Tyrosine Kinase Inhibitor Sunitinib Delays Platelet-Induced Coagulation: Additive Effects of Aspirin

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Abstract

Background Sunitinib is a multitarget tyrosine kinase inhibitor (TKI) used for cancer treatment. In platelets, sunitinib affects collagen-induced activation under noncoagulating conditions. We investigated (1) the effects of sunitinib on thrombus formation induced by other TK-dependent receptors, and (2) the effects under coagulating conditions. Cardiovascular disease is a comorbidity in cancer patients, resulting in possible aspirin treatment. Sunitinib and aspirin are associated with increased bleeding risk, and therefore we also investigated (3) 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

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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 glycoprotein VI-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.

Conclusion 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.

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 tumor 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 hemostasis remain subclinical.

Platelets contribute to hemostasis by signaling 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 noncoagulating 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 antiplatelet 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 anticancer 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 tissue factor (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.

Methods

Materials

Sunitinib malate (Sutent) was provided by Pfizer (New York, New York, United States). Aspirin, bovine serum albumin (BSA), D(+)-glucose, unfractionated heparin, and apyrase were purchased from Sigma-Aldrich (Saint Louis, Missouri, United States). The agonists collagen-related peptide cross-linked (CRP-XL) and vWF III (vWF-III) were obtained from CambCol Laboratories (Cambridge, United Kingdom), whereas thrombin was obtained from Enzyme Research Laboratories (South Bend, Indiana, United States) and Horm collagen type I from Takeda (Hoofddorp, The Netherlands). 2-Methylthioadenosine-diphosphate (2MeS-ADP) and D-phenylalanyl-prolyl-arginyl chloromethyl ketone were obtained from Santa Cruz Biotechnology (Dallas, Texas, United States). Recombinant human TF (Innovin) was purchased from Dade Behring (Deerfield, Illinois, United States). Laminin came from Octapharma (Berlin, Germany). Fura-2-AM was obtained from Invitrogen (Carlsbad, California, United States). Plastic syringes and fluorescein isothiocyanate (FITC)-labeled PAC1 monoclonal antibody (mAb) against activated human integrin $\alpha_{IIb}\beta_3$ were purchased from BD Bioscience (nr. 340507; Franklin Lakes, New Jersey, United States), while FITC-conjugated α-fibrinogen

mAb was purchased from DAKO (F0111; Santa Clara, California, United States). Alexa Fluor (AF) 647-labeled CD62-P mAb was obtained from Biolegend (London, United Kingdom), whereas the FITC-labeled 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, New York, United States).

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 (100×10^9 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. PS exposing platelets were labeled with FITC-conjugated annexin A5 and measured by flow cytometry using a BD Accuri C6 flow cytometer and accompanying software (Erembodegem, Belgium).

Cytosolic Ca^{2+} Measurements

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements in Fura-2 loaded platelets (200×10^9 platelets/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 platelet-rich plasma (PRP) (250×10^9 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, Pennsylvania, United States) and maximum amplitude was quantified.

PamGene Kinase Assay

Washed platelets (500×10^9/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 $10{,}000 \times g$ at 4°C. Supernatants were collected and protein content was quantified with a BioRad DC protein kit (Hercules, California, United States).

TK profiles were determined using the PamChip peptide TK microarray system on PamStation12 (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 10 × protein PTK reaction buffer (PK), 0.4 µL of 100 × BSA, 0.4 µL of 1 M dithiothreitol solution, 4 µL of 10 × PTK additive, 4 µL of 4 mM adenosine triphosphate, 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_2O). 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 PamChip. Then, the microarray assays were run for 94 cycles. An image was recorded by a CCD camera PamStation12 at kinetic read cycles 32 to 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). Upstream kinase analysis,[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 six 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, California, United States). All images were analyzed using specific (half-automated) scripts in the open-access Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, Madison, Wisconsin, United States) 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 to 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 1× standard deviation as indicated.

Statistical Analysis

Data are presented as mean ± standard error of the mean, or as median ± interquartile ranges for patient data. GraphPad Prism 8.3.0 software (La Jolla, California, United States) was used for statistical analyses, using the paired and nonparametric Wilcoxon test to compare means of two parallel experimental conditions and one-way nonparametric analysis of variance (ANOVA) (Kruskal–Wallis) for experiments with more than two conditions. In case of multiple experimental conditions and time points, a nonparametric two-way ANOVA was used. A p-value less than 0.05 was considered statistically significant in which * is $p \leq 0.05$; ** is $p \leq 0.01$; and *** is $p \leq 0.001$.

