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TRANSLATIONAL RESEARCH
Vascular biology and medicine

Matrix metalloproteinase-2 on activated platelets triggers endothelial PAR-1 initiating atherosclerosis

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Aims Platelets participate in atherogenesis with mechanisms not yet fully clarified. Vascular wall MMP-2 is involved in the arterial remodelling accompanying atherosclerosis. Platelets contain and release MMP-2 but no informations are available on its role in atherosclerotic lesion formation.

Methods and results We generated double knockout mice lacking the LDL receptor and MMP-2 only in circulating blood cells showing that they develop significantly lesser femoral intima thickening after photochemical-induced arterial damage and atherosclerotic lesions in the aorta, measured by the *en face* method, after 4 months of atherogenic diet. Moreover, repeated transfusions of autologous-activated platelets in $LDLR^{-/-}$ mice on atherogenic diet significantly enhanced the extension of aortic atherosclerotic lesions while transfusion of activated platelets from $MMP\text{-}2^{-/-}$ mice did not. In vitro coincubation studies showed that platelet-derived MMP-2 plays a pivotal role in the development and progression of atherosclerosis through a complex cross-talk between activated platelets, monocyte/macrophages, and endothelial cells. Translational studies in patients with CAD and chronic HIV infection showed that platelet surface expression of MMP-2 highly significantly correlated with the degree of carotid artery stenosis.

Conclusion We show a previously unknown mechanism of the pathway through which platelets expressing MMP-2 trigger the initial phases of atherosclerosis and provide a mechanism showing that they activate endothelial PAR-1 triggering endothelial p38MAPK signalling and the expression of adhesion molecules. The development of drugs blocking selectively platelet MMP-2 or its expression may represent a new approach to the prevention of atherosclerosis.

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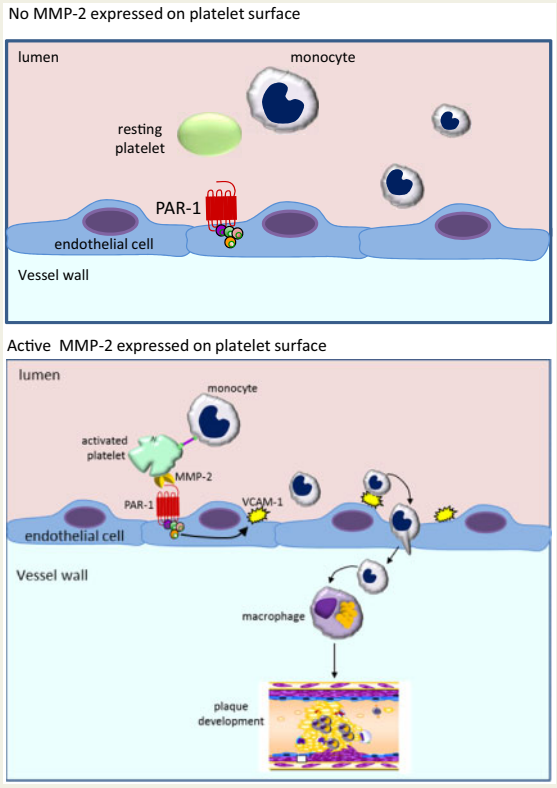
Graphical Abstract



Our study demonstrates that platelet-derived MMP-2 plays a pivotal role in the very early phases of atherogenesis in hypercholesterolemic mice. Circulating activated platelets expressing MMP-2 on their surface interact with PAR-1 of endothelial cells triggering signal transduction and activation with the consequent exposure of adhesion molecules (i.e. VCAM-1), the latter in turn facilitate the adhesion and transmigration of monocytes through the endothelial monolayer ultimately leading to atheroma formation. The interaction between MMP-2 expressed by activated platelets and endothelial cell PAR-1 may represent a novel therapeutic target for the prevention of atherosclerosis.

Keywords Platelets • Endothelial cells • PAR-1 • MMP-2 • Atherosclerosis

Translational Perspective

With this study, we demonstrate that platelet-derived MMP-2 plays a pivotal role in the very early phases of atherogenesis in hypercholesterolemic mice. Circulating activated platelets expressing MMP-2 on their surface interact with PAR-1 of endothelial cells (ECs) triggering signal transduction and activation with the consequent exposure of adhesion molecules, the latter in turn facilitate the adhesion and transmigration of monocytes through the endothelial monolayer ultimately leading to atheroma formation. The interaction between MMP-2 expressed by activated platelets and EC PAR-1 may represent a novel therapeutic target for the prevention of atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory disorder that involves innate and adaptive immune responses,[1] with immune cells playing a crucial role in plaque formation.[2] Circulating monocytes interact with dysfunctional endothelium that has lost its anti-adhesive properties to infiltrate the arterial wall, mature in macrophages, and generate foam cells internalizing and oxidizing LDL, leading to the fatty streak development.[3]

Platelets play an important role in recruiting inflammatory cells to the sites of atheroma formation by favouring their arrest onto altered endothelium.[4] In particular, activated platelets favour leucocyte binding to vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expressed on activated endothelium promoting their adhesiveness.[5] Moreover, platelet-released CD40L binds its cognate receptor CD40 on endothelial cells (ECs) triggering the release of inflammatory mediators, such as IL-8 and MCP-1, and the production of reactive oxygen species (ROS).[6–8]

It is held that circulating blood platelets do not interact with normal endothelium but strongly interact with activated EC through their GPIb engaging endothelial VWF and P-selectin.[9,10] The interaction between activated EC and platelets in turn leads to platelet activation, with consequent enhanced surface expression of molecules involved in intercellular adhesion processes, such as CD40L and P-selectin.[11] Platelet P-selectin in particular is required for platelet binding to monocytes and neutrophils[12] and brings about the delivery of platelet-derived proinflammatory mediators to the vessel wall favouring the exacerbation of atherosclerosis.[13] Indeed, the selective removal of platelet P-selectin, abrogating the interaction of platelets with endothelium, was shown to prevent atherosclerotic lesion development in hypercholesterolemic mice.[13] Consistently, repeated injections of activated wild-type platelets in hypercholesterolemic *ApoE*$^{-/-}$ mice increased aortic plaque formation while the injection of activated platelets from *P-selectin*$^{-/-}$ mice did not.[13] The direct interaction of platelets with the endothelium appears to be required, because when instead of platelets the supernatant of activated platelets was injected no increase of atherosclerotic lesions was observed.[13] Platelet/endothelium contact may also be relevant for the deposition of other platelet-derived mediators, such as RANTES and PF4, contributing to atherosclerosis.[13]

A crucial role in atherogenesis is played by matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes that degrade various components of the extracellular matrix, mediate tissue remodelling and facilitate the migration of smooth muscle cells from the media to the intima during atherogenesis.[14–16] Human platelets contain, release, and express on their surface upon activation several MMPs, including MMP-2.[17,18] Platelet-released MMP-2 exerts local collagenolytic activity[19] and is involved in the heterotypic interaction with other cells modifying the phenotype of the latter.[20] Thus, it is conceivable that platelet-derived MMP-2 may be one of the mediators involved in the progression of atherosclerosis.

Here, we provide the first genetic evidence that platelet-released MMP-2 plays a critical role in initiating atherogenesis in hypercholesterolemic mice by triggering endothelial PAR-1, thus favouring EC activation, showing that platelets act as major players in the very early stages of atherosclerosis development. Our findings identify platelet MMP-2 as a novel potential therapeutic target for the prevention of atherosclerosis development.

Methods

Methods are provided in detail in the *Supplementary material online*.

Results

Platelet-derived MMP-2 is required for atherosclerotic lesion development

Twelve weeks of atherogenic diet strikingly enhanced total serum cholesterol concentrations in *LDLR*$^{-/-}$ mice as well as in *LDLR*$^{-/-}$/*MMP-2*$^{-/-}$ double knockout (*dKO*) and in *LDLR*$^{-/-}$ mice transplanted with bone marrow from *MMP-2*$^{-/-}$ mice (*LDLR*$^{-/-}$-chimeric) but not in *MMP-2*$^{-/-}$ and in wild-type (WT) mice (Supplementary material online, *Figure S1*).

Femoral arteries of *LDLR*$^{-/-}$ mice on high-fat diet developed 3 weeks after photochemical damage significantly greater intimal hyperplasia as compared with wild-type mice, while *dKO, LDLR*$^{-/-}$-chimeric as well as *MMP-2*$^{-/-}$ mice developed a significantly lower intimal thickening (*Figure 1A and B*). Moreover, *LDLR*$^{-/-}$ developed a significantly greater extension of aortic atherosclerotic lesions as compared with wild-type, *dKO, MMP-2*$^{-/-}$, and *LDLR*$^{-/-}$-chimeric mice (*Figure 1C* and Supplementary material online, *Figure S2A*).

