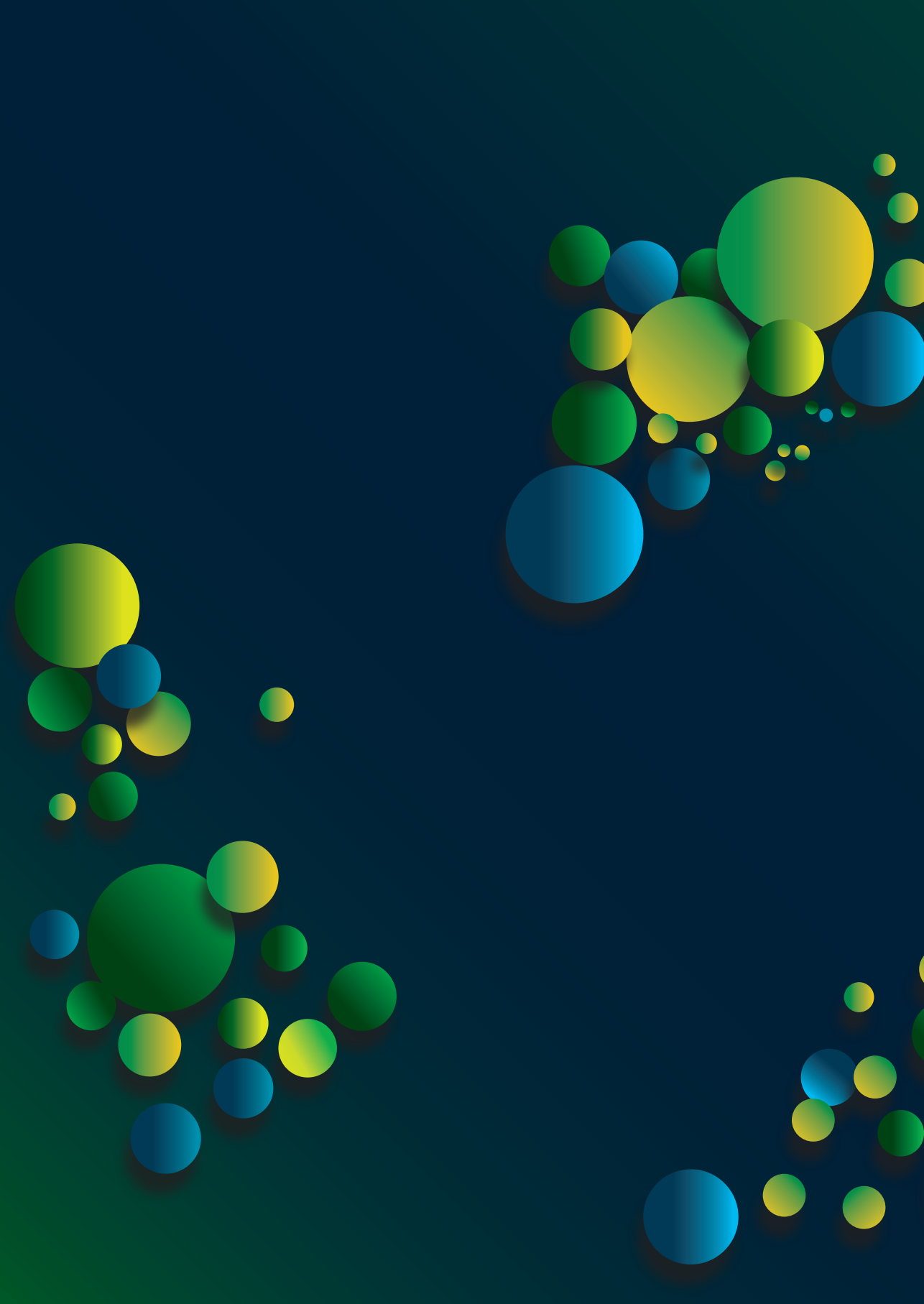

Chapter 2

Acquired platelet antagonism: off-target antiplatelet effects of malignancy treatment with tyrosine kinase inhibitors

Tullemans BME, Heemskerk JWM and Kuijpers MJE

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Abstract

Platelets can contribute to tumour progression and metastasis. Cancer patients are at increased risk of thrombosis, while advanced stages of cancer associate with thrombocytosis or increased platelet reactivity. Tyrosine kinase inhibitors (TKIs) are widely used as a targeted strategy for cancer treatment, aiming to prolong progression-free survival of the patients. Because of their broad kinase target spectrum, most TKIs inevitably have off-target effects. Platelets rely on on tyrosine kinase activity for their activation. Frequently observed side effects are lowering of platelet count and inhibition of platelet functions, whether or not accompanied by an increased bleeding risk. In this review we aim to give insight into: (i) 38 TKIs that are currently used for treatment of different types of cancer, either on the market or in clinical trials, (ii) how distinct TKIs can inhibit activation mechanisms in platelets, and (iii) the clinical consequences of the antiplatelet effects due to TKI treatment. For several TKIs, the knowledge on affinity for their targets does not align with published effects on platelets and reported bleeding events. Together, this review should raise awareness of the potential antiplatelet effects of several TKIs, which will be enhanced in the presence of antithrombotic drugs.

Introduction

Human blood platelets are required for haemostasis, and also contribute to pathological thrombosis[1]. Platelets normally circulate in the blood at a concentration of 150-450x10^9/L, with a life span of around 10 days. Thrombocytopenia, a low platelet concentration which increases the bleeding risks, is defined as a count <50x10^9/L. By implication, only a fraction of the normally circulating platelets seems to be required for proper haemostasis, as could be demonstrated in mice[2]. Hence, the majority of circulating platelets are likely to contribute to other physiological processes, which can include maintenance of vascular integrity, tissue repair, immune responses, and infection prevention. The existence of different populations of platelets, primed by their environment[3], may even suggest a certain degree of specialisation in these functions. Considering the 'overload' of circulating platelets, it is not a surprise that these a-nucleated cells also contribute to pathological processes, including cancer.

It is well known that cancer patients are at increased risk for thrombosis[4], and that advanced stages of cancer are associated with increased platelet reactivity[5]. Recent papers furthermore suggest that the growth factor composition and even the RNA profile of platelets can change due to the presence of cancer[6, 7]. On top of this, malignancy can lead to thrombocytosis (elevated platelet count), which is regarded as a negative predictor of survival[8]. The suggested mechanism is enhanced platelet formation by megakaryocytes, due to tumour-derived interleukin-6 production and high thrombopoietin production in the liver[9]. This points to a cycle of tumour-induced platelet formation and activation, followed by growth factor release and tumour promotion and metastasis. This reciprocal interaction between platelet activation and tumour growth has recently been reviewed by others[10]. The model proposed in 2000 is that tumours secrete chemokines which recruit blood cells, including platelets, and thus promote the process of angiogenesis, i.e., the formation of new blood vessels from pre-existing vessels, in order to secure tumour development and metastasis[11]. Platelets contain numerous pro-angiogenic (next to antiangiogenic) molecules in their α-granules, which are either synthesised by megakaryocytes in the bone marrow or are taken up via endocytosis[12].

In the majority of cancers, oncogene-encoded proteins or growth factor receptors are amplified or mutated, which carry tyrosine protein kinase activity or steer both tyrosine and serine/threonine kinases in the downstream signalling pathways. Accordingly, abnormal protein kinase activity is considered as a hallmark of tumour biology[13], resulting in altered cell proliferation, survival, motility, metabolism, angiogenesis, and evasion of immune responses[14]. This insight has prompted the search for tyrosine kinase inhibitors (TKIs) as targeted therapeutic drugs in oncology.

Protein tyrosine kinases are classified into the receptor-linked tyrosine kinases, e.g., the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptors; and the cytosolic non-receptor kinases, such as Src family kinases (SFK), Syk and Btk. In tumours,

often gain-of-function mutations are seen in the genes encoding for tyrosine kinases, resulting in constitutively active proteins. Examples of such aberrant oncoproteins are BCR-ABL and NPM-ALK. The latter are fusion genes that originate from reciprocal translocation of genetic material between two chromosomes, resulting in proteins that are 'always on' and leading to uncontrolled cell division. In the past fifteen years almost 40 TKIs have been developed and approved for cancer treatment (Table 2.1). In most cases, these are small molecules with relatively high affinity to the so-called targeted kinases, but often with similar affinities to other tyrosine kinases. As many of these tyrosine kinases are also expressed in non-proliferative cells, including platelets, TKIs clearly can have relevant off-target effects.

Blood platelets are unable to proliferate or differentiate but display high tyrosine kinase activities in comparison to other cell types. In platelet activation, tyrosine phosphorylation is one of the key signal transduction mechanisms. As described below, non-receptor tyrosine kinases are activated by the collagen receptor, glycoprotein (GP)VI; the C-type lectin-like receptor 2 (CLEC-2); the von Willebrand factor (vWF) receptor, GPIb-IX-V; and by adhesion-regulating integrins[15, 16]. In this review we aim to give insight into: (i) the TKIs that are currently used for treatment of different types of cancer, either on the market or in clinical trials, (ii) how distinct TKIs can inhibit activation mechanisms in platelets, and (iii) the clinical consequences of the antiplatelet effects due to TKI treatment.

Cancer treatment with tyrosine kinase inhibitors

TKIs are defined as pharmaceutical drugs that inhibit tyrosine kinases. The TKIs that are currently in use are in majority small molecules that can pass the cell membrane. Their common mode of action is by competition with ATP in the conserved catalytic binding site in the superfamily of (non)receptor tyrosine kinases. Because of the comparable structure of the catalytic pocket of tyrosine kinases, TKIs often target multiple kinases that play a role in several signalling pathways. At present, around 40 drugs with TKI activity have been approved by the USA Food and Drug Administration or have entered clinical trials for anticancer treatment (Table 2.1).

Based on their precise action, TKIs can be classified into four categories[17]. Type I inhibitors target the active conformation of the kinase and compete with the ATP-binding site (example: sunitinib). Type II inhibitors instead recognise the inactive conformation, and thereby indirectly compete with ATP by occupying a hydrophobic pocket near the ATP-binding site. Accordingly, type II inhibitors are considered to modify the kinase activity in an allosteric manner (examples are imatinib and sorafenib). Type III compounds are the so-called allosteric inhibitors. These bind more distantly from the ATP-binding site and inhibit kinase activity via classical allosteric interference (examples are MEK1 inhibitors such as selumetinib). Type IV inhibitors are also known as covalent inhibitors, forming an irreversible covalent bond near the active site of the kinase, usually by reacting with a cysteine residue. This blocks the binding of ATP and prevents activation of the

kinase (examples are ibrutinib and vantedanib).

Although directed against specific targets, almost all TKIs affect multiple kinases. Table 2.1 provides an overview of the currently used TKIs that are prescribed for which cancer type, together with their target kinases in tumour cells. The majority of the drugs are targeted at receptor tyrosine kinases for growth factors (EGF, FGF, PDGF, VEGF) or for differentiation/proliferation factors (Flt, Fms, Kit, Ret). Several of the drugs (in addition) target intracellular tyrosine kinases (ABL, B-Raf, Btk, Itk, MEK, SFK, Syk). Although affinities towards individual kinases will differ, essentially all TKIs have a more or less broad efficacy, which in some cases is intentional. For instance, both VEGF and PDGF are known to be important in tumour angiogenesis. Sunitinib, blocking signalling via both types of receptors, thus will have a broader action spectrum than vatalanib, targeting only VEGF receptors[18]. In general, the use of TKIs for treatment of specific tumours depends on the affinity for the targeted kinases[19] and the pharmacokinetics at indicated dosages and treatment regimens[20] (Table 2.1). As far as understood, the ultimate specificity is not strongly correlated with chemical structure or the subfamily of the targeted kinase[18].

Receptor-linked tyrosine kinases are also abundantly expressed in non-tumour cells, and treatment with TKIs will inevitably have off-target effects, thus interfering with the normal function of non-diseased cells and tissues. This can explain side effects of treatment, varying from general complications as fatigue, diarrhoea, and nausea, to specific complications like hand-foot syndrome[21]. In spite of drug-to-drug variations, many TKIs can cause skin toxicity, even in >50% of patients[22]. For antiangiogenic TKIs, 'blood-related' side effects have been reported, such as hypertension, myelosuppression and bleeding[23]. Incidentally, also on-treatment cardiovascular events have been reported, for instance linked to affected vascular integrity of the endothelium[24]. TKI effects on the vessel wall comprise endothelial dysfunction and increased capillary leakage[25], which may contribute to an increased bleeding tendency. Paradoxically, interfering with the integrity of endothelial cells can shift the haemostatic balance in favour of thrombosis, which might explain why treatment with TKIs can associate with arterial thromboembolic events in cancer patients[25, 26].

Despite clinical benefit of the prescribed TKIs, some of the patients who initially respond to the therapy experience a relapse, e.g., due to acquired drug resistance of the tumour[14]. In such cases, the treatment schedule is adjusted, or a switch is made to an alternative TKI as a second- or third-line treatment (Table 2.1). In specific cases, several TKIs can be combined for effective blockade of one or two signalling pathways[14, 21]. Furthermore, chemotherapy or radiation therapy can be complemented with TKI treatment[13].

Protein tyrosine kinases implicated in platelet activation

A global overview of platelet tyrosine kinases signalling underneath relevant receptors, as well as the downstream platelet responses is given in Table 2.2.

Table 2.1. Overview of tyrosine kinase inhibitors (TKIs) prescribed to cancer patients or evaluated in clinical trials

TKI*	Commercial name	Type of TKI	Reported targets	Used for treatment of	Line treatment †	Dosage (treatment regimen) †	Bleeding / thrombosis reported	Reference
Acalabrutinib	Calquence	IV	Btk, Tec	CLL, MCL, NHL	1 or 2	100-400 mg once daily 100 mg twice daily	bleeding	45
Afatinib	Gilotrif, Giotrif	I	EGFR, ErbB2-4	NSCLC	1	20-50 mg once daily (cdd)	bleeding	64, 65
Apatinib (phase III)	YN968D1	ND	VEGFR2	BC, GC, HCC, NSCLC, PC	NA	750 mg once daily (cdd)		83
Axitinib	Inlyta	I	VEGFR1-3	RCC	1	5 mg twice daily (cdd)	bleeding	20, 63
Bosutinib	Bosulif	I	Abl, Src	CML	1	500 mg once daily (cdd)	bleeding	59, 84
Brivanib	BMS-582664		FGFR1, VEGFR	HCC, MCRC, solid tumours	1	800 mg oncedaily (cdd)		85
Cabozantinib	Cabometyx, Cometriq	II	Axl, Flt3, Kit, MET, Ret, VEGFR2	TC RCC	1 2	140 mg once daily (cdd) 60 mg once daily (cdd)	bleeding	63, 86
Cediranib	Recentin	ND	Kit, PDGFR, VEGFR1-3	HCC, RCC	1 or 2	30-45 mg once daily (cdd)		87
Ceritinib	Zykadia	I	Alk	ALK^+ NSCLC	1	750 mg once daily (cdd)		88
Cobimetinib	Cotellic	ND	MEK1-2	melanoma	1	60 mg once daily, (3/1)		89
Crizotinib	Xalkori	I	Alk, MET, ROS1	NSCLC	1	250 mg twice daily (cdd)		20, 90
Dabrafenib	Tafinlar	I	B-Raf	Melanoma, NSCLC, TC	1	150 mg twice daily (cdd)		91
Dacomitinib (phase III)	PF-00299804	ND	EGFR, ErbB1-2, ErbB4	NSCLC	NA	45 mg once daily (cdd)		92
Dasatinib	Sprycel	I	BCR-ABL, EphA2, Kit, PDGFR, SFK	CP CML AP MB, LB CML	2	100-140 mg once daily (cdd) 70 mg twice daily (cdd)	bleeding	20, 93
Dovitinib	TKI258	ND	CMS1, FGFR1-3, Flt3, Kit, PDGFR, VEGFR1-3	GIST, melanoma, RCC	2 or 3	500 mg once daily (5/2)		94
Entospletinib (phase II)	GS-9973	ND	Syk	CLL	NA	800 mg twice daily (cdd)		95
Erlotinib	Tarceva	I	EGFR	NSCLC PC	2 or 3	100-150 mg once daily (cdd)		20, 96
Fostamatinib (phase II)	R788	ND	Syk	B-cell lymphoma, CLL	NA	200 mg twice daily (cdd)		97
Gefitinib	Iressa	I	EGFR	NSCLC	1	250 mg once daily (cdd)		20, 98
Ibrutinib	Imbruvica	IV	Btk, Tec	CLL, MCL, NHL	1 or 2	420 mg once daily (cdd)	bleeding	34, 37, 38
Icotinib (phase III)	BPI-2009H	ND	EGFR	brain cancer, NSCLC	NA	125 mg three times daily (cdd)		98
Imatinib	Gleevec, Glivec	II	BCR-ABL, DDR, Kit, PDGFR	ALL, AP CML, CP CML, KIT^+ GIST	1	400-600 mg once daily (cdd)	bleeding	20, 99
Ipatasertib (phase II)	RG7440	ND	Akt	BC, CRC, NSCLC, OC, PRC	NA	400 mg once daily (3/1)		100

TKI*	Commercial name	Type of TKI	Reported targets	Used for treatment of	Line treatment †	Dosage (treatment regimen) †	Bleeding / thrombosis reported	Reference
Lapatinib	Tykerb	II	EGFR, ErbB1-2	$ErbB2^+$ BC $ErbB2^+$ and HR^- BC $ErbB2^+$ and HR^+ BC	1 or 2	1000-1500 mg once daily (cdd)		20, 101
Lenvatinib	Lenvima	II	FGFR, Kit, PDGFRα, Ret, VEGFR	RCC, TC		24 mg once daily (cdd)		63, 102
Nilotinib	Tasigna	II	BCR-ABL, CSF-1R, DDR, Kit, PDGFR	CP CML, GIST	1	300-400 mg twice daily (cdd)	thrombosis	20, 99
Nintedanib	Ofev, Vargatef	II	FGFR, FMS, PDGFR, VEGFR	IPF, advanced NSCLC	1 2	150 mg twice daily (cdd)	bleeding	63, 103
Osimertinib	Tagrisso, Tagrix	ND	EGFR	NSCLC	2	80 mg once daily (cdd)		104
Pazopanib	Votrient	I	FGFR, FMS, Itk, Kit, Lek, PDGFR, VEGFR	NSCLC, OC, RCC, STS, TC	1	800 mg once daily (cdd)	bleeding	20, 63
Ponatinib	Iclusig	II	BCR-ABL, FGFR, KIT, PDGFR, Ret, Src, VEGFR	Ph^+ ALL, CML	1 or 2	30 mg once daily (cdd)	bleeding	105
Regorafinib	Stivarga	II	B-Raf, FGFR, Kit, PDGFR, Ret, Tie2, VEGFR1-3	advanced GIST, MCRC	3	160 mg once daily (3/1)		106
Selumetinib	AZD6244	III	MEK1-2	$KRAS^{mutated}$ NSCLC	2 or 3	75 mg twice daily (cdd)		107
Sorafenib	Nexavar	I	VEGFR	HCC, RCC	1-3	400 mg twice daily (cdd)	bleeding	20, 60, 63, 85
Sunitinib	Sutent	I	CSF-R, Flt3, Kit, PDGFR, VEGFR	RCC, GIST, PNET	1 or 2	37.5-50 mg once daily (4/2)	bleeding	20, 60, 61, 63
Trametinib	Mekinist	III	MEK1-2	BRAF $V600^+$ melanoma	1	2 mg once daily (cdd)	bleeding	108
Vandetanib	Caprelsa	IV	EGFR, Ret, VEGFR	MTC, NSCLC	1 or 2	300 mg once daily (cdd)		20, 109
Vatalanib (phase III)	PTK787/ ZK-222584	III	Kit, PDGFRβ, VEGFR	MCRC	1	1250 mg once daily (cdd)		110
Vemurafenib	Zelboraf	I	B-Raf	BRAF $V600^+$ melanoma	2 or 3	960 mg twice daily (cdd)		20, 111

ALK, anaplastic lymphoma kinase; ALL, acute lymphocytic leukaemia, AP, acute-phase; BC, breast cancer; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CP, chronic phase; CRC, colorectal cancer; CSF-R, colony-stimulating factor receptor; CSF-1R, colony-stimulating factor 1 receptor; EGFR, epidermal growth factor receptor; ErbB2, human epithelial growth factor 2; FGFR, fibroblast growth factor receptor; GC, gastric cancer; GIST, gastrointestinal stromal tumour; HCC, hepatocellular carcinoma; HR, hormone receptor; IPF, idiopathic pulmonary fibrosis; LB, lymphoid blast; MB, myeloid blast; MCL, mantle cell lymphoma; MCRC, metastatic colorectal cancer; MTC, medullary thyroid cancer; NA, not applicable; ND, not determined; NHL, Non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; OC, ovarian cancer; PC, pancreatic cancer; PDGFR, platelet-derived growth factor receptor; Ph+, Philadelphia-positive; PNET, primitive neuroectodermal tumour; PRC, prostate cancer; RA, rheumatoid arthritis; RCC, renal cell carcinoma; SFK, Src family kinase; STS, soft tissue sarcoma; TC, thyroid cancer; VEGFR, vascular endothelial growth factor. *Small-molecule inhibitors of kinase enzymes are indicated with the suffix 'nib'. TKIs are indicated with the suffix 'tinib'. Angiogenesis inhibitors are indicated with the suffix 'anib'. Rapidly accelerated fibrosarcoma (RAF) kinase inhibitors are indicated with the suffix 'rafenib'. †3/1, 3 weeks of dosing, followed by 1 week of rest; 4/2, 4 weeks of dosing, followed by 2 weeks of rest; 5/2, 5 days of dosing, followed by 2 days of rest; cdd, continuous daily dosing.

GPVI signalling. Collagen-induced platelet activation is established via the tyrosine kinase-linked receptor GPVI, a member of the immunoglobulin superfamily. GPVI is linked to the Fc receptor γ (FcRγ) chain, which contains two immunoreceptor tyrosine-based activation motives (ITAM) requiring phosphorylation to mediate platelet activation[27]. Ligand binding and dimerization of the GPVI-FcRγ complex leads to activation of the SFK isoforms Src, Fyn and Lyn, which in turn phosphorylate the FcRγ ITAM tyrosine residues to recruit and phosphorylate Syk[1, 28]. The tyrosine kinases SFK and Syk furthermore phosphorylate downstream targets, including the transmembrane adapter linker for activated T cells (LAT) and the Src homology 2 domain-containing leukocyte phosphoprotein (SLP76). The consequence is the formation of a large signalling complex, including LAT, SLP76, Btk, isoforms of phosphoinositide-3 kinase and Tec family kinases[29]. A key downstream event is the phosphorylation and activation of the second messenger-generating phospholipase (PLC)γ2, resulting in Ca^{2+} mobilisation and protein kinase C (PKC) activity. Further responses are integrin activation, thromboxane A_2 release, granule secretion and phosphatidylserine exposure.

CLEC-2 signalling. A similar powerful activation pathway of platelets is induced via CLEC-2, a C-type lectin receptor also acting through tyrosine phosphorylation. Known ligands of this receptor are podoplanin (expressed in tumour tissue among others) and the snake venom rhodocytin. Signalling occurs through a so-called hemITAM motif[30]. The clustering of CLEC-2 induces more or less similar events as described for GPVI. Starting with the tyrosine phosphorylation of SFK and Syk, a signalling complex is formed including Tec family tyrosine kinases, with as result PLCγ2 and PI3K activation. Consequences are, again, integrin activation, granule release, and thromboxane A_2 production[30].

GPIb-IX-V signalling. Interaction of vWF with the GPIb-IX-V receptor is one of the first steps in platelet tethering and adhesion under shear flow[1]. This interaction causes only weak signalling, e.g., leading to restructuring of the actin cytoskeleton, with under certain conditions phosphorylation of SFK (Src, Fyn, Lyn) and activation of PI3K isoforms[1]. Integrin $\alpha_{IIb}\beta_3$ activation and platelet spreading are a result of this.

Table 2.2. Protein tyrosine kinases implicated in platelet activation responses: global overview of tyrosine kinases implicated in signaling via key platelet receptors, as well as downstream platelet responses. Summarized from [1, 16, 18, 30]

	Signalling tyrosine kinases		
Receptor	**SFK**	**Other**	**Platelet response**
GPVI	Src, Fyn, Lyn, Fgr	Btk, Syk, Tec	Ca^{2+} mobilisation, integrin activation, degranulation
CLEC-2	Src, Fyn, Lyn	Btk, Syk, Tec	Ca^{2+} mobilisation, integrin activation, degranulation
GPIb-IX-V	Src, Fyn, Lyn	Btk	Integrin activation
Integrin $\alpha_{IIb}\beta_3$	Src, Fyn, Lyn	FAK, Syk, SLP76, Pyk2	Spreading, outside-in signaling, clot retraction

CLEC-2, C-type lectin-like receptor 2; GP, glycoprotein; SFK, Src family kinase; SLP76, Src homology 2 domain-containing leukocyte phosphoprotein

Integrin-dependent signalling. Platelet integrins, in particular $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and $\alpha V\beta_3$, regulate adhesion, aggregation and thrombus formation[28]. Especially regarding integrin $\alpha_{IIb}\beta_3$ (ligands: fibrinogen, vWF and other matrix proteins) much research has been performed to the outside-in signalling events triggered by the occupied, activated conformation. Several tyrosine kinases are implicated in this signalling pathway, including FAK, Pyk2, Src, SLP76 and Syk[29, 31].

Effects of TKIs on platelet function

Tyrosine kinases are targeted by TKIs as treatment for cancer, where their most important effect is the prolongation of progression free survival. Given the presence of on-target or related off-target tyrosine kinases in megakaryocytes and platelets, it is to be expected that several TKIs interfere with platelet formation and/or platelet activation processes.

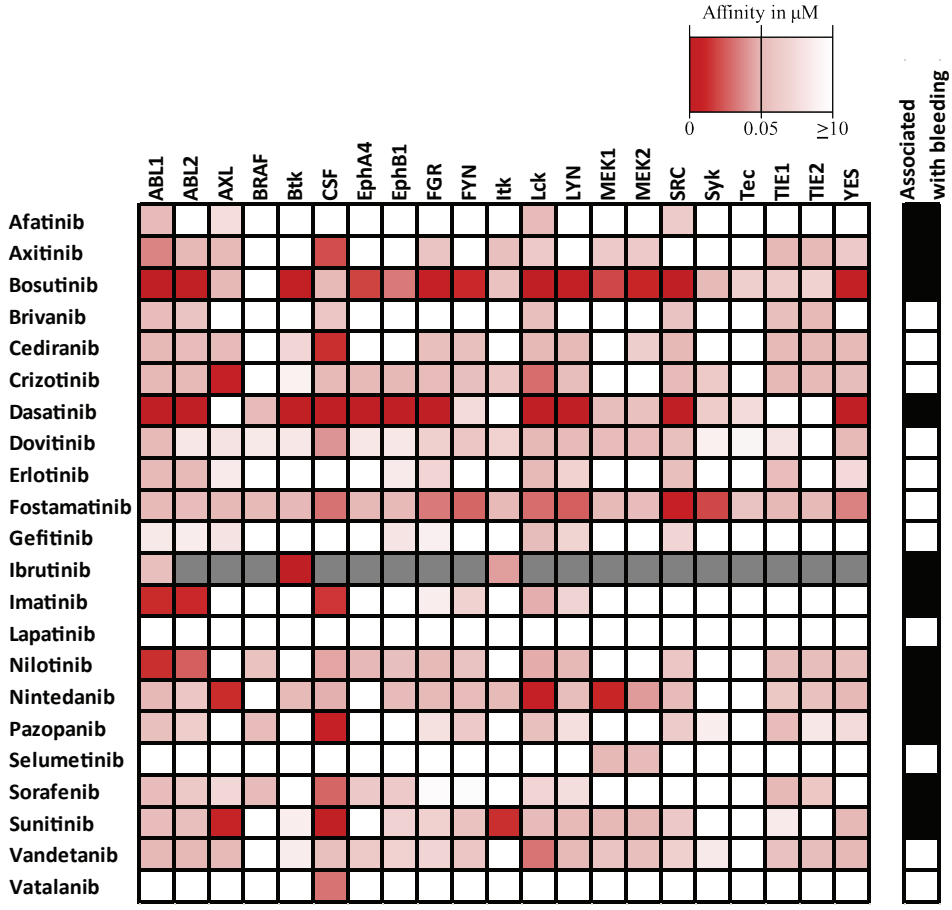

Figure 2.1. Heatmap of affinity profile of tyrosine kinase inhibitors (TKIs) with described targets that are known to be expressed in platelets. Shown are normalized affinity-based dissociation constants (K_d) of the indicated TKIs determined for purified kinases[18, 19, 33, 36], as far as they are present in platelets. Scaling is from 0 to 10, with the lowest K_d indicating the highest affinity (deep red is highest affinity = lowest K_d; white is lowest affinity = highest K_d; pink gradient represents intermediate K_d's; gray values have not been described). The right column indicates increased bleeding risk (black, yes; white, no or unknown); see Table 2.1.

To provide an overview, we evaluated the inhibition profiles of TKIs (22 in total) against target protein tyrosine kinases that are known to be expressed in platelets[32]. Markedly, the majority of these TKIs demonstrated relatively low affinities for kinases with crucial roles in platelet activation processes (SFK, Syk, Btk, MEK and Eph isoforms), with as exceptions bosutinib, dasatinib, fostamatinib, nintedanib and sunitinib (Figure 2.1). For such an *in vitro* affinity-based analysis[18, 19, 33], it should be realised that the presence of blood plasma and blood cells can profoundly change the bioavailability of a TKI, apart from its metabolic and pharmacokinetic profile. Markedly, bleeding symptoms have been reported for 11 of these TKIs. For individual TKIs, based on their known targets, we summarised in Table 2.3 which of these targets are expressed in platelets. Furthermore, we made an inventory of the published effects of these TKIs on platelet responses.

Ibrutinib is a covalently acting TKI that is targeted at the family members Btk and Tec, which probably explains the strong effects observed on platelets[34]. It is frequently used for the treatment of mantle cell lymphoma (MCL) and chronic lymphocytic leukaemia (CLL). Emerging data suggest that ibrutinib could also be used to treat solid tumours[35] and has been shown to inhibit several other kinases such as Itk, JAK3, Hck, Blk, EGFR, ErbB2 and ErbB4[36]. Ibrutinib treatment is associated with a risk of bleeding[34, 37, 38]. Being the most investigated TKI with regard to platelets, ibrutinib has been demonstrated to potently inhibit collagen-induced responses of platelets from patients on-treatment[39-44]. Efficient inhibition by ibrutinib of the GPVI pathway (in response to collagen or collagen-related peptide) has been demonstrated to proceed via reduced PLCγ2 phosphorylation[42]. In treated patients, the suppression of collagen-induced platelet aggregation correlated with the occurrence of bleeding events[42]. Subsequent studies showed that ibrutinib suppressed multiple (mostly) GPVI-dependent platelet responses, including adhesion, spreading, calcium fluxes, secretion, phosphatidylserine exposure and clot retraction[40, 43]. In addition, evidence was obtained for reduced $\alpha_{IIb}\beta_3$-dependent outside-in signalling, linked to thrombus instability *in vitro*[40]. Several studies confirmed that Btk can act as a central target of ibrutinib in GPVI-stimulated platelets, although downstream tyrosine kinases may be affected as well[40, 42, 44]. *In vitro*, ibrutinib was found to fully inhibit the tyrosine phosphorylation of Src and PLCγ2[42]. Other affected events were the phosphorylation of Fyn, Lyn, Btk, Tec and Syk[40, 44]. Taken together, this pointed to a potent suppression of ibrutinib of the GPVI signalosome with as major target the Tec family kinases.

The second-generation drug, acalabrutinib, is a more selective, irreversible Btk inhibitor. In a clinical trial with patients with relapsed CLL, acalabrutinib showed promising safety and efficacy profiles, although some minor bleedings were still reported[45]. A recent study comparing treatment with ibrutinib or acalabrutinib indicated that both compounds impaired the platelet aggregation responses after collagen receptor stimulation[46]. Both drugs inhibited platelet Btk and Tec at physiological concentrations, while only ibrutinib inhibited SFK isoforms. This provided

Table 2.3. Tyrosine kinase inhibitors (TKIs) targeting platelet activation processes Listed are TKIs known to target tyrosine kinases expressed in platelets and megakaryocytes (MKs), and described activation responses and assays, and agonist pathways.

		Effect on platelets													
TKI	Targets in platelets (MKs)	Platelet count	Integrin activation	Secretion	Procoagulant activity	Spreading	Aggregation	Adhesion	Thrombus formation	Tyrosine phosphorylation	Elevated $[Ca^{2+}]_i$	Clot retraction	PFA-100 closure times	Agonist pathway	Ref
Acalabrutinib	Btk, Tec		↓	↓			↓		↔		↓			GPVI	46
Afatinib	Akt		↓	↓	↓		↓				↓			GPVI	65
Axitinib	*n.d.*	↓												ND	63
Bosutinib	Src						↔							ND	52
														ND	53
Cabozantinib	Axl, Tie2 (Kit)													ND	
Canertinib	ErbB													ND	
Cediranib	(Kit)													ND	
Cobimetinib	MEK1-2													ND	
Dabrafenib	B-Raf													ND	
Dasatinib	Src (Kit)			↓			↓	↓	↓	↓				GPVI	51
		↓					↓						↑	GPVI	49
							↓							AA; epinephrine	52
							↓	↓						GPVI	56
Dovitinib	CMS1 (Kit)													ND	
Entospletinib	Syk													ND	
Fostamatinib	Syk													ND	
Ibrutinib	Btk, Tec, Src						↓							GPVI	39
							↓							GPVI	41
		↑		↓			↓						↑	GPVI	43
			↓		↓	↓	↓	↔	↓		↓	↓		GPVI	40
							↓	↓		↓				GPVI; VWF	42
				↓		↓	↓		↓	↓				collagen; fibrinogen	44
Imatinib	Lyn, Btk (Kit)			↔	↔		↔		↓	↔				GPVI	57
							↓		↔					GPVI	56
Ipatasertib	Akt													ND	
Nilotinib	Lyn, Btk			↔	↔		↔			↔				GPVI	57
	(Kit, Ret)		↔	↔			↔	↑	↑					GPVI	56
Nintedanib	FMS													ND	
Pazopanib	Itk, Lck (Kit)	↓												ND	63
Ponatinib	Src, Syk, Lyn,		↓	↓	↓	↓	↓	↓	↓	↓				collagen; fibrinogen	57
	Btk (Kit)						↓						↑	GPVI	58
Regorafinib	Tie2, B-Raf (Kit)													ND	
Selumetinib	MEK1-2													ND	
Sorafenib	B-Raf (Kit, Ret)	↓												ND	63
Sunitinib	(Kit, Ret)	↓												ND	62
		↓			↔		↓	↓	↓	↓				GPVI	61
		↓												ND	63
Trametinib	MEK1-2													ND	
Vemurafenib	MEK1-2													ND	

AA, Arachidonic Acid; $[Ca^{2+}]_i$, intracellular calcium concentration; GP, glycoprotein; ND, not determined; VWF, von Willebrand factor. Arrows: ↓ decreased; ↑ increased; ↔ not affected.

an explanation why only ibrutinib caused dysfunctional thrombus formation, in contrast to acalabrutinib[46].

Imatinib, targeting the oncogenic kinase BCR-ABL, was the first tyrosine kinase inhibitor developed for chronic myeloid leukaemia (CML). Imatinib is known to block Kit and PDGF receptors, as well as Erk and Akt isoforms[47]. This TKI has been reported to evoke thrombocytopenia in 18% of the treated patients, with haemorrhages being reported as well[48]. Despite a high efficacy of imatinib, 15% of patients appear to develop clinical resistance and relapse after initial response to therapy.

Dasatinib and nilotinib have been developed as second generation BCR-ABL inhibitors for the treatment of CML. Two case reports showed that dasatinib could induce a bleeding diathesis correlated to platelet dysfunction, which was reversed upon cessation of the dasatinib intake[49, 50]. Studies with blood from patients or healthy subjects showed an impairment of platelet aggregation by dasatinib given *in vivo* or *in vitro*[51, 52]. In particular, platelet aggregation in response to collagen, arachidonic acid and epinephrine was affected[52], along with collagen-induced thrombus formation[51, 53], thus suggesting interference with GPVI signalling and other pathways. The mechanism of dasatinib-induced inhibition in platelets was elucidated from changes in the protein phosphorylation profile after collagen stimulation. Herein, dasatinib appeared to interfere with SFK, PLC and PI3K activities[51]. Thrombocytopenia is a relatively common side effect of dasatinib treatment[54]. This is proposed to be due to reduced megakaryocytopoiesis, thus resulting in lower platelet production[55]. The combined quantitative (via megakaryocytes) and qualitative (via signalling) platelet dysfunction most likely is the reason of the relatively high bleeding incidence in dasatinib-treated patients. Compared to dasatinib, imatinib induces less antiplatelet activity and causes less bleeding at clinically achievable doses, likely due to the fact that it is less inhibitory for SFK isoforms. On the other hand, nilotinib is reported to potentiate thrombus formation and increase the risk of thrombosis[56].

Ponatinib and bosutinib are third generation BRC-ABL inhibitors for the treatment of CML, which are applicable when patients develop resistance to imatinib. The use of ponatinib has been associated with bleeding diatheses, along with changes in platelet count and function, which thus may explain the impaired haemostasis[57, 58]. One study describes inhibiting effects of ponatinib, imatinib and nilotinib on platelet activation, secretion, and aggregation in response to collagen[57]. Herein, ponatinib appeared to be the most potent platelet inhibitor. Ponatinib increased PFA-100 closure times, likely by suppressing ITAM signalling via several tyrosine kinases, including Src, Lyn, Syk, and Btk[57, 58]. In contrast, imatinib and nilotinib did not affect platelet granule secretion, procoagulant activity or aggregation, and only moderately reduced the tyrosine phosphorylation of Lyn and Btk, as compared to ponatinib[57]. Yet, whole blood thrombus formation was reduced by imatinib and ponatinib to a similar extent. For bosutinib (also targeting Src), bleeding has been reported as well[59], although in patients it did not affect platelet aggregation in response to

several agonists[52]. In microfluidic whole blood testing of platelet thrombus formation, bosutinib was found to be less potent than dasatinib[53]. Overall, it appears that BCR-ABL inhibitors used for leukaemia treatment have an inhibiting effect on platelet functions, with the possible exception of nilotinib.

Sunitinib, sorafenib and pazopanib are multi-target TKIs used for the treatment of renal cell carcinoma, aiming to inhibit tumour angiogenesis via interference with the receptors for VEGF and PDGF. Such multi-target TKIs may potentially also inhibit tyrosine kinases present in platelets. For sunitinib and sorafenib, a meta-analysis of 23 trials (>6,500 patients) showed an increased incidence of bleeding events with 16.7% on all grades of bleeding, and a 2.4% probability of high-grade bleeding events[60]. For sunitinib, both quantitative and qualitative effects on platelets are reported. The compound can be taken up by platelets from healthy donors and renal cell carcinoma patients, and hence reduce the platelet functionality in a way depending on protein tyrosine phosphorylation[61]. In patients on-treatment, this is accompanied by a decrease in platelet count[61, 62]. For sorafenib, similarly to other TKIs used for treatment of renal cell carcinoma such as pazopanib, an increased risk of bleeding has also been observed[63], but effects on platelet function have not been investigated.

Afatinib is used for the first-line treatment of particularly patients with non-small cell lung cancers[64]. It acts as an irreversible inhibitor of EGF and HER receptors. An increased bleeding risk of patients receiving afatinib has been linked to impaired platelet function and apoptosis[65]. In particular, afatinib impaired GPVI-induced platelet responses, including calcium fluxes, integrin activation, secretion, procoagulant activity and platelet aggregation (Table 2.3). On the other hand, thrombin-induced platelet responses were less strongly inhibited. Further investigation showed that the collagen-induced phosphorylation of Akt was decreased by afatinib[65].

Cabozantinib, cediranib, dovitinib, lenvatinib, regorafinib and other TKIs co-target the receptor kinase Kit, which acts as a receptor for stem cell factor in megakaryocytopoiesis, regulating platelet production. The Kit receptor is known to signal via SFK, PI3K, Ras/MEK and JAK/STAT pathways[66]. In an analogous way, the thrombopoietin receptor Mpl signals via JAK2 and Tyk2 as immediate effector kinases, resulting in the activation of STAT isoforms[67]. For these signalling pathways, key tyrosine kinases are also present in platelets and/or megakaryocytes (Table 2.2 and 2.3), which raises the possibility that Kit-targeted TKIs can also modulate platelet function as well as platelet count. So far, the signalling effects of these TKIs on platelets have not been investigated in detail. Cabozantinib intake has been associated with a higher bleeding risk[63]. Related drugs are ruxolitinib, pacritinib and lesaurtinib, which also target JAK1/2 and are usually prescribed for myeloproliferative disorders, such as essential thrombocythemia, characterised by an elevated platelet count (thrombocytosis). The latter compounds appear to lower (normalise) the concentration of circulating platelets[68], most likely via inhibition of the thrombopoietin pathway in megakaryocytes. However, effects on platelet function are still unclear. It is known that

thrombopoietin potentiates agonist-induced platelet activation via JAK2 and Tyk2 signalling[69]. This indicates that this pathway is not strong enough to induce platelet activation on its own. Inhibition of JAK2 in platelets has been reported to only attenuate collagen-induced responses[70].

For multiple prescribed TKIs with a broad target range, bleeding symptoms have not been reported (Table 2.1). Examples of these are fostamatinib, dovitinib and vantedanib, which based on their affinity profiles should affect SFK and/or MEK isoforms in platelets (Figure 2.1). In these cases, additional research is needed to verify effects on platelet count and activation, and to determine if there is a bleeding risk. Summarising the current knowledge of TKI effects on platelet activation responses, it appears that the majority of multi-target TKIs in particular suppresses GPVI-induced signalling pathways, but that they can also target kinase events downstream of other platelet receptors. As the inhibition of GPVI alone has been shown to prevent occlusive thrombus formation without causing bleeding[71], it is most likely that the targeting of pathways downstream of additional receptors is responsible for an increased bleeding risk with these compounds.

Clinical implications of platelet inhibition with TKIs

Platelets are known to interact with cancer cells in the blood. Tumours exposed to the blood stream can activate platelets, while platelets can stimulate tumour angiogenesis, growth, and metastasis. The question has been raised whether and how interfering in the platelet-cancer loop via antiplatelet treatment may also influence cancer progression. Several studies have reported that aspirin or $P2Y_{12}$ receptor inhibitors suppress tumour angiogenesis *in vitro*[72] and tumour growth *in vivo*[73]. A low daily aspirin intake has been associated with a reduced risk for the development of cancer as well as prevention of metastases[74]. The mechanism is not completely resolved, but it has been shown that inhibition of platelets by aspirin decreased their ability to stimulate cancer cell proliferation through modulation of the Myc oncoprotein[75]. For other antiplatelet drugs, clinical evidence of a beneficial effect on cancer is still lacking.

Of growing interest is the recent finding that platelets are capable to sequester bioactive compounds from the plasma, including growth factors[12, 76, 77] and RNA species[7]. In addition, platelets accumulate the anticancer drugs bevacizumab[78] and sunitinib (in granules)[61]. Other TKIs can also be taken up by platelets, which usually is a requirement for interaction with the intracellular kinases. Whether these TKIs can also secondarily be released by platelets and, perhaps, then contribute to tumour inhibition is matter of speculation. Another consideration is that uptake of TKIs by platelets (e.g., in patients with thrombocytosis) can reduce the availability of the compounds for tumour inhibition. On the other hand, it is likely that a decreased aggregation tendency of platelets (as induced by TKIs) also lowers tumour-induced platelet activation, and thereby reduces metastasis of tumour cells. However, the evidence for such a mechanism is at best indirect, and further investigation here is required. Taken together, it appears that platelets

can interfere with drug effects in different ways and by different mechanisms, which should be taken into account upon therapeutic drug monitoring.

Application of the TKIs axitinib, dasatinib, pazopanib, sorafenib and sunitinib – all of which have indications may cause bleeding – can lead to a reduction of the platelet concentration in blood (Table 2.3). In cancer patients, thrombocytosis has been associated with a worse overall prognosis, when compared to patients with a normal platelet count[8, 9]. In addition, in glioblastoma patients, who underwent concomitant radiotherapy and chemotherapy, a decrease in platelet count correlated with longer overall survival[79]. In renal cell carcinoma patients with metastasis, a reduction in platelet count after start of sunitinib treatment was associated with a better treatment outcome[62]. Although more research is needed, these studies suggest that platelet count can be used as one of the parameters to monitor anticancer treatment efficacy.

Besides platelet count, also platelet function can be affected by many TKIs, as indicated in Table 2.3. This suggests that combined platelet count and function tests could be used in clinical practice to assess the risk of bleeding upon TKI treatment. For the availability of such (point-of-care) tests, we like to refer to the expert literature[80]. In the future it may be possible to use flow-based assays for this purpose. Our laboratory recently found that this method detects additive effects of low platelet count and impaired platelet functionality in patients with a bleeding phenotype[81].

It is known that cancer patients are at increased risk of thrombosis, also indicated as Trousseau's syndrome[82]. As a consequence, many cancer patients have a history of cardiovascular disease and are treated with antiplatelet or anticoagulant drugs. When additional treatment with a TKI is started, the clinical problem of an enhanced bleeding risk may arise. Another treatment issue is that some oral anticoagulants and TKIs (ibrutinib for instance) have a common metabolic pathway (CYP3A4), which can affect the pharmacokinetics of either drug. These interactions between TKIs and antithrombotic treatments need to be further investigated.

Conclusions

Several of the TKIs used for the treatment of cancer can increase the risk of bleeding. For some but not all of these, the bleeding tendency is linked to a lowering of platelet count and/or an impairment of platelet function. Based on their binding profiles, some other TKIs are predicated to have an antiplatelet effect, although no bleeding side effects have been reported so far. For a third group of TKIs, no published data on platelets are available. Given the suspected role of platelets in tumour progression, platelet inhibition may be a clinically relevant side-effect of several TKIs. Together, the available knowledge asks for awareness of the potential antiplatelet effects of TKIs, which are likely to be further enhanced in combination with antithrombotic drugs.

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Author Contributions Statement

BMET reviewed the literature, compiled tables and Fig. 2.1, and wrote the manuscript. JWMH was responsible for the outline, and wrote and revised the manuscript. MJEK was responsible for the outline, reviewed the literature, and wrote and revised the manuscript.

Conflict of Interest Statement

MJEK and JWMH report receiving grants from Pfizer during the conduct of the study. BMET states that she has no conflict of interest.

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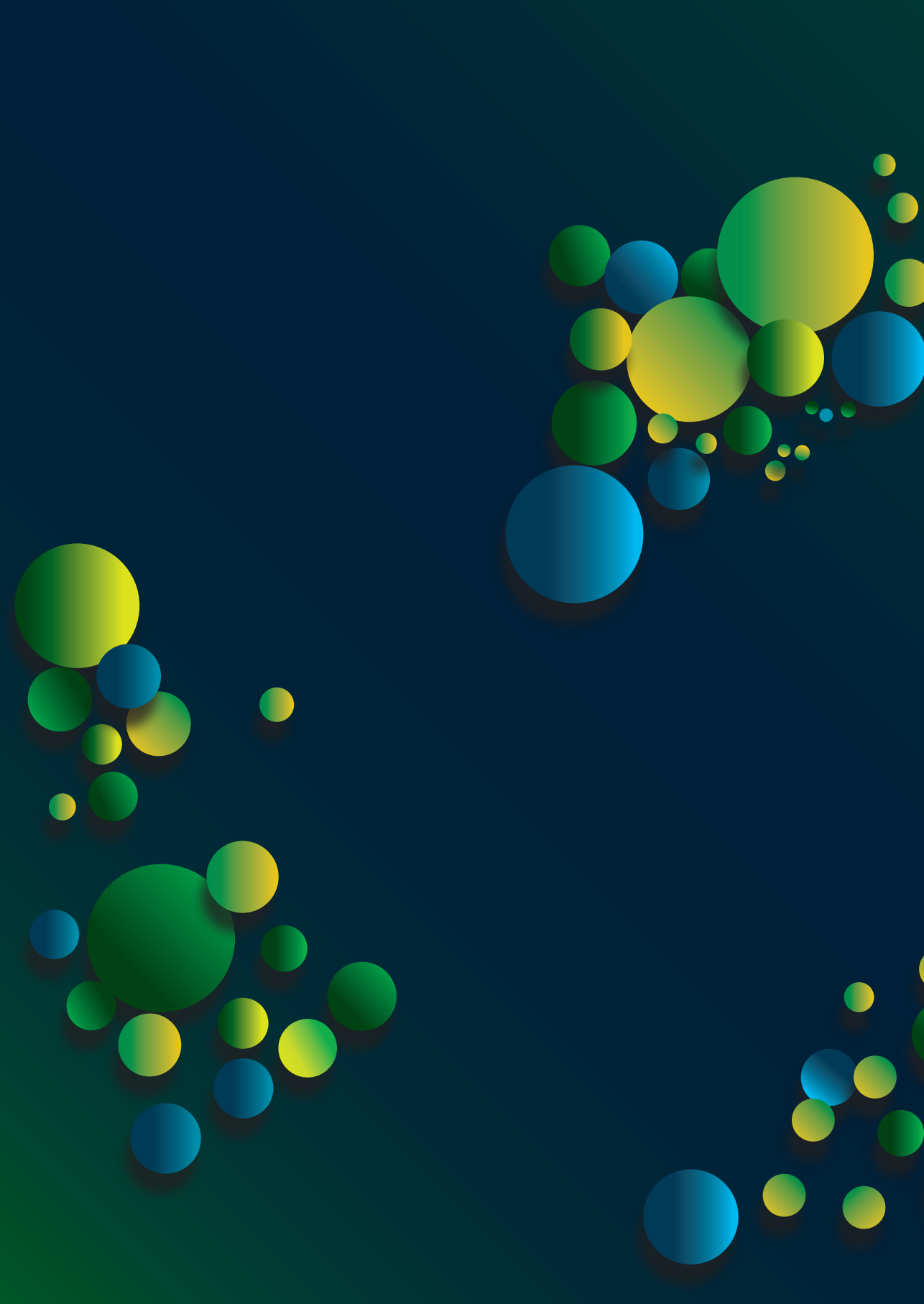

Chapter 3

Tyrosine kinase inhibitor pazopanib inhibits platelet procoagulant activity in renal cell carcinoma patients

Tullemans BME, Nagy M, Sabrkhany S, Griffioen AW, oude Egbrink MGA, Aarts MJB, Heemskerk JWM and Kuijpers MJE

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Abstract

Pazopanib is an angiostatic tyrosine kinase inhibitor (TKI) presently used for cancer treatment, particularly in patients with renal cell carcinoma (RCC). This treatment can be accompanied by mild bleeding as an adverse effect. Given the role of protein tyrosine kinases in platelet activation processes, we investigated whether and how pazopanib can affect platelet functions in purified systems and during treatment of advanced RCC patients. In isolated platelets from healthy volunteers, pazopanib dose-dependently reduced collagen-induced integrin activation and secretion, as well as platelet aggregation. Pazopanib addition diminished glycoprotein (GP)VI-dependent tyrosine phosphorylation of multiple platelet proteins, including the tyrosine kinase Syk. Furthermore, pazopanib inhibited GPVI-induced Ca^{2+} elevation, resulting in reduced exposure of the procoagulant phospholipid phosphatidylserine (PS). Upon perfusion of control blood over a collagen surface, pazopanib inhibited thrombus size as well as PS exposure. Blood samples from ten RCC patients were also analysed before and after 14 days of pazopanib treatment as monotherapy. This treatment caused an overall lowering in platelet count, with three out of ten patients experiencing mild bleeding. Platelets isolated from pazopanib-treated patients showed a significant lowering of PS exposure upon activation. In addition, platelet procoagulant activity was inhibited in thrombi formed under flow conditions. Control experiments indicated that higher pazopanib concentrations were required to inhibit GPVI-mediated PS exposure in the presence of plasma. Together, these results indicated that pazopanib suppresses GPVI-induced platelet activation responses in a way partly antagonized by the presence of plasma. In treated cancer patients, pazopanib effects were confined to a reduction in GPVI-dependent PS exposure. Together with the reduced platelet count, this may explain the mild bleeding tendency observed in pazopanib-treated patients.

Introduction

Tyrosine kinase inhibitors (TKIs) are widely approved drugs, aiming to target tyrosine kinase signalling pathways that regulate uncontrolled cellular growth and proliferation. Currently, several TKIs are in clinical use for the treatment of malignancies, such as lung, breast, kidney, and neuro-endocrine pancreatic cancers as well as gastro-intestinal stromal tumours and chronic myeloid leukaemia[1-3]. Their common way of action is by competition with adenosine triphosphate (ATP) in the conserved catalytic binding site in the protein tyrosine kinase superfamily. In spite of this action mechanism, individual TKIs can target partially different spectra of intracellular tyrosine kinases, can have different pharmacokinetics, and vary in their adverse effects[1]. Commonly, TKIs are clinically applied as a multi-target therapy to intervene in tumour proliferation[4, 5]. Specific targets are the receptors for vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), which are all involved in (tumour) angiogenesis[3, 6]. The expected effects are to reduce tumour lesions, delay disease development, and thus prolong the progression-free survival of patients[7].

Platelets contain several protein tyrosine kinases as key signal transducers, which regulate the function of platelets in haemostasis[8]. Downstream of glycoprotein (GP)VI (collagen receptor), GPIb-IX-V (von Willebrand receptor) and CLEC-2 (podoplanin receptor), Src family tyrosine kinases control the signalling routes to most platelet responses[8-10]. Activation of GPVI also implies tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) present on the Fc-receptor γ-chain, which is co-expressed with GPVI[11]. This results in activation of the protein tyrosine kinase Syk[10, 12], and further downstream Bruton tyrosine kinase (Btk), culminating in the phosphorylation and activation of phospholipase Cγ2 (PLCγ2), an event required for integrin activation and granule secretion. A similar set of protein tyrosine kinases (Src-family kinases, Syk and JAK isoforms) is known to play a critical role in megakaryocyte development and thrombocytopoiesis[13].

Considering the role of protein tyrosine kinases in both platelet activation and platelet formation, it can be expected that treatment of patients with a broad-spectrum TKI affects haemostasis. Indeed, for several TKIs, anti-haemostatic effects have been reported, like anaemia, neutropenia, thrombocytopenia, and bleeding incidences[14-19]. In particular, the TKIs sunitinib, ibrutinib, and ponatinib may cause a bleeding risk that relates to impaired GPVI-induced signalling, platelet aggregation, and thrombus formation[14, 15, 18]. Also for other TKIs a decrease in platelet count has been described upon treatment[18, 20-22].

Pazopanib is currently used as a first-line therapy for advanced clear-cell renal cell carcinoma (RCC), the most common type of kidney cancer in adults. Pazopanib is aimed to target the VEGF and PDGF receptors, stem-cell factor receptor cKit and Flt-3[23]. The inhibition of these receptors decreases tumour angiogenesis and growth, and hence prolongs patient survival[24, 25]. *In vitro* studies have indicated that pazopanib also inhibits several other tyrosine kinase-linked

receptors, including fibroblast growth factor receptor, IL-2 receptor inducible T-cell kinase (Itk), leukocyte-specific protein tyrosine kinase (Lck), and the glycoprotein receptor c-Fms[24]. In addition, other *in vitro* kinase targets of pazopanib have been described, of which ABL1, ABL2, FGR, Src, Fyn and Lck are present in platelets[26, 27]. Patients are commonly treated with a high daily dose of pazopanib (800 mg), resulting in a steady-state plasma concentration of up to 45 µg/mL after several weeks[24]. This treatment regimen can reduce the platelet count and lead to bleeding events[24]. Since effects of pazopanib on platelet function have not been reported, we aimed to investigate this *in vitro* and *ex vivo*, using blood from RCC patients and control subjects.

Materials and Methods

Materials

Pazopanib (Votrient) was obtained from LC Laboratories (Woburn MA, USA). Arachidonic acid was obtained from Bio/DATA Corporation (Horsham PA, USA), fibrillar Horm type I collagen from Takeda (Hoofddorp, The Netherlands), thrombin from Enzyme Research Laboratories (South Bend IN, USA), thrombin receptor activating peptide 6 (TRAP-6) from Bachem (Bubendorf, Switzerland) and U46619 (thromboxane A_2 receptor agonist) from Cayman Chemicals (Ann Arbor MI, USA). Fluorescein isothiocyanate (FITC)-labelled PAC-1 monoclonal antibody (mAb) against activated human integrin $\alpha_{IIb}\beta_3$ was from BD Bioscience (Franklin Lakes NJ, USA; nr. 340507), while FITC-labelled anti-human CD62 mAb was from Beckman Coulter (Sydney, Australia; nr. 65050). Collagen-related peptide (CRP-XL) was obtained from the University of Cambridge (Cambridge, UK). 2Methylthio-adenosine-diphosphate (2Me-S-ADP), D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) and mouse anti-Syk antibody were purchased from Santa Cruz Biotechnology (Dallas TX, USA). Fibrinogen and unfractionated heparin were obtained from Sigma-Aldrich (Saint Louis MO, USA). FITC-labelled annexin A5 was from Pharmatarget (Maastricht, The Netherlands). Fura-2-AM was from Invitrogen (Carlsbad CA, USA); Pluronic F-127 from Molecular Probes (Eugene OR, USA). For Western blotting, the following antibodies were used: mouse anti-phosphotyrosine mAb (clone 4G10) obtained from Millipore (Billerica MA, USA), rabbit anti-tubulin Ab from Abcam (Cambridge, UK), rabbit anti-phospho Syk Tyr525/526, HRP-conjugated anti-rabbit-IgG from Cell Signalling, (Leiden, The Netherlands) and HRP-conjugated anti-mouse-IgG from VWR international (Amsterdam, The Netherlands).

Blood collection from patients and healthy volunteers

The study was approved by the medical ethics committee of the Maastricht University Medical Centre+ (MUMC+, The Netherlands). All participants provided written informed consent in accordance with the Declaration of Helsinki. Blood was obtained from healthy volunteers and ten patients diagnosed with metastatic renal cell carcinoma at the Department of Medical Oncology of MUMC+. Patients were included, if eligible for treatment with pazopanib as a single

agent (800 mg/day). Excluded were subjects who used anticoagulants or platelet inhibitory drugs. From healthy volunteers, one blood sample was collected, while the patients donated two blood samples: one day before and at 14 days after starting pazopanib treatment, i.e., when a steady-state plasma concentration was reached[24]. Blood samples were collected from the antecubital vein into 3.2% trisodium citrate; the first 5 mL of blood was discarded after which 10 mL was collected.

Blood composition and platelet isolation

Haematological parameters, including platelet count, were determined with a Sysmex XP300 (Chuo-ku Kobe, Japan). Washed platelets were obtained as described[28]. In brief, platelet-rich plasma (PRP) was collected after 15 minutes centrifugation at 240 *g*, followed by a washing step. Washed platelets were resuspended into Hepes buffer pH 7.45 [10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% bovine serum albumin (BSA)]. Platelet count was adjusted, as required for the particular assay.

Blood from healthy volunteers was used for in vitro experiments of pazopanib effects. Samples of whole blood, PRP or washed platelets were pre-incubated with pazopanib or vehicle (dimethylsulfoxide) for 10 minutes at 37°C.

Light transmission aggregometry

Aggregation of platelets, washed or in PRP ($250x10^9$ platelets/L) was measured using a Chronolog aggregometer (Havertown PA, USA) under constant stirring (37°C); pazopanib (5, 10 or 30 µM) or vehicle was present as indicated. Aggregation responses were quantified as maximal amplitude in light transmission[29]. Aggregation of washed platelets was induced with collagen (1 µg/mL), 2Me-S-ADP (1 µM) in the presence of fibrinogen (25 µg/mL), thrombin (1 nM), TRAP-6 (10 µM), U46619 (1 µM), or arachidonic acid (10 µM). Aggregation of platelets in PRP was induced with collagen (1 µg/mL).

Flow cytometry

Washed platelets ($100x10^9$/L) were incubated for 10 minutes at 37°C with vehicle or pazopanib (10 µM). The cells in Hepes buffer pH 7.45 containing 2 mM $CaCl_2$ were then stimulated with CRP-XL (1 µg/mL), 2Me-S-ADP (1 µM), or thrombin (1 nM). Using described flow cytometry procedures, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were determined with FITC-conjugated PAC1 mAb (1:10) and FITC-conjugated anti-CD62P mAb (1:10), respectively[30]. For the measurement of PS exposure, platelets were stimulated with CRP-XL (5 µg/mL) and thrombin (4 nM) for 60 minutes at 37°C[31]. Exposure of PS was determined with FITC-conjugated annexin A5 (1 µg/mL).

To assess PS exposure in the presence of plasma, platelet count of washed platelets or PRP were adjusted to $100x10^9$ platelets/L. Washed platelet were diluted in Hepes buffer pH 7.45 with 2 mM $CaCl_2$; PRP was diluted with autologous plasma supplemented and 6.3 mM $CaCl_2$ plus 3.2 mM $MgCl_2$. Washed platelets and PRP were mixed in various ratios to obtain 0, 10, 30, 50 and 100% plasma. The mixed samples were preincubated with pazopanib (1, 5, 10, 30, 50, 75 or 100 µM) or vehicle for 10 minutes at 37°C, and then activated with CRP-XL (5 µg/mL) and TRAP-6 (15 µM) in the presence of PPACK (40 µM). Exposure of PS was determined after 60 minutes at 37°C with FITC-conjugated annexin A5 (1 µg/mL). Flow cytometric measurements were performed in duplicate using a BD Accuri C6TM flow cytometer and corresponding software (Erembodegem, Belgium).

Whole blood perfusion experiments

Whole blood perfusion experiments were performed as described before[32]. In short, citrate-anticoagulated blood samples were incubated with pazopanib (30 µM) or vehicle for 10 minutes at room temperature. After recalcification in the presence of thrombin inhibitor (40 µM PPACK, 6.3 mM $CaCl_2$, 3.2 mM $MgCl_2$, f.c.), the samples were perfused through a transparent parallel-plate flow chamber, containing a coverslip coated with type I collagen (50 µg/mL) at a wall-shear rate of 1000 s^{-1}. After 4 minutes, thrombi formed on coverslip were stained with FITC-conjugated annexin A5 (1 µg/mL in Hepes buffer pH 7.45, containing 2 mM $CaCl_2$ and 1 U/mL heparin). At least 10 random brightfield and fluorescence images were captured with an EVOS microscope (Bothell WA, USA). Microscopic digital images were analysed for platelet deposition (% of surface area coverage, SAC), multilayer % SAC, integrated feature size and staining for PS (% SAC), using ImageJ 1.45s software (ImageJ ecosystem, from imagej.nih.gov/ij/). The integrated feature size is a parameter of platelet aggregation, taking into account the proportional contribution of large and small thrombi on microspots[33]. Further details of image analysis are described elsewhere[34].

Cytosolic Ca^{2+} measurements

Washed platelets were incubated with Fura-2-AM (3 µM) in the presence of pluronic (600 µM) for 45 minutes at 37°C. After another wash step, the Fura-2 loaded platelets ($100x10^9$/L) were used for cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements, as described previously[35]. In brief, using polystyrene cuvettes, platelets in suspension (0.5 mL) were pre-incubated with pazopanib (10 µM) or vehicle for 8 minutes at room temperature and 2 minutes at 37°C. After baseline measurement, $CaCl_2$ (1 mM) was added, followed by an agonist. Changes in Fura-2 fluorescence were measured by ratio fluorometry at dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. After correction for background fluorescence, ratio values were converted into levels of $[Ca^{2+}]_i$. Maximal rises in $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$-time integrals (4 minutes) were determined[36].

Western blotting

Washed platelets (500x10^9/L) were pre-incubated with pazopanib (10 or 30 μM) or vehicle and stimulated under stirring conditions with CRP-XL (5 μg/mL) or left unstimulated. Samples were lysed with NP40 lysis buffer, supplemented with a cocktail of phosphatase inhibitors, and protein content was determined using a BioRad protein assay (Hercules CA, USA). Platelet lysates were separated by polyacrylamide gel electrophoresis and subjected to standard western blotting. Blots were stained for tyrosine phosphorylation profile as described[18], using anti-phosphotyrosine mAb 4G10 (1:2000) and secondary HRP-conjugated Ab (1:500). Specific phosphorylation of Syk was visualized, as described[37], using anti-Syk Tyr525/526 mAb (1:1000) and secondary HRP-conjugated secondary Ab (1:500). Total Syk was determined by reprobing with anti-Syk mAb (1:1000) and HRP-conjugated secondary Ab (1:1000). As a control for total platelet proteins, blots were also probed for α-tubulin (1:1000). The intensity of stained bands was analysed with ImageJ 1.45s software.

Statistical analysis

Data were checked for Gaussian distribution using the Kolmogorov-Smirnov Normality test. Normally distributed data are presented as means ± SEM; data that are not normally distributed are presented as median ± interquartile ranges. Statistical significance between in vitro datasets (vehicle vs. pazopanib-treated samples) was determined using paired t-test. Paired datasets of the patients (before and after pazopanib therapy) were compared using the Wilcoxon matched-pairs signed-rank test. When more than 2 conditions were compared, a one-way ANOVA was used. GraphPad Prism 5.0 software (La Jolla CA, USA) was used for statistical analyses. A P-value less than 0.05 was considered to be statistically significant.