Results

Sunitinib Reduces Thrombus Formation and PS Exposure in Whole Blood under Flow on Collagen Type I and III

To widely explore the multitarget 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 3× 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 (►**Supplementary Fig. S1**, available in the online version). 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 analyzed (►**Supplementary Fig. S2**, available in the online version), and a heatmap was generated for a more systematic analysis of the seven thrombus parameters, which were averaged and scaled per parameter (►**Fig. 1**). Parameters of thrombus formation were only consistently affected on GPVI-dependent surfaces, that is, collagen type I and III (►**Fig. 1A, B**). In the presence of sunitinib, these thrombi were smaller and less compact in structure as compared to the control condition (►**Supplementary Fig. S2A** and **S2B**, brightfield images [available in the online version]). Quantification showed a reduction in platelet deposition, thrombus height, and contraction (►**Fig. 1**). P-selectin exposure was significantly reduced in thrombi formed on collagen III and vWF co-coated with laminin, fibrinogen, and ristocetin (►**Supplementary Fig. S2B** and **S2C**, available in the online version). Notably, thrombus parameters for CLEC-2-, GPIb-, and integrin $\alpha_{IIb}\beta_3/\alpha_6\beta_1$-dependent surfaces were not affected by sunitinib (►**Fig. 1C–F**, ►**Supplementary Fig. S2C–S2F**, available in the online version), 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 (►**Fig. 2A**). Most relevant for GPVI signaling 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 multitarget nature of sunitinib with regard to TK inhibition in platelets.

GPVI signaling results in sustained elevation of intracellular Ca^{2+} 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 (►**Fig. 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 (►**Fig. 2C**). This was in agreement with the reduction of PS exposure in whole blood under flow, only on collagen type I (►**Fig. 1A**).

