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Platelet-associated matrix metalloproteinases regulate thrombus formation and exert local collagenolytic activity

Running head: Thrombus-MMPs exert local collagenolytic activity

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**Abstract**

**Objective.** Platelets are increasingly implicated in processes beyond hemostasis and thrombosis, such as vascular remodeling. Members of the matrix metalloproteinase (MMP) family not only remodel the extracellular matrix but also modulate platelet function. Here we made a systematic comparison of the roles of MMP family members in acute thrombus formation under flow conditions and assessed platelet-dependent collagenolytic activity over time. **Approach and Results.** Pharmacological inhibition of MMP-1 or -2 (human) or deficiency in MMP-2 (mouse) suppressed collagen-dependent platelet activation and thrombus formation under flow, whereas MMP-9 inhibition/deficiency stimulated these processes. Absence of MMP-3 was without effect. Interestingly, MMP-14 inhibition led to the formation of larger thrombi, which occurred independently of its capacity to activate MMP-2. Platelet thrombi exerted local collagenolytic activity capable of cleaving immobilized dye-quenched collagen and fibrillar collagen fibers within hours, with loss of the majority of the platelet adhesive properties of collagen as a consequence. This collagenolytic activity was redundantly mediated by platelet-associated MMP-1, -2, -9 and -14 but occurred independently of platelet α-granule release \((Nbeal2^{−/−}\) mice). The latter was in line with subcellular localization experiments, which indicated a granular distribution of MMP-1 and -2 in platelets, distinct from α-granules. Whereas MMP-9 protein could not be detected inside platelets, activated platelets did bind plasma-derived MMP-9 to their plasma membrane. Overall platelet MMP activity was predominantly membrane-associated and influenced by platelet activation status. **Conclusion.** Platelet-associated MMP-1, -2, -9, and -14 differentially modulate acute thrombus formation and at later time points limit thrombus formation by exerting collagenolytic activity.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>Cvx</td>
<td>convulxin</td>
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<tr>
<td>PAR</td>
<td>protease activated receptor</td>
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<tr>
<td>GPVI</td>
<td>glycoprotein VI</td>
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<tr>
<td>DQ</td>
<td>dye quenched</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>SAC</td>
<td>surface area coverage</td>
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Introduction
The last decades progress has been made in obtaining insight into the molecular mechanisms underlying platelet adhesion to exposed extracellular matrix components, platelet activation and subsequent thrombus formation. It is proposed that platelet function is modulated by members of the matrix metalloproteinase (MMP) family. MMPs are found in most vascular cells, including platelets, and in plasma. It has been demonstrated that the MMP-2 concentration in arterial blood from patients with acute coronary syndrome and in shed blood from a skin wound correlates with platelet activation and platelet number, which suggests that platelet MMPs can contribute considerably to total plasma MMP levels. Platelets contain MMP-1 (not in mouse), -2, -3 and -14. Over the last years the presence of relevant levels of MMP-9 in platelets has been a matter of extensive debate. The classical view in cell biology is that MMP-14, also called membrane type 1 MMP (MT1-MMP), is membrane-inserted and that MMP-1, -2 and -9 become secreted upon cell activation. However, there are clear indications that MMP-1, -2 but also MMP-9 can interact and associate with the surface of platelets. Although MMP-1 and -3 seem to be mainly present in platelet α-granules, conflicting evidence exists on the storage and release of platelet MMP-2, since it has been found in both the α-granules and the cytoplasm. The recent discovery that mutations in NBEAL2 underlie an inherited bleeding disorder characterized by a lack of α-granules within platelets (gray platelet syndrome), combined with the generation of Nbeal2−/− mice provides a means to investigate the subcellular localization and role of MMP family members in more detail. Most MMPs are produced in a latent pro-MMP form which can be enzymatically activated upon platelet activation. For instance, platelet MMP-14 activates MMP-2 on the platelet surface presumably by forming a complex with tissue inhibitor of metalloproteinases-2 (TIMP-2). Ample evidence exists that recombinant MMP-1 and -2 potentiate adhesion or aggregation of human washed platelets or platelet-rich plasma in vitro, whereas MMP-9 exhibits an inhibitory effect. Less well studied is the role of these MMP family members in platelet activation under physiological whole blood flow conditions. So far, only for MMP-2 a role in in vivo thrombus formation has been reported. In addition, even less is known about possible roles of MMP-3 and -14 in platelet activation.

In addition to their proposed effects on platelet function, MMPs are important in the physiological turnover and pathophysiological remodeling of the extracellular matrix. These vascular actions have been confined especially to the collagenase (MMP-1, -8, -13) and gelatinase (MMP-2 and -9) classes of MMPs that have, respectively, interstitial collagens I, II and III, and denatured collagens as major substrates. Although the collagenolytic activity of platelets has already been described in 1974, it is unclear which platelet-associated MMP family members exert this activity. We hypothesize that proteolytically active MMPs associate with platelets locally at the site of injury, where they can act on both thrombus formation and underlying matrix components. For the present study a systematic comparison was made of the roles of different MMP family members on platelet activation and thrombus formation under physiological arterial flow conditions, using an established
flow chamber model.\textsuperscript{26} Furthermore, platelet-collagenolytic activity was assessed over time using three different substrates in the presence of MMP-specific inhibitors.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Differential modulating effects of MMPs on platelet activation and thrombus formation under arterial flow conditions.**

Human whole blood pre-incubated with MMP-specific inhibitors or blood from mice deficient in MMP-2, -3, -9 was perfused over a fibrillar type I collagen surface at a vWF-dependent shear rate of 1000 s\(^{-1}\) to systematically compare the roles of these MMPs on platelet activation and thrombus formation. Pharmacological inhibition of MMP-1 or MMP-2 resulted in significantly diminished platelet activation and surface area covered with platelets, while MMP-9 or -14 inhibition promoted these processes (figure 1A-B). Strikingly, inhibition of the membrane-inserted MMP-14 led to the formation of fewer but larger thrombi (figure 2), which appeared to be more stable in time (visual inspection during real-time microscopic monitoring). Interestingly, MMP-9 inhibition appeared to be without significant effect on thrombus size (P=0.37), whereas platelet spreading was increased (visual inspection during real-time microscopic monitoring). No additive effect of combined MMP-9 and -14 inhibition was found on thrombus size when compared to single inhibition of MMP-14 (figure 2). This suggests that the action mechanisms by which MMP-9 and -14 potentiate platelet activation and/or thrombus formation may differ.