Lipid-rich lesions in *LDLR*$^{-/-}$ mice were localized especially in the aortic arch, where 71.2 ± 3.5% of the area was covered by plaques, while in *dKO* and *LDLR*$^{-/-}$-chimeric mice a strikingly lower aortic arch area was interested by plaque deposition (28.7 ± 3.2% and 21 ± 7.3%, respectively, $P < 0.0001$) (*Figure 1D* and Supplementary material online, *Figure S2B*).

Lipid-rich plaques in serial aortic sections show a massive infiltration of macrophages and vascular smooth muscle cells (VSMCs) in aortic plaques from *LDLR*$^{-/-}$ mice. The inflammatory cell infiltration is significantly reduced in *dKO* and, importantly, in chimeric mice lacking MMP-2 only in bone marrow-derived cells, and it is completely absent in WT and *MMP-2*$^{-/-}$ mice (Supplementary material online, *Figure S3*). To discriminate between classical or alternative macrophage activation, we evaluated the presence of M1 or M2 polarized cells in mouse aortic plaques. Interestingly, in aortic plaques of *LDLR*$^{-/-}$ mice M1 macrophages prevailed, according to the atherosclerotic phenotype, while in *dKO* and *LDLR*$^{-/-}$-chimeric mice M2 macrophages, associated with an antiinflammatory activity, were dominant (Supplementary material online, *Figure S4*). Picrosirius red staining showed that *LDLR*$^{-/-}$ aortas had lost the well-knitted collagen fibre structure in the aortic adventitia and in the media layer that were disorganized and formed intricate networks throughout the plaque. In contrast, Picrosirius red staining in aortic sections of dKO and chimeric *LDLR*$^{-/-}$ mice showed less marked alterations of collagen deposition (Supplementary material online, *Figure S3*).

Finally, chronic severe platelet depletion in *LDLR*$^{-/-}$ mice significantly reduced total aorta surface area covered by lipid plaques, confirming the importance of platelets for the atherogenic process, and repeated i.v. injection of activated platelets from *LDLR*$^{-/-}$ mice in platelet-depleted *LDLR*$^{-/-}$ mice not only restored but strikingly enhanced (+313 ± 12.5%) atherosclerotic lesions in mouse aortae. On the contrary, repeated transfusion of activated platelets from *MMP-2*$^{-/-}$ mice did not restore atherogenesis. Moreover, the injection of activated platelets from *dKO* mice or of resting *LDLR*$^{-/-}$ platelets did not favour atherosclerotic plaque formation (*Figure 1E*).

Altogether, these data show that the expression of MMP-2 by platelets is required for atherogenesis in hypercholesterolemic conditions.

Platelet hyperreactivity of *LDLR*$^{-/-}$ mice is abolished by MMP-2 deletion

The agonist-triggered expression of P-selectin on circulating platelets was significantly higher in *LDLR*$^{-/-}$ mice than in wild-type mice, while it was significantly lower in *MMP-2*$^{-/-}$, *dKO* as well as in *LDLR*$^{-/-}$-chimeric mice (*Figure 2A*).

Similarly, the formation of circulating platelet/leucocyte aggregates induced by i.v. collagen plus epinephrine was significantly greater in

