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Legumain is upregulated in acute cardiovascular events and associated with improved outcome - potentially related to anti-inflammatory effects on macrophages

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HIGHLIGHTS

- High circulating levels of legumain in patients with stable or acute cardiovascular disease.
- High circulating legumain in the acute phase is correlated with improved outcome.
- Platelets contain, release and could be sources of circulating legumain.
- Extracellular legumain mediates anti-inflammatory responses in primary monocytes.

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ABSTRACT

Background and aims: We have previously found increased levels of the cysteine protease legumain in plasma and plaques from patients with carotid atherosclerosis. This study further investigated legumain during acute cardiovascular events.
Methods: Circulating levels of legumain from patients and legumain released from platelets were assessed by enzyme-linked-immunosorbent assay. Quantitative PCR and immunoblotting were used to study expression,

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while localization was visualized by immunohistochemistry.
Results: In the *SUMMIT Malmö* cohort (n = 339 with or without type 2 diabetes and/or cardiovascular disease [CVD], and 64 healthy controls), the levels of circulating legumain were associated with the presence of CVD in non-diabetics, with no relation to outcome. In symptomatic carotid plaques and in samples from both coronary and intracerebral thrombi obtained during acute cardiovascular events, legumain was co-localized with macrophages in the same regions as platelets. *In vitro*, legumain was shown to be present in and released from platelets upon activation. In addition, THP-1 macrophages exposed to releasate from activated platelets showed increased legumain expression. Interestingly, primary peripheral blood mononuclear cells stimulated with recombinant legumain promoted anti-inflammatory responses. Finally, in a STEMI population (*POSTEMI*; n = 272), patients had significantly higher circulating legumain before and immediately after percutaneous coronary intervention compared with healthy controls (n = 67), and high levels were associated with improved outcome.
Conclusions: Our data demonstrate for the first time that legumain is upregulated during acute cardiovascular events and is associated with improved outcome.

1. Introduction

Atherosclerosis is the major underlying cause of cardiovascular disease (CVD), characterized by interaction between accumulated lipids and inflammation, resulting in persistent low-grade inflammation. Several immune cells contribute to this non-resolving inflammation including monocytes/macrophages and T cells. In addition to their role in thrombus formation, platelets and platelet-mediated inflammation seem to play a pathogenic role in atherosclerosis, both in the early and late stage [1].

Proteases secreted by macrophages play important roles in plaque progression and destabilization by degrading the extracellular matrix (ECM) in the fibrous cap. Matrix metalloproteases (MMPs) are well known markers of CVD progression [2]. However, the roles of other proteases, like members of the cysteine protease family, are less studied. The cysteine protease legumain (asparaginyl endopeptidase) is thought to promote ECM degradation by activation of proMMP-2 [3] and processing of cathepsins [4] or by direct proteolysis of ECM components like fibronectin [5,6]. Legumain is present intracellularly in lysosomes, where it participates in protein degradation, but is also extensively secreted and found in human serum and plasma [7,8]. Legumain has been shown to be highly upregulated in unstable carotid plaques and more in unstable than in stable regions of the same plaque [9,10]. Very recently, high plasma levels of legumain were measured in patients with complex coronary lesions [11]. We have recently shown that patients with carotid atherosclerosis have increased levels of legumain in plasma and plaques, with the highest level in patients with symptomatic disease [8]. Within the atherosclerotic lesion, legumain was co-localized with macrophages, and *in vitro*, pro-inflammatory macrophages (M1) secreted legumain especially after stimulation with cholesterol crystals [8]. To further extend our findings, we analyzed circulating legumain in two cohorts (*SUMMIT Malmö* and *POSTEMI*), representing patients with stable and acute CVD, respectively. Furthermore, legumain in thrombus materials and platelets was investigated, as well as the role of legumain in monocyte-macrophage inflammation.

2. Materials and methods

A detailed description of materials and methods are given in the Supplementary Data.

2.1. Study populations

The cross-sectional *SUrrogate markers for Micro- and Macrovascular hard endpoints for Innovative diabetes Tools (SUMMIT)* study was carried out at four European centers in order to identify vascular changes associated with clinically manifested CVD in patients with type-2 diabetes (T2D). The *SUMMIT Malmö* cohort consisted of 134 subjects with T2D and CVD, 134 subjects with T2D and no clinical history of CVD, 71 with CVD but no T2D, and 64 controls without T2D or CVD. The patients were recruited at Skåne University Hospital, Malmö, Sweden, between December 2010 and April 2013. The clinical characteristics of the cohort are shown in Supplementary Table 1 and have been described previously [12]. EDTA plasma were sampled and prepared by centrifugation for 10 min at 2000g and samples were stored at −80 °C until analyzed.

The Post conditioning in ST-Elevation Myocardial Infarction (POSTEMI) trial was a prospective, randomized, single-center, open-label clinical trial, investigating the effect of ischemic post-conditioning (IPost) on infarct size in patients with STEMI treated with primary percutaneous coronary intervention (PCI) [13] (Supplementary Table 2). The study design including a detailed cardiac magnetic resonance imaging (CMR) protocol has previously been reported [14]. Briefly, 272 patients with first-time STEMI and symptom duration < 6 h were included between January 2009 and August 2012 at Oslo University Hospital Ullevål, Norway. Blood sampling was performed before (median 2.8 h after symptom onset) and immediately after the PCI procedure and further at day 1 (median 18.3 h after PCI), and at 4 month follow-up. Serum and plasma was prepared by centrifugation for 10 min at 2000*g* and samples were stored at −80 °C until analyzed.