Similar to the findings in human blood, absence of MMP-2 and -9 in the murine system had an opposing effect. Thrombi formed with blood from Mmp2\(^{-/-}\) mice tended to be smaller (not shown) than wild type thrombi and were less active as P-selectin expression (CD62P, marker for platelet secretion) and PS exposure (marker for platelet procoagulant activity) were significantly reduced (P<0.05) (figure 1C). In contrast, perfusion of blood from Mmp9\(^{-/-}\) mice resulted in thrombi that covered an increased surface area (+39%, P=0.04). This was accompanied by higher levels of PS exposure (+42%, P=0.04) and P-selectin expression (+83%, P=0.33) compared to corresponding wild type mice. Deficiency of MMP-3 had no effect on platelet activation and thrombus formation (figure 1C) and this MMP family member was therefore not included in further experiments. Of note, the roles of MMP-1 and -14 could not be investigated in the murine system as murine platelets do not express MMP-1 and MMP-14 deficient mice suffer from postnatal lethality.\textsuperscript{27}

Based on their modulating effect on platelet activation and thrombus formation, we next investigated whether MMP-1, -2, -9 and -14 localize on the thrombus surface. Using fluorescently labeled MMP antibodies, we found that MMP-1, -2, -9 and -14 all associate with the membrane of a human platelet thrombus. Addition of isotype-matched control antibodies yielded no detectable levels of fluorescence (figure 3). MMP-1, -2 and -9 associated with the thrombus in a speckled
manner, whereas the membrane type MMP-14 appeared to be homogeneously distributed over the thrombus (figure 3).

**GPVI-activated platelets rapidly display high MMP activity on their surface.**

Currently, it is unclear how platelet MMP activity is triggered, as well as its relevance compared to other sources of MMP activity. Here, we used an OmniMMP fluorogenic substrate to measure platelet-derived MMP activity (membrane bound and/or secreted) from washed platelets after platelet stimulation with weak (thrombin, 2-MeSADP, thapsigargin) or strong (convulxin, ionomycin) Ca²⁺ mobilizing agents.²⁸ Interestingly, activation with ionomycin and the GPVI receptor agonist convulxin (whether or not in combination with thrombin) triggered the highest levels of fluorescent MMP substrate conversion, with a 9.6-fold and 5.2-fold increase, respectively, compared to resting platelets (figure 4A). Stimulation with the weak Ca²⁺ inducing agents showed a trend towards increased MMP activity, although this was not statistically significant. MMP activity was completely inhibited by the general MMP inhibitor phenanthroline (figure 4A), but not by the cysteine, serine and threonine protease inhibitor leupeptin (10.7 μg/mL, n=5), confirming selectivity of the OmniMMP assay for MMPs. A ten-fold dilution of plasma treated with 4-amino-phenyl mercuric acetate (activating latent MMPs) yielded an MMP activity of 8.5 μg/mL (n=7), which was comparable to GPVI-stimulated platelets (9.4 μg/mL).

Following our findings that MMP-1, -2, -9 and -14 can associate with the thrombus surface (figure 3) and GPVI agonists being the most potent trigger of platelet MMP activity, we next assessed the subcellular localization of these MMP family members in platelets using confocal microscopy. In resting platelets, MMP-1 and -2 displayed a granular distribution, without significant overlap with the α-granule marker P-selectin (Pearson’s correlation coefficient of 0.24 for MMP-1 and 0.11 for MMP-2, suppl. fig. I-II). Upon platelet activation with convulxin, MMP-1 and -2 relocated to the platelet plasma membrane (suppl. figure I). The membrane-type MMP-14 localized on the plasma membrane of both resting and activated platelets. Interestingly, we observed caps with high levels of MMP-14 fluorescence on the plasma membrane of activated platelets, possibly suggestive of clustering of MMP activity. In line with the most recent literature, we could not detect MMP-9 in resting or activated platelets. Interestingly though, activated platelets did bind plasma-derived MMP-9 within 5 minutes after their activation (suppl. figure III), which likely accounts for the MMP-9 binding to thrombi observed in figure 3.

Given our data that activated platelets rapidly accumulate MMPs on their surface, we next measured the MMP activity of the platelet membrane fraction in comparison to the supernatant of (non-)activated platelets. The vast majority of MMP activity after GPVI stimulation was indeed membrane-bound (11.3 μg/mL), compared to 0.5 μg/mL in the supernatant (figure 4B). Kinetic experiments indicated that OmniMMP activity rapidly increased after platelet activation and reached a maximum slope of 37 FU/min 10 minutes after activation, after which MMP activity slowly declined (figure 4C). Knowing that MMP-2 is reported to be activated on the platelet plasma membrane⁵, we specifically measured MMP-2 activity using a cell-based
MMP-2 activity assay with a reported sensitivity for MMP-2 in the nanomolar range. Platelet activation with convulxin led to a rapid increase in platelet-bound MMP-2 activity with maximal activity of 1.6 FU/min reached within 5 minutes after activation. Pre-incubation with MMP-2 inhibitor III completely inhibited MMP-2 activity (figure 4D). In sum, our data suggest that platelets can rapidly display high levels of MMP activity on their membrane. This activity is dependent on platelet stimulation by strong, high cytosolic Ca^{2+} mobilizing agents such as the GPVI receptor agonist convulxin.