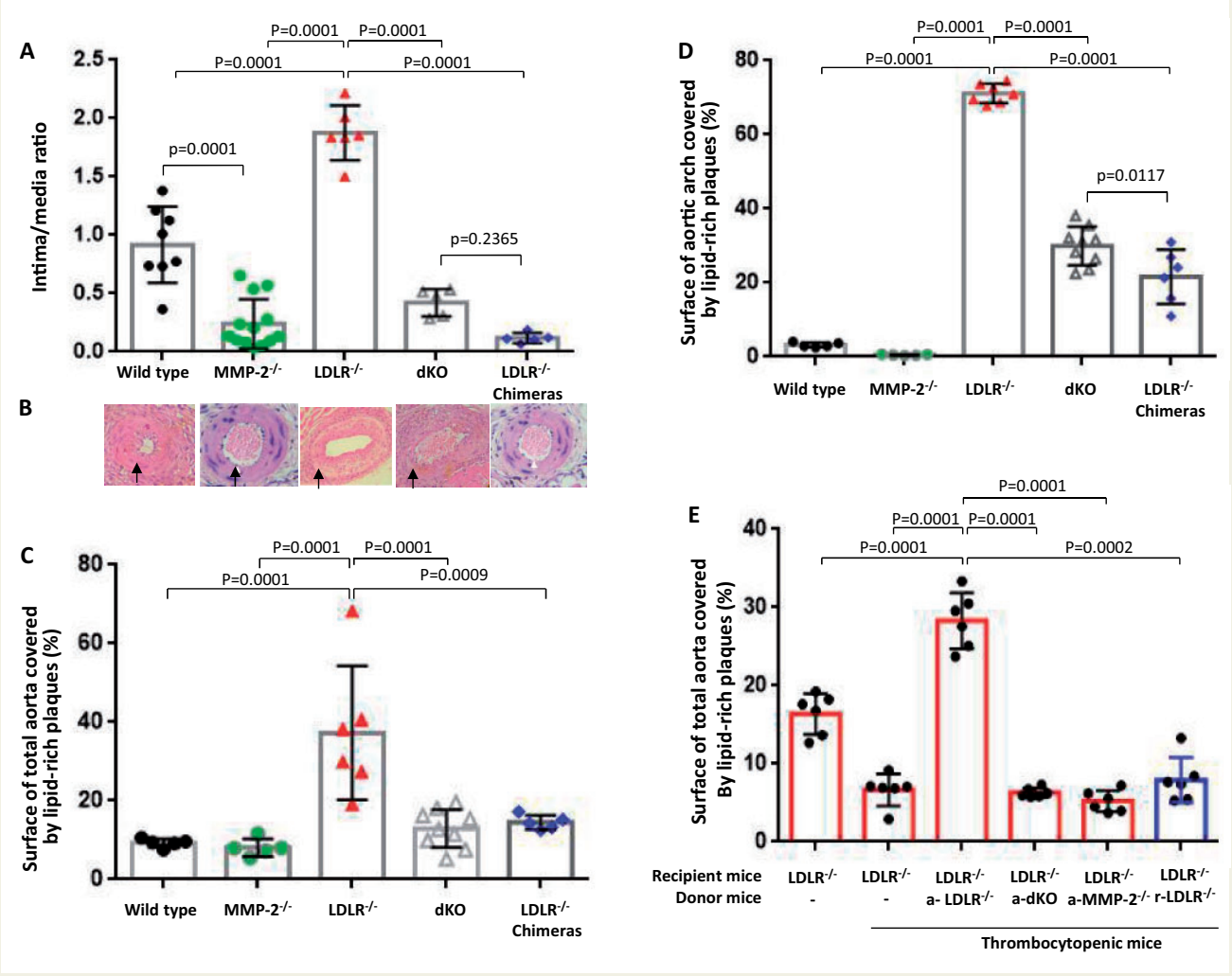


Figure 1 Platelet-derived MMP-2 is required for atherosclerotic lesion development. (*A*) Thickening of intimal/media of the femoral artery induced by photochemical damage in hyperlipidemic mice is abolished by MMP-2 deletion. Intimal/media ratio of the femoral artery 21 days after photochemical injury in wild-type ($n = 8$), *MMP-2*$^{-/-}$ ($n = 13$), *LDLR*$^{-/-}$ ($n = 6$), *dKO* ($n = 5$), and *LDLR*$^{-/-}$-chimeric ($n = 6$) mice kept on HFD for 12 weeks. Three sections for mouse were analysed. One-way analysis of variance with Tukey's multiple comparisons test was carried out. (*B*) Representative photomicrographs of haematoxylin and eosin staining of femoral artery sections. Magnification is 600×. Internal elastic lamina is indicated by arrowheads. At least 8 sections for each artery were analysed. I/M ratio in *LDLR*$^{-/-}$ sham-injured mice was 0.03 ± 0.007 ($n = 4$). (*C*) Hyperlipidaemia-induced aortic lipid plaque deposition is dependent on platelet MMP-2. Lipid-rich plaque deposition in total aorta (*C*) or in the aortic arch (*D*) of wild-type ($n = 5$), *MMP-2*$^{-/-}$ ($n = 5$), *LDLR*$^{-/-}$ ($n = 6$), *dKO* ($n = 9$), and *LDLR*$^{-/-}$-chimeric mice ($n = 5$). *$P < 0.0001$ vs. wild-type and *MMP-2*$^{-/-}$; $^{\#}P < 0.001$ vs. *LDLR*$^{-/-}$. One-way analysis of variance with Tukey's multiple comparisons test was carried out. The deletion of MMP-2 in all tissues (*dKO* mice) or only in platelets (*LDLR*$^{-/-}$-chimeric mice) significantly reduced the deposition of lipid plaques in the entire aorta (*C*) and in the aortic arch (*D*). The deletion of platelet-derived MMP-2 seems to be especially important for plaque deposition in the aortic arch. Repeated transfusion of activated platelets expressing MMP-2, but not of platelets deficient of MMP-2, enhances aortic plaque deposition. (*E*) Thrombin-activated (a) platelets from *LDLR*$^{-/-}$, *dKO* and *MMP-2*$^{-/-}$ mice or resting (r) platelets from *LDLR*$^{-/-}$mice were transfused i.v. every 4 days in thrombocytopenic *LDLR*$^{-/-}$ mice, for 6 weeks. Lipid-rich plaques were measured in the entire aorta using the *en face* method after staining with Sudan IV. Thrombocytopenia significantly reduced aortic lipid-rich plaques ($P < 0.01$ vs. *LDLR*$^{-/-}$ mice with normal platelet count). Repeated transfusion of activated platelets from *LDLR*$^{-/-}$ mice enhanced aortic plaque deposition while activated platelets from *MMP-2*$^{-/-}$ mice did not. $n = 6$ mice for each group. All data are expressed as mean ± standard deviation. One-way ANOVA with Tukey's multiple comparisons test was carried out.

LDLR$^{-/-}$ than in wild-type mice (*Figure 2B*) and significantly lower in all groups with deleted MMP-2 (*Figure 2B*).

Finally, ex vivo platelet adhesion to collagen under high shear rate flow conditions was significantly higher in *LDLR*$^{-/-}$ as compared with wild-type mice, an effect abolished in *dKO* and *LDLR*$^{-/-}$-chimeric mice, while it was significantly lower in *MMP-2*$^{-/-}$ mice (*Figure 2C* and Supplementary material online, *Figure S5*).

Therefore, deletion of platelet MMP-2 in hypercholesterolemic mice reduces in vivo and ex vivo platelet activation.

Activated platelets induce endothelial cell activation

Co-incubation of human umbilical vein endothelial cells (HUVECs) with thrombin-activated, but not with resting, platelets from *LDLR*$^{-/-}$ mice triggered endothelial activation, as shown by a significant increase of adhesive molecules such as ICAM-1 (data not shown) and VCAM-1. This effect was prevented by the pre-incubation of platelets with a P-selectin-blocking antibody while no further reduction of VCAM-1 expression was observed by blocking also platelet CD40L, suggesting that platelet P-selectin is important for endothelial activation (*Figure 3A*). On the other hand, co-incubation of HUVEC with thrombin-activated platelets from *MMP-2*$^{-/-}$ mice did not trigger endothelial activation despite expressing P-sel (*Figure 3B*), while the co-incubation with activated platelets from *P-sel*$^{-/-}$ mice, yet expressing MMP-2 at their surface, still induced a significant increase in endothelial VCAM-1 expression, although lower than that induced by *LDLR*$^{-/-}$ platelets, that was completely abolished by pre-treatment with the selective MMP-2 inhibitor TIMP-2 (*Figure 3A*). The lack of endothelial activation by *MMP-2*$^{-/-}$ platelets was not due to defective adhesion because the adhesion of platelets from *MMP-2*$^{-/-}$ mice to activated HUVEC is not different from that of normal platelets (Supplementary material online, *Figure S6*).

Activated, but not resting, platelets from *LDLR*$^{-/-}$ were able to trigger VCAM-1 expression also by human aortic endothelial cells (HAECs), an effect prevented by TIMP-2 (1 μg/mL) as well as by the blockade of PSGL-1 using a selective antibody (Supplementary material online, *Figure S7*).

EC activation initiated by thrombin-stimulated platelets required direct cell contact, in fact, when platelets were separated from HUVEC by interposing a filter, activated platelet-triggered endothelial VCAM-1 expression was strikingly reduced (Supplementary material online, *Figure S8*).

These findings show that activated platelets activate ECs through a mechanism involving both P-selectin and surface expressed MMP-2.

Platelet-derived MMP-2 facilitates transendothelial migration of monocytes/macrophages

To clarify the role of platelet MMP-2 in monocytes/macrophages recruitment in the arterial intima, we measured transendothelial migration of mixtures of monocytes/macrophages with activated platelets. Thrombin-stimulated *LDLR*$^{-/-}$-platelets strongly triggered the transendothelial migration of monocytes/macrophages derived from either *LDLR*$^{-/-}$ or *MMP-2*$^{-/-}$ mice (*Figure 4A*). Confirmatory experiments have been carried out using purified monocytes from *LDLR*$^{-/-}$ mice (Supplementary material online, *Figure S9*). On the contrary, activated platelets from *MMP-2*$^{-/-}$ mice did not elicit macrophage transmigration, thus platelets must express MMP-2 at their surface to facilitate transendothelial macrophage migration (*Figure 4B*). Furthermore, the inhibition of PAR-1 on ECs, by the selective PAR-1 antagonist RWJ-56610, abolished macrophage transmigration (*Figure 4C*). Thrombin-activated platelets from *LDLR*$^{-/-}$ mice favour the transmigration of purified *LDLR*$^{-/-}$ monocytes/macrophages though IL-1β activated *LDLR*$^{-/-}$ ECs but not from *PAR3*$^{-/-}$ ECs (Supplementary material online, *Figure S10*). On the other hand, the blockade of PSGL-1 on monocytes/macrophages

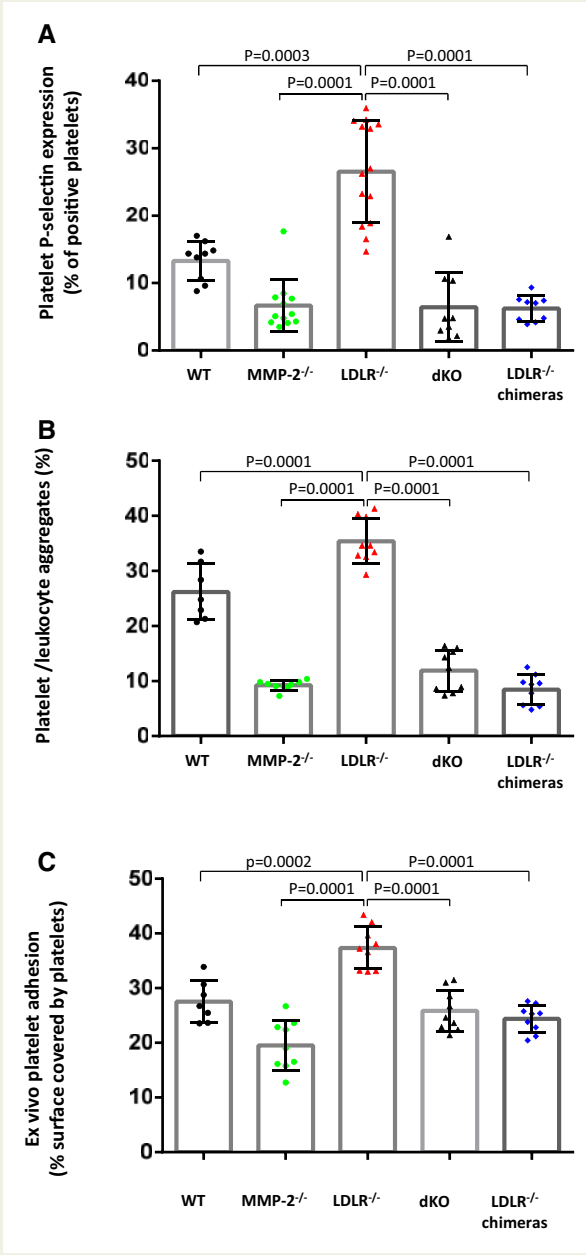
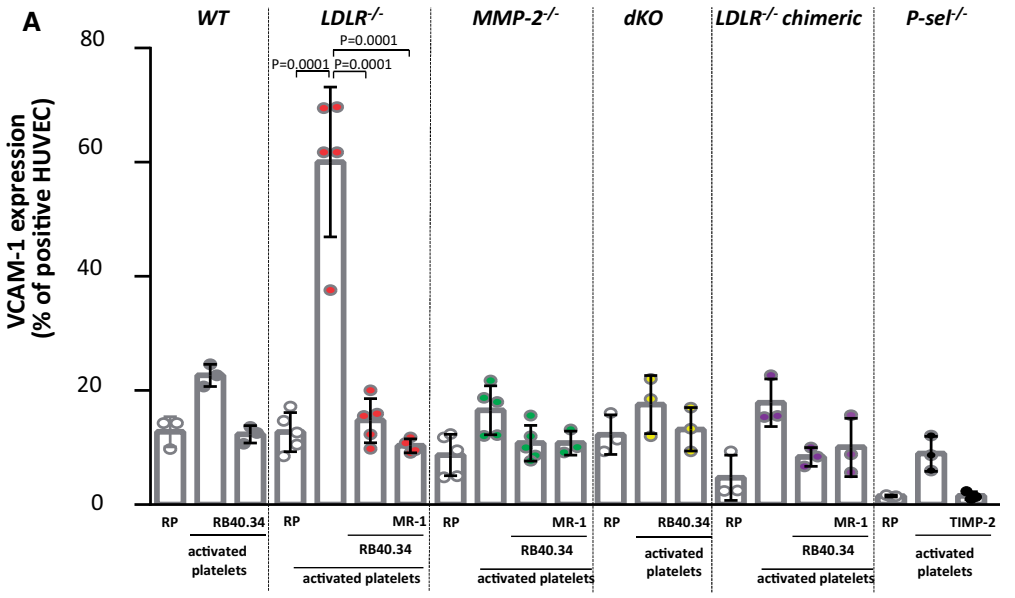