2.2. Ethics

All studies were approved by the local ethical committee and performed in accordance with the principles of the Declaration of Helsinki. The *POSTEMI* study which was an intervention trial was also registered at clinicaltrials.gov (registration number NCT00922675). Written informed consent was obtained from each individual participating in the studies.

3. Results

3.1. Elevated circulating legumain is associated with the presence of CVD in non-diabetic patients

In the *SUMMIT Malmö* cohort, there was a significant correlation between levels of circulating legumain and CVD (Fig. 1A, left panel), confirming our previous findings [8]. Higher levels of legumain were associated with the presence of CVD at baseline in non-diabetics (n = 135), but not in diabetics (n = 268) (Fig. 1A, right panel). Furthermore, the association between legumain and CVD in non-diabetics was independent of age, sex and HDL cholesterol (Fig. 1B). At baseline, levels of circulating legumain were also positively associated with increased right carotid maximal plaque thickness defined as previously described [12], arterial stiffness (pulse wave velocity) (Fig. 1C), serum creatinine levels and the width of carotid bulb (sinus) in non-diabetics (Supplemental Table 3). After 3 years of follow-up, 21 (16%) CV events (both fatal and non-fatal) were registered in the non-diabetics and 36 (14%) in the diabetics group, but no associations were found between legumain and CV events at follow-up (Supplemental Table 3).

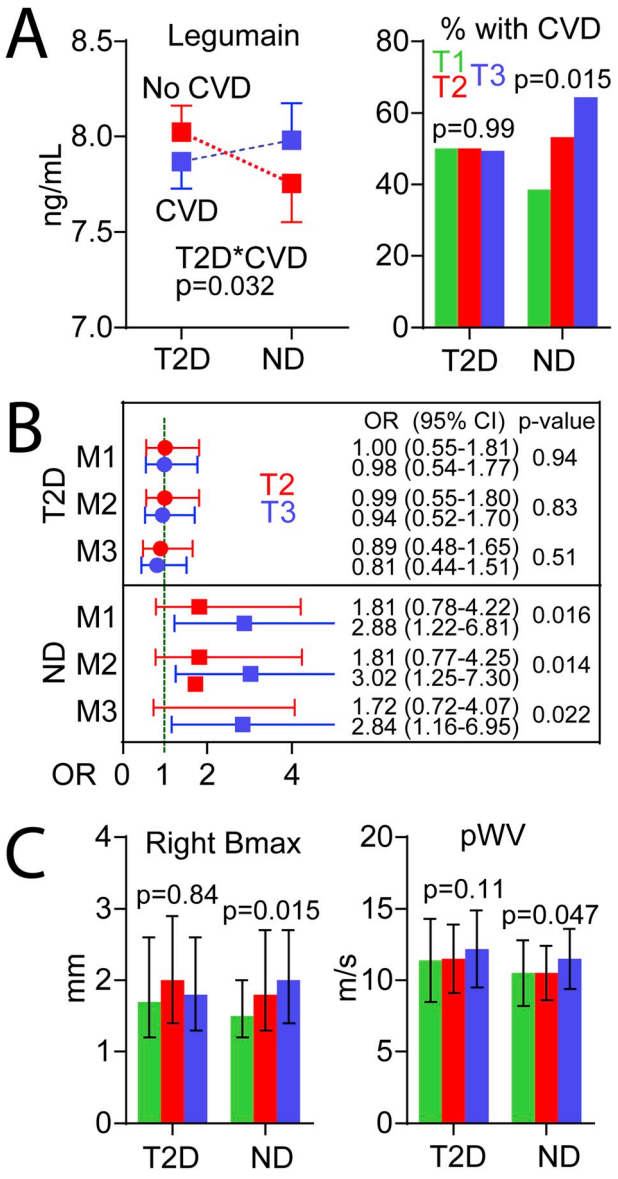


Fig. 1. Circulating levels of legumain in diabetics (T2D) and non-diabetics (ND) with or without CVD.
(A) General linear modelling (two-way ANOVA) was used to calculate significant associations between legumain and the main factors, T2D and CVD, and their interaction. The *SUMMIT Malmö* cohort consisted of 134 subjects with T2D and CVD, 134 subjects with T2D and no CVD, 71 with CVD and no T2D. Estimated marginal means of legumain levels and 95% confidence intervals are presented (left panel). The percentage of individuals with CVD across tertiles (T1-T3) of plasma legumain in all individuals, T2D and ND is shown in the right panel. Chi-square test was used to calculate *p* values and *p* values for linear trends across tertiles. (B) Logistic regression showing association between increasing legumain tertiles (Tertile [T] 1 as reference, T2 red, T3 blue) and CVD in T2D (top part) and ND (bottom part). M1, univariate; M2 adjusted with age and sex; M3 adjusted with age, sex and HDL. (C) Right maximal plaque thickness (Right Bmax, left) and arterial stiffness (pWV, right) according to tertiles of legumain in T2D and ND. *p*-values calculated with Kruskal-Wallis within each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Legumain is co-localized with platelets in carotid plaques and released upon platelet activation

We have previously shown co-localization of legumain with both pro-inflammatory M1 and anti-inflammatory M2 macrophages in carotid plaques [8]. Herein, we confirm the co-localization of legumain with macrophages (CD68), and also show that legumain is localized in the same regions as the platelet marker CD41 in symptomatic carotid plaques (Fig. 2A). By immunoblotting, legumain was actually detected in isolated platelets, both as the proform (56 kDa) and predominantly as the mature form (36 kDa) (Fig. 2B). The mature form was shown to be active, by binding to the legumain-selective activity-based probe (ABP) MP-L01 [15]. Furthermore, when platelet-rich plasma (PRP) was exposed to the PAR-1 agonist SFLLRN, a rapid and significant release (40% increase within 10 min) of legumain was induced (Fig. 2C). This legumain release was inhibited by the PAR-1 antagonist vorapaxar (Fig. 2D).

