Changes in thrombus composition and profilin-1 release in acute myocardial infarction

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Aim	Thrombus formation is a dynamic process regulated by flow, blood cells, and plasma proteins. The present study was performed to investigate the characteristics of human coronary thrombus in ST-segment elevation myocardial infarction (STEMI).
Methods and results	Patients admitted with ST-elevation myocardial infarction, in which thrombectomy was performed, were included ($n = 86$). Intracoronary thrombi and blood from the culprit coronary site and the systemic circulation were obtained during percutaneous coronary intervention (PCI). Thrombi were categorized by onset-of-pain-to-PCI elapsed time in thrombus of <3 (T3) and more than 6 h of evolution (T6). Clinical, morphological, and proteomic variables were investigated. While T3 were mainly composed by platelets and fibrin(ogen), T6 were characterized by a reduced platelet content, increased leucocytes infiltration (including monocytes, neutrophils, T-cells, and B-cells), and appearance of undifferentiated progenitor cells. Significant differences between T3 and T6 were found in the cell cytoskeleton-associated proteome (beta-actin and tropomyosin 3 and 4). By discovery proteomics, we have identified profilin-1 (Pfn-1) in the coronary thrombi and detected higher levels in T3 than in T6. While plasma Pfn-1 levels were low in T3 patients, levels significantly increased in both coronary and peripheral circulation in T6 patients indicating release. *In vitro* platelet aggregation studies showed that platelets secrete Pfn-1 upon complete activation.
Conclusion	Coronary thrombi show rapid dynamic changes both in structure and cell composition as a function of elapsed onset-of-pain-to-PCI time. Aged ischaemic thrombi were more likely to have reduced Pfn-1 content releasing Pfn-1 to the circulation. Onset-of-pain-to-PCI elapsed time in STEMI patients and hence age of occlusive thrombus can be profiled by Pfn-1 levels found in the peripheral circulation.
Keywords	PCI • Occlusive thrombus ageing • Platelets • Profilin-1

Introduction

Intracoronary thrombosis is the cause of the majority of acute coronary syndromes (ACSs).[1,2] Atherothrombosis is a dynamic process that, in addition to the atherosclerotic vessel wall, includes plasma proteins and blood cells with a direct influence on its morphology, evolution, and tentative resolution. Antithrombotic drugs have largely improved the treatment of ACS patients in the recent years and have shown to reduce mortality.[3–5]

Nevertheless, the composition of the occluding thrombus, its changes with time of evolution and its interaction and cross-talk with the other cells is not fully known. Therefore, profiling intracoronary thrombus cellular and soluble proteins may be a strategy to identify novel factors in thrombosis.

The mechanisms of thrombus formation on disrupted and eroded atherosclerotic plaques have been the subject of substantial investigation.[1,2,6–8] At the site of plaque disruption, platelets deposit and form aggregates that anchor the newly formed stable fibrin networks.[9,10]

Thrombosis has been usually studied in model systems, *in vitro* or *ex vivo*, in peripheral vessels and microcirculation, or post-mortem;[11–15] however, aspiration of coronary thrombus at percutaneous coronary interventions (PCI) allows studying *in vivo* retrieved samples of ongoing thrombosis in humans. Up to now, however, available

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information is mainly based on scanning electron microscope, magnetic resonance imaging, and immunohistochemistry.[16−20]

Here, we have applied protein discovery approaches[21,22] and advanced cellular microscopy[23] in order to expand our understanding of the characteristics of coronary thrombus. We have investigated the cellular composition and the proteomic signature of aspirated coronary thrombi from ST-segment elevation myocardial infarction (STEMI) patients, treated as per guidelines, and investigated the effect of onset-of-pain-to-PCI elapsed time on their evolving composition. By proteomics, we have identified among the differential proteins Pfn-1, a 15 kDa actin-binding protein that has been recently linked to atherosclerosis and vascular cell proliferation and remodelling.[24,25] Here, we report on the rapidly changing nature of coronary thrombi cell composition and on proteomic changes in relation to onset-of-the-pain-to-PCI elapsed time in the peripheral blood.

Methods

For expanded Methods, please refer to Supplementary material online.

Study population

The study design consisting in a discovery phase and a validation phase is shown in *Figure 1*. We analysed 86 patients admitted with ST-elevation myocardial infarction, in which thrombectomy was performed.