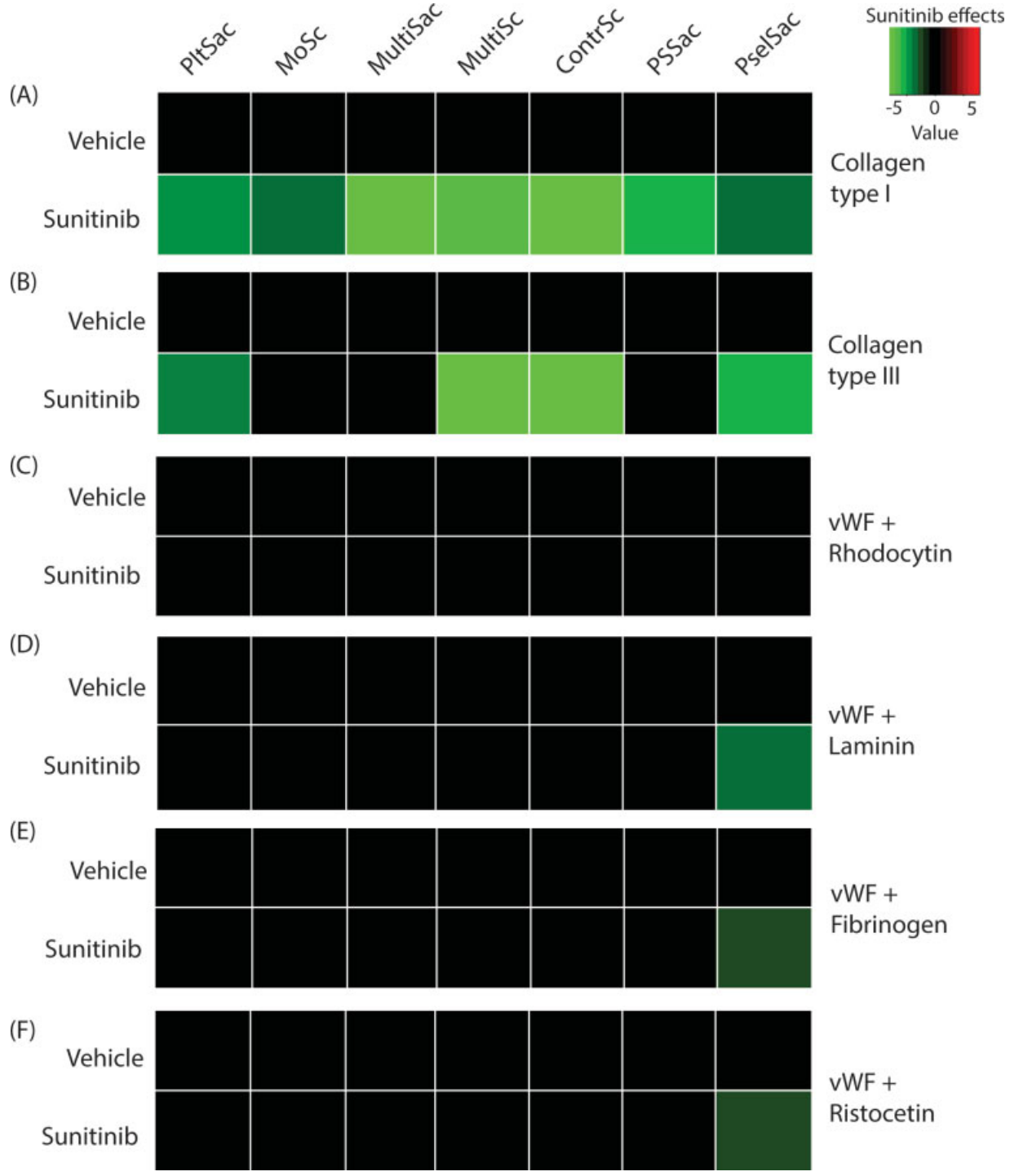

Fig. 1 Sunitinib strongly reduces thrombus formation on glycoprotein VI (GPVI)-dependent surfaces, as compared to surfaces for CLEC-2, GPIb, integrin $\alpha_6\beta_1$, and $\alpha_{IIb}\beta_3$. Whole blood from healthy volunteers was preincubated with vehicle (< 0.1% dimethyl sulfoxide [DMSO]) or sunitinib (30 μM) for 10 minutes 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, (**C**) von Willebrand factor (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), phosphatidylserine (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 ($p \leq 0.05$).

Considering that GPVI and CLEC-2 display many similarities in the downstream signaling 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 (►**Supplementary Fig. S3A, S3B**, available in the online version). Also, PS exposure as induced by