Results

Pazopanib in vitro suppresses collagen- and ADP-induced platelet aggregation, secretion and PS exposure

Platelet activation in haemostasis involves multiple agonists and their receptors[38]. Adhesion of platelets to extracellular matrix proteins, like collagen, is followed by platelet activation (characterized by integrin $\alpha_{IIb}\beta_3$ activation and secretion), aggregation (via released ADP and thromboxane A_2) and thrombus formation. Highly activated platelets also expose the procoagulant phospholipid PS, which promotes thrombin generation and fibrin formation[39, 40]. We first investigated the effect of pazopanib on the aggregation response of washed platelets to collagen (acting via GPVI) or ADP (acting via P2Y receptors). Dose-response curves indicated near-complete inhibition with either agonist already at a relatively low dose of 10 μM pazopanib (Figure 3.1A). In following experiments, the concentration of 10 μM appeared to suppress platelet aggregation with ADP, arachidonic acid and U46619 (thromboxane A_2 analogue). On the other

hand, platelet aggregation induced by thrombin or the PAR1 receptor agonist, TRAP-6, was not affected by pazopanib at this dose (Figure 3.1B).

Subsequently, flow cytometry was used to determine the effects of pazopanib on specific platelet responses, i.e. integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion (P-selectin expression). Markedly, pazopanib at 10 µM strongly inhibited integrin activation and granule secretion induced by the GPVI receptor agonist (CRP-XL), with no or limited effect in response to ADP or thrombin stimulation (Figure 3.1C). Pazopanib also reduced CRP-XL plus thrombin-induced PS exposure by >50% (Figure 3.1D). Taken together, these results indicate that, in washed platelets, pazopanib is

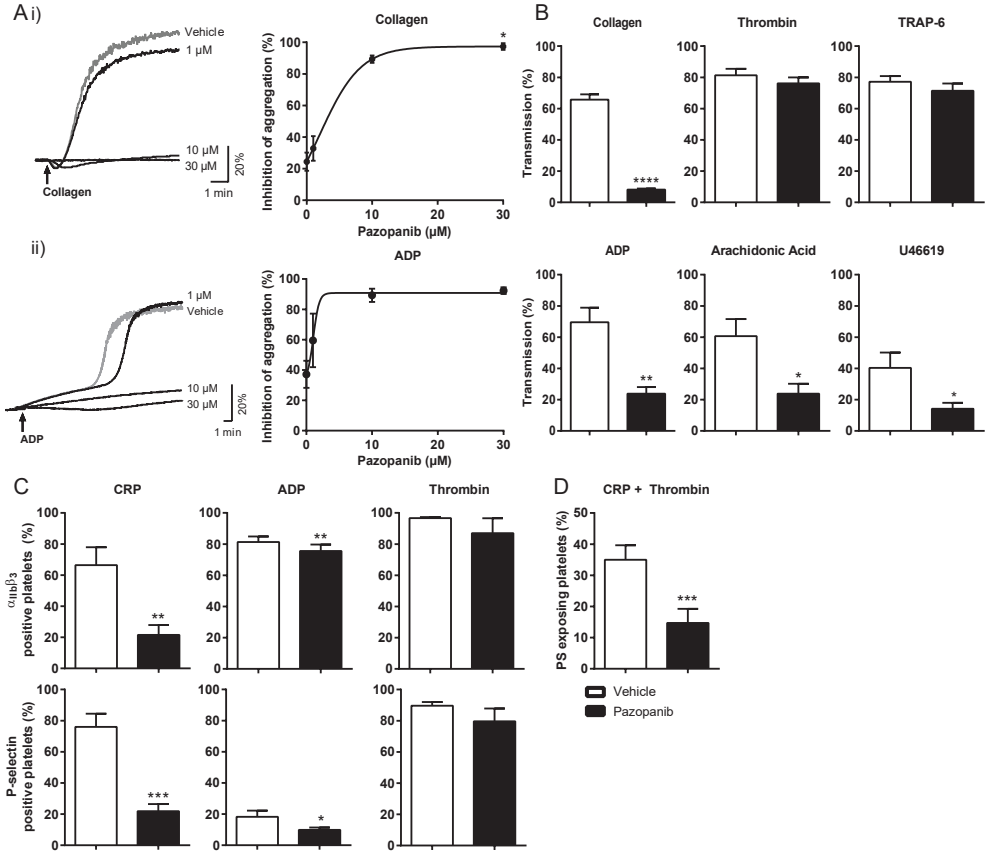

Figure 3.1. Pazopanib inhibits collagen- and ADP-induced platelet aggregation, integrin activation, secretion and PS exposure. Washed platelets from healthy donors were incubated with 1, 10, or 30 µM pazopanib or vehicle (0.1% DMSO) for 10 minutes. (A) Representative aggregation curves and dose-response graphs of inhibition of aggregation (n = 4). Arrow indicates addition of (i) 1 µg/mL collagen or (ii) 25 µg/mL fibrinogen and 1 µM Me-S-ADP. (B) Aggregation of washed platelets in presence (black bars) or absence (white bars) of 10 µM pazopanib was induced by 1 µg/mL collagen, 1 nM thrombin, 10 µM TRAP-6, 1 µM Me-S-ADP in presence of 25 µg/mL fibrinogen, 30 µM arachidonic acid or 1 µM U46619. Histograms indicate maximal amplitude of aggregation (n = 6). (C) Platelets, pre-treated with vehicle or pazopanib (10 µM), were stimulated with 5µg/mL CRP-XL, 1µM Me-S-ADP or 1 nM thrombin for 10–20min, and analysed by flow cytometry (n = 6). Shown are percentages of platelets binding FITC-labelled PAC-1 monoclonal antibody against integrin $a_{IIb}b_3$ or FITC-labelled anti-human CD62 mAb. (D) Platelets pre-treated with vehicle or pazopanib (10µM) were stimulated with 5 µg/mL CRP-XL and 4 nM thrombin for 60 minutes, and analysed by flow cytometry (n = 6). Exposure of PS was determined by FITC-labelled annexin A5. Histogram shows percentages of platelets binding FITC-annexin A5. Data are means ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

an efficient antagonist of platelet responses induced by GPVI agonists (collagen or CRP-XL), but is less effective in antagonizing responses induced by the G-protein coupled receptor agonists ADP, thromboxane A_2 or thrombin.

Pazopanib inhibits phosphorylation of Syk, and reduces platelet Ca^{2+} responses

The decreased platelet responses to GPVI agonists suggested that pazopanib influences platelet signalling via the tyrosine kinase-operating ITAM pathway. To investigate this in more detail, we assessed the effect of pazopanib on protein tyrosine phosphorylation patterns of GPVI-stimulated platelets using western blotting. Pre-incubation of $500x10^9$ platelets/L with 30 µM pazopanib (i.e., equivalent to 15 µM for $250x10^9$ platelets/L, used for aggregation experiments) strongly reduced the CRP-XL induced tyrosine phosphorylation of multiple proteins (Figure 3.2A). This suggested interference of pazopanib early in the GPVI signalling cascade. This was confirmed by the finding that pazopanib strongly inhibited the phosphorylation of Syk (Figure 3.2B).

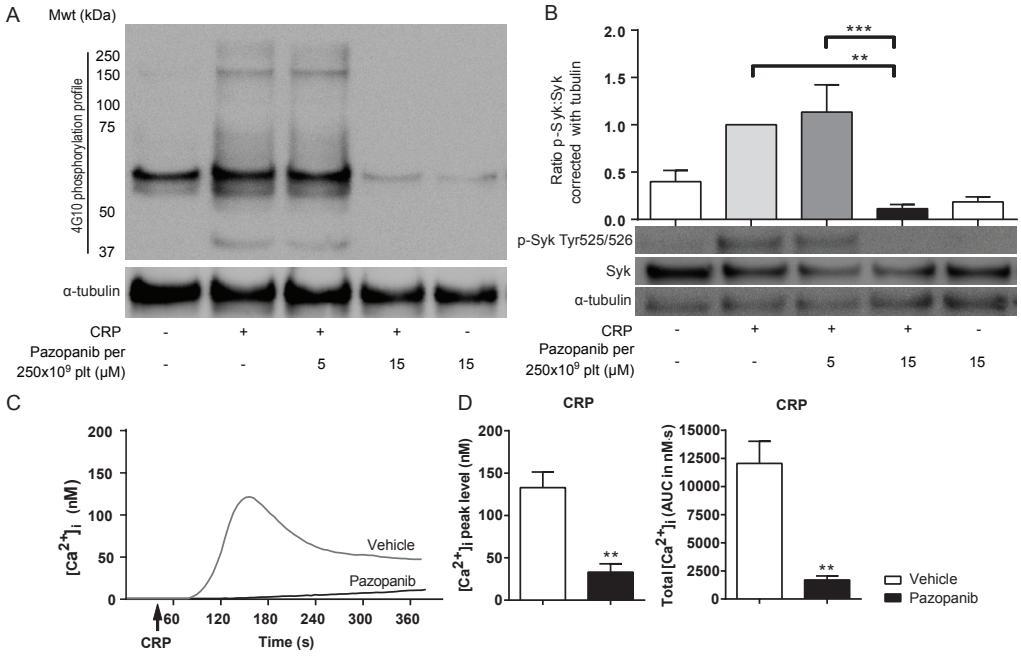

Figure 3.2. Pazopanib inhibits GPVI-induced protein tyrosine phosphorylation via Syk and cytosolic Ca^{2+} rises. (A,B) Representative western blots (n=4) with washed platelets ($500×10^9$/L) incubated with vehicle or pazopanib (10 or 30 µM) for 10 minutes, and stimulated with 5 µg/mL CRP-XL for 3 minutes. For comparison to Figure 1, pazopanib concentrations were re-calculated to match a platelet concentration of $250×10^9$/L. (A) Protein tyrosine phosphorylation was visualized with 4G10 mAb. Total amount of protein was visualized by staining for a-tubulin. (B) Protein phosphorylation of Syk (Tyr525/526) and Syk, and parallel staining for a-tubulin. Shown is the ratio of phospho-Syk/total Syk assessed by gray intensity analysis (n=4). (C) Representative traces of changes in cytosolic Ca^{2+} of Fura-2-loaded platelets ($100×10^9$/L), pre-incubated with vehicle or 10 µM pazopanib for 10 minutes, and stimulated with 1 µg/mL CRP-XL or 1 nM thrombin in the presence of 1 mM $CaCl_2$. (D) Histograms show maximal rise in $[Ca^{2+}]_i$ and total $[Ca^{2+}]_i$ (area-under-curve, AUC) (n=5). Data are means ± SEM, **p<0.01, ***p<0.001.

GPVI signalling via Syk results in elevation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) as a prerequisite for integrin activation, secretion and PS exposure. To investigate this, platelets were loaded with the Ca^{2+} probe Fura-2, and agonist-induced responses were measured. In platelets stimulated with CRP-XL, the presence of pazopanib resulted in a reduction of the maximal (peak height) and total (area-under-curve) Ca^{2+} rises (Figure 3.2C-D). Pazopanib did however not influence $[Ca^{2+}]_i$ elevation in response to thrombin stimulation (not shown). These results confirm that pazopanib interfered in the early GPVI-induced signalling cascade.

Pazopanib affects thrombus size and PS exposure in whole blood under flow

Platelet activation via GPVI is an essential step in collagen-induced thrombus formation in whole blood under flow conditions[41]. This method was used to determine the effect of pazopanib on platelets in a whole blood environment. Pre-incubation of blood with 30 µM pazopanib resulted in a small, but significant decrease in platelet deposition compared to vehicle-treated blood (Figure 3.3A-B). Furthermore, the height of thrombi with multi-layered platelets was reduced with pazopanib (Figure 3.3C). The aggregate-reducing effect was confirmed by measuring the size distribution of thrombi (integrated feature size), which was significantly decreased after treatment of the blood with pazopanib (Figure 3.3D). Post-staining with fluorescently labelled annexin A5 indicated a lower PS exposure (Figure 3.3E), as in agreement with the flow cytometric results using washed platelets.

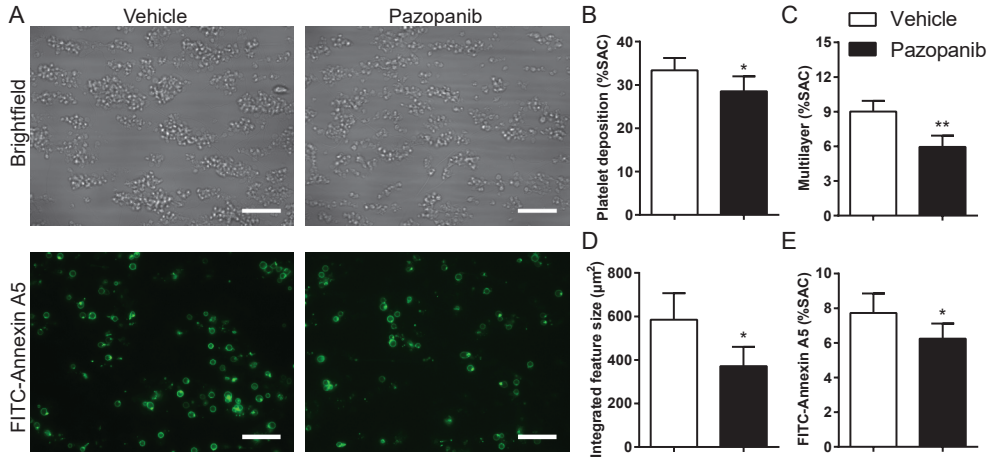

Figure 3.3. Pazopanib suppresses thrombus formation in whole blood perfused over collagen. Whole blood from healthy volunteers was perfused for 4 minutes at wall shear rate of 1,000 s^{-1} over microspots containing collagen-I. (A) Representative brightfield images (upper panel) and fluorescence images of PS exposure (lower panel) of control blood sample incubated with 30µM pazopanib. Bar=20 µm. (B–E) Quantification (n=6) of brightfield images of: platelet deposition (B), multilayered thrombus (C), cumulative size of thrombus (integrated feature size) (D), and fluorescence images of staining for PS exposure (E). Data are means ± SEM (n = 6), *p < 0.05, **p < 0.01.

Pazopanib treatment of renal cell carcinoma patients moderately affects platelet functions

To investigate the clinical relevance of these findings, blood samples were obtained from ten patients diagnosed with metastatic RCC, and eligible for pazopanib treatment (Table 3.1). The patients (6 females) had a mean age of 69 (range: 51-88) years. Blood samples were collected at one day prior and at 14 days after the start of pazopanib treatment. Median platelet count in the patients' blood before start of treatment was $230x10^9$/L, while this moderately, but significantly reduced to $201x10^9$/L after treatment (Figure 3.4A). Bleeding complications were reported for three patients, who all developed mild epistaxis (Table 3.1).

Using washed platelets from patients before and after pazopanib treatment, aggregation was determined by light transmission aggregometry. Strikingly, no treatment effect could be observed upon collagen stimulation (Figure 3.4B). Flow cytometry was used to assess $\alpha_{IIb}\beta_3$ integrin activation and secretion upon stimulation with CRP-XL, ADP or thrombin. Again, no effect of the treatment on these platelet responses could be observed (Figure 3.4C). On the other hand, platelet procoagulant activity, as determined from PS exposure in response to CRP-XL plus thrombin, was significantly decreased by 18% after treatment (Figure 3.4D).

In whole blood flow experiments over collagen, parameters of thrombus formation were investigated before and after pazopanib treatment. Whereas platelet deposition and thrombus buildup were not affected in the post-treatment blood samples (Figure 3.5A-D), a significant reduction of 48% was seen in PS exposure in the post-treatment samples, as compared to pre-treatment (Figure 3.5A, E).

Plasma impairs pazopanib effects on platelet function

In isolated platelets we observed a dose-dependent inhibiting effect of pazopanib on platelet function via the inhibition of tyrosine phosphorylation of Syk, and downstream intracellular Ca^{2+} signalling. This was accompanied by reduced integrin activation, secretion and PS exposure. In contrast, pazopanib treatment of RCC patients only resulted in reduction of platelet

Table 3.1. Characteristics of patients treated with pazopanib 800 mg/day.

Patient no.	Age range (years)	Platelet count day 0 ($x10^9$/L)	Platelet count day 14 ($x10^9$/L)	Bleeding
1	56-60	238	183	No
2	61-65	222	212	No
3	71-75	175	107	Yes, epistaxis
4	61-65	212	189	No
5	66-70	217	219	Yes, epistaxis
6	81-85	443	288	Yes, epistaxis
7	66-70	402	130	No
8	51-55	295	249	No
9	76-80	274	276	No
10	81-85	179	139	No

PS exposure. We hypothesized that the presence of plasma can interfere with the antiplatelet activity of pazopanib. To investigate this, experiments were performed with both PRP and washed platelets, which were incubated with different concentrations of pazopanib. In undiluted PRP, collagen-induced aggregation was not affected by pazopanib, whereas this response was strongly inhibited at 10 µM in washed platelets (Figure 3.6A). Pre-incubation of PRP with increasing doses of pazopanib until 20 µM did not inhibit PS exposure, in contrast to pre-incubation of washed platelets (Figure 3.6B).

It has been described that pazopanib is highly bound by plasma proteins at concentration ranges of 10-100 µg/mL[24]. This corresponds with a concentration of 23-230 µM. Hence, also higher concentrations of pazopanib (50-100 µM) were tested in platelets incubated at different

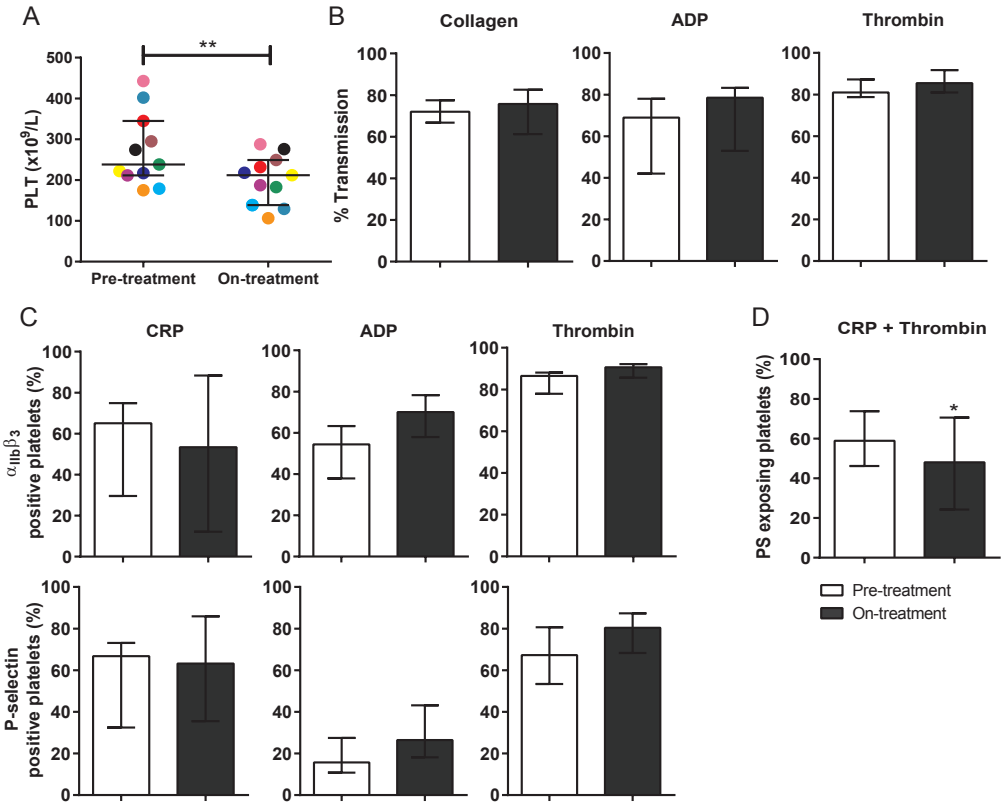

Figure 3.4. Treatment of carcinoma patients with pazopanib decreased platelet count and suppressed phosphatidylserine exposure in washed platelets. Blood from patients was collected the day before and at 2 weeks after pazopanib treatment. (A) Platelet count was measured in blood from RCC patients before and on treatment with pazopanib. Each dot represents a single patient. (B) Platelets were isolated and aggregation was induced by 1µg/mL collagen, 1µM Me-S-ADP (in the presence of 25µg/mL fibrinogen), or 1 nM thrombin. Histograms indicate maximal amplitude of aggregation (n = 6). (C) Platelets before and during treatment of patients were stimulated with 5µg/mL CRP-XL, 1µM 2Me-S-ADP or 1 nM thrombin for 10–20min, and analyzed by flow cytometry (n = 10). Shown are percentages of platelets binding FITC-PAC1 mAb (aIIbb3 expression) or FITC-anti-CD62P mAb (P-selectin expression). (D) Patient platelets were stimulated with 5 µg/mL CRP-XL and 4 nM thrombin for 60min, and analyzed by flow cytometry (n = 6). Histogram shows percentages of platelets binding FITC-annexin A5 (PS exposure). Data are medians ± interquartile ranges, *p<0.05, **p<0.01.

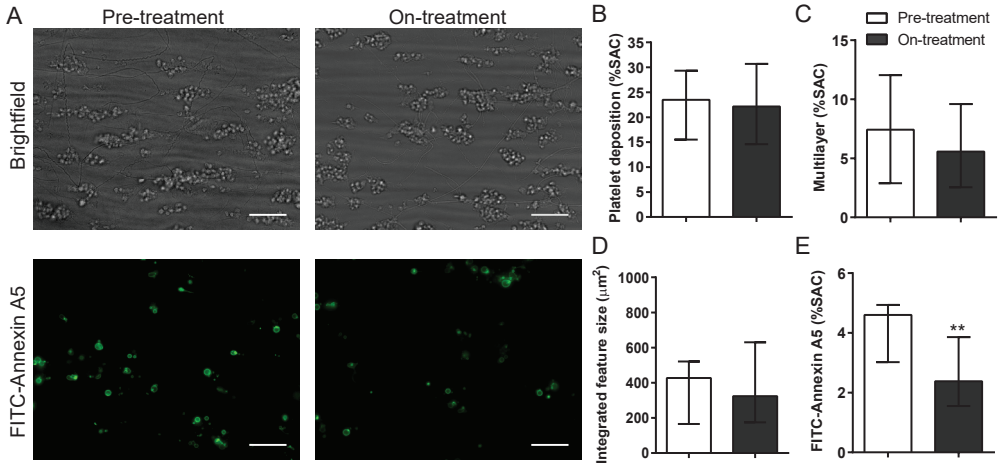

Figure 3.5. Treatment of pazopanib patients suppressed phosphatidylserine exposure upon thrombus formation on collagen. (A) Whole blood from patients was perfused for 4min at wall shear rate of 1,000 s^{-1} over a collagen surface. Shown are representative brightfield images (upper panel) and fluorescence images of PS exposure (lower panel) for blood samples taken before and after 2 weeks of pazopanib treatment. Bar=20 µm. (B–E) Quantification of brightfield images of platelet deposition (B), multilayered thrombus (C), cumulative size of thrombus (integrated feature size, IFS) (D), and fluorescence images of staining for PS exposure (E). Data are medians ± interquartile ranges (n=10), **p<0.01.

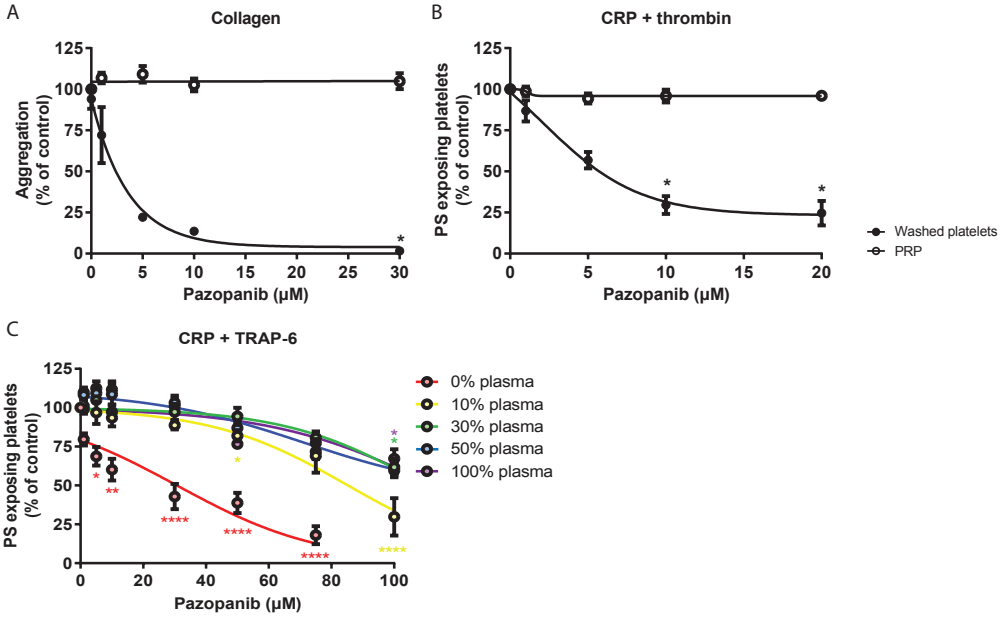

Figure 3.6. Plasma suppresses effects of pazopanib on platelet activation in vitro. Washed platelets or PRP from healthy donors were incubated with 1, 5, 10, or 30 µM pazopanib or vehicle (0.1% DMSO) for 10 minutes. (A) Aggregation of platelets in buffer or PRP in response to 1 µg/mL collagen. (B) Exposure of PS measured in washed platelets or in PRP, stimulated with 5 µg/mL CRP-XL and 4 nM thrombin for 60 minutes, and analyzed by flow cytometry using FITC-labeled annexin A5. (C) Platelets reconstituted at different plasma concentrations were pre-incubated with pazopanib (1–100 µM), and stimulated with 5 µg/mL CRP-XL plus 15 µM TRAP-6; PS exposure was quantified by flow cytometry using FITC-labeled annexin A5 (PS exposure). Data are means ± SEM (n=3), *p<0.05, **p<0.01, ****p<0.0001.

plasma concentrations (0, 10, 30, 50 or 100% plasma). It appeared that pazopanib treatment substantially suppressed PS exposure in response to CRP-XL plus TRAP-6 in the presence of low amounts of plasma (Figure 3.6C). However, at 100 µM pazopanib, PS exposure was still significantly reduced by 25% with 30-100% plasma (Figure 3.6C, $p<0.01$). This effect is comparable to the moderate inhibition of PS exposure in patients treated with pazopanib (see Figure 3.4D). These results suggest that the presence of plasma interferes with the incorporation of pazopanib into platelets.

Discussion

In the present study, we demonstrate that the multi-target TKI pazopanib markedly reduces the collagen-induced activation responses of isolated platelets, including aggregation, PS exposure and Ca^{2+} signalling through inhibition of tyrosine kinases, including Syk. In blood samples from RCC patients treated with pazopanib, these effects were confined to a suppression of GPVI-mediated PS exposure, observed in isolated platelets as well as in whole blood thrombus formation under flow.

Several protein tyrosine kinases are known to contribute to platelet activation processes and haemostasis[8, 10, 11]. An activity-based kinase profiling already showed that pazopanib can target various tyrosine kinases that are highly expressed in platelets[42]. Here we confirm that, in washed platelets, the GPVI-dependent phosphorylation of multiple proteins, including Syk, is suppressed by pazopanib, in a way accompanied by reduced platelet activation processes. Syk is known to have multiple sites of phosphorylation which both regulate activity and serve as docking motifs for other proteins[43]. These sites include Tyr-348 and Tyr-352 within the SH2-linker region, Tyr-525 and Tyr-526 within the activation loop of the kinase domain, Tyr-630 in the C terminus of Syk, and other sites such as Thr-384 and Ser-297. It has been shown that Tyr-525/526 is essential for Syk function[44]. Therefore, we selected this phosphorylation site to study the effect of pazopanib on platelet function. However, from the present data it cannot be concluded whether pazopanib inhibits Syk directly, or if its phosphorylation is reduced through inhibition of upstream tyrosine kinases. Most likely, it can be both as pazopanib has been shown to have affinity for multiple TKs in platelets. We have observed that the Syk inhibitor II completely inhibits PS-exposure in washed platelets[37], which was much stronger than the inhibitory effect of pazopanib (Figure 3.1D). Furthermore deficiency and selective inhibition of Syk has been shown to prevent platelet aggregation and activation in response to collagen-receptor stimulation in mice[45].

Interestingly, pazopanib also suppressed platelet responses to ADP, arachidonic acid and TxA_2 analogue, while responses to thrombin were unaffected. In agreement with this is the recent finding that three tyrosine kinases are phosphorylated upon platelet activation with ADP using phosphoproteomics[46]. These are JAK3, Btk and TNK2, indicating TKs are involved in signalling underneath ADP. Whether TKs are also phosphorylated under AA and thromboxane A_2 receptor

stimulation has not yet been investigated with phosphoproteomics, but this is not unlikely. Although it appears that the majority of the targets of pazopanib are underneath GPVI, these data indicate that pazopanib may also target kinase events downstream of other platelet receptors. Moreover, as it has been shown that inhibition of GPVI alone prevents occlusive thrombus formation without causing bleeding[47], these results support the hypothesis that the targeting of pathways downstream of additional receptors may be responsible for the increased bleeding risk with pazopanib treatment.

Pazopanib is known to have an extremely high plasma protein binding compared to other TKIs[24, 48]. Therefore, we explored if the presence of blood plasma affected its inhibitory effect on platelet responses. Upon increasing plasma concentrations, it appeared that the inhibition of collagen-induced aggregation became lost at pazopanib concentrations up to 100 µM, whereas the inhibition of PS exposure was still present albeit diminished. Albumin, as a major plasma protein (about 55% of plasma proteins) present at 35-50 mg/mL[49], is considered to be the main pazopanib-binding plasma component[50]. This is in agreement with the residual, but consistent inhibition of PS exposure observed in the platelets from patients treated with pazopanib, as well as in whole blood thrombus formation under flow.

Patients with advanced RCC are commonly treated with 800 mg pazopanib per day. The reported steady state, maximal concentration here is 45 µg/mL[51], which corresponds to a concentration of 100 µM. In phase III trials, pazopanib effectively delays disease progression and reduces tumour lesions[25, 52]. In spite of the only partial response rate[25], the affinity of pazopanib for VEGF and PDGF receptors is relatively high[42], and likely is higher than that for the intracellular tyrosine kinases implicated in platelet activation. This may explain why the pazopanib dose used for effective cancer treatment does not completely abolish platelet activation processes, but only platelet procoagulant activity (PS exposure), i.e. a response that is most sensitive to inhibition of the cytosolic Ca^{2+} rises.

We and others[24] observed a moderate decrease in platelet count upon pazopanib treatment. This by itself is not expected to result in bleeding, with values still within the normal range of 150–400x10^9 platelets/L[53]. This effect may suggest interference in platelet formation (megakaryocytopoiesis). This has not been reported so far, but there is evidence that megakaryocytic signalling via Src family and Syk kinases is required for megakaryocyte migration, and platelet formation[13].

The combination of a reduced platelet count and impaired PS exposure may explain the mostly minor bleeding events observed during pazopanib treatment. In the present study, this held for three out of ten patients, all experiencing epistaxis. This number is relatively high compared to a published clinical trial, reporting mild bleeding in only 13% of the patients[24]. However, we like to note that the power of our study is low. Mild bleeding has also been reported with the use of other TKIs in cancer therapy[18, 19]. Ibrutinib – affecting collagen- and von Willebrand factor-

dependent platelet functions[54] – can cause a risk of mild bleeding in about half of the treated patients, whilst 4-8% of these experiencing major haemorrhages[14]. Treatment with the drug ponatinib resulted in a prolonged PFA-100 closure times in patients' blood samples, indicating a loss of platelet function[15]. Bleeding occurred here in about 10% of the patients, who however occasionally used other anticoagulants or antiplatelet drugs[55]. Treatment with the TKI dasatinib was associated with mild thrombocytopenia and an increased risk of bleeding, likely due to combined effects on megakaryocytes and platelets[56]. Also in the latter case, the patients' platelets were less responsive to collagen stimulation, resulting in decreased thrombus formation[57]. To take this further, we recently reviewed how distinct TKIs inhibit platelet activation mechanisms, as well as the clinical consequences of antiplatelet effects due to TKI treatment[58]. Comparison of affinity profiles of TKIs for platelet targets, as well as literature regarding effects on platelet count, platelet function and bleeding, enabled us to distinguish three categories of TKIs. (i) For several TKIs the bleeding tendency is linked to a lowering of platelet count and/or an impairment of platelet function, (ii) other TKIs are predicated to have an antiplatelet effect, although no bleeding side effects have been reported so far, and (iii) for some TKIs no published data on platelets are available.

In comparison to pazopanib, treatment of RCC patients with sunitinib was found to result in a more profound inhibition of platelet activation[18]. Sunitinib is taken up by platelets, and can thus effectively reduce collagen-receptor induced aggregation and thrombus formation[18]. A comparative study of sunitinib versus pazopanib treatment indicated that both drugs provided a progression-free survival benefit when compared to placebo; however, pazopanib had a better safety and quality-of-life profile[52]. Sunitinib causes bleeding in up to 20% of patients, and life-threatening bleeding in 3% of the patients[16]. As indicated above, for pazopanib these numbers are lower, but still non-negligible[24]. Accordingly treatment of metastatic RCC with either pazopanib or sunitinib should be accompanied by special attention of the haemostatic condition, especially when the patients are also treated with antiplatelet drugs.

In summary, the present work demonstrates that platelet treatment with pazopanib *in vitro* results in strong inhibition of collagen-induced platelet activation, aggregation and PS exposure, whereas pazopanib treatment of RCC patients is restricted to inhibition of the platelet procoagulant activity. In combination with the reduction in platelet count, these effects are likely to contribute to the higher bleeding tendency in pazopanib treated RCC patients. Therefore, anti-platelet effects of TKIs should be taken into account in therapy decisions for patients, especially when prescribed in combination with antiplatelet drugs.

Acknowledgements

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Author Contributions Statement

BMET performed experiments, analysed data and wrote manuscript. MN performed experiments and critically reviewed manuscript. SS performed experiments, wrote medical ethical approval and critically reviewed manuscript. AWG provided essential tools, discussed results and critically reviewed manuscript, MGAoE discussed results and critically reviewed manuscript, MJBA included and took care of RCC patients and critically reviewed manuscript, JWMH designed and discussed experiments, critically reviewed and revised manuscript, MJEK designed and performed experiments, discussed results, wrote and revised manuscript.

Conflict of Interest Statement

The authors declare no personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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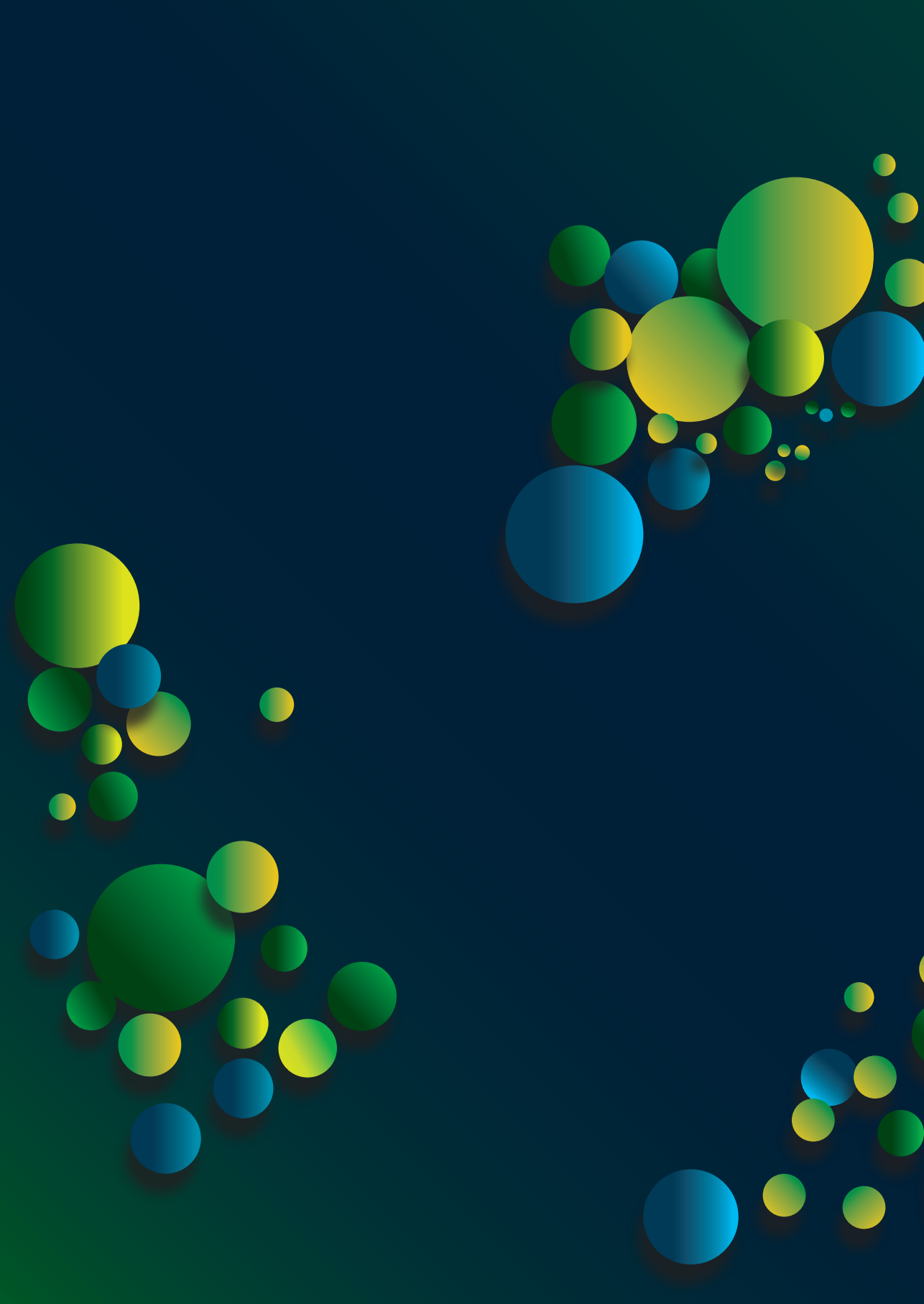

Chapter 4

Tyrosine kinase inhibitor sunitinib delays platelet-induced coagulation: additive effects of aspirin

Tullemans BME, Fernández DI*, Veninga A*, Baaten CCFMJ, Peters LJF, Aarts MJB, Eble JA, Campello E, Spiezia L, Simioni P, van der Vorst EPC, van der Meijden PEJ, Heemskerk JWM and Kuijpers MJE

(* equal contribution)

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Abstract

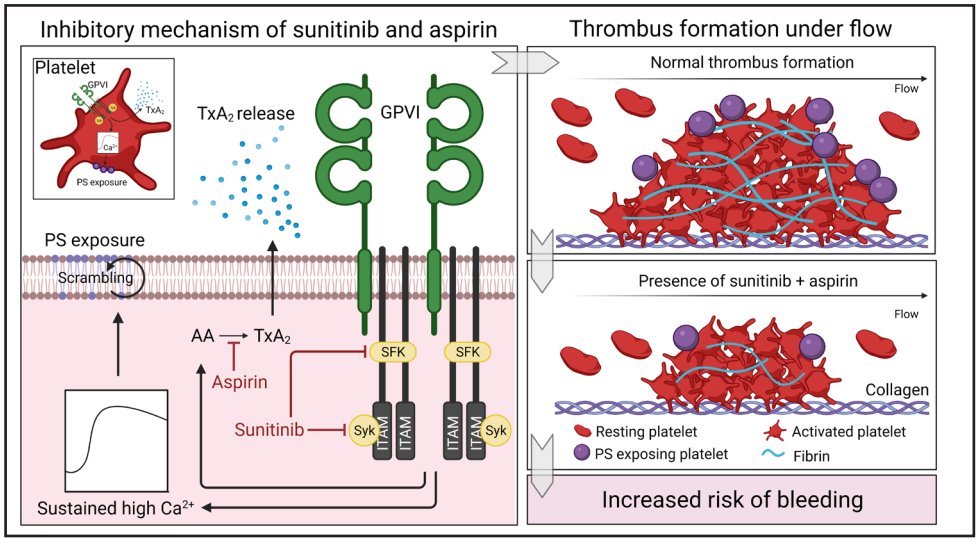
Background: Sunitinib is a multi-target tyrosine kinase inhibitor (TKI) used for cancer treatment. In platelets, sunitinib affects collagen-induced activation under non-coagulating conditions. We investigated (i) the effects of sunitinib on thrombus formation induced by other TK-dependent receptors, and (ii) the effects under coagulating conditions. Cardiovascular disease is a co-morbidity in cancer patients, resulting in possible aspirin treatment. Sunitinib and aspirin are associated with increased bleeding risk, and therefore we also investigated (iii) the synergistic effects of these compounds on thrombus and fibrin formation.

Methods: Blood or isolated platelets from healthy volunteers or cancer patients were incubated with sunitinib and/or aspirin or vehicle. Platelet activation was determined by TK phosphorylation, flow cytometry, changes in $[Ca^{2+}]_i$, aggregometry and whole blood perfusion over multiple surfaces, including collagen with(out) tissue factor (TF) was performed.

Results: Sunitinib reduced thrombus formation and phosphatidylserine (PS) exposure under flow on collagen type I and III. Also, sunitinib inhibited GPVI-induced TK phosphorylation and Ca^{2+} elevation. Upon TF-triggered coagulation, sunitinib decreased PS exposure and fibrin formation. In blood from cancer patients more pronounced effects of sunitinib were observed in lung and pancreatic as compared to neuroglioblastoma and other cancer types. Compared to sunitinib alone, sunitinib plus aspirin further reduced platelet aggregation, thrombus formation and PS exposure on collagen under flow with(out) coagulation.

Conclusions: Sunitinib suppresses collagen-induced procoagulant activity and delays fibrin formation, which was aggravated by aspirin. Therefore, we urge for awareness of the combined antiplatelet effects of TKIs with aspirin, as this may result in increased risk of bleeding.

Visual abstract



Introduction

Treatment of cancer patients with sunitinib results in anticancer effects and improves progression free survival[1, 2]. Sunitinib is a broad-spectrum tyrosine kinase inhibitor (TKI), affecting not only tumour cells, but also the blood vessel wall[3]. A side effect of sunitinib treatment is mild bleeding (mainly epistaxis, and mucocutaneous and gastrointestinal bleeding, which occurs in approximately 19% of sunitinib-treated patients)[3]. Fortunately, in the majority of cases, the effects on haemostasis remain sub-clinical.

Platelets contribute to haemostasis by signalling pathways involving multiple protein tyrosine kinases (TKs) such as Syk, Btk and Src family kinases (SFKs)[4]. We and other authors have previously shown that sunitinib is sequestered by platelets, which affects collagen-induced platelet function under non-coagulating conditions[5, 6]. TKs do not only signal under the collagen receptor glycoprotein (GP)VI, but also under the receptors GPIb, CLEC-2 and integrins $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ that bind von Willebrand factor (vWF), podoplanin, laminin and fibrinogen, respectively[4]. The effects of sunitinib on thrombus formation stimulated via these receptors has not been studied. Furthermore, the question arises whether the suppression of collagen-induced thrombus formation is also present under physiological conditions of coagulation.

The link between cancer and arterial thrombosis has been well-established[7, 8]. Cancer patients with a cardiovascular history receive treatment with anti-platelet drugs like aspirin to prevent recurrent events. Aspirin irreversibly inhibits platelet activation and is associated with an increased bleeding risk[9] by preventing the formation of thromboxane A_2 (TxA_2)[10]. TxA_2 acts as an important soluble agonist released by platelets upon GPVI stimulation. In this respect, aspirin and other platelet-targeted pharmacologic treatments are under investigation as adjuvant anti-cancer therapy[11, 12]. The combined effects of these drugs on platelet function have not been investigated thus far.

The aim of the present study was to further investigate the effect of sunitinib on thrombus formation by stimulation with multiple agonists, as well as under coagulating conditions stimulated by collagen plus TF. Furthermore, we assessed whether treatment with sunitinib in combination with aspirin increasingly inhibited collagen-induced thrombus formation as compared to either compound alone. The results indeed show an enhancing effect of this dual treatment of platelets with sunitinib and aspirin on collagen-induced platelet phosphatidylserine (PS) exposure and ensuing fibrin formation. Finally, platelet-dependent fibrin formation in whole blood from cancer patients was significantly inhibited by sunitinib.

Materials and Methods

Materials

Sunitinib malate (Sutent) was provided by Pfizer (New York NY, USA). Aspirin, bovine serum albumin (BSA), D(+)-glucose, unfractionated heparin, and apyrase were purchased from

Sigma-Aldrich (Saint Louis MO, USA). The agonists collagen-related peptide crosslinked (CRP-XL) and Von Willebrand factor III (vWF-III) were obtained from CambCol Laboratories (Cambridge, UK), whereas thrombin was obtained from Enzyme Research Laboratories (South Bend IN, USA) and Horm collagen type I from Takeda (Hoofddorp, The Netherlands). 2-Methylthio-adenosine-diphosphate (2MeS-ADP) and D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) were obtained from Santa Cruz Biotechnology (Dallas TX, USA). Recombinant human TF (Innovin) was purchased from Dade Behring (Deerfield IL, USA). Laminin came from Octapharma (Berlin, Germany). Fura-2-AM was obtained from Invitrogen (Carlsbad CA, USA). Plastic syringes and fluorescein isothiocyanate (FITC)-labelled PAC1 monoclonal antibody (mAb) against activated human integrin $\alpha_{IIb}\beta_3$ were purchased from BD Bioscience (nr. 340507; Franklin Lakes NJ, USA), while FITC-conjugated α-fibrinogen mAb was purchased from DAKO (F0111; Santa Clara CA, USA). Alexa Fluor (AF) 647-labelled CD62-P mAb was obtained from Biolegend (London, UK), whereas the FITC-labelled anti-human CD62-P mAb was obtained from Beckman Coulter (nr. 65050; Sydney, Australia). 3,3-dihexyloxacarbocyanine iodide (DiOC6) was purchased from Anaspec (Reeuwijk, The Netherlands). FITC-conjugated annexin A5 was from Pharmatarget (Maastricht, The Netherlands). AF568-conjugated annexin A5 and AF647-labelled fibrinogen were purchased from Molecular Probes, Life Technologies (New York NY, USA).

Blood collection and platelet isolation

With approval from the medical ethics committee from the Maastricht University Medical Centre+ (MUMC+) and informed consent in accordance with the Declaration of Helsinki, blood was collected from healthy volunteers. Furthermore, blood was collected from 11 patients diagnosed with several cancer types (lung, pancreas, neuroglioblastoma, ovarian or paraganglioma) at the University Hospital of Padua (Italy), also with approval of the local medical ethical committee and after informed consent. Blood was collected in 3.2% trisodium citrate tubes by venipuncture, after discarding the first 3 mL of blood. Platelets, plasma or serum were isolated from whole blood as described previously[13]. Whole blood or isolated platelets were incubated with either vehicle or sunitinib (10 or 30 μM as indicated) for 10 minutes at room temperature (unless stated otherwise) before measurements.

Flow cytometry

Washed platelets ($100x10^9$ platelets/L) were supplemented with 2 mM $CaCl_2$ and stimulated by a combination of CRP-XL (5 μg/mL) and thrombin (4 nM) for 1 hour at 37°C. Phosphatidyl-serine (PS) exposing platelets were labelled with FITC-conjugated annexin A5 and measured by flow cytometry using a BD Accuri C6TM flow cytometer and accompanying software (Erembodegem, Belgium).

Cytosolic Ca^{2+} measurements

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements in Fura-2 loaded platelets ($200x10^9$ platelet/L) were performed as described using a Shimadzo RF-5001PC spectrofluorophotometer (Kyoto, Japan)[14]. Levels of $[Ca^{2+}]_i$ were determined by the conversion of ratio values[15] with correction for background fluorescence and maximal values of $[Ca^{2+}]_i$ (peak level) were used as output.

Light transmission aggregometry

Washed platelets or PRP ($250x10^9$ platelets/L) were incubated with vehicle or sunitinib (10 or 30 µM as indicated) for 10 minutes at 37°C. Where indicated, aspirin (100 µM) was incubated in PRP for 30 minutes at 37°C before further platelet isolation. Platelet aggregation was induced by collagen type I (5 µg/mL), 2MeS-ADP (1 µM) or thrombin (1 nM) and aggregation responses were measured using a Chronolog optical aggregometer (Havertown PA, USA) and maximum amplitude was quantified.

Pam-Gene kinase assay

Washed platelets ($500x10^9$ platelets/L) were pretreated with vehicle (control) or 30 µM sunitinib for 10 minutes at 37°C and were subsequently stimulated with 5 µg/mL CRP-XL in the presence of 2 mM $CaCl_2$. Unstimulated, resting platelets served as control. After 90 seconds of stimulation, samples were lysed by adding 1:1 M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Samples were lysed for 15 minutes on ice and afterwards centrifuged for 15 minutes at 10,000 *g* at 4°C. Supernatants were collected and protein content was quantified with a BioRad DC protein kit (Hercules CA, USA).

Tyrosine kinase profiles were determined using the PamChip® peptide tyrosine kinase microarray system on PamStation®12 (PTK; PamGene International, ´s-Hertogenbosch, The Netherlands). Each PTK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for TKs. Each peptide on the chip builds a 15-amino acid sequence representing a putative endogenous phosphorylation site which functions as a TK substrate. The phosphorylation of the peptides is visualized by detection of the fluorescent signal which is emitted as a result of the binding of the FITC-conjugated PY20 anti-phosphotyrosine antibody.

For the PTK assay, 7.5 µg of protein was applied per array (N=3 per condition) and carried out using the standard protocol supplied by Pamgene. All reagents used for PTK activity profiling were supplied by Pamgene International B.V. Initially, to prepare the PTK Basic Mix, the freshly frozen lysate was added to 4 µL of 10x protein PTK reaction buffer (PK), 0.4 µL of 100x BSA, 0.4 µL of 1M dithiothreitol (DTT) solution, 4 µL of 10x PTK additive, 4 µL of 4 mM ATP and 0.6 µL of monoclonal anti-phosphotyrosine FITC-conjugate detection antibody (clone PY20). Total volume

of the PTK Basic Mix was adjusted to 40µL by adding distilled water (H_20). Before loading the PTK Basic Mix on the array, a blocking step was performed applying 30µL of 2% BSA to the middle of every array and washing with PTK solution for PamChip® preprocessing. Next, 40µL of PTK Basic Mix were applied to each array of the PamChips®. Then, the microarray assays were run for 94 cycles. An image was recorded by a CCD camera PamStation®12 at kinetic read cycles 32–93 at 10, 50 and 200ms and at end-level read cycle at 10, 20, 50, 100 and 200ms. The spot intensity at each time point was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands). Upstream Kinase Analysis (UKA)[16], a functional scoring method (PamGene) was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from 6 databases) and sensitivity scores (based on treatment-control differences).

Whole blood perfusion experiments with(out) coagulation and quantification

Whole blood perfusion experiments without coagulation were performed as described before[17]. Platelet activation properties were determined by staining for integrin activation, P-selectin expression and PS exposure using FITC-conjugated α-fibrinogen mAb (1:80), AF647-conjugated CD62-P (1:100), and AF568-conjugated annexin A5 (1:200).

Whole blood perfusion experiments under coagulating conditions were performed as described before[18]. Platelet activation properties were determined by supplementing blood samples with AF647-fibrinogen (16.5 µg/mL f.c), DiOC6 (0.5 µg/mL f.c.), and AF568-Annexin A5 (1:200).

Image capturing was performed using an EVOS microscope (Life Technologies, Carlsbad CA, USA). All images were analysed using specific (half-automated) scripts in the open-access Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison WI, USA) as described[17]. For comparative data analysis heatmaps were generated from the mean values per parameter for each surface, which were scaled to a range from 0-10 based on the highest value per parameter. In order to visualize effects more clearly, scaled data were subtracted from control data to obtain subtraction heatmaps. The effects in subtracted heatmaps were filtered based on significant differences or 1x standard deviation as indicated.

Statistical analysis

Data are presented as mean ± SEM. GraphPad Prism 8.3.0 software (La Jolla CA, USA) was used for statistical analyses, using the paired and nonparametric Wilcoxon test to compare means of 2 parallel experimental conditions and one-way non-parametric Anova (Kruskal-Wallis) for experiments with more than 2 conditions. In case of multiple experimental conditions and timepoints, a nonparametric 2-way Anova was used. A p-value less than 0.05 was considered statistically significant in which * is $p<0.05$; ** is $p<0.01$ and *** is $p<0.001$.

Results

Sunitinib reduces thrombus formation and PS exposure in whole blood under flow on collagen type I and III

To widely explore the multi-target inhibition of sunitinib on thrombus formation, we investigated thrombus formation induced by several TK-dependent receptors using multiple surfaces for coating in whole blood flow chamber experiments. First, we performed dose-response studies with sunitinib and showed that approximately 3x higher concentration of this compound was required to inhibit aggregation induced by collagen (1 µg/ml) in the presence of plasma when compared to washed platelets (Suppl. Figure 4.1). This agrees with the knowledge that sunitinib is highly protein-bound[1].We therefore assessed the effects on thrombus formation during whole blood perfusion under flow using 30 µM sunitinib. This dose is in range with the plasma concentrations observed in patients with higher peak levels of sunitinib[19],but exceeded the therapeutic target levels[1].

The flow studies were performed in a microspot setting, allowing the quasi-simultaneous measurement of sunitinib on adhesive surfaces consisting of collagen type I, collagen type III, vWF co-coated with rhodocytin, vWF co-coated with laminin, vWF co-coated with fibrinogen and vWF co-coated with ristocetin. Hence, roles of different platelet receptors, such as GPVI, CLEC-2, GPIb and integrin $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$, were investigated as described before[20-22]. Captured microscopic images were analysed (Suppl. Figure 4.2), and a heatmap was generated for a more systematic analysis of the seven thrombus parameters, which were averaged and scaled per parameter (Figure 4.1). Parameters of thrombus formation were only consistently affected on GPVI-dependent surfaces, i.e. collagen type I and III (Figure 4.1A, B). In the presence of sunitinib, these thrombi were smaller and less compact in structure as compared to the control condition (Suppl. Figure 4.2A and B, brightfield images). Quantification showed a reduction in platelet deposition, thrombus height and contraction (Figure 4.1). P-selectin exposure was significantly reduced in thrombi formed on collagen III and vWF co-coated with laminin, fibrinogen and ristocetin (Suppl. Figure 4.2B and 4.2C). Notably, thrombus parameters for CLEC-2, GPIb- and integrin $\alpha_{IIb}\beta_3/\alpha_6\beta_1$-dependent surfaces were not affected by sunitinib (Figure 4.1C-F, Suppl. Figure 4.2C-F), suggesting a mostly GPVI-specific effect.

Sunitinib inhibits multiple tyrosine kinases, intracellular Ca^{2+} responses and PS exposure upon GPVI stimulation

We have previously shown that overall tyrosine phosphorylation upon GPVI stimulation is inhibited by sunitinib[5]. To study the effect of sunitinib on GPVI-induced phosphorylation of multiple specific TKs simultaneously, we performed a PamGene kinase assay. We observed that in isolated platelets, in total 34 TKs were significantly phosphorylated by stimulation with CRP-XL, which were subsequently significantly inhibited by sunitinib preincubation (Figure 4.2A). Most

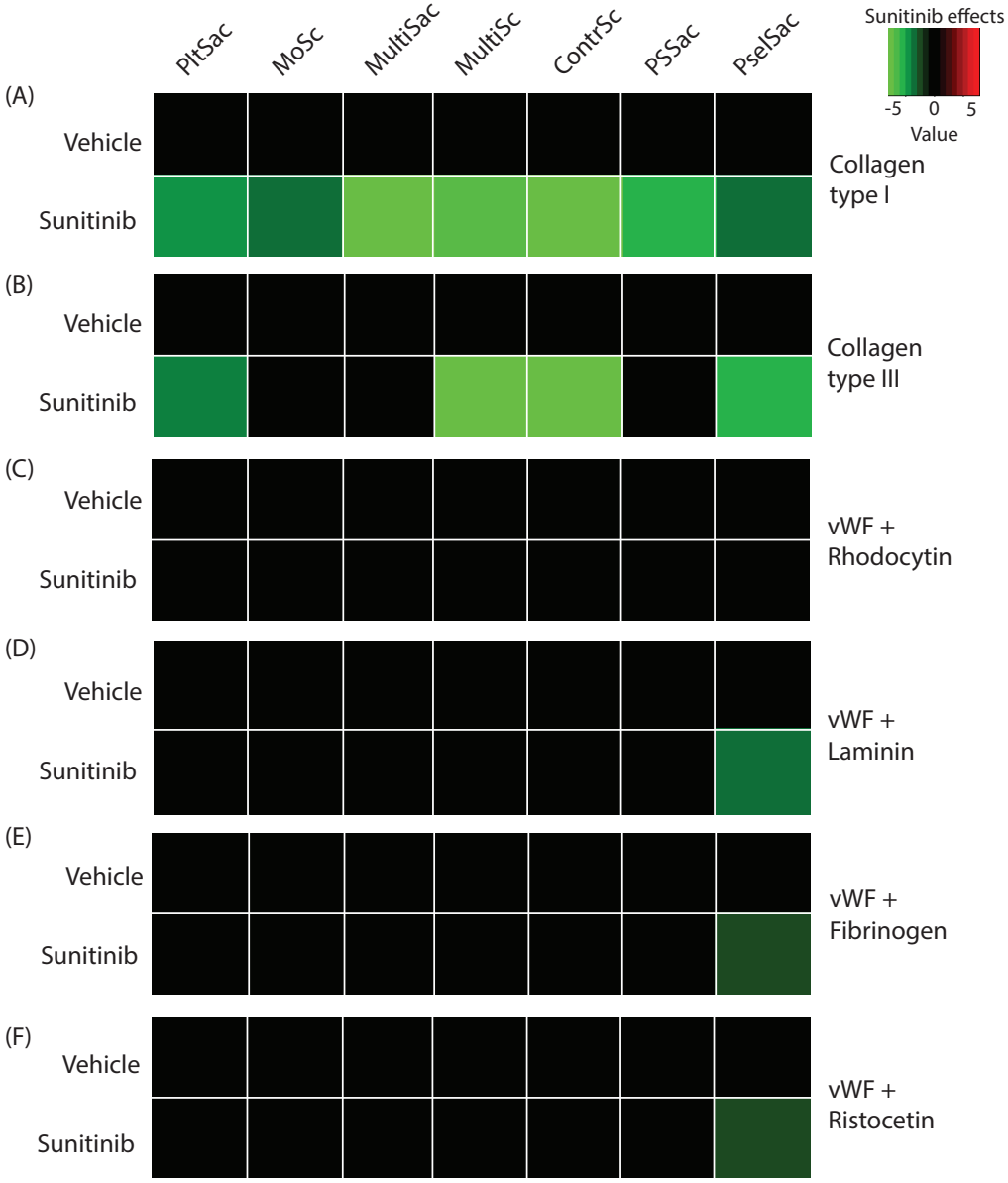


Figure 4.1. Sunitinib strongly reduces thrombus formation on GPVI-dependent surfaces, as compared to surfaces for CLEC-2, GPIb, integrin $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$. Whole blood from healthy volunteers was preincubated with vehicle (<0.1% DMSO) or sunitinib (30 µM) for 10 min and perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over coated microspots containing (A) collagen type I, (B) collagen type III and (C) vWF plus rhodocytin, (D) vWF plus laminin, (E) vWF plus fibrinogen and (F) vWF plus ristocetin. Subtraction heatmaps are shown representing significant effects of normalized values from the following parameters: platelet deposition (PltSac), morphological score (MoSc), multilayer deposition (MultiSac), multilayer score (MultiSc), contraction score (ContrSc), PS exposure deposition (PSSac) and P-selectin deposition (PselSac). Controls with addition of vehicle (<0.1% DMSO) were set at 0 for reference. Effects were filtered for significant changes ($p<0.05$).

relevant for GPVI signalling are Syk and the SFKs Src, Fyn, and Yes, which all show a high level of inhibition in the presence of sunitinib. Interestingly, the Focal Adhesion Kinases (FAK1 and 2) which signal mostly downstream of integrins[23], as well as the Gas6 receptor Tyro3/Sky showed a similar pattern of regulation. Furthermore, also other non-GPVI-linked TKs were activated upon GPVI stimulation, most likely via agonists secreted from the α-granules. Overall, this assay clearly shows the multi-target nature of sunitinib with regard to TK inhibition in platelets.

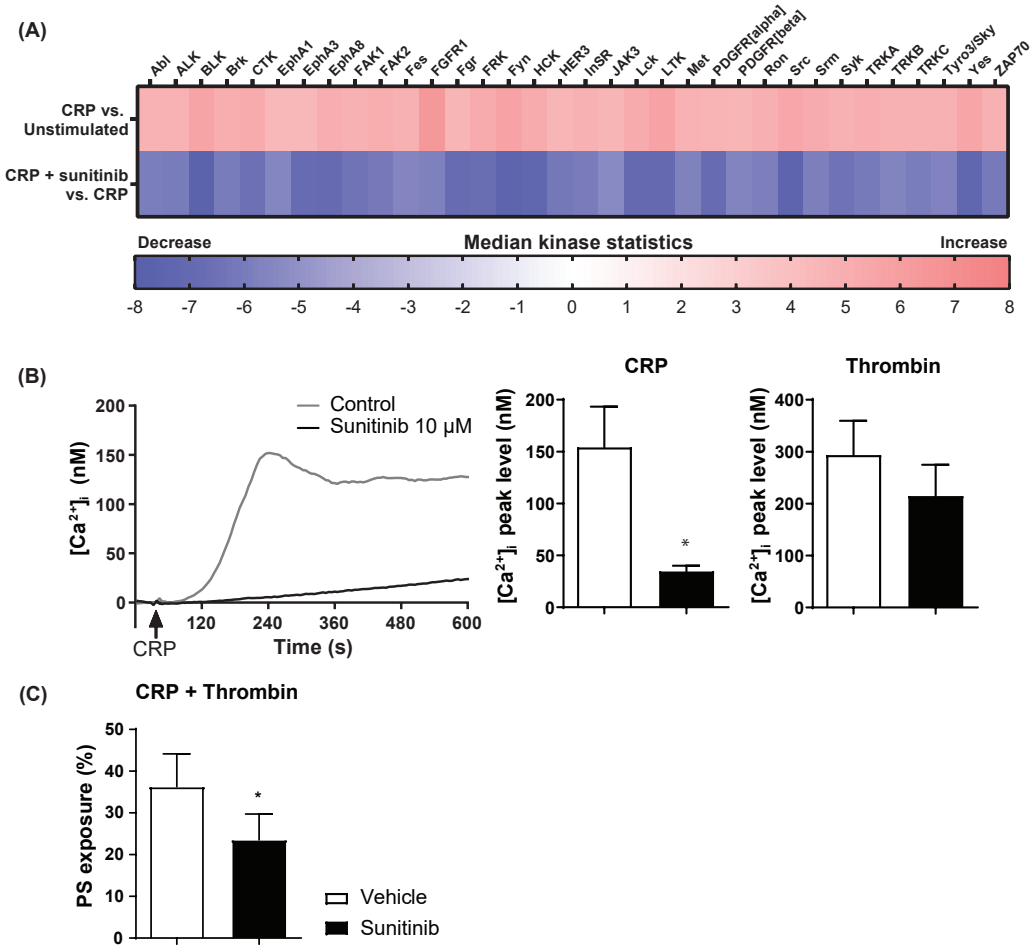


Figure 4.2. Sunitinib inhibits GPVI-induced tyrosine kinase activity, Ca^{2+} responses and PS exposure in washed platelets. (A) Heatmaps visualize all tyrosine kinases that are significantly up- or downregulated in both comparisons (sorted alphabetically). Cut-off has been set at a median final score of at least 1.2. Colour indicator reflects the calculated median kinase statistic reflecting effect size and directionality (red: upregulated; blue: downregulated). (B) Representative curve of a calcium response of Fura-2 loaded platelets ($200x10^9$/L); arrow indicates addition of CRP-XL (5 µg/mL). Bar graphs show quantification of maximum (peak) level of the calcium response induced by CRP-XL (5 µg/mL) or thrombin (1 nM) of Fura-2 loaded platelets in presence (black bars) or absence of sunitinib (white bars). All data are represented as means + SEM (n=5-7), *p<0.05. (C) Isolated platelets from healthy volunteers were incubated with vehicle (<0.1% DMSO) or sunitinib (10 µM) for 10 minutes at RT. Platelets ($100x10^9$/L) were activated in the presence (black bars) or absence of sunitinib (white bars) by CRP-XL (5 µg/mL) in combination with thrombin (4 nM) and incubated for 60 minutes at 37°C. PS exposure was detected by FITC-conjugated annexin A5 and measured by flow cytometry.