In an attempt to mimic the inflammatory milieu within atherosclerotic lesions, THP-1 monocytes were pre-activated with TNFα before addition of releasate from thrombin-activated platelets (sPRL) or unstimulated platelets (uPRL). As shown in Fig. 2E, sPRL significantly enhanced legumain mRNA expression in TNFα-pre-activated THP-1 cells compared to uPRL. Activation of TLR4 or TLR2 by LPS or Pam3Cys, respectively, or IL-1β, all potent inducers of monocyte activation, did not induce any change in legumain mRNA level.

3.3. Legumain is detected in thrombi materials obtained at the site of vascular occlusion

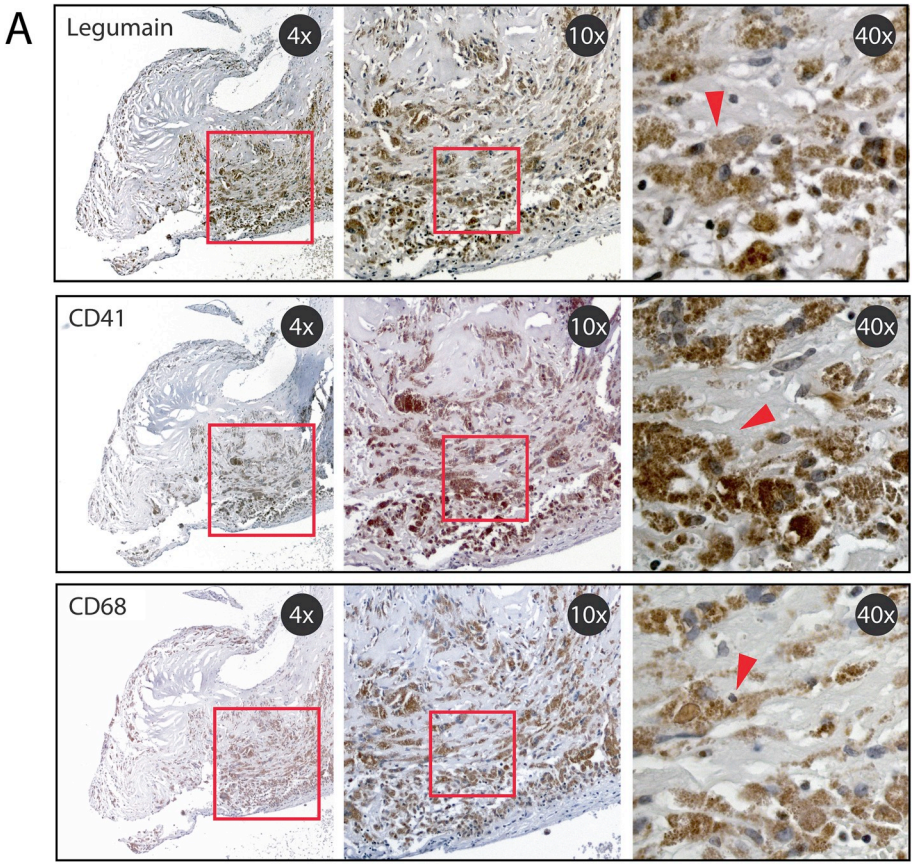
Examination of thrombus material removed from the site of the ruptured plaque showed positive legumain immunostaining, with the same pattern both in coronary and intracerebral thrombi (Fig. 3A). Within the thrombi, legumain was co-localized both with monocytes/macrophages (CD14) and the platelet marker CD41 within the same region (Fig. 3B). In thrombi analyzed by LC-MS/MS, legumain was also identified with high confidence (Fig. 3C).

3.4. Legumain promotes development of anti-inflammatory macrophages

We next examined whether recombinant human legumain (100 ng/mL) affected the mRNA expressions of proto-typical pro-inflammatory (MCP-1) or anti-inflammatory (IL-10) cytokines, as well as the M2 marker CD163 in primary monocytes from healthy individuals. Whereas legumain significantly enhanced IL-10 and CD163 mRNA expressions, it markedly decreased the expression of MCP-1 (Supplemental Fig. S1A). In the cell supernatant, a similar pattern was seen for MCP-1 and sCD163, whereas secretion of IL-10 was not modulated by legumain (Supplemental Fig. S1B). To explore if legumain is involved in macrophage polarization, we added recombinant legumain to primary monocytes during differentiation by M-CSF. Legumain significantly reduced the monocyte activation marker CD14 in both undifferentiating and differentiating monocytes, and the effect was stable for up to 48 h for the differentiating cells (Supplemental Fig. S2). Further, legumain reduced expression of M1 markers in undifferentiating monocytes (CD68, TLR2, TLR4, iNOS) and in differentiating monocytes (TLR2, TLR4, Fig. 4A); as well as increased the expression of M2 polarization markers in undifferentiating monocytes (CD36, CD136) and differentiating monocytes (MSR-1, CD36, CD136; Fig. 4B). Legumain-stimulation further reduced the release of pro-inflammatory MPO and MCP-1, without affecting the release of TNF from the same cells (Fig. 4C). In total, our results suggest a net anti-inflammatory effect of legumain on primary monocytes.