Figure 2 Platelet hyperreactivity of hyperlipemic mice is blunted by MMP-2 deletion. (*A*) Platelet P-selectin expression and (*B*) circulating platelet-leukocyte aggregates induced by ex vivo stimulation with collagen + epinephrine and (*C*) ex vivo platelet adhesion on a collagen-coated surface under high shear stress flow conditions (3000/s). Platelets from *LDLR*$^{-/-}$ mice ($n = 9$) are hyperreactive as compared with platelets from wild-type mice ($n = 7$), while platelets from *MMP-2*$^{-/-}$ ($n = 9$), *dKO* ($n = 9$), and *LDLR*$^{-/-}$-chimeric mice ($n = 9$) are hyporeactive. Platelet count did not differ between *LDLR*$^{-/-}$, *MMP-2*$^{-/-}$, *dKO*, and *LDLR*$^{-/-}$-chimeric mice. All data are expressed as mean ± standard deviation. One-way analysis of variance with Tukey's multiple comparisons test was carried out.

A



B

Thrombin (0.1U/ml)-stimulated platelets from:	Platelet P-selectin (% of positive platelets)	Platelet MMP-2 expression (% of positive platelets)
Wild type mice	78.2±10.8*	37.4±13.1*
LDLR$^{-/-}$ mice	90.8±6.2	41.5±2.7
MMP-2$^{-/-}$ mice	14.2±2.7*	3.3±2.13*
dKO mice	14.0±3.6*	4.9±3.1*
LDLR $^{-/-}$ *chimeric mice*	16.6±3.66*	6.13±1.82*
P-sel $^{-/-}$ mice	3.7±0.82*	25.3±2.22*

Figure 3 Activated platelets trigger VCAM-1 expression by HUVEC. (*A*) Co-incubation of HUVEC with resting and activated platelets from WT (grey), *LDLR*$^{-/-}$ (red), *MMP-2*$^{-/-}$ (green), *dKO* (yellow), *LDLR-chimeric* (purple), and *P-sel*$^{-/-}$ mice (black). VCAM-1 expression, an index of endothelial cell activation, was assessed by flow cytometry. White circles: resting platelets of each strain. In selected experiments, platelet P-selectin, CD40L, and active MMP-2 were blocked using, respectively, RB40.34 (100 μg/mL), MR1 (20 μg/mL), and TIMP-2 (1 μg/mL). Baseline VCAM-1 expression by HUVEC in the absence of platelets was 1.69±0.25% of positive cells. Data are expressed as mean ± standard deviation of at least three different experiments. *LDLR*$^{-/-}$ platelets: *$P < 0.0001$ vs. all groups. (*B*) The expression of P-selectin and MMP-2 at the surface of the activated platelets used in co-incubation experiments as assessed by flow cytometry. Data are expressed as mean ± standard deviation. *$P < 0.001$ vs *LDLR*$^{-/-}$ mice. One-way analysis of variance with Tukey's multiple comparisons test was carried out.

also abolished transendothelial migration triggered by activated platelets, suggesting that the interaction of platelet P-selectin with macrophage PSGL-1 is also relevant for this phenomenon (*Figure 4C*).

Thus, our data show that macrophage infiltration through endothelium is triggered by the direct interaction between platelet MMP-2 and endothelial PAR-1, with a mechanism involving also P-selectin.

Platelet-derived MMP-2 activates endothelial cell PAR-1

To clarify if the activation of ECs induced by activated platelets was mediated mainly by platelet-derived MMP-2 or by platelet-P-selectin we evaluated the phosphorylation of AKT and p38MAPK which are involved, respectively, in the signalling pathways triggered by PAR-1 and PSGL-1 activation. Co-incubation of HUVEC with thrombin-activated platelets significantly enhanced AKT phosphorylation, an effect abolished by TIMP-2 and by the selective PAR1 antagonist RWJ-56610 (*Figure 5A*). Moreover, the MMP-2-generated tethered ligand DPRSFLLRN[21] directly activated EC PAR-1, as shown by the enhancement of VCAM-1 expression assessed by flow cytometry (Supplementary material online, *Figure S11*). On the other hand, the phosphorylation of endothelial p38MAPK induced by thrombin-activated platelets was only partially inhibited by the blockade of PSGL-1 on ECs with an anti PSGL-1 MoAb (*Figure 5B*). Moreover, active MMP-2 induced the cleavage of PAR-1 in HAECs, similarly to thrombin (*Figure 6A and B*).

These data demonstrate that in the interaction between activated platelets and EC, platelet MMP-2 is the main trigger of endothelial activation acting on EC PAR-1.