GPVI signalling results in sustained elevation of intracellular calcium levels ($[Ca^{2+}]_i$) upon stimulation, resulting in PS-exposure[24]. In accordance with the effects on TK phosphorylation, also the Ca^{2+} increase in response to CRP-XL was nearly abolished in the presence of sunitinib (Figure 4.2B). Elevation in $[Ca^{2+}]_i$ upon thrombin stimulation was not significantly affected. Also, platelet procoagulant activity (PS exposure), was inhibited by sunitinib in isolated platelets stimulated with CRP plus thrombin (Figure 4.2C). This was in agreement the reduction of PS exposure in whole blood under flow, only on collagen type I (Figure 4.1A).

Considering that GPVI and CLEC-2 display many similarities in the downstream signalling pathways[25],we additionally determined the effects of sunitinib on CLEC-2 dependent platelet activation. We observed that in isolated platelets, both rhodocytin-induced aggregation, as well as activation of integrin $\alpha_{IIb}\beta_3$ and expression of P-selectin were significantly reduced by sunitinib (Suppl. Fig 4.3A, B). Also, PS exposure as induced by rhodocytin plus thrombin was significantly reduced by sunitinib (Suppl. Fig. 4.3C). However, we did not observe an effect of sunitinib in whole blood thrombus formation on rhodocytin plus vWF (Fig. 4.1C, Suppl. Fig 4.2C), suggesting that CLEC-2-induced thrombus formation was not affected by sunitinib in whole blood.

So far, all data showed that sunitinib most prominently reduced phosphorylation of TKs and rises of intracellular Ca^{2+} levels, resulting in inhibition of thrombus formation and PS exposure induced by the collagen receptor GPVI. Therefore, we used collagen type I for further experiments.

Aspirin enhances sunitinib-induced inhibition of platelet aggregation and thrombus formation

To investigate the combined effect of sunitinib and aspirin on platelet function, we started by measuring platelet aggregation. Sunitinib alone caused a minor inhibition (±14%) of the aggregation response induced by a high dose (5 µg/ml) of collagen, whereas aspirin inhibited this collagen-induced aggregation by ±70% (Figure 4.3A). The combination of sunitinib and aspirin reduced the aggregation response even further as compared to aspirin alone (>80%; Figure 4.3A). Similar results could be observed when platelets were stimulated with 2MeS-ADP (Figure 4.3B). However, both sunitinib and/or aspirin treatment did not affect the aggregation response elicited by thrombin (Figure 4.3C).

Next, we determined the effects of sunitinib and/or aspirin in whole blood thrombus formation over collagen type I. When aspirin was added *in vitro*, we only observed a significant effect on thrombus contraction, while other parameters were not affected (Figure 4.3D). No additional effects on thrombus formation in whole blood could be observed when both aspirin and sunitinib were added *in vitro* (Figure 4.3D). Subsequently, we obtained blood from healthy volunteers who had taken a single dose of aspirin (100 mg) one day before. Treatment with aspirin inhibited platelet deposition as compared to controls without aspirin intake (Figure 4.3E, Suppl. Figure 4.4). Addition of sunitinib to blood from aspirin-treated donors further enhanced the

effects that were observed with aspirin alone (Figure 4.3E). Especially thrombus height (multilayer) and PS exposure were reduced when both drugs were present, as compared to aspirin alone (Figure 4.3E). Altogether, these results suggest that dual treatment with anti-platelet therapy and sunitinib increases the inhibition of platelet function.

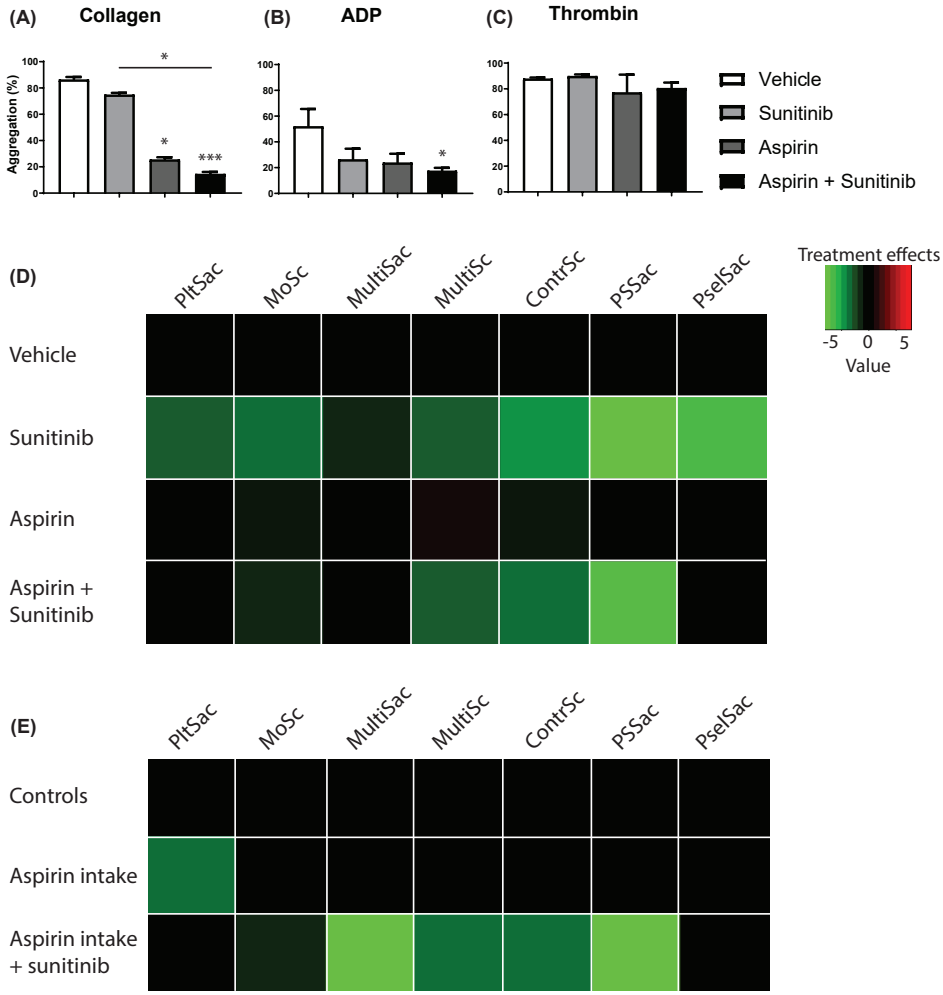


Figure 4.3. Aspirin enhances the effect of sunitinib in collagen-induced platelet aggregation and in *ex vivo* thrombus formation on collagen in whole blood after aspirin intake. Washed platelets ($250x10^9$/L) were incubated with vehicle (<0.1% DMSO), aspirin (100 µM) and/or sunitinib (10 µM). Aggregation of washed platelets was induced by (A) 5 µg/mL collagen, (B) 1 µM 2MeS-ADP or (C) 1 nM thrombin. Data are represented as means + SEM (n=3), *p<0.05, ***p<0.001. (D, E) Whole blood from healthy volunteers was preincubated with vehicle (<0.1% DMSO), sunitinib (30 µM) and/or aspirin (100 µM) for 10 min. Recalcified whole blood was perfused over a collagen surface at 1000 s^{-1} for 3.5 minutes, followed by perfusion with buffer supplemented with AF568-annexin A5 and AF647-α-CD62P to detect PS exposure and P-selectin expression, respectively. (D) Subtraction heatmaps representing the significant effects of normalized values of the parameters. Controls with addition of vehicle (<0.1% DMSO) were set at 0 for reference. Effects were filtered for significant alterations by addition of sunitinib and/or aspirin. (E) Subtraction heatmaps representing the significant effects of normalized values of parameters as for supplemental Figure 4.4B. Healthy controls were set at 0 for reference to aspirin intake or aspirin intake with *in vitro* addition of sunitinib. Effects were filtered for significant changes of the different platelet parameters (p≤0.05).

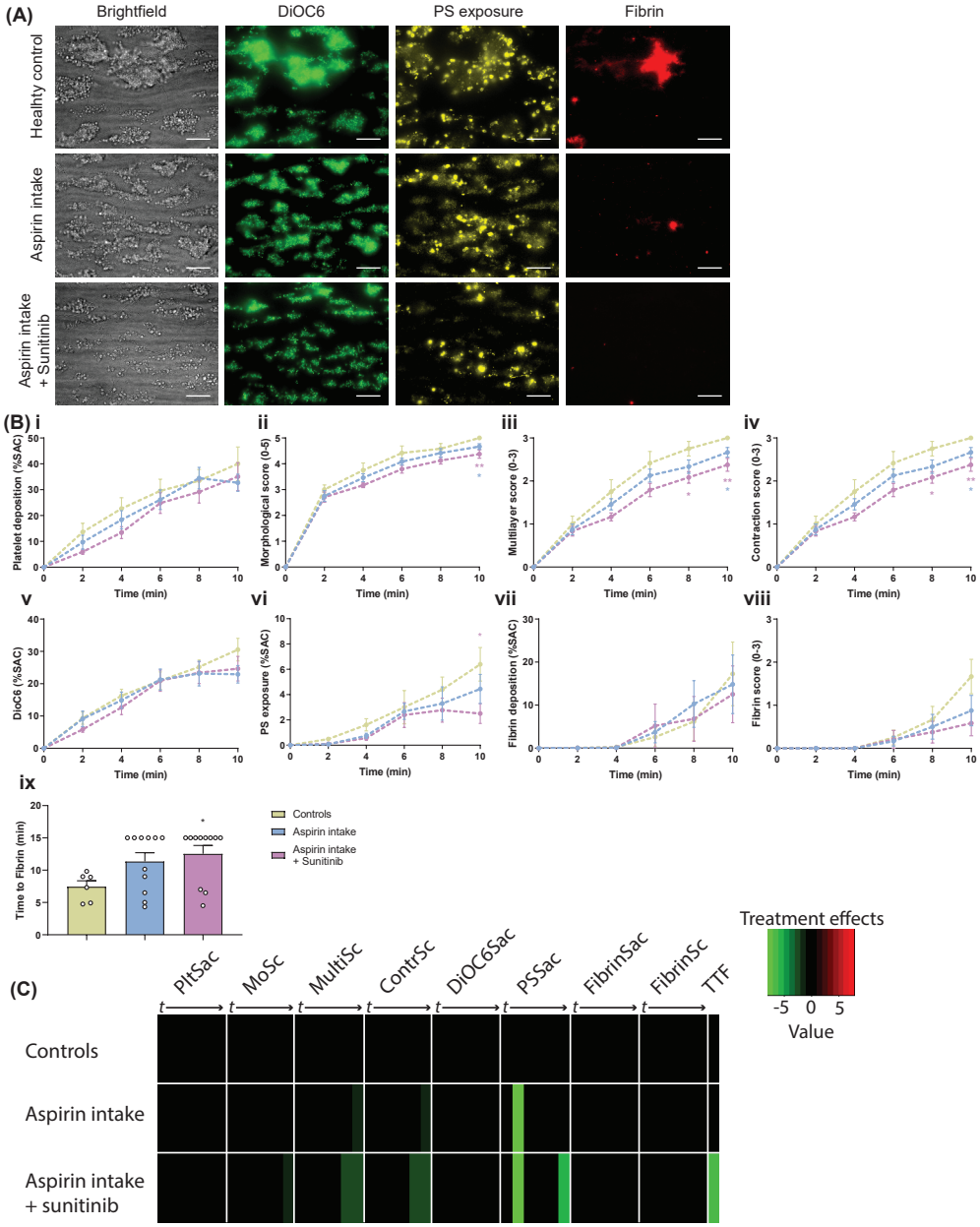


Figure 4.4. Aspirin partially enhances the inhibiting effect of sunitinib in platelet-dependent fibrin formation under flow. Citrated whole blood of healthy volunteers with single dose of aspirin was treated *in vitro* with vehicle (<0.1% DMSO) or sunitinib (30 µM) for 10 min followed by addition of DiOC6 (platelet deposition), AF568-annexin A5 (PS-exposure) and AF647-α-fibrinogen (fibrin). Blood was co-perfused with $CaCl_2$/$MgCl_2$ over collagen type I at a wall shear rate of 1000 s^{-1}. (A) Representative brightfield and fluorescence images taken after 10 minutes of blood perfusion. (B) Kinetic analysis of brightfield images of (i) platelet deposition (PltSac), (ii) morphological score (MoSc), (iii) multilayer score (MultiSc), and (iv) contraction score (ContrSc) and of fluorescence images of (v) DiOC6 platelet deposition (DiOC6Sac), (vi) PS exposure (PSSac), (vii) fibrin deposition (FibrinSac), (viii) fibrin score (FibrinSc) and (ix) time to fibrin (TTF). Data are represented as means + SEM (n=6), *p<0.05, **p<0.01. (C) Heatmap representing the significant effects of normalized values of parameters analysed in B. Healthy controls were set at 0 for reference to aspirin intake or aspirin intake with *in vitro* addition of sunitinib. Effects were filtered for significant changes of the different platelet parameters (p≤0.05).

Aspirin enhances sunitinib effects on thrombus fibrin formation

As we observed that the exposure of procoagulant PS was reduced by sunitinib (plus aspirin), we further investigated whether these effects extended to conditions of thrombin generation and fibrin formation. Hence, we assessed the kinetics of platelet-fibrin thrombus formation under flow on a collagen surface co-coated with TF[18]. Recalcified whole blood was labelled with DiOC6, AF568-Annexin A5 and AF647-fibrinogen, and was perfused over a microspot consisting of collagen type I plus TF. Using control blood samples, we observed time-dependent increases in platelet deposition and thrombus formation in time, with the start of fibrin formation after 7-8 minutes (Figure 4.4A). Preincubation of blood with sunitinib did not affect the parameters of platelet deposition and thrombus size in time, but sunitinib significantly reduced PS exposure with a concomitant delay in fibrin formation (Suppl. Figure 4.5). Of note, sunitinib did not affect thrombin generation under static conditions, as measured with the calibrated automatic thrombogram[26] in PRP stimulated with collagen (data not shown). Markedly, using blood from aspirin-treated healthy volunteers, we observed a reduction in thrombus size, height and density as compared to healthy volunteers without aspirin intake (Figure 4.4A, B). Furthermore, a slight decrease in PS exposure as well as a delay in fibrin formation was observed upon aspirin intake (Figure 4.4). These effects were further reduced by *in vitro* addition of sunitinib (Figure 4.4C). Especially, effects on thrombus height, contraction, PS exposure and time to fibrin generation were increased upon dual treatment with aspirin and sunitinib as compared to aspirin alone. Together, these results suggest that sunitinib reduces GPVI-induced activation of platelets resulting in decreased PS exposure and thereby delaying the time for fibrin to be formed, which is enhanced by aspirin intake.

Sunitinib reduces platelet-dependent fibrin formation under flow in whole blood from cancer patients

Previously, we demonstrated that sunitinib reduced thrombus formation and PS exposure in whole blood from renal cell carcinoma (RCC) patients under non-coagulating conditions[5]. To explore whether sunitinib could also reduce platelet-dependent fibrin formation under flow in blood from cancer patients, we obtained blood from 11 patients diagnosed with lung, pancreatic and (neuro)glioblastoma cancer (Suppl. Table 4.1). Sunitinib has been reported to be used as a (combination) therapeutic strategy for these types of cancer[27-29]. As a proof-of-principle, we preincubated recalcified whole blood from these patients with vehicle or sunitinib followed by labelling with DiOC6, AF568-Annexin A5 and AF647-fibrinogen. Upon perfusion of the vehicle-treated blood over collagen co-coated with TF, we observed increases in platelet deposition and thrombus formation in time, with a median start of fibrin formation after 7-8 minutes, comparable to healthy controls (Suppl. Figure 4.6A, B). Three patients were treated with anticoagulant or antiplatelet drugs (Suppl. Table 4.1), but this did not affect thrombus/fibrin formation as

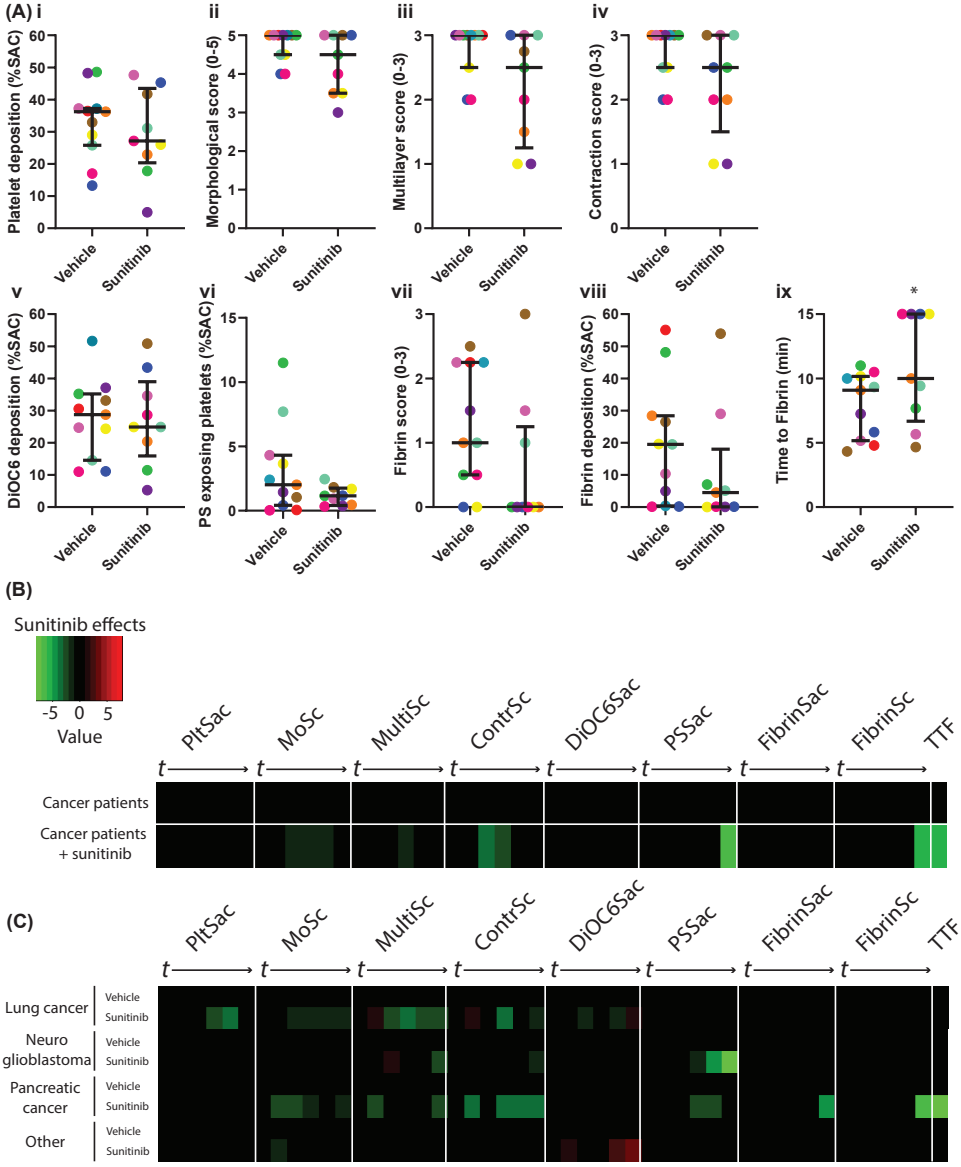


Figure 4.5. Sunitinib reduces platelet-dependent fibrin formation under flow in whole blood from cancer patients. Citrated whole blood from cancer patients (suppl. Table I) was treated *in vitro* with vehicle (<0.1% DMSO) or sunitinib (30 μM) for 10 min, followed by addition of DiOC6 (platelet deposition), AF568-annexin A5 (PS-exposure) and AF647-α-fibrinogen (fibrin). Blood was co-perfused with $CaCl_2/MgCl_2$ over collagen type I plus TF at a wall shear rate of 1000 s^{-1}. (A) Endpoint analysis of brightfield images of (i) platelet deposition (PltSac), (ii) morphological score (MoSc), (iii) multilayer score (MultiSc), and (iv) contraction score (ContrSc) and of fluorescence images of (v) DiOC6 platelet deposition (DiOC6Sac), (vi) PS exposure (PSSac), (vii) fibrin deposition (FibrinSac), (viii) fibrin score (FibrinSc) and (ix) time to fibrin (TTF). Data are represented as median ± interquartile range (n=9-11). Each dot represents an individual cancer patient (colour-coding in Suppl. Table 4.1), *p<0.05. (B) Subtraction heatmap representing the overall effect of sunitinib in cancer patients. The effects of normalized values of parameters during time are based on the analysis of Suppl. Figure 7. Vehicle treated blood of cancer patients was set at 0 for reference to *in vitro* addition of sunitinib. Effects were filtered for significant changes between the different platelet parameters (p≤0.05). (C) Subtraction heatmap of the effect of sunitinib per cancer type. Effects were filtered for changes greater than the 1x SD range of the different platelet parameters.

compared to the patients without this treatment. Preincubation of blood with sunitinib showed a trend in overall inhibition of thrombus size and height in time, as well as a reduction in PS exposure with a significant delay in fibrin formation (Figure 4.5A and Suppl. Figure 4.6A, B). To visualize the overall effects of sunitinib in blood from cancer patients, we generated a heatmap (Figure 4.5B). This heatmap clearly shows that sunitinib inhibits platelet PS-exposure and fibrin formation, but also some effects on thrombus formation parameters can be observed. We included patients with different types of cancer, which this may result in different cancer-specific responses. Therefore, we also generated a heatmap with the patients averaged per cancer type (Figure 4.5C). This heatmap shows that in lung and pancreatic cancer, more pronounced effects of sunitinib were observed on thrombus formation parameters (i.e. height, size and contraction) as compared to neuroglioblastoma and other cancer types. This variable response to sunitinib treatment was even more pronounced when a heatmap was generated per patient (suppl. Figure 4.6C). This heatmap shows that the effects of sunitinib on thrombus and fibrin formation under flow varied more between individual patients than between the types of cancer. Overall, in 6 of the 9 patients were sunitinib was added we observed clear inhibition of thrombus formation and/or PS exposure and/or fibrin generation. This suggested that the *in vitro* inhibitory effects of sunitinib were more pronounced in cancer patients (Figure 4.5C and suppl. Figure 4.6C) than in healthy controls (Suppl. Figure 4.5).

Discussion

In the present study we further explored the multi-target inhibition of sunitinib on thrombus formation, by investigating thrombus formation by stimulation with multiple agonists, as well as under coagulating conditions stimulated by collagen plus TF. We observed that sunitinib predominantly inhibited collagen-induced exposure of phosphatidylserine, resulting in delayed fibrin formation. Furthermore, we found an enhancing effect of dual treatment of platelets with sunitinib and aspirin in these processes. Finally, platelet-dependent fibrin formation in whole blood from cancer patients was significantly inhibited by sunitinib.

We have previously reported that platelets sequester sunitinib in vitro as well as in cancer patients and that this uptake inhibits platelet function in response to collagen[5]. As sunitinib is a multi-target TKI and TKs do not only signal under the collagen receptor GPVI, but also under the receptors GPIb, CLEC-2 and integrins $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$[21], we investigated the effects of sunitinib on thrombus formation in whole blood perfused over multiple agonists. However, sunitinib did not affect thrombus formation on CLEC-2, GPIb- and integrin $\alpha_{IIb}\beta_3/\alpha_6\beta_1$-dependent surfaces, which pointed to GPVI as the main signalling pathway inhibited by this TKI. We did observe that sunitinib inhibited CLEC-2-induced aggregation and activation responses in isolated platelets. This is in line with the many similarities in the downstream signalling pathways underneath GPVI and CLEC-2[25]. The inhibiting effects of sunitinib on CLEC-2-induced thrombus formation were however absent in

whole blood. This may be explained by a stronger dependence on Syk of CLEC-2 signalling, while GPVI signalling is more dependent on Src[25]. Inhibition of Src by sunitinib upon GPVI stimulation was more pronounced as compared to inhibition of Syk (Figure 4.2A), suggesting that the effects of sunitinib on CLEC-2 signalling may be less prominent, especially in whole blood conditions. Furthermore, we noted that higher collagen doses overrule the inhibiting effects of sunitinib at 10 µM. Hence, a higher concentration of sunitinib may be required to inhibit platelet activation at higher agonist concentrations. A possible role for integrin $\alpha_2\beta_1$, the other collagen receptor on platelets, should be mentioned here as well, as this receptor also signals via Src and Syk kinases[30]. It has been shown that integrin $\alpha_2\beta_1$ has a supporting role with regard to platelet activation and contributes to stable thrombus formation on collagen[31]. As we have shown that sunitinib strongly reduced phosphorylation of Syk and Src, the inhibiting effects observed in thrombus formation on collagen may be partly due to inhibition of integrin $\alpha_2\beta_1$ signalling. In addition, thrombus formation under flow on collagen type III was reduced by sunitinib treatment, although not as strong as compared to thrombus formation on collagen type I. Together, this is consistent with the multi-target nature of sunitinib as a broad-spectrum protein TKI, having lower affinity for multiple TKs rather than high affinity for one specific signalling protein[32, 33]. This also explains why the effects of sunitinib on platelet activation are not as strong as compared to specific inhibitors of Syk or Src[34, 35].

We showed that in total 34 TKs were activated upon GPVI stimulation, which were subsequently inhibited in the presence of sunitinib. Several TKs could be directly linked to GPVI signalling, such as Syk and the SFKs, but most TKs were most likely activated by agonists secreted from the α-granules upon GPVI stimulation, such as fibrinogen, activating FAK1/2 via integrin $\alpha_{IIb}\beta_3$[23], as well as Gas6, activating Tyro3/Sky. As platelets contain many bioactive molecules in their granules, most likely also the other non-ITAM-linked TKs that showed phosphorylation upon GPVI stimulation, have been activated via similar secondary events. As we have shown that platelet secretion is inhibited by sunitinib[5], the inhibiting effects of sunitinib on non-GPVI-linked TKs may also be due to reduced secretion of secondary mediators. Prolonged elevation of intracellular calcium levels is a prerequisite for platelet PS exposure, a hallmark of procoagulant activity[24]. The observed effects of sunitinib on intracellular calcium rises in response to GPVI stimulation fits with the currently observed reduction in TK phosphorylation and agrees with previously shown effects on collagen-induced tyrosine phosphorylation of multiple proteins[5]. This implies interference of sunitinib early in the signalling cascade used by the collagen receptor GPVI, e.g. with TKs in the GPVI signalosome or with Syk as a central regulator of collagen-induced platelet activation. In agreement, another study reported that collagen-induced tyrosine phosphorylation of c-Src is reduced in platelets treated with sunitinib[6]. Others have shown that also in cardiomyocytes sunitinib treatment results in reduced calcium responses accompanied with decreased sarcomere shortening[36]. We showed that sunitinib reduced platelet PS exposure in isolated platelets *in vitro*,

as well as during whole blood thrombus formation under flow in the presence and absence of coagulation. This reduction in platelet procoagulant activity upon sunitinib treatment resulted in delayed and reduced fibrin clot formation. An earlier study with other TKIs has also demonstrated that platelet tyrosine phosphorylation is required for procoagulant activity and fibrin formation[37].

As both aspirin and sunitinib are associated with increased bleeding risk[3, 9], we investigated if the combination of these drugs could reinforce the inhibiting effects on platelets. We observed that indeed this aspirin-sunitinib combination resulted in stronger inhibition of collagen-induced platelet aggregation as compared to either compound alone. However, in whole blood perfusion experiments (with and without coagulation) we did not observe these additive effects when aspirin and sunitinib were added *in vitro*. In contrast, platelet deposition and thrombus height were reduced in blood from aspirin-treated donors as compared to controls without aspirin intake. Moreover, addition of sunitinib further enhanced the effects that were observed with aspirin alone, accompanied by further reductions in PS exposure and fibrin formation. In an earlier study from our group with patients suffering from peripheral artery disease (PAD), no effects of aspirin intake were observed on thrombus formation under flow as compared to healthy controls without aspirin intake[38]. This could be due to the increased platelet reactivity observed in these PAD patients, that was counterbalanced by the aspirin intake. In contrast, in this study *in vitro* addition of aspirin to blood from healthy volunteers did reduce thrombus formation and PS exposure under flow[38], which was also observed by others[39, 40]. The present findings are compatible to the variable effects of aspirin intake alone on thrombus formation, while the combination of aspirin with other anti-platelet drugs causes a marked reduction[41-45]. Furthermore, a recent study has shown concomitant effects of aspirin and ibrutinib on collagen-induced aggregation[46]. Altogether, these results suggest that dual treatment with anti-platelet therapy and TKIs increase the inhibition of platelet function.

In the present study we included a limited number of cancer patients, who suffered from different cancer types. Also, we added the sunitinib *in vitro*, which may be a limitation to the current study. The effects of sunitinib on platelet-dependent coagulation in blood from patients treated with sunitinib requires further study. Also, the combined effects of sunitinib and aspirin or other anti-platelet drugs on platelets from patients on treatment in relation to bleeding should be further investigated. Despite these limitations, we clearly observed in this proof-of-principle study that the *in vitro* inhibitory effects of sunitinib on thrombus size and height under coagulating conditions were more pronounced in several individual cancer patients as compared to healthy controls. It is known that cancer patients have a procoagulant phenotype[47, 48], and therefore platelets from cancer patients may be more sensitive to inhibitory compounds as compared to platelets from healthy individuals. We also observed more pronounced effects of sunitinib on thrombus formation parameters in lung and pancreatic cancer as compared to neuroglioblastoma and other cancer types. It has been reported that tumour-induced changes in platelet proteins

and mRNA are tumour-type specific[49, 50], hence we hypothesize that these specific tumour-induced changes may influence the platelet response to inhibiting drugs.

Altogether, we conclude that sunitinib inhibited GPVI-induced phosphorylation of TKs (a.o. Syk and Src), with subsequent reduction of Ca^{2+} elevation, resulting in reduced platelet activation and PS-exposure. This was accompanied by delayed and reduced formation of fibrin, all of which were aggravated in the presence of aspirin. This asks for awareness among clinicians for the combined antiplatelet effects of TKIs together with anti-platelet drugs.

Acknowledgements

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Conflict of Interest Statement

JWMH is a cofounder and shareholder of FlowChamber B.V. The other authors declare no relevant conflicts of interest.

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Supplemental data

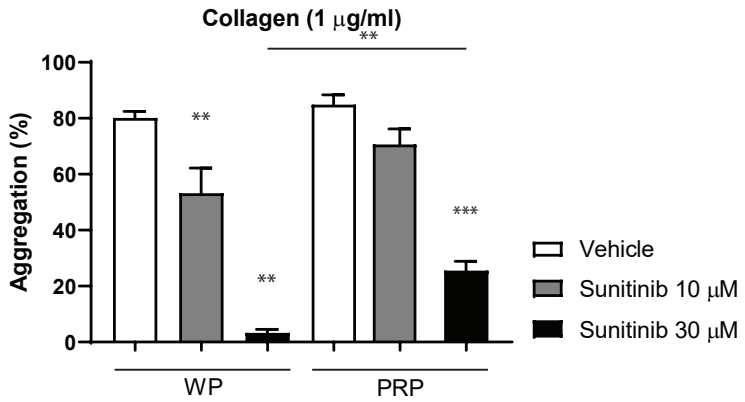
Supplemental Table

Supplemental Table 4.1. Characteristics of cancer patients

Patient no.	Gender	Age (year)	Cancer type	Chemotherapy treatment	Other drug treatment (mg/d)	Platelet count (x10^9/L)
1	Female	71	Lung	NA	NA	164
2	Male	72	Lung	Pembrolizumab	Aspirin (100)	105
3	NA	NA	Lung	NA	NA	203
4	Male	76	Neuroglioblastoma	-	-	118
5	Female	45	Neuroglioblastoma	-	-	93
6	Male	81	Neuroglioblastoma	-	Fondaparinux (5)	62
7	Male	76	Pancreatic	-	-	248
8	Male	82	Pancreatic	-	Dabigatran	168
9	Male	67	Pancreatic	Gemcitabine Abraxane	-	198
10	Female	61	Ovarian	Oxaliplatin CPL	-	301
11	Female	30	Paraganglioma (Neuroendocrine Neoplasm)	-	-	294

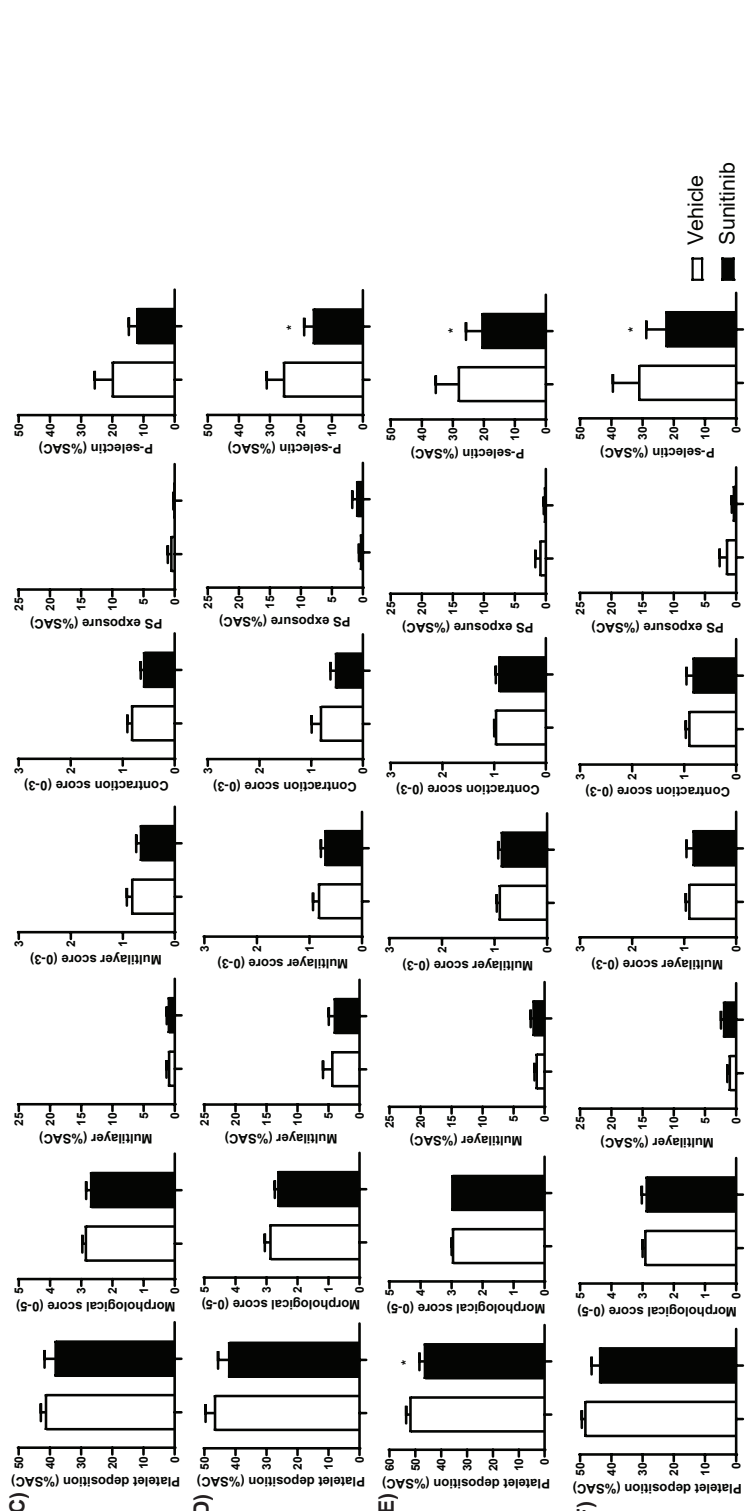
NA: Not available; CPL: Caelyx PEGylated liposomal; PEG: Polyethylene glycol

Supplemental Figures

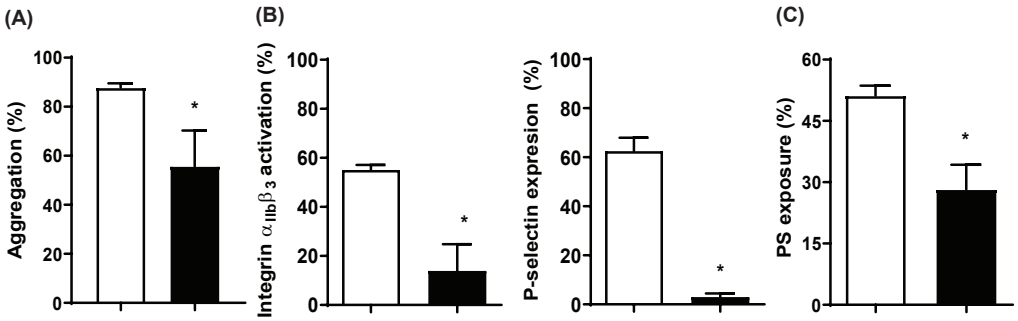


Supplemental Figure 4.1. Inhibition of aggregation with sunitinib is stronger in washed platelets compared to PRP. Washed platelets or PRP (250x10^9/L) from healthy volunteers was incubated with vehicle (<0.1% DMSO) or sunitinib (10 or 30 μM) for 10 minutes at 37°C. Shown are the percentages of aggregation of washed platelets (left) or PRP (right) in absence (white bar) or presence of sunitinib (grey / black bars) in response to collagen (1 μg/mL). Data are represented as means + SEM (n=4-10), **p<0.01, ***p<0.001.

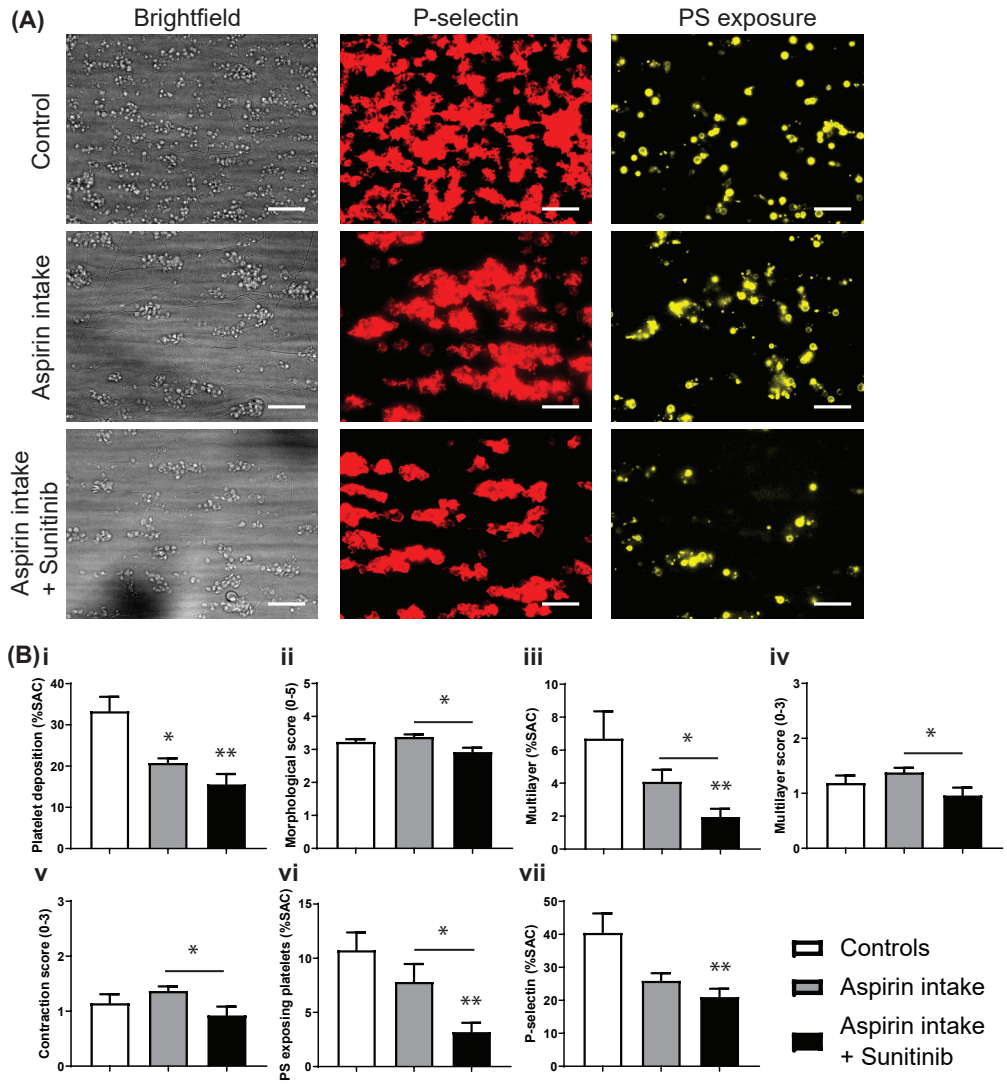
(A)
Collagen type I
Sunitinib
Vehicle
(B)
Collagen type III
Sunitinib
Vehicle
(C)
vWF + Rhodocytin
Sunitinib
Vehicle
(D)
vWF + Laminin
Sunitinib
Vehicle
(E)
vWF + Fibrinogen
Sunitinib
Vehicle
(F)
vWF + Ristocetin
Sunitinib
Vehicle
Brightfield
P-selectin
PS exposure
Platelet deposition (%SAC)
Morphological score (0-5)
Multilayer (%SAC)
Multilayer score (0-3)
Contraction score (0-3)
PS exposure (%SAC)
P-selectin (%SAC)



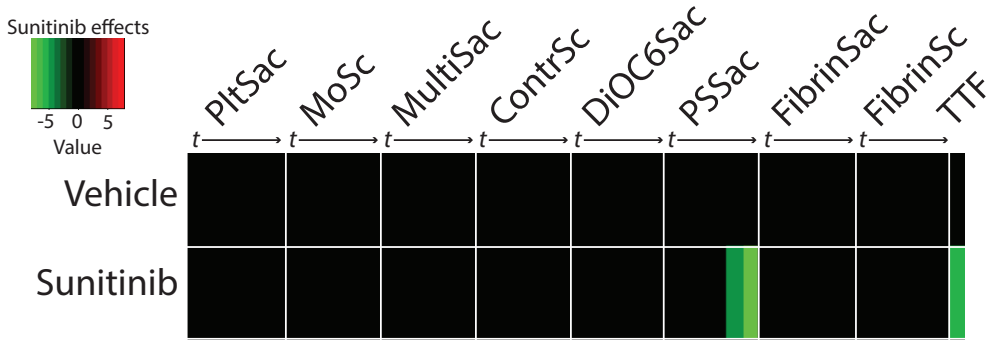
Supplemental Figure 4.2. Sunitinib reduces thrombus formation on GPVI-dependent surfaces, as compared to surfaces for CLEC-2, GPIb, integrin $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$. Whole blood from healthy volunteers was preincubated with vehicle (<0.1% DMSO) or sunitinib (30 µM) for 10 min and perfused for 3.5 minutes at wall shear rate of 1,000 s^{-1} over microspots containing (A) collagen type I, (B) collagen type III, (C) vWF co-coated with rhodocytin, (D) vWF co-coated with laminin, (E) vWF co-coated with fibrinogen and (F) vWF co-coated with ristocetin. Left: representative brightfield and fluorescence images after 10 minutes of flow. Right: Quantification of brightfield and fluorescence images of control (white bars) and sunitinib treatment (black bars). Data are represented as means + SEM (n=6-7), *p<0.05, **p<0.01.



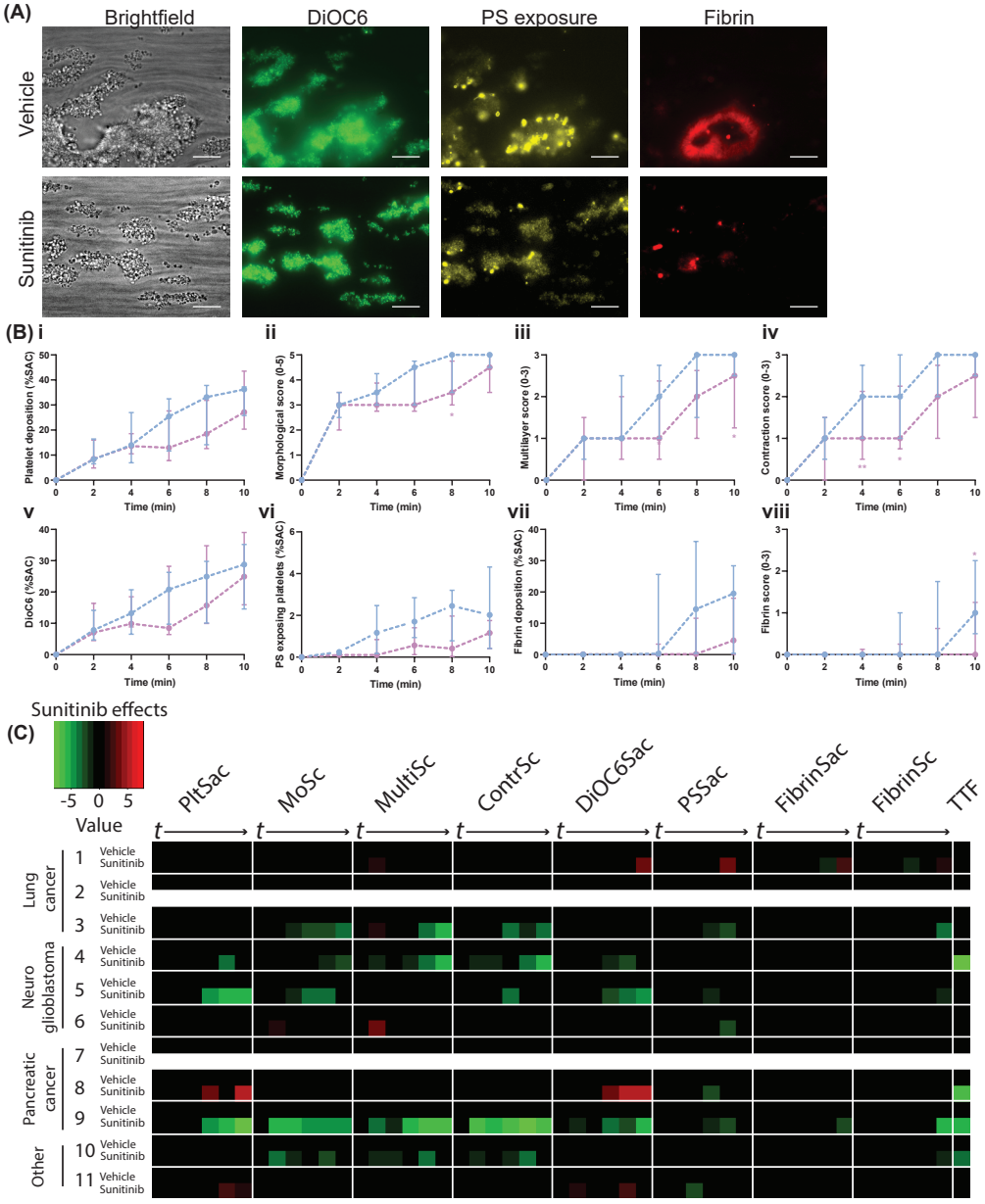
Supplemental Figure 4.3. Sunitinib inhibits CLEC-2 induced aggregation and activation in washed platelets. (A) Washed platelets (250x10^9/L) from healthy volunteers were incubated with vehicle (<0.1% DMSO) or sunitinib (10 or 30 µM) for 10 minutes at 37°C. Shown are the percentages of aggregation in the absence (white bar) or presence of sunitinib (black bars) in response to rhodocytin (1 µg/mL). (B) Washed platelets (100 x 10^9/L) were pre-incubated with vehicle (<0.1% DMSO) or sunitinib (10 µM) and stimulated rhodocytin (1 µg/mL) for 15 minutes and simultaneously stained for activated integrin $\alpha_{IIb}\beta_3$ and α-granule secretion (P-selectin). Histograms show the percentages of platelets binding FITC-labelled PAC-1 monoclonal antibody or AF647-labelled anti-human CD62 mAb. (C) Isolated platelets from healthy volunteers were incubated with vehicle (<0.1% DMSO) or sunitinib (10 µM) for 10 minutes at 37°C. Platelets (100x10^9/L) were activated in the presence (black bars) or absence of sunitinib (white bars) by rhodocytin (5 µg/mL) in combination with thrombin (4 nM) and incubated for 60 minutes at 37°C. PS exposure was detected by FITC-conjugated annexin A5 and measured by flow cytometry. Data are represented as means + SEM (n=4-6), *p<0.05.



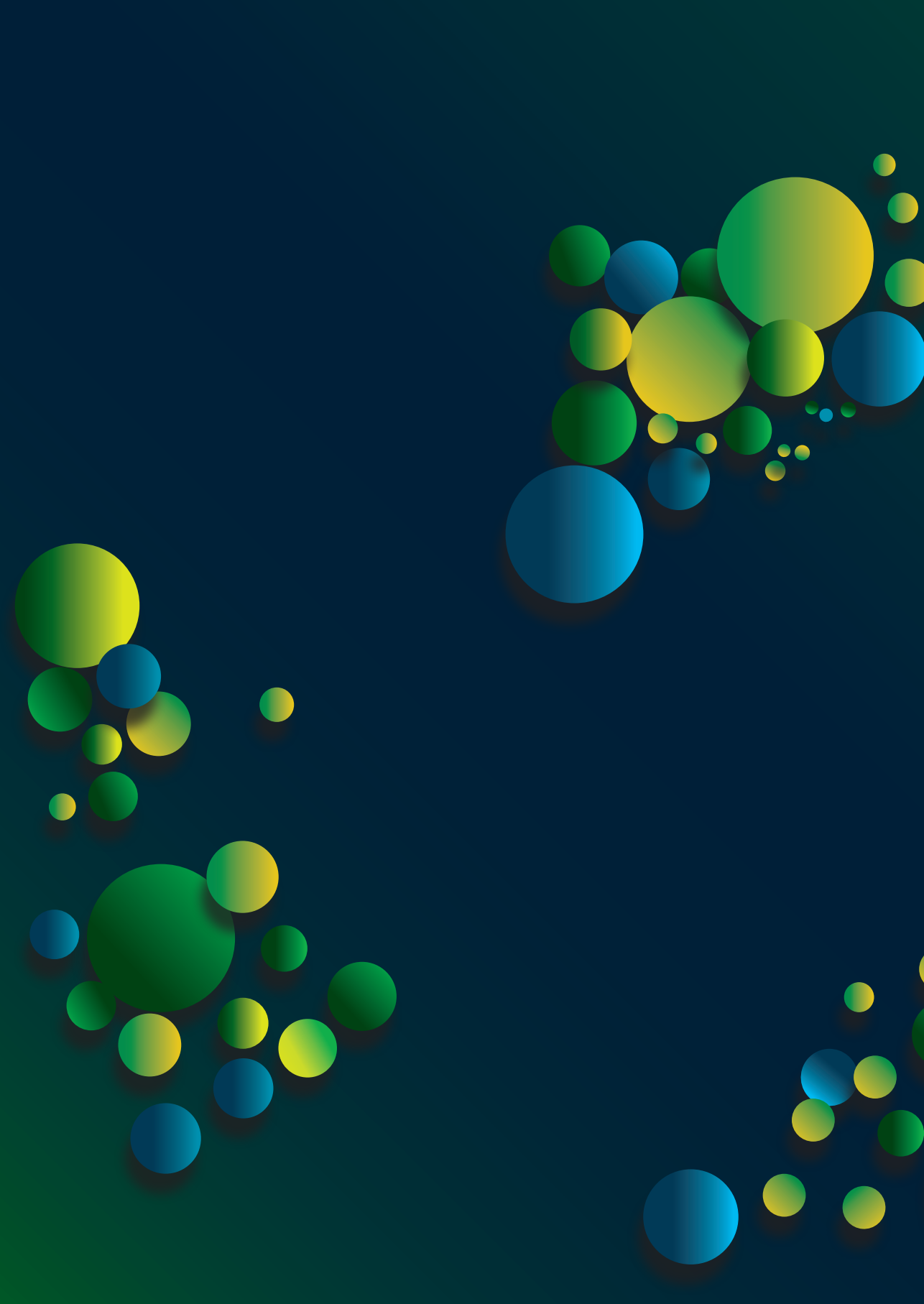
Supplemental Figure 4.4. Aspirin intake enhances the effect of sunitinib in *ex vivo* thrombus formation on collagen in whole blood. Recalcified whole blood of healthy volunteers or healthy volunteers with single dose of aspirin was treated *in vitro* with vehicle or sunitinib (30 µM) for 10 min. Subsequently, blood was perfused over a collagen surface at 1000 s^{-1} for 3.5 minutes, followed by perfusion with buffer supplemented with AF568-annexin A5 and AF647-α-CD62P to visualize PS exposure and P-selectin expression, respectively. (A) Representative brightfield and fluorescent images. (B) Quantification of brightfield images of (i) platelet deposition, (ii) morphological score, (iii) multilayer (%SAC), (iv) multilayer score, (v) contraction score, and fluorescence images of (vi) PS exposure and (vii) P-selectin expression of vehicle (white bar), aspirin intake (dark grey bars), or aspirin intake with *in vitro* sunitinib (black bar). Data are represented as means + SEM (n=3-6), *p<0.05, **p<0.01.



Supplemental Figure 4.5. The effects of sunitinib on platelet-dependent fibrin formation under flow. Whole blood from healthy volunteers was preincubated with vehicle (<0.1% DMSO) or sunitinib (30 µM) for 10 min. Subsequently, the blood was supplemented with DiOC6, AF568-annexin A5 and AF647-α-fibrinogen to visualize platelet deposition, PS-exposure and fibrin formation, respectively. Blood samples were co-perfused with $CaCl_2$/$MgCl_2$ over collagen type I co-coated with TF microspot at a wall shear rate of 1000 s^{-1}. Subtraction heatmaps representing the significant effects of normalized values of the parameters. Controls with addition of vehicle (<0.1% DMSO) were set at 0 for reference. Effects were filtered for significant alterations by addition of sunitinib ($p \leq 0.05$).



Supplemental Figure 4.6. Sunitinib reduces platelet-dependent thrombus-fibrin formation under flow in whole blood from cancer patients. Citrated whole blood from cancer patients (suppl. Table I) was treated *in vitro* with vehicle (<0.01% DMSO) or sunitinib (30 µM) for 10 min, followed by addition of DiOC6 (platelet deposition), AF568-annexin A5 (PS-exposure) and AF647-α-fibrinogen (fibrin). Blood was co-perfused with $CaCl_2$/$MgCl_2$ over collagen type I co-coated with TF at a wall shear rate of 1000 s^{-1}. (A) Representative brightfield and fluorescent images taken after 10 min of flow. (B) Kinetic analysis of brightfield images of (i) platelet deposition (PltSac), (ii) morphological score (MoSc), (iii) multilayer score (MultiSc), and (iv) contraction score (ContrSc) and of fluorescence images of (v) DiOC6 platelet deposition (DiOC6Sac), (vi) PS exposure (PSSac), (vii) fibrin deposition (FibrinSac), and (viii) fibrin score (FibrinSc). Data are represented as median ± interquartile range (n=9-11), *p<0.05, **p<0.01. (C) Subtraction heatmap representing the effect of sunitinib in individual cancer patients. Vehicle treated blood of cancer patients was set at 0 for reference to *in vitro* addition of sunitinib. Effects were filtered for changes greater than the 1x SD range of the different platelet parameters.



Chapter 5

Quantitative and qualitative changes in platelet traits of sunitinib-treated patients with renal cell carcinoma in relation to circulating sunitinib levels: a proof-of-concept study

Tullemans BME, Brouns SLN, Swieringa F, Sabrkhany S, van den Berkmortel FWPJ, Peters NJB, de Bruijn P, Koolen SLW, Heemskerk JWM, Aarts MJB and Kuijpers MJE

Submitted

Abstract

Background: Tyrosine kinase inhibitors (TKIs), such as sunitinib, are used for cancer treatment, but may also affect platelet count and function with possible hemostatic consequences. Here, we investigated how patient treatment with the TKI sunitinib affected quantitative and qualitative platelet traits as a function of the sunitinib level and the occurrence of bleeding.

Methods: Blood was collected from 20 metastatic renal cell carcinoma (mRCC) patients before treatment, and at 2 weeks, 4 weeks and 3 months after sunitinib administration. We measured blood cell counts, platelet aggregation, and the concentrations of sunitinib as well as its N-desethyl metabolite in plasma, serum and isolated platelets. Progression of disease (PD) and bleeding were monitored after 3 months.

Results: In sunitinib-treated mRCC patients, concentrations of (N-desethyl)sunitinib in plasma and serum were highly correlated. In the patients' platelets the active metabolite levels were relatively increased as compared to sunitinib. On average, a sustained reduction in platelet count was observed on-treatment, which was significantly related to the inhibitor levels in plasma/serum. Principal component and correlational analysis showed that the (N-desethyl-) sunitinib levels in plasma/serum were linked to a reduction in both platelet count and collagen-induced platelet aggregation. The reduced aggregation associated in part with reported bleeding, but did not correlate to PD.

Conclusions: The sunitinib-induced reduction in quantitative and qualitative platelet traits may reflect the effective sunitinib levels in the patient. These results may serve as a proof-of-principle for other TKI-related drugs, where both platelet count and functions are affected, which could be used therapeutic drug monitoring.

Introduction

In the past two decades, over 40 tyrosine kinase inhibitors (TKIs) have been developed and approved for the treatment of many cancer types[1]. The majority of these drugs target the receptor-linked tyrosine kinases for growth factors, e.g., receptors for vascular endothelial growth factor, and/or differentiation/proliferation factors (e.g., Flt, Fms, Kit, and Ret). Several other TKIs target intracellular tyrosine kinases (Abl, B-Raf, Btk, Itk, Src-family kinases and Syk). However, in spite of the intended specific targets, most TKIs used for treatment also have broad off-target effects, invariably affecting a range of protein tyrosine kinases. It is debated whether these off-target effects help to improve progression-free survival (PFS) and overall survival (OS) of the treated patients[2].

Until shortly, the survival of metastatic clear cell renal cell carcinoma (ccRCC) patients was poor with a 5-year survival rate of 12%[3]. However, in recent years treatment options have increased by the availability of immune- and targeted therapies, using anti-PD1 and anti-CTLA4 antibodies, as well as new TKIs. Randomized controlled trials testing the targeted therapies showed an overall improvement in response rates, PFS and OS, especially for the intermediate and poor risk groups[4-7]. For the favorable risk patients, it appeared that sunitinib, a broad-spectrum TKI, was superior to the immuno-agents nivolumab/ipilimumab, and was equivalent to pembrolizumab plus axitinib[4, 5]. Therefore, sunitinib has become a mainstay not only for first-line treatment in this particular risk group, but also in second line treatment after nivolumab/ipilimumab.

Upon activation of platelets via non-G-protein coupled receptors, tyrosine phosphorylation, via Src-family kinases and Syk, is one of the key signal transduction mechanisms[8]. Especially, platelet activation via the collagen receptor glycoprotein (GP) VI fully relies on this tyrosine kinase pathway. Clinical studies have shown that several TKIs, next to affecting platelet function, also affect platelet count. Both effects on platelets can contribute to the increased bleeding risk, regularly observed in patients treated with TKIs[1, 9]. Also for sunitinib treatment, mild bleeding diathesis has been described as a side effect. This was observed as epistaxis, or mucocutaneous and gastrointestinal bleeding, which occurred in approximately 19% of the sunitinib-treated patients[10].

Recently, we have demonstrated a rapid uptake of sunitinib by platelets, which was accompanied by a reduced collagen receptor-induced aggregation, secretion of α-granules and thrombus formation under flow conditions, both *in vitro* and *ex vivo* in RCC patients on treatment[11]. Furthermore, we and others have shown that the platelet concentration decreases upon sunitinib treatment[11-13], an effect that has been postulated as a prognostic factor for the sunitinib treatment response in RCC[12]. However, to which extent these sunitinib effects are related to each other and to the circulating sunitinib concentration and/or bleeding is unclear.

In the present study with 20 patients, we investigated as a proof-of-concept with regards to other TKIs, whether the effects of sunitinib on quantitative (count) and qualitative (aggregation)

platelet traits is associated with the circulating inhibitor level. Furthermore, we set out to deduce how this relates to reported bleeding symptoms.

Materials and Methods

Materials

Sunitinib malate (Sutent) was provided by Pfizer (New York NY, USA). Bovine serum albumin (BSA), D(+)-glucose, and apyrase were purchased from Sigma-Aldrich (Saint Louis MO, USA). Horm collagen type I was from Takeda (Hoofddorp, the Netherlands). Ilomedin (iloprost) injection fluid was obtained from Bayer (Mijdrecht, the Netherlands).

Study population and blood collection

The study was performed in accordance with the declaration of Helsinki and approved by the local medical ethical committee of Maastricht University Medical Center+ (MUMC+). Full informed consent was obtained from all participants. Blood samples were collected from 20 patients diagnosed with metastatic renal cell carcinoma (mRCC) at the Department of Medical Oncology of MUMC+ (Maastricht, the Netherlands), Zuyderland Medical Centre (Sittard-Geleen, the Netherlands) and SJG Hospital (Weert, the Netherlands). Blood was also collected from 10 healthy donors of similar age and gender. Patients were included, when eligible for treatment with sunitinib as a single agent (50 mg/day) with a treatment schedule of 4 weeks on and 2 weeks off medication. Patient blood samples were collected at four different timepoints: (i) before start with sunitinib, and after (ii) 2 weeks, (iii) 4 weeks and (iv) 3 months of sunitinib administration.

Blood samples were obtained from the antecubital vein. At each timepoint; 10 ml was collected using a vacuum container containing 3.2% trisodium citrate, and a second blood sample of 10 ml was collected in a Clot Activator Tube (CAT) Serum Separator (Greiner Bio-One, Alphen a/d Rijn, the Netherlands). Blood cell counts and haematological parameters were assessed in patient and healthy control samples, using a Sysmex XP300 (Kobe, Japan).

Reporting of bleeding

Patients were asked to fill out a self-assessment bleeding form after 2 weeks, 4 weeks and 3 months of sunitinib administration, as was used earlier[14]. The physicians provided additional information with regards to bleeding. Any bleeding was scored as 1, no bleeding was scored as 0.

Response evaluation

A response to sunitinib treatment was defined as partial response (PR), stable disease (SD) or progressive disease (PD) according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines, based on a CT scan performed 3 months after initiating sunitinib, as compared to the CT scan prior to treatment. For some of the 20 patients, values were only collected at

timepoints 0 and 2 weeks. Missing values at 4 weeks and/or 3 months were due to dose reduction (in 3 patients, after severe side effects of medication), hospital admission as a result of thrombocytopenia requiring blood transfusion (2 patients) or death (4 patients).

Platelet isolation

Platelets were isolated from whole blood, as described previously[15]. Platelet-rich plasma (PRP) was isolated from citrate-anticoagulated blood by centrifugation at 240 *g* for 15 minutes. The PRP was supplemented with 1:10 acidic citrate dextrose (ACD; 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) and centrifuged for 2 minutes at 2,230 *g*. The supernatant plasma was retained in case of patient samples, and was further processed as described below. The platelet pellet was resuspended into Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl and 2 mM $MgCl_2$) supplemented with 5 mM glucose and 0.1% bovine serum albumin (BSA). After addition of 1:15 ACD and 1 U/mL apyrase, the platelets were centrifuged for 2 minutes at 2,230 *g*, followed by resuspension into Hepes buffer pH 7.45 with 5 mM glucose and 0.1% BSA. Platelet count was determined using a Sysmex XP300 (Kobe, Japan) and adjusted as stated per assay.

Patient blood sample preparations

Serum was isolated from CAT tubes by centrifugation at 2,200 *g* for 10 minutes at room temperature. Both serum and plasma (obtained from PRP) were centrifuged a second time at 22,500 *g* for 5 minutes to remove possible debris. The collected plasma and serum samples were frozen, and stored at –80°C until further use.

Isolated platelets ($250x10^9$ platelets/L) were incubated for 5 minutes at room temperature with 50 nM iloprost, and centrifuged for 2 minutes at 2,230 *g*. Supernatant was discarded, and the pellet was frozen and stored at-80°C until further use.

Light transmission aggregometry

Washed platelets ($250x10^9$ platelets/L) were incubated for 5 minutes at 37°C before stimulation. Aggregation response was induced by addition of 1 µg/mL collagen type I, except for patients with anti-platelet drugs where aggregation was induced by 5 µg/mL collagen. The collagen concentration was kept the same at all timepoints per patient. Platelet aggregation was recorded using a Chronolog optical aggregometer (Havertown PA, USA), and maximum aggregation amplitude was quantified at 8 minutes after collagen addition.

Pharmacokinetic analysis of sunitinib in plasma and serum samples

Sunitinib and the N-desethyl metabolite (SU12662) were quantified, as described previously[16] in plasma, serum and platelet pellets obtained from patients after 2 and 4 weeks on

sunitinib treatment. The concentration of either compound in isolated platelets was corrected for the number of platelets in the pellet, and normalized to ng per $2.5x10^8$ platelets.

Statistical analysis

Data are presented as median ± interquartile ranges. Datasets within patients were compared using the Wilcoxon matched pairs test, whereas comparisons to controls were determined using the Kruskal-Wallis test. Correlation analysis was performed using a nonparametric Spearman correlation (2-tailed) using GraphPad Prism 8 software. To identify associations within the dataset, variables were quantile normalized and a rotated principal component analysis, based on an eigenvalue over 1, was performed using the statistical package for social sciences (SPSS version 24). A P-value less than 0.05 was considered to be statistically significant.