These patients had to satisfy always criteria 1 and 2 and either 3 or 4 as follows: (i) presence of new or presumably new ST-elevation >0.1 mV in 2 or more contiguous ECG leads felt to be ischaemic and without other explanation of ST elevation such as acute pericarditis or ventricular aneurysm (ii) clinical scenario consistent with myocardial infarction (iii) elevated enzymes (CK-MB >ULN or in the absence of CK-MB a total CK > 2x ULN or elevation of troponin) within 24 h of the onset of ischaemic discomfort (iv) new Q-waves distinct from time of presentation.

Percutaneous coronary intervention was performed according to guidelines. Manual aspiration was performed with either the 6F Export catheter (Medtronic) or Pronto catheter (Vascular Solution). Aspiration was started 2 cm before lesion with thrombus. Then the thrombectomy catheter was moved forward very slowly with continuous aspiration and the lesion was crossed. Usage of a second or third syringe was recommended. The thrombectomy catheter was removed with aspiration even into the guiding catheter, allowing the aspiration of the blood from the guiding catheter. Additional passages were recommended until no signs of thrombus were observed on angiography.

Loading dose of clopidogrel was 600 mg and maintenance was 75 mg/day. Aspirin was administered as a 300 mg loading dose followed by 100 mg/day. All the patients received full dose of i.v. heparin before PCI (1 mg/kg) or 0.7 mg/kg in those patients who received abciximab. Dosages of GPIIb/IIIa were bolus of 0.25 mg/kg i.v. and 0.125 µg/kg/min infusion (maximum 10 µg/min) for 12 h.

The median inter-quartile range (IQR) for onset-of-pain-to-PCI time was 210 (128−360) min. To investigate the effect of onset-of-pain-to-PCI time on the composition of STEMI thrombus, two groups were selected: <3 h onset-of-pain-to-PCI (T3 group, $n = 33$) and >6 h onset-of-pain-to-PCI up to 24 h (T6 group, $n = 32$). Supported by previous investigation,[16] the range time between 3 and 6 h (T3−6 group, $n = 21$) was excluded in the initial stage of the study in order to clearly differentiate between early (<3 h) and late (>6 h) phases of ischaemia. For validation studies and in order to analyse Pfn-1 control levels, we included a control group of 28 subjects without any cardiovascular event before recruitment composed by patients with cardiovascular risk factors (C-RF, $n = 14$) and without risk factors (C-NoRF, $n = 14$). Also STEMI patients in the range time between 3 and 6 h ($n = 21$) were included in the validation phase.

Demographic and clinical characteristics of the STEMI and control groups are provided in Supplementary material online, *Table S1*.

Sample collection

All thrombi were routinely macroscopically estimated in appearance for size and colour. Thrombi were collected in one or more segments, but total weight of the thrombi and their macroscopic appearance were similar between the both groups under study.

Figure 1 Schematic diagram representing the overall approaches of this study.
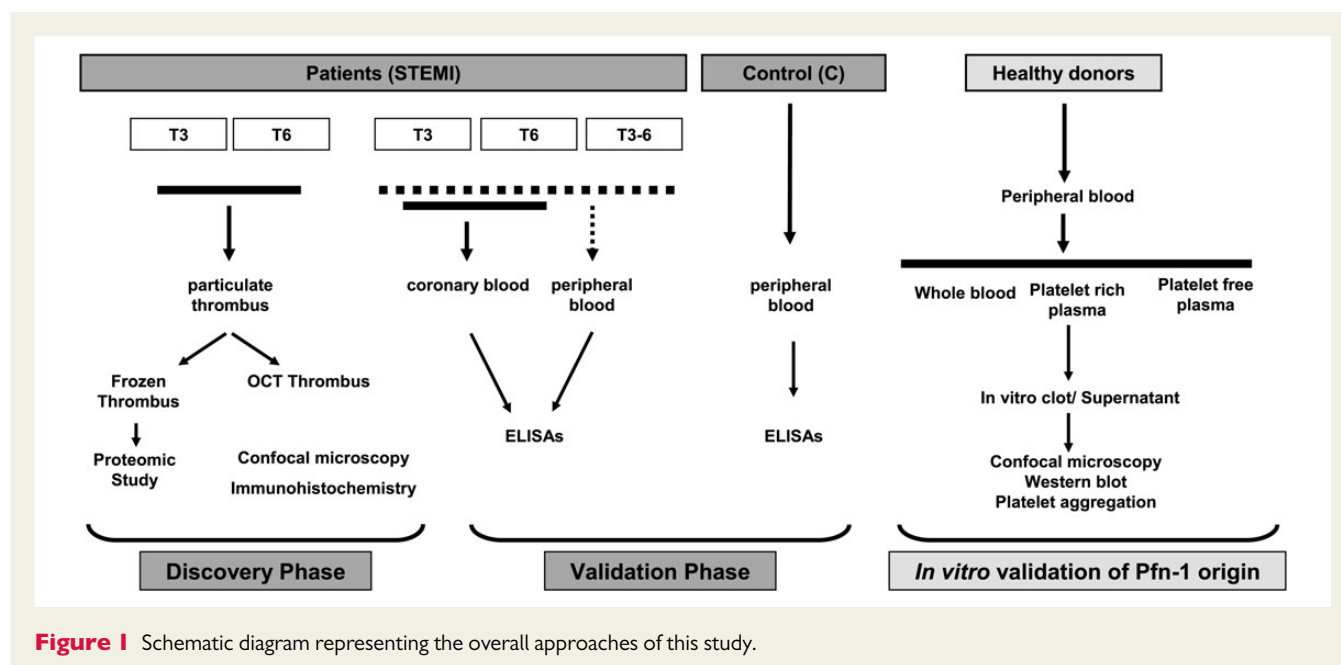
Aspirated thrombi were immediately washed to eliminate attached red blood cells and divided in two parts. One part was snap-frozen and stored at −80°C for further experiments and the other was fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound and stored at −80°C for immunohistochemical and confocal analysis. Coronary and peripheral blood was collected at the time of PCI (for more details please refer to Supplementary material online, Methods).