3.5. Elevated circulating legumain during the acute phase of STEMI

As legumain was found in and released from platelets *in vitro* and also co-localized with platelets in thrombi materials, circulating legumain was examined in an acute CVD cohort consisting of 272 patients with STEMI. Patients treated with IPost and conventional PCI had a similar temporal legumain profile (Supplemental Fig. S3), consequently, the study population was analyzed as a whole. Interestingly, patients with STEMI had significantly higher serum legumain before and immediately after PCI (n = 245 and n = 249, respectively) compared with healthy controls (n = 67), followed by a significant decline



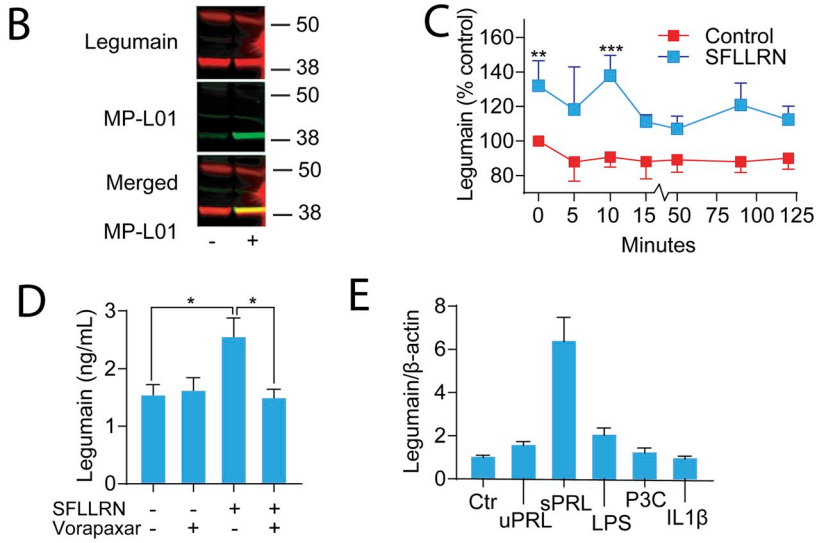


Fig. 2. Legumain is co-localized with platelets in carotid plaques and released upon platelet activation. (A) Immunohistochemistry of legumain, CD41 (platelet marker) and CD68 (monocyte/macrophage marker) in sequential sections of a symptomatic carotid plaque. The red squares indicate the same area at 10X and 40X magnification. (B) One representative immunoblot of legumain (red) without (left lane) or with (right lane) the legumain-selective activity-based probe MP-L01 (green) in human platelet lysate (merged; yellow) (n = 3). (C) Concentration of legumain in platelet-rich plasma (PRP) after platelet activation using 100 μM SFLLRN compared to control and measured by ELISA (n = 4–8). (D) Release of legumain from platelets preincubated for 90 min with or without 1 μM vorapaxar before 10 min stimulation with or without 100 μM SFLLRN (n = 8). Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to controls. (E) THP-1 monocytes were pre-activated with 5 ng/mL TNFα for 96 h prior to culturing for 6 h with platelet releasate from unstimulated (uPRL), or thrombin-activated (sPRL) platelets. For comparison, TNFα -treated THP-1 cells were also stimulated with recombinant human IL-1β (5 ng/mL), LPS (TLR4 agonist; 5 ng/mL) or P3C (Pam3Cys, TLR2 agonist; 1 μg/mL). Legumain mRNA expression was measured by real-time PCR and calculated towards β-actin. Data are presented as mean ± SEM, n = 3–4. *p < 0.05 *vs* unstimulated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

at one day and 4 months after PCI reaching levels comparable to the healthy controls (Fig. 5A). This pattern was independent of the presence of diabetes (data not shown). However, although markedly elevated at baseline, circulating legumain was not associated with infarct size, left ventricular ejection fraction (LVEF) or myocardial salvage as determined by CMR at 4 months after STEMI. Also, there was a moderate inverse correlation between legumain measured at admission, before PCI, and LVEF measured by CMR during the acute phase (Supplemental Table 5). Heparin was administered to all patients before PCI, and as heparin has been shown to influence circulating levels of certain cytokines [16], we examined circulating legumain from nine patients with suspected stable coronary artery disease undergoing elective angiography. These analyses showed lower legumain levels after heparin administration (p = 0.0039) (Fig. 5B), indicating that heparin treatment might actually underestimate the measured legumain levels.

3.6. Circulating legumain levels are correlated with markers of platelet activation

Platelet-derived mediators should ideally be measured in platelet-poor plasma and we therefore measured legumain levels in both plasma and serum from the same individuals in a sub-group of the STEMI patients (n = 42) and healthy controls (n = 18). Noteably, legumain levels were significantly elevated in both plasma and serum in patients