Results

Patient demographics and clinical characteristics

In the present study, 20 patients were included with mRCC, who were eligible for treatment with 50 mg sunitinib per day with a treatment schedule of 4 weeks on and 2 weeks off medication (Table 5.1). Median age of the mRCC patients was 65 years (range: 52-83). Of the patients, 6 were females (median age 65; range: 59-83) and 14 were male (median age 65.5; range: 52-80). All patients presented with ccRCC, except for two patients who were diagnosed with papillary RCC. Three patients required dose reduction of sunitinib during the study follow-up, due to side effects (Table 5.1). Four of the patients additionally received daily aspirin as an anti-platelet drug, one of whom received heparin during the 3-month follow-up. Two patients received the cholesterol-lowering drug atorvastatin, which can also affect platelet function[17, 18]. Furthermore, 10 healthy individuals were included with similar median age of 63.5 (range: 53-85), of whom 3 were female.

Plasma and serum levels of sunitinib and N-desethyl-sunitinib are highly correlated, while platelets take up more N-desethyl-sunitinib as compared to sunitinib

Sunitinib and its active metabolite N-desethyl-sunitinib were determined in both plasma and serum from mRCC patients after 2 and 4 weeks of treatment. The concentration of N-desethyl-sunitinib appeared to be significantly lower in serum and plasma than that of sunitinib (Figure 5.1A vs 5.1B, plasma $P<0.0001$ and serum $P<0.0006$). Comparison of plasma and serum levels of sunitinib or N-desethyl-sunitinib did not show marked differences at 2 or 4 weeks (Figure 5.1A, B). On the other hand, the concentration of sunitinib, but not of its metabolite, was slightly, but significantly, decreased after 4 weeks in both plasma and serum, as compared to 2 weeks on treatment (Figure 5.1A). This is compatible with a 14-days peak level upon sunitinib administration, after which this reduced to a steady-state level.

Because platelets are known to sequester sunitinib, we also measured the concentrations

Table 5.1. Demographics and clinical characteristics of RCC patients treated with sunitinib with a 3-month follow-up

Patient no.	Gender	Age (year)	Type RCC	Sunitinib treatment (mg/d)	Other drug treatment (mg/d)	Platelet count (x10^9/L) Day 0	Week 2	Week 4	Month 3	Bleeding		Progression	
1	Male	58	Clear cell	50	ASA (100); ATOR (40)	360	385	244	-	No	-	NA	†
2	Male	66	Clear cell	50	-	164	102	79	121	No	-	Yes	20% increase
3	Female	66	Clear cell	50	-	455	155	-	-	No	-	NA	†
4	Female	59	Clear cell	50	-	146	24	-	-	Yes	Hematuria, GI bleeding	NA	†
5	Male	56	Clear cell	50	-	260	51	202	204	No	-	No	-
6	Female	83	NA	50	-	301	199	137	162	Yes	Epistaxis, gingivitis	No	20% decrease
7	Female	64	Clear cell	50	-	218	182	95	201	Yes	Hematuria	No	32% decrease
8	Male	80	Papillary	50	ASA (80)	309	-	100	-	Yes	GI bleeding after heparin treatment	Yes	35% increase
9	Female	60	Clear cell	50	-	225	29	-	-	No	-	NA	Hospital uptake
10	Male	52	Clear cell	50	-	169	128	128	203	Yes	GI bleeding, gingivitis	No	-
11	Male	72	Clear cell	50	-	157	200	49	-	No	-	NA	↓ Dosage
12	Male	63	Papillary	50	-	446	114	-	-	No	-	NA	↓ Dosage
13	Male	75	NA	50	ASA (80)	221	142	148	273	No	-	Yes	35% increase
14	Male	68	Clear cell	50	ATOR (40)	426	194	165	326	No	-	No	30% decrease
15	Male	55	Clear cell	50	-	148	151	139	142	Yes	Epistaxis, hemoptysis, GI bleeding	No	-
16	Male	54	Clear cell	50	-	170	116	161	-	Yes	Hematuria, gingivitis	Yes	16% increase, ↓Dosage
17	Male	68	Clear cell	50	-	195	177	187	-	Yes	Hemoptysis	Yes	8% increase
18	Female	82	Clear cell	50	-	286	212	98	134	Yes	Epistaxis, gingivitis, hemoptysis, vaginal bleeding	No	23% decrease
19	Male	70	Clear cell	50	ASA (80)	233	99	36	99	No	-	No	-
20	Male	65	Clear cell	50	-	201	133	56	-	No	-	NA	†

ASA: acetylsalicylic acid (aspirin); ATOR: atorvastatin; GI: gastrointestinal; NA: not available; RCC: renal cell carcinoma; †: patient deceased during follow-up period; ↓ Dosage: decreased dosage of sunitinib due to side effects.

of sunitinib and the active metabolite in isolated platelets from mRCC patients. Markedly, the concentration of the metabolite was significantly higher in platelets after 2 and 4 weeks on treatment, as compared to that of sunitinib (Figure 5.1C). This contrasted to the lower concentration of the metabolite in the plasma and serum samples, suggesting a preferential uptake of the metabolite by platelets. Furthermore, the concentrations of sunitinib and metabolite after 2 and 4 weeks of treatment showed strong correlations between the plasma, serum and platelet samples ($r>0.67033$, $P<0.01$).

Prolonged changes in platelet count by sunitinib treatment of mRCC patients are related to inhibitor levels in plasma and serum

Whole blood cell counts, haemoglobin and haematocrit were determined in blood samples from mRCC patients at four time points (Figure 5.2), i.e., the day before start of sunitinib treatment, after 2 and 4 weeks (1 treatment cycle) and after 3 months (2 complete cycles). Before start of sunitinib treatment, no significant differences were observed in these parameters

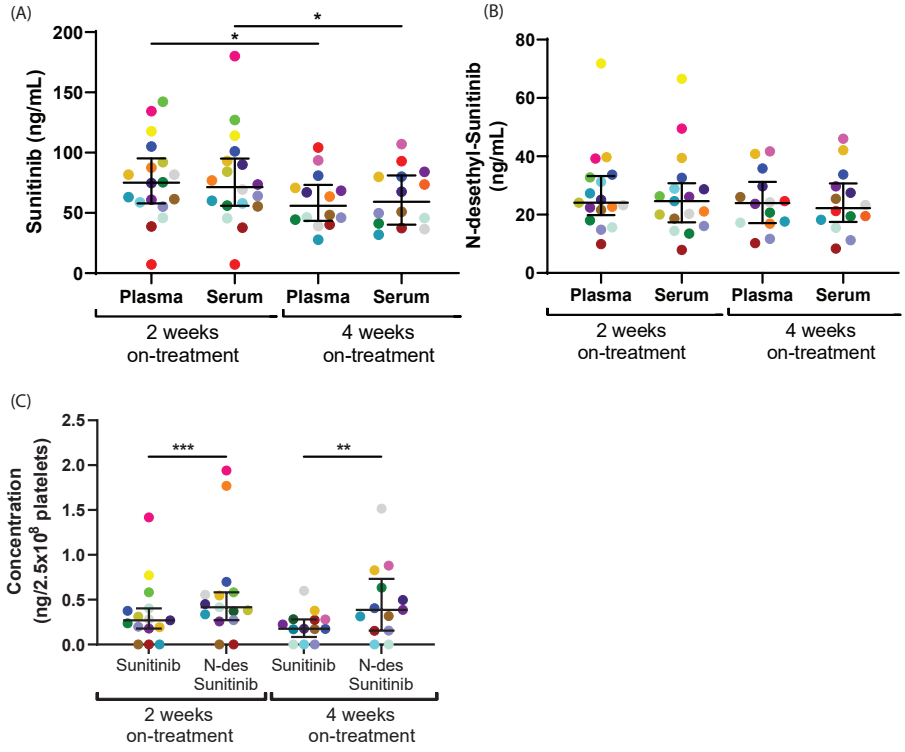


Figure 5.1. Comparison of sunitinib and N-desethyl-sunitinib levels in plasma, serum and isolated platelets from mRCC patients on 2 and 4 weeks on treatment. The concentration of sunitinib (A) and N-desethyl-sunitinib (B) were measured in plasma and serum samples obtained from mRCC patients at 2 and 4 weeks of sunitinib treatment. (C) The concentrations of both compounds were determined in isolated platelets, normalized to ng per $2.5x10^8$ platelets. Individual dots in the scatterplots represent one patient, for color coding see Table 5.1. Lines and error bars represent median ± interquartile range (n=14-20). *P<0.05. **P<0.01, ***P<0.001

between patients and healthy controls (Figure 5.2). However, after 2 weeks of treatment platelet counts were decreased (>10%) in 15 patients (range: 17-87% reduction), while 5 patients showed no decrease or even an increase (5-10%, Figure 5.2A). Seven of the patients who presented a reduced platelet count showed an even further reduction after 4 weeks, which effect for six of them persisted for 3 months. The median platelet concentration of all patients increased after 3 months as compared to 4 weeks, yet was still lower than before the start of treatment (Figure 5.2A). In total, fifteen patients had a platelet count between 24-148x10^9/L (Table 5.1), which was below the normal range (150-400x10^9/L, according to the Dutch Society of Hematology, NVH). Of note, 5 patients presented with formal thrombocytopenia (<50x10^9 platelets/L) after 2-4 weeks of treatment. White blood cell (WBC) counts decreased (>10%) in 13 patients (range: 19-60% reduction) after 2 weeks of sunitinib treatment (Figure 5.2B), which decrease persisted in most patients for 3 months. According to the Common Terminology Criteria 7 patients presented with grade 2 neutropenia during the study period with no more than 3 patients per time point. No patients had grade 3 or 4 neutropenia. After two weeks of sunitinib administration, overall red

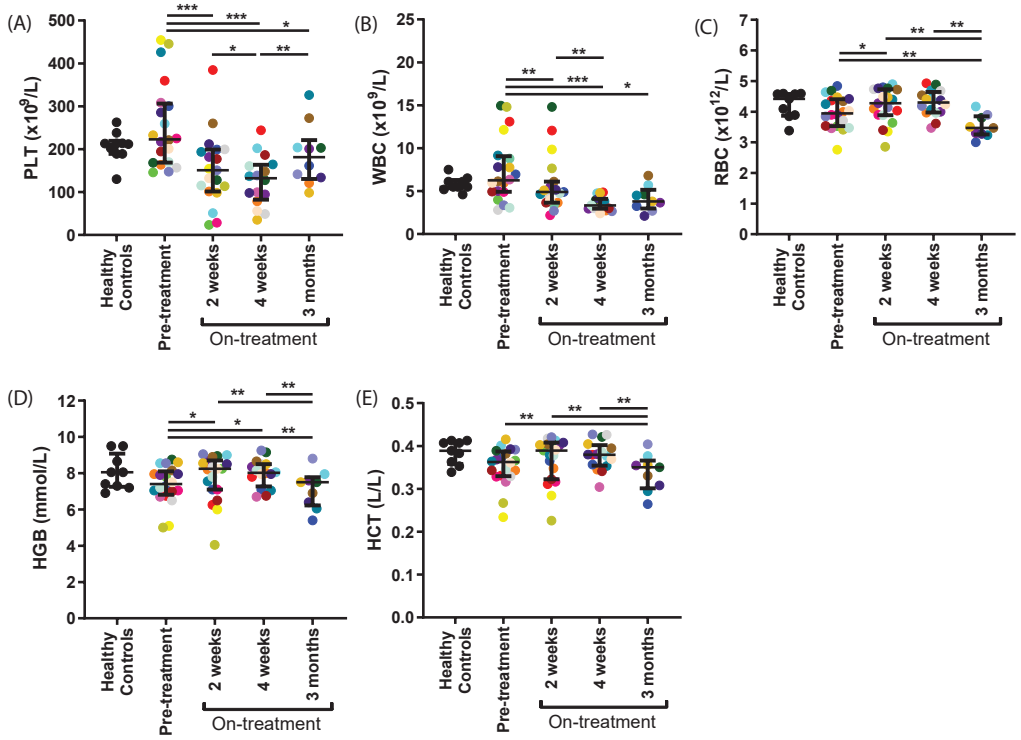


Figure 5.2. Treatment of mRCC patients with sunitinib decreases platelet count and other hematological parameters over time. Blood was collected from 10 healthy controls and 20 mRCC patients the day before, after 2 weeks, 4 weeks and 3 months on sunitinib treatment. Hematological parameters were measured in whole blood. Scatterplots show (A) platelet count, (B) white blood cell count (WBC), (C) red blood cell count (RBC), (D) hemoglobin concentration (HGB) and (E) hematocrit levels (HCT). Individual dots in the scatterplots represent one patient, for color coding see Table 1. Lines and error bars represent median ± interquartile range (n=10-20). *P<0.05. **P<0.01, ***P<0.001.

blood cell count (RBC) and hemoglobin were slightly, but significantly increased (Figure 5.2C-D). After 4 weeks and 3 months, these values were decreased, as well as the hematocrit (Figure 5.2E).

We examined whether the concentrations of (N-desethyl-)sunitinib were correlated with the reduction in platelet count. The levels of either compound in plasma and serum appeared to be correlated with the count reductions after 2 weeks treatment (Table 5.2, Figure 5.3A), and for the metabolite this correlation was even stronger after 4 weeks (Table 5.2). For the compound levels in platelets, we noticed a borderline significant negative correlation (Table 5.2, Figure 5.3B). Together, these data suggested that the changes in platelet count are related to the sunitinib levels in plasma and serum, thereby possibly reflecting the effective sunitinib dose in the patient.

Table 5.2. Concentrations of (N-desethyl-)sunitinib in plasma and serum correlate with changes in platelet count and aggregation in RCC patients at different time points.

			Change in platelet count				Correlation	
Variables		Time point	-ΔPlt median (IQR)	n	+ΔPlt median (IQR)	n	R	P-value
Sunitinib	Plasma	Week 2	78.5 (60.5-108.3)	n=14	61.4 (55.3-81.8)	n=3	-0.4966	0.0443*
	Serum		80.6 (57.4-104.3)	n=14	64.0 (55.4-69.4)	n=3	-0.5221	0.0336*
	Pellet		0.29 (0.18-0.54)	n=12	0.20 (0.00-0.28)	n=3	-0.4480	0.0960
	Plasma	Week 4	56.0 (43.0-73.5)	n=14	-	n=0	-0.4066	0.1505
	Serum		59.3 (40.3-81.0)	n=14	-	n=0	-0.3846	0.1755
	Pellet		0.18 (0.09-0.28)	n=13	-	n=0	-0.2707	0.3701
N-desethyl-Sunitinib	Plasma	Week 2	26.0 (21.0-35.3)	n=14	21.6 (14.8-23.2)	n=3	-0.7353	0.0011**
	Serum		26.2 (18.6-34.3)	n=14	18.6 (16.1-20.3)	n=3	-0.6152	0.0100*
	Pellet		0.43 (0.35-0.67)	n=12	0.27 (0.00-0.56)	n=3	-0.5147	0.0517
	Plasma	Week 4	24.0 (17.0-31.5)	n=14	-	n=0	-0.7099	0.0058**
	Serum		22.3 (17.5-30.7)	n=14	-	n=0	-0.7143	0.0054**
	Pellet		0.39 (0.15-0.73)	n=13	-	n=0	-0.5557	0.0515
ΔAggregation		Week 2	-9.8 (-24.3 - -1.9)	n=11	-5.7 (-6.6 - 35.6)	n=3	0.6249	0.0192*
		Week 4	-7.1 (-44.7 - 4.4)	n=13	-	n=0	0.1099	0.7231
		Month 3	-6.6 (-17.3 - 0.3)	n=10	-17.6 (-18.1 - -17.1)	n=2	-0.5515	0.1049

IQR: interquartile range; ΔPlt count = Platelet count at indicated timepoint on treatment – platelet count before treatment. Negative value (-ΔPlt) indicates a reduction in platelet count; Positive value (+ΔPlt) indicates an increase in platelet count. Correlations are based on all changes in platelet count.

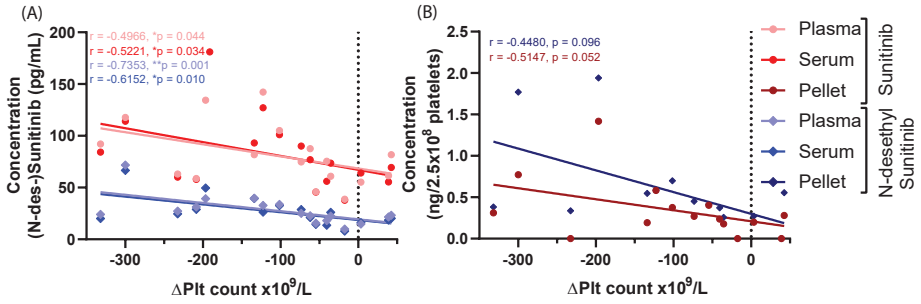


Figure 5.3. Concentrations of (N-desethyl-)sunitinib correlate with the reduction in platelet count. (A) Correlations between the concentration of sunitinib and N-desethyl-sunitinib in plasma and serum and difference in platelet count (ΔPlt count = Plt count at 2 weeks on treatment – platelet count before treatment). (B) Correlation between the concentration of sunitinib and N-desethyl-sunitinib in isolated platelets (normalized to ng/2.5x10^8 platelets) and difference in platelet count.

Comparative changes in quantitative and qualitative platelet traits by sunitinib treatment of mRCC patients

For adequate dosing, the collagen concentration required for maximal platelet aggregation was determined using washed patient platelets before the sunitinib treatment. Either 1 or 5 µg/mL collagen was used, dependent on intake of aspirin or atorvastatin (see Table 5.1). This dose was maintained for all follow-up measurements per patient. Platelet aggregation responses on treatment were compared to pre-treatment measurements for each patient. This resulted in an overall decrease in aggregation (range: 5-45% decrease) in 10 mRCC patients after 2 weeks sunitinib treatment (Figure 5.4A), despite of the observed variation between patients. Platelet aggregation was continued to be decreased in 10 patients (in 7 patients >10%) after 4 weeks, while in 5 patients there was no decrease or even increased aggregation as compared to pre-treatment. Altogether, on average no differences in aggregation were observed after 4 weeks. After 3 months, again no differences in aggregation were observed, due to substantial variation. Remarkably, platelets from 6 patients showed a long-term reduction (>10% decrease) in collagen-induced aggregation until 3 months, while platelets from other patients were not or only minimally affected during this time-period (Figure 5.4A). The five mRCC patients who were treated with both sunitinib and aspirin and/or atorvastatin, revealed no significant differences in platelet aggregation, as compared to patients on sunitinib monotherapy.

We investigated whether an observed reduction in platelet aggregation was correlated to a reduction in platelet count at all time points (Table 5.2). It appeared that the difference in platelet count (ΔPlt = Platelet count at 2 weeks on treatment - platelet count before treatment) correlated with the difference in aggregation (ΔPlt aggregation = maximum amplitude at 2 weeks on treatment- maximum amplitude before treatment) at two weeks on treatment only (Table 5.2, Figure 5.4B). Next, we compared the reduction in platelet aggregation with the inhibitor levels in plasma, serum and platelets, though no correlation was observed between these parameters (Figure 5.4C).

To further unravel the relationship between the quantitative and qualitative platelet traits and sunitinib levels in patients, we performed a principal component analysis (PCA). The resulting heatmap (Figure 5.4D) indicates which parameters tended to cluster together per component. Four components were uncovered, which combined accounted for 95% of the variance of this data. It is observed that the parameters contributing to component 1 (C1) included aggregation, count and platelet concentration of (N-desethyl-)sunitinib at 2 and 4 weeks. C2 was composed of the parameters of plasma, serum and platelet levels of (N-desethyl-)sunitinib, and C3 showed the related count levels and metabolite concentrations in plasma and serum at 2 and 4 weeks. C4 revealed an association of aggregation and sunitinib levels in plasma and serum at 4 weeks. Altogether, this PCA underlined that the (N-desethyl-)sunitinib levels in either serum or plasma from mRCC patients associated with the reduction in both platelet count and aggregation,

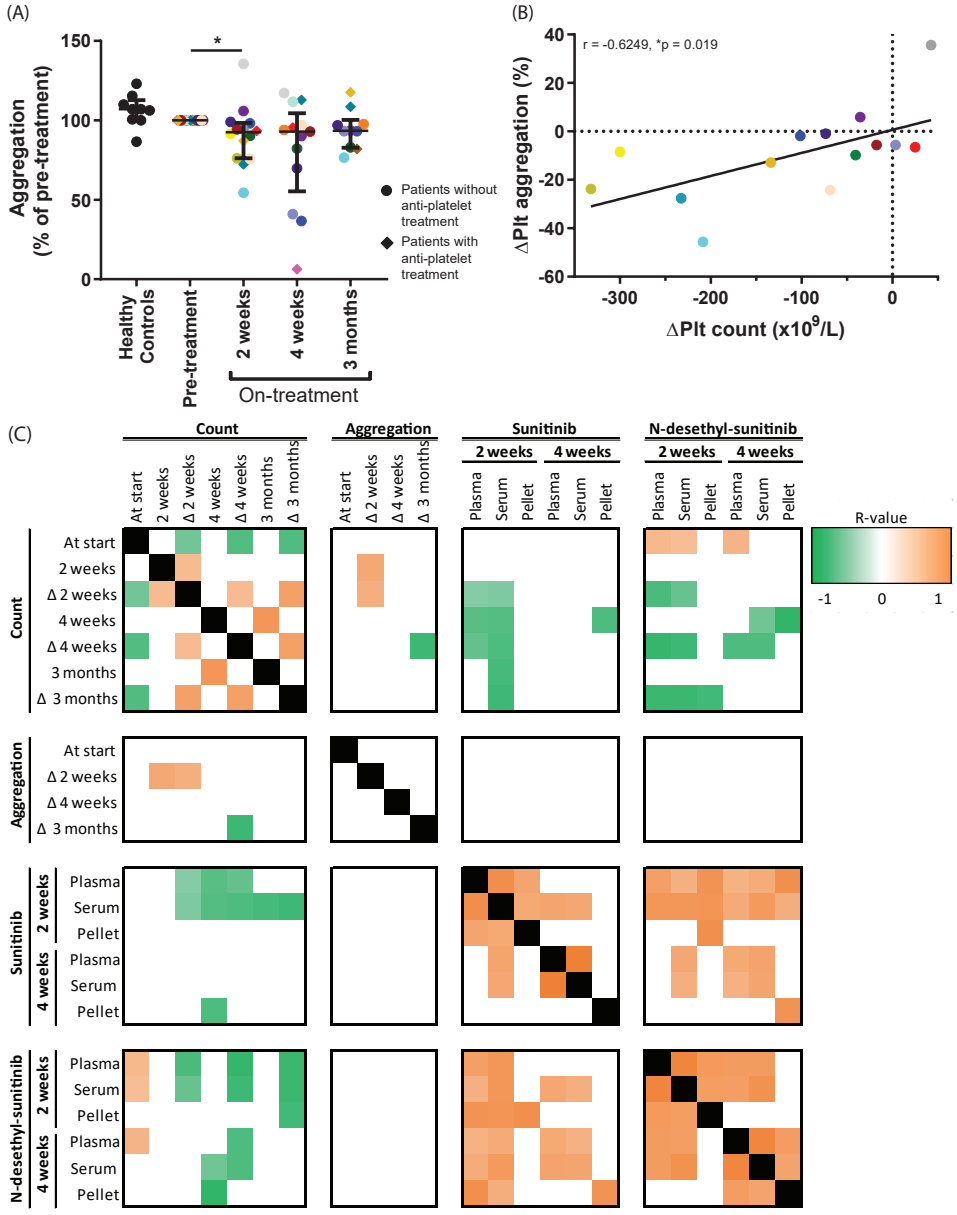


Figure 5.4. Platelet aggregation is reduced in RCC patients after 2 weeks of sunitinib treatment and correlates with reduction in platelet count. Blood was collected from 10 healthy controls and 20 RCC patients the day before, after 2 weeks, 4 weeks and 3 months on sunitinib treatment. (A) Light transmission aggregometry was induced in isolated platelets (250x10^9/L) by collagen type I. Scatterplots indicate percentage of aggregation normalized to aggregation pre-treatment. Circles indicate platelets from patients treated with sunitinib, stimulated with 1 µg/mL collagen; diamonds indicate platelets from patients treated with sunitinib and anti-platelet drugs, stimulated with 5 µg/mL collagen. Individual dots in the scatterplots represent one patient, for color coding see Table 5.1. Lines and error bars represent median ± interquartile range (n=10-20), *P<0.05. (B) Correlation between difference in platelet count (ΔPlt count = Plt count at 2 weeks on treatment – platelet count before treatment) and difference in platelet aggregation (ΔPlt aggregation = Maximum amplitude at 2 weeks on treatment – maximum amplitude before treatment). Individual dots in the scatterplots represent one patient, for color coding see Table 5.1. (C) Correlation of significant R values for (changes in) platelet count, change in aggregation response and (N-desethyl-)sunitnib concentration parameters, with a highly negative correlation in green, a highly positive correlation in orange and no correlation in white.

suggesting that the quantitative and qualitative platelet traits were jointly affected by the relative exposure to this TKI.

Changes in quantitative and qualitative platelet traits by sunitinib treatment of mRCC patients in relation to reported bleeding and disease progression

Of the 20 mRCC patients treated with sunitinib, 9 reported bleeding complications during the 3-month follow-up. Events included minor nose bleeds (epistaxis) or bleeding gums (gingivitis), blood in urine (haematuria), stool (gastrointestinal bleeding) or mucus (haemoptysis) (Table 5.1).

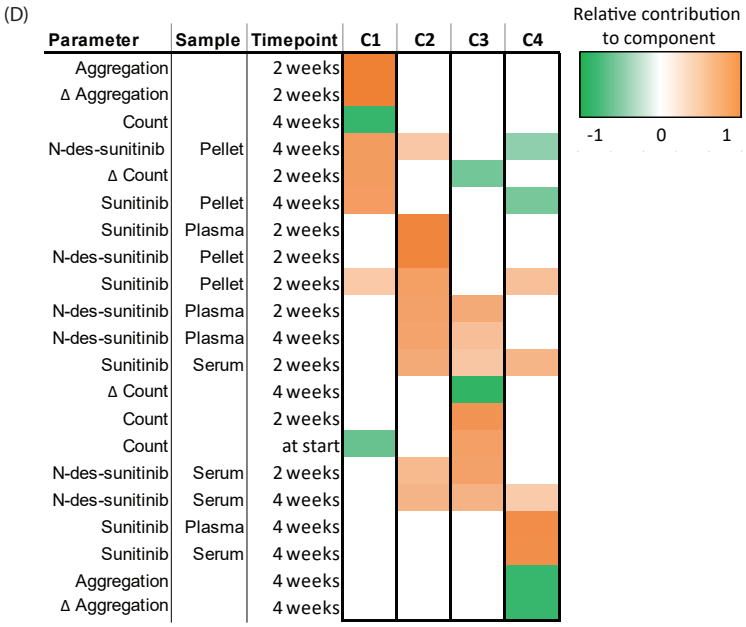


Figure 5.4. Continued. (D) Rotated component matrix determined by principal component analysis. Heatmap shows relative contribution of parameters to the four components (C1-4) with an eigenvalue over 1. Heatmap was filtered to only include values greater than 0.4 or less than-0.4 as important contributors to the determined component. Colors refer to a highly negative contribution in green, a highly positive contribution in orange and no correlation in white.

Table 5.3. Reported bleeding correlates with platelet aggregation in RCC patients at 4 weeks on treatment.

		Reported bleeding				Correlation	
Variables	Time point	No median (IQR)	n	Yes median (IQR)	n	R	P-value
Platelet count	Week 2	132.5 (75.8-229.8)	n=9	164.0 (119.0-194.8)	n=8	-0.0515	0.8498
	Week 4	78.5 (49.0-202.0)	n=7	132.3 (98.4-155.0)	n=8	0.0201	0.9508
	Month 3	162.5 (104.5-255.8)	n=4	161.5 (137.8-202.0)	n=5	-0.3651	0.3786
ΔAggregation	Week 2	-10.7 (-29.6 - 4.0)	n=6	-3.8 (-6.7 - 0.8)	n=6	0.2612	0.4072
	Week 4	-3.8 (-5.7 - 12.2)	n=4	-24.0 (-62.3 - -7.8)	n=8	-0.6680	0.0206*
	Month 3	-10.2 (-22.1 - 12.7)	n=4	-6.8 (-12.0 - -4.8)	n=5	0.02623	0.9516
Progression %	-	10.0 (0.0 - 31.3)	n=4	0 (-22.3 - 14)	n=8	-0.2500	0.4285

IQR: interquartile range.

Interestingly, of the 5 patients who were on dual treatment of sunitinib and aspirin, only 1 patient (also treated with heparin) reported bleeding events during the first cycle of sunitinib treatment.

We analysed whether the altered platelet count correlated with the reported bleeding. As shown in Table 5.3, this was not the case. On the other hand, we observed that platelet function, as measured by collagen-induced aggregation, was negatively correlated with bleeding at 4 weeks on-treatment (Table 5.3), although the number of patients included in this analysis was relatively small (n=4-8). At the other time points this correlation was not observed.

After three months on treatment, CT scans were evaluated for cancer response in 15 patients (Table 5.1). In 5 of these patients progression of the disease was established, with an average of 23% increase in tumor size. In the other 10 patients no progression was observed (Table 5.1). In this limited group of patients, no correlation was observed between disease progression and reduction in blood cell counts or platelet aggregation (Suppl. Table 5.1).

Discussion

In the present study with patients treated with sunitinib for mRCC, we observed that changes in platelet count were related to the inhibitor levels in plasma and serum. The reduction in platelet count may thus reflect the effective sunitinib levels in the patient, which may be used for therapeutic drug monitoring[19]. Furthermore, we observed a strong correlation between effects on platelet count and collagen-induced platelet aggregation, regardless of the treatment time point. This pointed to a common effect of this TKI on quantitative and qualitative platelet traits, which was confirmed by PCA. Next to RCC, sunitinib is approved for the treatment of gastrointestinal stromal tumor (GIST) and pancreatic neuroendocrine tumors. Furthermore, phase 3 clinical trials with sunitinib have been completed with positive outcome for non-small cell lung cancer[20, 21]. Hence, the results of the present study may also be applicable to other cancer types. In addition, platelet traits have been reported to be affected by other TKIs used for treatment of a large variety of malignancies[1, 9]. Also, other anti-cancer drugs can be taken up by platelets[22]. Therefore, the results of the present study with sunitinib may serve as a proof-of-concept with regards to platelet trait effects of other drugs.

We observed that the patients on-treatment who showed a stronger inhibition in platelet aggregation (at normalized platelet count), also displayed a lower platelet count in whole blood. This can be explained by two separate mechanisms elicited by sunitinib, being (i) an effect on megakaryocyte level, culminating in a lower platelet count, and (ii) an effect on platelet function (aggregation), likely via an impaired tyrosine kinase-mediated signaling. Regarding the first mechanism, sunitinib may affect megakaryocytes or precursor cells directly in the bone marrow via interference in the proliferation of megakaryocytes and the production of proplatelets, which processes are dependent on tyrosine kinase activity[23]. Alternatively, the effects on megakaryocytes may be indirect via altered thrombopoietin production. Tumors can stimulate

platelet production via de secretion of various cytokines, especially interleukin-6, which can in turn enhance thrombopoietin expression in the liver[24]. A direct effect of sunitinib on tumor proliferation may result in reduced levels of thrombopoietin and thereby lowering of the platelet count. Besides platelet count, we confirmed that sunitinib also reduced the WBC count (up to grade 2) in the mRCC patients. Furthermore, also the RBC count, and hemoglobin and hematocrit levels were affected upon sunitinib treatment. These results point to an effect of sunitinib on different hematopoietic cell populations in the bone marrow. Other studies with larger patient numbers indeed concluded that a reduced neutrophil count could be a predictor of PFS and OS probability[13, 25]. With regard to RBCs, sunitinib has been reported to affect erythropoiesis in both directions, resulting in either anemia[26] or in erythrocytosis[27]. Markedly, in our small patient group, we observed a transient increase in RBC count, hemoglobin and hematocrit during the first cycle of sunitinib treatment, as reported previously and explained by cycling kinetics during the dosing schedule[28].

Regarding the second mechanism of sunitinib affecting tyrosine kinase-mediated platelet signaling, we observed that collagen-induced platelet aggregation was reduced in mRCC patients after 14 days of sunitinib treatment. Others have shown that this inhibition was already present after 24 hours of sunitinib treatment[29]. Besides aggregation, sunitinib also inhibits collagen-induced thrombus formation, exposure of phosphatidylserine under flow, as well as α-granule secretion[11, 29]. As discussed before, the protein tyrosine kinases that are affected by sunitinib are c-Src[29], as well as other kinases expressed in platelets (e.g., Axl, CSF and Itk)[1].

As platelets are able to take up sunitinib[11], we hypothesized that this affects the outcome of sunitinib plasma measurements. Therefore, we compared the concentrations of sunitinib and N-desethyl-sunitinib (active metabolite of sunitinib) in plasma, serum and isolated platelets from mRCC patients at 2 and 4 weeks of treatment. The concentration of N-desethyl-sunitinib was much lower than sunitinib in both serum and plasma, which is in line with previous results[30]. Furthermore, there was no difference between serum and plasma levels of either sunitinib or N-desethyl-sunitinib at 2 or 4 weeks of sunitinib administration. Honeywell et al. also reported no differences for sunitinib in plasma as compared to serum for 5 patients on sunitinib treatment after 24 hours and 3 weeks[31]. In contrast, another study demonstrated lower concentrations of sunitinib in plasma as compared to serum in patients treated with sunitinib[29]. These studies demonstrated a high variation in sunitinib plasma concentrations, which could only partly be explained by patient- or medication-related factors[32]. This highlights the importance for further research of therapeutic drug monitoring for individual dosage adjustments, especially in cases of toxicity[19]. Interestingly, the concentrations of the active metabolite were significantly higher in isolated platelets from mRCC patients at 2 and 4 weeks as compared to that of sunitinib itself. The only difference between the two molecules is an ethyl (CH2-CH3) group that is removed from sunitinib by cytochrome P450[33]. As this enzyme not present in platelets, these results suggest

preferential uptake of the metabolite by these cells. Overall, we concluded that although the concentration of the active metabolite in platelets was higher, the uptake of sunitinib in platelets did not affect the sunitinib concentration in plasma versus serum. The serum concentration of N-desethyl-sunitinib negatively correlated with platelet count at 4 weeks of treatment, suggesting that higher platelet counts result in a lower concentration of metabolite present in serum. Together with the observation that metabolite concentrations in isolated platelets were higher, this may suggest that platelets do not secrete sunitinib after uptake.

Mild bleeding was reported in 9 of the 20 investigated cancer patients on sunitinib. No correlation existed between the platelet count at any time point on treatment and the occurrence of bleeding during the 3-month study period. This agrees with earlier reports that showed that the platelet count is a poor predictor for bleeding[34, 35]. Hence, the reported bleeding events may be explained by an accompanying inhibition of platelet activation by sunitinib. We observed a correlation between platelet aggregation responses and bleeding during sunitinib treatment, meaning that stronger reduction in collagen-induced platelet aggregation was observed in patients that reported bleeding episodes. Of note, only a small number of patients was included in this analysis, and at no other time points a correlation was observed between platelet aggregation and reported bleeding. Also, Walraven et al. did not observe this correlation between bleeding and platelet aggregation after 3 weeks of treatment in 5 patients[29]. As normal platelet functions can be considered to be required for effective control of hemostasis, correlations between bleeding and TKI-induced effects on platelet function should be investigated in larger clinical studies. Of note, in patients treated with sunitinib, also endothelial dysfunction and increased capillary leakage can be induced by treatment with sunitinib[36], which may also play a role in the increased bleeding tendency. Paradoxically, interfering with endothelial cell integrity can also shift the hemostatic balance in favor of thrombosis, and treatment with sunitinib and other TKIs have also been associated with an increase in arterial thromboembolic events in RCC patients[36, 37].

A correlation between platelet count and disease progression in this study could not be observed due to a relatively small number of patients. However, it has already been demonstrated in large, retrospective patient studies that a decrease in platelet count following sunitinib treatment more likely resulted in a response to therapy and longer OS in mRCC[12] and GIST[13]. In the present study we clearly showed that a reduction in platelet count is accompanied by an inhibition of platelet aggregation, which were correlated to the (N-desethyl-)sunitinib levels in either serum or plasma of mRCC patients (component 1 of the PCA). Altogether this suggested that both quantitative and qualitative platelet traits were linked to the exposure to this TKI.

Renal cancer is a disease of the elderly, with most patients being diagnosed between 65 and 74[38, 39]. Older cancer patients generally have more comorbidities, especially cardio-vascular disease, and therefore often receive anti-coagulant or anti-platelet drugs. In the present study, five patients were treated with sunitinib in combination with aspirin and/or atorvastatin, both

affecting platelet functions[17, 18, 40]. In our tests, this did not result in significant differences in platelet aggregation as compared to patients on sunitinib without comedication. One explanation may be an increased platelet reactivity observed in cancer patients[41], which compensates for the intake of the anti-platelet drugs. On the other hand, a recent study has shown combined effects of ibrutinib and aspirin on collagen-induced aggregation[42]. Therefore, the effects of dual anti-platelet and TKIs treatments on the inhibition of platelet function should be further investigated.

Our study encounters some limitations. Due to the limited number of patients and some missing values, no (clear) correlations were observed between platelet count and function on the one hand and bleeding and disease progression on the other hand. Also, only one platelet function test could be performed due to the limited amount of blood obtained from the patients. Out of multiple function tests, we opted for light transmission aggregometry as this is still the gold standard for platelet function testing. Due to ethical restrictions, the relation between platelet dysfunction after sunitinib treatment and abnormal (pro)platelet formation from megakaryocytes in the bone marrow could not be assessed. For the TKI dasatinib it has been reported that it promoted megakaryocyte differentiation, while it impaired migration and proplatelet formation[43]. The effects of TKI treatment on megakaryocyte proliferation and function should therefore be investigated in future studies.

In summary, in this study with mRCC patients treated with sunitinib a strong correlation was found between the effects on platelet count and inhibition of aggregation as well as (N-desethyl) sunitinib levels in plasma and serum. The effects on count and function may thus reflect the relative exposure of the patient to sunitinib. This pointed to an association between the effect of this TKI on quantitative and qualitative platelet traits. These results may serve as a proof-of-principle with regards to other drugs, indicating that TKI treatment can affect both platelet count and function, which could be used as a measure for relative drug exposure and therapeutic drug monitoring.

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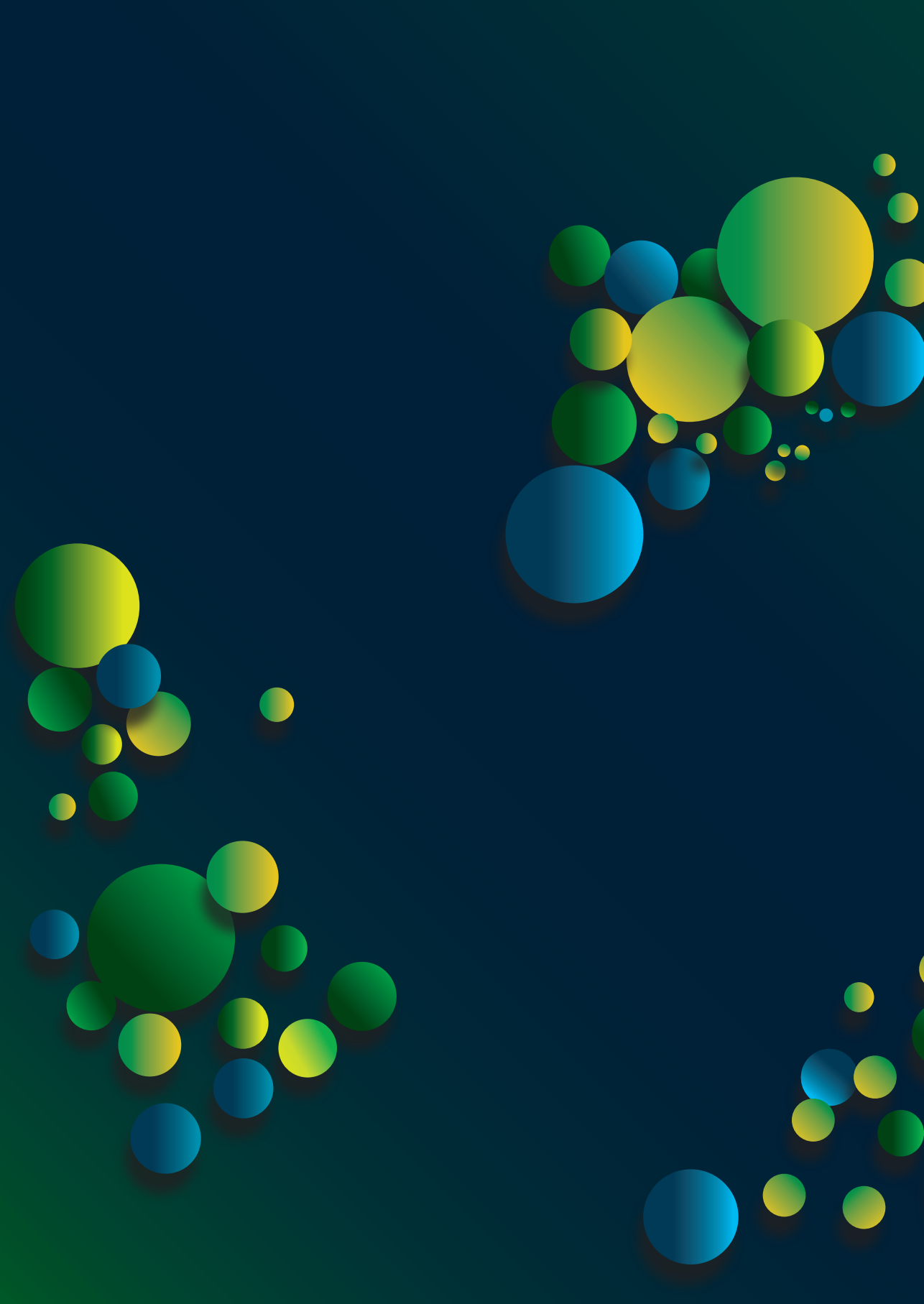
Supplemental data

Supplemental Table 5.1. No correlation of disease progression with changes in blood cell counts and platelet aggregation in mRCC patients

		Reported bleeding				Correlation	
Variables	Time point	No (PR + SD) median (IQR)	n	Yes (PD) median (IQR)	n	R	P-value
ΔPlatelet count	Week 2	-87.5 (-189.9 - -36.8)	n=8	-36.5 (-60.3 - 24.8)	n=4	0.4609	0.1535
	Week 4	-143.3 (-194.6 - -45.1)	n=8	-73.0 (-147.0 - -8.8)	n=5	0.2535	0.4351
	Month 3	-77.8 (-137.8 - -8.6)	n=8	4.5 (-42.5 - 51.5)	n=2	0.4352	0.2667
ΔWhite blood cell count	Week 2	-1.68 (-4.04 - -0.08)	n=8	-0.68 (-2.05 - 1.26)	n=4	0.4104	0.198
	Week 4	-3.65 (-5.03 - -2.02)	n=8	-2.65 (-3.18 - -0.83)	n=5	0.4655	0.1181
	Month 3	-3.70 (-5.70 - -1.35)	n=8	0.65	n=1	0.5477	0.2222
ΔRed blood cell count	Week 2	0.21 (0.20 - 0.33)	n=8	0.25 (-0.07 - 0.41)	n=4	0.1282	0.7152
	Week 4	-0.06 (-0.17 - 0.18)	n=8	0.08 (-0.01 - 0.35)	n=5	0.3803	0.2222
	Month 3	-0.93 (-1.73 - -0.50)	n=8	-0.87	n=1	0.1369	0.8889
ΔAggregation	Week 2	-7.7 (-24.0 - -1.2)	n=8	-5.7	n=1	0.0688	1.0000
	Week 4	-24.0 (-60.2 - -4.2)	n=8	-6.6 (-72.1 - 7.3)	n=4	0.2132	0.6095
	Month 3	-6.6 (-14.5 - 5.6)	n=6	-10.2 (-18.1 - -2.4)	n=2	-0.0870	0.8889

IQR: interquartile range; PR: partial response; PD: progressive disease; SD: stable disease.



Chapter 6

Comparison of inhibitory effects of irreversible and reversible Btk inhibitors on platelet function

Tullemans BME, Karel MFA, Léopold V, ten Brink MS, Baaten CCFMJ, Maas SL, de Vos AF, Eble JA, Nijziel MR, van der Vorst EPC, Cosemans JMEM, Heemskerk JWM, Claushuis TAM* and Kuijpers MJE*

(* equal contribution)

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Abstract

All irreversible Bruton tyrosine kinase (Btk) inhibitors, including ibrutinib and acalabrutinib induce platelet dysfunction and increased bleeding risk. New reversible Btk inhibitors were developed, like MK-1026. The mechanism underlying increased bleeding tendency with Btk inhibitors remains unclear. We investigated the effects of ibrutinib, acalabrutinib and MK-1026 on platelet function in healthy volunteers, patients and Btk-deficient mice, together with off-target effects on tyrosine kinase phosphorylation. All inhibitors suppressed GPVI- and CLEC-2-mediated platelet aggregation, activation and secretion in a dose-dependent manner. Only ibrutinib inhibited thrombus formation on vWF-co-coated surfaces, while on collagen this was not affected. In blood from Btk-deficient mice, collagen-induced thrombus formation under flow was reduced, but preincubation with either inhibitor was without additional effects. MK-1026 showed less off-target effects upon GPVI-induced TK phosphorylation as compared to ibrutinib and acalabrutinib. In ibrutinib-treated patients, GPVI-stimulated platelet activation and adhesion on vWF-co-coated surfaces were inhibited, while CLEC-2 stimulation induced variable responses. The dual inhibition of GPVI and CLEC-2 signalling by Btk inhibitors might account for the increased bleeding tendency, with ibrutinib causing more high-grade bleedings due to additional inhibition of platelet-vWF interaction. As MK-1026 showed less off-target effects and only affected activation of isolated platelets, it might be promising for future treatment.

Introduction

Ibrutinib, an irreversible Bruton's tyrosine kinase (Btk) inhibitor, is widely used to treat multiple haematological malignancies[1-5]. Ibrutinib treatment shows high response rates and improved progression-free survival, with decreased toxicity when compared to conventional treatment options[1-6]. However, ibrutinib is prescribed life-long (or until progression) and requires side effect management. These toxicities are responsible for discontinuation in up to 20% of patients[6-9]. A prominent side effect of ibrutinib is bleeding, with over 50% of patients experiencing bleeding events within 3 years, ranging from mild mucocutaneous bleeding to life-threatening haemorrhage[3, 6, 8, 10]. Studies have suggested that ibrutinib-associated bleeding tendency is caused by interference with platelet GPVI signalling pathway through off-target effects on Src kinases[11, 12].

Recently, multiple other Btk inhibitors have been developed, including the irreversible Btk inhibitor acalabrutinib and the reversible Btk inhibitor MK-1026 (formerly known as ARQ-531)[13, 14]. Opposed to data suggesting that acalabrutinib shows less off-target effects, it still causes an increased bleeding tendency in patients, albeit showing mainly mild bleeding[15]. Studies investigating GPVI inhibitors as potential antithrombotic drugs have shown that inhibition on receptor level does not impair haemostasis[16]. Also, GPVI-deficient patients exhibit only a mild bleeding diathesis[17] and many potential patients are undiagnosed as they remain asymptomatic[18]. In agreement, studies in mice have shown that GPVI deficiency alone does not impair haemostasis[19-21], while combined GPVI and CLEC-2 deficiency does[21]. Furthermore, Btk was shown to be involved in GPIb-vWF mediated platelet adhesion, as well as integrin $\alpha_{IIb}\beta_3$ outside-in activation which were inhibited by ibrutinib[6, 22, 23]. Therefore, the current mechanism of increased bleeding tendency in patients using Btk inhibitors is still unclear and underlines the importance for treatment decisions and the development of novel Btk inhibitors. We therefore investigated the effect of multiple Btk inhibitors on platelet function pathways in healthy volunteers, patients and Btk knock-out (KO) mice.

Materials and Methods

Materials and methods are available in Supplemental Data.

Results

Ibrutinib and acalabrutinib inhibit platelet activation and aggregation by GPVI and CLEC-2 in a dose-dependent manner

Treatment with ibrutinib, but also acalabrutinib, can increase bleeding tendency[6, 15]. To assess the underlying pathways, we investigated platelet activation mediated by multiple agonists in presence of ibrutinib or acalabrutinib.

Both ibrutinib and acalabrutinib inhibited aggregation of washed platelets and platelet-rich plasma induced by 1 µg/ml collagen in a dose-dependent manner (Fig 6.1A). In isolated

platelets, a significant inhibition was observed at 1 µM and higher for both compounds (Fig 6.1Ai). In the presence of plasma, a higher concentration of 5-10 µM was required to significantly inhibit collagen-induced platelet aggregation (Fig 6.1Aii). The IC_{50} values for ibrutinib and acalabrutinib were 7.7 and 6.0 times lower in washed platelets as compared to PRP (Suppl Table 6.1). At a higher collagen concentration, aggregation was not affected by either inhibitor. (Suppl Fig 6.1Aii). CLEC-2-induced platelet aggregation was also inhibited by both acalabrutinib and ibrutinib in a similar dose-dependent manner (Fig 6.1B). GPVI-induced integrin $\alpha_{IIb}\beta_3$ activation, P-selectin expression, and PS-exposure were also dose-dependently reduced by both inhibitors, although lower

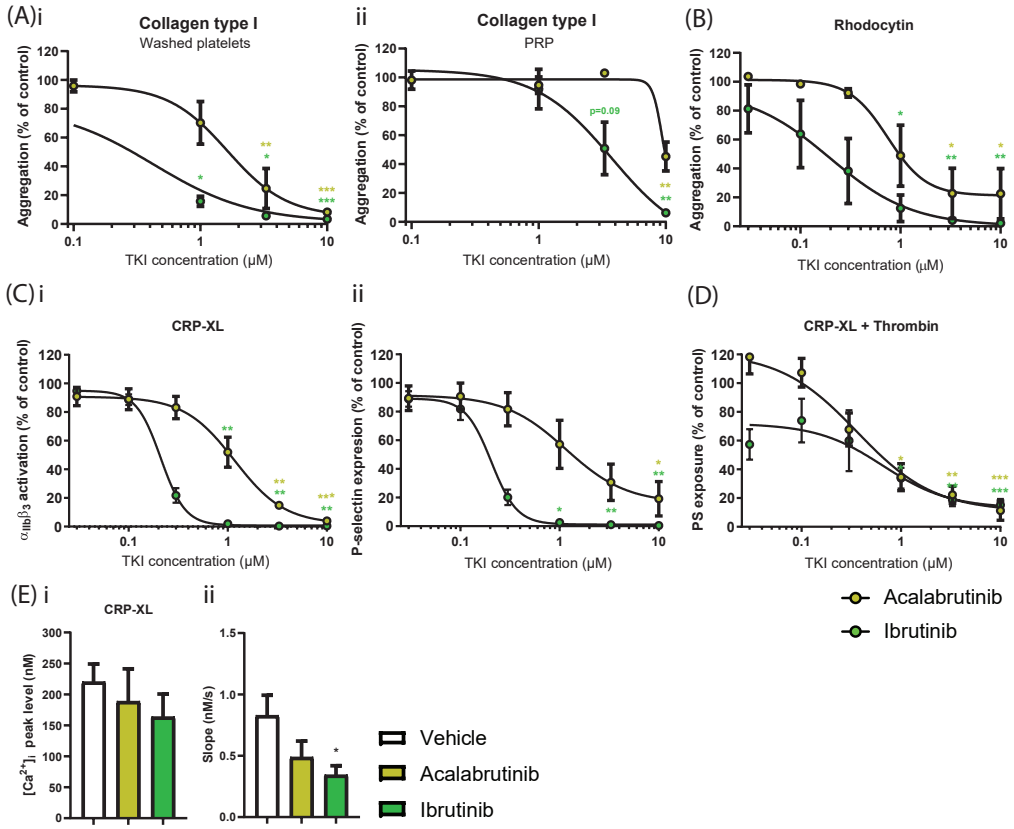

Figure 6.1. Ibrutinib and acalabrutinib inhibit GPVI- and CLEC-2-induced platelet activation and aggregation in a dose-dependent manner. Washed platelets (Ai, B) or PRP (Aii) ($250x10^9$/L) from healthy volunteers were incubated with different concentrations of acalabrutinib or ibrutinib for 10 minutes at 37°C. Shown are dose-response curves of aggregation induced by collagen type I (A, 1 µg/ml) or rhodocytin (B, 1 µg/ml) in presence of acalabrutinib (lime circles) or ibrutinib (green circles) normalised to control. (C) Washed platelets ($100x10^9$/L) were pre-incubated with different concentrations of acalabrutinib or ibrutinib and stimulated with collagen-related peptide (CRP-XL, 5 µg/mL) for 15 minutes and simultaneously stained for activated integrin $\alpha_{IIb}\beta_3$ (i) and α-granule secretion (P-selectin, ii). Dose response curves show the percentages of platelets binding FITC-labelled PAC-1 monoclonal antibody or AF647-labelled anti-human CD62 mAb normalized to control expression. (D) Platelets pre-treated with different concentrations of acalabrutinib or ibrutinib were stimulated using both CRP-XL (5 µg/mL) and thrombin (4 nM) for 60 minutes at 37°C and PS-exposure was determined by flow cytometry. Histogram shows percentage of platelets binding FITC-labelled annexin A5 normalized to control expression. (E) Fura-2-loaded platelets ($200x10^9$/L) pre-incubated with vehicle, acalabrutinib (1 µM) or ibrutinib (1 µM) were stimulated with CRP-XL (10 µg/mL) in presence of 2 mM $CaCl_2$. Histograms show (i) maximal rise in $[Ca^{2+}]_i$ and (ii) slope. Data are shown as means ± SEM (n=6-7). * p<0.05, ** p<0.01 vs control (vehicle).

concentrations of ibrutinib (<1 µM) resulted in significant inhibition as compared to acalabrutinib (Fig 6.1C-D). The IC_{50} values of ibrutinib for the inhibition of integrin $\alpha_{IIb}\beta_3$ activation, P-selectin expression and PS exposure were 5.0, 8.2 and 2.2 times lower, respectively, than for acalabrutinib (Suppl. Table 6.1). Furthermore, both compounds did not alter the peak level of intracellular Ca^{2+} elevations, but the slope of this response was reduced in ibrutinib-treated platelets (Fig 6.1E).

Aggregation induced by the $P2Y_{12}$ agonist 2MeS-ADP, the thromboxane A_2 analogue U46619, and thrombin (Suppl Fig 6.1A) as well as ristocetin remained unaffected in presence of Btk inhibitors (Suppl Fig 6.1B). ADP-stimulated $\alpha_{IIb}\beta_3$ activation and α-granule secretion were mildly inhibited by both Btk inhibitors (Suppl Fig 6.1C). No effects on $\alpha_{IIb}\beta_3$ activation or α-granule secretion were observed upon thrombin activation (Suppl Fig 6.1C). Altogether, these results suggest that ibrutinib and acalabrutinib inhibit both GPVI- and CLEC-2-mediated platelet aggregation, albeit lower concentrations of ibrutinib were required. This might explain the increased bleeding tendency seen with both inhibitors.

Ibrutinib, but not acalabrutinib, impairs thrombus formation in whole blood on surfaces co-coated with vWF

We for the first time investigated the effects of ibrutinib and acalabrutinib on whole blood thrombus formation under flow, simultaneously over multiple surfaces[24]. This allowed systematic analysis of platelet activation and thrombus formation on collagen type I and III and vWF co-coated with laminin, rhodocytin, ristocetin or fibrinogen. In the presence of plasma, concentrations higher than 3.3 µM were required to significantly inhibit collagen-induced aggregation (Fig 6.1A). Considering previous *in vitro* studies, as well as plasma concentrations of ibrutinib and acalabrutinib in patients for inhibition of B-cell carcinomas[15, 25], we selected 1 µM to inhibit washed platelets and 5 µM to inhibit platelets in the presence of plasma.

Microscopic visualization of platelet adhesion, activation and thrombus formation on collagen showed that neither of the two inhibitors affected these processes, except for reduced PS-exposure by ibrutinib (Fig 6.2A). This was confirmed by image analysis resulting in five thrombus parameters (P1-P5) and three activation markers (P6-8, Suppl Fig 6.2). To systematically summarize the effects on all parameters, cumulative histograms were generated showing that ibrutinib, but not acalabrutinib, reduced PS-exposure on collagen type I and III (Fig 2B, P8). Upon blood perfusion over surfaces that trigger GPIb alone (vWF+ristocetin) or in combination with CLEC-2 (vWF+rhodocytin), $\alpha_6\beta_1$ (vWF+laminin) or $\alpha_{IIb}\beta_3$ (vWF+fibrinogen), only ibrutinib decreased almost all parameters of thrombus formation (Fig 6.2B, Suppl Fig 6.2). In comparison to control, thrombi were smaller and less compact in structure with ibrutinib and showed reduced expression of activated integrin $\alpha_{IIb}\beta_3$ and P-selectin (α-granule release) (Suppl Fig 6.2). These results show that, in contrast to acalabrutinib, platelet adhesion to surfaces co-coated with vWF was strongly impaired by ibrutinib.

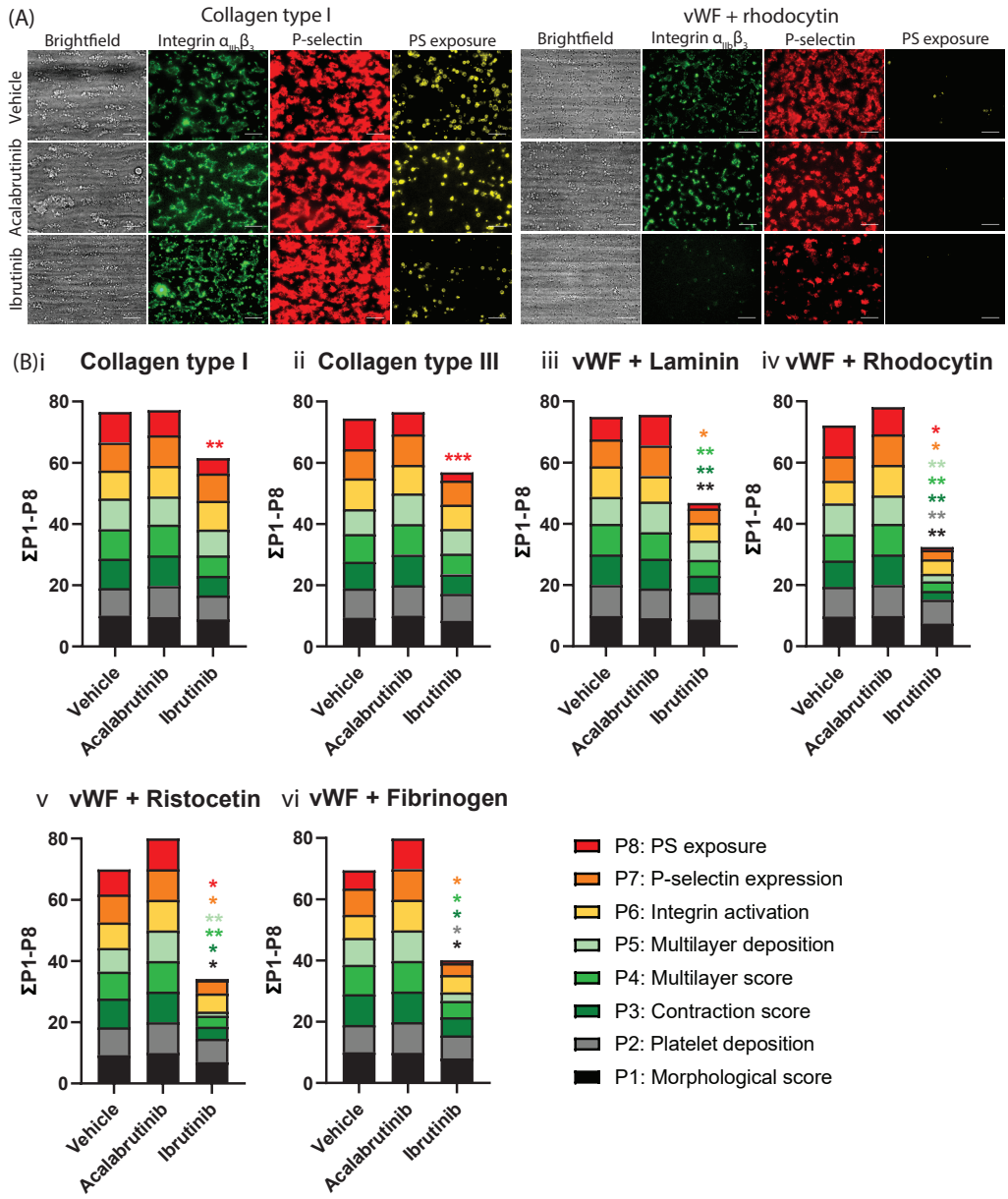

Figure 6.2. Ibrutinib, but not acalabrutinib, affected thrombus formation in whole blood perfused over six different surfaces. Pre-incubated blood samples from healthy donors with vehicle (<0.1% DMSO), acalabrutinib (5 µM) or ibrutinib (5 µM) were recalcified in presence of PPACK. Blood was perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over six different surfaces. After perfusion, thrombi were stained with AF568-annexin A5, FITC-α-fibrinogen and AF647-α-CD62P to detect PS-exposure, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression. (A) Representative images of thrombi formed on collagen type I and vWF co-coated with rhodocytin. (B) Histograms show the cumulative representation of scaled values from 0-10 for each parameter over different surfaces consisting of (i) collagen type I, (ii) collagen type III, (iii) vWF co-coated with laminin, (iv) vWF co-coated with rhodocytin, (v) vWF co-coated with ristocetin and (vi) vWF co-coated with fibrinogen in presence of acalabrutinib or ibrutinib. Colours reflect the adhesion parameters (P1-2; black, grey), aggregation parameters (P3-5; shades of green), and activation parameters (P6-8; yellow, orange and red). * p<0.05, ** p<0.01, *** p<0.001 vs vehicle.

Effects of ibrutinib and acalabrutinib on wild-type and Btk-deficient mouse platelets

To further examine the effects of ibrutinib and acalabrutinib on platelets, thrombus formation and platelet activation were determined in blood from WT and Btk-KO mice. Blood samples were incubated with ibrutinib or acalabrutinib before perfusion over collagen type I. In control conditions (vehicle), thrombus formation was reduced in blood from Btk-KO mice with regard to platelet deposition, multilayer formation (P1-2, P5) and PS-exposure (P8, Fig 6.3A-B, Suppl Fig 6.3A). In WT blood, presence of acalabrutinib gave a similar inhibitory pattern of thrombus formation (Fig 6.3A, C). Comparable to human blood, incubation of WT mouse blood with ibrutinib resulted in limited effects on thrombus formation, only a decrease in PS-exposure was observed (Fig 6.3A, C, Suppl Fig 6.3A). When Btk inhibitors were pre-incubated in blood from Btk-KO mice, no additional inhibition on thrombus formation was observed (Figure 6.3A, C, suppl Fig 6.3A). The only observed effect, was a reduction in P-selectin expression in the presence of ibrutinib, as compared to vehicle, in Btk-KO mice (Figure 6.3A, C, Suppl Fig 6.3).

GPVI-induced platelet activation by CRP-XL was strongly reduced in Btk-KO platelets as compared to WT with regard to $\alpha_{IIb}\beta_3$ activation, α-granule release and PS-exposure, as determined by flow cytometry (Fig 6.3D-E). This was in agreement with the results obtained from whole blood perfusion experiments. Interestingly, $\alpha_{IIb}\beta_3$ activation and P-selectin expression were also moderately reduced in Btk-KO mice upon PAR4 stimulation, while activation with ADP was not affected (Suppl Fig 6.3B-C). When blood was preincubated with Btk inhibitors, we observed significant reduction of all platelet activation markers in WT platelets upon GPVI stimulation (Fig 6.3D-E). In platelets from Btk-KO mice, both inhibitors did not further reduce $\alpha_{IIb}\beta_3$ activation and P-selectin expression regardless of the agonist. We only observed significant reduction of PS-exposure in Btk-KO platelets in the presence of ibrutinib, as compared to vehicle (Fig 6.3E).

Overall, preincubation of whole blood from Btk-KO mice with acalabrutinib and ibrutinib did not result in major additional effects on thrombus formation on collagen under flow, as well as on platelet activation.

Btk inhibitors reduce GPVI-induced phosphorylation of multiple platelet tyrosine kinases

To evaluate off-target effects of the Btk inhibitors, we performed a PamGene kinase assay to visualize which tyrosine kinases were regulated upon platelet stimulation via GPVI, as well as those tyrosine kinases that were subsequently influenced upon pre-incubation of the platelets with 1 μM ibrutinib or acalabrutinib. We observed that in isolated platelets, in total 73 tyrosine kinases were significantly phosphorylated by stimulation with CRP[65], of which were significantly inhibited by ibrutinib and acalabrutinib preincubation (Fig 6.4). Median kinase statistics showed that ibrutinib inhibited tyrosine phosphorylation of these proteins on average 4.4-fold, while acalabrutinib was less strong and reduced this response 1.8-fold, i.e. 2.5-fold weaker as compared to ibrutinib.

