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The renin-angiotensin system in healthy human platelets: expressed but inactive

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ABSTRACT

Platelets play a crucial role in arterial thrombus formation, offering potential for new antiplatelet therapies with reduced bleeding risk. Here, we investigated the role of the renin-angiotensin system (RAS) in human platelets and explored its potential link to COVID-19 coagulopathy. Experiments were performed *ex vivo* on healthy human platelets. The expression of RAS receptors (Mas, MrgD, ACE, ACE2, AT1 and AT2) was evaluated using western blot and immunofluorescence. Platelets were incubated *in vitro* with either Captopril or different RAS peptides including Alamandine, Angiotensin-I, Angiotensin-II, Angiotensin-(1–7), and Angiotensin-(1–9). Platelet adhesion was measured by spectrophotometry using BCECF fluorescence. Platelet activation and aggregation were analyzed using aggregometry after stimulation with extracellular matrix proteins. ACE and ACE2 activity were assessed using Fluorescent Peptides (FPS). We demonstrated that healthy human platelets express all the tested RAS receptors. However, RAS peptides did not modulate platelet adhesion or aggregation despite a wide range of concentrations tested. ACE activity was detected in platelet lysates, but it was not inhibited by Captopril, while ACE2 activity was undetectable. Our findings suggest that while RAS receptors are expressed in platelets, RAS peptides do not impact platelet function, at least in our experimental setting. COVID-19 coagulopathy may occur independently of the RAS.

PLAIN LANGUAGE SUMMARY

Platelets play a crucial role in the formation of a blood clot after vessel injury. However, blood clots can also obstruct arteries (= arterial thrombosis), resulting in heart attack, stroke, or peripheral ischemia. As platelets play such an important role in the pathological development of arterial thrombosis, antiplatelet therapies aim to reduce the risk of recurrence of these diseases. Antiplatelet therapies usually work by targeting specific receptors to reduce platelet activity, i.e. platelet adhesion, aggregation and activation. As current antiplatelet agents can cause bleeding, research aims to develop new strategies to target only pathological platelet conditions. In this study, we tried to characterize the effect of different peptides belonging to the renin-angiotensin system (RAS) on platelets. As the RAS plays an important role in blood pressure regulation, we sought to establish whether it also plays a role in platelet activity. Furthermore, as SARS-CoV-2 usually enters cells via ACE2, an enzyme belonging to the RAS, we questioned whether the RAS could play a role in peripheral thrombosis associated with severe COVID-19 disease (COVID-19 coagulopathy). We observed that RAS receptors are expressed by platelets, while RAS peptides did not affect platelet adhesion, aggregation, and activation in our experiments. Consequently, we do not think that the RAS plays a significant role in platelet dynamics and COVID-19 coagulopathy.

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Introduction

Platelets play a critical role in hemostasis and blood clot formation, and are strongly involved in arterial thrombosis, leading to ischemic heart disease and stroke.[1–4] While antiplatelet therapies are effective in secondary prevention of acute ischemic events and during percutaneous interventions, cardiovascular diseases remain the leading cause of death worldwide.[5] A major challenge with current antithrombotic agents is the risk of a potentially life-threatening bleeding. Therefore, there is a pressing need for new therapeutic strategies that prevent thrombosis while minimizing bleeding risk.

In search of new antiplatelet therapies, platelet physiology has been extensively studied and is commonly separated in platelet adhesion, activation and aggregation. Upon vascular injury, platelets interact with the exposed extracellular matrix (ECM) mainly through GPIb-IX-V, GPVI, and α2β1, leading to platelet adhesion and subsequent activation. Platelet activation triggers shape change, granule release, and the recruitment of additional platelets through signaling pathways involving ADP, thromboxane A2, collagen, and thrombin. Aggregation is mediated by the GP IIb/IIIa receptor, which binds fibrinogen, linking platelets together to form a stable thrombus.[6]

Since the discovery that platelets are sensitive to Angiotensin (Ang) II,[7] research has also focused on the relationship between the renin-angiotensin system (RAS) and platelets. The RAS is a peptidergic hormone system that regulates blood pressure, fluid balance and systemic vascular resistance. The RAS exerts its effect mainly via Ang-II and its receptors AT1 and AT2, but also involves alternative pathways such as the ACE2/Ang-(1–7)/MAS-axis,[8,9] and the Alamandine/MrgD-axis.[10] As some of these RAS peptides also appear to have effects on platelet aggregation,[11,12] the RAS could represent a potential target for novel antiplatelet drugs.[13,14]

This study aims to demonstrate the presence of various RAS receptors and enzymes on healthy human platelets, and to characterize the *in vitro* effects of RAS peptides on platelet function, particularly aggregation and adhesion.

Materials and method

Experiments were performed *ex vivo* on healthy human platelets. Initial experiments aimed to demonstrate the presence of RAS receptors on platelets (western blot and immunofluorescence). Subsequent experiments aimed to assess the impact of RAS peptides and Captopril on platelet function (aggregation and adhesion). Finally, the extra- and intracellular activity of ACE or ACE2 was assessed using platelet lysates and fluorescent peptides. Figure 1 shows a graphical representation of the methodology used to isolate platelets and assess platelet function.

Platelet isolation

Washed platelets (WP) were prepared as previously described.[15] Blood was collected from healthy volunteers (who had not taken any medication in the previous 10 days), using a 10 ml tube (SARSTEDT, S-Monovette) with a 6:1 ratio of whole blood to ACD (117 mM sodium citrate, 78 mM citric acid, and 282 mM dextrose). Platelet-rich plasma (PRP) was obtained by centrifugation at 250 g for 2 × 15 min at room temperature (ALC® PK 121 R). Platelet-poor plasma (PPP) was obtained by centrifugation at 1125 g. To prepare WP, aliquots of PRP (1 ml) were distributed in 2 ml centrifuges tubes containing 22.5 µM Prostaglandin E1 (PGE1, Enzo Life Sciences, BML-PG006-0010) and centrifuged at 10 000 g for 30 sec (Eppendorf ® miniSpin plus). The platelet pellet was resuspended in magnesium- and calcium-free Tyrode buffer at pH 6.2 (17 µM PGE1, 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, 10 mM Hepes, 1.25 mM $NaHCO_3$, and 5.6 mM dextrose). Centrifugation and resuspension were repeated twice. Finally, the platelets were resuspended in Tyrode buffer, pH 7.4, with 2 mM $CaCl_2$ and 1 mM $MgCl_2$. In accordance with the Helsinki Declaration, all participants provided informed consent, and the Swiss ethics committee approved all protocols (project-ID 2017–00732).

Western blot

Platelet lysates (50 µg) were loaded on Mini-PROTEAN TGX pre-cast electrophoresis gels (BioRad, USA). Proteins were subsequently transferred to a PVDF membrane by semi-dry transfer according to the