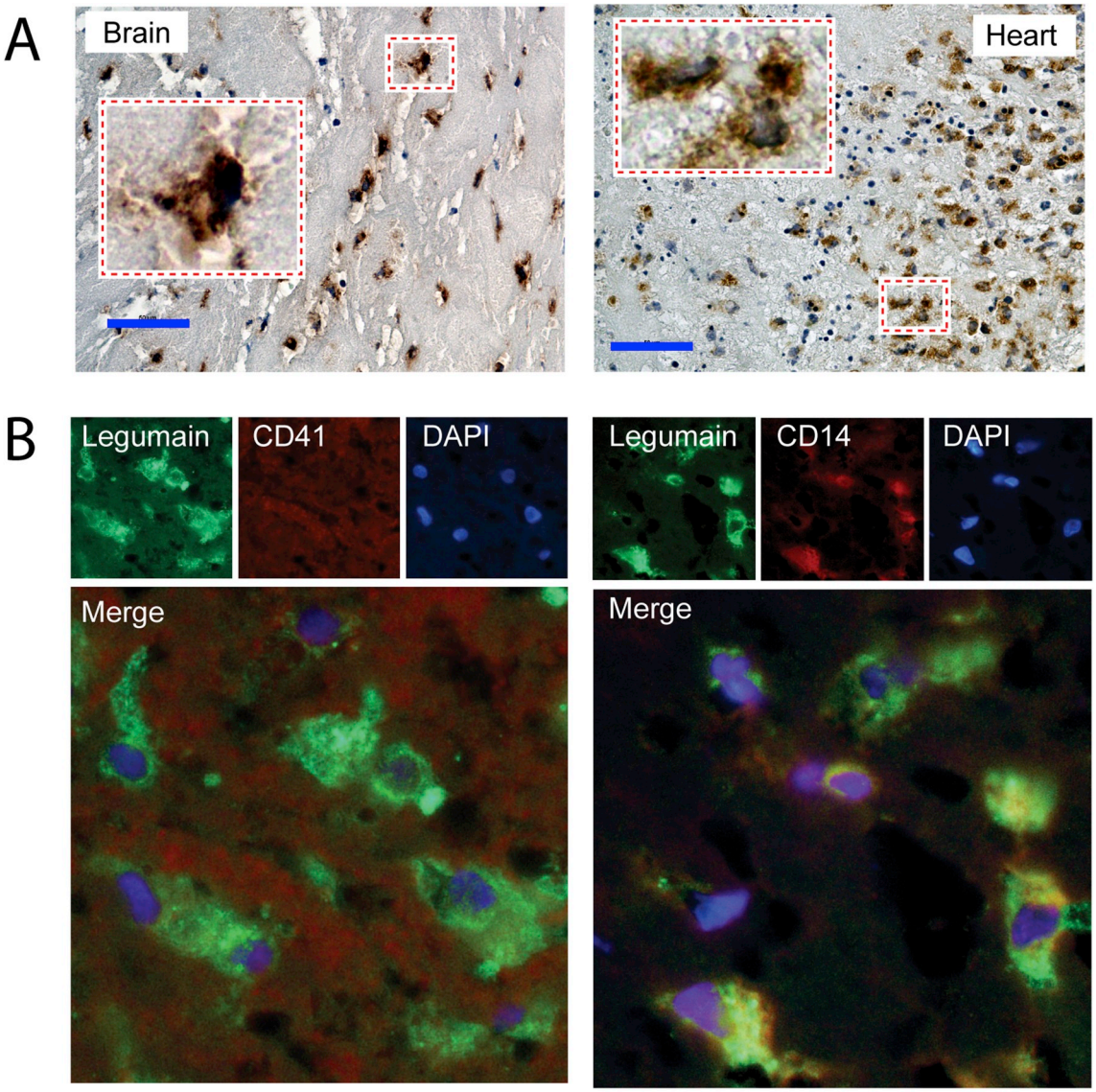


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Fig. 3. Legumain is present cerebrovascular and coronary thrombus material.
(A) Immunostaining of legumain in thrombus material from one representative patient (n = 5) with acute ischemic stroke (left) removed from the site of plaque rupture; and from one representative patient (n = 5) with STEMI undergoing PCI (right). The red squares indicate the same area at 10X and 40X magnification. (B) Fluorescent double-staining of legumain (green) and CD41 (platelet marker; red, left), and CD14 (macrophage marker; red, right). Nuclei are stained blue by DAPI. Merge of the individual stainings. One representative staining of n = 4. (C) Protein identification by LC-MS/MS from intracranial thrombus material identified two unique peptides for legumain (upper panels show the annotated MS/MS spectra for the identified peptides and lower panel shows the identified peptides highlighted from the full protein sequences). The overall sequence coverage for legumain was 7.1% and Mascot identification score was 120. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared to controls (Fig. 5C). As expected, legumain levels as well as levels of P-selectin and platelet factor 4 (PF4), two established platelet-derived markers, were higher in serum compared to plasma, in both patients and controls (Fig. 5D). In addition, plasma levels of these three platelet-derived molecules were significantly correlated in the STEMI patients, but not in healthy controls (Fig. 5E).