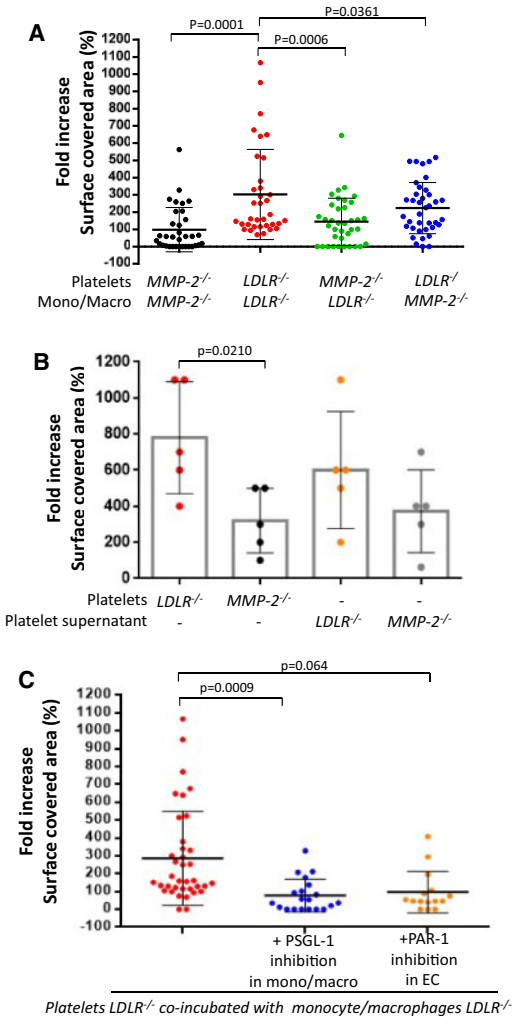


Figure 4 Platelet MMP-2 facilitates transendothelial migration of monocytes/macrophages. (*A*) Activated platelets from *LDLR*$^{-/-}$ mice significantly favoured transmigration of monocytes/macrophages from both *LDLR*$^{-/-}$ and *MMP-2*$^{-/-}$ mice while platelets from *MMP-2*$^{-/-}$ did not. $N = 3$ experiments, at least eight slides for each experiment were analysed. (*B*) Activated platelets from *LDLR*$^{-/-}$ mice expressing MMP-2 at their surface induce macrophage transendothelial migration while activated platelet supernatants (containing 200±18 pg/mL of active MMP-2) do not. Platelets from *MMP-2*$^{-/-}$ mice are ineffective. Surface MMP-2 expression activated by platelets from *LDLR*$^{-/-}$ and *MMP-2*$^{-/-}$ mice was, respectively, 17.1±0.21% and 1.7±0.7% of positive cells. $N = 5$ experiments. (*C*) Platelet-induced macrophage transendothelial migration was inhibited either by the preincubation of monocytes/macrophages from *LDLR*$^{-/-}$ mice with the inhibitor of PSGL-1 and by the preincubation of endothelial cells with a selective inhibitor of PAR-1. In these experiments, platelets and monocytes/macrophages were from *LDLR*$^{-/-}$ mice. $N = 3$ experiments, at least five slides for each experiment were analysed. (*D*) Thrombin-activated platelets from *LDLR*$^{-/-}$ induced purified *LDLR*$^{-/-}$ monocyte transmigration though IL-1β activated endothelial cells isolated from *LDLR*$^{-/-}$ but not from *PAR3*$^{-/-}$ mice. $N = 3$ experiments, at least five slides for each experiment were analysed. One-way analysis of variance with Tukey's multiple comparisons test was carried out.

Circulating platelet expression of MMP-2 is associated with atherosclerosis extent in humans

MMP-2 expressed on circulating platelets was significantly higher in CAD and chronic HIV patients compared to healthy controls (*Figure 7A*). Expression of MMP-2 on platelets significantly and positively correlated with the degree of carotid artery stenosis at ultrasound imaging ($r = 0.5285$, $P < 0.0001$, $n = 25$) (*Figure 7B*), with a higher percentage of platelets expressing MMP-2 in subjects with higher degree of carotid stenosis. Degree of carotid stenosis did not correlate instead with total cholesterol ($r = 0.21$, $n = 25$, $P = 0.5$) or with alpha-2-globulin ($r = 0.17$, $n = 25$, $P = 0.9$) and neutrophil-to-lymphocyte ratio ($r = 0.22$, $n = 25$, $P = 0.3$), inflammatory biomarkers.[22]

Discussion

Our data show a previously unrecognized crucial role of platelet-derived MMP-2 in the very early phases of atherogenesis in hypercholesterolemic mice. Circulating activated platelets expressing MMP-2 on their surface interact with PAR-1 of EC triggering their activation with the consequent exposure of adhesion molecules, the latter in turn facilitate the adhesion and transmigration of monocytes which ultimately lead to atheroma formation. Thus, circulating activated platelets play a role in endothelial activation which is the very initial trigger of the cascade of events involved in atherogenesis (*Graphical abstract*).

Turbulence in arterial blood flow, such as that occurring at branches, bifurcations, and curvatures, favours the interaction of platelets with the vessel wall,[4,23] probably explaining the preferential location of atheromas at sites of altered blood flow.[24] Activated platelets deliver to ECs, upon flow-favoured contact with the latter, cytokines, and growth factors, which contribute to the adhesion and transmigration of monocytes/macrophages.[13,25,26]

The heterotypic interaction of platelets with white blood cells leading to the activation of the latter and the deposition of platelet-derived chemokines on ECs triggering leucocyte adhesion were considered so far the main mechanisms through which platelets contribute to atherogenesis.[4,13] Here, we show that activated platelets by expressing MMP-2 act at an earlier stage in atherogenesis by turning on EC PAR-1 and consequently the expression of adhesion molecules on the endothelium.

Considerable evidence supports a central role of MMP-2 in the development of atherosclerosis.[27–29] MMP-2 degrades elastin leading to the production of peptideswhich participate in atherosclerosis progression by accelerating LDL oxidation and calcification of the vascular wall.[30] Both human and experimental atherosclerotic plaques contain high amounts of MMP-2[27,31–33] and enhanced MMP-2 expression has been associated with a higher rate of subsequent ischaemic cerebrovascular events[31]by favouring platelet activation, plaque rupture, and thrombus formation.[28,31] We show here that the deletion of MMP-2 in *LDLR*$^{-/-}$ mice markedly reduced neointima formation triggered by femoral artery injury, an effect evident also when the deletion of MMP-2 concerned only circulating blood cells, suggesting a pivotal role of platelet- rather than vascular-derived MMP-2 in atherosclerosis development. We also show that circulating activated

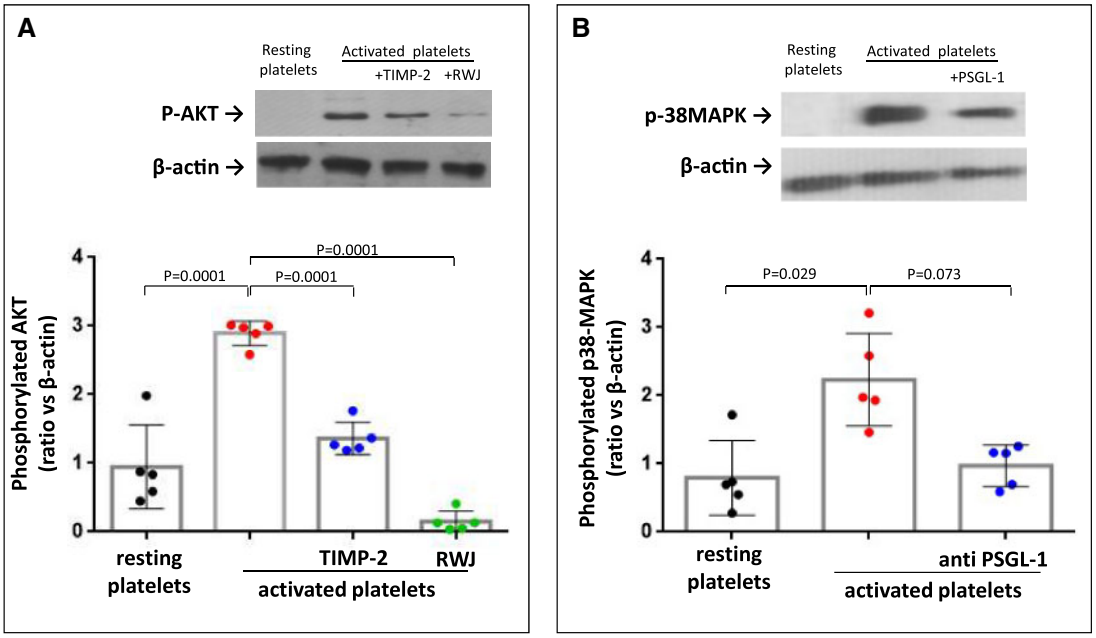


Figure 5 Phosphorylation of endothelial cell AKT induced by coincubation with activated platelets is abolished by TIMP-2 or by a selective PAR-1 antagonist. Expression of phospho-p38-MAP Kinase (T180/Y182) (*B*) and phospho-AKT Ser^{473} (*B*) in endothelial cell extracts, assessed by western blotting, after co-incubation with resting or thrombin-activated platelets from *LDLR*$^{-/-}$ mice. Enhanced EC-AKT phosphorylation induced by activated platelets was abolished by TIMP-2 (10 μg/mL) (Millipore, Darmstadt, Germany) and strikingly inhibited by the selective PAR-1 antagonist RWJ-56610 (5 μM). In the upper panels, representative western blotting gels are shown. Data from five different experiments were quantified by densitometry and expressed as arbitrary units. One-way analysis of variance with Tukey's multiple comparisons test was carried out.

platelets expressing surface MMP-2 contribute directly to atherosclerotic lesion formation as demonstrated by the exacerbation of atherosclerosis produced by the repeated transfusion of activated platelets expressing MMP-2 in thrombocytopenic *LDLR*$^{-/-}$ mice but not of activated platelets lacking MMP-2.