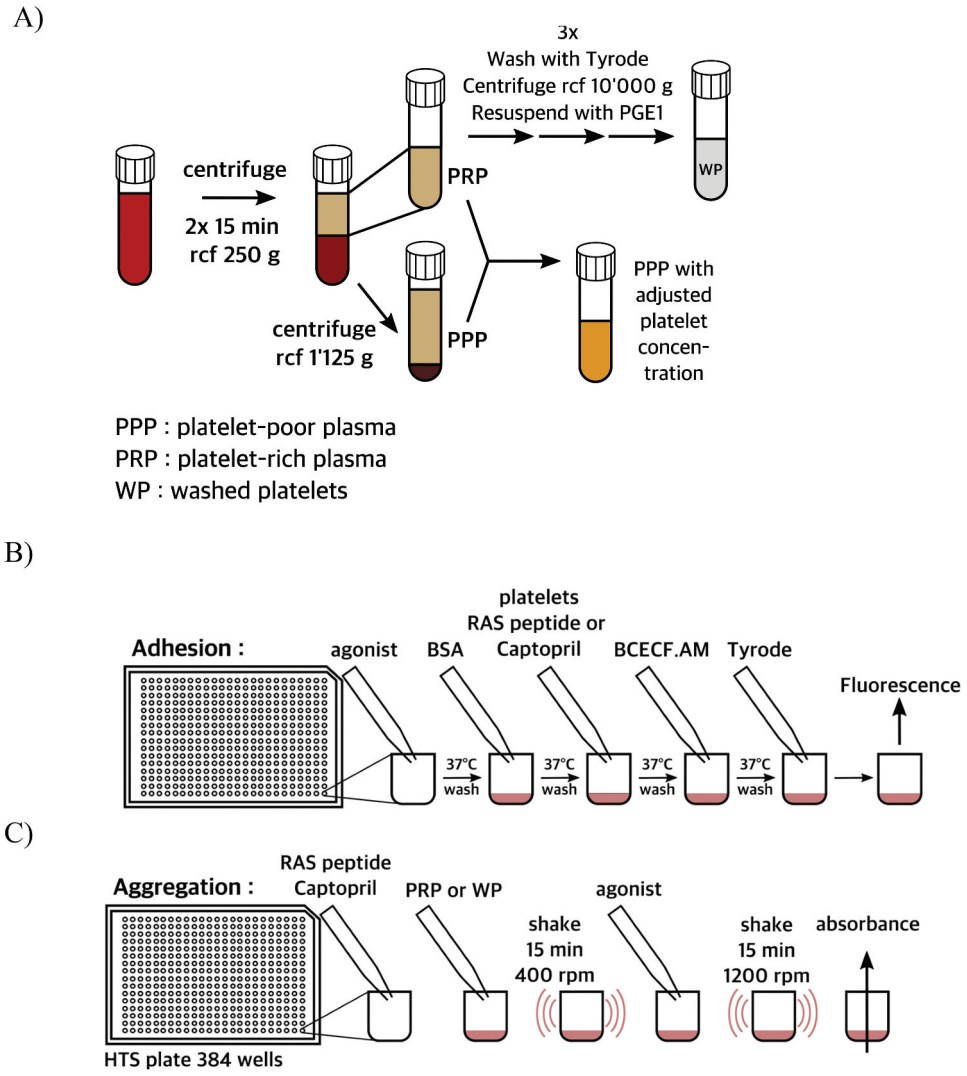


Figure 1. Main experimental protocols. A) platelet isolation, B) adhesion study design, C) aggregation study design. PRP: platelet-poor plasma, PRP: platelet-rich plasma, WP: washed platelets, rpm: revolutions per minute, rcf: relative centrifugal force, BSA: bovine serum albumin, RAS: renin-angiotensin system, HTS: high-throughput screening.

manufacturer recommendations (BioRad, USA). The membrane was incubated in a blocking solution (Odyssey Blocking Buffer PBS – 927–40000) for 1 hour at room temperature followed by overnight incubation at 4°C under stirring, with a primary antibody diluted in 1/3 blocking solution + 2/3 TBST. After incubation, the membrane was washed three times for 10 min in a solution containing TBS added to 0.1% Tween 20 (TBST) and incubated for 2 h under stirring and at room temperature with a secondary antibody, diluted in 1/3 blocking solution + 2/3 TBST. The membrane was rewashed (five times for 10 min) from the excess antibody and the bands were detected using fluorescence and revealed by Odyssey Imaging System (Li-Cor Biosciences). The antibodies used were anti-ACE (Santa Cruz -20 791), anti-AT1 (abcam 18 801), anti-AT2 (abcam 92 445), anti-ACE2 (abcam 15 348), anti-MAS (abcam 66 030), anti-MrgD (abcam 155 099), anti-GAPDH (Santa Cruz 47 724). Secondary antibodies used were iRDye 680RD Donkey Anti-Rabbit (LiCor) and iRDye 800CW Donkey Anti-Goat (LiCor), both of them at the concentration of 1:6000.

Immunofluorescence

Uncoated Ibidi slides (12 Well Chamber, removable 81 201) containing WP at the concentration of 80 000/µl were incubated at 37°C – 5% CO_2 for 1.5 hours. Non-adherent platelets were washed out with Tyrode buffer at 37°C. Platelets were fixed with cold 1% paraformaldehyde for 30 min at 4°C followed by washing

with PBS (three times, 10 min intervals). Platelets were permeabilized with 0.1% Triton 10 for 5 minutes. A control condition with no permeabilization was performed, with PBS instead of Triton. After permeabilization, platelets were washed three times with PBS and 0.1% BSA for 10 min. Nonspecific binding was blocked with 5% BSA for 1 hour at room temperature. Platelets were then incubated for 2 hours at 37°C with primary antibodies against human ACE (Santa Cruz 20 791), AT2 (SAB2900440), ACE2 (Santa Cruz 20 998), MAS (abcam 66 030), MrgD (Thermo Fisher Scientific PA5–33942), GpIb (Thermo Fisher Scientific MA511642), diluted in 2% BSA. After incubation, the samples were washed with 2% BSA and incubated with 1:300 diluted Alexa Fluor 488 and 555 (Invitrogen, A21206 and A21422, respectively) secondary antibodies for 2 hours at room temperature. After incubation, platelets were washed with 2% BSA and PBS and chambers were mounted with an aqueous mounting medium. Images were acquired with Nikon Eclipse Ti2 confocal microscope 60x objective.

Adhesion assay

The adhesion assay followed a similar method as previously described by our lab.[16] Briefly, the wells of a 384-well plate (Greiner Bio-One 781 186) were coated with ECM proteins: Collagen-related peptide (CRP, 10 µg/mL, University of Cambridge), non-fibrillar Collagen I (8 µg/mL, Sigma, C7661), Collagen III (8 µg/mL, Sigma, C4407), Laminin 411 (15 µg/mL, BioLamina, LN411–02), Laminin 511 (10 µg/mL, BioLamina, LN511–02), Fibrinogen (1 mg/mL, Sigma, F8630) and Fibronectin (20 µg/mL, Sigma, F4759). To prevent nonspecific adhesion of platelets to plastic, BSA 0.03% (Sigma, A7906) was added and incubated for 1 hour at 37°C. The plate was refrigerated until the platelets were isolated, or frozen if the experiment was performed another day. Platelets (80,000/µL) and the peptides were added to each well and incubated for 1 hour at 37°C to allow adherence of the platelets. BCECF.AM solution (4 µg/mL, Sigma, B8806) was added and the plate was incubated for 30 minutes at 37°C. Fluorescence was measured with a microplate reader spectrophotometer (PerkinElmer, Victor X3) with 485 nm as the excitation wavelength and 535 nm as the emission wavelength.

Aggregation assay

Either 10 µl of WP (450'000/µl) or 20 µl of PRP (Tyrode buffer or PPP for the control) were added to each well of a 384-well plate (GreinerBio), containing 10 µl of peptide dilutions : Captopril (1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM), Ang-I (10 aM, 100 aM, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM), Ang-II (100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM), Ang-(1–7) (100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM), Ang-(1–9) (100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM), Alamandine (100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM). The preparation was incubated for 15 minutes, at 400 rpm at room temperature, and then stimulated with 10 µl of different ECM proteins: fibrillar collagen 1 (6.67 µg/ml for WP, 5 µg/ml for PRP, Mölab 0203009), Thrombin (0.83 U/ml, Sigma, T7009), Fibrinogen (0.42 mg/ml, Sigma, F8630), ADP (0.31 µg/ml, Sigma, A2754), Epinephrin (0.63 µg/ml, Sigma, E4250), Collagen-related peptide (0.83 µg/ml for WP, 0.63 µg/ml for PRP, University of Cambridge). The plates were stirred for 15 minutes at 1200 rpm at room temperature (Eppendorf, Mixmate). Platelet function was measured using a plate reader (PerkinElmer, Victor X3) with the absorbance parameter at wavelength 595 nm for the PRP condition and 405 nm for the WP condition. Results were compared to a positive stimulated control with Collagen-I (5 µg/ml for PRP, 6.67 µg/ml for WP).

FPS-based activity assay

WP were centrifuged for 30 seconds at 10 000 rpm and the supernatant was discarded. 150 µl of RIPA Lysis Buffer (150 mM Sodium Chloride, 1% NP-40 or Triton x-100, 0.5% Sodium Deoxicholate, 0.1% SDS) was added, and the samples were incubated overnight at 4°C under constant agitation. After incubation, samples were centrifuged at 12 000 rpm for 20 minutes at 4°C, and the supernatant was collected and kept on ice. The Assay Buffer (100 mM TrisBase, 50 mM NaCl, 10 µM $ZnCl_2$, 10 mM $CaCl_2$, pH 8) was added to a 384-well black plate (Greiner Bio-One 781 900). Captopril dilutions (1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, Sigma, C4042) and platelet lysates (60 µg/ml) were added to the corresponding wells. Finally, the Fluorogenic Substrate FPS (10 µM Mca-RPPGFSAFK(Dnp), BML-P227-0001 - Enzo Life Sciences, to detect

ACE and 40 µM Mca-YVADAPK(Dnp)-OH, ES007 - RnD systems to detect ACE2) was added to the plate. The plates were incubated at 37°C and fluorescence intensity was measured every 300 seconds for 37 cycles, (excitation wavelength: 320 nm and emission wavelength: 405 nm) with a plate reader (VictorX, PerkinElmer).