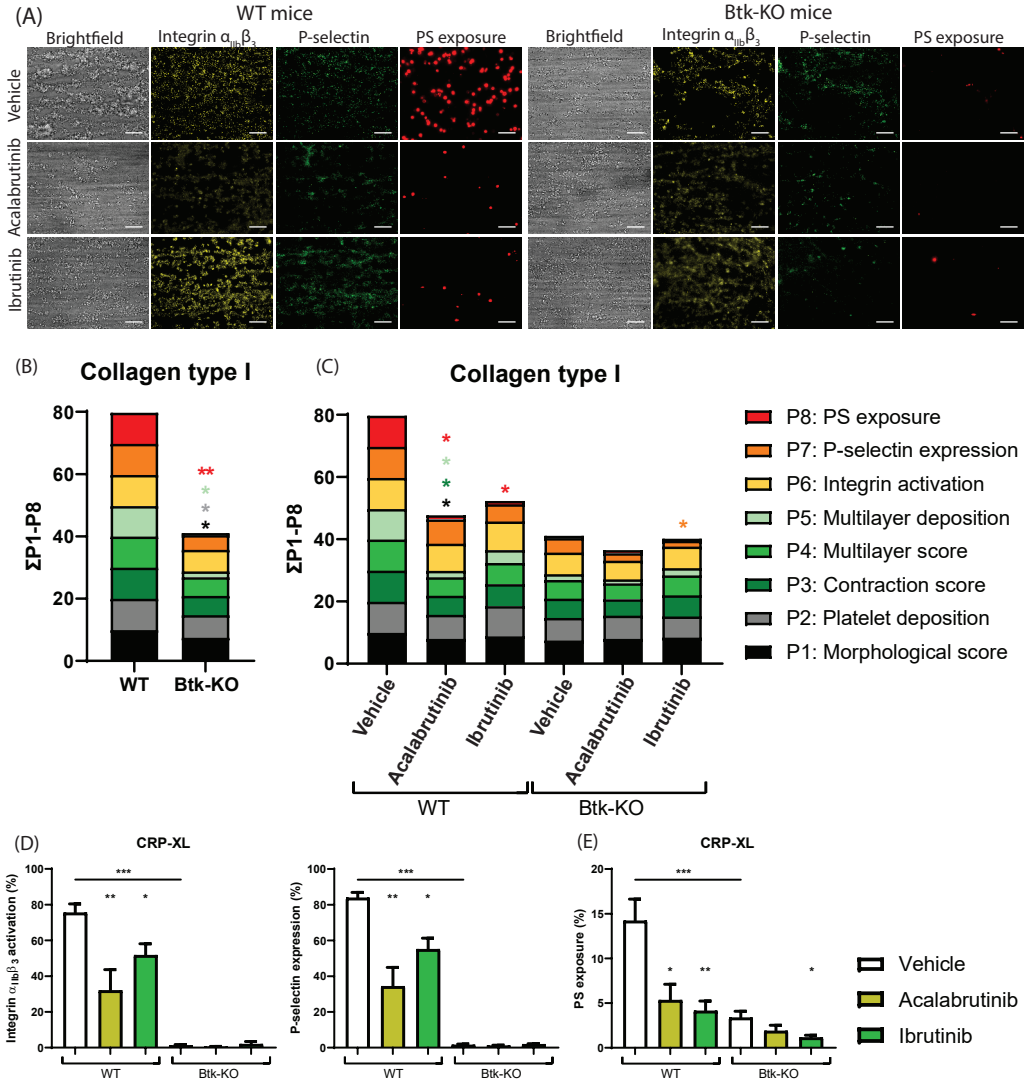

Figure 6.3. Acalabrutinib and ibrutinib reduced thrombus formation and PS-exposure in whole blood under flow, as well as activation of isolated platelets from WT but not Btk-KO mice. Pooled citrated whole blood samples from two WT or Btk-KO mice were pre-incubated with vehicle (<0.1% DMSO), acalabrutinib (5 μM) or ibrutinib (5 μM) in the presence of PPACK and fragmin for 10 minutes at 37°C. After incubation, blood samples were recalcified and perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over a collagen type I surface. Thrombi were post-stained with FITC-labelled anti-CD62P mAb, PE-labelled JonA mAb and AF647-labelled Annexin-A5 to detect P-selectin expression, integrin activation and PS-exposure respectively. (A) Representative brightfield and fluorescence images from thrombus formation in blood from WT and Btk-KO mice for each inhibitor. (B) Histograms show the cumulative representation of scaled values from 0-10 for each parameter on a collagen type I surface of thrombus formation in WT and Btk-KO mice blood samples. Colours reflect the adhesion parameters (P1-2; black, grey), aggregation parameters (P3-5; shades of green), and activation parameters (P6-8; yellow, orange and red). (C) Histograms of cumulative scaled values of multiple parameters in WT or Btk-KO mice with addition of vehicle (<0.1% DMSO), acalabrutinib or ibrutinib. (D) Platelet activation in whole blood induced by CRP-XL (1 μg/mL). Histograms show the percentages of platelets binding PE-labelled JON/A monoclonal antibody against activated $\alpha_{IIb}\beta_3$ integrin or BB700-labelled CD62P mAb in WT and Btk-KO mice. (E) PS-exposure of platelets induced by CRP-XL (1 μg/mL). Histogram shows percentage of platelets binding FITC-labelled lactadherin. Data are shown as means ± SEM (n=6-8). * p<0.05. ** p<0.01, *** p<0.001 vs vehicle or otherwise indicated.

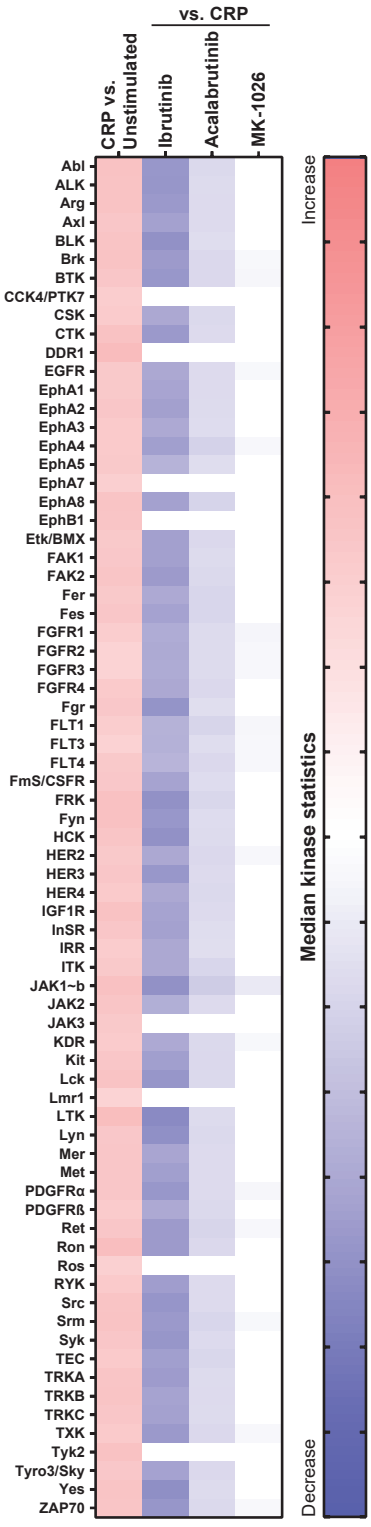

Figure 6.4. Effect of ibrutinib, acalabrutinib and MK-1026 on GPVI-induced tyrosine kinase activity. Washed platelets ($500x10^9$/L) were pretreated with vehicle (control) or 1 µM ibrutinib, acalabrutinib or MK-1026 for 10 minutes at 37°C and were subsequently stimulated for 90 seconds with 5 µg/mL CRP-XL in the presence of 2 mM $CaCl_2$. Unstimulated, resting platelets served as control. Heatmap visualizes all tyrosine kinases that are significantly up- or downregulated upon CRP stimulation (sorted alphabetically). Cut-off has been set at a median final score of at least 1.2. Colour reflects the calculated median kinase statistic reflecting effect size and directionality (red: upregulated; blue: downregulated).

In comparison to these irreversible inhibitors, we investigated if the reversible Btk inhibitor MK-1026[14] showed a more favourable anti-platelet effect. First, we evaluated which platelet tyrosine kinases were affected by this compound upon GPVI stimulation. Only 18 tyrosine kinases were significantly inhibited by MK-1026 (Fig 6.4). Median kinase statistics showed that MK-1026 inhibited tyrosine phosphorylation of these proteins on average 0.52-fold, which was 8.3-fold lower as compared to ibrutinib. As expected, all three inhibitors significantly inhibited the phosphorylation of Btk, and ibrutinib and acalabrutinib also inhibited Tec. Furthermore, ibrutinib and acalabrutinib also inhibited the phosphorylation of Src family kinase (SFKs) and other downstream proteins as previously described[11, 12].

These results show that ibrutinib and acalabrutinib showed the same off-target effects on GPVI-induced tyrosine kinase phosphorylation in platelets, although the effects of acalabrutinib were less strong. The reversible inhibitor MK-1026 appeared to have less off-target effects in platelets upon GPVI stimulation, in combination with a less strong inhibition profile as compared to the irreversible inhibitors.

The reversible Btk inhibitor MK-1026 shows only limited anti-platelet effects in human and mouse platelets in vitro

As we demonstrated that irreversible inhibition of Btk can impair platelet function, we also investigated the effects of the reversible Btk inhibitor MK-1026 on platelet function. First, we performed collagen- and rhodocytin-induced dose-response aggregation experiments in PRP and washed platelets. Similar to the irreversible Btk inhibitors, MK-1026 inhibited platelet aggregation (with more prominent effects in washed platelets compared to PRP) at multiple concentrations induced by both agonists (Fig 6.5A). GPVI-induced integrin $\alpha_{IIb}\beta_3$ activation, P-selectin expression, and PS-exposure were also dose-dependently reduced by MK-1026 (Fig 6.5B). The calculated IC_{50} values for MK-1026 in the presence of plasma were similar as for acalabrutinib (Suppl Table 6.1). Based on these results, in combination with reported *in vivo* plasma concentrations[26], we selected 5 µM for further experiments. Aggregation induced by ADP and U46619 were slightly but significantly reduced at this concentration of MK-1026, while responses to thrombin and ristocetin were unaffected (Suppl Fig 6.4A-B). Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were not inhibited by MK-1026 in platelets stimulated with ADP or thrombin (Suppl Fig 6.4C). Also, MK-1026 did not alter platelet adhesion and thrombus formation on six different surfaces, as seen with acalabrutinib (Fig 6.5D, Suppl Fig 6.5).

We also investigated the effects of MK-1026 using blood from Btk-KO mice. Similarly as for acalabrutinib and ibrutinib, we did not observe additional inhibition of whole blood thrombus formation on collagen with MK-1026 in blood samples from WT or Btk-KO mice (Fig 6.6A-B, Suppl Fig 6.6A). Also, no inhibitory effect of MK-1026 was found upon platelet activation or PS-exposure from WT or Btk-KO mice with CRP-XL, ADP or PAR4 agonist (Fig 6.6C-D, Suppl Fig 6.6B-C).

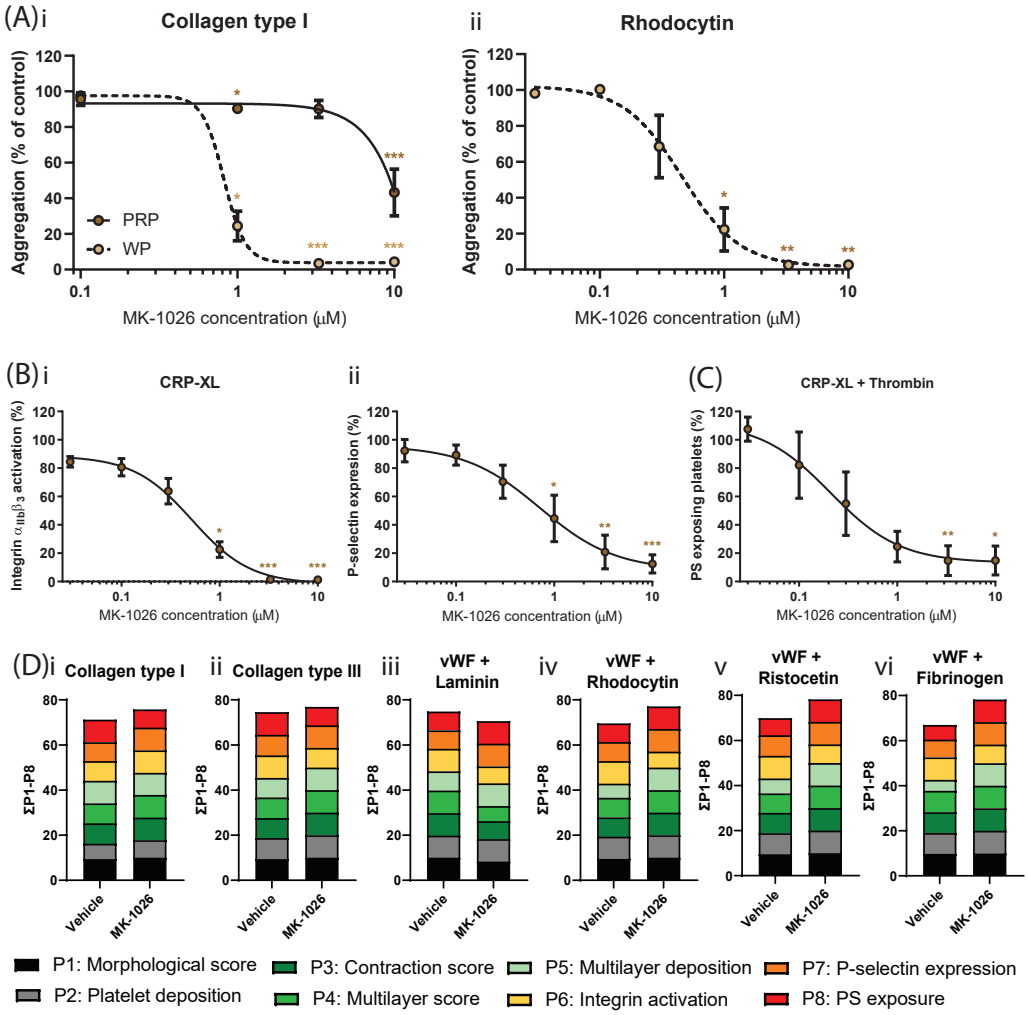

Figure 6.5. Effects of MK-1026 on aggregation and activation of isolated platelets in response to several agonists as well as whole blood thrombus formation under flow. (A) PRP or washed platelets (250x10^9/L) from healthy volunteers were incubated with different concentrations of MK-1026 for 10 minutes at 37°C. Shown are dose-response curves of aggregation induced by (i) collagen type I (1 μg/mL), or (ii) rhodocytin (1 μg/mL) in the presence of MK-1026 normalised to control expression. (B) Washed platelets (100x10^9/L) were pre-incubated with different concentrations of MK-1026 and stimulated with collagen-related peptide (CRP-XL, 5 μg/mL) for 15 minutes and simultaneously stained for activated integrin $\alpha_{IIb}\beta_3$ and α-granule secretion (P-selectin). Histograms show the percentages of platelets binding FITC-labelled PAC-1 monoclonal antibody or AF647-labelled anti-human CD62 mAb normalized to control expression. (C) Platelets pre-treated with different concentrations of MK-1026 were stimulated using both CRP-XL (5 μg/mL) and thrombin (4 nM) for 60 minutes at 37°C and PS-exposure was determined by flow cytometry. Histogram shows percentage of platelets binding FITC-labelled annexin A5 normalized to control expression. Data are shown as means ± SEM (n=6-7). (D) Pre-incubated blood samples from healthy donors with vehicle (<0.1% DMSO) or MK-1026 (5 μM) were recalcified in the presence of PPACK. Blood was perfused for 3.5 minutes at wall shear rate of 1,000 s^{-1} over six different coated surfaces. Thrombi were post-stained with AF568-annexin A5, FITC-α-fibrinogen and AF647-α-CD62P to detect PS-exposure, integrin activation and P-selectin expression. Cumulative histograms represent the scaled values (0-10) for each of the parameters. Colours reflect the adhesion parameters (P1-2; black, grey), aggregation parameters (P3-5; shades of green), and activation parameters (P6-8; yellow, orange and red). * p<0.05, ** p<0.01, *** p<0.001 vs vehicle.

These data show that the reversible Btk inhibitor MK-1026 inhibited GPVI- and CLEC-2-mediated aggregation of washed platelets, but did not impair platelet adhesion or activation in whole blood perfused over collagen.

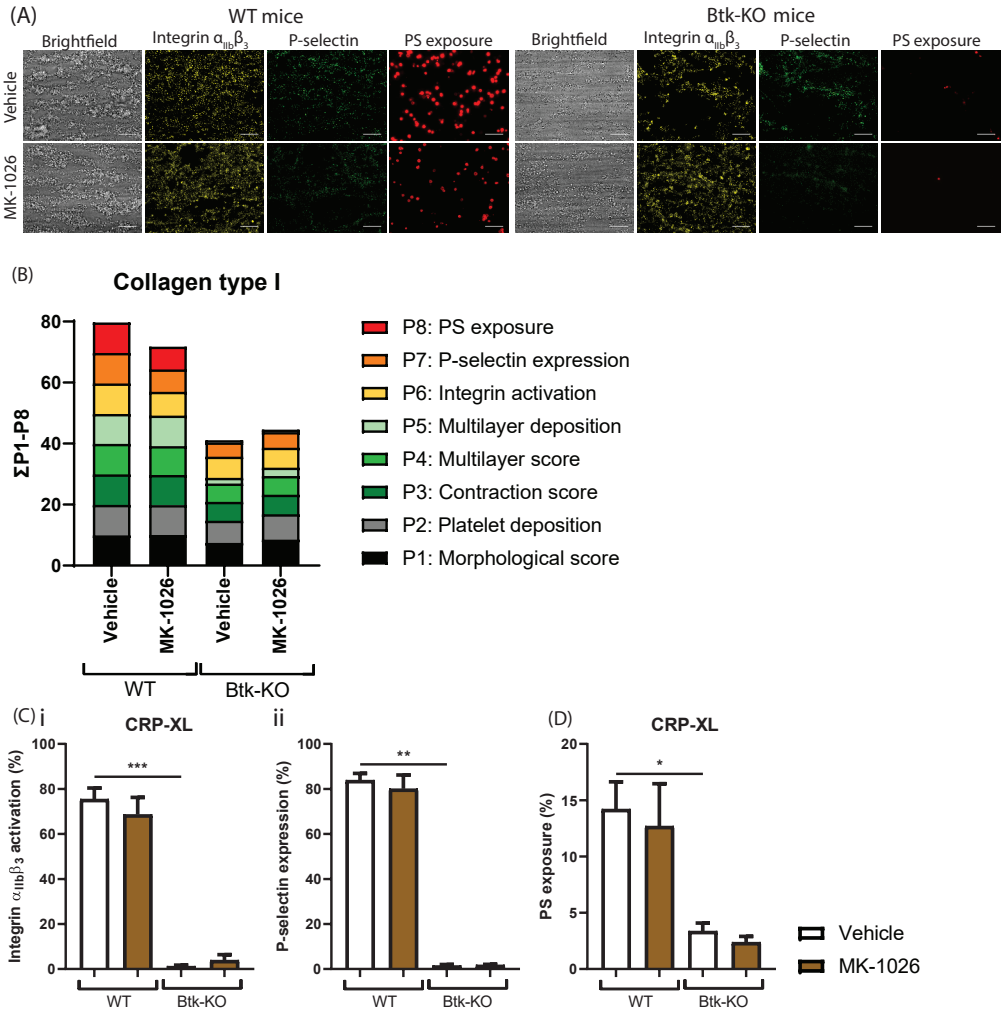

Figure 6.6. Thrombus formation, platelet activation and PS-exposure in blood from WT and Btk-KO mice was unaffected by MK-1026. Pooled citrated whole blood samples from two WT or Btk-KO mice were pre-incubated with vehicle (<0.1% DMSO) or MK-1026 (5 µM) in the presence of PPACK and fragmin for 10 minutes at 37°C. After incubation, blood samples were recalcified and perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over a collagen type I surface. Thrombi were post-stained with FITC-labelled anti-CD62P mAb, PE-labelled JON/A mAb and AF647-labelled Annexin-A5. (A) Representative brightfield and fluorescence images from thrombus formation in blood from WT and Btk-KO mice. (B) Histograms of cumulative scaled values of multiple parameters in WT or Btk-KO mice with addition of vehicle (<0.1% DMSO) or MK-1026. Colours reflect the adhesion parameters (P1-2; black, grey), aggregation parameters (P3-5; shades of green), and activation parameters (P6-8; yellow, orange and red). (C) Platelet activation in whole blood induced by CRP-XL (1 µg/mL). Histograms show the percentages of platelets binding PE-labelled JON/A monoclonal antibody or BB700-labelled CD62P mAb in WT and Btk-KO mice. (D) PS-exposure of platelets induced by CRP-XL (1 µg/mL). Histogram shows percentage of platelets binding FITC-labelled lactadherin. Data are shown as means ± SEM (n=6-8). * p<0.05, ** p<0.01, *** p<0.001.

Ibrutinib inhibits thrombus formation under flow on multiple surfaces and GPVI-induced platelet activation responses in patients

Next, we assessed which platelet activation pathways were altered in patients using ibrutinib. Sixteen patients were included, of which 7 did not receive ibrutinib and 9 were treated with 420-560 mg ibrutinib once a day (Table 6.1). Baseline characteristics were largely similar between groups. Importantly, none of the patients were thrombocytopenic, except one patient with a platelet count of $50x10^9$/L (Table 6.1). Of the ibrutinib-treated patients, 66% (6/9) reported bleeding symptoms with a median ISTH-BAT score of 2 (range 1-7, Table 6.1). Of patients without ibrutinib treatment, none reported bleeding symptoms.

Whole blood from patients was perfused over collagen type I or vWF plus rhodocytin or laminin. No differences in thrombus formation were observed between patients without ibrutinib and healthy controls (Suppl Fig 6.7). On collagen, treatment with ibrutinib reduced thrombus contraction and height (multilayer) compared to non-treated patients. Furthermore, $\alpha_{IIb}\beta_3$ activation, P-selectin expression and PS-exposure were significantly reduced (Fig 6.7A, Suppl Fig 6.7A). For thrombi formed on vWF plus rhodocytin or laminin, $\alpha_{IIb}\beta_3$ activation and α-granule secretion were reduced upon ibrutinib treatment (Fig 6.7A, Suppl Fig 6.7B-C).

Aggregation of isolated platelets from ibrutinib-treated patients showed on average no significant inhibition upon stimulation with any agonist (Fig 6.7B). However, platelets from 5 ibrutinib-treated patients were highly nonresponsive to collagen stimulation, while platelets from 2 patients responded normally to collagen. Also, with rhodocytin, platelets from 4 patients receiving ibrutinib did not aggregate, while platelets from 4 patients showed normal aggregation. When platelet activation was examined by flow cytometry, $\alpha_{IIb}\beta_3$ activation and P-selectin expression were inhibited in CRP-XL-stimulated platelets from all ibrutinib-treated patients (Fig 6.7C). Upon ADP stimulation, platelets from patients on treatment showed a slight increase in $\alpha_{IIb}\beta_3$ activation, while no effects on secretion were observed (Fig 6.7C). PS-exposure by thrombin plus CRP-XL, was unchanged for all patients' platelets (Fig 6.7D).

As 66% of our patients reported bleeding symptoms, we compared patients with significant bleeding (>2 ISTH-BAT score) with patients with no/mild bleeding (0-1 ISTH-BAT score). Generally, no significant changes were observed in platelet aggregation and thrombus formation in patients with or without bleeding (Suppl Fig 6.8).

In summary, platelets from patients on ibrutinib treatment displayed inhibition of activation by GPVI as well as reduced adhesion and thrombus formation under flow over collagen and surfaces co-coated with vWF. Furthermore, variable responses were seen with CLEC-2 stimulation.

Table 6.1. Characteristics of the included patients

						Haematological parameters			Bleeding	
Group	Gender	Age (year)	Disease	Ibrutinib treatment (mg/d)	Anti-platelet drugs	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	PLT ($x10^9$/L)	Yes/No	ISTH BAT score
Patients	Male	73	WM	-	-	3.6	4.3	140	No	0
	Male	45	WM	-	-	6.9	3.7	200	No	0
	Male	68	CLL	-	-	7.7	4.2	190	No	0
	Female	74	CLL	-	-	48.5	3.6	160	No	0
	Male	86	MCL	-	ASA, CLOP	6.8	3.8	160	No	0
	Male	65	WM	-	-	4.4	3.6	220	No	0
	Male	64	MCL	-	ASA	3.9	4.1	200	No	0
Median (±IQR)		68 (64-74)		-	-	6.8 (3.9-7.7)	3.8 (3.6-4.2)	190 (160-200)	0	
Patients + ibrutinib	Female	75	DLBCL leg type	560	-	5.7	4.5	380	Yes	1
	Male	90	MCL	560	-	0.9	2.5	50	Yes	1
	Female	66	CLL	420	-	3.9	4.3	160	Yes	3
	Male	51	WM	420	-	6.9	3.7	210	No	0
	Female	62	WM	420	-	4.5	4	180	No	0
	Male	80	MCL	280	-	8.6	3.5	240	Yes	7
	Male	86	MCL	560	-	5.1	3.5	180	Yes	4
	Female	73	MCL	560	-	8.2	3.9	260	Yes	1
	Male	72	WM	420	-	6	3.7	260	No	0
Median (±IQR)		73 (64-83)		-	-	5.7 (4.2-7.5)	3.7 (3.5-4.1)	210 (170-260)	2* (0.25-3.75)	

ASA: acetylsalicylic acid; CLL: chronic lymphocytic leukaemia; CLOP: clopidogrel; DLBCL: diffuse large B-cell lymphoma; ISTH-BAT: International Society on Thrombosis and Haemostasis bleeding assessment tool; IQR: interquartile range; MCL: mantle cell lymphoma; MPV: mean platelet volume; PLT: platelet count; RBC: red blood cell count; WBC: white blood cell count; WM: Waldenstroms macroglobulemia; *p<0.05, patients vs patients + ibrutinib

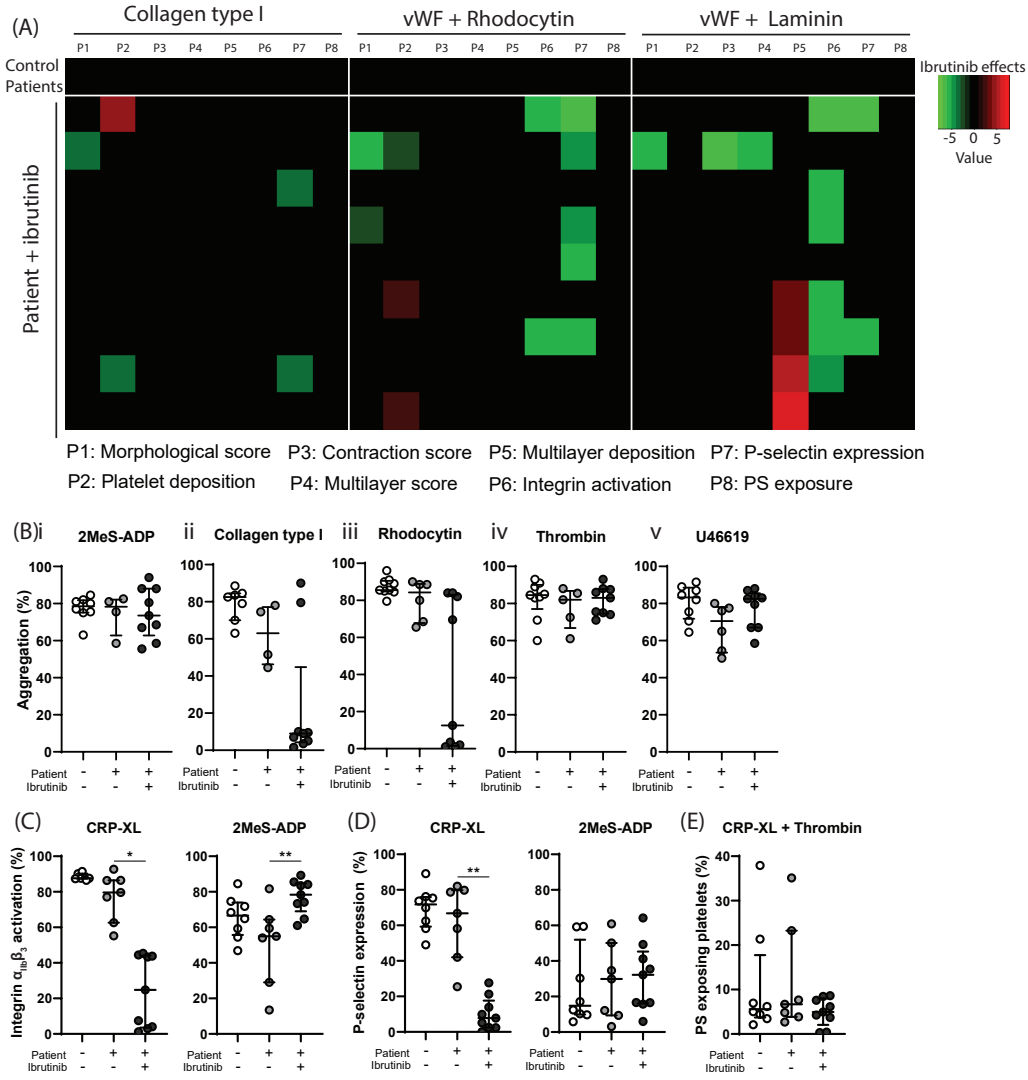

Figure 6.7. Ibrutinib inhibits whole blood thrombus formation under flow on multiple surfaces and GPVI-induced platelet aggregation and activation responses in patients. Citrated whole blood was collected from healthy volunteers and 16 patients with(out) ibrutinib treatment. (A) Whole blood from healthy controls and patients was perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over coated microspots containing collagen type I, vWF co-coated with rhodocytin or vWF co-coated with laminin. Subtraction heatmap representing the effect of ibrutinib treatment in individual patients. Average values obtained with blood of patients without ibrutinib treatment was set at 0 for reference. Effects were filtered for changes greater than the 2x SD range of the different platelet parameters. (B) Light transmission aggregometry was induced in isolated platelets (250x10^9 /L) by collagen (1 µg/mL), rhodocytin (1 µg/mL), 2MeS-ADP (1 µM), U46619 (1 µM) or thrombin (1 nM). Scatter plots show the percentage of aggregation of healthy controls (white circles), patients (grey circles) or patients receiving ibrutinib (black circles). Each circle represents one individual. (C, D) Washed platelets (100x10^9 /L) were stimulated with collagen-related peptide (CRP-XL, 5 µg/mL) or 2MeS-ADP (1 µM) for 15 minutes and analysed by flow cytometry. Shown are percentages of platelets binding FITC-labelled PAC1 mAb against integrin $\alpha_{IIb}\beta_3$ (C) and AF647-labelled CD62P mAb for α-granule secretion (D), respectively. (E) PS-exposure was stimulated using both CRP-XL (5 µg/mL) and thrombin (4 nM) for 60 minutes at 37°C. Scatter plots show the percentage of platelets binding FITC-labelled Annexin A5. Data are represented as median ± interquartile range (n=4-9), * p<0.05, ** p<0.01.

Discussion

Due to their promising effects on progression-free survival, Btk inhibitors are increasingly prescribed in haematological malignancies[4, 5]. Btk inhibitors require lifelong treatment, and the high percentage of discontinuation due to side effects (including bleeding tendency) stresses the importance of understanding and managing these[6-8]. We found that ibrutinib and acalabrutinib inhibit both GPVI- and CLEC-2-mediated platelet aggregation in a dose-dependent manner. Also, all inhibitors dose-dependently impaired GPVI-induced $\alpha_{IIb}\beta_3$ activation and α-granule secretion. Ibrutinib reduced thrombus formation on surfaces co-coated with vWF, but not on collagen. Moreover, we are the first to study the effects on platelet function of MK-1026, a reversible Btk inhibitor.

Other studies investigating effects of Btk inhibitors on platelet function have described different findings. Several papers have shown inhibition of the GPVI pathway by ibrutinib or acalabrutinib, either directly via Btk or due to off-target effects on Src kinases or other downstream proteins[11, 12, 23, 27-31]. Others have implicated that GPIb-[29, 32] or $\alpha_{IIb}\beta_3$-mediated signalling[28, 31] were affected by ibrutinib. In agreement, in the present study ibrutinib also inhibited thrombus formation on ristocetin and fibrinogen co-coated with vWF. Levade et al. have also shown that ibrutinib affected platelet adhesion to vWF only under flow, but did not assess acalabrutinib[22]. Furthermore, we found that both ibrutinib and acalabrutinib impaired GPVI-mediated aggregation, but not platelet adhesion on collagen under flow. Previous studies found conflicting results with regards to the effects of ibrutinib on collagen adhesion, with some who did find inhibition[12,33] and others that could not[34]. A possible explanation for this is that platelet adhesion to collagen is also regulated by integrin $\alpha_2\beta_1$, which does not signal via Btk[34]. Our data now directly compared the differences between ibrutinib and acalabrutinib in whole blood thrombus formation under flow on six different surfaces, and shows for the first time that ibrutinib, but not acalabrutinib impairs platelet adhesion surfaces co-coated with vWF. This might play a role in the increased incidence of major bleeding seen in ibrutinib treatment compared to acalabrutinib.

Although GPVI is important for platelet adhesion and activation on collagen, recent studies have shown that GPVI inhibitors do not impair haemostasis[16]. GPVI-deficient patients exhibit only a mild bleeding diathesis[17] and GPVI depletion in mice did not increase bleeding tendency[19,20]. Therefore, the bleeding tendency seen with Btk inhibitors cannot solely be mediated by GPVI. Depletion of both GPVI and CLEC-2 from mouse platelets did impair haemostasis[21]. In line with this, we found that Btk inhibitors inhibited both GPVI- and CLEC-2-mediated aggregation.

Only few studies have investigated Btk-KO mice or X-linked agammaglobulinemia (XLA) patients, a Btk deficiency in humans. Btk-KO mice showed impaired GPVI, CLEC-2 and GPIb signalling[35-38]. In line with this, we found decreased GPVI-induced platelet granule secretion and thrombus formation in Btk-KO mice. XLA patients demonstrated impaired GPVI- or CLEC-2-induced PLCγ2 phosphorylation and platelet aggregation[39, 40]. As compared to XLA patients, ibrutinib has

similar effects on GPVI, CLEC-2 and GPIb signalling. However, XLA patients are not associated with an increased bleeding risk[12]. This suggests that Btk inhibitors affect other targets, most notably Tec, which can substitute for Btk[35] and is also inhibited by ibrutinib and acalabrutinib[12]. Also, off-target effects on Src kinases have been implicated in the bleeding tendency[11, 12]. We assessed off-target effects on GPVI-induced tyrosine kinase phosphorylation in platelets by using the PamGene assay. These results showed that ibrutinib and acalabrutinib showed the same off-target effects, although the effects of acalabrutinib were less strong. Importantly both inhibitors had an off-target effect on Tec and Src kinases, questioning the previous conclusions that these were responsible for the increased severe bleeding seen with ibrutinib compared to acalabrutinib.

When comparing results found *in vitro* in human and mice, we found that in human samples, Btk inhibitors did not alter collagen-mediated adhesion under flow in agreement with Zheng et al.[31], whereas in mice, Btk inhibition (either by KO mice or acalabrutinib) reduced this response. Btk-dependent signalling is completely absent in KO mice, and therefore, this may result in stronger effects as compared to pharmacological inhibition. Btk proteins display 99.4% similarity between human and mice[41], so a species-dependent interaction with an inhibitor cannot explain the observed differences. A possibility could be different bioavailability between mouse and man. Ibrutinib and acalabrutinib are highly plasma protein-bound in human blood, 97.3% and 97.5%, respectively[42, 43], while acalabrutinib was reported to have lower protein binding in mouse blood (75.4%)[43]. This may contribute to the more pronounced effects of acalabrutinib in mouse blood as compared to human blood. We also observed different results between human and mouse platelets with MK-1026 (same concentration) in flow cytometric experiments. This may be explained by the use of murine whole blood in these experiments. Mouse blood contains ~three times more platelets as compared to human blood, and plasma proteins are also likely to bind MK-1026. Altogether, this may explain the absent effect of MK-1026 on platelet functions of wild-type mice.

As our data propose a direct role for Btk in impaired platelet function, we tested the reversible Btk inhibitor MK-1026. MK-1026 showed promising effects on ibrutinib resistant CLL cells *in vitro* and is currently being tested in clinical trials[14]. In the PamGene kinase assay MK-1026 appeared to have less off-target effects in platelets upon GPVI stimulation, in combination with a less strong inhibition profile as compared to the irreversible inhibitors. MK-1026 reduced GPVI- and CLEC-2-mediated aggregation, as well as GPVI-induced platelet activation in washed platelets, largely similar to acalabrutinib. In human and mouse blood, MK-1026 did not impair collagen-induced thrombus formation under flow, in contrast to acalabrutinib which did affect this response in mouse blood. Hence, this reversible inhibitor had less inhibitory effects on platelets as compared to ibrutinib, in agreement with a recent study[31]. Hence, MK-1026 could be expected to show a slightly reduced or comparable bleeding tendency compared to acalabrutinib.

The present dose-response experiments, in line with previous studies[11, 33], show that at

similar dose, ibrutinib is a more effective platelet inhibitor compared to other Btk inhibitors. This has been attributed to the inhibition of drug efflux pumps by ibrutinib[33]. We observed that higher concentrations of inhibitors are required to inhibit platelet aggregation in the presence of plasma, as compared to washed platelets. This in line with previous observations with other TKIs[44, 45]. This is most likely caused by binding of ibrutinib and acalabrutinib to albumin[46-48]. It has been reported that prolongation of the incubation time lowered the IC_{50} values of ibrutinib and acalabrutinib for GPVI-mediated aggregation[49]. However, in that study a much lower collagen concentration was used, which may be more sensitive to longer incubation with low doses of inhibitors. Furthermore, that study did not include CLEC-2-dependent platelet responses[49]. Although inhibitor concentration could play a role in the extent of inhibition, we showed that ibrutinib and acalabrutinib influence additional platelet pathways as compared to MK-1026, suggesting that the extent of Btk inhibition might not be the reason for the increased bleeding tendency.

Previous studies assessing which platelet pathways are involved in bleeding tendency in patients using ibrutinib found conflicting results. Some showed a correlation with collagen-induced aggregation[12, 22, 50], and some with platelet adhesion to collagen[12], whereas others could not find this[22]. Bleeding tendency was also associated with adhesion to vWF under flow[22] and one study found a correlation with ristocetin-induced platelet aggregation[32], which another study could not replicate[28]. Ibrutinib can also induce shedding of GPIbα, GPIX and integrin $\alpha_{IIb}\beta_3$ in patients, but the correlation with bleeding remains unknown[51].

A recent study assessed platelet parameters in patients with CLL and mantle cell lymphoma (MCL)[52], showing correlations between bleeding tendency, thrombocytopenia and decreased ADP-induced platelet aggregation. However, a drawback of this study was that a large patient proportion was thrombocytopenic, which can directly influence platelet aggregation, as platelet concentration in PRP was not reported to be adjusted. This impaired the establishment if ibrutinib or platelet count affected aggregation response. In our patients with (in general) normal platelet counts, we also observed significant differences in platelet responses to several stimuli of patients using ibrutinib. Furthermore, 66% of the included patients with ibrutinib treatment reported bleeding symptoms. Although we could show that in patients, similar to healthy volunteers, ibrutinib inhibited GPVI signalling, with variable effects on CLEC-2, as well as reduced thrombus formation to surfaces co-coated with vWF, this could not differentiate for the bleeding tendency. Generally, the patients showed a large variation in measurement outcomes, which might be influenced by clinical factors. However, with patients on ibrutinib treatment, we have directly compared whole blood thrombus formation under flow on multiple surfaces, including vWF plus rhodocytin and laminin, which has not been reported with ibrutinib-treated patients thus far.

In conclusion, the present work demonstrated that ibrutinib, acalabrutinib and MK-1026 inhibited GPVI- and CLEC-2-mediated platelet aggregation, but only ibrutinib also inhibited GPVI-induced platelet activation and thrombus formation on surfaces co-coated with vWF. The novel

reversible BTK inhibitor MK-1026 might therefore be promising for future treatment in patients at risk for bleeding.

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Author contributions

BMET, MFAK, VL, MStB, CCFMJB, SLM and EPCvdV acquired, analysed and interpreted data. AFdV provided Btk-deficient mice. JAE contributed essential tools. MRN and TAMC provided ethical approval and included patients. JMEMC and JWMH provided lab space with equipment and supervision. TAMC and MJEK designed and supervised the research, and interpreted data. BMET, TAMC and MJEK wrote and revised the paper. All the authors provided critical feedback, edited, and approved the final manuscript.

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Supplemental Data

Materials & methods

Materials

Acalabrutinib was obtained from Selleckchem (Houston, TX, USA), MK-1026 (formerly ARQ-531) was a provided by ArQule, Inc., a wholly-owned subsidiary of Merck & Co. (Kenilworth NJ, USA) and ibrutinib was purchased from Toronto Research Chemicals (North York, Canada). Bovine serum albumin (BSA), D(+)-glucose, unfractionated heparin and apyrase were purchased from Sigma-Aldrich (Saint Louis MO, USA). Low molecular weight heparin (Fragmin®) was from Prizer (Capelle a/d IJssel, The Netherlands). Horm® collagen type I came from Takeda (Hoofddorp, The Netherlands), whereas the agonists collagen-related peptide crosslinked (CRP-XL) and von Willebrand factor III (vWF-III) were obtained from CambCol Laboratories (Cambridge, UK). Thrombin was from Enzyme Research Laboratories Inc (South Bend, IN, USA) and protease-activated receptor 4 activating peptide (PAR4-AP) was obtained from Bachem (Bubendorf, Switzerland). U46619 (thromboxane A_2 receptor agonist) came from Cayman Chemicals (Ann Arbor MI, USA). 2-Methylthio-adenosine-diphosphate (2MeS-ADP) and D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) were obtained from Santa Cruz Biotechnology (Dallas TX, USA). Laminin was purchased from Octapharma (Lachen, Switzerland), whereas rhodocytin was purified from Calloselasma rhodostoma venom as described previously[1]. Fura-2-AM was obtained from Invitrogen (Carlsbad CA, USA). Fluorescein isothiocyanate (FITC)-labelled PAC1 monoclonal antibody (mAb) against activated human integrin $\alpha_{IIb}\beta_3$ were purchased from BD Bioscience (nr. 340507; Franklin Lakes NJ, USA). FITC-conjugated α-fibrinogen mAb was purchased from DAKO (F0111; Santa Clara CA, USA). Alexa Fluor (AF)568 and AF647-conjugated annexin A5 were purchased from Molecular Probes, Life Technologies (New York NY, USA). AF647-labelled CD62-P mAb was obtained from Biolegend (London, UK). FITC-conjugated annexin A5 was from Pharmatarget (Maastricht, The Netherlands), whereas FITC-conjugated lactadherin came from Haematologic Technologies (Essex Junction VT, USA). FITC-labelled rat anti-mouse CD62P mAb and phycoerythrin (PE) labelled rat anti-mouse JON/A mAb were obtained from Emfret Analytics (Würzburg, Germany).

Blood collection and platelet isolation from healthy volunteers and patients

With approval from the medical ethics committee from the Maastricht University Medical Centre+ (MUMC+), blood was collected from healthy volunteers. Furthermore, blood was collected from patients diagnosed with chronic lymphocytic leukaemia (CLL), mantle cell lymphoma and Waldenstroms macroglobulinemia with or without ibrutinib treatment at the Catharina Hospital in Eindhoven (the Netherlands) with approval of the local medical ethical committee. This study was performed in accordance with the declaration of Helsinki and informed consent was obtained

from all participants. Blood was collected in 3.2% trisodium citrate tubes by venipuncture, after discarding the first 3 mL of blood. Blood cell counts and haematological parameters were assessed of all blood samples using a Sysmex XP300 (Kobe, Japan). Patients characteristic and ISTH-BAT score2 were acquired for each patient.

Platelet-rich plasma (PRP) or platelets were isolated from whole blood as described previously[3, 4]. Washed platelets were resuspended in Hepes buffer, pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mg/mL glucose, and 1 mg/ml BSA). Platelet concentrations were adjusted as stated per assay.

Blood collection from animals

Specific-pathogen-free Btk-KO mice[5] and littermate controls (wild-types, WT) were housed in the Animal Research Institute Amsterdam facility under standard care. Studies were reviewed and approved by the Central Authority for Scientific Procedures on Animals (CCD) and the Animal Welfare Body (IvD) of the Academic Medical Centre, University of Amsterdam (ID number: 17-4125-1-80). The animal care and use protocol adhered to the Dutch Experiments on Animals Act (WOD) and European Directive of 22 September 2010 (Directive 2010/63/EU) in addition to the Directive of 6 May 2009 (Directive 2009/41/EC).

Only male mice between 8 and 12 weeks of age were used for experiments. Citrated blood was drawn from WT and Btk-KO mice from the vena cava inferior under full anaesthesia induced by intraperitoneal injection of ketamine (125 mg/kg bodyweight) and dexmedetomidine (300 µg/kg bodyweight). After blood collection mice were sacrificed by severing the diaphragm.

Whole blood thrombus formation under flow

Thrombus formation under flow with human blood was performed as described 6. Briefly, glass coverslips were coated with 3 microspots of collagen type I (100 µg/mL), collagen type III (100 µg/mL), vWF (50 µg/mL) co-coated with laminin (100 µg/mL), vWF co-coated with rhodocytin (250 µg/mL), vWF co-coated with ristocetin (250 µg/mL), or vWF co-coated with fibrinogen (250 µg/mL), and mounted in a parallel plate flow chamber. Citrated blood samples from healthy donors were incubated with vehicle or 5 µM of Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at room temperature. Blood samples from CLL patients were used directly. Whole blood from healthy volunteers or CLL patients was recalcified in the presence of PPACK (40 µM), and perfused at a wall shear rate of 1000 s^{-1} for 3.5 minutes. Platelet activation properties were determined by post-perfusion with Hepes buffer (with additional 2 mM $CaCl_2$ and 1 U/mL heparin) containing FITC-conjugated α-fibrinogen mAb (1:80), AF647-conjugated CD62-P (1:100), and AF568-conjugated annexin A5 (1:200).

Thrombus formation under flow with mouse blood was performed as described[7]. Glass coverslips were coated with collagen type I (100 µg/mL) and blood samples, in the presence of

PPACK (57.1 µM) and Fragmin (35.7 µM), were incubated with 5 µM Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at 37°C. Next, the blood samples were recalcified and perfused at a shear rate of 1000 s^{-1} for 3.5 minutes. To measure platelet activation status, thrombi were post-stained with Tyrode Hepes buffer, pH 7.45 (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 1 mg/mL glucose, 1 mg/ml BSA, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1 U/mL heparin) containing PE-labelled Jon-A (1:20) and FITC-conjugated anti-P-selectin mAb (1:40) and AF647-labelled Annexin-A5 (1:200).

For both human and mouse experiments, brightfield and fluorescent images were captured using an EVOS microscope (Bothel, WA, USA). Images were analysed using specific scripts in the open-access Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, WI, USA) as described[6, 8]. Values for the following parameters of thrombus formation and platelet activation were obtained: morphological score of platelet adhesion and thrombus formation (P1; scale 0-5), surface area coverage of adhered platelets (P2; %SAC), platelet aggregate contraction score (P3; scale 0-3), platelet aggregate multilayer score (P4; scale 0-3), coverage of multi-layered platelet aggregation (P5; %SAC), integrin $\alpha_{IIb}\beta_3$ activation (P6; %SAC), P-selectin expression (P7; %SAC) and PS exposure (P8; %SAC). For comparative data analysis, cumulative histograms were generated of scaled values from 0-10 for each parameter. For patient data subtraction heatmap representing the effect of ibrutinib treatment in individual patients. Average values obtained with blood of patients without ibrutinib treatment was set at 0 for reference. Effects were filtered for changes greater than the 2x SD range of the different platelet parameters.

Light transmission aggregometry

Isolated platelets ($250x10^9$ platelets/L) from healthy volunteers were incubated with vehicle or 3.3 µM of Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at 37°C, whereas platelets from CLL patients were used directly after preheating to 37°C for 10 minutes. Aggregation response was induced by collagen type I (1 µg/mL), 2MeS-ADP (1 µM), U46619 (1 µM), rhodocytin (1 µg/mL) or thrombin (1 nM). Platelet aggregation was recorded using a Chronolog optical aggregometer (Havertown PA, USA) and maximum amplitude was quantified at 8 minutes after agonist addition.

Flow cytometry

Washed platelets ($100x10^9$ platelets/L) from healthy volunteers or CLL patients were supplemented with 2 mM $CaCl_2$. Samples from healthy volunteers were incubated with vehicle or 1 µM of Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at room temperature, whereas patient samples were used directly. Platelets were stimulated by CRP-XL (5 µg/mL), 2MeS-ADP (1 µM), or thrombin (1 nM) for 15 minutes at RT. Activation of integrin $\alpha_{IIb}\beta_3$ and P-selectin

expression were determined using FITC-conjugated PAC1 mAb (1:20) and AF647-conjugated CD62-P mAb (1:40) respectively. For measuring phosphatidyl-serine (PS) exposure platelets were activated with a combination of CRP-XL (5 µg/mL) and thrombin (4 nM) for 1 hour at 37°C and labelled with FITC-conjugated annexin A5. Flow cytometry was performed in duplicates using a BD Accuri C6TM flow cytometer and accompanying software (Erembodegem, Belgium).

Platelet activation in whole blood from WT and Btk-KO mice was performed using pooled blood samples (5 µl), in the presence of PPACK (57.1 µM) and Fragmin (35.7 µM), incubated with 5 µM Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at 37°C. Platelets were then stimulated for 30 minutes at room temperature with control buffer (PBS), CRP-XL (1 µg/ml), 2MeS-ADP (1 µM) or PAR4-AP (0.1 mg/ml). Platelet activation was assessed by flow cytometry using APC-conjugated anti-CD61, BB700-labelled anti-CD62P, PE-conjugated JON/A and FITC-labelled lactadherin. The labelling was terminated by addition of 40 volumes of buffer. Analysis was carried out immediately by flow cytometry using a CytoFLEX (Beckman Coulter, Brea, CA) until 10,000 CD61+ events were recorded. All stimulations and staining were done in duplicate. Compensation settings were validated using single-stained controls. Percentage of positivity was determined using unstimulated controls. Data analysis was done using FlowJoTM v10 (BD Biosciences, San Jose, CA).

Cytosolic Ca^{2+} measurements

Washed platelets ($200x10^9$ platelets/L) were loaded with Fura-2 acetoxymethyl ester and changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were measured in 96-well plates using a FlexStation 3 (Molecular Devices, San Jose, CA, USA) as described[9,10]. In brief, platelet suspension was preincubated with vehicle or 1 µM Btk inhibitor (acalabrutinib, ibrutinib or MK-1026) for 10 minutes at RT. Fura-2 loaded platelets in presence of $CaCl_2$ were stimulated by CRP-XL (10 µg/mL). Changes in Fura-2 fluorescence were measured in duplicate and ratio values were calculated and are presented as $[Ca+]_i$ in nM.

PamGene kinase assay

Washed platelets ($500x10^9$ platelets/L) were pretreated with vehicle (control) or 1 µM ibrutinib, acalabrutinib or MK-1026 for 10 minutes at 37°C and were subsequently stimulated with 5 µg/mL CRP-XL in the presence of 2 mM $CaCl_2$. Unstimulated, resting platelets served as control. After 90 seconds of stimulation, samples were lysed by adding 1:1 M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Samples were lysed for 15 minutes on ice and afterwards centrifuged for 15 minutes at 10,000g at 4°C. Supernatants were collected and protein content was quantified with a BioRad DC protein kit (Hercules CA, USA).

Tyrosine kinase profiles were determined using the PamChip® peptide tyrosine kinase

microarray system on PamStation®12 (PTK; PamGene International, ´s-Hertogenbosch, The Netherlands). Each PTK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for TKs. Each peptide on the chip builds a 15-amino acid sequence representing a putative endogenous phosphorylation site which functions as a TK substrate. The phosphorylation of the peptides is visualized by detection of the fluorescent signal which is emitted as a result of the binding of the FITC-conjugated PY20 anti-phosphotyrosine antibody.

For the PTK assay, 7.5 µg of protein was applied per array (N=4 per condition) and carried out using the standard protocol supplied by Pamgene. All reagents used for PTK activity profiling were supplied by Pamgene International B.V. Initially, to prepare the PTK Basic Mix, the freshly frozen lysate was added to 4 µL of 10 x protein PTK reaction buffer (PK), 0.4 µL of 100 x bovine serum albumin (BSA), 0.4 µL of 1 M dithiothreitol (DTT) solution, 4 µL of 10 x PTK additive, 4 µL of 4 mM ATP and 0.6 µL of monoclonal anti-phosphotyrosine FITC-conjugate detection antibody (clone PY20). Total volume of the PTK Basic Mix was adjusted to 40 µL by adding distilled water (H_20). Before loading the PTK Basic Mix on the array, a blocking step was performed applying 30 µL of 2% BSA to the middle of every array and washing with PTK solution for PamChip® preprocessing. Next, 40 µL of PTK Basic Mix were applied to each array of the PamChips®. Then, the microarray assays were run for 94 cycles. An image was recorded by a CCD camera PamStation®12 at kinetic read cycles 32–93 at 10, 50 and 200 ms and at end-level read cycle at 10, 20, 50, 100 and 200 ms. The spot intensity at each time point was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands). Upstream Kinase Analysis (UKA)[11], a functional scoring method (PamGene) was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from 6 databases) and sensitivity scores (based on treatment-control differences).

Statistical analysis

Data for healthy volunteers are shown as mean ± standard error of mean (SEM), whereas CLL patients are presented as median ± interquartile ranges. GraphPad Prism 8.3.0 software (La Jolla, CA, USA) was used for statistical analysis. For healthy donor and CLL patient samples a non-parametric and unpaired t-test (Mann-Whitney) was used to compare vehicle and inhibitor treatment in vitro, whereas for mouse samples statistics were calculated using a one-way non-parametric Anova (Kruskal-Wallis) to compare WT versus Btk-KO samples, as well as the comparison between vehicle and inhibitor treatment in both WT and Btk-KO mice. A p-value less than 0.05 was considered to be statistically significant in which * is $p<0.05$, ** is $p<0.01$ and *** is $p<0.001$.

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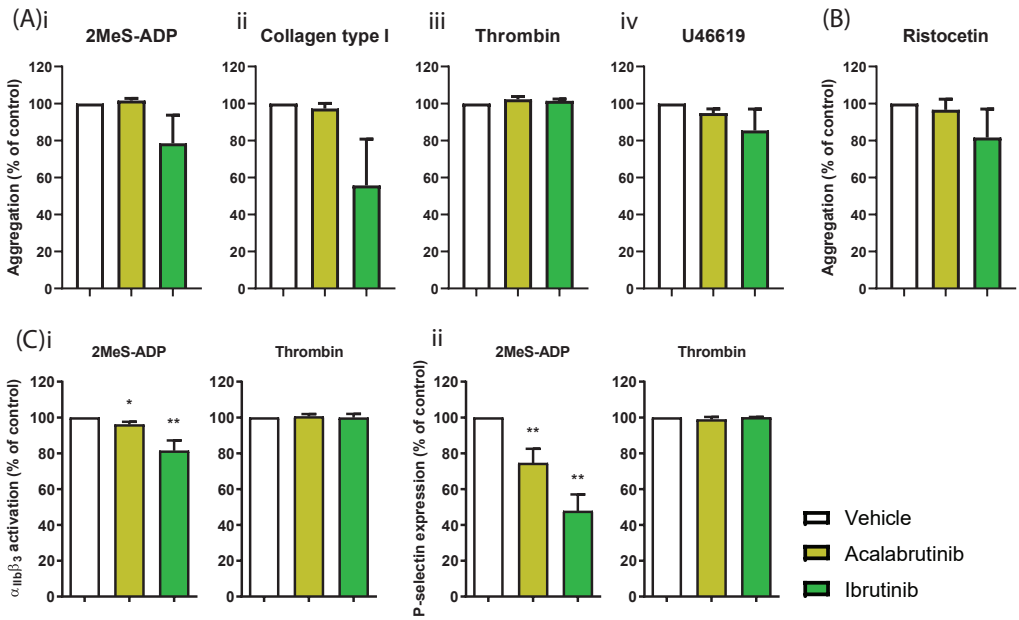
Supplemental table

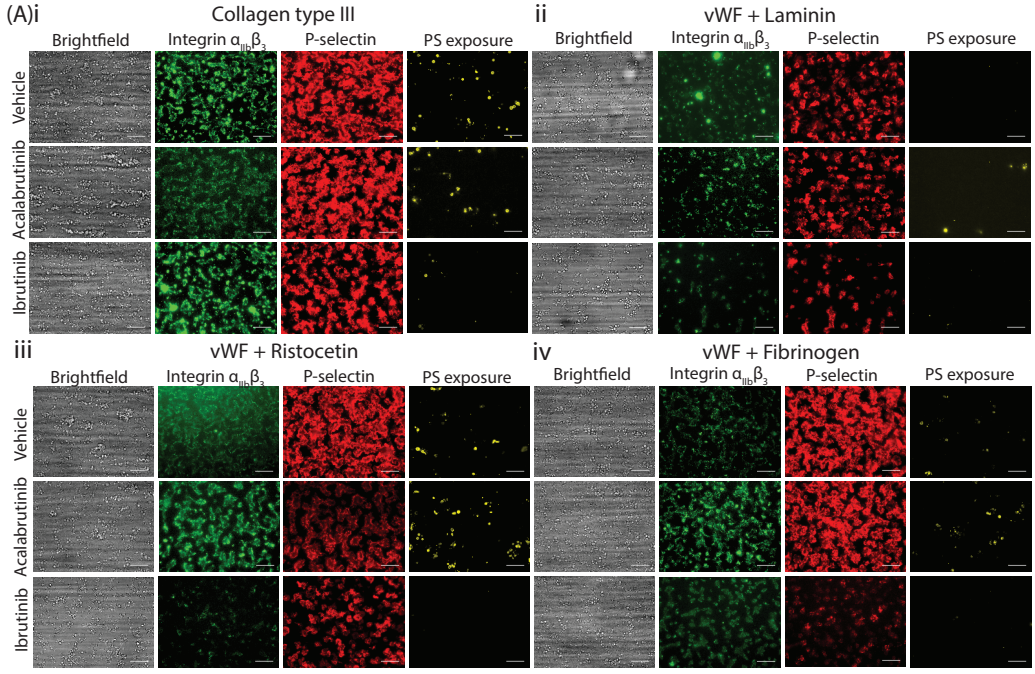
Supplemental Table 6.1. IC_{50} values (μM) of Btk inhibitors for inhibition of GPVI- and CLEC-2 induced platelet aggregation and activation in presence (PRP) or absence of plasma (WP).

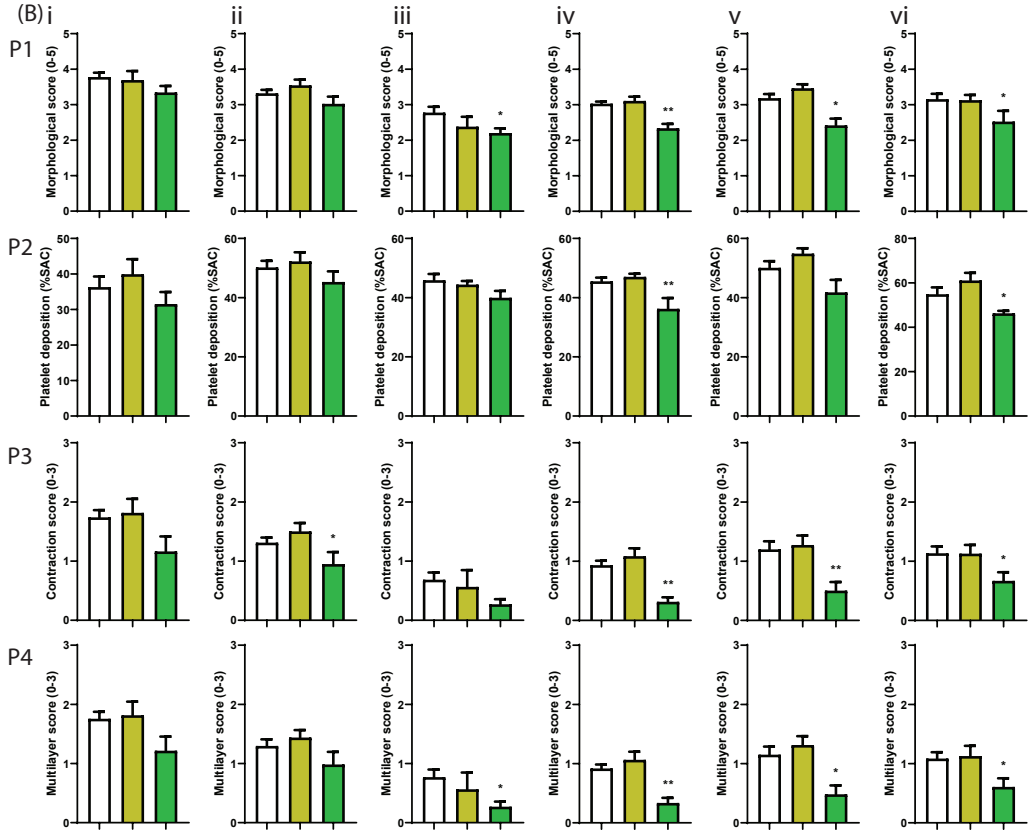
	Aggregation			Activation		
	GPVI		CLEC-2	GPVI		GPVI + PAR
Btk inhibitor	PRP	WP	WP	Integrin activation	Secretion	PS exposure
Ibrutinib	3.31	0.425	0.170	0.196	0.176	0.332
Acalabrutinib	9.85	1.635	1.275	0.982	1.439	0.723
MK-1026	8.84	0.820	0.487	0.388	0.807	0.410

Supplemental figures



Supplemental Figure 6.1. Platelet aggregation and activation of blood samples from healthy volunteers with ibrutinib and acalabrutinib, as well as dose-response of collagen-induced aggregation. (A) Washed platelets ($250x10^9$/L) from healthy volunteers were incubated with vehicle, ibrutinib (3.3 μM) or acalabrutinib (3.3 μM). Aggregation was induced by (i) 2MeS-ADP (1 μM), (ii) collagen type I (5 μg/mL), (iii) thrombin (1 nM) or (iv) U46619 (1 μM). Aggregation responses were measured during 8 minutes. (B) PRP ($250x10^9$/L) was incubated with vehicle, ibrutinib (5 μM) or acalabrutinib (5 μM) for 10 minutes at 37°C and stimulated with ristocetin (2 mg/mL). Aggregation responses were measured during 8 minutes. (C) Washed platelets ($100x10^9$/L) were stimulated with 2MeS-ADP (1 μM) or thrombin (1 nM) for 15 minutes and analysed by flow cytometry. Shown are percentages of platelets binding FITC-labelled PAC1 mAb against integrin $\alpha_{IIb}\beta_3$ and AF647-labelled CD62P mAb for α-granule secretion, respectively. Data are shown as means ± SEM (n=5-6). * p<0.05, ** p<0.01, *** p<0.001 vs vehicle.