**MMP-dependent collagen degradation is localized at the site of the thrombus and affected by platelet activation status.**

Given the presence of MMP activity on the membrane of activated platelets, we next investigated the collagen matrix degrading potential of platelet thrombi. Therefore, thrombi were formed *ex vivo* in a flow device on a combined surface of DQ collagen (homogeneous coating on surface) and Horm collagen (fibrillar coating) at arterial shear rate. Plasma and unbound cells were removed by careful rinsing. An absence of white blood cells was confirmed by CD45 staining (not shown). Within 1.5 h after thrombus formation fluorescent signals were detected due to cleavage of DQ collagen locally at the site of the thrombi, indicating collagenolytic activity, which was predominantly present at the thrombus core (figure 5A). Similar patterns of thrombus-related fluorescence were obtained when DQ gelatin was used instead of DQ collagen (data not shown). Interestingly, upon post-addition of thrombin, thrombus collagenolytic activity was predominantly detected at the thrombus shell of both human and murine thrombi (DQ collagen fluorescence pattern in figure 5C). Collagenolytic activity was also found on the fibrillar Horm collagen fibers and platelet-membrane tethers, which was most prominent after post-stimulation with thrombin (representative fluorescence images figure 5C). Post-addition of phenanthroline immediately after the thrombi were formed led to an almost complete suppression of DQ collagen fluorescence (figure 5A-B). Post-addition of MMP-specific inhibitors, resulted in a significant suppression of DQ collagen fluorescence of 40-60% depending on the inhibitor used (figure 5B). The comparable effects found with the different MMP-specific inhibitors, suggest a redundancy in the collagenolytic capacity of platelet MMP-1, -2, -9 and -14. A similar reduced thrombus-dependent collagenolytic activity was observed with thrombi made from WT, *Mmp2^-/-* or *Mmp9^-/-* blood (figure 5C-D). Interestingly, no difference was observed with *Nbeal2^-/-* thrombi, indicating that thrombus-dependent collagenolytic activity is not mediated via α-granule release (figure 5D). This fits with our data that platelet-MMPs are predominantly localized outside platelet α-granules (suppl. fig. 1).

Alternatively, thrombus-dependent degradation of fibrillar type I collagen was visualized over time using scanning electron microscopy. Type I collagen fibers became visibly thinner within 3 h of incubation with thrombi, started to fragment at 4 h and were nearly completely cleared after 17 h. Adherent and spread platelets were still present at the site of the thrombus (figure 6). This process was dependent on MMP activity as addition of phenanthroline prevented degradation of collagen by
75% (supplemental figure IV). To investigate the ability of platelet-degraded collagen to sustain platelet deposition pre-formed thrombi (T=0 h) were incubated with HEPES buffer supplemented with 2-MeSADP/thrombin for 6 h and then rinsed. Freshly taken DiOC6-labeled whole blood was then flowed over the same surface for 4 minutes. Whereas initial thrombus surface area coverage was 22.2 ± 4.5% (T=0h); surface area covered by fresh DiOC6-labeled blood was greatly reduced to 1.5 ± 0.7% (P<0.001), indicating a loss of platelet-adhesive properties of platelet-degraded collagen (suppl. figure V). Of note, labeling with the membrane probe DiOC6 did not affect platelet adhesive capacity (not shown). In sum, these results show that platelet-associated MMP family members are capable of locally degrading an immobilized collagen matrix in a redundant manner, thereby restricting future thrombus formation in vitro.

Discussion
This study demonstrates, for the first time, that platelet-associated MMP-1, -2, -9 and -14 play a dual role in collagen-dependent thrombus formation. They directly modulate platelet activation and thrombus formation under flow, and are capable of degrading the underlying collagen matrix, thereby restricting future thrombus formation.

By employing the established multiparameter flow chamber assay, we made a systematic comparison of the role of different MMP family members in whole blood platelet activation and thrombus formation under arterial flow conditions. Interestingly, whereas MMP-1 and -2 enhance, MMP-9 and -14 inhibit platelet P-selectin expression (marker for platelet secretion), platelet procoagulant activity and thrombus formation. Deficiency in MMP-3 was without effect on these parameters, which is in line with a report that similarly found no effect of MMP-3 inhibition on platelet aggregation in the human system. Our results regarding the stimulatory function of MMP-1 and -2 extend the finding that MMP-1 can bind to platelet β3 thereby priming platelets for aggregation and that MMP-2 is involved in the modification of platelet glycoprotein Ib-V-IX and αIIbβ3 resulting in enhanced platelet adhesion and aggregation. MMP-14 has also been reported to cleave the N-terminal exodomain of the thrombin receptor PAR-1, resulting in a tethered ligand that activates G-protein-coupled receptor pathways in platelets. However, this manuscript received no follow-up in literature so far.

The thrombus inhibiting effects of MMP-9 and MMP-14 appear to be more potent than the stimulatory effects of MMP-1 and -2. We propose that MMP-9 interferes with platelet adhesive properties since we observed increased platelet spreading and surface area covered by thrombi upon MMP-9 inhibition, while thrombus size was comparable to the vehicle control. Cleavage of platelet CD40L by MMP-9 may affect thrombus stability at a later stage. In contrast, MMP-14 was shown to primarily limit thrombus growth and stabilization as both the surface area coverage and the thrombus size were increased in the presence of the MMP-14 inhibitor. The employed pharmacological inhibitor of MMP-14 (NSC-405020) does not interfere with the capacity to activate MMP-2, suggesting that other targets for
MMP-14 on the platelet surface may exist. As NSC-405020 directly interacts with the hemopexin domain of MMP-14, possible targets for MMP-14 could be linked to the homodimerization of MMP-14 or its collagenolytic activity.33

Our observation that MMP-1, -2, -9 and -14 associate with thrombi formed on collagen under flow was substantiated by our finding that MMP-1 and -2 rapidly relocate from granule structures inside the platelet to the plasma membrane of activated platelets. The membrane-type MMP-14 localized on the plasma membrane of both unstimulated and activated platelets. Although we could not detect MMP-9 protein in resting or activated platelets, we did observe that activated platelets bind plasma-derived MMP-9, which likely accounts for the MMP-9 binding to thrombi observed in figure 3. Based on our findings it is tempting to conclude that MMP-9 is most likely absent or present in neglectable amounts in platelets. However, given the persistent debate in literature on this topic we decided to perform an extensive literature search and found 26 original research reports in which the presence of MMP-9 was examined in human, mouse or rat platelets. Interestingly, in 11 out of 26 reports MMP-9 was detected in platelets, whereas in the other 15 reports it remained undetectable (Suppl. Table I). We here summarize key underlying reasons for this discrepancy and provide several recommendations for future studies and reporting findings: I) Platelet MMP-9 levels might be below the detection limit of some of the assays used. It is therefore of importance that authors state both the detection limit of their assay as well as the final sample concentration; II) Buffers containing EDTA or sodium orthovanadate (Na3VO4) have inhibitory effects on MMP activity and should be avoided when determining MMP-9 activity using zymography11; III) Potential contamination of the platelet sample with CD45+ leucocytes could lead to a false positive signal as CD45+ leucocytes contain considerable levels of MMP-9.9, 29 Analysis of the purity of the platelet sample (e.g. by flow cytometry) is thus highly recommended; V) When detecting platelet MMP-9 levels in a plasma environment it should be taken into account that plasma MMP-9 can bind to activated platelets (this manuscript). In line with this finding, platelet pre-activation during blood drawing and sample preparation should be minimized to avoid plasma MMP-9 binding to the platelet plasma membrane.