Proteomic analysis and mass spectrometry

Analysis of differential protein patterns was performed by a proteomic approach using two-dimensional electrophoresis followed by mass-spectrometry for protein identification, as previously described[21] in T3 ($n = 15$) and T6 ($n = 13$) thrombi. STEMI patients for the proteomic study were selected based on the available amount of thrombus sample to perform the technique. For analytical and preparative gels, 100 and 300 μg of protein of the urea/chaps thrombi extracts were, respectively, loaded in 17-cm dry strips (pH 3–10 linear range), and were separated by electrofocusing according to their isoelectric point (pI). Second dimension was resolved in 12% SDS−PAGE gels. Electrophoresis was performed using an Ettan Daltsix System and gels were developed by fluorescent Flamingo staining using a Typhoon. For mass spectrometry analysis proteins were identified as previously described[21] by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF.

Immunohistochemistry

For immunohistochemical (IHC) analysis, serial sections (5 μm thick) of OCT embedded thrombi were placed on poly-L-lysine-coated slides and treated with of H_2O_2 for inhibition of endogenous peroxidase activity and with horse or goat serum to block non-specific bindings. Therefore, slides were incubated for 2 h with antibodies against CD61 (platelets), CD3 (T-lymphocytes), CD20 (B-lymphocytes) CD105 and CD34 (undifferentiated cells), and with antibodies anti-monocyte/macrophage, anti-neutrophils, and anti-fibri(ogen), and detected with avidin−biotin immunoperoxidase technique. The chromogen used was 3.3′-dia minobenzidine.

Haematoxylin and eosin (H&E) staining was routinely performed in all thrombi as a first approach, previously to the IHC analysis, to identify sample morphology (see Supplementary material online, *Figure S1* for representative T3 and T6 thrombi). The images were captured by a Nikon Eclipse 80i microscope and digitized by a Retiga 1300i Fast camera at ×400. Positive-staining areas for each antigen were calculated from an average of 5-fields/sample with the ImageJ 1.37v software. The values were given as a percentage of the total thrombus area [content (%) = (positive stained area/total thrombus area) × 100].

Western blot

Protein extracts of coronary thrombi, *in vitro* platelet-free plasma (PFP)- and platelet-rich plasma (PRP)-generated clots, and supernatant from aggregation experiments were resolved in 15% SDS−PAGE gels under reducing conditions. Protein detection was performed using an anti-human profilin antibody combined with the Dye Double Western Blot kit.

Confocal microscopy

Serial sections of collected coronary thrombi and of *in vitro* generated whole blood clots were incubated with anti-profilin, anti-fibrinogen, anti-CD61-FITC (fluorescein isothiocyanate conjugated) and anti-CD105 antibodies for confocal analysis. Coverslides were washed and incubated with the appropriate secondary antibody Alexa Fluor 488, Alexa Fluor 633 IgG (H+L) and Hoechst (for nuclei labelling). Immunostained coverslides were washed and covered with Prolong Gold antifade reagent. Images were recorded on a Leica inverted fluorescence confocal microscope with HCX PL APO 63x/1.2W CORR/ 0.175 objective.