(A)i
Collagen type III
ii
vWF + Laminin
iii
vWF + Ristocetin
iv
vWF + Fibrinogen
Brightfield
Integrin $\alpha_{IIb}\beta_3$
P-selectin
PS exposure
Vehicle
Acalabrutinib
Ibrutinib


(B)
i
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iv
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P1
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Morphological score (0-5)
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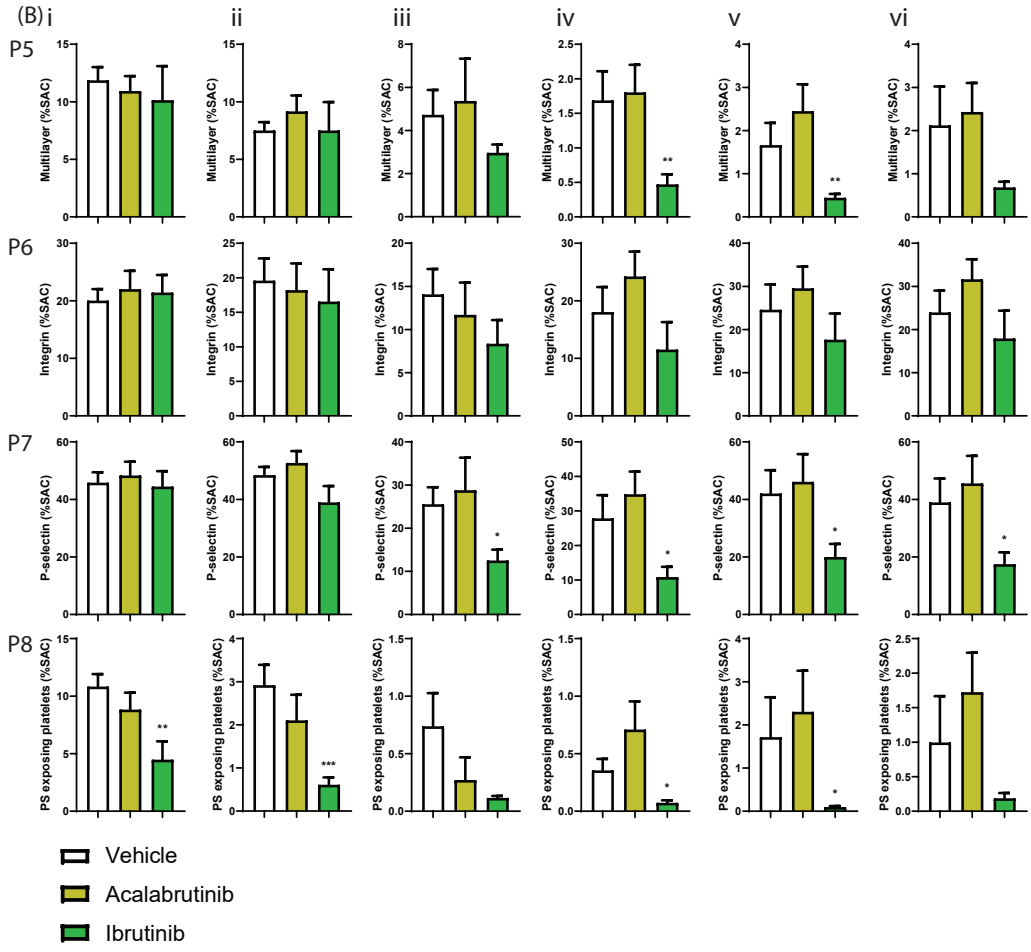
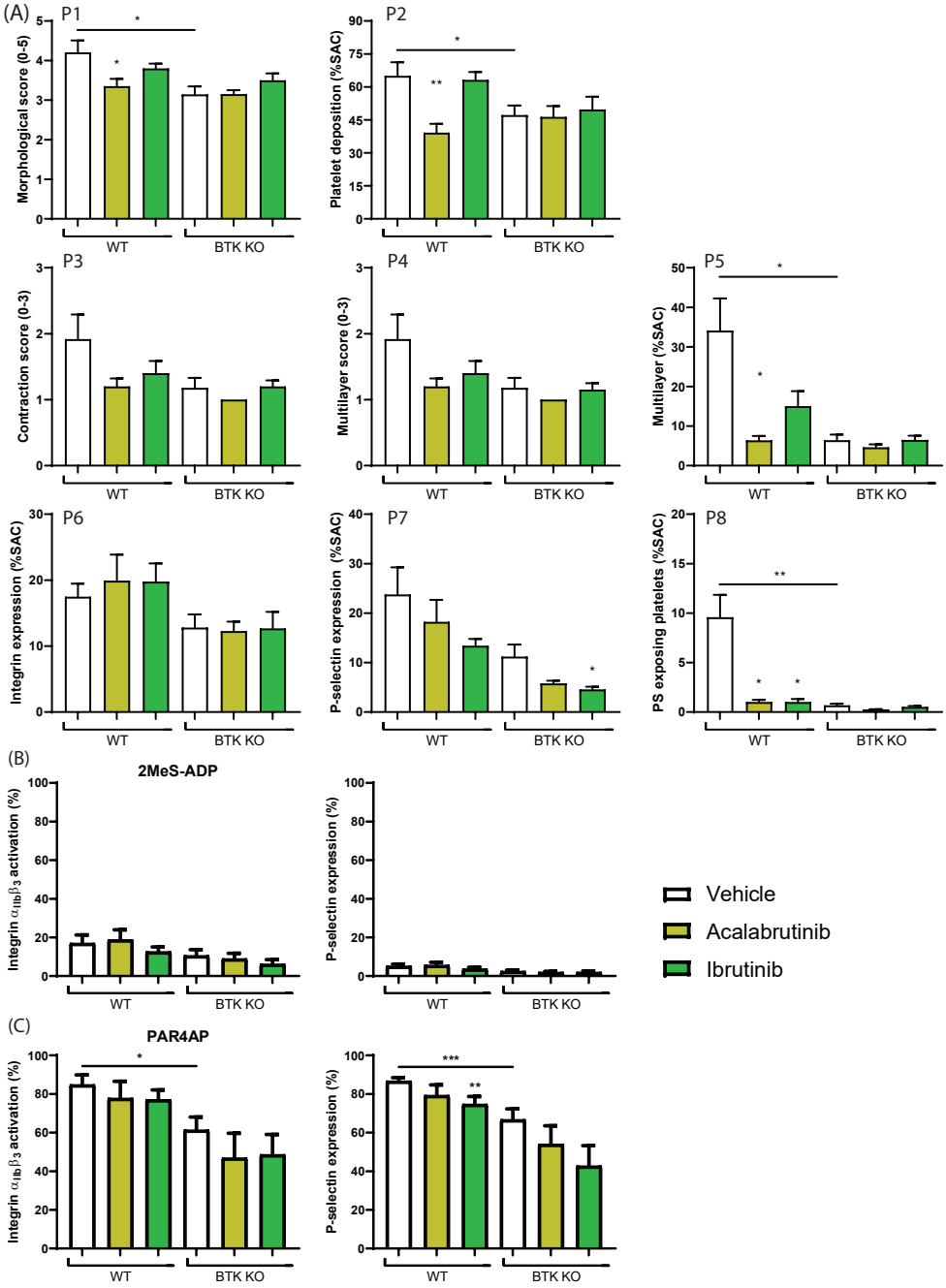
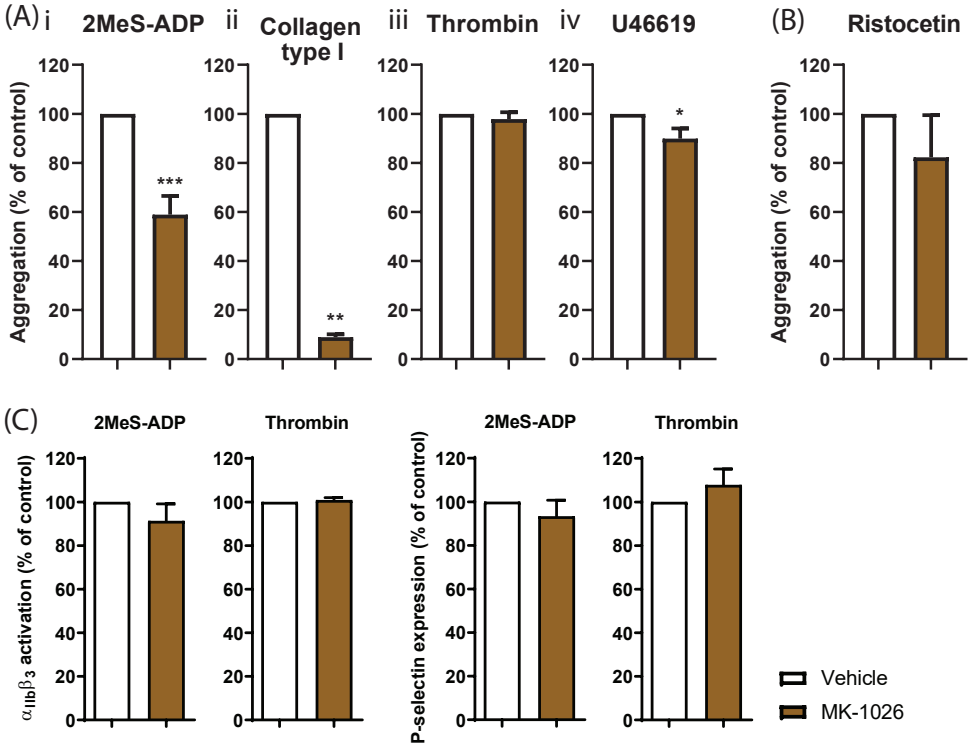
Contraction score (0-3)
Multilayer score (0-3)



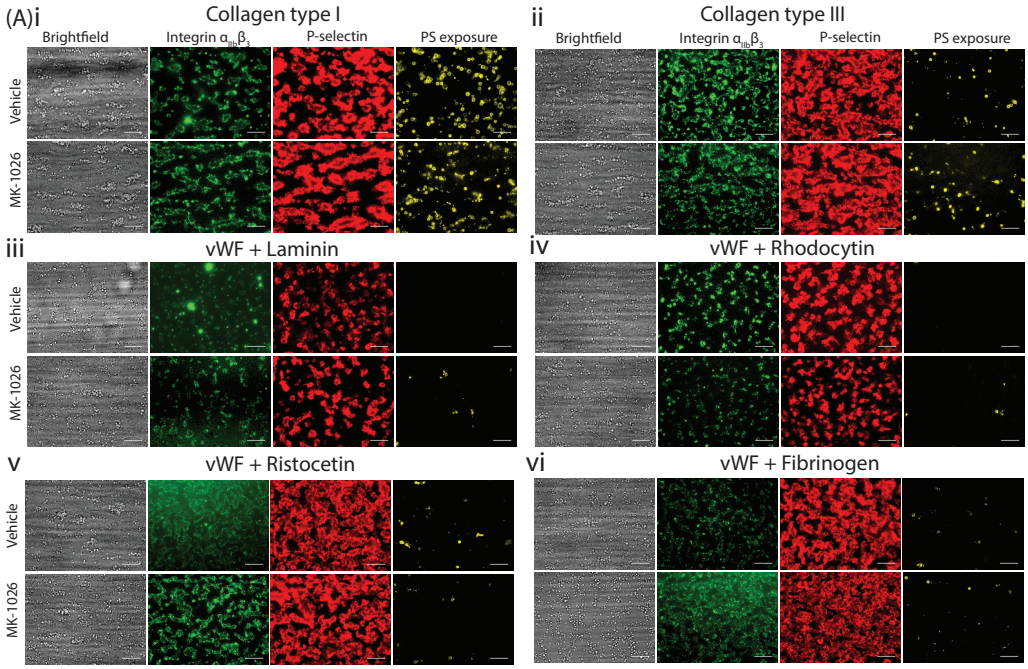
Supplemental Figure 6.2. Thrombus formation under flow with blood from healthy donors in presence of acalabrutinib or ibrutinib over multiple microspots. (A) Representative brightfield and fluorescence images and (B) quantification of control (vehicle, white bars), acalabrutinib (5 µM, grey bars) or ibrutinib (5 µM, black bars) over (i) collagen type I, (ii) collagen type III, (iii) vWF co-coated with rhodocytin, (iv) vWF co-coated with laminin, (v) vWF co-coated with ristocetin and (vi) vWF co-coated with fibrinogen. P1 = morphological score of platelet adhesion and thrombus formation, P2 = surface area coverage of adhered platelets, P3 = platelet aggregate contraction score, P4 = platelet aggregate multilayer score, P5 = coverage of multi-layered platelet aggregation, P6 = integrin $\alpha_{IIb}\beta_3$ activation, P7 = P-selectin expression and P8 = PS exposure. Data are shown as means ± SEM (n=6-8). * p<0.05, ** p<0.01, *** p<0.001.



Supplemental Figure 6.3. Thrombus formation and platelet activation with blood of WT or Btk-KO mice. Citrated whole blood from WT or Btk-KO mice pre-incubated with control (vehicle, white bars), acalabrutinib (5 µM, grey bars) or ibrutinib (5 µM, black bars) was perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over a collagen type I surface. (A) Quantification of brightfield and fluorescence images. (B-C) Platelet activation in whole blood induced by (B) 2MeS-ADP (1 µM) or (C) PAR4AP (100 µg/mL). Histograms show the percentages of platelets binding PE-labelled JON/A monoclonal antibody or PerCP-labelled CD62P mAb in WT and Btk-KO mice. Data are presented as means ± SEM (n=5-6), * p<0.05, ** p<0.01 vs vehicle or indicated otherwise.



Supplemental Figure 6.4. Platelet aggregation and activation of blood samples from healthy volunteers in presence of MK-1026. (A) Washed platelets ($250x10^9$/L) from healthy volunteers were incubated with vehicle or MK-1026 (3.3 µM). Aggregation was induced by (i) 2MeS-ADP (1 µM), (ii) collagen type I (5 µg/mL), (iii) thrombin (1 nM) or (iv) U46619 (1 µM). Aggregation responses were measured during 8 minutes. (B) PRP ($250x10^9$/L) from healthy volunteers were incubated with vehicle or MK-1026 (5 µM) for 10 minutes at 37°C and stimulated with ristocetin (2 mg/mL). Aggregation responses were measured during 8 minutes. (C) Washed platelets ($100x10^9$/L) were stimulated with 2MeS-ADP (1 µM) or thrombin (1 nM) for 15 minutes and analysed by flow cytometry. Shown are percentages of platelets binding FITC-labelled PAC1 mAb against integrin $\alpha_{IIb}\beta_3$ and AF647-labelled CD62P mAb for α-granule secretion, respectively. Data are represented as means ± SEM (n=5-6), * $p<0.05$.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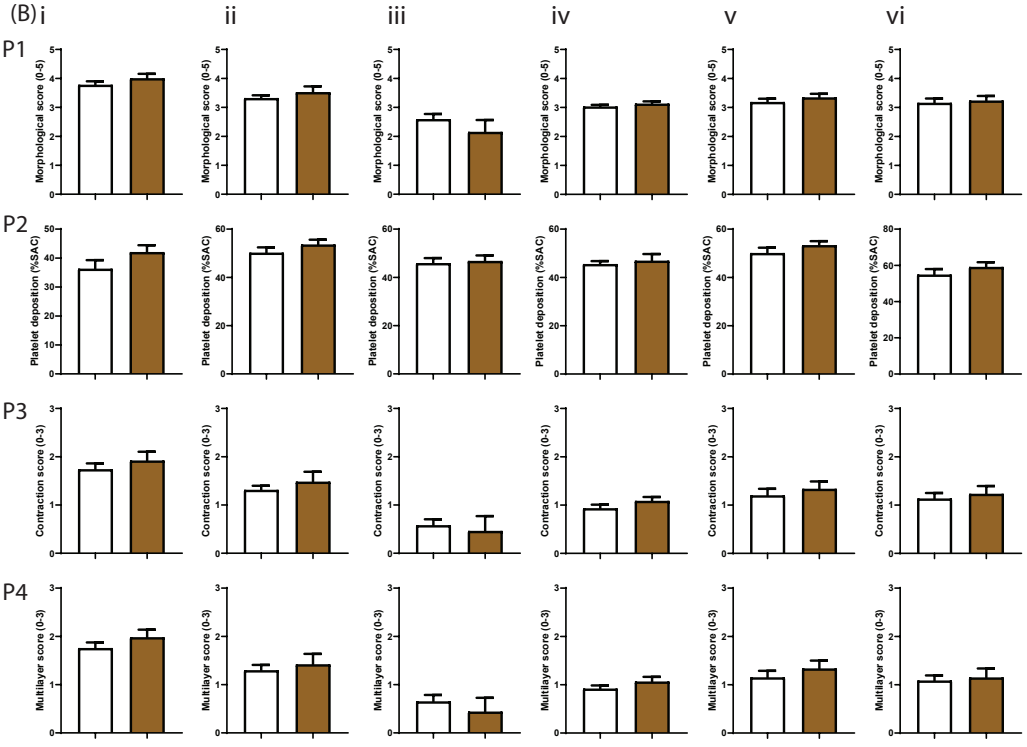
Brightfield
Integrin $\alpha_{IIb}\beta_3$
P-selectin
PS exposure
Vehicle
MK-1026
ii
Collagen type III
Brightfield
Integrin $\alpha_{IIb}\beta_3$
P-selectin
PS exposure
iii
vWF + Laminin
Vehicle
MK-1026
iv
vWF + Rhodocytin
v
vWF + Ristocetin
Vehicle
MK-1026
vi
vWF + Fibrinogen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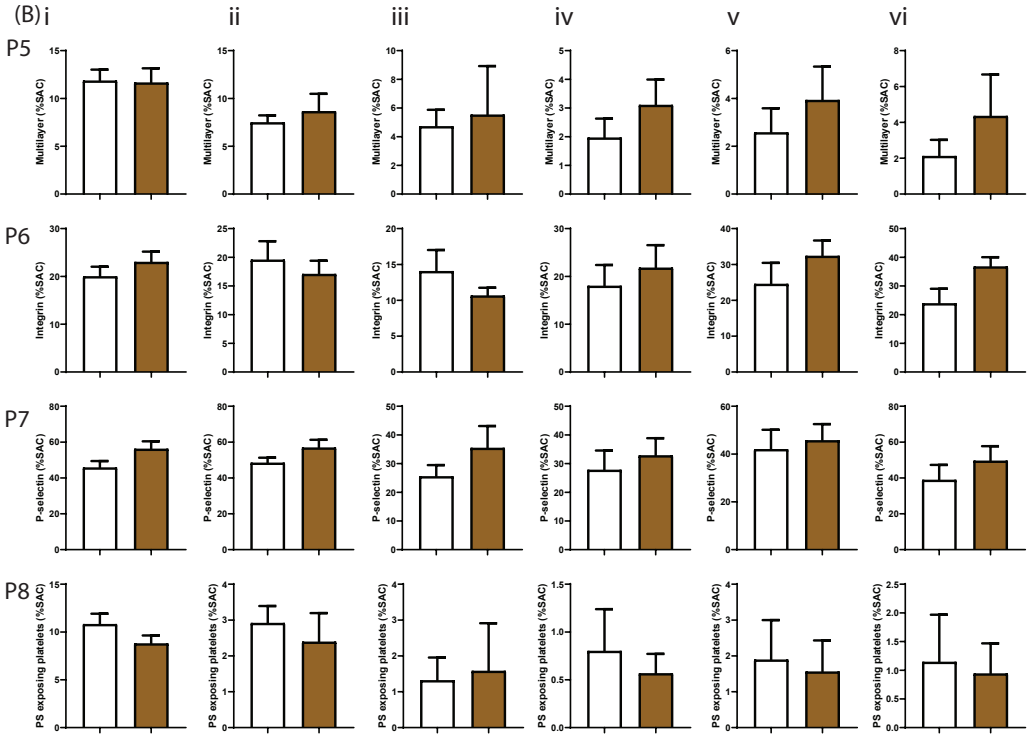
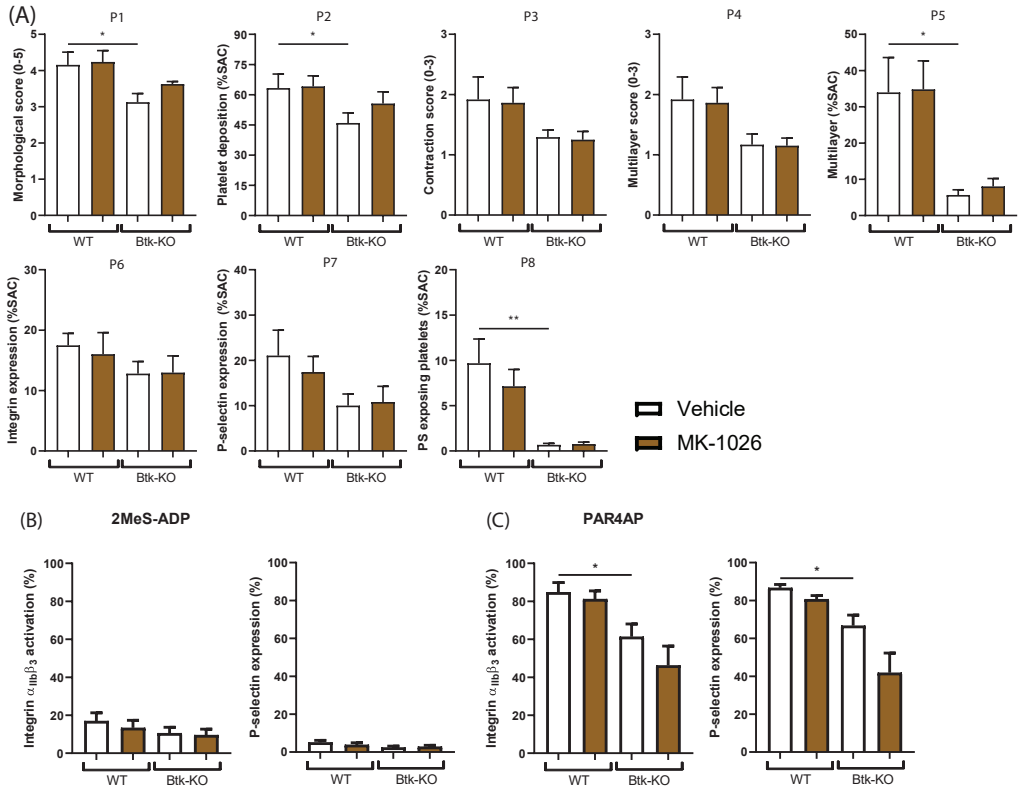
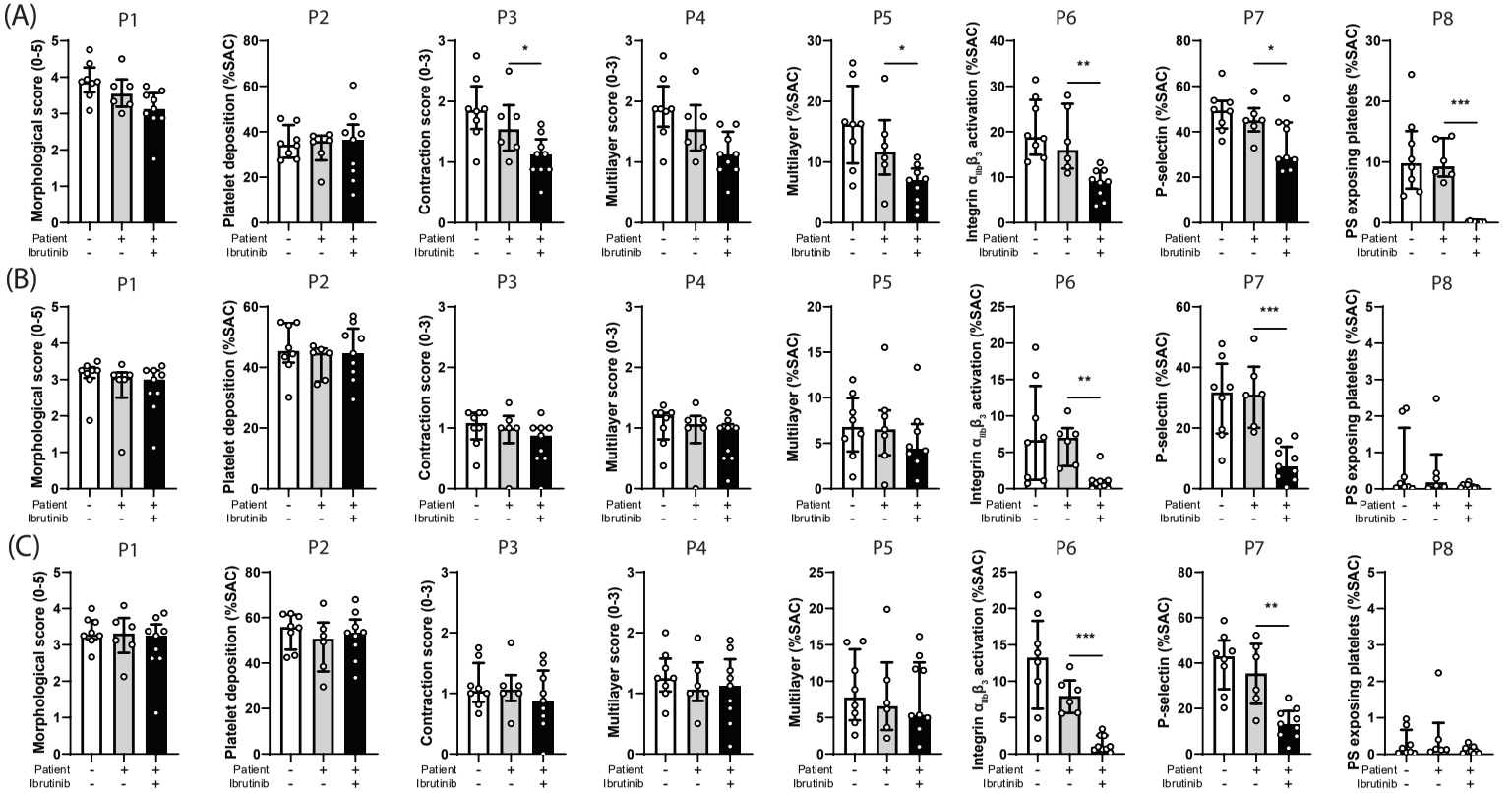
Multilayer score (0-3)



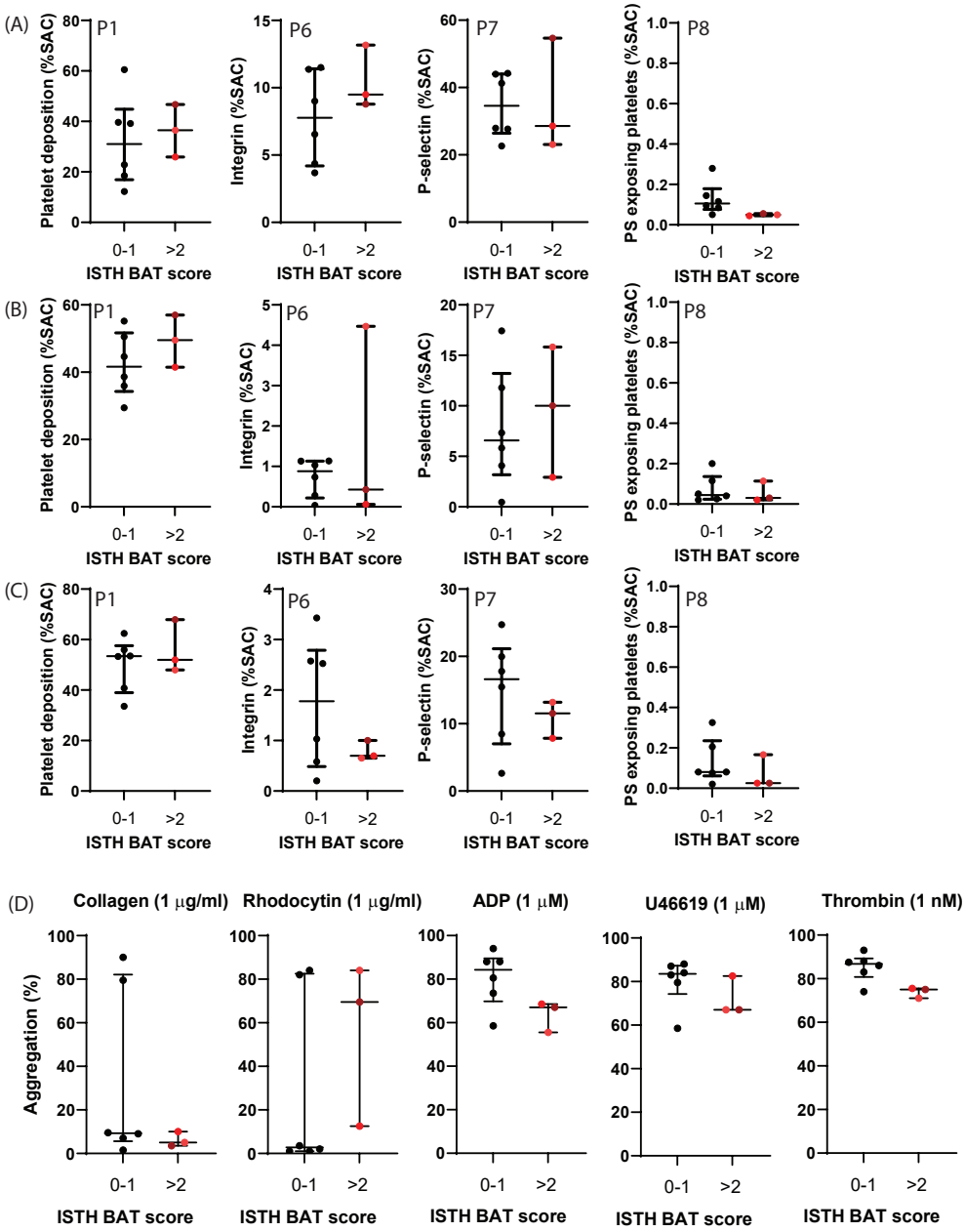
Supplemental Figure 6.5. Thrombus formation under flow with blood from healthy volunteers in presence of MK-1026 over multiple microspots. (A) Representative brightfield and fluorescence images and (B) quantification of control (white bars), MK-1026 (sand-coloured bars) over (i) collagen type I, (ii) collagen type III, (iii) vWF co-coated with laminin, (iv) vWF co-coated with rhodocytin, (v) vWF co-coated with ristocetin and (vi) vWF co-coated with fibrinogen. Data are shown as means ± SEM (n=6).



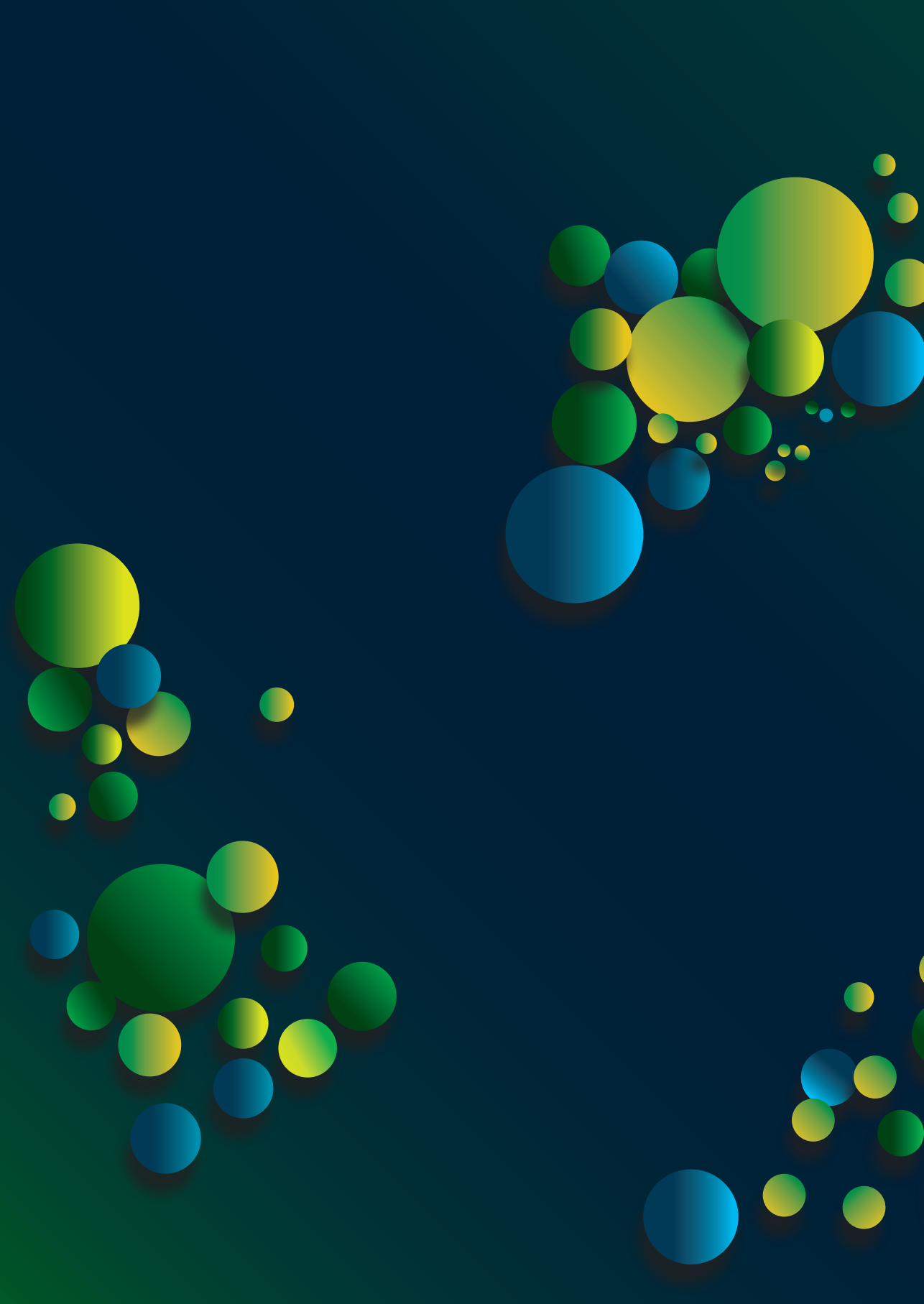
Supplemental Figure 6.6. Thrombus formation under flow with blood of WT or Btk-KO mice over collagen type I surface. (A) Quantification of brightfield and fluorescence images of control (white bars) or MK-1026 (sand-coloured bars) over collagen type I. Parameter 1-8 are as described for Supplement Figure 6.2. (B-C) Platelet activation in whole blood induced by (B) 2MeS-ADP (1 µM) or (C) PAR4AP (100 µg/mL). Histograms show the percentages of platelets binding PE-labelled JON/A monoclonal antibody or PerCP-labelled CD62P mAb in WT and Btk-KO mice. Data are presented as means ± SEM (n=5-6), * p<0.05, ** p<0.01 vs vehicle or indicated otherwise.



Supplemental Figure 6.7. Thrombus formation under flow with blood samples of patients with or without ibrutinib treatment. Citrated whole blood from healthy controls and patients (with or without ibrutinib treatment) was perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over coated microspots. Quantification of brightfield and fluorescence images of healthy donors (white bars), patients (light grey bars) or patients receiving ibrutinib (black bars) over (A) collagen type I, (B) vWF co-coated with rhodocytin and (C) vWF co-coated with laminin. Parameter 1-8 are as described for Supplement Figure 2. Data are presented as medians ± interquartile ranges (n=6-9), * p<0.05, ** p<0.01, *** p<0.001 vs patient without treatment.



Supplemental Figure 6.8. Thrombus formation and aggregation with blood samples of ibrutinib-treated patients with or without bleeding. Citrated whole blood from patients receiving ibrutinib treatment was perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over coated microspots. Quantification of brightfield and fluorescence images of ibrutinib-treated patients with a ISTH BAT score of 0-1 (black dots) or a ISTH BAT score over a value of 2 (red dots) over (A) collagen type I, (B) vWF co-coated with rhodocytin and (C) vWF co-coated with laminin. Parameters 1, 6-8 are as described for Supplement Figure 6.2. (D) Light transmission aggregometry was induced in isolated platelets ($250x10^9$/L) by collagen (1 μg/mL), rhodocytin (1 μg/mL), 2MeS-ADP (1 μM), U46619 (1 μM) or thrombin (1 nM). Scatter plots show the percentage of aggregation of patients receiving ibrutinib with a low bleeding score (0-1, black dots) or a bleeding score of 2 or higher (red dots). Each dot represents one individual patient. Data are presented as medians ± interquartile ranges (n=3-6).



Chapter 7

Multiparameter screening for platelet-inhibitory effects of tyrosine kinase inhibitors used for cancer treatment

Tullemans BME, Veninga A, Fernandez DI, Aarts MJB, Eble JA, van der Meijden PEJ, Heemskerk JWM and Kuijpers MJE

In revision

Abstract

Background: Currently antiplatelet drugs, used for treatment of arterial thrombosis, often coincide with an increased risk of bleeding. Hence, platelet inhibitors that preserve haemostasis are warranted. Multiple tyrosine kinase inhibitors (TKIs), used for cancer treatment, have been reported to inhibit platelet function, with minor bleeding symptoms. In this study, we investigated the antiplatelet properties of several clinically used TKIs as exploration of possible repurposing these compounds as antiplatelet drugs.

Methods: Eight TKIs were selected based on the affinity for tyrosine kinases expressed in platelets and limited bleeding symptoms. Whole blood samples, platelet-rich plasma (PRP) or isolated platelets from healthy donors were pre-incubated with the TKI or vehicle. Platelet aggregation, activation, intracellular calcium mobilisation and whole blood thrombus formation under flow were determined.

Results: Overall, the TKIs with the highest affinities for platelet-expressed targets strongly inhibited platelet functions. Dasatinib and sunitinib dose-dependently reduced collagen-induced aggregation in PRP and washed platelets, while pazopanib, cabozantinib and vatalanib inhibited this response in washed platelets only, and fostamatinib, axitinib and lapatinib showed no or limited effects. Fostamatinib effectively reduced whole blood thrombus formation under flow on collagen, as well as other substrates. Pazopanib, sunitinib, dasatinib, axitinib and vatalanib mildly reduced thrombus formation on collagen. Intracellular calcium measurements and activation responses in isolated platelets were inhibited by dasatinib, fostamatinib, sunitinib and pazopanib. After glycoprotein-VI receptor-stimulation fostamatinib, cabozantinib and vatalanib decreased the highly activated platelet population, whereas negative, resting populations were increased.

Conclusions: Strong inhibitory effects of dasatinib, fostamatinib, sunitinib and pazopanib pointed to interference in early collagen receptor-induced signalling events, when compared to cabozantinib and vatalanib. Since Fostamatinib, sunitinib, pazopanib and vatalanib are reported to cause no more than mild bleeding symptoms, these are promising candidates to explore further as antiplatelet drugs.

Introduction

Ischaemic heart disease and stroke accounted >15 million deaths worldwide in 2019, making arterial thrombosis a leading global cause of death[1]. The major underlying cause are the thrombotic complications of atherosclerosis, either via plaque rupture or erosion[2, 3]. Current treatment is mostly (dual) antiplatelet therapy, such as aspirin and clopidogrel that prevent thromboxane A_2 release and ADP receptor activation, respectively[4]. However, these inhibitors mostly have an irreversible mode of action on platelet G-protein coupled receptor (GPCRs) signalling pathways and coincide with an increased risk of bleeding[5]. Therefore, novel antiplatelet drugs are being developed that inhibit thrombosis, but do not interfere in haemostasis[5]. In this respect glycoprotein (GP)VI and C-type lectin-like receptor 2 (CLEC-2) receptors, as well as downstream tyrosine kinases, have been postulated as interesting antithrombotic targets[6].

GPVI is an immunoreceptor tyrosine-based activation motif (ITAM)-linked receptor, activated by collagen and fibrin(ogen), resulting in initial platelet activation and subsequent thrombus formation[7-9]. Deficiency of GPVI in mice suppressed arterial thrombosis without substantial prolongation of bleeding time[10, 11]. Inhibition of GPVI has also been reported to protect from thrombosis upon plaque rupture in mice[12, 13]. Furthermore, patients with defects in GPVI have only a mild bleeding diathesis[14] or remain asymptomatic[15]. Two GPVI inhibitors showing promising antithrombotic effects, are currently being tested in phase II clinical trials: the Fab fragment ACT017 and Revacept, a collagen binding dimeric GPVI-Fc fusion protein[6]. However, a disadvantage of these treatments is the administration via intravenous injections.

CLEC-2 is a hemITAM-linked receptor, activated by the endogenous ligand podoplanin[16]. CLEC-2 deficient mice are protected from arterial thrombus formation and displayed only a minor effect on haemostasis[17, 18]. In contrast, deficiency of both GPVI and CLEC-2 resulted in lack of cessation of tail bleeding, as well as profound reduction of arterial thrombosis[19]. In human blood, a monoclonal antibody (mAb) to CLEC-2 has been shown to block CLEC-2 induced platelet activation *in vitro*[20]. However, no clinical trials targeting CLEC-2 have started until now.

Both GPVI and CLEC-2 heavily depend on tyrosine kinase signalling including Syk, Btk and Src family kinases (SFK)[21]. In addition, other receptor signalling pathways, likely to a lesser extent, use include tyrosine kinases, such as those evoked by GPIb, integrin $\alpha_{IIb}\beta_3$, Axl and Tie, which are receptors for vWF, fibrinogen, Gas6 and angiopoietin, respectively[22, 23]. With such a wide mode of action on platelet activation, while leaving GPCR signalling mostly intact, tyrosine kinase inhibitors (TKIs) may provide an alternative way to inhibit platelet activation[6]. During the past decades, many orally available TKIs have been developed for cancer treatment[24, 25], with different inhibitory profiles against tyrosine kinases (Table 7.1). These compounds have been shown to be well tolerated, and in general cause only a mild increase in risk of bleeding. The Syk inhibitor fostamatinib, used for treatment of chronic immune thrombocytopenic purpura (ITP), has been reported to reduce GPVI- and CLEC-2 stimulated platelet aggregation, with only

minor effects on bleeding time in mice[26, 27]. Notably, TKIs targeting a broad spectrum of tyrosine kinases including the receptors for vascular endothelial growth factor (VEGFR), platelet-derived growth factor (PDGFR) and Kit are used for the treatment of renal cell carcinoma. These include sunitinib, pazopanib, axitinib and cabozantinib (Table 7.1). We have recently shown that sunitinib and pazopanib inhibit collagen-induced platelet function both *in vitro* and in patients receiving treatment[28, 29]. Axitinib and cabozantinib also have targets that are present in platelets[24] and have been associated with increased risk of bleeding[30], but no effects on platelet function have been reported. On the contrary, several TKIs used for treatment of breast and colon cancer have limited affinity for tyrosine kinases present in platelets, without reported bleeding[24]. These include lapatinib and vatalanib (Table 7.1).

The aim of the present study was to systematically evaluate the antiplatelet properties of eight clinically used TKIs, and to assess their potential for repurposing of these compounds as antiplatelet drugs. Therefore, we investigated the effects of these compounds on platelet function in isolated platelets and in whole blood under flow, using different agonists to include multiple signalling pathways.

Materials and Methods

Materials

The active metabolite of fostamatinib (R406) was obtained from InvivoGen (San Diego CA, USA). Active metabolites of axitinib (A-1107), cabozantinib, dasatinib and pazopanib were

Table 7.1: Characterization of TKIs used for cancer treatment with targets present in platelets.

TKI	Reported Targets	Targets in platelets (MKs)	Used for treatment of	Bleeding reported	Ref
Dasatinib	PDGFR, EFGR, BCR-ABL, EphA2, Kit, SFK	SFK: Src, Fyn, Lck, Lyn, Yes, Btk, (Kit)	CP CML, AP MB, LB CML	Yes	66, 67
Fostamatinib	Syk	Syk, Src, Fgr, Fyn, lck, Lyn, Yes	B-cell lymphoma, CLL	No	54
Sunitinib	VEGFR, PDGFR, CSF-R, Ret, Kit, Flt3	(Ret, Kit)	RCC, GIST, PNET	Yes	30, 66, 68, 69
Pazopanib	VEGFR, PDGFR, FGFR, Kit, Fms, Itk, Lck	Lck (Kit)	NSCLC, OC, RCC, STS, TC	Yes	30, 66
Axitinib	VEGFR, PDGFRβ	Lck, Yes, Axl, Tie	RCC	Yes	30, 66
Cabozantinib	VEGFR, Met, Ret, Kit, Flt3, Axl, Tie	Axl, Tie (Ret, Kit)	TC, RCC	Yes	30, 70
Lapatinib	EGFR, ErbB1-2	ND	$ErbB2^{+}HR^{-}$ or HR^{+} BC	No	66, 71
Vatalanib	VEGFR, PDGFRβ, Kit	(Kit)	MCRC	No	72

AP: acute-phase; BC: breast cancer; CLL: chronic lymphocytic leukaemia; CML: chronic myeloid leukaemia; CP: chronic-phase; CSF-R: colony-stimulating factor receptor; EGFR: epidermal growth factor receptor; ErbB2: human epithelial growth factor 2; FGFR: fibroblast growth factor receptor; GIST: gastrointestinal stromal tumour; HR: hormone receptor; LB: lymphoid blast; MB: myeloid blast; MCRC: metastatic colorectal cancer; MKs, megakaryocytes; ND: not determined; NSCLC: non-small-cell lung cancer; OC: ovarian cancer; PDGFR: platelet-derived growth factor receptor; PNET: primitive neuroectodermal tumour; RCC: renal cell carcinoma; SFK: Src family kinase; STS: soft tissue sarcoma; TC: thyroid cancer; VEGFR: vascular endothelial growth factor receptor.

purchased from LC Laboratories (Woburn MA, USA). The active metabolite of lapatinib (GW-572016) was purchased from Selleckchem (Houston TX, USA), whereas the active metabolite of sunitinib came from Pfizer (New York NY, USA). The active metabolite of vatalanib (PTK787) was obtained from Adooq Bioscience (Irvine CA, USA). Bovine serum albumin (BSA), D(+)-glucose, unfractionated heparin and apyrase were purchased from Sigma-Aldrich (Saint Louis MO, USA). Horm collagen type I came from Takeda (Hoofddorp, the Netherlands), whereas the agonists collagen-related peptide crosslinked (CRP-XL) and von Willebrand factor III (vWF-III) were obtained from CambCol Laboratories (Cambridge, UK). Rhodocytin was purified from *Calloselasma rhodostoma* venom as described previously[31]. Thrombin was from Enzyme Research Laboratories Inc (South Bend, IN, USA). U46619 (thromboxane A_2 receptor agonist) came from Cayman Chemicals (Ann Arbor MI, USA). 2-Methylthio-adenosine-diphosphate (2MeS-ADP) and D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) were obtained from Santa Cruz Biotechnology (Dallas TX, USA). Laminin was purchased from Octapharma (Lachen, Switzerland), whereas Fura-2-AM and allophycocyanin (APC)-labelled mouse anti-human CD63 monoclonal antibody (mAb, clone MEM-259) were obtained from Invitrogen, Fisher Scientific (Carlsbad CA, USA). Fluorescein isothiocyanate (FITC)-labelled PAC1 mAb against activated human integrin $\alpha_{IIb}\beta_3$ (340507), Phycoerythrin (PE)-labelled annexin A5, Brilliant Violet (BV)510-conjugated mouse anti-human CD42b mAb (clone HIP1) and BV421-labelled rat anti-human TLT-1 mAb (clone 268420), were purchased from BD Bioscience (Franklin Lakes NJ, USA). FITC-conjugated α-fibrinogen mAb was purchased from DAKO (F0111; Santa Clara CA, USA). Alexa Fluor (AF)568-conjugated annexin A5 was purchased from Molecular Probes, Life Technologies (New York NY, USA). Peridinin-chlorophyll-protein cyanine5.5 (PerCP Cy5.5)-conjugated mouse anti-human CD62-P mAb (clone AK4) and AF647-labelled mouse anti-human CD62-P mAb (clone AK4) were obtained from Biolegend (London, UK). FITC-conjugated annexin A5 was from Pharmatarget (Maastricht, the Netherlands).

Blood collection and platelet isolation

In accordance with the declaration of Helsinki and approval of the local medical ethical committee (Maastricht University Medical Centre+, MUMC+), we obtained full informed consent from all participants. Blood from healthy volunteers was collected in 3.2% trisodium citrate tubes by venipuncture, after discarding the first 3 mL of blood. Blood cell counts and haematological parameters were assessed, using a Sysmex XP300 (Kobe, Japan).

Platelet-rich plasma (PRP) or platelets were isolated from whole blood as described previously[32, 33]. Washed platelets were resuspended in Hepes buffer, pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mg/mL glucose, and 1 mg/mL bovine serum albumin). Platelet count was adjusted as stated per assay.

Thrombus formation

Thrombus formation under flow was determined as described[34]. Briefly, glass coverslips were coated with 3 microspots of collagen type I (100 µg/mL), collagen type III (100 µg/mL), vWF (50 µg/mL) co-coated with laminin (100 µg/mL) or vWF co-coated with rhodocytin (250 µg/mL), vWF co-coated with ristocetin (250 µg/mL), or vWF co-coated with fibrinogen (250 µg/mL) and mounted in a parallel plate flow chamber. Citrated blood samples were incubated with the TKIs axitinib, cabozantinib, dasatinib, fostamatinib, lapatinib, pazopanib, sunitinib, vatalanib or vehicle for 10 minutes at room temperature. Pre-incubated whole blood samples were recalcified in presence of PPACK (40 µM) and perfused at a wall shear rate of 1000 s^{-1} for 3.5 minutes. Platelet activation properties were determined by post-perfusion with Hepes buffer (supplemented with 2 mM $CaCl_2$ and 1 U/mL heparin) containing FITC-conjugated α-fibrinogen mAb (1:80), AF647-conjugated αCD62P mAb (1:100), and AF568-conjugated annexin A5 (1:200). Brightfield and fluorescence images were captured using an EVOS microscope (Bothel WA, USA).

Analysis of microscopic data

Microscopic images were analysed using specific scripts in the open-access Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, WI, USA) as described[34, 35]. Multiple thrombus formation parameters were obtained from brightfield images including P1: morphological score of platelet adhesion and thrombus formation (scale 0-5), P2: surface area coverage of adhered platelet (% SAC), P3: platelet aggregate contraction score (scale 0-3), P4: platelet aggregate multilayer score (scale 0-3) and P5: coverage of multi-layered platelet aggregation (% SAC). From fluorescence images, platelet activation parameters were obtained by determining P6: integrin $\alpha_{IIb}\beta_3$ activation (% SAC), P7: P-selectin expression (% SAC) and P8: phosphatidylserine (PS) exposure (% SAC). Heatmaps were generated from the mean values per parameter for each surface for comparative data analysis. Values were scaled from 0-10 based on the highest value of each parameter for each surface. For visualization of the effects, subtraction heatmaps were generated by subtraction of scaled control data from each TKI condition and heatmaps were filtered based on significant differences ($p<0.05$). Heatmaps were generated using R Core Team (i386 3.2.5; Vienna, Austria).

Platelet aggregation by light transmission aggregometry

Isolated platelets or PRP ($250x10^9$ platelets/L) were incubated with TKIs or vehicle for 10 minutes at 37°C. Maximal aggregation was induced by collagen type I (1 µg/mL), 2MeS-ADP (1 µM), U46619 (1 µM), rhodocytin (1 µg/mL) or thrombin (1 nM). Platelet aggregation was recorded using a Chronolog optical aggregometer (Havertown PA, USA). The maximum amplitude was quantified at 8 minutes after agonist addition.

Cytosolic Ca^{2+} measurements

Washed platelets ($200x10^9$ platelets/L) were loaded with Fura-2-AM and changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were measured in 96-well plates using a FlexStation 3 (Molecular Devices, San Jose, CA, USA), as described[36, 37]. Briefly, platelets in suspension were preincubated with TKIs or vehicle for 10 minutes at room temperature. Pre-loaded and -incubated samples were stimulated by CRP-XL (10 µg/mL). Changes in Fura-2 fluorescence were measured in duplicate, and ratio values were calculated. Data are presented as $[Ca^{2+}]_i$ in nM or area under the curve (AUC) in nMs.

Platelet activation by flow cytometry

Washed platelets ($25x10^9$ platelets/L) were incubated with TKIs or vehicle for 10 minutes at room temperature. Platelets were stimulated, in the presence of 2 mM $CaCl_2$, by CRP-XL (5 µg/mL), 2MeS-ADP (2 µM), or thrombin (4 nM) for 15 minutes, and simultaneously stained for multiple activation markers using an antibody mix consisting of FITC-conjugated PAC1 (1:20), BV510-conjugated αCD42b (1:50), BV421-conjugated αTLT1 (1:50), APC-conjugated αCD63 (1:20), PerCP-Cy5.5-conjugated αCD62P (1:500) mAbs. For measuring phosphatidyl-serine (PS) exposure, platelets were activated with a combination of CRP-XL (5 µg/mL) and thrombin (4 nM) for 1 hour at 37°C, and post-labelled with PE-conjugated annexin A5. Samples for activation markers as well as PS exposure were fixed after stimulation for at least 30 minutes at 4°C in the dark using a 0.3% fixation buffer, before measuring 10,000 events per sample on a BD FACSCanto II (BD bioscience, Franklin Lakes, NJ, USA).

Analysis of flow cytometric data

Separate FCS files were opened in FlowJo V10 software (Treestar, Ashland OR, USA) and checked for data anomalies by FlowAI[38]. Before concatenation, event count per sample was set at 5,000 events by DownSample for equal weighing of every sample. Resulting file was subjected to t-distributed stochastic neighbour embedding (tSNE) analysis to reduce the high dimensionality to a 2D plot. FlowSOM analysis was used to identify five clusters[39]. Fractions of platelets present in the resulting FlowSOM populations were determined per original sample. Each platelet population was characterised by the expression level of the different markers.

Statistical analysis

Data are shown as mean ± standard error of mean (SEM). GraphPad Prism 8.3.0 software (La Jolla, CA, USA) was used for statistical analysis with an unpaired non-parametric t-test (Mann-Whitney) or one-way ANOVA. A p-value less than 0.05 was considered to be statistically significant, in which * is $p<0.05$, ** is $p<0.01$ and *** is $p<0.001$.

Results

Characteristics of selected TKIs

For this study, eight reversible multi-target TKIs were selected based on published affinities for tyrosine kinases (TKs) expressed in platelets, and reported minor bleeding risk (Table 7.1, Figure 7.1)[24]. Dasatinib and fostamatinib display high affinity for platelet-expressed Syk and Src-family kinases (SFKs), while sunitinib, pazopanib, axitinib and cabozantinib show moderate to mild affinity for only some of these platelet-expressed targets (Figure 7.1). Dasatinib and fostamatinib are known as inhibitors of the GPVI, CLEC-2 and $\alpha_{IIb}\beta_3$ activation pathways in platelets[26, 40], but have not been systematically evaluated. Lapatinib and vatalanib have unknown affinities for platelet-expressed kinases (Figure 7.1, Table 7.1).

Distinct effects of selected TKIs on collagen-induced platelet aggregation in the presence or absence of plasma

Impaired platelet function by TKIs is described to be due to inhibition of in particular the ITAM-linked receptor GPVI[24, 25]. Therefore, we first investigated the effects of the active metabolites of different TKIs on collagen-induced aggregation responses. Dose-response curves with isolated platelets showed distinct effects of the different TKIs (Figure 7.2, dotted lines). The most potent aggregation inhibition was observed with dasatinib (>90% inhibition with 1 µM, Figure 7.2A). Surprisingly, the combined SFK and Syk-inhibitor fostamatinib, with several targets in platelets, did not show a relevant inhibitory effect (Figure 7.2B). For the weaker SFK inhibitors sunitinib, pazopanib, and cabozantinib, aggregation in washed platelets became nearly abolished with 33 µM (Figure 7.2C-D, F) Unexpectedly, vatalanib without assumed targets in platelets also inhibited this response at 33 µM (Figure 7.2H). IC_{50} values of these four TKIs were between 6-13 µM and a

	SFK							TFK		Others			
TKI	SRC	Fgr	Fyn	Lck	Lyn	Yes	**Syk**	Btk	Tec	Axl	Tie1	MEK1	MEK2
Dasatinib	+++	+++	±	+++	+++	+++	±	+++	±	-	-	±	±
Fostamatinib	+++	++	+++	++	++	++	+++	+	±	+	+	+	+
Sunitinib	±	±	±	+	+	+	-	±	-	+++	±	+	+
Pazopanib	±	±	±	±	±	±	±	-	-	-	±	-	-
Axitinib	-	±	-	±	-	±	-	-	-	+	+	-	-
Cabozantinib	-	-	-	-	-	-	-	-	-	+	±	-	-
Lapatinib	-	-	-	-	-	-	-	-	-	-	-	-	-
Vatalanib	-	-	-	-	-	-	-	-	-	-	-	-	-

Interaction strength		Kd (nM)	IC50 (nM)
+++	Very strong	<0.01	<0.1
++	Strong	<0.1	<1
+	Moderate	<1	<10
±	Mild	<10	<100
-	No / ND	>10	ND

Figure 7.1. Affinity profile of common tyrosine kinase inhibitors (TKIs) for relevant tyrosine kinases implicated in platelet activation. Shown is a heatmap of the affinity-based dissociation constants (Kd)[62-64] or IC_{50} values[65] of the indicated TKIs. Values are from binding experiments with indicated purified kinases, including Src family kinases (SFK), Syk, Tec family kinases (TFK), focal adhesion kinases (FAK) and MEK. Colour coding with highest affinity (lowest Kd or IC_{50}) shown in deep red and lowest affinity (highest Kd or IC_{50}) shown in white. Data are based on ref [24].

concentration of 10 µM was used for further experiments (Table 7.2). Axitinib and lapatinib with limited or no TK targets slightly affected the aggregation response to collagen at the highest dose tested of 100 µM (Figure 7.2E, G).

As most TKIs are protein-bound in plasma, dose response curves were also determined in platelet-rich plasma (PRP; Figure 7.2, solid lines). Upon collagen stimulation, the strongest inhibitory effect in PRP was also observed with dasatinib (IC_{50} of 0.4 µM, and >90% inhibition at 3.3 µM, Figure 7.2A). In patient studies, plasma concentrations up to 0.1 µM dasatinib are reported[41], which concentration thus was used in following experiments (Table 7.2). Inhibition with fostamatinib, also resulted in a small but significant decrease of the aggregation response (±20% inhibition, Figure 7.2B). For sunitinib, significant inhibition (60%) was reached at 33 µM, which was higher than required in washed platelets (Figure 7.2C). The IC_{50} value of 33 µM sunitinib

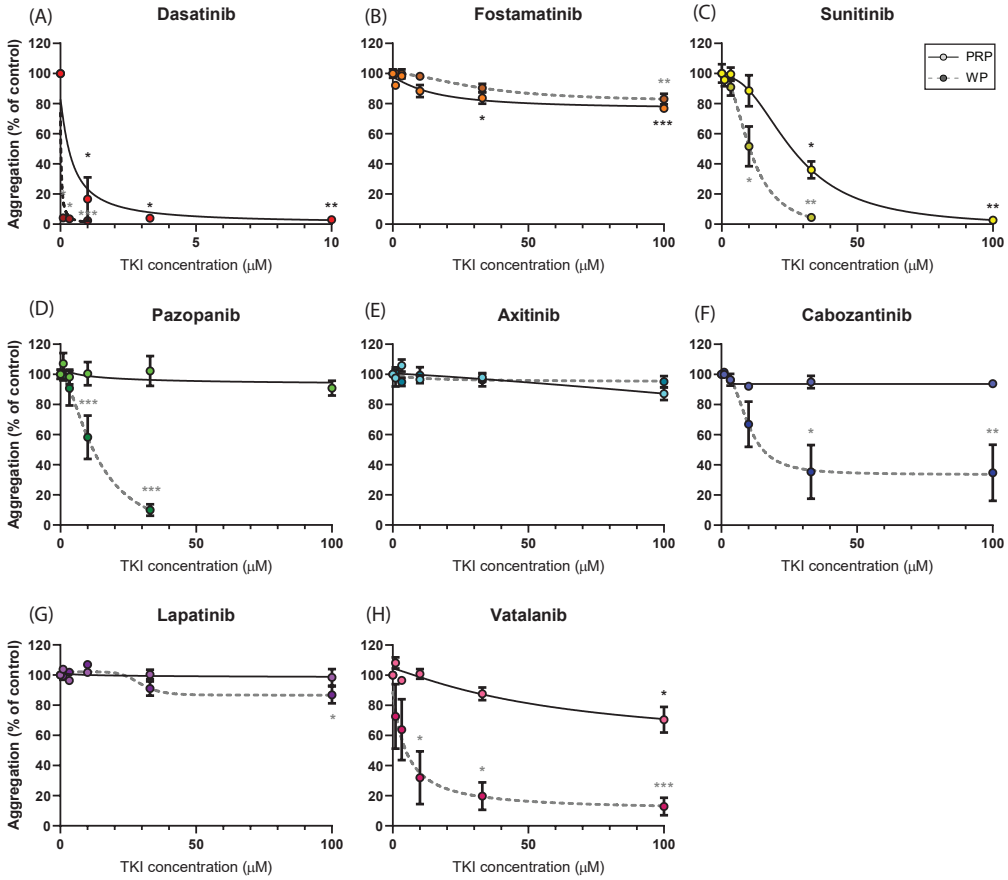


Figure 7.2. Dose-dependent effects of TKIs on platelet aggregation induced by collagen. Washed platelets or PRP (250x10^9/L) were incubated with vehicle (<0.1% DMSO) or TKI for 10 minutes. Dose response curves of inhibition of aggregation induced by collagen type I (1 µg/mL) for (A) dasatinib, (B) fostamatinib, (C) sunitinib, (D) pazopanib, (E) axitinib, (F) cabozantinib, (G) lapatinib or (H) vatalanib, measured in either PRP (solid line) or washed platelets (dotted line). Maximum aggregation responses were determined at 8 minutes; data are shown as means ± SEM (n = 6). * p<0.05. ** p<0.01, *** p<0.001.

was used for further analysis in whole blood experiments. In contrast to washed platelets, the aggregation responses in the presence of pazopanib, cabozantinib or vatalanib were not, or barely inhibited in PRP (Figure 7.2D, F, H). In agreement with the responses in washed platelets, axitinib and lapatinib did not show altered collagen-induced aggregation in PRP (Figure 7.2E, F). For the latter set of compounds, no more than 20% inhibition was observed in PRP, for which reason the highest concentration of 33 µM was used for plasma-containing experiments (Table 7.2).

Whole blood thrombus formation over (non)collagen surfaces u nder flow is most strongly affected by high affinity TKIs

To explore the effects of the TKIs under physiologically relevant circumstances, we investigated platelet activation and thrombus formation under flow on coated surfaces, triggering a range of platelet receptors, i.e., GPVI, GPIb, CLEC-2, and integrins $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$. Blood samples were pre-incubated with one of the TKIs (applied at mostly 33 µM, see Table 7.2), and perfused over multiple microspots, containing collagen type I or III, or vWF co-coated with either laminin, rhodocytin, ristocetin or fibrinogen. Microscopic analysis of brightfield and fluorescence images resulted in five different thrombus parameters (P1-5) and three platelet activation markers (P6-8) (Suppl. Figure 7.1). In agreement with previous results[34], different types of platelet thrombi were formed on the various surfaces with the control condition (Figure 7.3A). A systematic analysis was performed for evaluating the effects of each TKI by generating a subtraction heatmap for the eight different parameters, which were scaled (0-10) per parameter and per surface (Figure 7.3B). In contrast to the limited effect on collagen-induced aggregation, fostamatinib at 33 µM was most potent in suppressing thrombus formation and platelet activation under flow, on collagen as well as non-collagen surfaces (Figure 7.3A, B). Dasatinib at 0.1 µM also reduced collagen-induced thrombus formation under flow. On the vWF co-coated surfaces, this concentration of dasatinib did not affect the thrombus parameters (Figure 7.3B). Sunitinib in particular affected multiple parameters of thrombus formation on the two collagen surfaces, but showed limited effects on other surfaces. Pazopanib, axitinib and vatalanib showed weak inhibition, only affecting 1-2

Table 7.2. Concentrations of TKIs used in platelet function assays

	Concentration (µM)	
TKI	WB or PRP	WP
Dasatinib	0.1	0.1
Fostamatinib	33	33
Sunitinib	33	10
Pazopanib	33	10
Axitinib	75	33
Cabozantinib	33	10 or 33
Lapatinib	33	33
Vatalanib	33	10 or 33

Abbreviations: PRP: platelet-rich plasma; WB: whole blood; WP: washed platelets

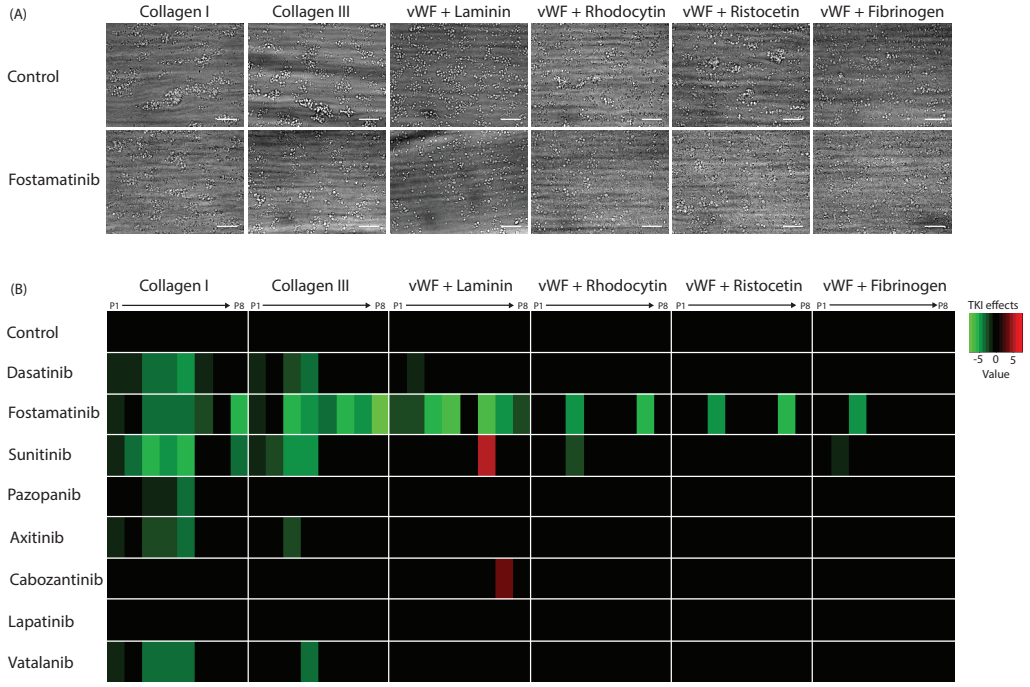


Figure 7.3. Effects of eight TKIs on whole blood thrombus formation under flow on different thrombogenic surfaces. Blood samples were pre-incubated with vehicle or TKI for 10 minutes. Samples were then recalcified in the presence of PPACK and perfused for 3.5 minutes at a wall shear rate of 1000 s^{-1} over six different coated surfaces. After perfusion, the formed thrombi were stained with AF568-annexin A5, FITC-α-fibrinogen and AF647-α-CD62P to detect PS exposure, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression, respectively. (A) Representative brightfield images of control or fostamatinib-treated blood after flow over collagen type I, collagen type III, vWF plus laminin, vWF plus rhodocytin, vWF plus ristocetin or vWF plus fibrinogen. (B) Subtraction heatmap representing significantly altered effects of parameters P1-P8 (normalized and analysed as in Suppl. Figure 7.1). P1 = morphological score of platelet adhesion and thrombus formation, P2 = surface area coverage of adhered platelets, P3 = platelet aggregate contraction score, P4 = platelet aggregate multilayer score, P5 = coverage of multi-layered platelet aggregation, P6 = αIIbβ3 activation, P7 = P-selectin expression, and P8 = PS exposure. Results from vehicle control runs were set at 0. Effects were filtered for significant alterations ($p<0.05$) with TKIs.