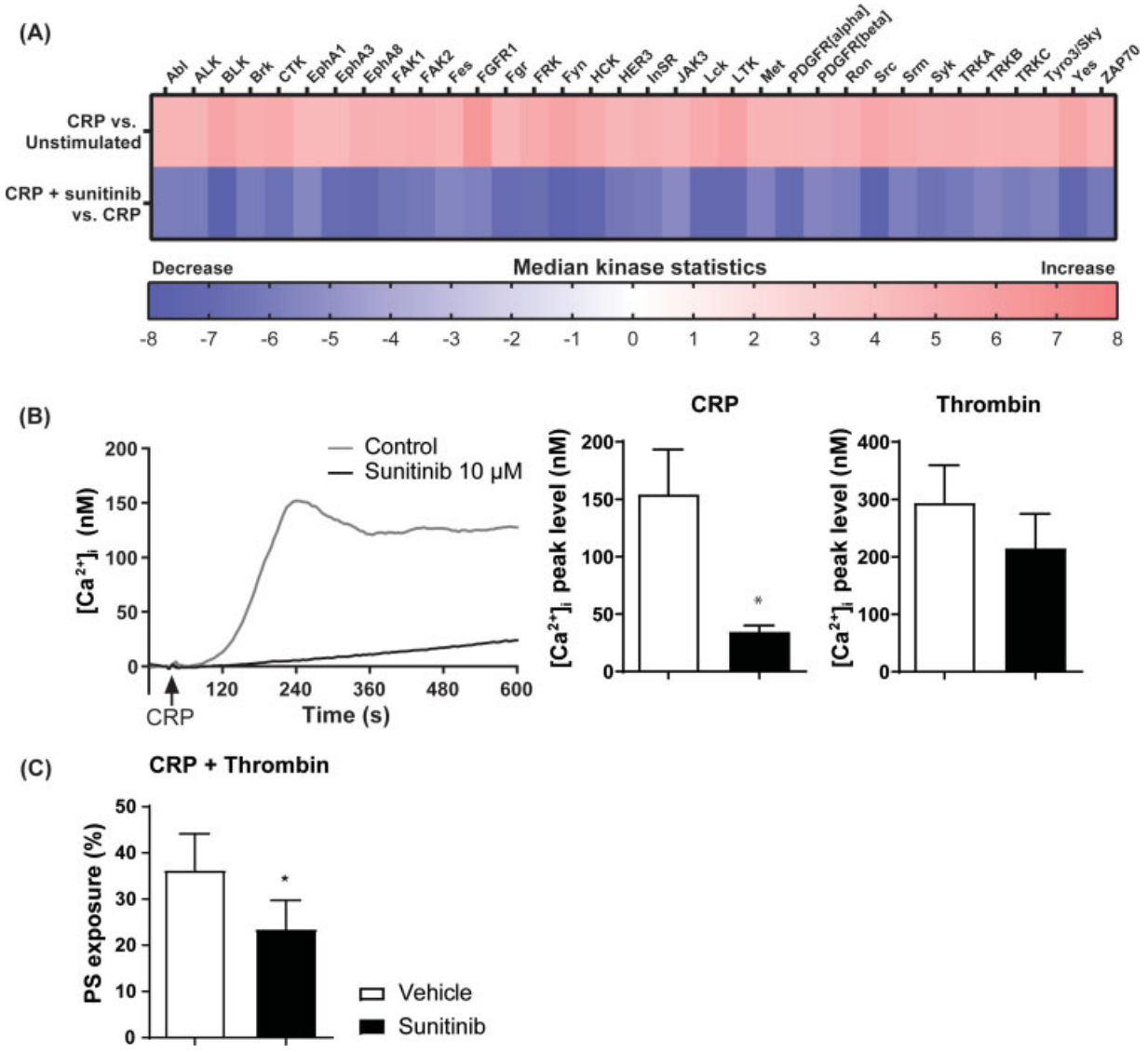

Fig. 2 Sunitinib inhibits glycoprotein VI (GPVI)-induced tyrosine kinase activity, Ca^{2+} responses, and phosphatidylserine (PS) exposure in washed platelets. (**A**) Heatmaps visualize all tyrosine kinases that are significantly up- or downregulated in both comparisons (sorted alphabetically). Cut-off has been set at a median final score of at least 1.2. Color 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 (200×10^9 /L); arrow indicates addition of collagen-related peptide crosslinked (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 + standard error of the mean (SEM) ($n = 5–7$), $^*p \leq 0.05$. (**C**) Isolated platelets from healthy volunteers were incubated with vehicle (< 0.1% dimethyl sulfoxide [DMSO]) or sunitinib (10 µM) for 10 minutes at room temperature (RT). Platelets (100×10^9 /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 fluorescein isothiocyanate (FITC)-conjugated annexin A5 and measured by flow cytometry.

rhodocytin plus thrombin was significantly reduced by sunitinib (►**Supplementary Fig. S3C**, available in the online version). However, we did not observe an effect of sunitinib in whole blood thrombus formation on rhodocytin plus vWF (►**Fig. 1C**, ►**Supplementary Fig S2C**, available in the online version), 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 ($\pm$ 14%) of the aggregation response induced by a high dose (5 µg/mL) of collagen, whereas aspirin inhibited this collagen-induced aggregation by $\pm$ 70% (►**Fig. 3A**). The combination of sunitinib and aspirin reduced the aggregation response even further as compared to aspirin alone (> 80%; ►**Fig. 3A**). Similar results could be observed when platelets were stimulated with 2MeS-ADP (►**Fig. 3B**). However, both