Statistical analysis

Differences between groups were evaluated by One-way analysis of variance (ANOVA) followed by Dunnett's test post hoc test. A $p < .05$ was considered statistically significant. GraphPad Prism 7.0 (GraphPad Software, Boston, MA, USA) was used to perform statistical analysis.

Results

Human platelets express RAS receptors on the extracellular membrane

To demonstrate the presence of RAS receptors, we performed western blot and immunofluorescence on healthy human platelets. Figure 2A shows a western blot with antibodies directed against MrgD, Mas, ACE, ACE2, and AT2. The bands were detected around 35 kDa for MrgD and Mas, consistent with the reference provided by the antibody manufacturer. ACE2-antibodies formed a band around 100 kDa (predicted non-glycosylated value 92 kDa), which we assigned as possibly partially glycosylated ACE2. The presence of ACE was detected around 70 kDa (predicted value 76 kDa). Finally, the AT2 receptor was detected at 55 kDa, as predicted by the reference. We conducted the same experiment confirming the presence of AT1 (Supplemental Figure S1). To validate these results, an immunofluorescence analysis was performed (Figure 2B) with different antibodies directed at the same RAS receptors. Glycoprotein Ib (GpIb) was used as a positive control to confirm the absence of contamination by other cells in the platelet sample. The fluorescence signals of the GpIb and RAS receptor antibodies co-localize, confirming the presence of these receptors at the surface of platelets. We conducted the same experiment with permeabilized and non-permeabilized platelets to determine whether the RAS receptors are integrated into the membrane or expressed intracellularly. The signal shows similar fluorescence signals between permeabilized and non-permeabilized platelets (Figure 2C). We conclude that RAS receptors MrgD, Mas, ACE2, ACE, AT1, and AT2 are expressed on the extracellular membrane.

Platelet adhesion to ECM proteins is not altered by the RAS

Using BCECF.AM fluorescence assays, we measured platelet adhesion to different ECM proteins (Fibrinogen, Fibronectin, Collagen-I, Collagen-III, Laminin-411, Laminin-511, and CRP) with BSA representing the background signal (non-coated control without ECM) (Figure 3A–F). We first used Captopril and RAS peptides including Alamandine, Ang-I, Ang-II, Ang-(1–7), and Ang-(1–9) to understand if they would affect platelet adhesion without ECM proteins. We observed that none of the peptides altered platelet adhesion (Figure 3A–F, BSA only). We subsequently tested various combinations of RAS peptides and the forementioned ECM proteins. In each case, fluorescence signal was increased as a result of platelet adhesion to the ECM, but the RAS peptides had no additional effect on platelet adhesion.

No significant effect of RAS peptides on platelet aggregation in absence of agonists

Using a high-throughput 384-well plate method, we first measured platelet aggregation of PRP and WP incubated with Captopril, Alamandine, Ang-I, Ang-II, Ang-(1–7), and Ang-(1–9) (Figure 4). The peptides were added to platelets under stirring conditions to determine if they would induce aggregation in the absence of agonists. Tyrode buffer was used as a negative control and Collagen-I as a positive control agonist. Aggregation is represented as normalized absorbance (PPP = 100%). For Alamandine, Ang-(1–7), Ang-(1–9), Ang-I, and Ang-II, the RAS peptides alone do not significantly change baseline platelet aggregation. For Captopril, we observe an aggregation decrease of 20% at the concentration of 10 µM ($p < .05$ for WP). At lower Captopril concentrations, platelet aggregation remains unchanged.

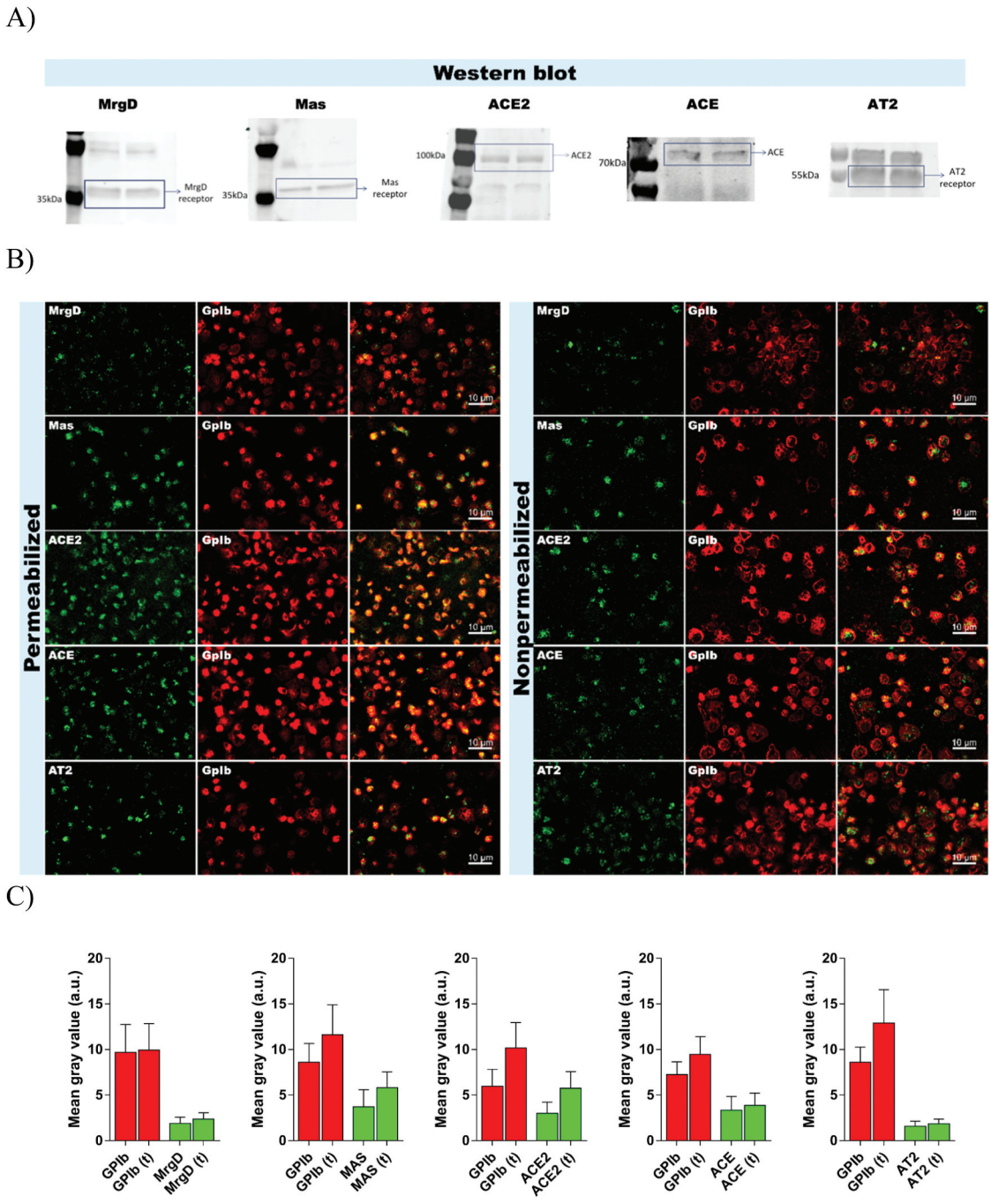
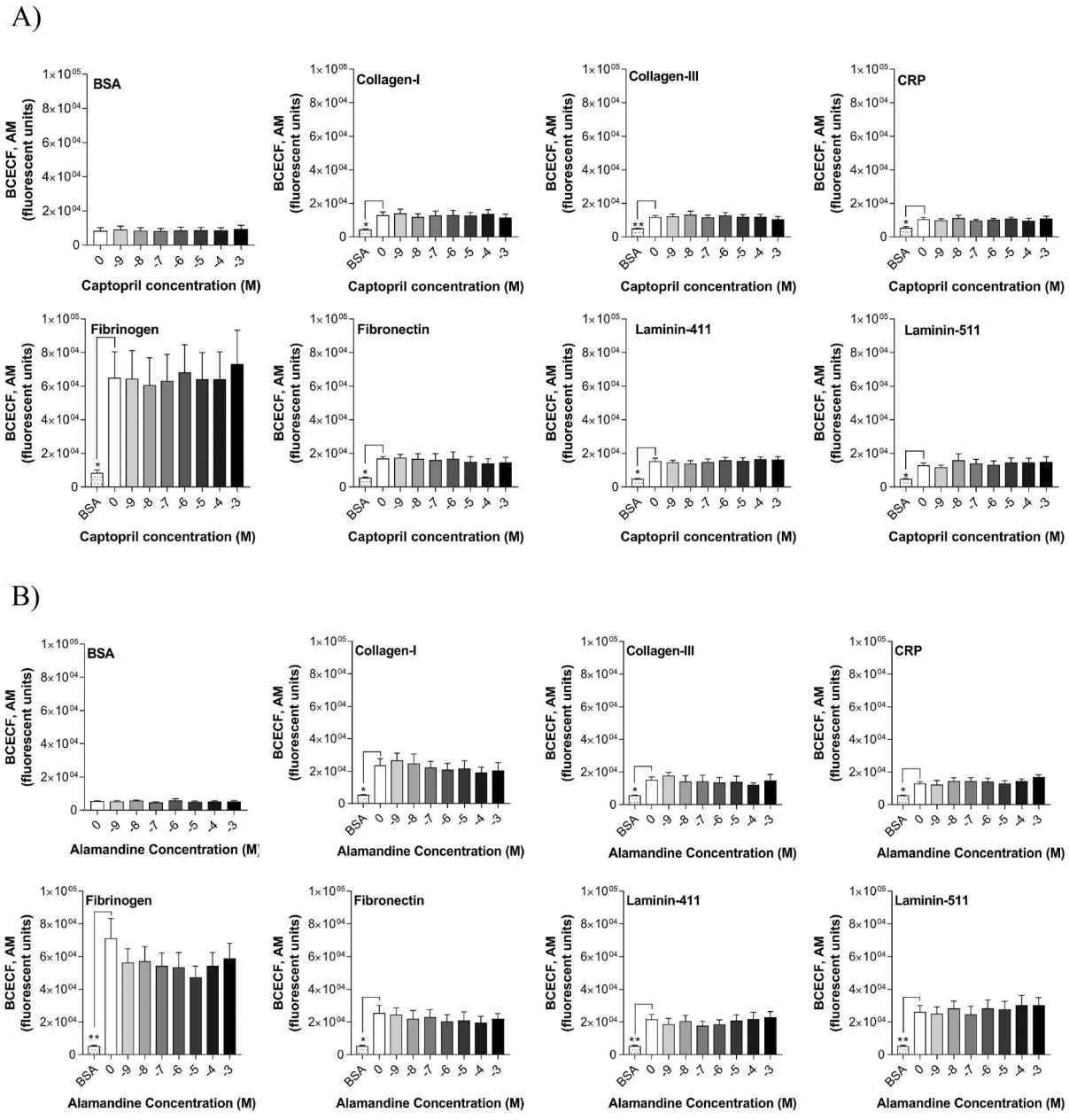