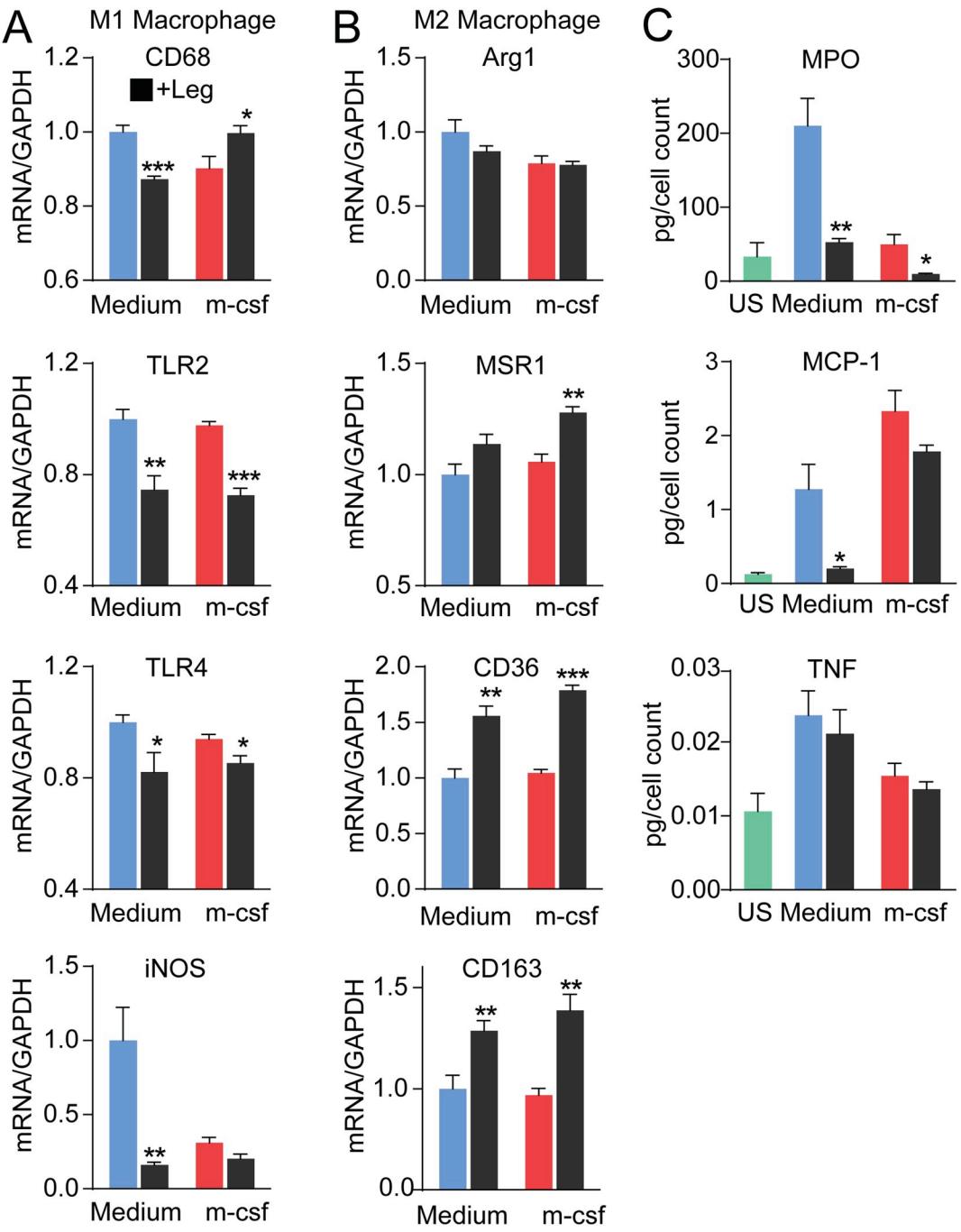

Fig. 4. Primary monocytes (medium) and differentiating primary monocytes (M-CSF, 20 ng/mL; R&D) were stimulated with 100 ng/mL recombinant human prolegumain (black bars) or PBS (control, red and blue bars) and evaluated for gene expression of M1 (*CD68, TLR2, TLR4, iNOS*) and M2 (*Arg1, MSR1, CD36, CD163*) macrophage markers after 6 h (A and B, respectively) and release of inflammatory mediators after 72 h (C). ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ *versus* control (red or blue bars). US, unstimulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