These results demonstrate an important role of platelet-derived MMP-2 in the cross-talk between platelets, inflammatory cells, and endothelium. Mice lacking MMP-2 display defective in vivo platelet activation and form less circulating platelet/leucocyte aggregates,[34] due to defective MMP-2-mediated potentiation of platelet activation.[21] Consistent with this, in the present study we show that circulating platelet/leucocyte aggregates and platelet P-selectin expression were reduced in *LDLR/MMP-2* double knockout mice as well as in *LDLR*$^{-/-}$ chimeric mice. Thus, in vivo platelet activation with the consequent expression of MMP-2 by platelets seems to be required for atherosclerosis initiation. Circulating activated platelets are commonly observed not only in patients with atherosclerosis[12,35,36] but also in subjects with known risk factors for atherosclerosis, like type 2 diabetes mellitus,[37,38] hypertension,[39] and hyperlipidaemia.[40] Moreover, platelet hyperreactivity and in vivo platelet activation are predictive of the development and extension of atherosclerosis in humans.[41–45] Several observations suggest that in vivo platelet activation actually precedes atherosclerotic plaque development, and in fact, the control of risk factors favouring atherosclerosis progression reduces in vivo platelet activation and hyperreactivity.[44,46]

To assess the translational value of our model data, we studied the expression of MMP-2 on the surface of circulating platelets and its possible association with the extent of atherosclerotic disease in a group of patients with stable CAD or chronic HIV infection. We show here that platelet MMP-2 was enhanced in patients with CAD and HIV and it significantly and positively correlated with carotid artery stenosis. A prospective study is ongoing to further define the pathogenic role of platelet-expressed MMP-2 for atherosclerosis progression and CV events in patients at CV risk.

Our observation that the deletion of MMP-2 reduced in a particularly striking way the size of atherosclerotic lesions in the aortic arch, the site where atherosclerotic lesions initially develop in mice, supports a role of platelet MMP-2 in the initial steps of atherogenesis.[47]

Our data show that platelet MMP-2 activates the endothelium by a mechanism involving endothelial PAR-1. In fact, blockade of active MMP-2 on platelets or of PAR-1 on the endothelium abolished AKT phosphorylation, the signalling pathway downstream of PAR-1, in ECs while blockade of PSGL-1 on the endothelium, the ligand of platelet P-selectin, did not abolish p38MAPK phosphorylation. Moreover, we show that active MMP-2 cleaves endothelial PAR-1, similarly to thrombin.[48] Upon activation, PAR-1 causes cytoskeletal rearrangement in ECs that destabilizes cell–cell contacts causing an increase in vascular permeability, which facilitates the passage of molecules and inflammatory cells from blood into subendothelium.[49,50] Moreover, activated platelets bind circulating monocytes/macrophages through a mechanism involving P-selectin/PSGL-1 and favour

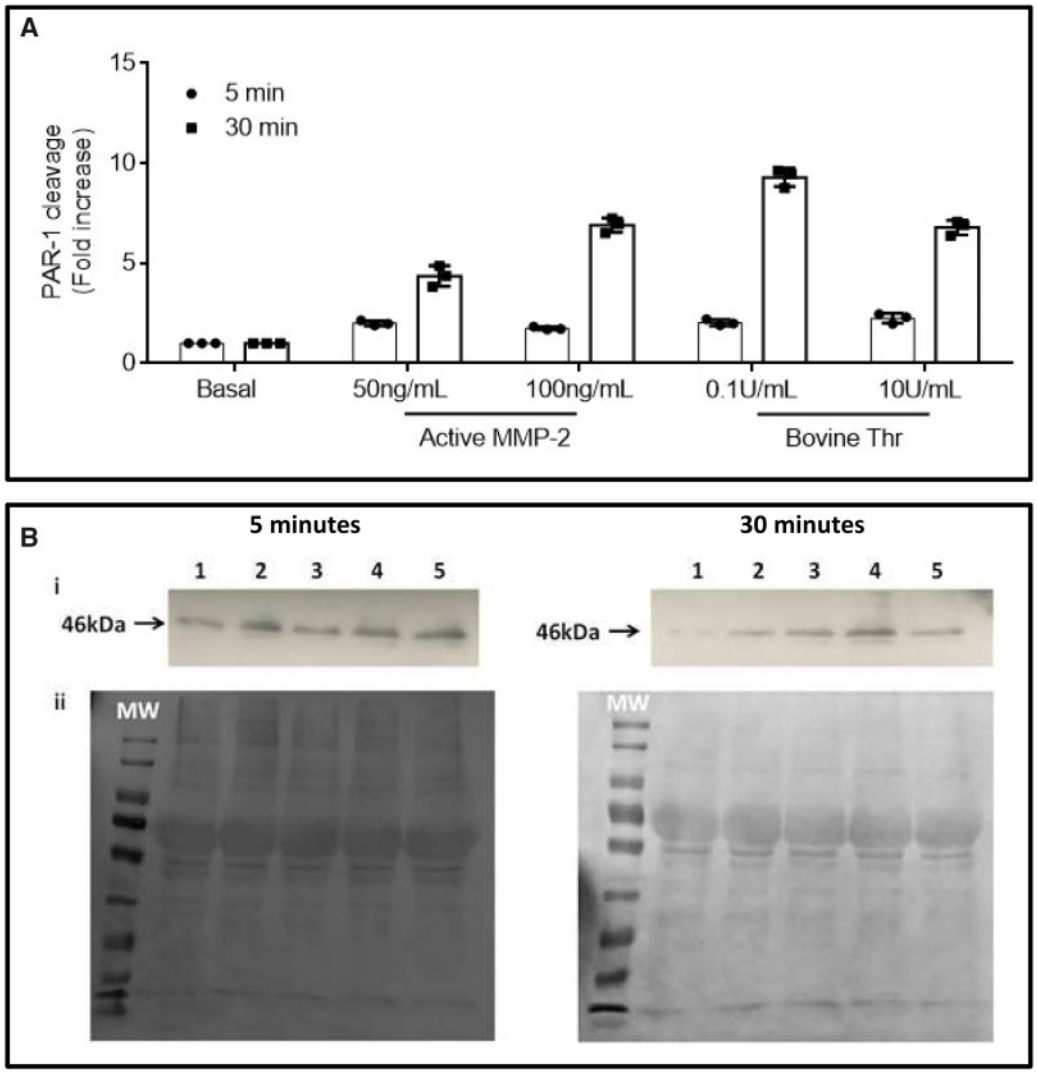


Figure 6 Active MMP-2 induced endothelial PAR-1 cleavage. (*A*) Cleavage of endothelial cell PAR-1. A. Time course of the cleavage of PAR-1 induced by active MMP-2 50 and 100 ng/mL. Bovine thrombin was used as positive control. The cleavage was assessed after 5 and 30 min of incubation of endothelial cells with active MMP-2 or thrombin. Cleavage of PAR-1 was assessed by western blotting using a primary antibody against cleaved PAR-1 and bands were analysed using Image J 1.51t software. Results are normalized as ratio vs. Ponceau S stain and reported as fold increase versus basal level of PAR1 (lane 1). (*B*) Representative (i) western blotting and (ii) Ponceau S staining of the cleavage of PAR-1 expressed on endothelial cells. On the left, PAR-1 cleavage upon 5-min incubation with active MMP-2 and bovine thrombin has been reported; on the right, PAR-1 cleavage upon 30-min incubation with active MMP-2 and bovine thrombin has been reported. MW, molecular weight; Lane 1, human aortic endothelial cells (baseline); Lane 2, human aortic endothelial cells + MMP-2 50 ng/mL; Lane 3, human aortic endothelial cells + MMP-2 100 ng/mL; Lane 4, human aortic endothelial cells + thrombin 0.1 U/mL; Lane 5, human aortic endothelial cells + thrombin 10 U/mL.

their transendothelial migration via the platelet MMP-2/endothelial PAR-1 pathway. In this way, platelets expressing MMP-2 promote macrophage infiltration into vascular tissue favouring the development of atherosclerotic plaques. Our study has some limitations. We did not use a tissue specific gene deletion approach by generating conditional strains MMP-$2^{-/-}$/PF4-Cre/LDLR$^{-/-}$ and MMP-$2^{-/-}$/PF4-Cre/wild type while to unravel platelet MMP-2 contribution we adopted repeated cross-transfusions of platelets from *MMP-2*$^{-/-}$ mice into thrombocytopenic *LDLR*$^{-/-}$ mice. Although the Cre/loxP strategy might elegantly support our conclusions, the cross transfusion model we used, with all the appropriate controls, and the large amount of confirmatory and mechanistic in vitro experiments using cells/tissues from specific knockout mice, exclude significant technical/biological bias in the interpretation of our results. Indeed, this approach has been used with success and is still used, in several important studies assessing the involvement of specific platelet and leucocyte molecules in atherosclerosis.[13,51,52] Another limitation is represented by the lack of studies with specific pharmacological inhibition of MMP-2, which could have strenghtened our results. Unfortunately, all chemical agents inhibiting MMP-2 are not