Figure 2. A) expression of MrgD, Mas, ACE2, ACE, AT2 was demonstrated by western blot of human platelet extracts. The two columns represent different runs from the same donor (n=3 donors for AT2, MAS, MrgD and ACE; n=5 for ACE2). Approximate ladder reference molecular weight on the left. B) immunofluorescence analysis confirmed extracellular MrgD, MAS, ACE2, ACE, and AT2 expression on isolated platelets from human blood samples. C) MrgD, MAS, ACE2, ACE, and AT2 show similar immunofluorescence activity between permeabilized and non-permeabilized platelets (mean ± SEM; n = 4).

No effect of RAS on platelet aggregation induced by different ECM agonists

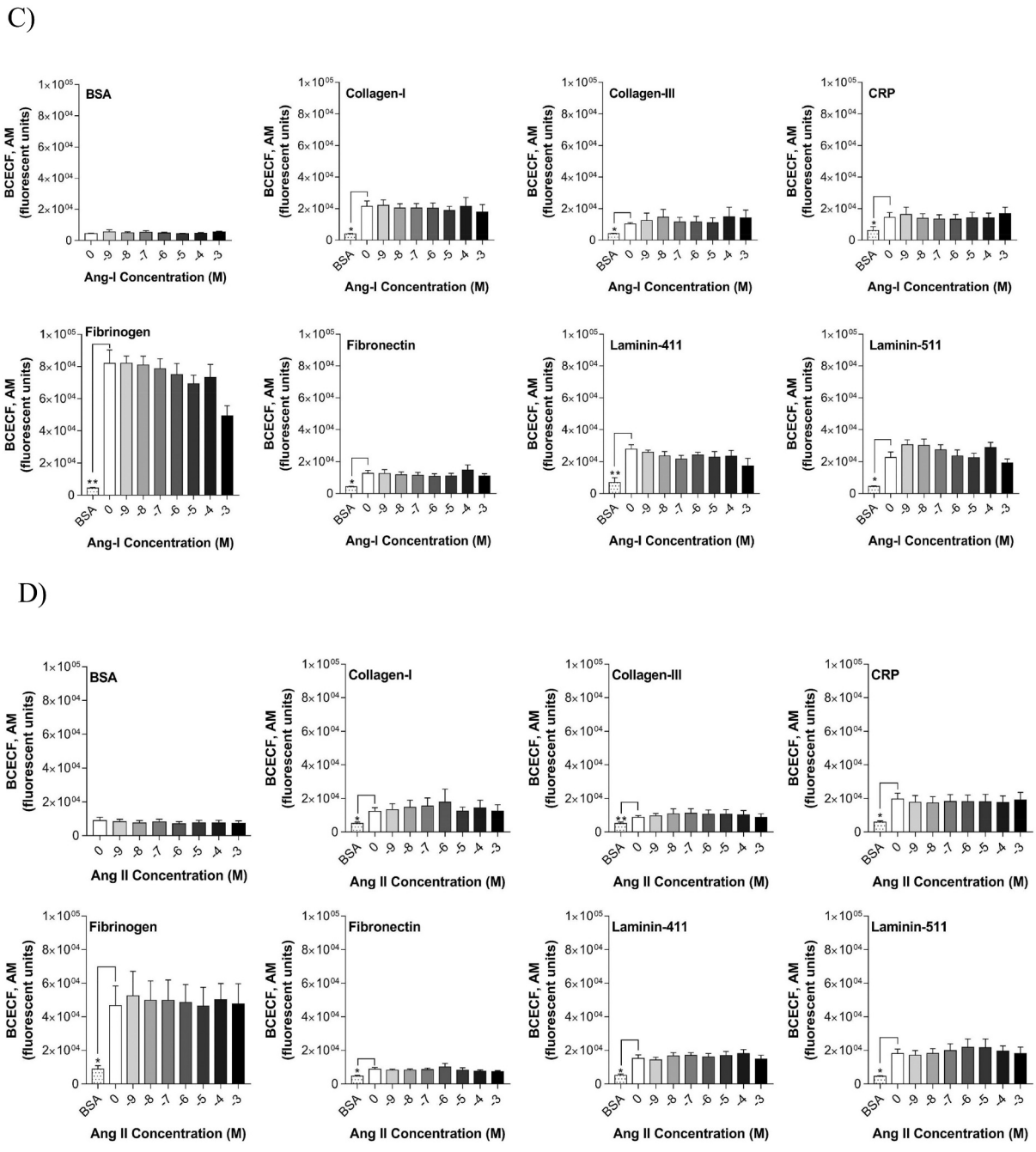
As RAS peptides alone did not affect platelet aggregation in stirring conditions, we investigated whether RAS peptides or Captopril modulate ECM agonist-triggered platelet aggregation. The microwells of a 384-well plate were coated with Captopril, Alamandine, Ang-I, Ang-II, Ang-(1–7), and Ang-(1–9) dilutions (for each 10 µl, 10^{-10} to 10^{-5} M). Tyrode buffer was used as a negative control and Collagen-I as a positive control agonist. Different ECM proteins were used, including Collagen, CRP, Thrombin, and Fibrinogen for



WP conditions, and Collagen, CRP, ADP, and Epinephrine for PRP conditions. Aggregation is represented as normalized absorbance (PPP = 100%). Treating WP with Captopril at a concentration of 10 μM, we observe a decrease of nearly 40% of aggregation induced by Collagen-I ($p < .01$), a decrease of > 50% of aggregation induced by CRP ($p < .05$), and a moderate but significant decrease in platelet aggregation induced by Thrombin ($p < .0001$) (Figure 5A). We did not observe any change in platelet aggregation induced by agonists for Alamandine, Ang-I, Ang-II, Ang-(1–7), and Ang-(1–9) (Figure 5B–F).

No ACE and ACE2 activity on human platelets

To further investigate a potential role of the detected ACE and ACE2 receptors, we performed additional experiments with platelet lysates to measure extra- or intracellular ACE and ACE2 activity (Figure 6). We observed a marked time-dependent increase of ACE activity to about 11 000 fluorescence units. The experiments were repeated with Captopril at increasing concentrations to determine if ACE activity could be blocked. Captopril did not inhibit ACE activity, except at concentrations over 50 mM.