We demonstrate that MMPs not only accumulate on the platelet plasma membrane but also display MMP activity. Platelet MMP activity was highest after stimulation with strong high calcium-mobilizing agents, such the platelet collagen GPVI receptor agonist convulxin, and reached a maximal activity after 5-10 minutes of activation (MMP-2 and OmniMMP substrate, respectively). At first sight our findings may challenge reports showing that platelet-derived MMP activity after collagen stimulation is mainly confined to the supernatant21 and not the platelet-membrane fraction.5, 15 However, others that demonstrated release of MMP-2 and -9, simultaneously also showed the association of these MMP family members with the platelet surface.5, 13, 14, 18, 23 In addition, Chesney et al.25 found that collagenase exhibits its highest activity in the platelet membrane fraction, which is in line with our findings. We postulate that non-uniformity in the use of platelet agonists and in the definition of platelet supernatant may explain part of the discrepancies in literature
regarding platelet-derived MMP activity. In our hands, agonists evoking sustained high cytosolic Ca\textsuperscript{2+} levels, such as collagen, are strong inducers of platelet-exposed MMP activity. Additionally, here we define supernatant as the fraction that is secreted from (non-)activated platelets, i.e. the platelet releasate. This differs from the study by Trivedi et al.\textsuperscript{15} in which supernatant is referred to as the lysate of centrifugated (non-)activated platelet pellet. In this case it is expected that the actual platelet releasate is lost during the procedure and is only a fraction of the “supernatant”\textsuperscript{15}.

Next, we investigated a potential physiological role for platelet-membrane associated MMP activity and found that in vitro-formed thrombi effectively cleave immobilized dye-quenched collagen or degraded fibrillar type I collagen within hours after formation. This led to a marked restriction of future thrombus formation in vitro. Hereby our findings add to emerging knowledge on the existence and nature of persistent thrombus activities.\textsuperscript{34} Interestingly, the core of the thrombus appeared to be most active in cleaving dye-quenched collagen. However, upon post-addition of thrombin the thrombus-mediated MMP activity was predominantly present at the outer shell of the thrombi. Platelet-associated MMP-1, -2, -9 and -14 (human/mouse) mediated this collagenolytic activity in a redundant manner. An absence of platelet α-granules (\textit{Nbeal2}\textsuperscript{-/-}) did not affect thrombus-mediated collagenolytic activity, which fits with our observation that subcellular localization of MMP-1, -2 and -14 does not significantly overlap with the α-granule constituent P-selectin. Hereby our data shed new light on the contrasting findings reported in literature regarding proposed colocalization of MMP-1 and -2 with α-granules.\textsuperscript{12, 16}

In sum, we show that MMP-1 and -2 stimulate platelet activation and thrombus formation under arterial flow, whereas MMP-9 and -14 act inhibitory on these processes. Secondly, we demonstrate that the membrane fraction of collagen-stimulated platelets exerts high levels of collagenolytic activity, which leads to localized degradation of immobilized collagen fibers and restriction of future thrombus formation. Platelet-associated MMP-1, -2, -9 and -14 have redundant roles herein.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Significance
This study demonstrates, for the first time, that platelet-associated MMP-1, -2, -9 and -14 play a dual role in collagen-dependent thrombus formation. We show that MMP-1 and -2 stimulate platelet activation and thrombus formation under arterial flow, whereas MMP-9 and -14 act inhibitory on these processes. In addition, we demonstrate that the membrane fraction of collagen-stimulated platelets exerts high levels of collagenolytic activity, which leads to localized degradation of immobilized collagen fibers and restriction of future thrombus formation. Platelet-associated MMP-1, -2, -9 and -14 have redundant roles herein. We hypothesize that this localized matrix degradation could result in increased accessibility for blood cells to infiltrate into the underlying matrix. This provides a novel and highly relevant mechanism in which can be intervened to prevent pathological matrix remodeling, thrombosis or bleeding.
Figure Legends

Figure 1. MMP-1 and -2 promote collagen-dependent platelet activation and thrombus formation under flow whereas MMP-9 and -14 suppress these processes. Blood from healthy human controls was pre-incubated with inhibitors of MMP-1/8 (FN439), MMP-2 (MMP-2 inhibitor III), MMP-9 (MMP-9 inhibitor I) and MMP-14 (NSC-405020) for 10 min (A-B). Alternatively, murine blood from Mmp2, 3, or 9 deficient mice or matched wild types was used (C). Blood was perfused over immobilized Horm collagen at a shear rate of 1000 s⁻¹ for 4 min. Formed thrombi were stained for PS exposure and CD62P expression by post-perfusion with AF647-annexin A5 and FITC-labeled αCD62P mAb respectively. (A) Representative phase contrast and fluorescence images of human thrombi pre-treated with MMP-specific inhibitors. (B) Bar graphs represent analysis of surface area coverage of human (labeled) thrombi pre-treated with MMP-specific inhibitors. (C) Bar graphs represent analysis of surface area coverage of (labeled) Mmp2, 3 or 9 deficient murine thrombi. Scale bar= 50 μm, data are mean ± SEM (n=4-7); *P<0.05, **P<0.01.

Figure 2. Inhibition of human MMP family members differentially affects thrombus size under flow. Thrombi were formed as described for figure 1. Bar graph represents thrombus size, expressed as integrated feature size. Data are mean ± SEM (n=4-7); *P<0.05, **P<0.01.