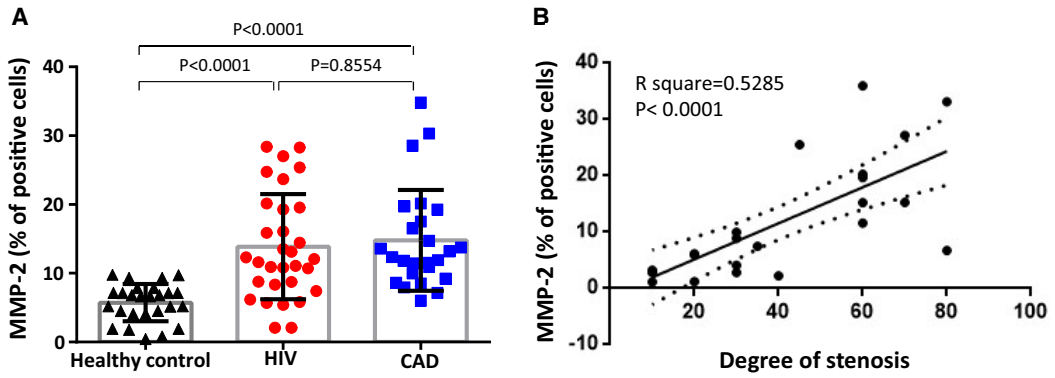


Figure 7 MMP-2 expression on platelet surface in HIV and CAD study population. (*A*) The MMP-2 levels in the HIV group and CAD group were significantly higher than in the healthy control group ($P < 0.0001$ for both); no significant difference between HIV and CAD groups. Data are reported as mean ± standard deviation. One-way analysis of variance with Tukey's multiple comparisons test was carried out. (*B*) Correlation between platelet surface expression of MMP-2 and degree of carotid stenosis. MMP-2 had a positive correlation with percentage of stenosed vessel, indicating that with increase in this parameter the MMP-2 on platelet surface increased. Linear regression was carried out (GraphPad Prism 6.01).

completely selective for MMP-2. We are currently intensively working on this issue and, in particular, we are working on the development of MMP-2-specific nanobodies.[53] Unfortunately, nanobody activity with mouse platelets seems to be weaker than with human platelets.[54]

In conclusion, our findings have considerable impact on the understanding of the inflammatory mechanisms starting atherogenesis throwing new light on the role of platelets in the very initial steps which trigger leucocyte infiltration in the arterial wall, and have potential implications for the design of novel, selective anti-atherogenic drugs considering that, in contrast to the current focus on slowing the progression of advanced plaques after middle age, future therapeutic options should be targeted at earlier stages of the disease process with the intent to preempting the progression of cardiovascular disease.[55]

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Authors' contributions

S.M. and P.G. designed experiments. S.M., E.F., G.C.T., A.O. and E.P. performed experiments, collected and analyzed data. S.M. and P.G. wrote the manuscript, which was edited by all co-authors. P.G. supervised, directed and managed the study.

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References

1. Chavez-Sanchez L, Espinosa-Luna JE, Chávez-Rueda K, Legorreta-Haquet MV, Montoya-Díaz E, Blanco-Favela F. Innate immune system cells in atherosclerosis. *Arch Med Res* 2014;**45**:1–14.
2. Libby P, Hansson GK. Inflammation and immunity in atherosclerosis. *Circ Res* 2015;**116**:307–311.
3. Ross R. Atherosclerosis—an inflammatory disease. *New Engl J Med* 1999;**340**:115–126.
4. Massberg SF, Korbinian B, Grüner S, Page S, Müller E, Müller I, Bergmeier W, Richter T, Lorenz M, Konrad I, Nieswandt B, Gawaz M. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med* 2002;**196**:887–896.
5. da Costa Martins P, García-Vallejo JJ, van Thienen JV, Fernandez-Borja M, van Gils JM, Beckers C, Horrevoets AJ, Hordijk PL, Zwaginga JJ. P-selectin Glycoprotein Ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium. *Arterioscler Thromb Vasc Biol* 2007;**27**:1023–1029.
6. Dole VS, Bergmeier W, Patten IS, Hirahashi J, Mayadas TN, Wagner DD. PSGL-1 regulates platelet P-selectin-mediated endothelial activation and shedding of P-selectin from activated platelets. *Thromb Haemost* 2007;**98**:806–812.
7. Giannini S, Falcinelli E, Bury L, Guglielmini G, Rossi R, Momi S, Gresele P. Interaction with damaged vessel wall in vivo in humans induces platelets to express CD40L resulting in endothelial activation with no effect of aspirin intake. *Am J Physiol Heart Circ Physiol* 2011;**300**:H2072–2079.
8. Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;**391**:591–594.
9. Andrè P, Denis CV, Ware J, Saffaripour S, Hynes RO, Ruggeri ZM, Wagner DD. Platelets Adhere to and Translocate on Von Willebrand factor presented by endothelium in stimulated veins. *Blood* 2000;**96**:3322–3328.
10. Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, McIntire LV, Berndt MC, López JA. The glycoprotein Ib-IX-complex is a platelet counterreceptor for P-selectin. *J Exp Med* 1999;**190**:803–813.
11. Van Gils JM, Zwaginga JJ, Hordijk PL. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. *J Leukoc Biol* 2009;**85**:195–204.
12. Berger G, Hartwell DW, Wagner DD. P-selectin and platelet clearance. *Blood* 1998;**92**:4446–4452.

13. Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;**9**:61–67.
14. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 2002;**90**:251–262.
15. Kanda S, Kuzuya M, Ramos MA, Koike T, Yoshino K, Ikeda S, Iguchi A. Matrix metalloproteinase and alphavbeta3 integrin-dependent vascular smooth muscle cell invasion through a type I collagen lattice. *Arterioscler Thromb Vasc Biol* 2000; **20**:998–1005.
16. Kuzuya K, Kanda S, Sasaki T, Tamaya-Mori N, Cheng XW, Itoh T, Itohara S, Iguchi A. Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. *Circulation* 2003;**108**:1375–1381.
17. Falcinelli E, Giannini S, Boschetti E, Gresele P. Platelets release active matrix metalloproteinase-2 in vivo in humans at a site of vascular injury. Lack of inhibition by aspirin. *Brit J Haematol* 2007;**138**:221–230.
18. Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW. Release of gelatinase A during platelet aggregation. *Nature* 1997;**386**:616–619.
19. Mastenbroek TG, Feijge MAH, Kremers RMW, van den Bosch MTJ, Swieringa F, De Groef L, Moons L, Bennett C, Ghevaert C, Johnson JL, van der Meijden PEJ, Cosemans JMEM. Platelet-associated matrix metalloproteinases regulate thrombus formation and exert local collagenolytic activity. *Arterioscler Thromb Vasc Biol* 2015;**35**:2554–2561.
20. Alunno A, Falcinelli E, Luccioli F, van den Bosch MTJ, Swieringa F, De Groef 1 L, Moons L, Bennett C, Ghevaert C, Johnson JL, van der Meijden PEJ, Cosemans JMEM. Platelets contribute to the accumulation of matrix metalloproteinase type 2 in synovial fluid in osteoarthritis. *Thromb Haemost* 2017;**117**:2116–2124.
21. Sebastiano M, Momi S, Falcinelli E, Bury L, Hoylaerts MF, Gresele P. MMP-2 mediates PAR-1 biased signaling in human platelets: a novel mechanism regulating platelet activation. *Blood* 2017;**129**:883–895.
22. Adamstein NH, MacFadyen JG, Rose LM, Glynn RJ, Dey AK, Libby P, Tabas IA, Mehta NN, Ridker PM. The neutrophil-lymphocyte ratio and incident atherosclerotic events: analyses from five contemporary randomized trials. *Eur Heart J* 2021;**42**:896–903.
23. Igawa T, Nagamura Y, Ozeki Y, Itoh H, Unemi F. Stenosis enhances role of platelets in growth of regional thrombus and intimal wall thickening in rat carotid arteries. *Circ Res* 1995;**77**:310–316.
24. Antoniadis AP, Giannopoulos AA, Wentzel JJ, Joner M, Giannoglou GD, Virmani R, Chatzizisis YS. Impact of Local flow haemodynamics on atherosclerosis in coronary artery bifurcations. *EuroIntervention* 2015;**11**:V18–22.
25. Lin J, Kakkar V, Lu X. Impact of matrix metalloproteinases on atherosclerosis. *Curr Drug Targets* 2014;**15**:442–453.
26. Vajen T, Benedikter B, Heinzmann ACA, Vasina EM, Henskens Y, Parsons M, Maguire PB, Stassen FR, Heemskerk JWM, Schurgers LJ, Koenen RR. Platelet extracellular vesicles induce a pro-inflammatory smooth muscle cell phenotype. *J Extracell Vesicles* 2017;**6**:1322454.
27. Busti C, Falcinelli E, Momi S, Gresele P. Matrix metalloproteinases and peripheral arterial disease. *Intern Emerg Med* 2010;**5**:13–25.
28. Gresele P, Falcinelli E, Sebastiano M, Momi S. Matrix metalloproteinases and platelet function. *Prog Mol Biol Transl Sci* 2017;**147**:133–165.
29. King SM, McNamee RA, Houng AK, Patel R, Brands M, Reed GL. Platelet dense-granule secretion plays a critical role in thrombosis and subsequent vascular remodeling in atherosclerotic mice. *Circulation* 2009;**120**:785–791.
30. Maurice P, Blaise S, Gayral S, Debelle L, Laffargue M, Hornebeck W, Duca L. Elastin fragmentation and atherosclerosis progression: the elastokine concept. *Trends Cardiovasc Med* 2013;**23**:211–221.
31. Lenti M, Falcinelli E, Pompili M, de Rango P, Conti V, Guglielmini G, Momi S, Corazzi T, Giordano G, Gresele P. Matrix metalloproteinase-2 of human carotid atherosclerotic plaques promotes platelet activation. Correlation with ischaemic events. *Thromb Haemost* 2014;**111**:1089–1101.
32. Li Z, Li L, Zielke HR, Cheng L, Xiao R, Crow MT, Stetler-Stevenson WG, Froehlich J, Lakatta EG. Increased expression of 72-kd type IV collagenase (MMP-2) in human aortic atherosclerotic lesions. *Am J Pathol* 1996;**148**:121–128.
33. Zhai X, Chi J, Tang W, Ji Z, Zhao F, Jiang C, Lv H, Guo H. Yellow wine polyphenolic compounds inhibit matrix metalloproteinase-2, -9 expression and improve atherosclerotic plaque in LDL-receptor-knockout mice. *J Pharmacol Sci* 2014;**125**:132–141.
34. Momi S, Falcinelli E, Giannini S, Ruggeri L, Cecchetti L, Corazzi T, Libert C, Gresele P. Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo. *J Exp Med* 2009;**206**:2365–2379.
35. Lukasik M, Rozalski M, Luzak B, Michalak M, Ambrosius W, Watala C, Kozubski W. Enhanced platelet-derived microparticles formation is associated with carotid atherosclerosis in convalescent stroke patients. *Platelets* 2013;**24**:63–70.
36. van Lammeren GW, Pasterkamp G, de Vries J-PPM, Bosch L, de Haan JJ, de Kleijn DPV, Moll FL, Vink A. Platelets enter atherosclerotic plaque via intraplaque microvascular leakage and intraplaque hemorrhage: a histopathological study in carotid plaques. *Atherosclerosis* 2012;**222**:355–359.
37. Davì G, Catalano I, Averna M, Notarbartolo A, Strano A, Ciabattoni G, Patrono C. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med* 1990;**322**:1769–1774.
38. Davì G, Gresele P, Violi F, Basili S, Catalano M, Giammarresi C, Volpato R, Nenci GG, Ciabattoni G, Patrono C. Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease. *Circulation* 1997;**96**:69–75.
39. Minuz P, Patrignani P, Gaino G, Seta F, Capone ML, Tacconelli S, Degan M, Faccini G, Fornasiero A, Talamini G, Tommasoli R, Arosio E, Lechi Santonastaso C, Lechi A, Patrono C. Determinants of platelet activation in human essential hypertension. *Hypertension* 2004;**43**:64–70.
40. Korporaal SJA, Akkerman JWN. Platelet activation by low density lipoprotein and high density lipoprotein. *Pathophysiol Haemost Thromb* 2006;**35**:270–280.
41. Csongrádi E, Nagy B, Jr, Fulop T, Varga Z, Karányi Z, Magyar MT, Oláh L, Papp M, Facskó A, Kappelmayer J, Paragh G, Káplár M. Increased levels of platelet activation markers are positively associated with carotid wall thickness and other atherosclerotic risk factors in obese patients. *Thromb Haemost* 2011;**106**: 683–692.
42. Keating FK, Whitaker DA, Kabbani SS, Ricci MA, Sobel BE, Schneider DJ. Relation of augmented platelet reactivity to the magnitude of distribution of atherosclerosis. *Am J Cardiol* 2004;**94**:725–728.
43. Koyama H, Maeno T, Fukumoto S, Shoji T, Yamane T, Yokoyama H, Emoto M, Shoji T, Tahara H, Inaba M, Hino M, Shioi A, Miki T, Nishizawa Y. Platelet P-selectin expression is associated with atherosclerosis wall thickness in carotid artery in humans. *Circulation* 2003;**108**:524–529.
44. Nomura S, Suzuki M, Katsura K, Xie GL, Miyazaki Y, Miyake T, Kido H, Kagawa H, Fukuhara S. Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis* 1995;**116**:235–240.
45. Shoji T, Koyama H, Fukumoto S, Maeno T, Yokoyama H, Shinohara K, Emoto M, Shoji T, Inaba M, Nishizawa Y. Platelet-monocyte aggregates are independently associated with occurrence of carotid plaques in type 2 diabetic patients. *J Atheroscler Thromb* 2005;**12**:344–352.
46. Gresele P, Guglielmini G, De Angelis M, Ciferri S, Ciofetta M, Falcinelli E, Lalli C, Ciabattoni G, Davì G, Bolli GB. Acute, short-term hyperglycemia enhances shear stress-induced platelet activation in patients with type II diabetes mellitus. *J Am Coll Cardiol* 2003;**41**:1013–1020.
47. Frenette PS, Moyna C, Hartwell DW, Lowe JB, Hynes RO, Wagner DD. Platelet-endothelial interactions in inflamed mesenteric venules. *Blood* 1998;**91**: 1318–1324.
48. Shea BS, Probst CK, Brazee PL, Rotile NJ, Blasi F, Weinreb PH, Black KE, Sosnovik DE, Van Cott EM, Violette SM, Caravan P, Tager AM. Uncoupling of the profibrotic and hemostatic effects of thrombin in lung fibrosis. *JCI Insight* 2017;**2**:e86608.
49. Garcia JG, Davis HW, Patterson CE. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J Cell Physiol* 1995;**163**:510–522.
50. Wilcox JN, Rodriguez J, Subramanian R, Ollerenshaw J, Zhong C, Hayzer DJ, Horaist C, Hanson SR, Lumsden A, Salam TA. Characterization of thrombin receptor expression during vascular lesion formation. *Circ Res* 1994;**75**:1029–1038.
51. Luo H, Wei L, Lu L, Kang L, Cao Y, Yang X, Bai X, Fan W, Zhao BQ. Transfusion of resting platelets reduces brain hemorrhage after intracerebral hemorrhage and tPA-induced hemorrhage after cerebral ischemia. *Front Neurosci* 2019;**13**:338.
52. Soehnlein O, Drechsler M, Döring Y, Lievens D, Hartwig H, Kemmerich K, Ortega-Gómez A, Mandl M, Vijayan S, Projahn D, Garlichs CD, Koenen RR, Hristov M, Lutgens E, Zernecke A, Weber C. Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. *EMBO Mol Med* 2013;**5**:471–481.
53. Marturano A, Hendrickx MLV, Falcinelli E, Sebastiano M, Guglielmini G, Hassanzadeh-Ghassabeh G, Muyldermans S, Declerck PJ, Gresele P. Development of anti-matrix metalloproteinase-2 (MMP-2) nanobodies as potential therapeutic and diagnostic tools. *Nanomedicine* 2020;**24**:102103.
54. Momi S, Tantucci M, Van Roy M, Ulrichts H, Ricci G, Gresele P. Reperfusion of cerebral artery thrombosis by the GPIb-VWF blockade with the Nanobody ALX-0081 reduces brain infarct size in guinea pigs. *Blood* 2013;**121**: 5088–5097.
55. Gibbons GH, Seidman CE, Topol EJ. Conquering atherosclerotic cardiovascular disease—50 years of progress. *N Engl J Med* 2021;**384**:785–788.