To validate our subsequent conclusion that the measured ACE activity is nonspecific, we assessed cytotoxicity of Captopril and observed that Captopril is toxic for platelets at concentrations of 10 and 100 mM (Supplemental Figure S2). The FPS experiment was repeated for ACE2, but no activity was measured. As a positive control, we used recombinant rACE2 (0.5 ng/µL), for which we obtained a marked increase in fluorescence to about 9000 fluorescence units.

Discussion

In this study, we demonstrated the expression of AT1, AT2, ACE, ACE2, MrgD and MAS on platelets using western blot and immunofluorescence. According to our permeabilized vs. non-permeabilized assay, the receptors are expressed on the extracellular membrane. In previous studies, AT1 and AT2 had already been observed on platelets using 125-I-Ang-II,[17] RT-PCR[18] and western blot.[19] The presence of Mas receptor has

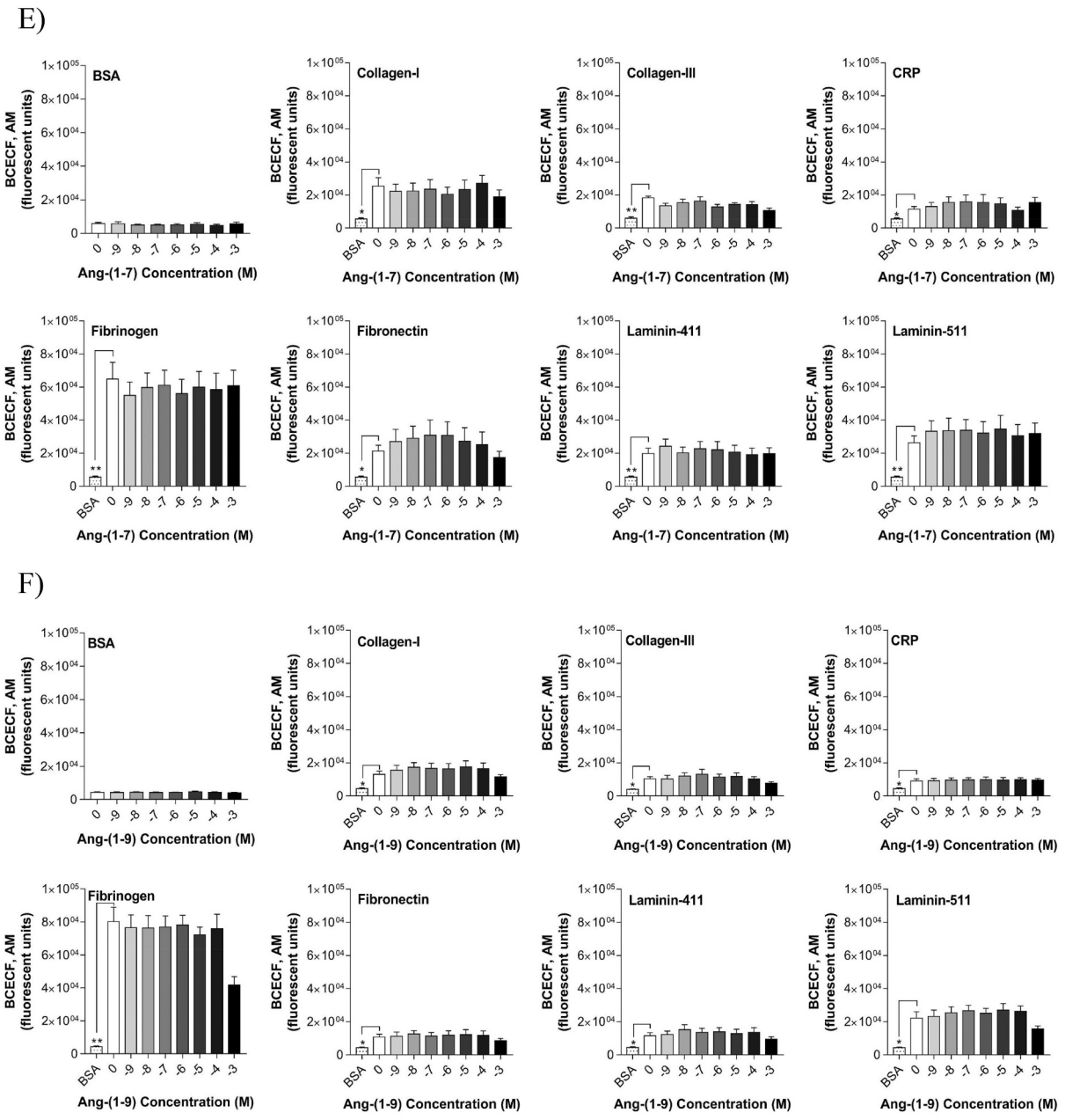


Figure 3. Adhesion assay to ECM proteins of WP treated with: A) Captopril, B) Alamandine, C) Ang-I, D) Ang-II, E) Ang-(1–7), F) Ang-(1–9) (10^{-9} to 10^{-3} M each). The treated platelets were added to wells coated with ECM proteins: fibrinogen (1 mg/mL); Fibronectin (20 µg/mL); non-fibrillar Collagen-I (8 µg/mL); Collagen-III (8 µg/mL); Laminin-411 (15 µg/mL); Laminin-511 (10 µg/mL) and Collagen-related peptide (CRP) (10 µg/mL). The X-axis represents the concentration of Captopril or RAS peptide in 10^{x} M, while the Y-axis represents fluorescence. Absolute fluorescence values were compared with the non-coated control (BSA) condition by one-way ANOVA followed by Dunnett's test. Results are expressed in mean ± SEM, $n = 4$, * $p < .05$, ** $p < .01$, *** $p < .001$ and **** $p < .0001$.

been demonstrated in mice,[11] but not yet in human platelets. MrgD and ACE have not been studied in platelets until now, to our knowledge.

As ACE2 permits cellular entry to SARS-CoV-2,[20,21] a few studies recently investigated the expression of ACE2 on platelets but reported conflicting results. In some studies, ACE2 mRNA was not detected by RNA-seq in platelets from healthy donors and COVID-19 patients.[22,23] Similarly, no expression of ACE2 in healthy human platelets was detected using immunofluorescence assay and western blot.[23,24] On the other hand, other studies detected expression of ACE2 in platelets by RT-PCR (RNA and protein levels) and western blot.[25,26] Campbell and Zaid have tried to find explanations for the contradictory results,