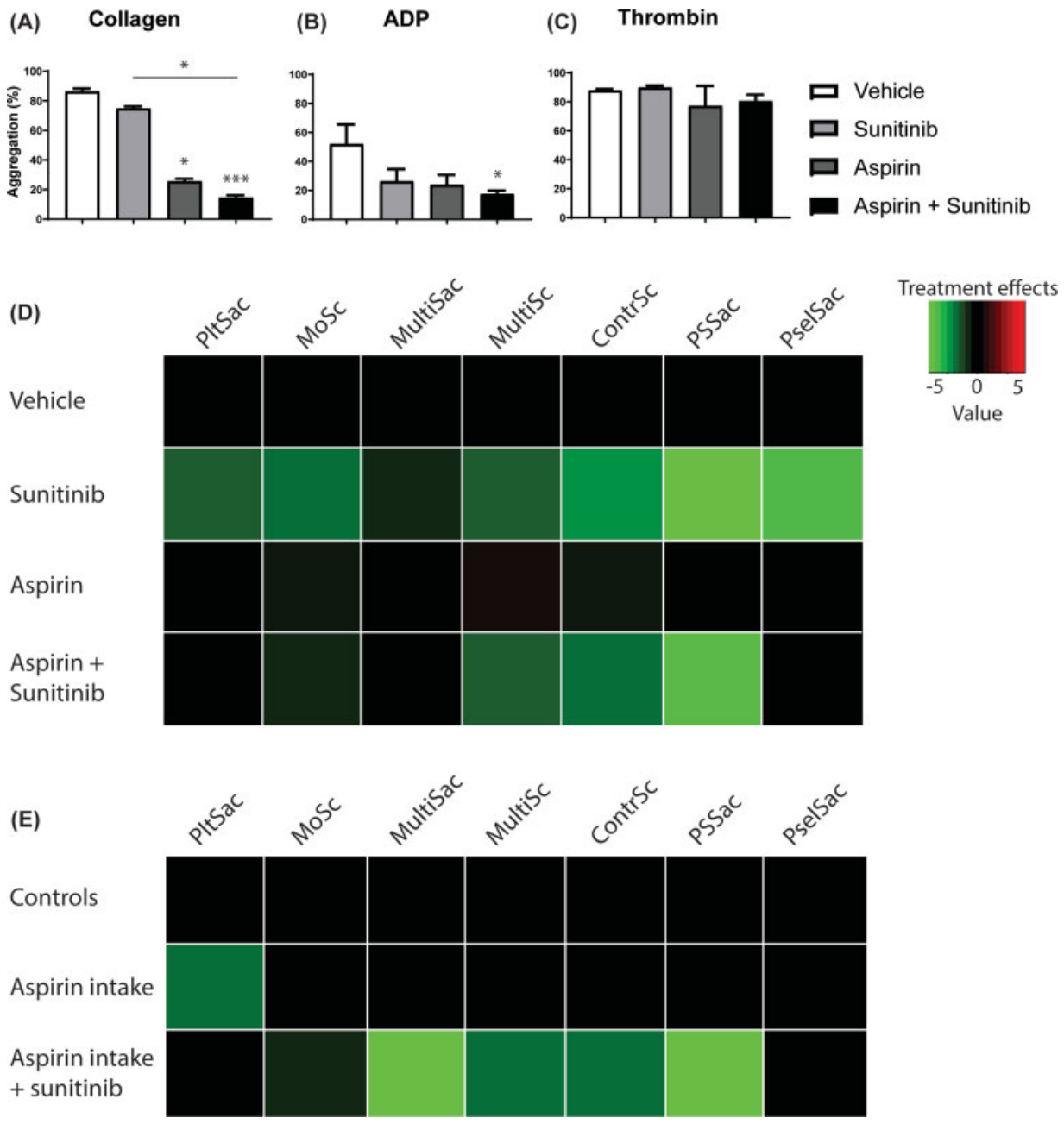

Fig. 3 Aspirin enhances the effect of sunitinib in collagen-induced platelet aggregation and in ex vivo thrombus formation on collagen in whole blood after aspirin intake. Washed platelets (250×10^9/L) were incubated with vehicle (< 0.1% dimethyl sulfoxide [DMSO]), aspirin (100 µM), and/or sunitinib (10 µM). Aggregation of washed platelets was induced by (**A**) 5 µg/mL collagen, (**B**) 1 µM 2-methylthio-adenosine-diphosphate (2MeS-ADP), or (**C**) 1 nM thrombin. Data are represented as means + standard error of the mean (SEM) ($n = 3$), $^{*}p \leq 0.05$, $^{***}p \leq 0.001$. (**D**, **E**) Whole blood from healthy volunteers was preincubated with vehicle (< 0.1% DMSO), sunitinib (30 µM), and/or aspirin (100 µM) for 10 minutes. Recalcified whole blood was perfused over a collagen surface at 1,000 s^{-1} for 3.5 minutes, followed by perfusion with buffer supplemented with AF568-annexin A5 and AF647-α-CD62P to detect phosphatidylserine (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 ►**Supplementary Fig. S4B**. 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 ($p \leq 0.05$).

sunitinib and/or aspirin treatment did not affect the aggregation response elicited by thrombin (►**Fig. 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 (►**Fig. 3D**). No additional effects on thrombus formation in whole blood could be observed when both aspirin and sunitinib were added in vitro (►**Fig. 3D**). Subsequently, we obtained blood from healthy volunteers who had taken a single dose of aspirin (100 mg) 1 day before. Treatment with aspirin inhibited

platelet deposition as compared to controls without aspirin intake (►**Fig. 3E**, ►**Supplementary Fig. S4**, available in the online version). Addition of sunitinib to blood from aspirin-treated donors further enhanced the effects that were observed with aspirin alone (►**Fig. 3E**). Especially thrombus height (multilayer) and PS exposure were reduced when both drugs were present, as compared to aspirin alone (►**Fig. 3E**). Altogether, these results suggest that dual treatment with antiplatelet therapy and sunitinib increases the inhibition of platelet function.