Figure 3. Human MMP-1, -2, -9 and -14 are localized on the thrombus surface. Human blood was perfused over immobilized collagen at a shear rate of 1000 s⁻¹ for 4 min. Formed thrombi on coverslips were rinsed with HEPES buffer and post-stained for MMP-1, -2, -9 by single step conjugated mAbs. MMP-14 was stained with MMP-14 mAbs followed by AF488-conjugated secondary mAbs. Shown are a representative DIC image of formed thrombi and representative fluorescence images of MMP-1, -2, -9 and -14 against an IgG background. Data are representative of three independent experiments. Scale bar = 50 μm.

Figure 4. Platelet-derived MMP activity, which is released within minutes, is highest after GPVI-stimulation and predominantly membrane-associated. Human resting washed platelets (1 x 10⁹/mL) were activated as indicated and MMP activity was measured with OmniMMP substrate or selective MMP-2 EnSens substrate. (A) Bar graph shows OmniMMP activity after 10 min stimulation of platelets with different agonists. (B) Bar graph of MMP activity of the membrane bound and secreted fraction of unstimulated or convulxin-activated platelets. (C) Slope of OmniMMP activity in time (FU/min) of unstimulated and convulxin-activated platelets (n=5); *P<0.05. (D) Slope of MMP-2 activity in time (FU/min) of unstimulated and convulxin-activated platelets (n=3) with(out) MMP-2 inhibitor (n=1). Data are mean ± SEM.
Figure 5. MMP-dependent collagen degradation is localized at the site of the thrombus and is redundantly regulated by MMPs but not platelet α-granule release. Human or murine blood was incubated with AF647-αGPIbα mAb to label platelets and perfused over a combined surface of immobilized Horm and DQ collagen at a shear rate of 1000 s⁻¹ for 4 min. Formed thrombi were post-incubated with HEPES buffer containing 2-MeSADP with(out) phenanthroline or MMP-specific inhibitors. MMP activity was visualized as fluorescence signal by confocal microscopy. (A, C) Representative fluorescence profiles and images taken 1.5 h after thrombus formation. White dotted line indicates area of the fluorescence profile. (B) Bar graph expresses DQ collagen fluorescence as the percentage of collagen degradation in the presence of general and selective MMP-specific inhibitors compared to corresponding vehicle controls. (C) Representative fluorescence profiles and images of human and murine DQ collagen and AF647-αGPIbα taken 1.5 h after thrombus formation in presence of thrombin. (D) Bar graph expresses DQ collagen degradation for Mmp2, Mmp9 or Nbeal2 deficient murine blood or matched wild types. Scale bar = 30 μm, data are mean ± SEM (n=4-9); *P<0.05, **P<0.01, ***P<0.001.

Figure 6. Thrombi degrade underlying collagen fibers in a time-dependent manner. Human whole blood was perfused over immobilized Horm collagen at a shear rate of 1000 s⁻¹ for 4 min. Formed thrombi were rinsed and incubated in HEPES buffer containing 2-MeSADP. At indicated time points, thrombi were fixed and prepared for SEM. (A) Representative SEM pictures of Horm collagen coating before thrombus formation and of formed thrombi on collagen at T= 0, 2, 4, 7 and 17 h. (B) Insets of indicated squares in (A). Arrows indicate partly degraded collagen fibers. Representative SEM images of 9 experiments are depicted. Scale bar = 10 μm.
Figure 1

(A) Surface area coverage (% of vehicle) for different treatments: Vehicle, MMP-1/8 inh., MMP-2 inh., MMP-9 inh., MMP-14 inh.

(B) Surface area coverage (% of vehicle) for different treatments: Vehicle, MMP-1/8 inh., MMP-2 inh., MMP-9 inh., MMP-14 inh.

(C) Surface area coverage (% of WT) for different treatments: Mmp^{+/+}, Mmp^{2/-}, Mmp^{3/-}, Mmp^{9/-}.
Figure 2

Integrated feature size ($\mu m^2$)

- H$_2$O control
- MMP-1/8 inh.
- DMSO control
- MMP-2 inh.
- MMP-9 inh.
- MMP-14 inh.
- MMP-9 + -14 inh.

* p < 0.05
** p < 0.01
Figure 4

(A) MMP activity (µg/ml bacterial collagenase) for various stimuli:
- Unstimulated
- Thrombin
- ADP
- Thapsigargin
- Convulxin (Cvx)
- Cvx + thrombin
- Ionomycin
- Cvx + phenanthroline

(B) MMP activity (µg/ml bacterial collagenase) for Cvx and Cvx + MMP-2 inh. III:
- Membrane-bound
- Released

(C) MMP activity slope (FU/min) over time (min):
- Cvx
- Unstimulated

(D) MMP-2 activity slope (FU/min) over time (min):
- Cvx
- Unstimulated
- Cvx + MMP-2 inh. III
Figure 5

(A) Fluorescence intensity (AU) graphs for + Vehicle, + Phenanthroline, Human, + thrombin, and Murine, + thrombin.

(B) Bar graph showing degradation (% of control) for Vehicle, Phenanthroline, MMP-1/8 inh., MMP-2 inh., MMP-9 inh., MMP-14 inh.

(C) Human, + thrombin and Murine, + thrombin graphs.