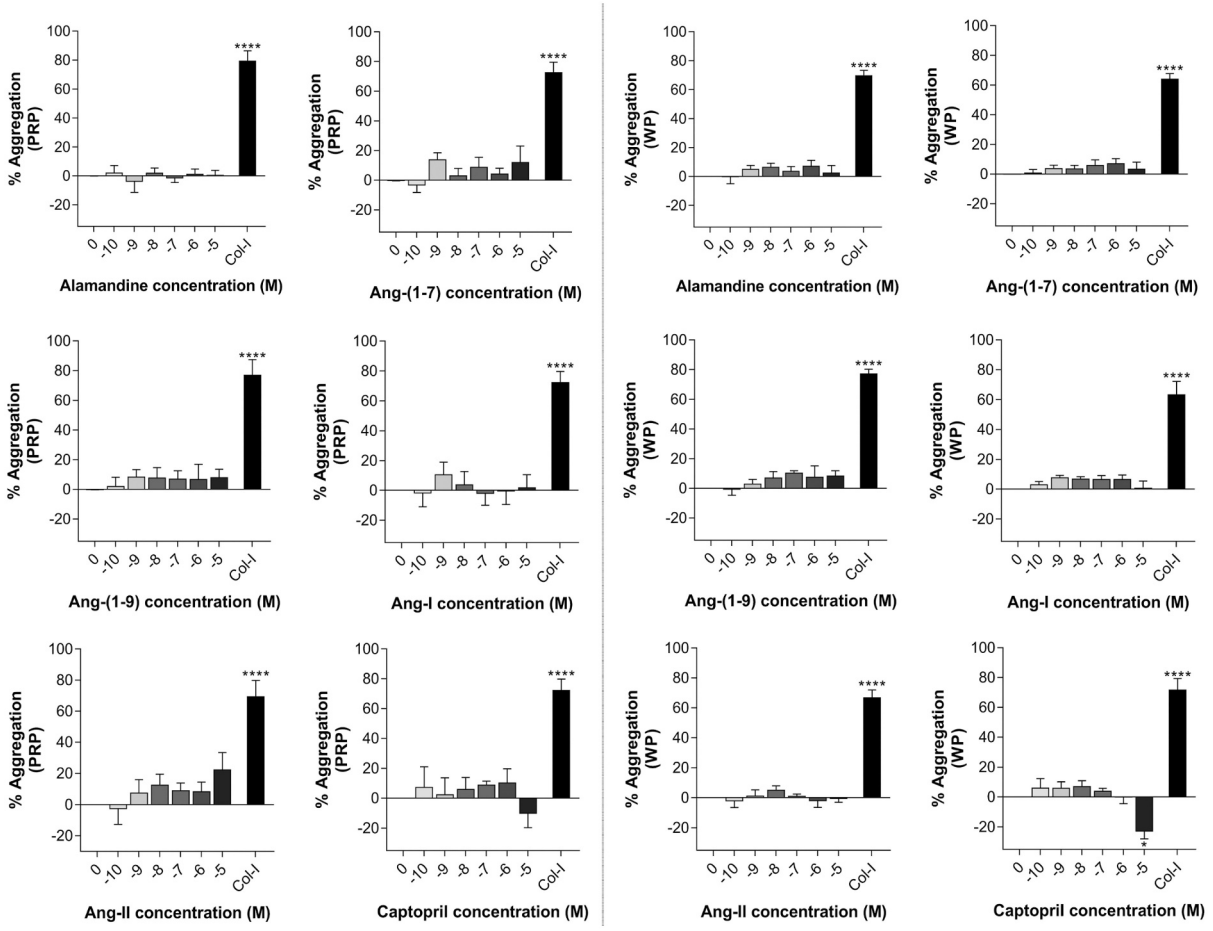
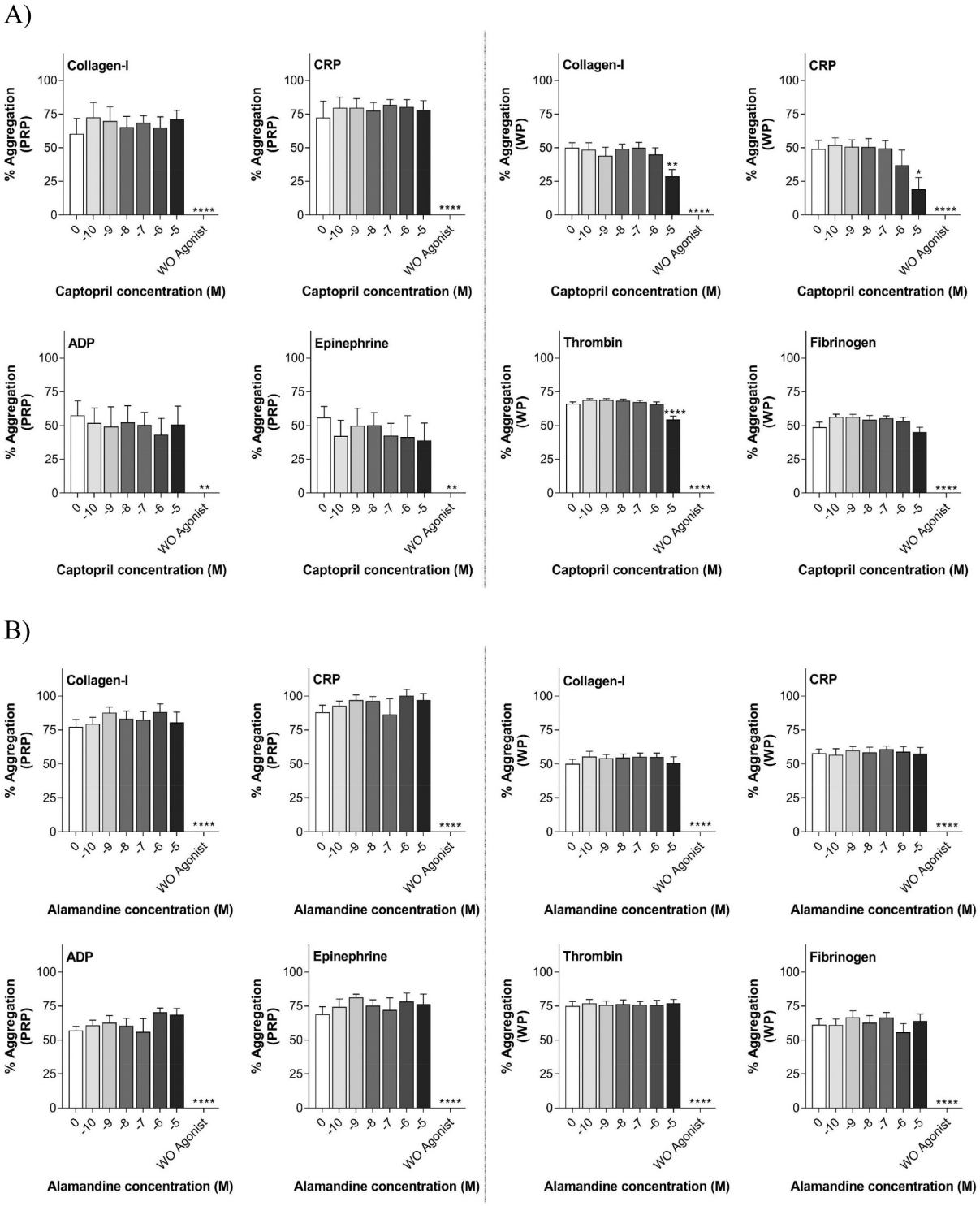


Figure 4. Aggregation assay analysis of PRP is shown on the left panel and isolated washed platelets (WP) on the right panel. PRP and WP were treated with Alamandine, Ang-(1–7), Ang-(1–9), Ang-I, Ang-II and Captopril, and compared with a positive stimulated control with Collagen-I (5 µg/ml for PRP, 6.67 µg/ml for WP). The X-axis represents the concentration of the added RAS peptide or Captopril in 10^x M. The Y-axis represents the mean aggregation using normalized absorbance. The bars represent mean ± SEM, n =4, * $p < .05$, ** $p < .01$, *** $p < .001$ and **** $p < .0001$.

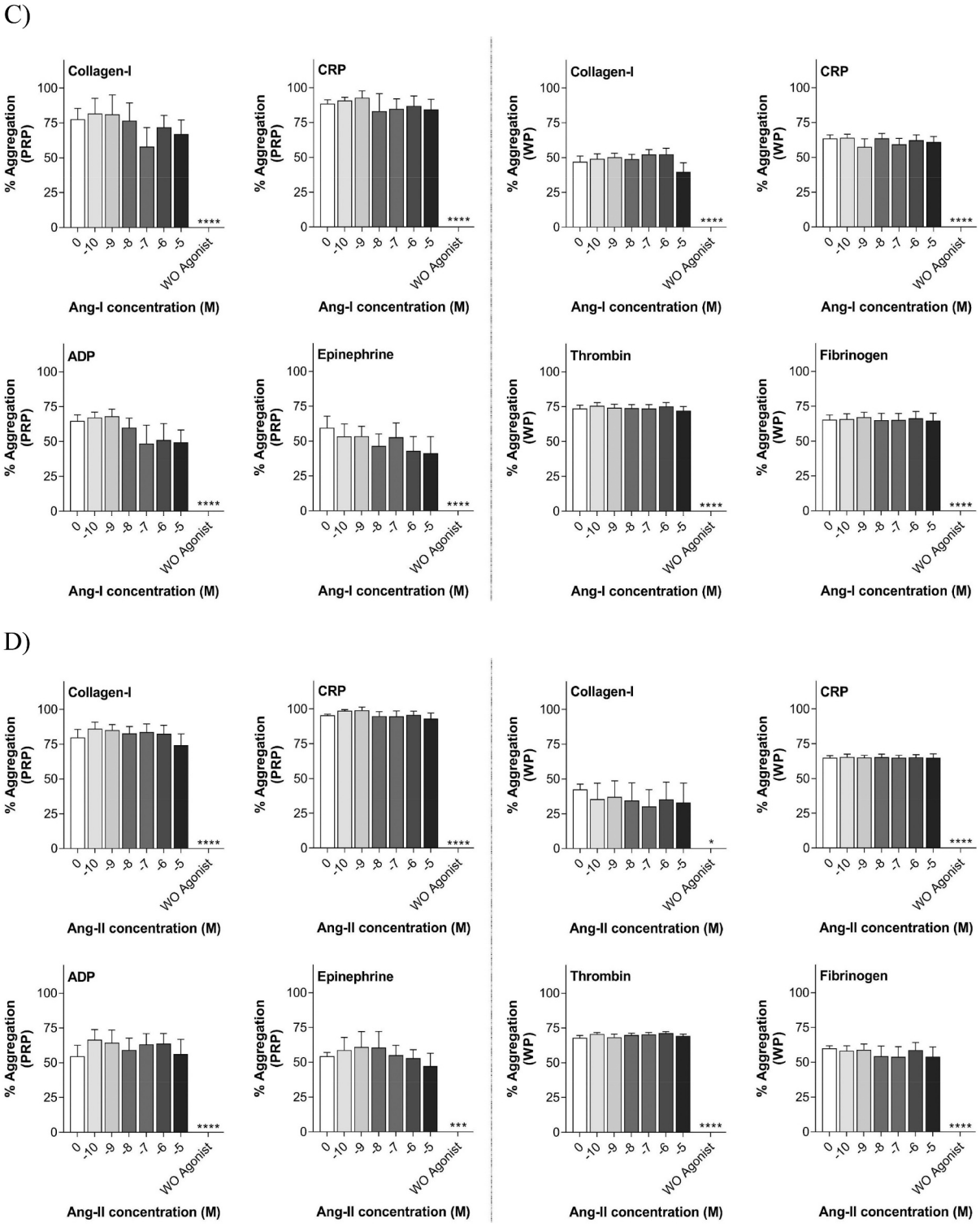
questioning the cell markers used to exclude contamination by white blood cells.[27,28] We confirmed our results using the GpIb receptor which is specific to platelets. In our opinion, the discrepancy between the platelet transcriptome (measured by RNA-seq) and the platelet proteome may also explain the conflicting previous results, as about 16% of the platelet proteome have no corresponding reference mRNA.[29]