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 labeled 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 to 8 minutes (►**Fig. 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 (►**Supplementary Fig. S5**, available in the online version). 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 (►**Fig. 4A, B**). Furthermore, a slight decrease in PS exposure as well as a delay in fibrin formation was observed upon aspirin intake (►**Fig. 4**). These effects were further reduced by in vitro addition of sunitinib (►**Fig. 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 patients under noncoagulating 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 (►**Supplementary Table S1**, available in the online version). 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 labeling 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 to 8 minutes, comparable to healthy controls (►**Supplementary Fig. S6A, S6B**, available in the online version). Three patients were treated with anticoagulant or antiplatelet drugs (►**Supplementary Table S1**, available in the online version), but this did not affect thrombus/fibrin formation as 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 (►**Fig. 5A** and ►**Supplementary Fig. S6A, S6B**, available in the online version). To visualize the overall effects of sunitinib in blood from cancer patients, we generated a heatmap (►**Fig. 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 may result in different cancer-specific responses. Therefore, we also generated a heatmap with the patients averaged per cancer type (►**Fig. 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 (►**Supplementary Fig. S6C**, available in the online version). 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 where 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 (►**Fig. 5C** and ►**Supplementary Fig. S6C** [available in the online version]) than in healthy controls (►**Supplementary Fig. S5**, available in the online version).

Discussion

In the present study, we further explored the multitarget 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 PS, 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