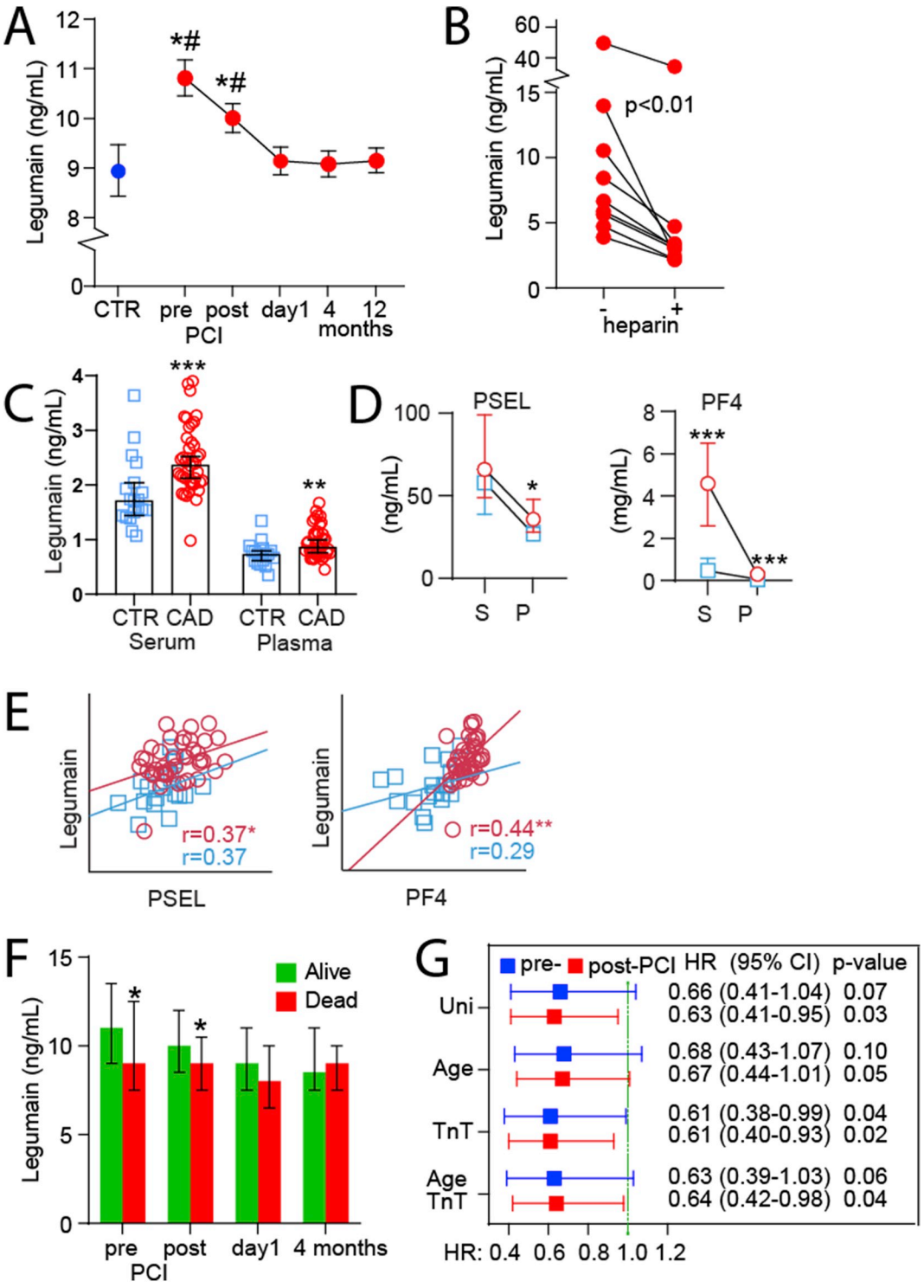

Fig. 5. Circulating legumain during STEMI in the *POSTEMI* cohort.
(A) Legumain was measured in serum from patients with STEMI collected before (pre; n = 245) and immediately after (post; n = 249) the PCI procedure, at day 1 (median 18.3 h after PCI, n = 243), and 4 (n = 249) and 12 months (n = 241) follow-up and in 67 controls (CTR). Data are presented as geometric means with 95% CI. *$p < 0.001$ *vs* controls, obtained by Kruskal-Wallis test, #$p < 0.001$ *vs* day 1, 4 and 12 months, respectively, obtained by Wilcoxon signed rank test. (B) Legumain serum levels in patients with stable coronary artery disease (n = 9) referred to elective coronary angiography before and after heparin administration. The first sample was drawn before the coronary angiography procedure and administration of unfractionated heparin and the second sample was drawn after administration of heparin at the end of the procedure. Data are presented for each subject. The *p*-value is obtained by Wilcoxon matched-pairs signed rank test. (C) Legumain, (D) P-selectin (PSEL) and platelet factor 4 (PF4) in plasma (P) and serum (S) from STEMI patients (red; n = 42) and healthy controls (blue; n = 18) measured by ELISA. (E) Correlation between plasma legumain and P-selectin and platelet factor 4. (F) Legumain levels in survivors (alive) and non-survivors (dead) measured pre- and post-PCI, at 1 day and after 4 months, and compared by the Mann Whitney *U* test. *$p < 0.05$. (G) Cox regression analyses of legumain (log transformed expressed per SD) levels pre- and post-PCI and all-cause mortality. Hazard ratio (HR) and 95% CI are shown for univariate and multi-variate (adjusted for age and troponin T (TnT)) analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Low circulating legumain levels are associated with increased mortality in the STEMI population

A total of 26 deaths (10% of the total cohort) were registered after a median of 70 months of follow-up after STEMI. Interestingly, legumain levels in blood samples drawn before and after PCI were lower in patients who died during follow-up (Fig. 5F). Furthermore, evaluated as a continuous variable, legumain levels both before and after PCI were associated with a reduced hazard ratio of all-cause mortality that was more or less unmodified when adjusting for age and TnT release (Fig. 5G).

4. Discussion

We have previously shown enhanced expression of legumain within symptomatic carotid plaques [8], and in the current study we present novel data on legumain regulation during acute cardiovascular events and the presence of active legumain in platelets. We found that circulating legumain levels were upregulated in the stable and particular in the acute phase of CVD (STEMI). In the latter population, high legumain levels were associated with improved outcome at follow-up. Moreover, legumain was co-localized with macrophages and platelets in symptomatic carotid plaques, as well as in both coronary and intracerebral thrombi obtained during acute cardiovascular events. Furthermore, legumain was released from platelets upon PAR-1 activation, and was upregulated in THP-1 monocytes when exposed to releasate from activated platelets. Interestingly, legumain reduced the monocyte activation marker CD14 in primary monocytes, and reduced the release of proinflammatory cytokines from both undifferentiated primary monocytes, and from monocytes undergoing macrophage differentiation. Legumain further downregulated M1 markers and upregulated M2 markers in both undifferentiated monocytes and differentiating monocytes, stimulating the development of an anti-inflammatory macrophage phenotype. This suggest that legumain could mediate platelet-monocyte interactions that could involve anti-inflammatory responses. Our findings of strong legumain immunostaining at the site of thrombus formation in STEMI patients and in patients with acute ischemic stroke further support a role for legumain in modulation of plaque stability.

A major finding in the present study was the observation of platelets as a source of legumain. Legumain is expressed in a wide range of cell types including various immune, stromal and tumor cells. We have recently reported increased secretion of legumain from M1 macrophages [8], indicating multiple sources of legumain. One study using a proteomic approach has previously reported legumain as one of approximately 4500 proteins stored in human platelets [17]. Our study is, to the best of our knowledge, the first report showing that platelets not only contain legumain, but also release this cysteine protease upon activation. In platelets, both the pro- and mature form were detected by immunoblotting, with highest level of the mature form. Notably, the mature form is active as it binds the legumain-selective ABP MP-L01 [15]. Active legumain in platelets might indicate lysosomal storage, as legumain is a lysosomal protease and low pH is required for activation and proteolytic activity of legumain and other proteases, and shown for cathepsin D in platelets [18]. However, after secretion, prolegumain can be activated in less acidic environments when stabilized by the cell surface $\alpha v\beta_3$ integrin, glycosaminoglycans or cystatins present extracellularly [15,19]. Also, the inflamed arterial wall in atherosclerosis creates a local acidic milieu that could activate prolegumain extracellularly. In carotid plaques, we have previously shown high expression of mature legumain [8]. However, legumain could also be stored in α-granules since these vesicles are fully released within a few minutes [20], which fits with the release pattern of legumain from platelets. The ability of the selective PAR-1 antagonist vorapaxar to block release of legumain, suggests a direct involvement of PAR-1 in legumain secretion from platelets.