Flow cytometry

Purity of the PRP suspension (absence of leucocytes) was proved by flow cytometry (Supplementary material online, *Figure S2*).

ELISA assay

Double antibody sandwich enzyme-linked immunoassays were used to measure profilin-1 (Pfn-1), P-selectin, and hsC-reactive protein in plasma.

Platelet aggregation

Platelet aggregation was assessed on the Multiplate Analyzer, using adenosine diphosphate (ADP: 10 μM), collagen (2 μg/mL), and thrombin receptor-activating peptide (TRAP-6: 64 μM) in citrate-anticoagulated whole blood as previously described.[26]

Experiments were reproduced stopping the reaction 10 s and 4 min after the agonist addition. The samples were centrifuged to obtain platelet poor plasma and were stored at −80°C until western blot analysis was performed.

Statistical analysis

Data are expressed as median and IQR except when indicated. A test for normality was performed using the Shapiro−Wilks test. Plasma Pfn-1 levels were transformed (logarithmic) to normalize the asymmetry. Group differences were determined by the Mann−Whitney test or *t*-test for non-normal and normal data, respectively. Categorical variables were compared using the χ^2 test or Fisher exact test. Bivariate correlations between variables were determined by Spearman or Pearson correlation coefficients. To determine correlation between Pfn-1 levels, antropometric parameters (age and gender), risk factors (diabetes, hypertension, obesity, dyslipidaemia, tobacco), antithrombotic treatments, and levels of hsC-reactive protein and P-selectin, we performed a bivariate analysis (correlation or *t*-test) followed by a multiple lineal regression model (stepwise selection of variables) including those variables statistically significant in the bivariate analysis to assess the most parsimonious model. Minimal required sample size was calculated and validated using the JavaScript-based method for simple power/sample size calculation when two independent groups are compared, provided in http://www.stat.ubc.ca/~rollin/stats/ssize/ n2.html. Based on the mean value of T3 and T6 groups and the standard deviation of the studied population, a minimal sample size of 31 patients per group (study power: 0.80, type I error = 0.05, two-sided test) was obtained. Receiver-operating characteristic (ROC) analysis was used to obtain sensitivity and specificity of the assays. Statistical analysis was performed with the Stat View 5.0.1 and IBM SPSS Statistics v 19.0 software. *N* indicates the number of subjects tested. A *P*-value ≤0.05 was considered significant.

Results

Clinical characteristics

The STEMI patients with onset-of-pain-to-PCI elapsed times of <3 h (T3) or >6 h (T6) did not show significant differences in demographic characteristics, cardiovascular risk factors nor in clinical treatment. The sample size used for the comparative analysis is larger than the

minimal required sample size for the study calculated as described in methods (T3 group: $n = 33$ vs. T6 group: $n = 32$).

The only significant difference between both groups was gender (male in T3 82% vs. male T6 56%; $P = 0.02$). Aspirin was administered in 95% of patients and 60% received a loading dose of clopidogrel on arrival at the cath lab; in the remaining cases, clopidogrel was administered immediately after the procedure. At PCI patients were anticoagulated with low-molecular weight or unfractionated heparin, and GPIIb/IIIa antagonists were used in 30% of the patients.

Median (IQR) ischaemic time from onset-of-the pain-to-PCI was 110 min (94−150) in T3 and 615 min (449−825) in T6. The initial TIMI flow grade (thrombolysis in myocardial Infarction: 0 = no perfusion, 1 = penetration without perfusion, 2 = partial reperfusion, 3 = complete reperfusion) was 0−1 in 81% of the STEMI-population included in the study (T3 88%, T6 74%, χ^2 test; $P = 0.9$). The 84% of PCI procedures were successful (TIMI flow grade 3; T3 88% and T6 80%, χ^2 test; $P = 0.4$; *Table 1*).

Coagulation tests including reptilase time, cephaline ratio, and international normalized ratio (INR) were in the normal range and similar among groups. Thrombin time (TT) was prolonged in all groups reflecting heparin treatment (average values TT range from 41 ± 20 to 33 ± 16 s). Details of haematological data are reported in Supplementary material online, *Table S2* and no significant differences were found among groups, except for red blood cells and haematocrit.