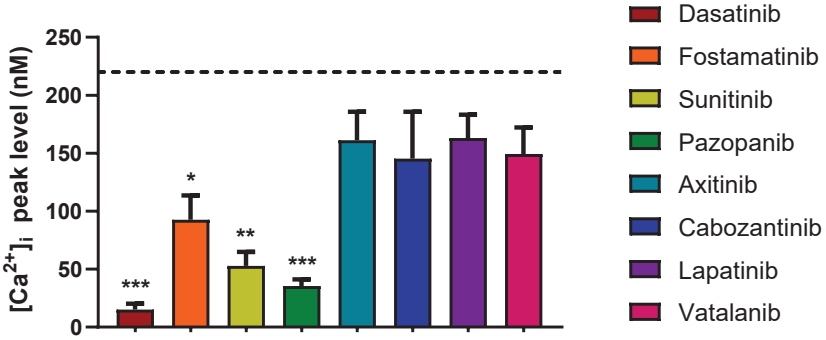


Figure 7.4. Effects of different TKIs on GPVI-induced intracellular calcium responses. Fura-2-loaded platelets ($200x10^9$ /L) were pre-incubated with vehicle or TKI for 10 minutes and stimulated with CRP-XL (10 µg/mL) in presence of 2 mM $CaCl_2$. Histograms show maximal rises in $[Ca^{2+}]_i$ (n=6). Black dotted lines indicate average of control values. Data are shown as means + SEM, * $p<0.05$, *** $p<0.001$.

parameters on collagen type I and III. Mainly the size, height and density of the thrombi were reduced (Figure 7.3B, Suppl. Figure 7.1). The inhibition of thrombus formation with dasatinib or sunitinib was in line with the effects seen in aggregation of PRP. Also, the weaker effects of the other TKIs (pazopanib, axitinib, cabozantinib, lapatinib and vatalanib) (Figure 7.3B) were in agreement with the PRP aggregation results. Together, this indicated that the TKIs with highest affinities for kinase targets in platelets (dasatinib, fostamatinib and sunitinib) had the largest impact on whole blood thrombus formation under flow.

Differential effects of TKIs on GPVI-induced calcium signalling

To further explore the inhibitory effects of the TKIs on platelet signalling, we measured the GPVI-induced intracellular calcium responses. Platelets were loaded with the probe Fura-2, preincubated with vehicle or TKI, and then stimulated with the GPVI agonist CRP-XL. Markedly, dasatinib, fostamatinib, sunitinib and pazopanib significantly decreased or even abolished the maximum intracellular calcium signal (Figure 7.4). In contrast, intracellular calcium rises were unchanged in the presence of axitinib, cabozantinib, lapatinib and vatalanib (Figure 7.4). Similar effects were seen when comparing the slope and area-under-the curve of these responses (not shown). In general, these results are in line with the observed effects on collagen-induced aggregation. However, cabozantinib and vatalanib showed 30-70% inhibition of platelet aggregation platelets. This suggested that for dasatinib, fostamatinib, sunitinib and pazopanib the effects were mediated by early GPVI-induced signalling events in accordance with Syk or SFK inhibition, whereas for cabozantinib and vatalanib the anti-aggregatory effects are downstream of the calcium rises.

Diverse effects of fostamatinib, cabozantinib and vatalanib on platelet activation markers and platelet populations

In order to further explain the selective anti-aggregatory effects of fostamatinib, cabozantinib and vatalanib, we investigated their effects on GPVI-induced integrin $\alpha_{IIb}\beta_3$ activation, secretion of α- and δ-granules and lysosomes. In general, all platelet activation markers were reduced in the presence of cabozantinib (33 μM), vatalanib (33 μM) and fostamatinib (33 μM) (Figure 7.5A). Fostamatinib consistently inhibited all GPVI-induced activation responses. In contrast, PS exposure was not inhibited by fostamatinib in isolated platelets (Figure 7.5A). The effects of cabozantinib and vatalanib on CRP-induced platelet activation were more restricted. Cabozantinib and vatalanib both inhibited integrin $\alpha_{IIb}\beta_3$ activation, in line with the inhibition of collagen-induced aggregation in washed platelets. Furthermore, cabozantinib inhibited δ-granule and lysosome secretion, whereas vatalanib resulted in reduction of α-granule secretion. PS exposure was also not affected by cabozantinib and vatalanib. As sustained high calcium rises are a prerequisite for PS exposure, the absence of effects on PS exposure by the TKIs is in agreement

with the calcium responses that remained unchanged by cabozantinib and vatalanib. Hence, cabozantinib and vatalanib suppress platelet integrin activation and secretion at doses that do not influence the calcium response.

To evaluate a differential sensitivity of platelets towards these TKIs in terms of activation markers, we determined how the formation of activated platelet populations was influenced

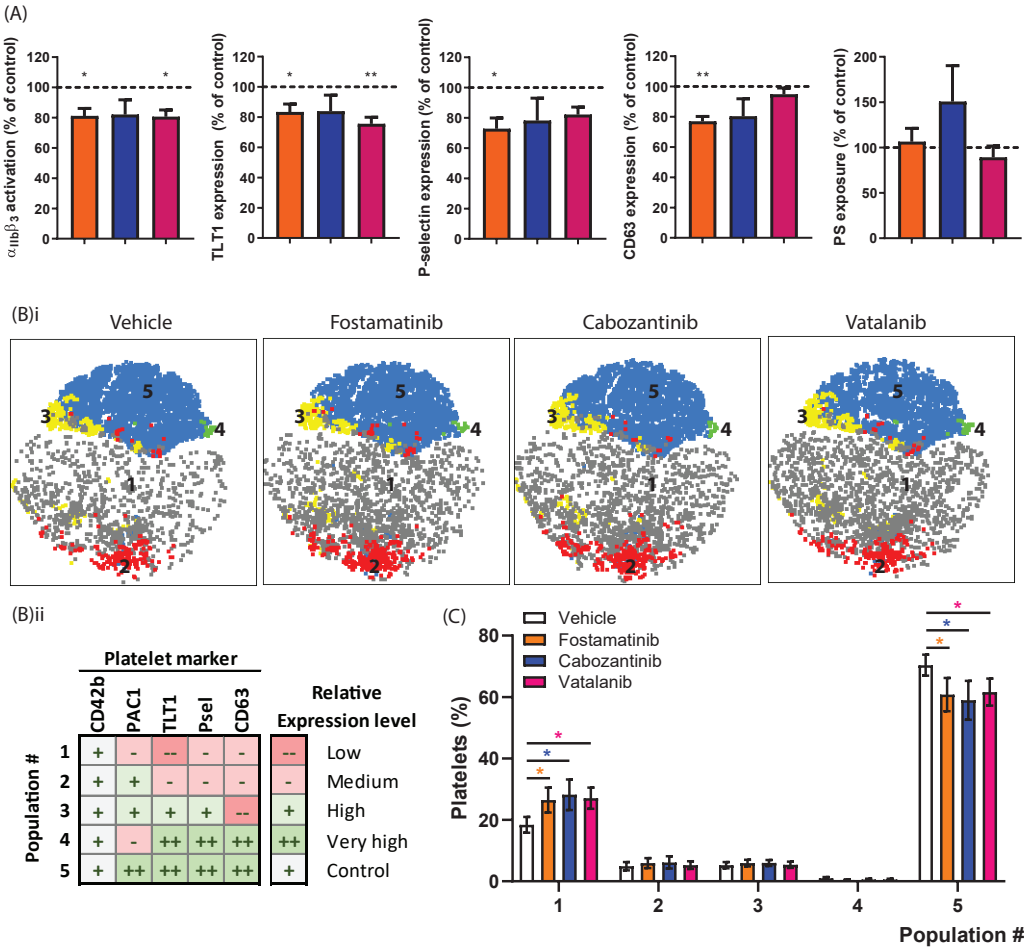


Figure 7.5. Effect of TKIs on populations of activated platelets in response of GPVI. Washed platelets ($25x10^9$/L) were incubated with vehicle, fostamatinib (33 μM), cabozantinib (33 μM) or vatalanib (33 μM) for 10 minutes. Platelet activation was induced by 5 μg/mL CRP-XL for 15 minutes and simultaneously labelled for activated integrin $\alpha_{IIb}\beta_3$ (PAC1-FITC), α-granule secretion (αTLT1-BV421, αCD62P-PerCP Cy5.5) and δ-granule/lysosome secretion (αCD63-APC). Separately, PS exposure was induced using 5 μg/mL CRP-XL plus 4 nM thrombin for 60 minutes at 37°C, labelling was with annexin A5-PE. (A) Histograms of integrin $\alpha_{IIb}\beta_3$ activation, TLT1, P-selectin and CD63 expression as well as PS exposure as percentages of control condition. (B) Distribution profiles of platelet populations after stimulation with CRP-XL. (i) 2D plots visualising the platelet fractions per population in the presence of vehicle, fostamatinib, cabozantinib or vatalanib. (ii) Characterisation of CRP-XL-induced platelet populations based on relative expression levels of activation markers determined by FlowJo analysis, using tSNE and FlowSOM plugins. (C) Histograms visualising the platelet fractions per population in the presence of vehicle (white bars), or fostamatinib, cabozantinib or vatalanib (coloured bars). Data are shown as means ± SEM (n=6), * p<0.05, ** p<0.01.

by the presence of fostamatinib, cabozantinib or vatalanib. Comparison of the four different activation markers, reflecting stages of granular secretion and integrin activation, rendered up to five different populations (Figure 7.5Bii) upon stimulation with CRP-XL. These were ranging from low up to very high expression levels for all markers with an equal expression of the control marker GPIb throughout the samples. The changes in distribution of these populations in the presence of the TKIs were illustrated by 2D scatterplots that visualised the distribution of the populations (Figure 7.5Bi). Population 1 with low-level platelet activation (resting platelets), appeared to be significantly increased with fostamatinib, cabozantinib or vatalanib upon GPVI stimulation. On the other hand, population 5 with high activation of $\alpha_{IIb}\beta_3$ and high secretion of α-, δ-granules and lysosomes was decreased in the presence of these TKIs (Figure 7.5C), whereas no changes were observed in other specialised platelet populations. This approach demonstrated

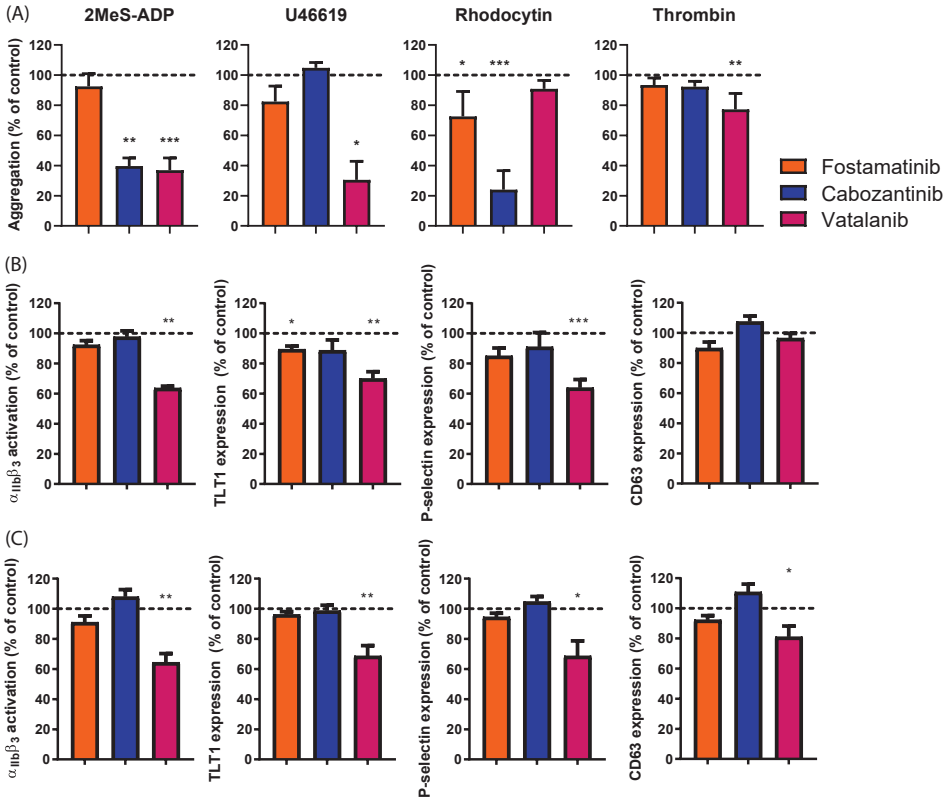


Figure 7.6. Platelet aggregation and activation induced by different activation pathways in presence of different TKIs. (A) Washed platelets ($250x10^9$/L) were incubated with vehicle (control), fostamatinib (33 μM), cabozantinib (33 μM) or vatalanib (33 μM) for 10 minutes. Platelet aggregation was then induced with 1 μM 2MeS-ADP, 1 μM U46619 (thromboxane A_2 analogue), 1 μg/mL rhodocytin or 4 nM thrombin. Aggregation responses were measured for 8 minutes. Histograms show aggregation as percentage of control (dotted line). (B-C) Washed platelets ($25x10^9$/L) were incubated with vehicle (control) or TKI for 10 minutes. Platelets were activated by (B) 2 μM 2MeS-ADP, or (C) 4 nM thrombin for 15 minutes. Simultaneous labelling was for activated integrin $\alpha_{IIb}\beta_3$ (PAC1-FITC), α-granule secretion (αTLT1-BV421, αCD62P-PerCP Cy5.5) and δ-granule/lysosome secretion (αCD63-APC). Histograms of integrin $\alpha_{IIb}\beta_3$ activation, TLT1, P-selectin and CD63 expression, as percentages of control condition (dotted line). Data are shown as means ± SEM (n = 6), * p<0.05. ** p<0.01, *** p<0.001.

the simultaneous inhibition of secretory and integrin-activating platelet responses by these TKIs.

In whole blood perfusion experiments, we observed inhibitory effects of fostamatinib on thrombus formation on vWF co-coated surfaces. To determine effects of fostamatinib, cabozantinib and vatalanib on platelet activation induced by other agonists, platelet aggregation was investigated in response to ADP, the thromboxane A_2 analogue U46619, the CLEC-2 agonist rhodocytin or thrombin. Fostamatinib only reduced rhodocytin-induced aggregation (Figure 7.6A), in agreement with Syk signalling underneath CLEC-2[26]. In contrast, cabozantinib significantly inhibited the aggregation responses to ADP and rhodocytin, while vatalanib prominently reduced the responses to ADP and U46619, and slightly to thrombin (Figure 7.6A). In line with these aggregation results, vatalanib suppressed ADP- and thrombin-induced integrin $\alpha_{IIb}\beta_3$ activation, TLT1 expression and P-selectin expression (Figure 7.6B, C). Upon thrombin stimulation also δ-granule and lysosome secretion were partially inhibited (Figure 7.6C). In contrast to the observed reduction in ADP-induced aggregation with cabozantinib, no inhibition of platelet activation was observed in response to ADP (Figure 7.6B, C). Taken together, this suggested that the latter two compounds inhibited signalling entities in pathways other than induced by GPVI.

Discussion

In this work we explored the antiplatelet properties of the TKIs dasatinib, fostamatinib, sunitinib, pazopanib, cabozantinib, axitinib, lapatinib, and vatalanib, to investigate the possible repurposing of these compounds as antiplatelet drugs. Overall, we observed that the TKIs with the highest reported affinities for targets in platelets (dasatinib, fostamatinib and sunitinib) at doses used showed the strongest inhibition on collagen-dependent whole blood thrombus formation under flow. Furthermore, fostamatinib was the most effective inhibitor of thrombus formation also on vWF plus rhodocytin, or vWF plus laminin. In the presence of other TKIs thrombus formation was no more than mildly reduced only on collagen. Intracellular calcium measurements and activation responses in isolated platelets showed that the inhibitory effects of dasatinib, fostamatinib, sunitinib and pazopanib were in line with proposed inhibitory effects on Syk and/or SFK, i.e., downstream of GPVI-induced platelet activation. At the doses tested, calcium measurements indicated that effects of the low-affinity inhibitors cabozantinib and vatalanib are downstream of phospholipase Cγ phosphorylation.

The present strong effects of the active metabolite of fostamatinib (R406) with high affinity for Syk on thrombus formation and PS exposure under flow are in line with a previous study using the selective Syk inhibitor PRT-060318[37]. However, our results obtained in isolated platelets are in contrast to other studies[26, 42]. These studies demonstrated significant inhibition of collagen- or rhodocytin-induced platelet aggregation with low concentrations of R406, whereas we observed only minor inhibition at higher concentrations. The difference may be due to longer incubation time of the platelets with the inhibitor in our study. As fostamatinib is a reversible

inhibitor, the incubation time could be of importance, however, we did observe strong inhibition in whole blood perfusion experiments using the same pre-incubation time. Furthermore, others have incubated with fostamatinib for even longer time-periods in platelets as well as other cell types, making transient inhibition unlikely[27, 43]. In addition, fostamatinib strongly inhibited GPVI-induced PS exposure in whole blood thrombus formation under flow, however, in isolated platelets this response was not affected. PS exposure is dependent on sustained high intracellular calcium levels[44], which we showed to be inhibited by fostamatinib. As PS exposure is triggered by dual stimulation of CRP and thrombin in our setting, this might overcome the partially decreased calcium signal induced by GPVI-stimulation in the presence of fostamatinib and thereby resulting in normal PS exposure.

Several of the investigated TKIs are used for treatment of metastatic renal cell carcinoma (mRCC) patients, i.e., sunitinib, pazopanib, cabozantinib and axitinib. Our results with sunitinib and pazopanib are consistent with a moderate inhibition of SFK, Syk and other relevant tyrosine kinases in platelets (Figure 7.1). For both compounds only mild bleeding effects have been reported[30]. Cabozantinib is used mainly as second- or third-line treatment for mRCC patients, and axitinib (combined with pembrolizumab) in first line mRCC, but their effects on platelets have not been investigated thus far. For cabozantinib, we report a reduced collagen-, CLEC-2- and ADP-induced aggregation in washed platelets, but no effect on thrombus formation. This is in line with no more than limited reported bleeding[30]. Axitinib at the dose of 33 µM used was essentially ineffective in most assays. We did observe limited effects on thrombus height and contraction in whole blood perfused over collagen. Notably, axitinib is described to induce mild (and in rare cases severe) thrombocytopenia in 15-20% of the patients[45-47], which points to a megakaryocytic rather than platelet function defect contributing to the reported bleeding[48, 49].

Lapatinib and vatalanib have been reported to have limited affinity for tyrosine kinases in platelets and remain without reported bleeding[24]. In agreement with Li et al.[50], lapatinib did not alter thrombus formation in whole blood perfusion, as well as intracellular calcium responses and aggregation in isolated platelets. Surprisingly, in the presence of vatalanib, we did observe inhibition of thrombus size, height, and contraction on collagen under flow at 33 µM, which is in contrast to the observations of Li et al., but the used concentration was not reported[50]. In addition, in isolated platelets vatalanib inhibited collagen-, ADP-, TxA_2- and thrombin-induced aggregation, as well as platelet activation. As GPVI-induced intracellular calcium rises were not affected by vatalanib, this suggested that vatalanib affects platelet signalling downstream of calcium. Vatalanib was developed to inhibit all VEGF receptors, and is mostly used for treatment of patients with colorectal cancer[51]. As no platelet tyrosine kinases are reported to be affected by this compound, further research is required to investigate the inhibitory mechanism in platelets.

In washed platelets, dasatinib, sunitinib, pazopanib, cabozantinib, and vatalanib prominently inhibited collagen-induced aggregation, while in PRP this response was unaffected

(cabozantinib and pazopanib) or required a higher concentration of the TKI to achieve inhibition (dasatinib, sunitinib and vatalanib). For pazopanib, this is in line with previous findings[28], and explained by high plasma binding[52-55]. Indeed, in patients with TKI treatment, the bioavailability of the drug is considered to be influenced by plasma binding with implications for drug toxicity and response[56]. In line with this, for the TKIs that did not or limitedly inhibited collagen-induced aggregation in PRP, we also observed no or only limited effects on thrombus formation under flow.

For the doses used of fostamatinib, cabozantinib and vatalanib we investigated effects on secretion and integrin activation in more detail. Most consistent effects were observed with fostamatinib, which moderately inhibited all GPVI-induced activation responses. Cabozantinib and vatalanib inhibited integrin $\alpha_{IIb}\beta_3$ activation along with aggregation. Interestingly, cabozantinib mildly suppressed δ-granule and lysosome secretion, whereas vatalanib reduced the α-granule secretion. The reason for this difference is unclear. Furthermore, we used two markers to measure of α-granule secretion, namely P-selectin (CD62P) and TLT-1. Although, in general we observed similar results using both markers with several agonists as well as inhibitors, it has been reported that TLT1 is more sensitive marker for platelet activation than P-selectin[57]. When a soluble TLT-1 is released in the plasma, it enhances platelet aggregation[58, 59] and has been demonstrated to regulate early clot formation though the stabilization of $\alpha_{IIb}\beta_3$ outside-in signalling[60]. Hence, this may provide an additional mechanism of the inhibitory effect of vatalanib.

We determined the distribution of the single platelet activation markers among different populations to further zoom in on the effects of fostamatinib, cabozantinib or vatalanib on GPVI-induced platelet activation. In general, in the presence of TKIs the population with highly expressed activation markers was decreased, whereas the population with negative expression of these markers was increased. This integrated approach suggested that pre-incubation with TKIs may affect the general responsiveness of the heterogeneous platelet populations[61].

In conclusion, we observed that fostamatinib was the most effective inhibitor of thrombus formation on collagen under flow, and that pazopanib, sunitinib, dasatinib, axitinib, and vatalanib also mildly reduced this process. Furthermore, the TKIs showed variable inhibiting effects in isolated platelets stimulated with several agonists. As especially fostamatinib, sunitinib, pazopanib, and vatalanib have been associated with mostly manageable or no bleeding events, these may be promising candidates to explore further as antiplatelet drugs. Clinicians should be aware of the antiplatelet properties of TKIs in general, especially when patients are already treated with antiplatelet or anticoagulant drugs.

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Author contributions

Conceptualization, MJEK, JWMH and PEJM; formal analysis, BMET, AV and DIF; investigation, BMET, AV and DIF; resources, JAE and MJBA; writing—original draft preparation, BMET and MJEK; writing—review and editing, BMET and MJEK; visualization BMET; supervision, MJEK, JWMH and PEJM; project administration, MJEK; funding acquisition, MJEK, JWMH and PEJM. All authors have read and agreed to the published version of the manuscript.

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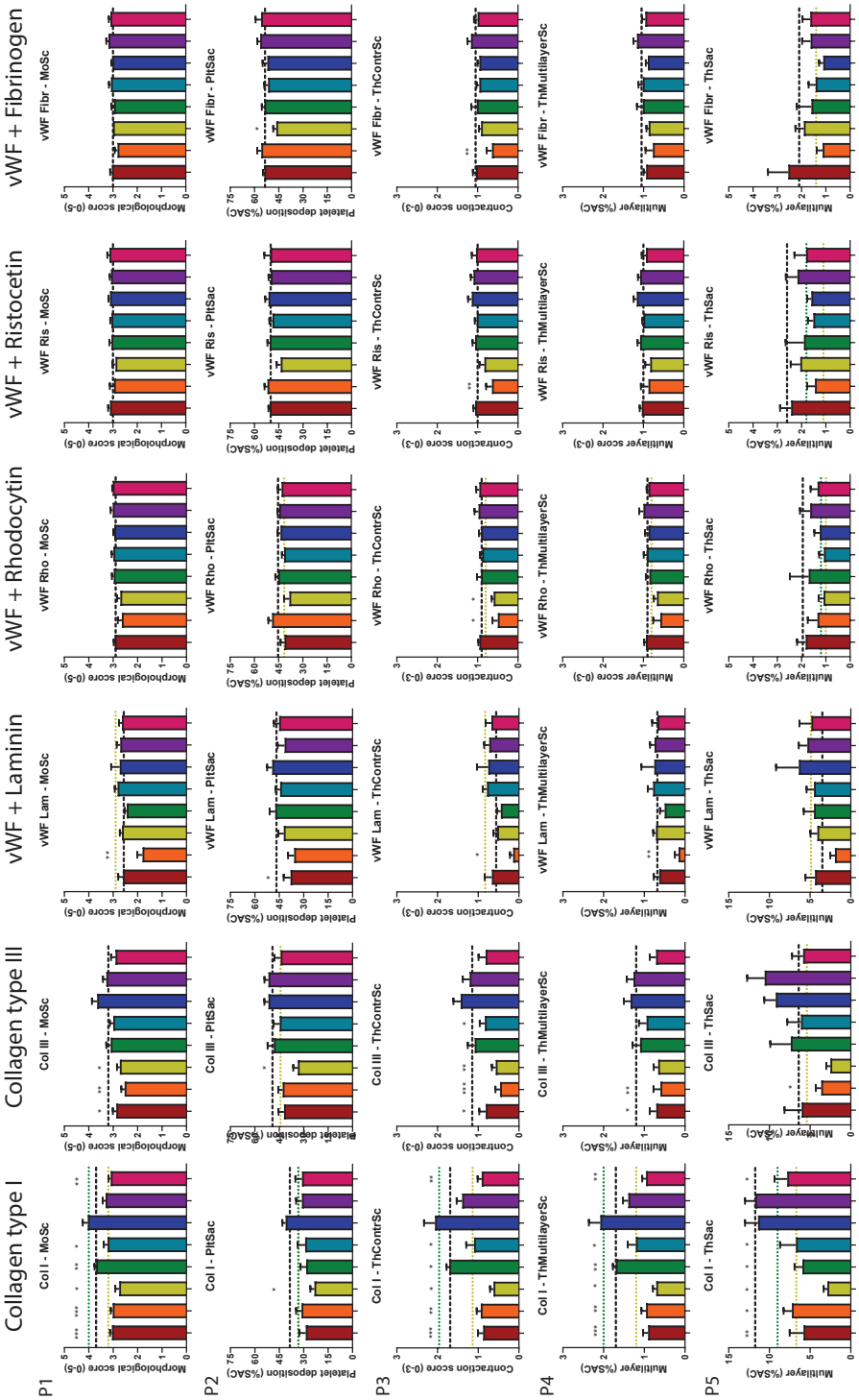
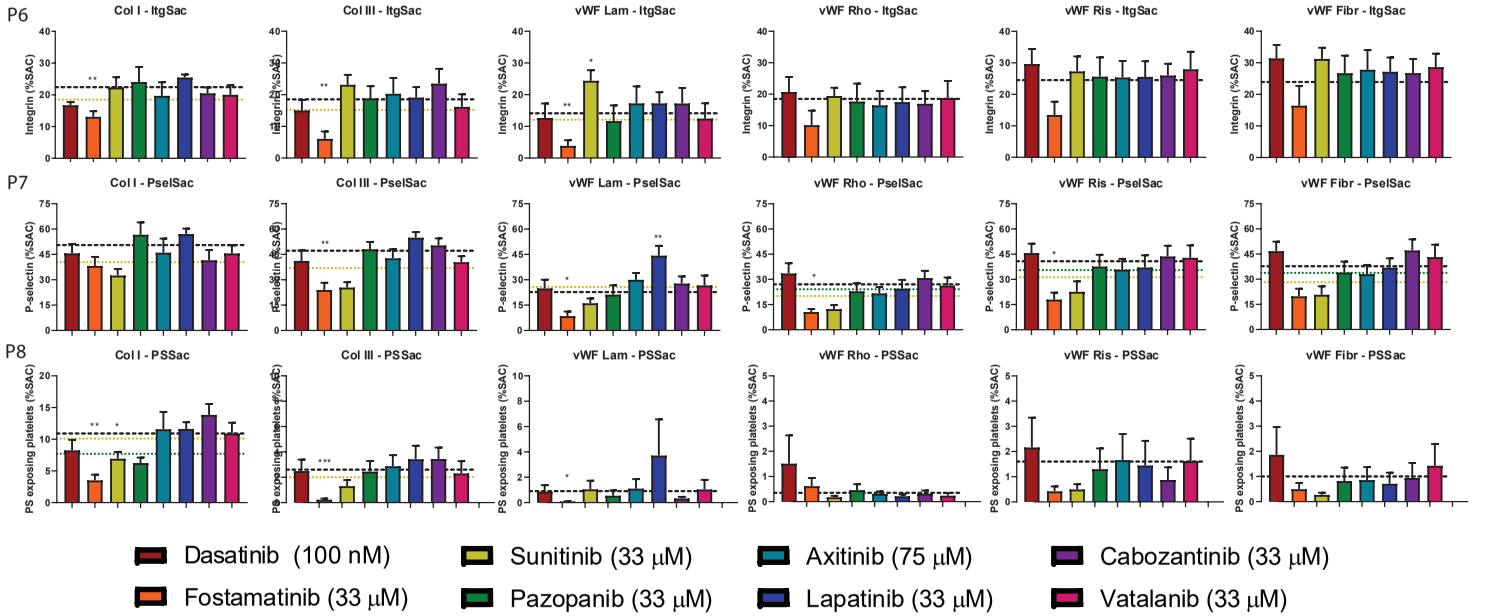
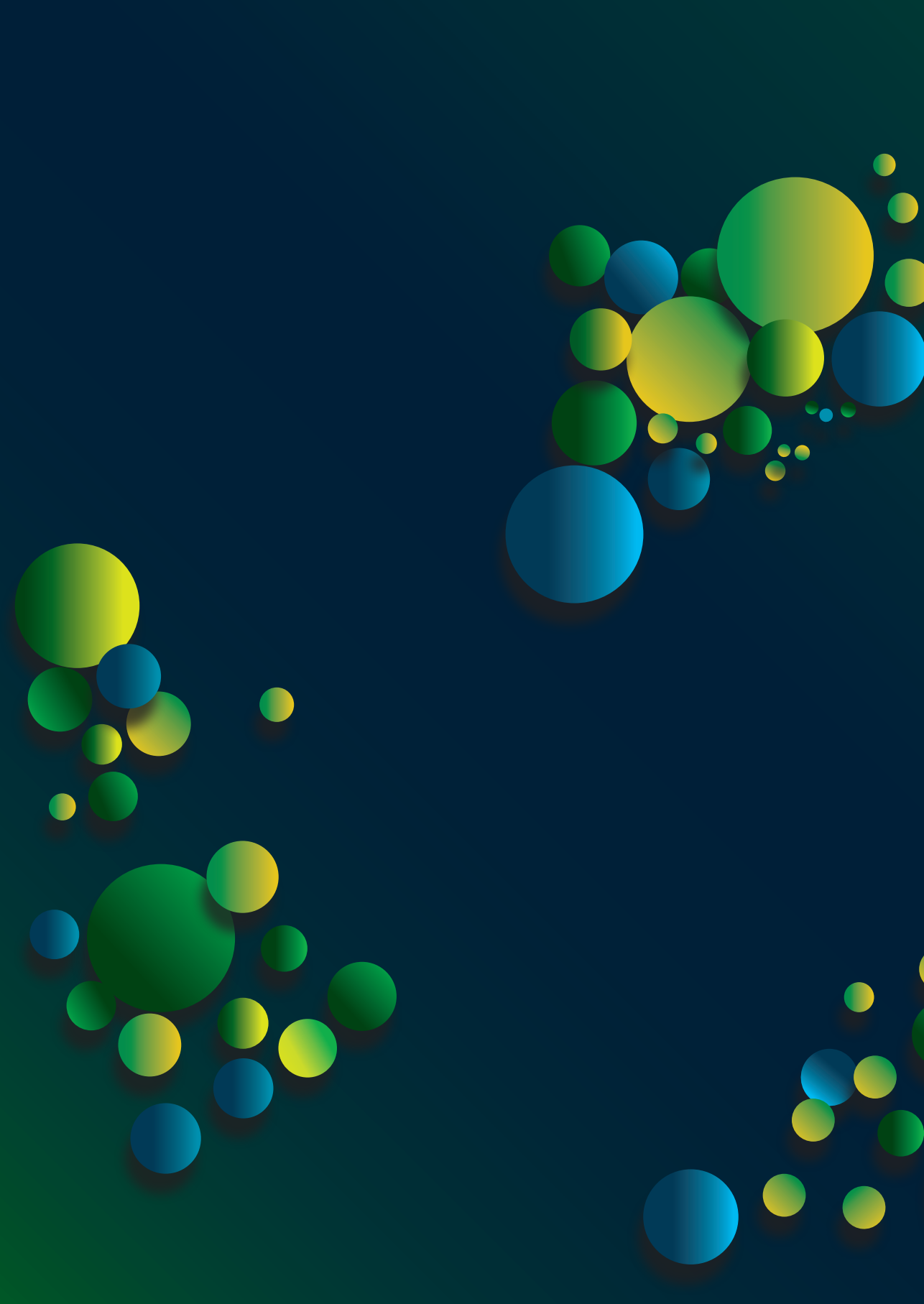
Supplemental data





Supplemental Figure 7.1. Thrombus formation under flow affected by eight TKIs over six different surfaces. Blood samples were pre-incubated with vehicle or TKI for 10 minutes at room temperature. Blood treated with indicated TKI was recalcified in the presence of PPACK, and perfused for 3.5 minutes at a wall shear rate of 1,000 s^{-1} over coated surfaces A-F. Thrombi were post-stained with AF568-annexin A5, FITC-α-fibrinogen and AF647-α-CD62P mAbs to detect PS exposure, integrin activation and P-selectin expression, respectively. Quantification of brightfield and fluorescence images of control and TKI-treated samples with the surfaces (A) collagen type I, (B) collagen type III, (C) vWF co-coated with laminin, (D) vWF co-coated with rhodocytin, (E) vWF co-coated with ristocetin and (F) vWF co-coated with fibrinogen. P1 = morphological score of platelet adhesion and thrombus formation, P2 = surface area coverage of adhered platelets, P3 = platelet aggregate contraction score, P4 = platelet aggregate multilayer score, P5 = coverage of multi-layered platelet aggregation, P6 = integrin $\alpha_{IIb}\beta_3$ activation, P7 = P-selectin expression and P8 = PS exposure. Data are shown as means ± SEM (n=6-7). Black dotted lines indicate average of control values (<0.1% DMSO); yellow or green dotted lines indicate average of control values (>0.1% DMSO) for sunitinib or pazopanib. * p<0.05



Chapter 8

General Discussion

Since the beginning of this century, a new class of anti-cancer agents, tyrosine kinase inhibitors (TKIs), has been developed and approved for the treatment of several malignancies[1, 2]. These TKIs inhibit growth factor receptors (tyrosine kinases, Table 8.1) involving angiogenesis and tumour development and thereby improving progression free survival (PFS) and overall survival (OS)[3]. As most TKIs inhibit multiple protein tyrosine kinases (Table 8.1), they inevitably also affect other cell types. One of the side effects described for several TKIs is (mild) bleeding or thrombosis[4]. Platelets are well known for their involvement in haemostasis, and they also contain multiple protein tyrosine kinases (Syk, Src family kinases (SFK), Btk), which are essential for their activation. Hence, these TKIs may also affect platelet function. Therefore, the overall aim of this thesis was to provide more insights in the off-target effects of TKIs on platelet function (Chapter 1). In the next paragraphs of this chapter, the observations described throughout this thesis are discussed in relation to the current literature, as well as clinical implications and future perspectives are given. Altogether, the findings reported in this thesis may provide relevant insights for monitoring the bleeding risk in patients receiving TKI treatment by assessing quantitative and qualitive platelet traits, especially in the presence of anti-coagulant or anti-platelet drugs.

Platelet signalling and TKIs

It has been described that several TKIs, for different cancer types, inhibit platelet function upon treatment, which can contribute to the risk of bleeding[5-7]. Chapter 2 shows an overview

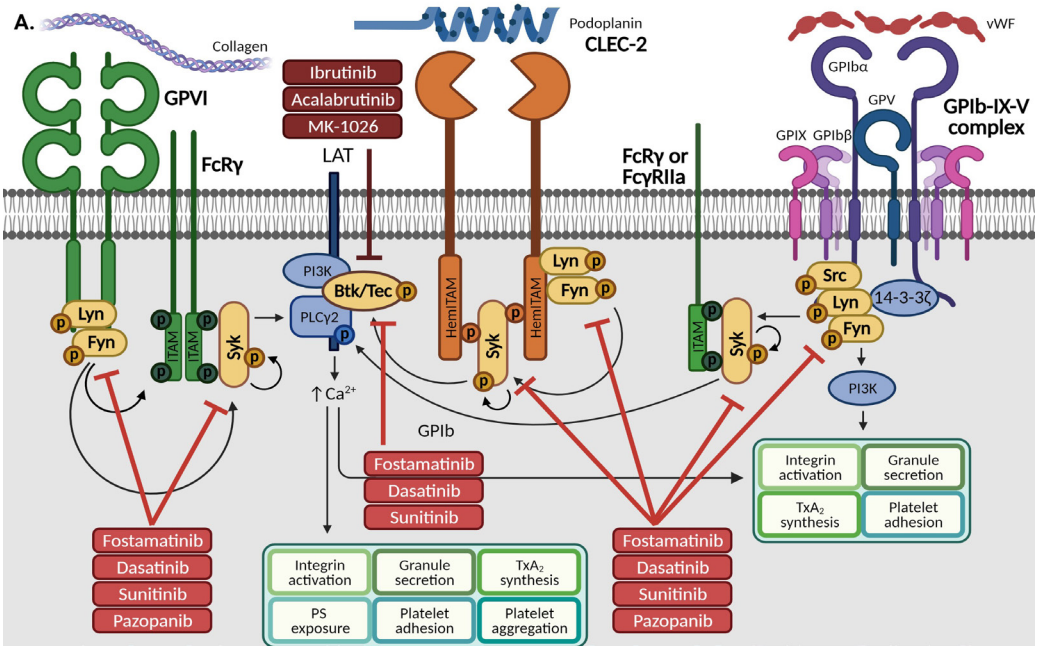


Figure 8.1. Overview of the tyrosine kinases expressed in platelet signalling (in yellow) downstream of multiple platelet receptors, resulting in different platelet responses. The intervention points of the TKIs (based on the Kd values described in Chapter 2) investigated in this thesis are indicated in red.

of the currently used TKIs in cancer treatment and the effects on platelet function. Throughout the Chapters 3-7, we used in total 11 TKIs (acalabrutinib, axitinib, cabozantinib, dasatinib, fostamatinib, ibrutinib, lapatinib, MK-1026, pazopanib, sunitinib and vatalanib) to explore the effects on platelet function *in vitro* and in cancer patients. Table 8.1 gives an overview of the anti-platelet effects from different assays, that were observed after *in vitro* treatment with these 11 TKIs. Overall, the majority of inhibited responses were demonstrated to affect the GPVI-pathway, and for several TKIs (ibrutinib and fostamatinib), (also) effects upon CLEC-2 or GPIb stimulation

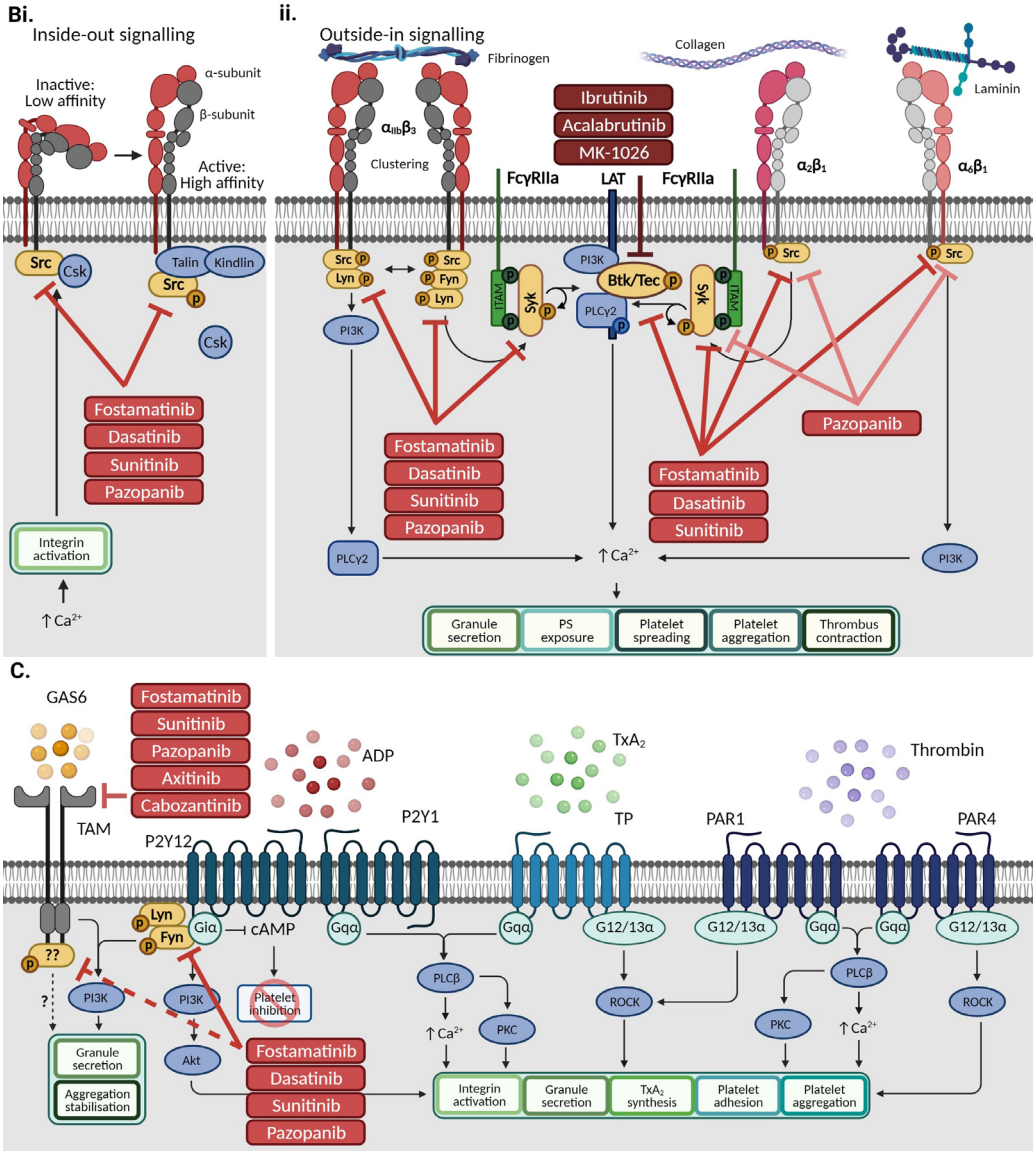


Figure 8.1. Continued. The intervention points of the TKIs (based on the Kd values described in Chapter 2) investigated in this thesis are indicated in red.

could be observed. Given the defined affinities in our review in combination with the results obtained for the 11 TKIs tested here, we visualised the proposed interference of these TKIs in the platelet signalling pathways (Figure 8.1).

In whole blood perfusion, effects on thrombus formation were mainly restricted to the glycoprotein (GP)VI-pathway as observed for dasatinib, sunitinib, pazopanib and vatalanib. Only ibrutinib and fostamatinib showed to be effective towards GPIb (combined with CLEC-2 or $\alpha_6\beta_1$)-induced thrombus formation (Table 8.1, Chapter 3, 4, 6 and 7). Ibrutinib and fostamatinib specifically inhibit Btk/Tec or Syk, respectively. Both proteins are important mediators in coupling cell surface receptors to downstream signalling events and are in close proximity in most signalling pathways[8, 9]. This suggests that direct targeting of Btk or Syk in platelet signalling by TKIs is more effective to inhibit platelet function as compared to TKIs primary targeting growth factor receptors. Also, a difference can be observed between inhibition of Btk and Syk. Btk inhibition by ibrutinib mainly inhibits GPIb-associated thrombus formation, whereas Syk inhibition with fostamatinib shows to affect thrombus formation with GPIb, as well as GPVI. As binding and activation of Syk initiates downstream signalling involving Btk in GPVI, CLEC-2 and GPIb-signalling, this hints towards the concept that inhibition higher upstream in platelet signalling results in more efficient inhibition.

In isolated platelets almost all inhibitors more prominently inhibited GPVI-induced aggregation, except fostamatinib, axitinib and lapatinib. For fostamatinib this contrasts to the papers of Spalton et al. and Lhermusier et al.[10, 11], who observed complete inhibition of collagen- and/or rhodocytin-induced platelet aggregation with already low concentrations of the active metabolite of fostamatinib. The only difference between these studies and the study in chapter 7, is the incubation time of the platelets with the inhibitor. We pre-incubated for 10 minutes, while in the earlier studies platelets were incubated for 1-5 minutes with fostamatinib. As fostamatinib is a reversible inhibitor, the incubation time could be of importance, however, we did observe strong inhibition in whole blood perfusion experiments using 10 minutes pre-incubation of whole blood.

The lack of GPVI-induced aggregation for axitinib and lapatinib is in line with literature findings. Although axitinib is associated with bleeding[7, 12], no inhibition of platelet function has been reported. In about 15-20% of the patients, axitinib is described to induce mild (grade 1-2) thrombocytopenia[13, 14], and in rare cases (<1%) severe (≥ grade 3) thrombocytopenia is reported[15]. For axitinib it is proposed that increased bleeding in these patients is caused by a decrease in platelet count rather than platelet inhibition. For lapatinib increased bleeding risk has not been reported and, furthermore, thrombus formation under flow (*in vitro*) was also unaffected[16]. Hence, these observations show that axitinib and lapatinib do not have off-target effects on platelets.

All other TKIs investigated in this thesis (acalabrutinib, cabozantinib, dasatinib, MK-1026, pazopanib, sunitinib and vatalanib) show most pronounced effects in platelets upon collagen stimulation. Inhibition in thrombus formation under flow was restricted to GPVI-induced

activation for pazopanib, sunitinib, dasatinib and vatalanib. This suggests that the GPVI pathway is most sensitive for inhibition with TKIs. GPVI, GPIb-IX-V and CLEC-2 are most potent receptors for thrombus formation at high-shear stress conditions[17]. They share several common tyrosine kinases (Fyn, Lyn, Syk, Btk, etc) in signal transduction in platelets[17, 18]. As TKIs are able to affect multiple of these tyrosine kinases, it is expected that more pathways should be inhibited. However, a possible explanation for the restriction of effects to the GPVI pathway could be the amount of copy numbers present in platelets. Of these receptors, GPIIbIIa (integrin $\alpha_{IIb}\beta_3$) and GPIb are the most abundantly expressed receptor on platelets with around 80,000 and 25,000 copies, respectively[19-21]. GPVI and CLEC-2 receptors are far less expressed with only 6,000-10,000 and 2,000 copies reported, respectively[19, 22, 23]. Therefore, it could be expected that GPVI and CLEC-2 are relatively more affected as compared to GPIb as less receptors (lower copy number) remain active. Signalling pathways of receptors with a higher expression, such as GPIIbIIa and GPIb, might still be partially functional as more receptors are available, making it more difficult to observe inhibition. Moreover, it is indicated by Spalton et al.[10] that GPVI is more dependent on Src family kinase (SFK) phosphorylation and FcγR signalling, whereas the CLEC-2 pathway is more reliant on Syk phosphorylation. Together this encourages that TKIs have probably a higher affinity for SFKs rather that Syk, resulting in more specific inhibition of the GPVI pathway.

Plasma protein binding of TKIs influencing drug availability and platelet inhibition

In (almost) all chapters of this thesis inhibition of platelet traits, qualitative and/or quantitative, by different TKIs is the central point of view. Although *in vitro* effects were present in almost all conditions tested, inhibition by TKIs was more pronounced in isolated platelets as compared to the presence of plasma (platelet-rich plasma or whole blood) (Table 8.1). This meant that a higher dose of a TKI was often needed to elicit similar inhibition in PRP or whole blood as compared to washed platelets. Strikingly, pazopanib and cabozantinib prominently inhibited aggregation of washed platelets to several agonists, while in PRP this response was unaffected (Table 8.1). This phenomenon is caused by high plasma binding of the TKIs (>95%)[24-27]. In patients with TKI treatment, the bioavailability of the drug is likely to be influenced by this plasma binding with implications for drug toxicity and response[28]. This shows the importance of the comparison between *in vitro* testing of these agents in presence or absence of plasma, as well as measuring effects present in patients receiving treatment. For clinical implications it is might point to adapting dosing regimens based on the pharmacokinetic/dynamic characteristics of TKIs[29] or more ideally by monitoring the unbound plasma concentration of a TKI during treatment[30]. For example, we measured levels of sunitinib and its metabolite in plasma, serum and isolated platelets at 2 and 4 weeks on treatment in patients with a 4/2 scheme (4 weeks dosing, 2 weeks rest) in Chapter 5. We showed that in plasma and serum, the unbound concentrations were comparable for

Table 8.1. In vitro effects of different multi-target TKIs on platelet function in blood from healthy volunteers

Chapter	TKI	Targets	Effects on platelets: Without presence of plasma: Signalling (% change)	Without presence of plasma: Activation (% change)	Without presence of plasma: Aggregation (% change)	With presence of plasma: Aggregation (% change)	With presence of plasma: Thrombus formation under flow (% change)
3 & 7	Pazopanib	VEGFR PDGFR FGFR FMS Itk, Kit Lek	↓ $[Ca^{2+}]_i$ peak level • GPVI (>70%) ↓ Tyrosine (Syk) phosphorylation • GPVI (80%)	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (70%) ↓ Granule secretion • GPVI (70%) • $P2Y_{12}$ (50%) ↓ PS exposure • GPVI + PAR (55%)	↓ Aggregation • GPVI (90%) • $P2Y_{12}$ (70%) • TP (65%)	No effect	↓ Platelet adhesion •GPVI (20%) ↓ Thrombus height & size •GPVI (30%) ↓ PS exposure •GPVI (20%)
4 & 7	Sunitinib	VEGFR PDGFR CSF-R Flt3 Kit	↓ $[Ca^{2+}]_i$ peak level • GPVI (80%) ↓ Tyrosine (4G10) phosphorylation • GPVI (75%)[77] ↓ PAM-kinase assay phosphorylation (Src, Syk, Fyn, Yes) • GPVI	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (80%) • CLEC-2 (70%) • $P2Y_{12}$ (20%) ↓ Granule secretion • GPVI (80%) • CLEC-2 (90%) ↓ PS exposure • GPVI + PAR (40%)	↓ Aggregation •GPVI (>95%) •$P2Y_{12}$ (50%)	↓ Aggregation • GPVI (60%)	↓ Adhesion & thrombus formation • GPVI (30-50%) ↓ Granule secretion • GPVI (30%) ↓ PS exposure • GPVI (35%) ↑ Time to fibrin (2 min)
6	Ibrutinib	Btk Tec	↓ $[Ca^{2+}]_i$ slope • GPVI (50%)	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (90%) • $P2Y_{12}$ (20%) ↓ Granule secretion • GPVI (85%) • $P2Y_{12}$ (50%) ↓ PS exposure • GPVI + PAR (70%)	↓ Aggregation •GPVI (95%) •CLEC-2 (>95%)	↓ Aggregation • GPVI (60%)	↓ Adhesion & thrombus formation • GPIb ± CLEC-2/$\alpha_6\beta_1$/$\alpha_{IIb}\beta_3$ (20-50%) ↓ Granule secretion • GPIb ± CLEC-2/$\alpha_6\beta_1$/$\alpha_{IIb}\beta_3$ (>50%) ↓ PS exposure • GPVI (65%) • GPIb ± CLEC-2 (>75%)
	Acalabrutinib	Btk Tec	No effect ($[Ca^{2+}]_i$)	↓ Granule secretion • GPVI (30%)	↓ Aggregation •GPVI (90%) •CLEC-2 (90%)	↓ Aggregation • GPVI (20%)	No effect
	MK-1026	Btk Tec	No effect ($[Ca^{2+}]_i$)	No effect	↓ Aggregation • GPVI (90%) • CLEC-2 (>95%) • $P2Y_{12}$ (40%)	↓ Aggregation •GPVI (50%)	No effect

Chapter	TKI	Targets	Effects on platelets				
			Without presence of plasma			With presence of plasma	
			Signalling (% change)	Activation (% change)	Aggregation (% change)	Aggregation (% change)	Thrombus formation under flow (% change)
7	Dasatinib	PDGFR BCR-ABL EphA2 Kit SFK	↓ $[Ca^{2+}]_i$ peak level • GPVI (>90%)	-	↓ Aggregation • GPVI (>95%)	↓ Aggregation • GPVI (50%)	↓ Thrombus height & size • GPVI (35-55%) ↓ $\alpha_{IIb}\beta_3$ activation • GPVI (15%)
	Fostamatinib	Syk	↓ $[Ca^{2+}]_i$ peak level • GPVI (55%) • GPVI (55%)	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (20%) ↓ Granule secretion • GPVI (20-30%)	↓ Aggregation • GPVI (20%)	↓ Aggregation • GPVI (20%)	↓ All parameters (except adhesion) • GPVI (15-70%) • GPIb + $\alpha_6\beta_1$ (30-90%) ↓ Thrombus contraction • GPIb ± CLEC-2/$\alpha_{IIb}\beta_3$ (30-45%) ↓ Granule secretion • GPIb ± CLEC-2/$\alpha_{IIb}\beta_3$ (50-65%)
	Axitinib	VEGFR	No effect ($[Ca^{2+}]_i$)	-	↓ Aggregation • GPVI (15%)	No effect	No effect
	Cabozantinib	VEGFR2 Axl Flt3 Kit MET Ret	No effect ($[Ca^{2+}]_i$)	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (20%) ↓ Lysosome secretion • GPVI (20%)	↓ Aggregation • GPVI (60%) • CLEC-2 (80%) • $P2Y_{12}$ (60%)	No effect	No effect
	Lapatinib	EGFR ErbB1-2	No effect ($[Ca^{2+}]_i$)	-	↓ Aggregation • GPVI (15%)	No effect	No effect
	Vatalanib	VEGFR PDGFRb Kit	No effect ($[Ca^{2+}]_i$)	↓ $\alpha_{IIb}\beta_3$ activation • GPVI (20%) ↓ Granule secretion • GPVI (20%)	↓ Aggregation • GPVI (80%) • $P2Y_{12}$ (60%) • TP (70%) • PAR1/4 (30%)	↓ Aggregation • GPVI (20%)	↓ Adhesion & aggregation • GPVI

SFK: Src family kinases; TKI: Tyrosine kinase inhibitor

sunitinib and its metabolite at both timepoints, however, a remarkable increase of metabolite concentration was observed in isolated platelets as compared to sunitinib concentration. This suggested a higher uptake of the active form of sunitinib by platelets in their granules, though both compounds were equally present in plasma. Therefore, it might also be of interest to not only monitor plasma concentrations at 2 of 4 weeks on treatment (dependent on treatment schedule)[31], but also measure the uptake of (active) compound by platelets as this might predict the platelet inhibitory effects.

Involvement of quantitative and quantitative platelet traits in bleeding tendency in patients

In the review in Chapter 2, 11 out of almost 40 TKIs are reported with an increased risk of bleeding or bleeding events. Given the key role of platelets in haemostasis, both platelet count and platelet function are relevant to prevent bleeding[32, 33]. In Chapters 3 and 5, we have shown that not only platelet function, but also platelet count is already decreased after 2 weeks after pazopanib or sunitinib treatment, as compared to pre-treatment (Table 8.2). Although TKIs are known to be associated with platelet dysfunction[6, 34], impaired platelet function is not always found to be the cause of increased bleeding tendency[35, 36]. For multiple TKIs, including dasatinib (Chapter 7), an association is found with drug-induced immune thrombocytopenia, defined as a platelet count below $50x10^9/L$[37-39], resulting in an increased risk of bleeding. While most of the patients included in Chapters 3 and 5 did not show thrombocytopenia, we did observe an overall decrease in platelet count upon pazopanib or sunitinib treatment (Table 8.2). This change in platelet count correlated with the inhibition of platelet function after sunitinib treatment, as well as with the plasma concentration of the metabolite of sunitinib (Chapter 5). This point to the idea that platelets are exposed to significant levels of sunitinib resulting in (mild) anti-platelet effects. Alternatively, sunitinib may act on the megakaryocytes, thereby decreasing platelet count and possibly also affect platelet function. As plasma concentration and plasma protein binding of sunitinib (or its metabolite) could be of importance in toxicity and response to a TKI treatment[30], this pointed toward a monitoring tool for off-target effects and/or progression-free survival.

Whilst several studies propose that TKI-associated platelet dysfunction does not cause bleeding[35, 36], almost all the patients included in Chapter 3, 5 and 6 did report (mild) bleeding episodes upon TKI treatment in the absence of thrombocytopenia (Table 8.2). This may imply that pharmacological platelet inhibition does play a role in the increase in bleeding risk. In agreement, we see impairment of GPVI-induced platelet activation, aggregation or thrombus formation upon treatment *in vitro* and/or in patients, some with additional effects on CLEC-2- or GPIb-induced platelet responses. It has been reported that targeting GPVI via GPVI inhibitors or GPVI depletion in mice or patients does not (significantly) impair haemostasis[40-45]. However, a combined deficiency of GPVI and CLEC-2 in mice does show impaired haemostasis[43]. These

Table 8.2. Overview of the effects of TKI treatment in patients on quantitative and qualitative platelet traits and reported bleeding during treatment period as compared to a control group

Chapter	Patients	TKI treatment	Dosage (mg/day)	Timepoint (weeks)	Effects on platelets				Bleeding (Yes/No)
					Quantitative	Qualitative			
					(% change)	Activation (% change)	Aggregation (% change)	Thrombus formation under flow (% change)	
3	RCC (n=10)	Pazopanib	800	2	↓ Platelet count (15%)	↓ PS exposure • GPVI (20%)	No effect	↓ PS exposure • GPVI (50%)	Yes (3/10)
5	RCC (n=20) and (n=8)[77]	Sunitinib	50	2	↓ Platelet count (40%), (30%)[77]	↓ GPVI-induced $\alpha_{IIb}\beta_3$ Activation (15%)[77] ↓ GPVI-induced granule secretion (15-50%)[77]	↓ GPVI-induced aggregation (10%) and (40%)[77]	↓ GPVI-induced platelet adhesion (20%)[77] ↓ GPVI-induced PS exposure (75%)[77]	Yes (9/20) and (4/8)[77]
				4	↓ Platelet count (50%)	-	↓ GPVI-induced aggregation (15%)	-	
				12	↓ Platelet count (25%)	-	No effect	-	
6	CLL, MCL, WM (n=16)	Ibrutinib (n=9)	420-560	-	↑ Platelet volume (20%)	↓ GPVI-induced $\alpha_{IIb}\beta_3$ activation (65%) ↓ GPVI-induced granule secretion (85%) ↑ $P2Y_{12}$-induced $\alpha_{IIb}\beta_3$ activation (35%)	No effect	↓ GPVI-induced thrombus height & density (30%) ↓ $\alpha_{IIb}\beta_3$ activation (>50% for GPVI, vWF-CLEC-2, and vWF-$\alpha_6\beta_1$ surface) ↓ PS exposure (20% for GPVI surface)	Yes (6/9)

CLL: Chronic lymphocytic leukaemia; MCL: Mantle cell lymphoma; RCC: Renal cell carcinoma; TKI: Tyrosine kinase inhibitor; WM: Waldenstrom macroglobulinemia

studies mainly determined the contribution of platelet GPVI- and CLEC-2 receptors themselves in haemostasis[40, 43]. However, in case of several TKIs, the inhibition in platelet signalling underneath these receptors is described to be via SFKs (Fyn, Lyn) or Syk[11, 46-49]. As the GPVI, CLEC-2 and GPIb pathways all require SFKs and/or Syk in its signalling, it overall suggests that multi-target TKIs can elicit combined inhibition of GPVI plus CLEC-2 or possibly GPIb signalling pathways. This could be more potent than targeting solely one receptor and, hence, relevant for bleeding tendency. In combination with a decrease in platelet count, this might even further increase the bleeding risk.

As cancer patients often have a cardiovascular history[50, 51], anti-platelet or anti-coagulant drugs are prescribed to prevent recurrent cardiovascular events. Anti-platelet therapies, such as clopidogrel and aspirin, are reported to be associated with an increased risk of bleeding[52-54]. As TKIs are described to increase this risk as well, in Chapter 5, we investigated the synergistic effects of sunitinib and aspirin as anti-platelet medication on thrombus formation and fibrin formation to elucidate the impact on haemostasis. We demonstrated that sunitinib suppressed procoagulant activity and prolonged the time to fibrin formation, which was aggravated by aspirin treatment. Therefore, a combination of TKIs and anti-platelet therapies may be expected to increase the frequency or grade of bleeding. Though such dual treatment of TKI with anti-platelet or anti-coagulant drugs could be increasing the bleeding risk, this might also be beneficial for the patients. It has been shown that anti-platelet therapies have anti-tumour effects in hepatocellular and colorectal cancer[55, 56]. Hence a combination of TKI with anti-platelet therapy could enhance the anti-tumour effects. For example, erlotinib together with aspirin is described to enhance sensitivity of the cancer as compared to aspirin alone, thereby increasing the anti-cancer activity[57]. The application of anti-platelet drugs is even more favourable, as it was shown that low doses of aspirin could be used as treatment for off-target effects, such as skin rash caused by erlotinib-treatment[58]. Additionally, for some TKIs, anti-platelet treatments can be useful as nilotinib is associated with an increased risk of thrombosis[59]. Yet, not all anti-platelet drugs show a positive correlation in combination with TKI treatment. The study of Haoula et al.[60] highlights the importance of drug-drug interactions between clopidogrel and other anticoagulants in combination with TKIs. This urges for better understanding of dual treatment with TKIs and anti-platelet or anti-coagulant therapies to monitor patients.

Overall, we showed that multiple TKIs inhibit platelet function via different pathways and that this combination may contribute to the reported bleeding risk in patients. In addition, for other TKIs inhibiting the GPVI-induced platelet function together with a decrease in platelet count, as seen with pazopanib and sunitinib, might also explain the mild bleeding tendency observed during treatment. Hence, the combination of the inhibitory effects on platelet traits together with anti-platelet or anti-coagulant drugs urges for better understanding and possibly more strict monitoring of patients as this may further increase the risk of bleeding.

Clinical implications

Platelets not only have an important role in haemostasis, they have also been described to be implicated in tumour development and growth[61-64]. Platelets are known to take up various pro- and antiangiogenic proteins into their α-granules[61, 62, 65]. These granules can be secreted upon platelet activation by activating factors expressed or secreted by tumour vasculature such as thrombin and ADP. In turn, the secreted proangiogenic factors can stimulate tumour growth and metastasis by stimulating angiogenesis[61, 66], improving vasculature integrity[67, 68] and inducing resistance to therapy[69, 70]. Due to this promoting role of platelets in tumour angiogenesis and progression, as well as the correlation with reduced survival, platelets are an interesting target for cancer therapy. Although numerous drugs have been developed to target platelet receptors[71], limitations for implications occur due the role of platelets in haemostasis associated with increased bleeding tendency[52-54]. Yet anti-platelet treatment have been described to reduce tumour angiogenesis, growth and progression[72, 73] by direct inhibition of the tumour or via indirect via inhibition of platelet secretion, thereby decreasing proangiogenic protein concentrations in the tumour environment[74]. Hence, the TKIs investigated in this thesis could also indirectly affect tumour growth via the inhibiting effects on platelet function, as we did observe inhibition of platelet activation and/or secretion upon GPVI stimulation with acalabrutinib, cabozantinib, fostamatinib, ibrutinib, pazopanib, sunitinib and vatalanib in isolated platelets. However, the principle of platelet-mediated anti-cancer effect of TKIs needs to be further investigated. Platelet inhibition remains contradictory and needs to be balanced, as it can improve cancer prognosis, as well as increase the risk of bleeding. In line with the inhibition of platelet function with TKIs observed throughout this thesis, one might argue that monitoring patients is needed to profit from the anti-tumour effects and keep the risk of undesirable bleeding to a minimum.