In the platelet adhesion assays, we did not observe any significant effect of the RAS on platelet adhesion. Enhanced platelet adhesion to leukocytes and endothelial cells has been observed in mice treated with Ang-II,[30] as well as in AT2-deficient mice.[31] Furthermore, the ACE inhibitor Quinapril reduced the adhesion of hypertensive rat platelets in response to collagen *ex vivo* and *in vitro*, without affecting bleeding time.[32] Interestingly, no effect was observed with platelets from normotensive rats, in accordance with our experiments on healthy human platelets. In another study, treatment of platelets with Ang-(1–7) and Losartan led to reduced Collagen-induced platelet adhesion in hypertensive rats,[33] a result we were not able to confirm in normotensive human platelets. Jiménez et al. observed that Losartan (but not Candesartan and Valsartan) inhibited platelet adhesion alone, as well as U46619 (TxA_2 agonist)-induced platelet adhesion.[34] Given these results, we suggest that Losartan inhibits platelet activation via TxA_2 or AT2.

RAS peptides alone did not induce aggregation in our experiments, as already reported for Ang-II.[35,36] Subsequently, Ang-II did not alter agonist-induced platelet aggregation while previous studies reported conflicting results (Summary table, Appendix). Ang-II has been reported to induce Collagen- and ADP-stimulated platelet aggregation in one study, but unfortunately the authors do not describe the time course



of aggregation, although recorded every 10 seconds.[37] Furthermore, Ang-II was shown to enhance both collagen-induced platelet aggregation measured by TxB_2 production,[38] and thrombin-induced platelet aggregation measured by aggregometry.[39] On the other hand, and in line with our study, numerous other studies did not find any effect of Ang-II on platelet aggregation stimulated by Collagen and ADP.[36,40] From the receptor perspective, Grothusen et al. demonstrated that the ARB Losartan reduces platelet aggregation *in vitro* using PRP from healthy humans.[41] In clinical trials, Valsartan, Candesartan and Losartan showed antiplatelet properties in hypertensive patients.[42,43] However, it has been shown that ARBs also inhibit TxA_2



-induced platelet aggregation,[19,44–47] while Ang-II enhances platelet aggregation induced by U46619, a TxA_2 agonist.[48] Losartan was also found to inhibit collagen-induced platelet activation via GPVI.[49] As our results using Ang-I or Ang-II *in vitro* do not show any effect on platelet aggregation, we propose that the effects of Ang-II and ARBs may be independent of AT1, as previously suggested by Sato et al..[43]

In our study, we also tested Ang analogs, which had no effect on platelet aggregation. Fraga-Silva et al. demonstrated an anti-thrombotic effect in rats and mice,[11] but Ang-(1–7) did not induce platelet aggregation in our experiments, in accordance with known literature.[48] Ang-(1–9) was shown to

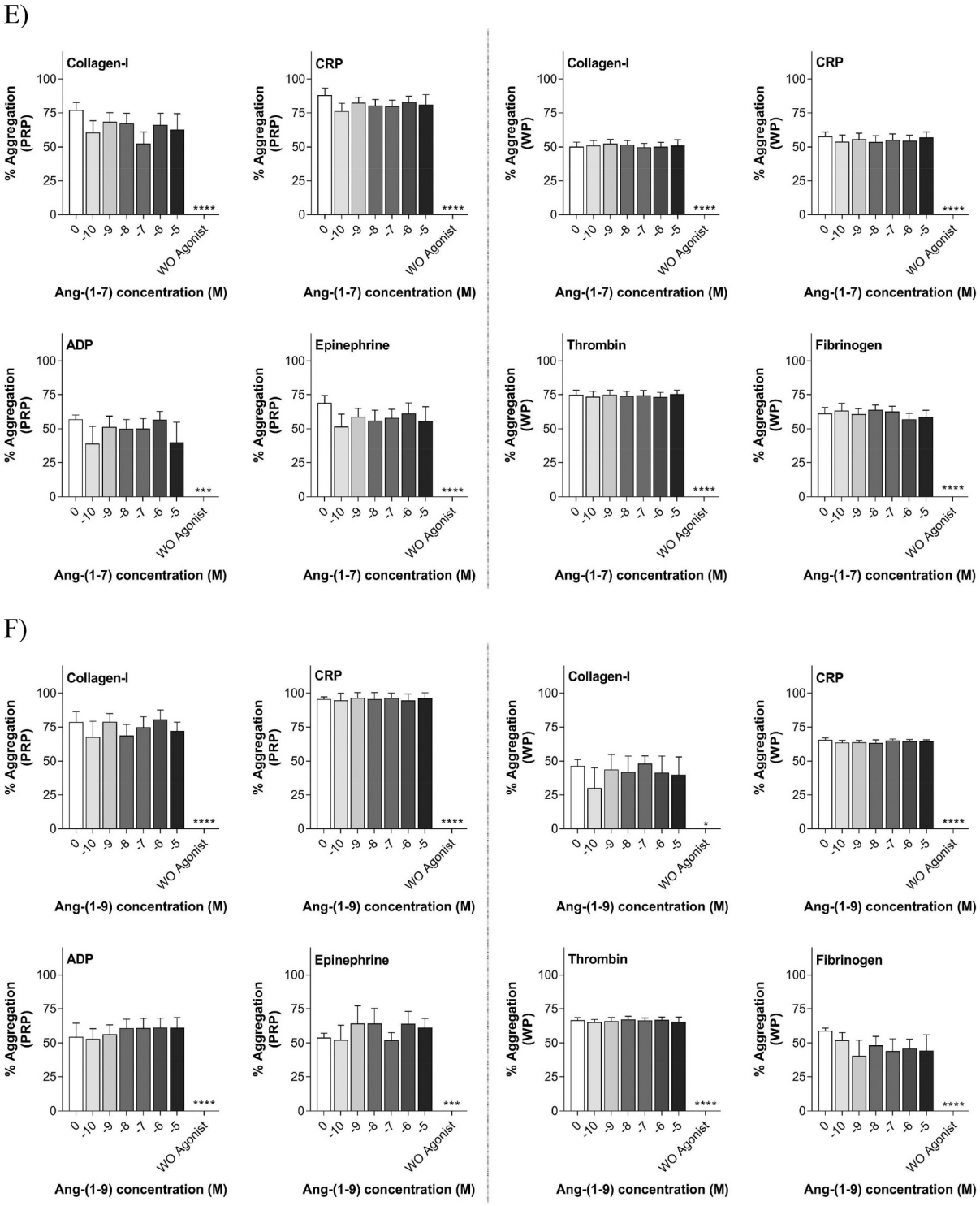


Figure 5. Aggregation assay analysis of WP and PRP pre-incubated with: A) Captopril, B) Alamandine, C) Ang-I, D) Ang-II, E) Ang-(1–7), F) Ang-(1–9) at concentrations of 10^{-10} to 10^{-5} M each and stimulated with Collagen (6.67 µg/ml), CRP (0.83 µg/ml), Thrombin (0.83 U/ml) and Fibrinogen (0.42 mg/ml) for WP, and Collagen (5 µg/ml), CRP (0.63 µg/ml), ADP (0.31 µg/ml) and Epinephrin (0.63 µg/ml) for PRP. The X-axis represents the Captopril or RAS peptide concentration in 10^{x} M. The Y-axis represents the mean aggregation using normalized absorbance. The bars represent mean ± SEM, $n = 4$, * $p < .05$, ** $p < .01$, *** $p < .001$ and **** $p < .0001$.