(D) Bar graph showing degradation (% of WT) for WT, Mmp2−/−, Mmp9−/−, Nbeal2−/−.
Material and methods

Reagents. 1,10-Phenanthroline monohydrate, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and collagenase derived from Clostridium histolyticum were from Sigma-Aldrich (St. Louis, Missouri, USA). H-Phe-Pro-Arg chloromethyl ketone (PPACK), FN439, MMP-2 inhibitor I and MMP-9 inhibitor I were from Calbiochem (La Jolla, California, USA). MT1-MMP inhibitor (NSC-405020) was purchased from Merck KGaA (Darmstadt, Germany). Leupeptin hemisulphate (Ac-Leu-Leu-Arg-al hemisulphate) and ionomycin were obtained from VWR International (Radnor, Pennsylvania, USA). The glycoprotein VI agonist, convulxin came from Stago BNL (Leiden, The Netherlands). Human thrombin was obtained from Enzyme Research Laboratories (Leiden, The Netherlands). Thapsigargin was purchased from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA) and 2-methylthioADP (2-MeSADP) from Bioconnect (Huissen, The Netherlands). Horm collagen came from Nycomed (Munich, Germany). Annexin A5 labeled with Alexa Fluor (AF)-647 was obtained from Molecular Probes (Leiden, The Netherlands). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse P-selectin (CD62P) monoclonal antibody (mAb) was from Emfret Analytics (Würzburg, Germany). AF647 goat anti-mouse IgG1, dye-quenched (DQ) collagen (type I collagen from bovine skin, fluorescein-conjugated) and DQ gelatin (from pig skin, fluorescein-conjugated) were from Invitrogen (Carlsbad, California, USA). AF647-αGPIbα human mAb was from MBL International (Woburn, Massachusetts, USA). Human MMP-1 phycoerythrin (PE)-conjugated mAb (clone 36607), human MMP-2 PE-conjugated mAb (clone 1A10), human MMP-9 fluorescein-conjugated mAb (clone 56129), human MMP-14/MT1-MMP mAb (clone #5H2) and IgG isotype control PE-conjugated Ab (IC002P) were obtained from R&D Systems (Minneapolis, Minnesota, USA). Human IgG1 isotype control (26-787-275125) was obtained from GenWay Biotech (San Diego, California, USA). The OmniMMP substrate was purchased from Enzo Life Sciences (Anvers, Belgium) and EnSens MMP-2 activity detection kit from Enzium (McNeil, Philadelphia, USA).

Blood collection from healthy volunteers and mice. Human blood was drawn from healthy volunteers after full informed consent according to the declaration of Helsinki. The volunteers were free from antithrombotic medication for at least 2 weeks. The blood was collected into 10% 129 mM trisodium citrate for the preparation of platelet-free plasma, 17% acid-citrate-dextrose (80 mM trisodium citrate, 52 mM citric acid, 183 mM D-glucose) for the isolation of washed platelets or 40 μM PPACK and 40 U/mL fragmin for flow perfusion experiments.

Mmp2−/− mice were generated as described1 and bred against a C57BL/6 background. Mmp13−/− mice2 were provided by Drs. M. Byrne and S. Krane (Center for Immunology and Inflammatory Diseases and Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, USA) and bred against a C57BL/6 background. Mmp-3−/− and Mmp9−/− mice3,4 were bred against a B10.RIII and CD1 background,
respectively. Nbeal2−/− mice were bred against a C57BL/6N background as described.5 Wild-type littermate mice matching genetic background, age and sex were obtained from the host institutions and used as controls. Mice were anesthetized by a subcutaneous injection of 0.1 mg/g body weight ketamine and 0.02 mg/g body weight xylazine and bled retro-orbitally. Mouse blood was collected into 10% 129 mM trisodium citrate and 5 U/mL heparin. Platelet counts were in the normal range for all mice. Animal experiments were approved by the research ethics committees of Maastricht University, The Netherlands.

**Contribution of MMPs to real-time thrombus formation under flow.** Flow perfusion experiments were performed with anticoagulated whole blood from mice deficient in MMP-2, -3 or -9 with blood from healthy volunteers. Prior to the experiment murine blood was recalcified with 7.5 mM CaCl2 and 3.75 mM MgCl2, to obtain physiological (millimolar) concentrations of Ca2+ and Mg2+, in the presence of 40 μM PPACK. Human blood, already containing physiological Ca2+ and Mg2+ levels, was pre-incubated for 10 min at 37 °C with selective MMP inhibitors or respective vehicle: FN439 (MMP-1/8 inhibitor), MMP-2 inhibitor I (MMP-2 inhibitor), MMP-9 inhibitor I (MMP-9 inhibitor) and/or NSC-405020 (MMP-14 inhibitor). Dose-response experiments revealed the optimal inhibitory/stimulatory effect of MMP-1/8 inhibitor at 5 μM, of MMP-2 inhibitor at 10 μM, of MMP-9 inhibitor at 50 nM and of MMP-14 inhibitor at 100 μM (data not shown). Murine and human blood was perfused over a collagen-coated coverslip that was mounted in a parallel-plate flow chamber at a shear rate of 1000 s−1 for 4 min, as described.6 Coverslips with murine thrombi were examined for surface coverage with an inverted microscope (Nikon Diaphot 22, Nikon; Tokyo, Japan). P-selectin expression (anti-CD62P) and phosphatidylserine (PS) exposure (annexin A5 binding) were assessed with a Bio-Rad 2100 multiphoton microscopic system (Carl Zeiss; Jena, Germany), as described.6, 7 Coverslips with human thrombi were examined for the same parameters using a single-photon, LSM7 Live line-scanning confocal system from Carl Zeiss (Oberkochen, Germany). Excitation was at 488 nm and emission between 540 to 625 nm. Images were taking with a 60x/1.4 NA oil emersion objective. Analysis of confocal images was performed with ImagePro/LaserPix software (Media Cybernetics; Silver Spring, Maryland, USA). Thrombus sizes was investigated as integrated feature size and was calculated as described previously.8

**Localization of MMP family members on thrombus surface.** Thrombi were formed with blood from healthy volunteers as described above and were devoid of leukocytes (CD45). Coverslips with thrombi were post-stained with PE-conjugated mAbs against MMP-1, -2, or -9 (1:50 v/v in HEPES pH 7.45) for 20 min. Alternatively, αMMP-14 (MT1-MMP) (1 µg/mL) was added for 10 min, followed by AF647 goat anti-mouse IgG1 (4 µg/mL) for 20 min. All mAbs were titrated in preliminary experiments and mouse IgG1 PE-conjugated antibody or human purified IgG served as a control.
Microscopic images were taken with a LSM7 Live line-scanning confocal system from Carl Zeiss, as described above.