In addition, platelet production can be (greatly) enhanced by malignant (solid) tumours[62, 75]. An increased platelet count or thrombocytosis (platelet count of >400x10^9/L) is recognized as a predictor of poor prognosis in patients with different cancer types[75, 76]. As platelets are able to take up all sorts of compounds into their granules, it is not unlikely for anti-cancer agents to be sequestered as well. The study of Sabrkhany et al.[77] showed that platelets take up sunitinib in their granules *in vitro* and in patients. In turn, we showed in Chapter 5 that both sunitinib and its metabolite were present in isolated platelet of sunitinib-treated patients and that platelet function is impaired. Besides high plasma binding of anti-cancer or anti-platelet agents, the ability of platelets to sequester these compounds might influence the bioavailability for the tumour, especially in thrombocytosis, i.e. increased amount of platelets. This might result in reduced inhibition of tumour growth as a lower concentration would be circulating in the blood. Also, thrombocytosis might not only be a consequence of cancer, it might be a paraneoplastic abnormality that is associated with a vicious cycle of promoting tumour cells, platelet activation and stimulating thrombocytosis[75]. Therefore, reducing the thrombocytosis before starting

treatment by interfering with this feedback-loop could be relevant. This could be accomplished via inhibition of platelet function with anti-platelet agents or heparins, however this could result in bleeding implications[78, 79]. Another approach to intervene with tumour growth and metastasis is inhibition of interleukin-6 (IL-6) by the IL-6 antibody siltuximab. This will deplete the increased growth factor levels present and additionally decrease platelet count[80]. Clinical trials with ovarian cancer, renal cell carcinoma and castration resistant prostate cancer already show beneficial effects[80-82].

Clinical significance for renal cell carcinoma patients

The TKIs used for treatment of patients with metastatic renal cell carcinoma (mRCC), i.e. sunitinib and pazopanib, have been the focus of Chapters 3, 4 and 5 of this thesis. Furthermore, we also studied the effects of cabozantinib, used mainly as 2nd or 3rd line treatment for mRCC patients, and axitinib, combined with pembrolizumab in first line mRCC[83, 84], on platelet functions in Chapter 7. In the Netherlands, good risk mRCC patients are treated with a TKI when metastases have occurred. Patients often have a large tumour thrombus in the renal vein, which means that local surgery is no longer possible. However, this is usually no consideration to start anticoagulation therapy. When started with 50 mg/day sunitinib, systemic concentrations of sunitinib should be 50-100 ng/mL[85]. However, for a substantial group of patients, this dosing schedule may result in much higher peak levels, resulting in toxicity[86]. In daily practice, sunitinib levels (steady state) in serum could be determined if patients experience serious side effects and if the levels are too high (>100 ng/mL[31]), the treatment dose could be adjusted accordingly. In addition, sunitinib levels could also be determined if patients have no side effects at all, as the sunitinib level is probably too low (<50 ng/mL[31]).

Hamilton et al.[87] have shown that the decrease in platelet count upon sunitinib treatment may serve as a prognostic value with regard to tumour development. In Chapter 5, we have demonstrated that this reduction in platelet count also coincides with a reduction in platelet function as well as sunitinib levels in plasma and serum. Together, this implies that both qualitative and quantitative platelet traits may serve as a monitoring tool with regards to sunitinib treatment in mRCC patients. Furthermore, similar observations have been reported for neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte (PLR) ratio. A NLR greater than 1.9 but below 3 and PLR over 160 are described to be favourable prognostic factors[88, 89]. Also, an increase in eosinophils have been reported to be a beneficial factor upon therapy in patients with renal cell carcinoma upon treatment[90, 91].

Also relevant for mRCC, although no longer used, is the classification of metastatic disease into groups with regards to overall survival, according to the Memorial Sloan-Kettering Cancer Centre (MSKCC)/Motzer citeria[92]; good, intermediate and poor risk. Currently, a switch has been made to the International Metastatic RCC Database Consortium (IMDC) criteria to make group

classification. For this purpose, 6 parts of pre-treatment are scored, with one point per item, looking back one year before starting systemic treatment: Time from diagnosis to treatment initiation <1 year, Karnofsky performance status (KPS) <80%, haemoglobin (Hb) concentration <12 g/L, serum calcium concentration >upper limit of normal (ULN), neutrophil count >ULN and platelets count> ULN[93]. With no points, the patient has a good risk for survival, at 1-2 points intermediate risk and from 3 on the risk is poor. For the good risk patients, it appeared that sunitinib was superior to the immuno-agents nivolumab/ipilimumab, and was equivalent to pembrolizumab plus axitinib[94, 95]. Therefore, sunitinib has become a mainstay not only for first-line treatment in this particular risk group, but also in second line treatment after nivolumab/ipilimumab. The results of the present thesis with regard to the effects of sunitinib on platelet count and function, are thus in agreement with the incorporation of platelets into this IMDC classification.

Future perspectives

To summarise, this thesis increased the knowledge of how TKIs can influence platelet function and contribute to reported bleeding events. In brief, *in vitro* experiments throughout this thesis demonstrate that impaired platelet function is more pronounced in isolated platelets as compared to whole blood perfusion, mainly affecting GPVI-induced platelet activation in presence of plasma. Only ibrutinib and fostamatinib showed (additional) inhibition of CLEC-2 and/or GPIb-induced platelet activation and aggregation. In addition, the observed suppression of aggregation and thrombus formation with sunitinib was aggravated by anti-platelet (aspirin) therapy. Our studies involving patients demonstrated that pazopanib or sunitinib treatment slightly decreased platelet count together with (limited) effects on the qualitative platelet traits, whereas for patients treated with ibrutinib impaired collagen-, rhodocytin- and vWF-induced aggregation and thrombus formation was observed. Moreover, we showed that for sunitinib the inhibition in quantitative and qualitative platelet traits could be correlated with the (effective) sunitinib levels in patients. Altogether, the inhibition of multiple platelet activation pathways, the reduction in platelet count, or TKI treatment combined with antiplatelet agents might contribute or even increase the reported bleeding tendencies of these TKIs. Therefore, patients should be monitored by measuring platelet function or (active) compound levels in serum, plasma or isolated platelets 2-4 weeks after start treatment or additionally in case of changes like dose reduction, reoccurrence of side effects or altering treatment schedule. Though, more research is needed to determine effects on sunitinib concentration in long term and whether possible effects extinguish over time, as our study in Chapter 6 is limited to follow-up period of maximal 12 weeks and limited in patient numbers due to dose reduction or discontinuation of medication (upon decease).

To further develop the findings of this thesis towards better and improved patient care,

more research is needed. Our patient study with sunitinib showed the correlation between quantitative and qualitative platelet traits with (active) sunitinib levels. Due a limited number of patients, no correlation could be observed between these parameters and bleeding and/or progression. Therefore, it would be interesting to investigate whether changes in platelet traits or concentration of unbound sunitinib (or other TKIs) in plasma or isolated platelets could predict response to therapy or bleeding in a larger cohort. This knowledge might lead to a way to follow patients more closely to prevent bleeding or monitor progression. As we observed aggravated effects on platelet function of sunitinib together with aspirin, more research into the combined anti-platelet effects of different anti-platelet or anti-coagulant drugs with TKIs is necessary. Especially patients receiving both anti-cancer and anti-platelet therapies could be at increased risk for bleeding.

The application of TKIs in treatment of other diseases, e.g. thrombosis, would also be fascinating to study. TKIs already have inhibiting effects on platelets mainly via the GPVI pathway, therefore, these drugs might be possible to use as anti-platelet drugs in (deep vein) thrombosis, which is also suggested recently by other studies[96, 97]. These TKIs are already approved in the clinic, ergo easier to test in other diseases involving thrombosis or thrombo-inflammation, in a short period of time as side effects are already reported. Additionally, due to the relative high affinity described for tyrosine kinases, relatively low therapeutic concentrations of TKI could be able to selectively inhibit platelet function without possibly increasing bleeding risk.

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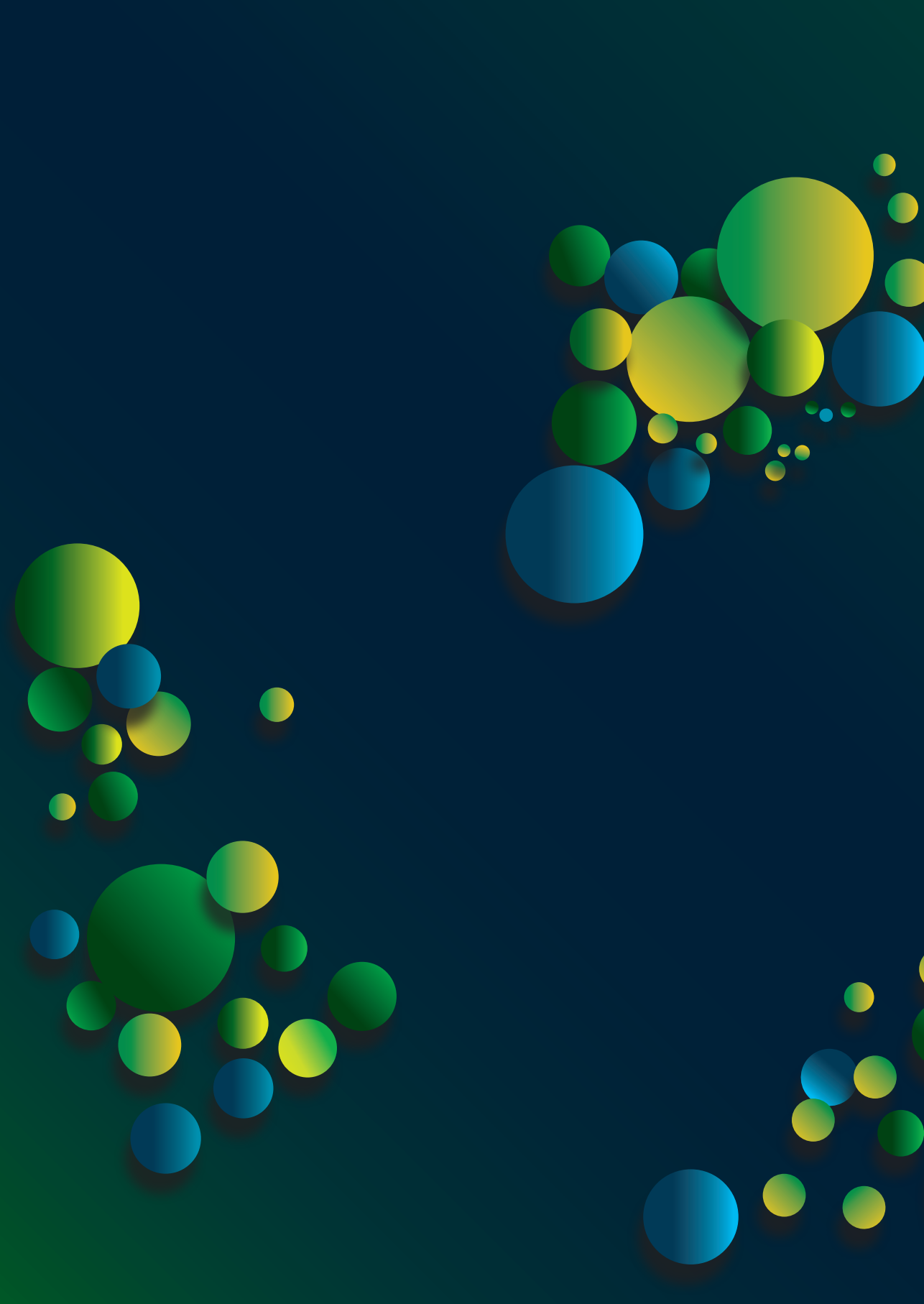

Chapter 9

Summary

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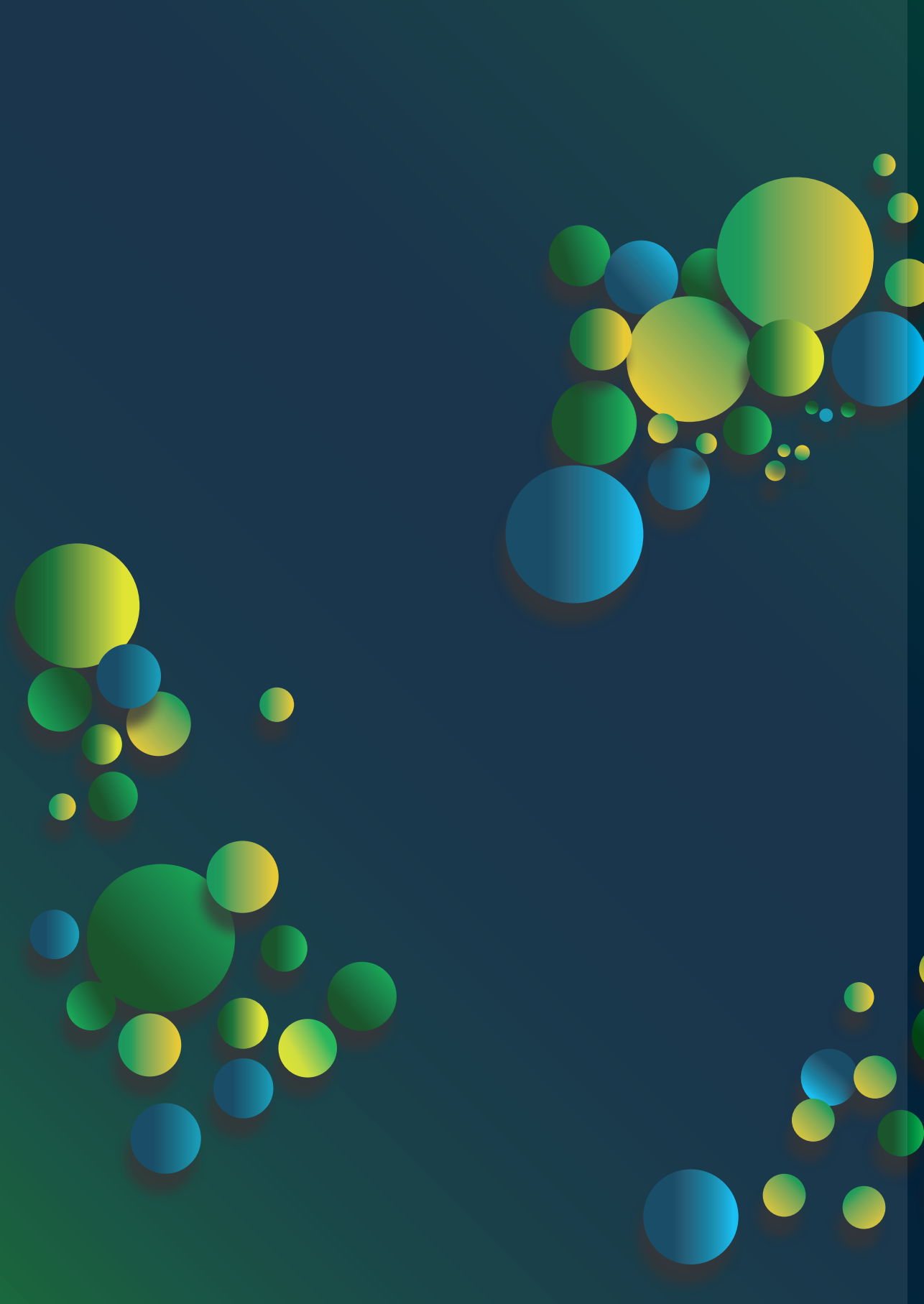
Impact

Curriculum Vitae

Publications

Acknowledgements



Summary

In the last decades, tyrosine kinase inhibitors (TKIs) have been developed as a new type of anti-cancer agents. These TKIs inhibit growth factor receptors (tyrosine kinases) involved in angiogenesis and tumour development, thereby improving progression free survival. As tyrosine kinases are expressed in many cell-types, and most TKIs inhibit multiple protein tyrosine kinases, they inevitably have off-target effects. One of the side effects described for several TKIs is (mild) bleeding. Blood platelets, well-known for their involvement in haemostasis, also contain multiple protein tyrosine kinases, which are essential for their activation. Therefore, the overall aim of this thesis was to provide more insights in the unintended effects of TKIs on platelet function. **Chapter 1** provides a brief background on platelet activation processes and thrombus formation, with additional focus on the involvement of tyrosine kinases in the different activation pathways.

To look more closely into the different TKIs and their anti-platelet effects, in **Chapter 2** a detailed literature-based review is given, which provides an extended overview and mechanistic insight into the action of TKIs in current clinical use. The targets of these TKIs were compared with the tyrosine kinases present in platelets and the possibility to be linked to anti-platelet effects. This revealed that (for some TKIs) the knowledge on affinity for their targets does not completely align with published effects on platelets and reported bleeding events. In addition, clinical consequences of platelet inhibition by treatment with TKIs were discussed. Conversely, given the role of platelets in tumour progression, we discussed whether platelet inhibition can be an interesting strategy to inhibit tumour growth and monitor response to therapy.

Pazopanib is an oral TKI used for treatment of advanced renal cell carcinoma (RCC). In **Chapter 3**, we investigated the effects of pazopanib on platelet function *in vitro* and in RCC patients on-treatment. We found that pazopanib (dose-dependently) reduced GPVI-induced platelet responses and thrombus formation under flow in blood from healthy volunteers. Pazopanib inhibited tyrosine phosphorylation and intracellular calcium signalling in platelets stimulated via the collagen receptor glycoprotein (GP)VI, subsequently leading to reduced exposure of phosphatidylserine (PS) as a marker for procoagulant activity. In addition, we analysed blood samples from 10 RCC patients before and 14 days after receiving pazopanib as monotherapy. This treatment caused an overall lowering in platelet count, with 3 patients experiencing mild bleeding. The effects of pazopanib on these patients' platelets were mainly confined to a reduction in GPVI-dependent procoagulant activity. Control experiments indicated that higher pazopanib concentrations were required to inhibit GPVI-mediated PS exposure in the presence of plasma. Together, these results indicated that pazopanib suppresses GPVI-induced platelet activation responses in a way partly antagonized by the presence of plasma.

Another multi-target TKI used for the treatment of advanced RCC is sunitinib. This compound was reported to affect collagen-induced activation under non-coagulating conditions. In **Chapter 4**, we investigated the effects of sunitinib on thrombus formation induced by other tyrosine kinase-dependent receptors, as well as the effects under coagulating conditions. We

observed that both thrombus formation and PS exposure under flow were affected by sunitinib on collagen type I and III. Upon tissue factor-triggered coagulation, sunitinib decreased PS exposure and fibrin formation. In blood from cancer patients more pronounced effects of sunitinib were observed in lung and pancreatic as compared to neuroglioblastoma and other cancer types. Cancer patients commonly suffer from cardiovascular disease as a co-morbidity, and may be at risk for (secondary) thrombotic events. Therefore, these patients are treated with anti-platelet drugs such as aspirin, which irreversibly inhibits platelet activation and is associated with an increased bleeding risk. As both sunitinib and aspirin are associated with bleeding, we further investigated the synergistic effects of both compounds in this setting. Compared to sunitinib alone, the combination of sunitinib with aspirin resulted in further reduction of platelet aggregation, thrombus formation and PS exposure on collagen under flow with(out) coagulation. In conclusion, sunitinib suppressed collagen-induced procoagulant activity and delayed fibrin formation, which was aggravated by aspirin treatment.

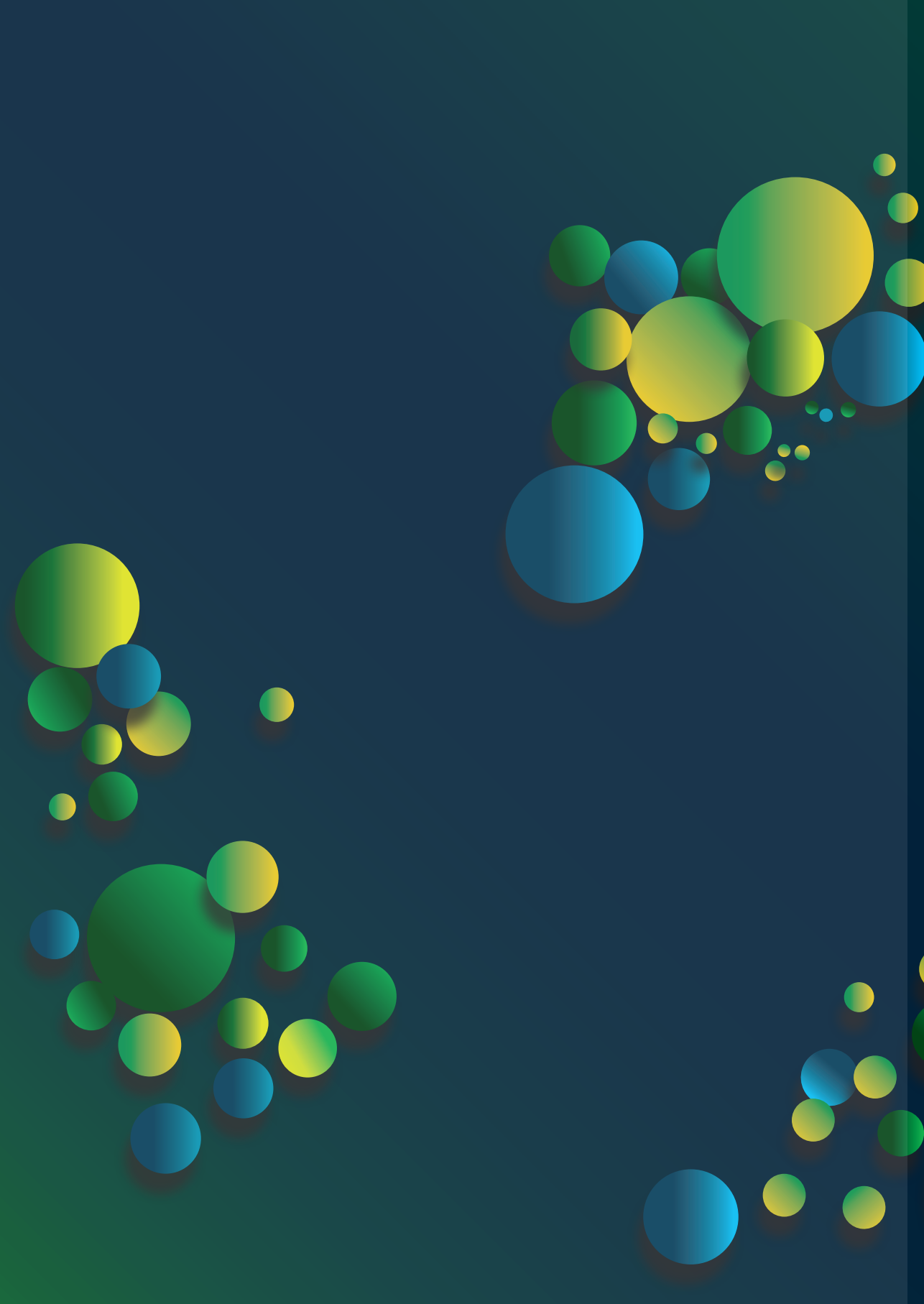
To investigate the effects of sunitinib not only *in vitro*, but also in cancer patients, in **Chapter 5** we investigated the quantitative and qualitative changes in platelet traits as a function of the sunitinib levels and the occurrence of bleeding in RCC patients on treatment with sunitinib. Blood was collected from 20 metastatic RCC (mRCC) patients before treatment, and at 2 weeks, 4 weeks and 3 months after sunitinib administration. In sunitinib-treated mRCC patients, concentrations of sunitinib and its active metabolite in plasma and serum were highly correlated. The active metabolite was relatively increased in the patients' platelets compared to sunitinib. On average, a continued reduction in platelet count was observed on-treatment, which was significantly related to the inhibitor levels in plasma/serum. Principal component and correlational analysis showed that the concentration of (active) sunitinib in plasma/serum was linked with a reduction in both platelet count and collagen-induced platelet aggregation. The reduced aggregation in part associated with reported bleeding, but did not correlate to disease progression. In conclusion, sunitinib induced reduction in quantitative and qualitative platelet traits may reflect the effective sunitinib levels in the patient.

The mechanism underlying the increased bleeding tendency with TKIs often remains unclear. Therefore, in **Chapter 6** we investigated the effects of three Btk inhibitors, two irreversible (ibrutinib and acalabrutinib) and one novel reversible inhibitor (MK-1026), on platelet function in healthy volunteers, patients and Btk deficient mice, together with off-target effects on tyrosine kinase phosphorylation. All three inhibitors impaired GPVI- and CLEC-2-mediated aggregation, activation and secretion in isolated platelets in a dose-dependent manner. Only ibrutinib inhibited thrombus formation on von Willebrand Factor (vWF) co-coated surfaces, while on collagen this process was not affected. In blood from Btk-deficient mice, collagen-induced thrombus formation under flow was reduced, but preincubation with either inhibitor was without additional effects. MK-1026 showed less off-target effects upon GPVI-induced tyrosine kinase phosphorylation as

compared to ibrutinib and acalabrutinib. In ibrutinib-treated patients, GPVI-stimulated platelet activation and adhesion on vWF-co-coated surfaces were inhibited, while CLEC-2 stimulation induced variable responses. The dual inhibition of GPVI and CLEC-2 signalling by Btk inhibitors might account for the increased bleeding tendency, with ibrutinib causing more high-grade bleedings due to additional inhibition of platelet-vWF interaction. As MK-1026 showed less off-target effects and only affected activation of isolated platelets, it might be promising for future treatment.

Although multiple TKIs are reported to increase the risk of bleeding, mainly minor bleeding symptoms are described. The currently used anti-platelet drugs for treatment of arterial thrombosis coincide with a greater risk of bleeding. Hence, platelet inhibitors that preserve haemostasis are warranted. In **Chapter 7**, we investigated the anti-platelet properties of several clinically used TKIs as exploration of possible repurposing of these compounds as anti-platelet drugs. Eight TKIs were selected based on the affinity for tyrosine kinases expressed in platelets and published bleeding symptoms. Overall, the TKIs with the highest affinities for platelet targets most strongly inhibited platelet function. Dasatinib and sunitinib dose-dependently reduced collagen-induced aggregation in platelet-rich plasma and washed platelets, while pazopanib, cabozantinib and vatalanib inhibited this response in washed platelets only, and fostamatinib, axitinib and lapatinib showed no or limited effects. Fostamatinib effectively reduced whole blood thrombus formation on collagen under flow, as well as on vWF plus rhodocytin or laminin. Pazopanib, sunitinib, dasatinib, axitinib and vatalanib (mildly) reduced thrombus formation only on collagen. Intracellular calcium measurements and activation responses in isolated platelets were inhibited by dasatinib, fostamatinib, sunitinib and pazopanib. Simultaneous measurement of different platelet populations raised by collagen receptor-stimulation showed that fostamatinib, cabozantinib and vatalanib decreased highly activated platelet populations, whereas negative, resting populations were increased. Altogether, the inhibitory effects of dasatinib, fostamatinib, sunitinib and pazopanib suggested interference in early collagen receptor-induced signalling events, whereas for cabozantinib and vatalanib the effects may be more downstream. Fostamatinib, sunitinib, pazopanib and vatalanib have been associated with mild or no bleeding events and, hence, may be promising candidates to explore further as potential anti-platelet drugs.

In **Chapter 8** are the most important findings of this thesis are discussed in perspective of the current literature. With this thesis we would like to raise awareness for the potential anti-platelet effects of several TKIs during cancer treatment, which could be enhanced in the presence of antithrombotic drugs, thereby increasing the bleeding risk. Changes in platelet features and unbound plasma concentrations of TKIs upon treatment could provide tools for monitoring and managing (these) side effects.



Samenvatting

In de afgelopen decennia zijn tyrosine-kinaseremmers (TKI's) ontwikkeld als een nieuw type antikankerbehandeling. Deze TKI's remmen in de eerste plaats groeifactor-receptoren (tyrosine kinasen) op tumor- en vaatwandcellen. Op deze manier worden angiogenese en tumorontwikkeling geremd, waardoor de progressie-vrije overleving bij patiënten wordt verbeterd. Omdat tyrosine kinasen in veel celtypen in het lichaam tot expressie komen, hebben ze onvermijdelijk bijwerkingen. Een van de beschreven bijwerkingen bij meerdere TKI's is (milde) bloedingen. Bloedplaatjes, die bekend staan om hun betrokkenheid bij hemostase, bevatten ook meerdere tyrosine kinasen die essentieel zijn voor hun activering. Het algemene doel van dit proefschrift was om meer inzicht te geven in de onbedoelde effecten van TKI's op de functie van bloedplaatjes. **Hoofdstuk 1** geeft een korte achtergrond over de processen van activering van bloedplaatjes en daaropvolgende trombusvorming, waarin we extra focus leggen op de betrokkenheid van tyrosine kinasen in de verschillende activeringsroutes.

Om meer inzicht te krijgen in de verschillende TKI's en hun effecten op bloedplaatjes effecten, wordt in **Hoofdstuk 2** een gedetailleerd literatuuronderzoek beschreven. Hierin tonen we een uitgebreid overzicht van TKI's die in de huidige kliniek gebruikt worden voor de behandeling van verschillende soorten kanker, almede de cellulaire aangrijpingspunten voor hun werking. De doelwitten van deze TKI's zijn vergeleken met de tyrosine kinasen die aanwezig zijn in bloedplaatjes, en indien mogelijk gekoppeld aan anti-bloedplaatjes effecten. Hieruit bleek dat (voor sommige TKI's) de kennis over affiniteit voor hun doelwitten niet volledig overeenkomt met gepubliceerde effecten op bloedplaatjes en de gerapporteerde bloedingen. Daarnaast zijn de klinische gevolgen van bloedplaatjesremming door behandeling met TKI's besproken. Omgekeerd, gezien de rol van bloedplaatjes in tumorprogressie, vroegen we ons af of bloedplaatjesremming een interessante strategie zou kunnen zijn om tumorgroei te remmen, danwel de respons op therapie te volgen.

Pazopanib is een orale TKI die gebruikt wordt voor de behandeling van gevorderd niercelcarcinoom (RCC). In **Hoofdstuk 3** onderzochten we de effecten van pazopanib op bloedplaatjesfunctie *in vitro* en bij RCC-patiënten tijdens hun behandeling. De resultaten lieten zien dat pazopanib (dosisafhankelijk) de bloedplaatjesreacties en trombusvorming onder stromingscondities na stimulatie van de collageen receptor glycoproteïne VI (GPVI) verminderde in het bloed van gezonde vrijwilligers. Pazopanib remde de fosforylering van tyrosine kinasen en de intracellulaire calciumsignalering in bloedplaatjes gestimuleerd via GPVI. Dit leidde vervolgens tot verminderde expressie van fosfatidylserine (PS) welke belangrijk is voor het bevorderen van de bloedstolling, de zogenaamde procoagulante activiteit. Daarnaast analyseerden we bloedmonsters van 10 RCC-patiënten vóór en 14 dagen na inname van pazopanib als monotherapie. Deze behandeling veroorzaakte een algehele verlaging van het aantal bloedplaatjes, waarbij 3 patiënten lichte bloedingen ervaarden. De effecten van pazopanib op de bloedplaatjes van deze patiënten waren voornamelijk beperkt tot een vermindering van

de GPVI-afhankelijke bevordering van de procoagulante activiteit. Controle experimenten lieten zien dat hogere pazopanib concentraties nodig waren om de GPVI-gemedieerde PS-expressie in aanwezigheid van plasma te kunnen remmen. Samen gaven deze resultaten aan dat pazopanib de GPVI-geïnduceerde activeringsreacties van bloedplaatjes onderdrukt op een manier die gedeeltelijk wordt tegengewerkt door de aanwezigheid van plasma.

Een andere TKI die wordt gebruikt voor de behandeling van gevorderd RCC is sunitinib. Van dit geneesmiddel is beschreven dat deze de collageen-geïnduceerde activering van bloedplaatjes onder niet-stollende omstandigheden beïnvloedt. In **Hoofdstuk 4** onderzochten we de effecten van sunitinib op trombusvorming geïnduceerd door andere tyrosine kinase-afhankelijke receptoren, evenals de effecten onder stollende condities. We hebben waargenomen dat zowel trombusvorming als PS-expressie onder stromingscondities werden geremd door sunitinib op collageen type I en III. Bij aanwezigheid van weefselfactor om bloedstolling te induceren, verminderde sunitinib de PS-expressie op bloedplaatjes en de vorming van fibrine. In bloed van kankerpatiënten werden meer uitgesproken effecten van sunitinib waargenomen in long- en alvleesklierkanker in vergelijking tot hersenkanker (neuroglioblastoma) en andere typen van kanker. Kankerpatiënten lijden vaak mede aan hart- en vaatziekten en hebben mogelijk verhoogd risico op (secundaire) trombotische gebeurtenissen. Daarom worden deze patiënten behandeld met medicatie tegen bloedplaatjesaggregatie zoals aspirine, wat de activering van bloedplaatjes onomkeerbaar remt en gepaard gaat met een verhoogd bloedingsrisico. Omdat zowel sunitinib als aspirine geassocieerd zijn met een verhoogd risico op bloedingen, hebben we de synergistische effecten van beide geneesmiddelen in deze setting verder onderzocht. Vergeleken met sunitinib alleen, resulteerde de combinatie van sunitinib en aspirine in een verdere vermindering van de aggregatie, trombusvorming en PS-expressie van bloedplaatjes op het collageen oppervlak onder stromingscondities, zowel in aan- als afwezigheid van bloedstolling. Samenvattend, sunitinib onderdrukte de collageen-geïnduceerde bevordering van de bloedstolling en vertraagde de vorming van fibrine; deze onderdrukking werd versterkt door behandeling met aspirine.

In **Hoofdstuk 5** hebben we de kwantitatieve en kwalitatieve veranderingen in eigenschappen van bloedplaatjes onderzocht in relatie tot de sunitinib concentratie in het bloed, en het optreden van bloedingen bij RCC-patiënten na behandeling met sunitinib. Bloed werd afgenomen van 20 patiënten met gevorderd RCC vóór start met behandeling en 2 weken, 4 weken en 3 maanden na behandeling met sunitinib. De concentraties van sunitinib en de actieve metaboliet in het plasma en serum van RCC-patiënten behandeld met sunitinib waren sterk gecorreleerd. De concentratie van de actieve metaboliet was relatief verhoogd in de bloedplaatjes van de patiënten in vergelijking met sunitinib. Gemiddeld werd er tijdens de behandeling een aanhoudende afname van het aantal bloedplaatjes waargenomen, wat significant verband hield met de concentratie van de remmers in het plasma en/of serum. Principal component- en correlatieanalyses toonden aan dat de concentratie van (actief) sunitinib in het plasma en/of

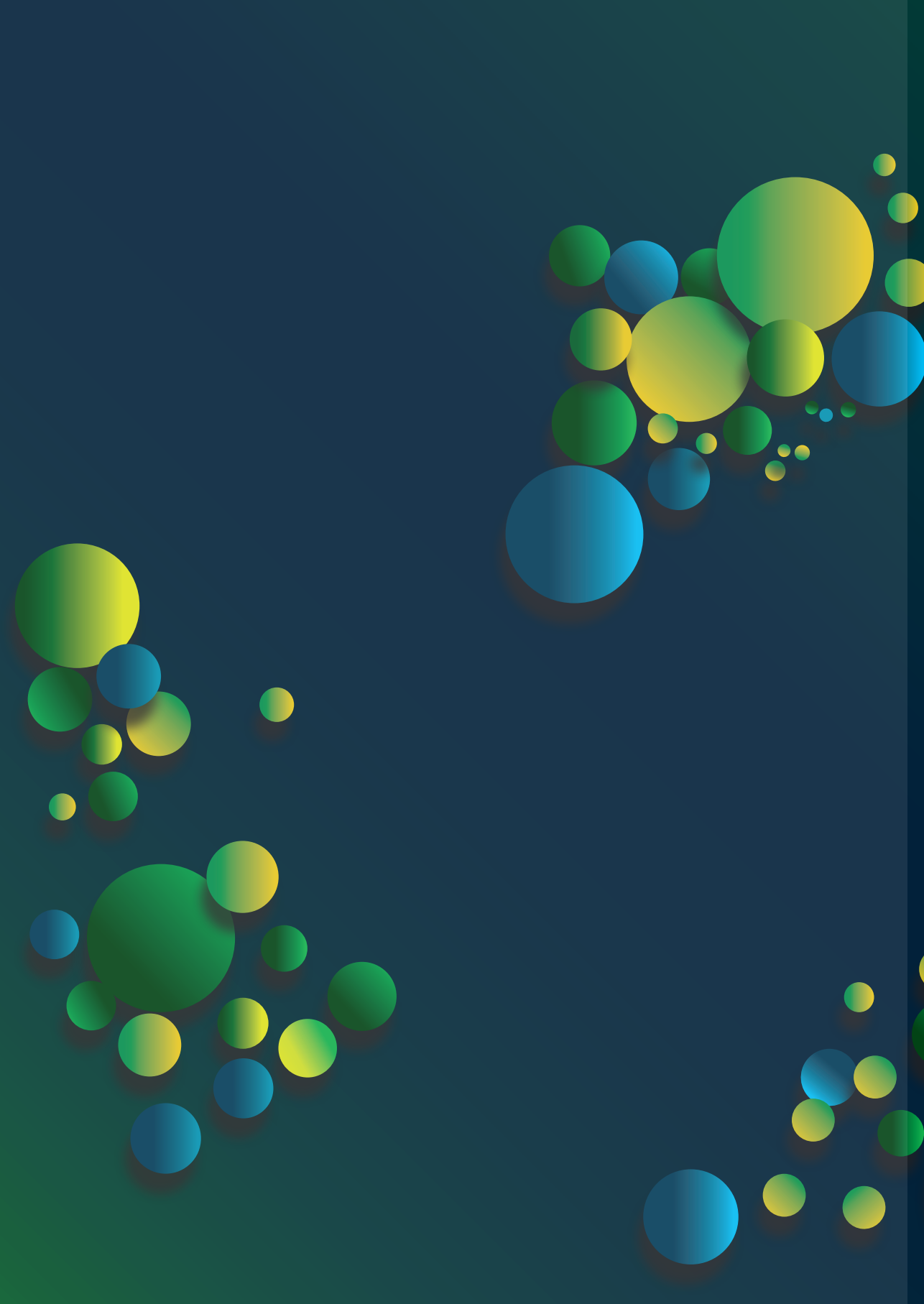
serum verband hield met een vermindering van zowel het aantal bloedplaatjes als de collageen-geïnduceerde aggregatie. De verminderde aggregatie was gedeeltelijk geassocieerd met de gerapporteerde bloedingen, maar correleerde niet met de progressie van de ziekte. Concluderend kan de door sunitinib geïnduceerde vermindering van kwantitatieve en kwalitatieve kenmerken van bloedplaatjes een weerspiegeling zijn van de effectieve sunitinib concentratie in de patiënt.

Het mechanisme dat ten grondslag ligt aan de verhoogde bloedingsneiging bij TKI's blijft vaak onduidelijk. Daarom hebben we in **Hoofdstuk 6** de (onbedoelde) effecten onderzocht van drie Btk-remmers op de functie van bloedplaatjes in het bloed van gezonde vrijwilligers, patiënten en Btk-deficiënte muizen. Het betreft twee irreversibele remmers (ibrutinib en acalabrutinib), en een nieuwe, reversibele remmer (MK-1026). Alle drie de remmers verminderden de GPVI- en CLEC-2-gemedieerde aggregatie, activatie en secretie van geïsoleerde bloedplaatjes op een dosisafhankelijke manier. Alleen ibrutinib verminderde trombusvorming op von Willebrand Factor (vWF) in combinatie met rhodocytine, laminine of fibrinogeen, terwijl op collageen geen effecten werden gezien. In het bloed van Btk-deficiënte muizen was de collageen-geïnduceerde trombusvorming onder stromingscondities verminderd, maar er werden geen additionele effecten waargenomen na toevoeging van de Btk-remmers. MK-1026 liet minder onbedoelde bijwerkingen zien bij GPVI-gestimuleerde tyrosine kinase fosforylatie in vergelijking tot ibrutinib en acalabrutinib. Bij patiënten onder behandeling met ibrutinib werden de GPVI-geïnduceerde activering en adhesie van bloedplaatjes geremd op de oppervlakken gecombineerd met vWF, terwijl variabele responsen werden waargenomen met CLEC-2 stimulatie. De duale remming van GPVI- en CLEC-2-signalering door Btk-remmers kan verantwoordelijk zijn voor de verhoogde bloedingsneiging, met meer hoogwaardige bloedingen bij behandeling met ibrutinib als gevolg van extra remming van bloedplaatjes adhesie aan vWF. Omdat MK-1026 minder off-target effecten liet zien en alleen de activatie van geïsoleerde bloedplaatjes beïnvloedde, zou dit een veelbelovende toekomstige behandeling kunnen zijn.

Hoewel het is beschreven dat meerdere TKI's het risico op bloedingen verhogen, betreft dit voornamelijk milde bloedingssymptomen. De momenteel voorgeschreven medicatie tegen aggregatie van bloedplaatjes ter behandeling van arteriële trombose geven een groter risico op bloedingen. Daarom zijn bloedplaatjes remmers noodzakelijk die de hemostase behouden. In **Hoofdstuk 7** hebben we van verschillende klinisch gebruikte TKI's de remmende effecten op bloedplaatjes onderzocht ter verkenning van het gebruik van deze geneesmiddelen als anti-bloedplaatjes medicatie. Acht TKI's werden geselecteerd op basis van de affiniteit voor tyrosine kinasen die tot expressie komen in bloedplaatjes en de gepubliceerde bloedingssymptomen. Over het algemeen remden de TKI's met de hoogste affiniteit voor tyrosine kinasen in bloedplaatjes, de functie van bloedplaatjes het sterkst. Dasatinib en sunitinib verminderden dosisafhankelijk de collageen-geïnduceerde aggregatie in zowel de aanwezigheid van plasma, als in geïsoleerde bloedplaatjes. Terwijl pazopanib, cabozantinib en vatalanib deze respons alleen in geïsoleerde

bloedplaatjes remden. Fostamatinib, axitinib en lapatinib vertoonden geen of beperkte effecten. Fostamatinib verminderde effectief de vorming van een trombus in bloed dat werd gestroomd over een collageen oppervlak, evenals over een oppervlak van vWF samen met rhodocytine of laminine. Pazopanib, sunitinib, dasatinib, axitinib en valatalanib verminderden (licht) de vorming van de trombus alleen op het collageen oppervlak. Intracellulaire calciummetingen en activeringsreacties in geïsoleerde bloedplaatjes werden geremd door dasatinib, fostamatinib, sunitinib en pazopanib. Gelijktijdige meting van verschillende populaties van bloedplaatjes opgewekt door stimulatie van de collageen receptor toonde aan dat fostamatinib, cabozantinib en vatalanib de sterk geactiveerde populatie van bloedplaatjes verlaagden, terwijl de negatieve, rustende populaties toenamen. Al met al suggereerden de remmende effecten van dasatinib, fostamatinib, sunitinib en pazopanib interferentie in de vroege signalering van de collageen receptor-geïnduceerde activering, terwijl voor cabozantinib en vatalanib de effecten verder stroomafwaarts kunnen zijn. Fostamatinib, sunitinib, pazopanib en vatalanib zijn in verband gebracht met milde of geen bloedingen en kunnen daarom veelbelovende kandidaten zijn om verder te onderzoeken als potentiële geneesmiddelen om bloedplaatjes te remmen.

In **Hoofdstuk 8** worden de belangrijkste bevindingen van dit proefschrift besproken in het perspectief van de huidige literatuur. Met dit proefschrift willen we het bewustzijn vergroten voor de mogelijke remmende effecten van verschillende TKI's op bloedplaatjes tijdens de behandeling van kanker. Deze zouden bovendien versterkt kunnen worden in aanwezigheid van antitrombotische middelen, waardoor het risico op bloedingen verder toeneemt. Veranderingen in de kenmerken van bloedplaatjes en ongebonden plasmaconcentraties na behandeling met TKI's kunnen handvaten bieden voor het monitoren en beheersen van (deze) bijwerkingen.



Impact

Cancer is a leading cause of death worldwide, with almost 10 million deaths in 2020[1]. One of the hallmarks of cancer is to chronically sustain cell proliferation caused by mutations in or amplification of oncogene-encoded proteins or growth factor receptors[2]. As these proteins include (tyrosine) kinases, cancer cells are described to have abnormal kinase activity which is known to drive processes such as cell proliferation, differentiation, migration, survival and angiogenesis[3, 4]. In the past two decades, increased understanding of this process has resulted in the development of many small molecule tyrosine kinase inhibitors (TKIs) as targeted therapy for cancer. In current settings of cancer treatment, TKIs significantly increase the progression-free survival of patients upon (lifelong) treatment. As tyrosine kinases are present in many cell-types, long-term treatment is often associated with (serious) adverse events including bleeding.

Platelets are important players in haemostasis and platelet production and function rely on several tyrosine kinases, suggesting that platelet count and function may be affected by TKIs. Hence, this may play a role in the increased risk of bleeding observed upon treatment with several TKIs. Yet, knowledge on whether and/or how platelets are affected by TKIs is limited. Therefore, we aimed to provide more insights in the effects of different TKIs on platelet function. To define what was known on the relation between TKIs and platelets at the start of this research, in **Chapter 2** an overview is given of the currently used TKIs in cancer treatment and the effects on platelet function. In order to extent the existing knowledge, we investigated platelet responses in the presence of different TKIs *in vitro* and in cancer patients (**Chapter 3-7**). Throughout this thesis, we showed that the majority of TKIs inhibit platelet responses mediated by the collagen receptor glycoprotein (GP)VI, e.g. activation, aggregation and/or thrombus formation. In some cases (additionally) CLEC-2- or GPIb-induced thrombus formation was inhibited by TKI treatment. These results unravelled parts of the underlying mechanism of platelet inhibition by these TKIs. As these mechanisms still remain incomplete or unknown for some TKIs, this could provide new fields of interest for fellow researchers. Furthermore, with our studies involving (specific) cancer patients upon TKI treatment (**Chapter 3, 5 and 6**), we were able to gain knowledge on the interactions between platelet traits and TKI levels in the blood of these patients. We found that in renal cell carcinoma patients not only platelet function, but also platelet count, was affected by the TKIs sunitinib and pazopanib. For sunitinib, we discovered that the (active) compound levels in plasma or serum correlated with platelet count and function in these patients. This effect was measured already two weeks after the start of treatment, while response to therapy is normally not monitored until three months after start of treatment by a CT-scan. These plasma concentrations of sunitinib (or its metabolite) could be of importance for toxicity and the response to treatment, pointing towards platelets as a monitoring tool for compound effects, both on- and off-target. These results could be of interest for clinicians and cancer patients (hopefully) resulting in (early) therapeutic drug monitoring and increased quality of life during treatment. Therefore, more studies on the unintended side effects/off-target effects of drugs (not only on platelets, but

also other cells) could be of great importance to provide ways to monitor and improve treatment. These studies would enhance our understanding of the exact mechanism underlying the side effects, which could lead to the development of new drugs with reduced side effects.

Cancer patients often have a cardiovascular history[5, 6] and are prescribed with anti-platelet or anti-coagulant drugs to prevent recurrent cardiovascular events. As anti-platelet medications, such as clopidogrel and aspirin, are also associated with an increased bleeding risk bleeding[7-9], in **Chapter 4** we examined whether there are synergistic effects on platelet function of dual treatment that could further increase the bleeding risk in TKI-treated cancer patients. We showed that aspirin aggravated the effects of sunitinib in platelet aggregation as well as delaying platelet-dependent coagulation in whole blood perfusion over a collagen surface. With these results we would like to raise awareness among clinicians of combining anti-platelet therapies and TKIs as this may result in an increased bleeding risk in patients.

As current anti-platelet therapies are associated with bleeding and thrombotic events may still occur, there is a need of better anti-thrombotic drugs. The platelet GPVI and CLEC-2 receptors are important players in arterial thrombosis and thrombo-inflammation with supporting roles in haemostasis[10]. A deficiency of GPVI or CLEC-2 does not significantly influence haemostasis[11, 12], making these receptors interesting targets for anti-thrombotic drugs[10, 13]. As these receptors rely on tyrosine kinases for their underlying signalling pathways, we studied the anti-platelet properties of several clinically used TKIs with the aim to explore possible repurposing of these compounds as anti-platelet drugs. As several of the tested TKIs are described with only minor bleeding events, the GPVI inhibition observed with these TKIs might suggest that these could be promising candidates as anti-platelet drugs. However, more research with regard to dosage to estimate efficacy and toxicity in thrombotic patients is needed. With additional research, repurposing TKIs could be of great interest for clinicians, in particular cardiologists, as these TKIs are well-tolerated and already orally available in the clinic.

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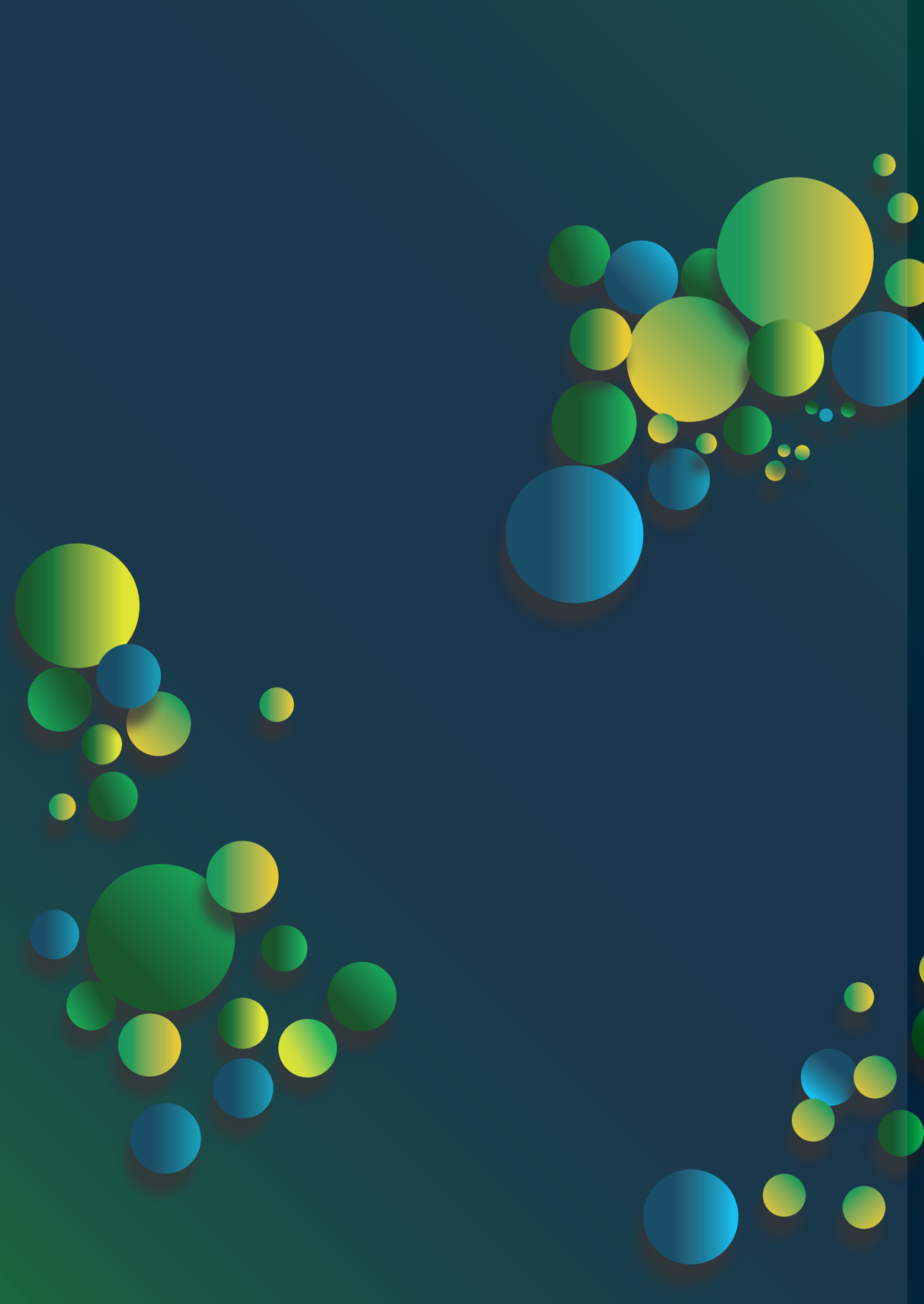
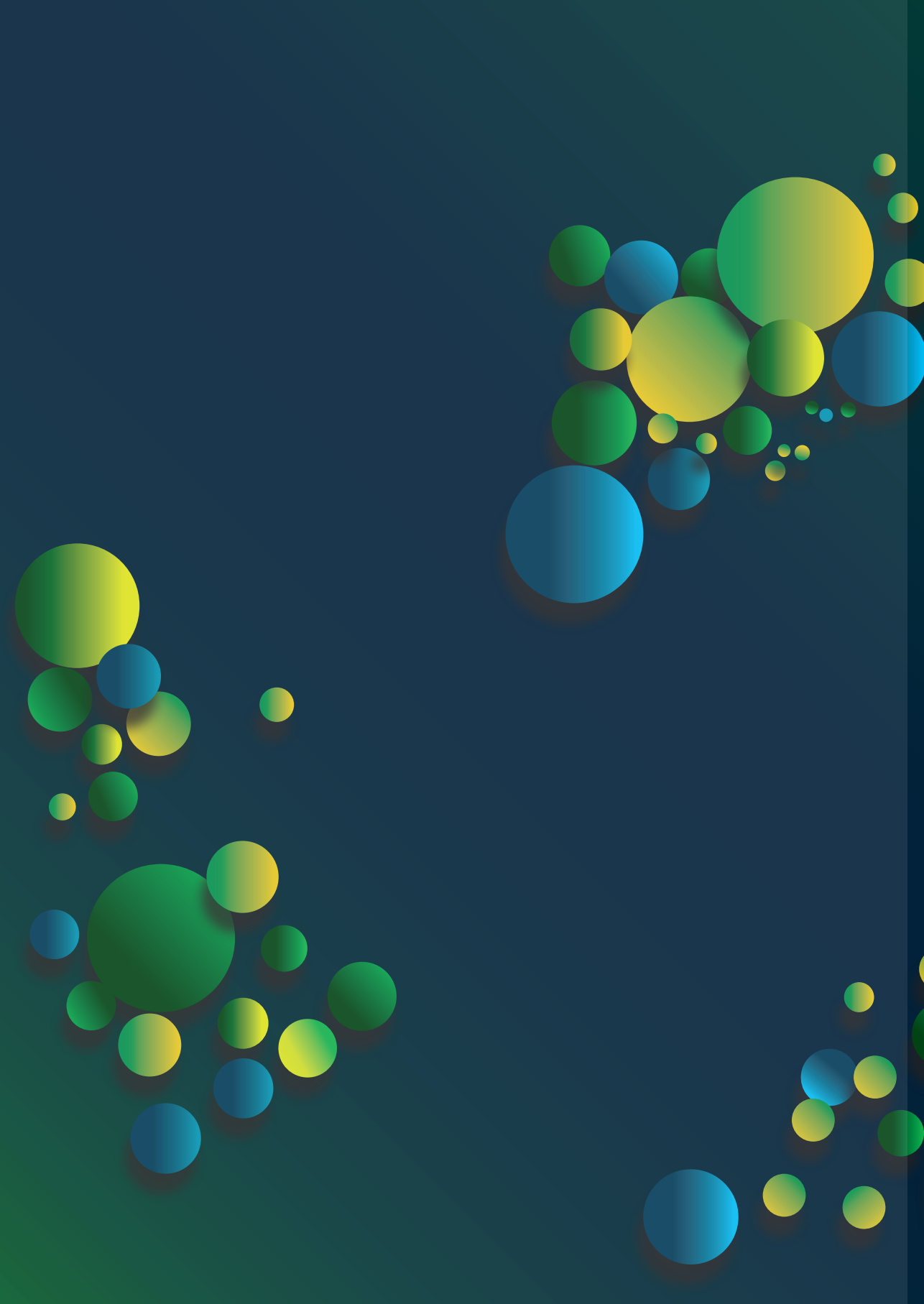


Curriculum Vitae

Beatrice Tullemans, roepnaam Bibian, werd geboren op 27 oktober 1992 te Weert, Nederland. In 2005 begon ze haar middelbare schoolopleiding aan de Philips van Horne SG te Weert, waar ze het HAVO diploma behaalde in 2010 (profiel Natuur & Gezondheid en Natuur & Techniek). Vervolgens startte ze een bacheloropleiding Applied Sciences, in de richting Life Sciences, aan de Fontys Hogeschool te Eindhoven. Na het behalen van het bachelorsdiploma in 2014, volgde ze de masteropleiding Biomedical Sciences aan de Universiteit van Maastricht, welke ze met succes afrondde in 2016. Tijdens deze opleidingen liep zij onderzoekstages met als onderwerp immuniteit bij koeien (Vakgroep Infectieziekten en Immunolgie, Universiteit Utrecht), ontwikkeling van immuniteit bij de mens (Vakgroep Interne Geneeskunde, Universiteit Maastricht), invloed van macrofagen op de atherosclerose (Vakgroep Pathologie, Universiteit Maastricht) en de invloed van kankerbehandelingen op de hemostase (Vakgroep Biochemie, Universiteit Maastricht). In november 2016 begon zij als PhD-student in het Onderzoeksschool Hart- en Vaatziekten Maastricht (CARIM) aan de Universiteit Maastricht binnen de Vakgroep Biochemie. Hier heeft ze wetenschappelijk onderzoek uitgevoerd op het gebied van bloedplaatjes, hemostase en kankerbehandeling, zoals in dit proefschrift beschreven. Zij werd hierin begeleid door Dr. Marijke J.E. Kuijpers, Dr. Maureen J.B. Aarts en Prof. Dr. Johan W.M. Heemskerk. Tijdens haar promotie-onderzoek voerde zij projecten uit in de laboratoria van Prof. Dr. C. Oury (Liège, België), Prof. Dr. A. Greinacher (Greifswald, Duitsland), Prof. Dr. W. Ouwehand (Cambridge, Verenigd Koninkrijk) en Prof. Dr. P. Simioni (Padua, Italië). Haar promotieonderzoek werd gepresenteerd op verschillende (inter)nationale congressen.

Beatrice Tullemans, called Bibian, was born on 27th of October 1992 in Weert, the Netherlands. In 2005 she started her secondary school at the Philips van Horne SG in Weert, where she obtained her HAVO diploma in 2010 (specialization Nature & Health and Nature & Technics). In the same year she started a bachelor study in Applied Sciences, with Life Sciences as specialization, at the Fontys University in Eindhoven. After graduating for her bachelor's degree in 2014, she continued education with a master in Biomedical Sciences at Maastricht University, where she obtained her degree in 2016. During this education, she performed internships on immunity in cows (Department of Infectious Diseases and Immunology, Utrecht University), development of immune cells in humans (Department of Internal Medicine, Maastricht University), effects of macrophages in atherosclerosis (Department of Pathology, Maastricht University) and effects of anti-cancer drugs on platelets in haemostasis (Department of Biochemistry, Maastricht University). In November 2016, she started as a PhD-student in the Cardiovascular Research Institute Maastricht (CARIM) at the Department of Biochemistry of Maastricht University. As a PhD-student, she performed research on platelets, hemostasis and anti-cancer drugs, such as described in this thesis. She performed this research under the supervision of Dr. Marijke J.E. Kuijpers, Dr. Maureen J.B. Aarts and Prof. Dr. Johan W.M. Heemskerk. As a PhD student, she worked in the laboratories of Prof. Dr. C. Oury (Liège, Belgium), Prof. Dr. A. Greinacher (Greifswald, Germany), Prof. Dr. W. Ouwehand (Cambridge, United Kingdom) and Prof. Dr. P. Simioni (Padova, Italy). She presented her research at several (inter)national scientific conferences.



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5. **Tullemans BME**, Nagy M, Sabrkhany S, Griffioen AW, oude Egbrink MGA, Aarts M, Heemskerk JWM, Kuijpers MJE. Tyrosine Kinase Inhibitor Pazopanib Inhibits Platelet Procoagulant Activity in Renal Cell Carcinoma Patients. ***Front Cardiovasc Med** 2018*; 5:142.
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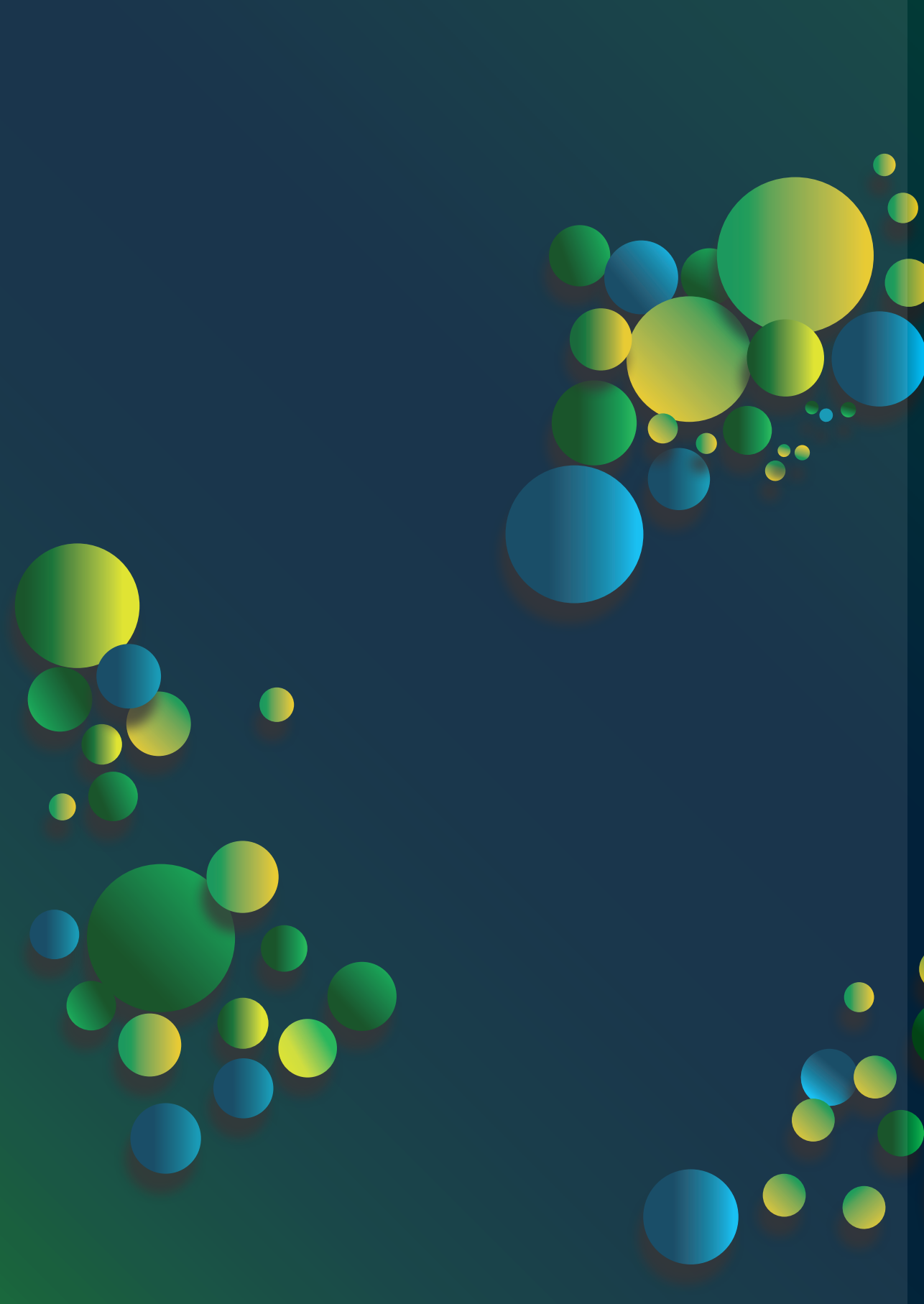
1. **Tullemans BME**. Platelet procoagulant activity is inhibited by the tyrosine kinase inhibitor pazopanib in vitro and in renal cell carcinoma patients. Centre for Thrombosis and Haemostasis (CTH) retreat, Mainz, Germany, 2017 (oral).
2. **Tullemans BME**. Platelet procoagulant activity is inhibited by tyrosine kinase inhibitor pazopanib in vitro and in renal cell carcinoma patients. 1st Italian-UK platelet meeting, Bath, United Kingdom, 2017 (poster).
3. **Tullemans BME**. Tyrosine kinase inhibitor pazopanib inhibits platelets procoagulant activity in vitro and in cancer patients. Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany, 2017 (poster).
4. **Tullemans BME**. Anti-platelet effects of tyrosine kinase inhibitors used for cancer treatment: pazopanib as a proof of principle. 4th European Platelet Network Conference (EUPLAN), Bruges, Belgium, 2018 (poster).
5. **Tullemans BME**. Multiparameter screening for platelet-inhibitory effects of tyrosine kinase inhibitors used for cancer treatment. The Platelet Society Meeting, Cambridge, United Kingdom, 2019 (poster).
6. **Tullemans BME**. Tyrosine kinase inhibitor sunitinib delays platelet-induced coagulation: additive effects of aspirin. Congress of the International Society on Thrombosis and Haemostasis (ISTH), Milan, Italy, 2020 (poster).
7. **Tullemans BME**. Quantitative and qualitative changes in platelet traits of sunitinib-treated patients with renal cell carcinoma in relation to circulating sunitinib levels: a proof-of-concept study. Congress of the international Society on Thrombosis and Haemostasis (ISTH), Philadelphia, USA, 2021 (poster).



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Thank you!