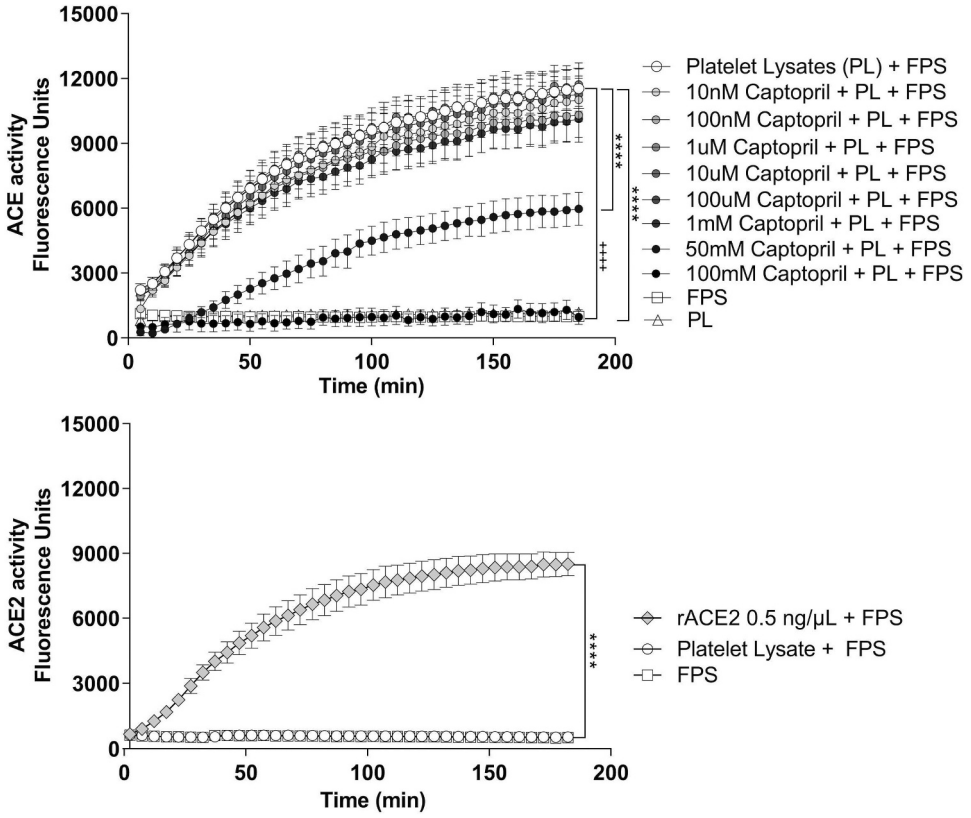


Figure 6. Time courses for FPS-peptide-based assay detecting ACE and ACE2 activity in platelet lysates treated with different concentrations of Captopril at pH 8. (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$, data are mean ± SEM; $n = 4$).

enhance collagen-induced platelet aggregation in rats by Kramkowski et al.[12] an effect we did not observe in human platelets.

In our experiments with the ACE inhibitor Captopril, we noted a decrease in aggregation for platelets treated with Captopril *in vitro*. However, as this was also measured in absence of agonists in stirring conditions, the observed effect does not seem to be linked with the agonists. In other studies, Captopril, Ramipril and Enalapril did not show any effect on ADP-induced platelet aggregation *in vitro*.[40,50,51] By contrast, an inhibitory effect was interestingly noted *in vivo* for Captopril and Ramipril (but not Enalapril).[40,52–55]

We subsequently used fluorescent proteins to measure the activity of ACE and ACE2 on platelets and platelet lysates. The detected ACE activity was not inhibited by Captopril except for cytotoxic concentrations over 50 mM (supplementary data). We therefore conclude that the used FPS was not specific to ACE which is probably inactive in platelets. As this is contradictory to other *in vivo* studies, we suggest that an indirect action mechanism may be responsible for the previously described effect of ACE inhibitors. Finally, ACE2 activity was undetectable and we conclude that ACE2 is present but inactive in healthy human platelets.

While SARS-CoV-2 infection mainly results in a respiratory disease, severe coronavirus disease (COVID-19) can also be associated with systemic coagulopathy. Although numerous pathways seem to be involved in COVID-19-associated coagulopathy, the underlying mechanisms remain complex and the role of the RAS and platelets still uncertain.[56] Various independent groups have demonstrated that SARS-CoV-2 can infect platelets, but it remains debated through which receptor.[22,25,28] It has first been assumed that the main cellular receptor for SARS-CoV-2 is ACE2, but other proposed pathways for SARS-CoV-2 uptake include the αIIbβ3 integrin and TLR4.[24,57] It has been reported that SARS-CoV-2 May directly bind to ACE2 on platelets, while other groups suggested that SARS-CoV-2 May enter platelets independently of ACE2, although increased platelet reactivity was measured.[22,25] In our opinion, as we observed ACE2 expression on platelets, SARS-CoV-2 May infect

platelets via ACE2. However, as RAS peptides had no significant effect on platelet function in our experiments, we do not think that the RAS is the main pathway for platelet hyperreactivity in severe COVID-19 disease. This would be in line with studies that have demonstrated that ACE inhibitors or Angiotensin receptor blockers (ARBs) do not increase the risk of infection nor severe COVID-19 disease.[58,59]

The present study has a number of limitations. As only agonists of the RAS system were used, we cannot exclude that these agonists were degraded by enzymes such as ACE and ACE2. To confirm the pathway of any measurable effect of RAS peptides, further studies should use specific receptor- and enzyme-blockers. Furthermore, our study focuses only on platelets from healthy donors, treated *in vitro*. Multiple study settings need to be established to further characterize a potential effect of RAS peptides. Platelets may be taken from patients with cardiovascular diseases or diabetes. For instance, a reduced density of Ang-II receptor on platelets from patients with insulin-dependent diabetes has been shown[60] and the effect on platelet adhesion by Quinapril was only seen in platelets from hypertensive rats.[61] According to Ding et al, binding of Ang-II to platelets is unchanged in hypertensive patients, but it remains unclear whether other pathways of the RAS system are affected.[62] Platelets from hypertensive patients treated with ARBs, ACE blockers, or aliskiren may also be used for experiments. Such a study would be particularly interesting because ACE blockers have multiple effects on the RAS system, which include binding to platelet receptors like GPVI [63], and intracellular accumulation of Ang-I and Ang-(1–9) among others. Furthermore, it is important to keep in mind that platelets from patients taking long-term antihypertensive medication may not necessarily yield the same results as platelets treated *in vitro* with antihypertensive drugs. Finally, our conclusions about platelet hyperreactivity in COVID-19 diseases were drawn by extrapolation using healthy platelets. Further studies should use platelets from patients infected by SARS-CoV-2 to characterize platelet function in COVID-19-related coagulopathy.

Conclusion

From this study, we conclude that healthy human platelets express the main receptors of the RAS, including MrgD, Mas, ACE, ACE2, AT1, and AT2. However, no effect was observed on platelet adhesion and aggregation by Captopril and RAS peptides including Alamandine, Ang-I, Ang-II, Ang-(1–7), Ang-(1–9) *in vitro* despite a large range of concentrations tested. ACE activity was detected in platelet lysates, but it was not inhibited by Captopril, while ACE2 activity was undetectable. We hypothesize that in severe COVID-19, thrombosis is most probably induced through a RAS- and ACE2-independent pathway. As this study focuses on healthy patients and platelets, further studies should investigate the effect of the RAS peptides on platelets treated with antihypertensive drugs and in patients with hypertension or COVID-19.

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Disclosure statement

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Author contributions

AML and NS conceived the study, obtained funding and supervised the work. FP, FMC, DSA, ADN, PH, CM, MM, FJ, GS, RG, YZ and AML were involved in participants' recruitment, did the data collection and performed the

experiments. FP, FMC and AML did the statistical analysis. FP wrote the manuscript, AML, ADN, AMC, CB, CM, RS, RF and FMC provided corrections. All authors read and approved the final manuscript. This work was used as FP's doctoral thesis (MD) at Bern University.

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