**Fluorescence measurements of platelet-derived MMP activity.** Human platelet-free plasma and washed platelets were obtained, as described. Platelets were diluted to 2 x 10^9/mL and activated for 15 minutes with thrombin (8 nM), convulxin (Cvx, 0.1 µg/mL), ionomycin (20 µM), thapsigargin (0.5 µM) or 2-MeSADP (40 µM). Where indicated, the general MMP inhibitor phenanthroline (2 mM) was added immediately after platelet activation. Control experiments indicated that a lower concentration phenanthroline (100 µM) was similarly effective. OmniMMP substrate (40 µM) was added 1:1 v/v to the (activated) platelet sample in a 96-well plate. Fluorescence was measured in a Spectra max M2 plate reader (Molecular Devices; Sunnyval, California, USA) at 405 nm for 10 h at 37 °C. Bacterial collagenase was used as a positive control. MMP activity was determined by the maximal slope of the fluorescence signal (between T=0-1.5 h) and normalized to the bacterial collagenase activity. To determine membrane-bound MMP activity, activated platelets were centrifuged at 2240 g for 2 min. Both membrane-bound and supernatant fractions (i.e. the platelet releasate) were collected and used immediately.

To perform kinetic measurements, platelets were activated with Cvx (1 µg/ml) plus thrombin (25 nM) for 0, 1, 2, 5, 10, 30 or 60 minutes and directly mixed with CCCP (100 µg/ml) to stop all ATP-dependent processes in platelets, such as platelet secretion. OmniMMP activity was then measured for 10 minutes as described above. Alternatively, a highly sensitive and specific cell-based MMP-2 activity assay was used according to manufacturer’s conditions. In brief, washed platelets (1 x 10^9/ml) were activated with Cvx (1 µg/ml) plus thrombin (25 nM) for 2 minutes and then mixed with Ensense MMP-2 detection mix. Fluorescent signal was detected with a LSM7 Live line-scanning non-confocal system from Carl Zeiss (Oberkochen, Germany). MMP-2 activity over time was determined by measuring the average pixel intensity per microscopic image and subsequent calculation of the first derivative.

**Collagen matrix-degrading potential of thrombus-derived MMPs.** Glass coverslips were coated for 60 min with a mixed 10 µl spot of DQ collagen (25 µg/mL) and Horm collagen (50 µg/mL). After coating, the coverslips were blocked, rinsed and mounted into a parallel plate flow chamber. Thrombi were formed with blood from healthy volunteers as described above and were devoid of leukocytes (CD45). Specific MMP inhibitors FN439 (5 µM), MMP-2 inhibitor I (10 µM), MMP-9 inhibitor I (50 nM), NSC-405020 (100 µM) or phenanthroline (1 mM, with 100 µM yielding the same results) were added to HEPES buffer (including 5 mM CaCl₂, 5 mM MgCl₂, 10 µM 2-MeSADP and, optionally, 25 nM human thrombin) and incubated with the pre-formed thrombi as indicated. Alternatively, thrombi were made with Mmp-2−/−, -9−/− or Nbeal2−/− blood during 4 min whole blood perfusion at 1000 s⁻¹ and carefully rinsed with HEPES buffer (including 5 mM CaCl₂, 5 mM MgCl₂, 10 µM 2-MeSADP and 25 nM human thrombin) to remove unbound blood components. Human thrombin (25
nM) and 2-MeSADP (10 µM) was added to the incubation buffer to prevent thrombus embolization during the incubation time. After 1.5 h of incubation, DQ collagen degradation was visualized using a single-photon, LSM7 Live line-scanning confocal system from Carl Zeiss (Oberkochen, Germany) at an excitation wavelength of 488 nm and emission wavelength of 540 to 625 nm.

**Imaging of thrombus-dependent collagen matrix degradation by scanning electron microscopy.** Thrombi were formed with blood from healthy volunteers as described above, rinsed to remove unbound blood components and then incubated at 37 °C with HEPES buffer supplemented with the antibiotics penicillin plus streptomycin. Where indicated phenanthroline (1 mM) was added to the HEPES buffer. At time point 0, 2, 4, 7 and 17 h thrombus- and collagen containing coverslips were carefully removed from the flow chamber and gently washed in HEPES buffer. Samples were fixed overnight with 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer (pH 7.4) at 4 °C. After rinsing with 0.1 M phosphate buffer, followed by dehydration in graded concentrations of ethanol, samples were critical point dried, mounted with silver paint on specimen stubs and coated with gold. Thrombi on (partly degraded) collagen fibers were visualized with a scanning electron microscope (Philips XL 30, Eindhoven, The Netherlands) at 10 kV.

**Statistics.** Groups were compared using the Mann-Whitney U-test or the independent samples t-test, as appropriate (Graphpad v5.0; La Jolla, California, USA). P<0.05 was considered statistically significant.

**References**


6. Cosemans JM, Schols SE, Stefanini L, de Witt S, Feijge MA, Hamulyak K, Deckmyn H, Bergmeier W, Heemskerk JW. Key role of glycoprotein ib/v/ix and
von willebrand factor in platelet activation-dependent fibrin formation at low shear flow. *Blood*. 2011;117:651-660


Supplemental material

Supplemental methods

Reagents. Poly-L-Lysine, 1,10-Phenanthroline monohydrate and saponin were from Sigma-Aldrich (St. Louis, Missouri, USA). The glycoprotein VI agonist, convulxin came from Stago BNL (Leiden, The Netherlands). Human thrombin was obtained from Enzyme Research Laboratories (Leiden, The Netherlands). 2-methylthioADP (2-MeSADP) from Bioconnect (Huissen, The Netherlands). Horm collagen came from Nycomed (Munich, Germany). Human MMP-1 phycoerythrin (PE) conjugated mAb (clone 36607), human MMP-2 PE conjugated mAb (clone 1A10), human MMP-9 fluorescein conjugated mAb (clone 56129), human MMP-14/MT1-MMP mAb (clone #5H2) and IgG isotype control PE conjugated Ab (IC002P) were obtained from R&D Systems (Minneapolis, Minnesota, USA). Human IgG1 isotype control (26-787-275125) was obtained from GenWay Biotech (San Diego, California, USA). DiOC₆ was purchased from AnaSpec (Waddinxveen, The Netherlands). Imsol Mount was from immunologic (Duiven, The Netherlands).