The platelet releasate comprises a multitude of inflammatory and vasoactive substances which can modulate endothelial cells and monocytes/macrophages within the atherosclerotic lesion [21]. We show that the releasate from activated platelets significantly enhances legumain mRNA expression in TNFα-pre-activated THP-1 monocytes, in a setting mimicking the *in vivo* situation within an inflammatory atherosclerotic lesion. Notably, well-known activators of monocyte/macrophage (i.e. TLR2/4 agonists and IL-1β) had no effects on legumain expression in these cells. Our findings suggest that legumain could be operating in the interaction between monocytes/macrophages and platelets to modulate plaque stability. Indeed, we found co-localization of legumain with both monocytes/macrophages and platelets within symptomatic carotid plaques and in thrombus material obtained at the site of thrombus formation in STEMI patients and in patients with ischemic stroke. This observation suggests that legumain, as a novel player in platelet-monocyte interactions, also operate *in vivo* during acute cardiovascular events.

The association between diabetes and cardiovascular disease is well documented and type 2 diabetes doubles the risk of developing coronary artery disease. As far as we know, this is the first study investigating legumain in a diabetic population. In patients with stable CVD, an association between high circulating legumain and cardiovascular events was only seen in the non-diabetic patients. Plasma levels of other proteases, like MMP-7 and -12, have been shown to be elevated in type-2 diabetes and to be associated with more severe atherosclerosis and increased incidence of coronary events [22]. The origin of circulating legumain in diabetics compared to non-diabetics is of interest. Circulating legumain in diabetics could originate from other tissues than atherosclerotic lesions, as suggested for the mentioned MMPs. As observed for legumain, the association between MMP-12 and pulse wave velocity was stronger in non-diabetics than in diabetics, although diabetics had higher plasma levels of MMP-12 than the non-diabetics. The different observations of legumain in non-diabetic and diabetic subjects need further investigations. However, these associations with outcome were not found in the STEMI study and our data should be interpreted with caution and need to be confirmed in larger study populations. Interestingly, during acute CVD (STEMI population), low circulating legumain levels during the acute phase were associated with increased mortality during follow-up. The reasons for these apparent discrepancies are at present not clear, but could reflect that legumain is differently regulated during stable compared to acute cardiovascular events. In fact, the rapid increase of circulating legumain during STEMI, with normalization within 24 h could suggest that legumain is rapidly cleared from the circulation, and that legumain is not an optimal biomarker in patients with stable disease. During an acute cardiovascular event, however, measurement of circulating legumain could potentially give additional prognostic information. Higher levels of legumain in serum compared to plasma further suggest that legumain is secreted upon platelet activation. Thus, measurements of platelet-derived markers in serum samples may in some degree also reflect the release of these molecules during coagulation *ex vivo*, which will not be an issue when using platelet-poor plasma. In a subset of STEMI patients, we did however see increased levels of legumain also in plasma samples, and plasma legumain significantly correlated with P-selectin and PF4, two soluble markers of platelet activation. Taken together, these analyses suggest that the raised levels of legumain in STEMI patients are not merely an *ex vivo* phenomenon, but reflect enhanced *in vivo* release of this molecule from platelets in the circulation of STEMI patients.

A potential role of legumain during modulation of plaque stability is at present not clear. Legumain has been shown to activate innate immunity, promote apoptosis, disturb immune tolerance and modulate ECM degradation and is also involved in disorders like Alzheimer's disease and cancer [19]. In STEMI, however, we found that high circulating legumain levels were associated with improved outcome. This may be contradictory to the suggested detrimental role in inflammation and ECM remodeling, but there are also data suggesting a role for legumain in tissue repair [23]. Moreover, in an experimental mouse model legumain was shown to mediate anti-inflammatory and pro-resolving effects of M2 macrophages by attenuating renal interstitial

fibrosis in obstructive nephropathy [24]. Indeed, the present study indicates that legumain induces polarization of anti-inflammatory macrophages, shown by an upregulation of several genes such as CD163, which could potentially reflect polarization of monocytes towards the M2 phenotype, while downregulating a M1 phenotype. Also, legumain may contribute to reduce inflammation by dampning monocyte activation through downregulation of the activation marker CD14, which was found to be downregulated up to 48 h after legumain stimulation. Thus, although some data may suggest that legumain could contribute to plaque destabilization, forthcoming studies should more thoroughly examine the consequences of enhanced legumain levels during STEMI and other acute cardiovascular events. The present study has some limitations such as lack of functional legumain data and the fact that size of both study populations yields relatively low numbers of events during follow-up.

Nonetheless, our data demonstrate for the first time that legumain is markedly upregulated during acute cardiovascular events. Also, the presence of legumain in acute thrombus material suggests that legumain could be a novel player in modulation of plaque stability, operating in the interaction between platelets and monocytes/macrophages, potentially mediating anti-inflammatory effects.

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Author contributions

NNL, IG, HTJ, RS, PA and BH conceived and design the study. NNL, IG, TU, SH, XYK, AEM, KO, TAN, BS, TE, HTJ and BH performed cell –and platelet experiments and analysis, and analysis of patient samples. CS, AY, KB, LG, BB, JE, PH, KS, IGo, JN, MG, IS, MS, HB, GØA were involved in clinical samples. MP and MD provided the probe. NNL, TU, CS, HB, BH performed statistical analysis. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

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