Thrombus composition

The morphological characterization of the thrombi showed that elapsed onset-of-pain-to-PCI time had a significant effect on the cellular and fibrinogen content of the thrombi (*Figure 2* and Supplementary material online, *Figure S3*). In thrombi of <3 h, the fibrin(ogen) was loosely distributed with fibrin fibres poorly structured (*Figure 2a* and Supplementary material online, *Figure S4*), while in thrombi of >6 h, the fibrin mesh was more compact (*Figure 2g* and Supplementary material online, *Figure S4*). As previously reported,[16] platelet content was high in T3 and much lower in T6 (*Figure 2b* and *h*; $P = 0.02$). Neutrophils and monocytes were rapidly recruited to the thrombus already appearing at T3 and remaining high at T6. Interestingly, T-lymphocytes (CD3$^+$) and B-lymphocytes (CD20$^+$), that were rarely seen in T3, showed a significant increase at T6 ($P = 0.05$). More than 90% of the coronary

Table 1 Background description of STEMI patients

	T3 (*n* = 33)	T6 (*n* = 32)	*P*-value
Past medical history of (%)			
PCI	9	6	0.67
CABG	3	3	0.98
Peripheral vasculopathy	0	0	>0.9999
Time delay in minutes [median (IQR)]			
Symptom onset to medical contact	24 (15−44)	272 (100−455)	≤0.001
Hospital admission to PCI	78 (45−103)	310 (265−744)	≤0.001
Symptom onset to PCI	110 (94−150)	615 (449−825)	≤0.001
Clinical presentation			
Killip (mean ± SD)	1.3 ± 0.8	1.4 ± 0.9	0.74
TIMI flow grade (mean ± SD)	0.3 ± 0.9	0.7 ± 1	0.17
Infarct-related artery (%)			
LAD	64	66	0.87
RCA	67	66	0.93
Cx	24	22	0.82
Antithrombotic treatment (%)			
ASA	100	91	0.11
Clopidogrel	52	68	0.25
Heparin	100	100	>0.9999
GpIIb/IIIa inhibitor	33	27	0.12
Reperfusion (%)			
TIMI flow grade 3 before PCI	6	13	0.35
TIMI flow grade 3 after PCI	88	80	0.39