Subcellular MMP localization and MMP-9 binding

Human blood was drawn from healthy volunteers after full informed consent according to the declaration of Helsinki. The blood was collected in 17% acide-citrate-dextrose (80 mM trisodium citrate, 52 mM citric acid, 183 mM D-glucose), after which washed platelets were prepared as described.¹ Resting or convulxin (1 µg/ml, 10 min) activated platelets (1 x 10⁸/mL) were fixed with phosphate buffered saline (PBS) + 4% formaldehyde for 20 minutes and washed twice in PBS + 0.2% bovine serum albumin (BSA). Platelets were permeabilized in 0.1% saponin in PBS + 0.2% BSA for 30 minutes and all consequent steps also contained 0.1% saponin. Non-specific sites were blocked with 2% BSA and 1 µg/ml non-specific IgG for 1h after which platelets were washed. Platelet samples were then stained overnight with PE- or FITC conjugated mAbs against MMP-1, -2, or -9 (1:20 v/v in PBS + 2% BSA) at 4 °C. Alternatively, platelet samples were incubated overnight with αMMP-14 (MT1-MMP) (5 µg/mL) at 4 °C, washed with PBS and then stained with AF647 goat anti-mouse IgG1 (4 µg/mL) for 1 h. To examine colocalization with the alpha-granule constituent P-selectin, all samples were again washed and stained with AF647-αCD62P (1:20). Samples were spun down at 250 g for 10min on poly-l-lysine coated coverslips (0.01%) and subsequently mounted using Imsol Mount for microscopic examination using the EVOS® Cell Imaging System. Colocalization of MMP family members with P-selectin was determined by using the “Colocalization Threshold” analysis option in Fiji v.1.48g software.² Herein, a standardized method was used to set a threshold to correct for background signal across images obtained with the different antibodies.
**Thrombogenicity of thrombus-degraded collagen fibers.**

Human blood was perfused over a collagen-coated coverslip that was mounted in a parallel-plate flow chamber at a shear rate of 1000 s\(^{-1}\) for 4 min, as described.\(^3\) Formed thrombi were rinsed with HEPES buffer to remove unbound blood components and then incubated overnight at 37 °C with HEPES buffer supplemented with 5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 10 µM 2-MeSADP, 25 nM human thrombin and penicillin plus streptomycin. Where indicated phenanthroline (1 mM) was added to the HEPES buffer. After overnight incubation thrombi were lysed with 0.1% triton X-100 to enable microscopic visualization of the underlying collagen. Microscopic images from >10 different fields per sample were collected with an inverted microscope (Nikon Diaphot 22, Nikon; Tokyo, Japan). The mean visible collagen fiber length before and after 24 h incubation was determined in pixels using Metamorph software (Molecular Devices; Sunnyvale, California, USA) and compared as an estimate of the percentage of collagen degradation.

Alternatively, after overnight incubation the flow chamber was rinsed with HEPES buffer to remove unbound cellular components. Triton X-100 was not used in this case. After rinsing, freshly drawn DiOC\(_6\) labeled blood from the same donor was perfused through the chamber for 4 minutes at 1000 s\(^{-1}\). Adhesion of DiOC\(_6\) labeled platelets was visualized using a single-photon, LSM7 Live line-scanning confocal system from Carl Zeiss (Oberkochen, Germany).
Supplemental results

**Suppl. figure I. Subcellular localization of MMP-1, -2 and -14 in unstimulated and convulxin-activated platelets.** Unstimulated or convulxin-activated platelets were fixed, permeabilized and stained for MMP family members in combination with P-selectin. Shown are representative microscopic images of (A) resting platelets and (B) convulxin-activated platelets stained for MMP-1 (PE-αMMP-1), MMP-2 (PE-αMMP-2), MMP-9 (FITC-αMMP-9) or MMP-14 (αMMP-14+AF488-IgG1); and P-selectin (AF647-αCD62P) with corresponding overlay. Note the granular distribution of MMP-1 and -2 in unstimulated platelets, distinctive from the fluorescent signal obtained with AF647-αCD62P. n=3, scale bar = 5 μm.

**Suppl. figure II. Quantitative determination of MMP-1, -2 and -14 colocalization with the alpha-granule marker P-selectin.** Washed human platelets were stained for MMP-1, -2 and -14 as described for suppl. fig. I. Shown is a bar graph with Pearson’s correlation coefficients determined by thresholding microscopic images >50 platelets and Fiji software. Data are mean ± SEM (n=3).
Suppl. figure III. MMP-9 is not present in platelets but binds to the surface of activated platelets in a plasma environment. Washed human platelets were activated in HEPES buffer or plasma for 5 min with convulxin. Platelets were then washed, fixed, permeabilized and stained for MMP-9. (A) Bar graph represents the analysis of MMP-9 fluorescence normalized to surface area coverage in the phase contrast image. (B) Representative fluorescence and phase contrast images of a single activated platelet. Scale bar = 5 μm, data are mean ± SEM (n=3); **p<0.01.

Suppl. figure IV. Thrombus-mediated collagen degradation is MMP-dependent. Human blood was perfused over immobilized Horm collagen at a shear rate of 1000 s⁻¹ for 4 min. Formed thrombi were post-incubated with HEPES buffer with(out) the MMP inhibitor phenanthroline. At indicated time points, thrombi were lysed with 0.1% triton X-100 to enable microscopic visualization of the underlying collagen. Shown is the effect of phenanthroline (24 h) on collagen fiber length normalized to vehicle T=0. Data are mean ± SEM (n=3); ***p<0.001.

Suppl. figure V. Thrombus-mediated collagen degradation restricts further thrombus formation. Human thrombi were formed as described for suppl. fig. IV and post-incubated with HEPES buffer at 37 °C. After overnight incubation the flow chamber was rinsed with HEPES buffer. Freshly drawn DiOC₆-labeled blood was perfused through the chamber for 4 min at 1000 s⁻¹. Bar graph represents the analysis of surface area coverage of DiOC₆-labeled platelets. Data are mean ± SEM (n=3); ***p<0.001.
### Suppl. Table 1 Literature on whether MMP-9 is present in platelets

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**Abbreviations:** CZE, capillary zone electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; mAb, monoclonal antibody; pAb, polyclonal antibody; N/A, not applicable; non perm., non permeabilized; perm., permeabilized; PRP, platelet rich plasma.
Supplemental references