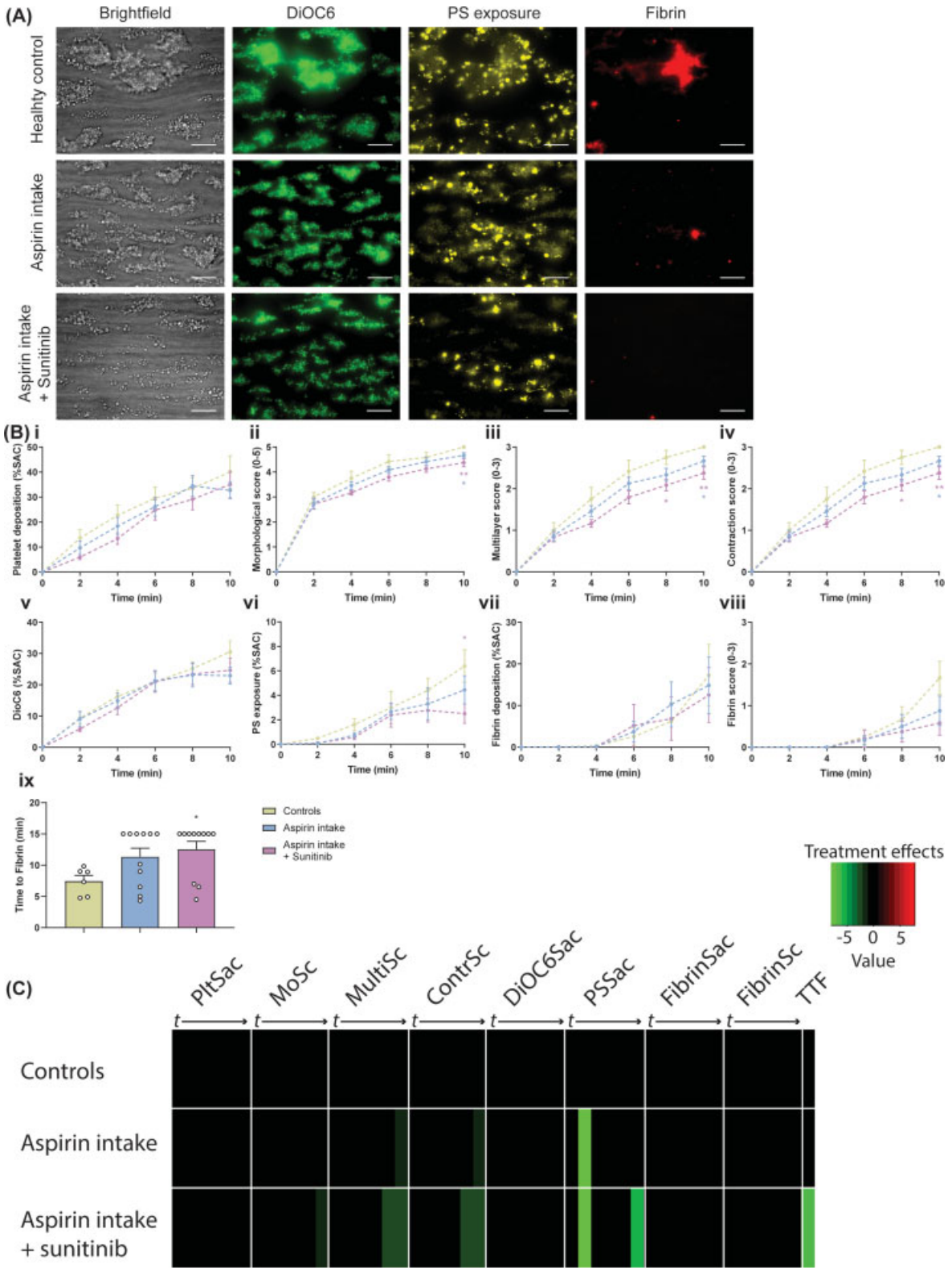

Fig. 4 Aspirin partially enhances the inhibiting effect of sunitinib in platelet-dependent fibrin formation under flow. Citrated whole blood of healthy volunteers with single dose of aspirin was treated in vitro with vehicle (< 0.1% dimethyl sulfoxide [DMSO]) or sunitinib (30 μM) for 10 minutes followed by addition of DiOC6 (platelet deposition), AF568-annexin A5 (phosphatidylserine [PS]-exposure), and AF647-α-fibrinogen (fibrin). Blood was coperfused with $CaCl_2/MgCl_2$ over collagen type I at a wall shear rate of 1,000 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 + standard error of the mean (SEM) ($n = 6$), $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. (**C**) Heatmap representing the significant effects of normalized values of parameters analyzed 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 ($p \leq 0.05$).

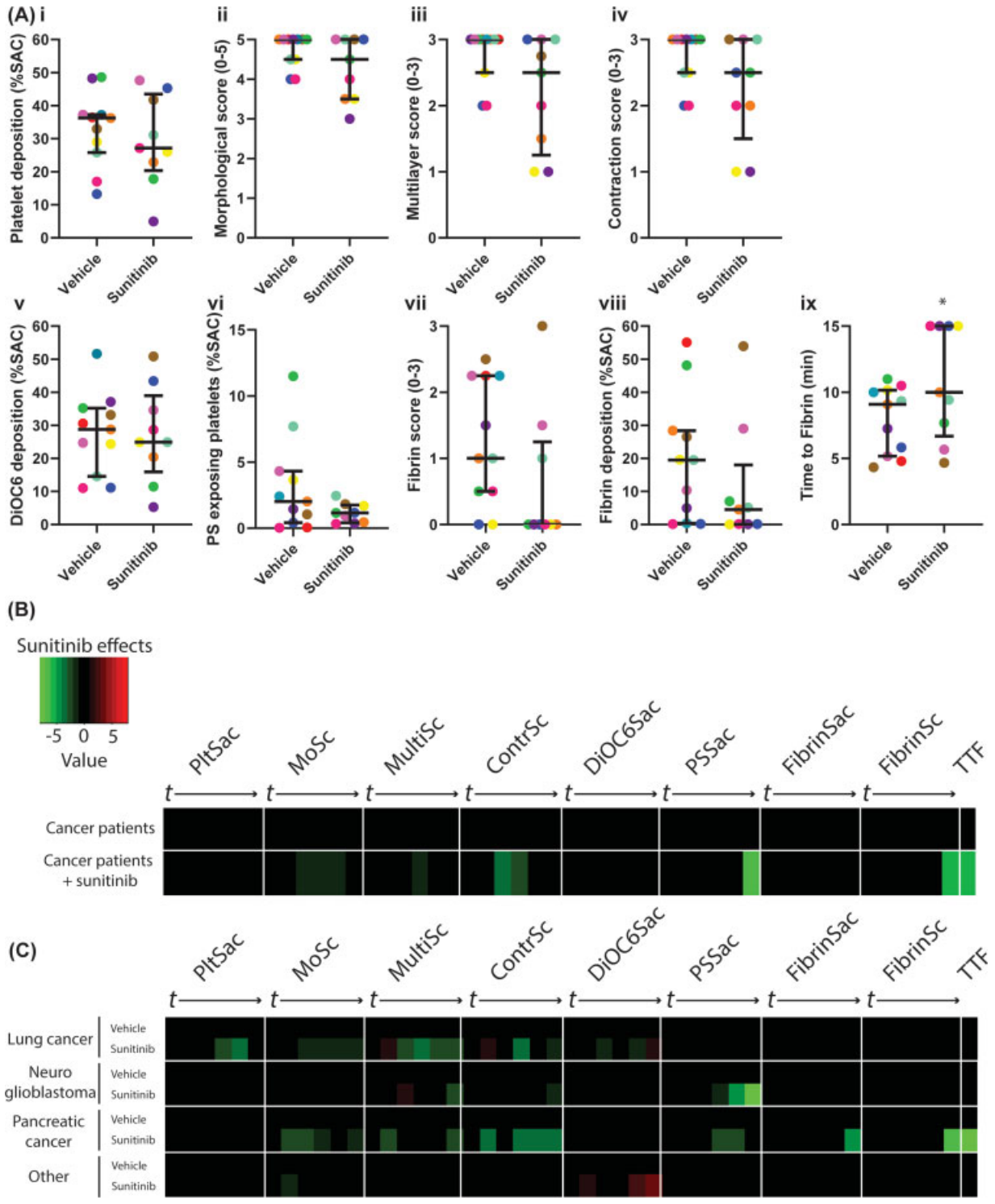

Fig. 5 Sunitinib reduces platelet-dependent fibrin formation under flow in whole blood from cancer patients. Citrated whole blood from cancer patients (►**Supplementary Table S1**) was treated in vitro with vehicle (<0.1% dimethyl sulfoxide [DMSO]) or sunitinib (30 μM) for 10 minutes, followed by addition of DiOC6 (platelet deposition), AF568-annexin A5 (phosphatidylserine [PS]-exposure), and AF647-α-fibrinogen (fibrin). Blood was coperfused with $CaCl_2/MgCl_2$ over collagen type I plus tissue factor (TF) at a wall shear rate of 1,000 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 (color-coding in ►**Supplementary Table S1**), $^{*}p \leq 0.05$. (**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 ►**Supplementary Fig. S7**. 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 ($p \leq 0.05$). (**C**) Subtraction heatmap of the effect of sunitinib per cancer type. Effects were filtered for changes greater than the 1× standard deviation (SD) range of the different platelet parameters.

sunitinib is a multitarget 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 signaling 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 signaling 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 signaling, while GPVI signaling is more dependent on Src.[25] Inhibition of Src by sunitinib upon GPVI stimulation was more pronounced as compared to inhibition of Syk (►**Fig. 2A**), suggesting that the effects of sunitinib on CLEC-2 signaling 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 $\alpha_2\beta_1$ signaling. 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 multitarget nature of sunitinib as a broad-spectrum protein TKI, having lower affinity for multiple TKs rather than high affinity for one specific signaling 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 signaling, 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 signaling cascade used by the collagen receptor GPVI, for example, 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 antiplatelet 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 antiplatelet 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 require further study. Also, the combined effects of sunitinib and aspirin or other antiplatelet 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 tumor-induced changes in platelet proteins and messenger ribonucleic acid are tumor-type specific,[49,50] hence we hypothesize that these specific tumor-induced changes may influence the platelet response to inhibiting drugs.

Altogether, we conclude that sunitinib inhibited GPVI-induced phosphorylation of TKs (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 antiplatelet drugs.

What is known about this topic?

- Sunitinib is a broad-spectrum tyrosine kinase inhibitor used for cancer treatment, with mild bleeding as a reported side effect.
- Platelets sequester sunitinib in vitro as well as in cancer patients.
- Sunitinib affects collagen-induced platelet activation under non-coagulating conditions.

What does this paper add?

- In coagulating whole blood under flow, sunitinib suppresses collagen-induced platelet procoagulant activity and delays fibrin formation.
- Dual treatment of platelets with sunitinib and aspirin further enhanced these processes.
- Platelet-dependent fibrin formation in whole blood from cancer patients was significantly inhibited by sunitinib.

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Conflict of Interest
J.W.M.H. is a cofounder and shareholder of FlowChamber B.V. The other authors declare no relevant conflicts of interest.

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