ASA, acetylsalicylic acid; CABG, coronary artery bypass grafting; Cx, circumflex artery; LAD, left anterior-descending artery; PCI, percutaneous coronary intervention; RCA, right coronary artery; TIMI, thrombolysis in myocardial infarction (0 = no perfusion, 1 = penetration without perfusion, 2 = partial reperfusion, 3 = complete reperfusion). T3, <3 h onset-of-pain-to-PCI; T6, >6 h onset-of-pain-to-PCI.
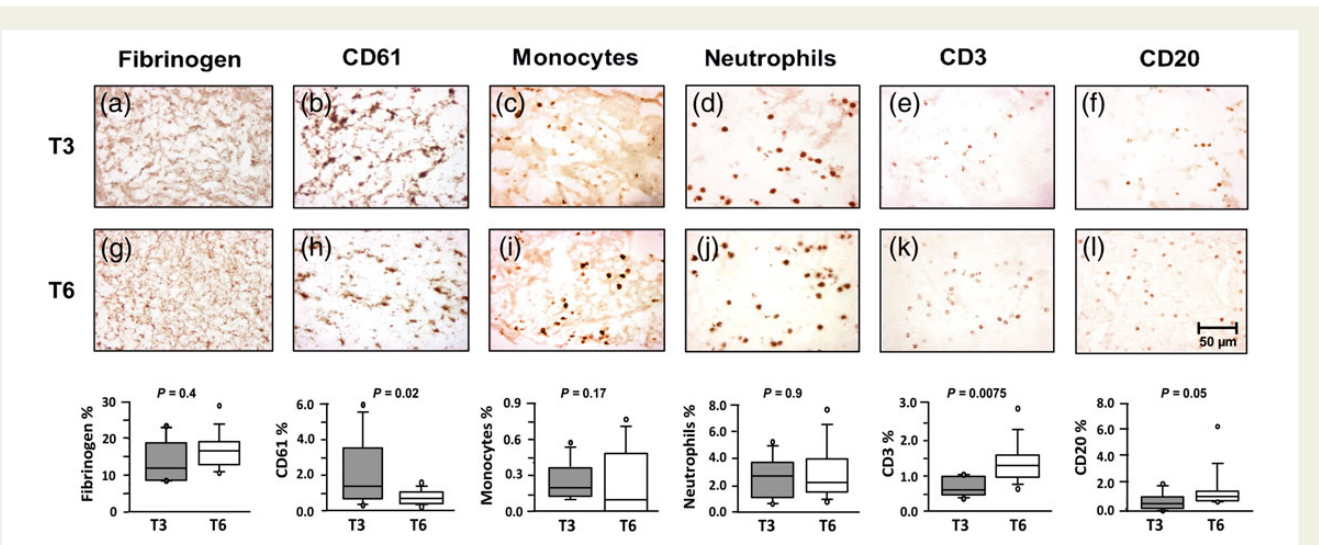
Figure 2 Fibrinogen, platelet, and cellular content in coronary thrombi in relation to the duration of ischaemia (onset-of-pain-to-PCI). Representative immunohistochemical image of thrombi sections stained with an anti-fibrinogen, anti-CD61, and anti-monocytes/macrophages, neutrophils, T-lymphocytes and B-lymphocytes antibodies comparing thrombi at short (T3) and long-time of ischaemia (T6). Box plot diagrams [median (interquartile range)] represent the staining percentage of the total thrombus area. Images were quantified using the ImageJ 1.37v software. Scale bar = 50µm.
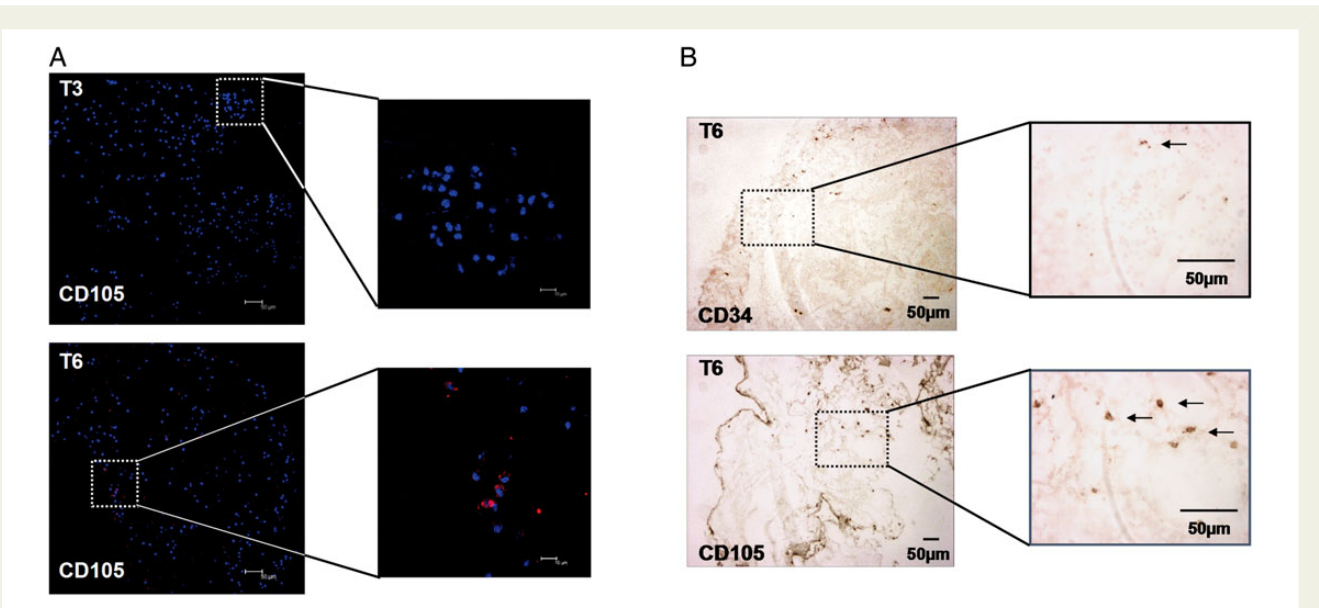

Figure 3 Endoglin and CD34 content in human coronary thrombi: relation with the time of ischaemia. (A) Confocal microscopy image of non-permeabilized coronary thrombus section stained with an anti-CD105 antibody (red) and Hoechst (blue) at short (T3) and long ischaemia time (T6). Low-magnification scale bar = 50 µm; High-magnification scale bar = 10 µm. (B) Immunohistochemical image of T6 thrombi sections stained with an anti-CD34 and anti-CD105 antibodies. Scale bar = 50 µm for both, low and high magnifications.

thrombi were positive for macrophage staining, without statistical differences between T3 and T6 groups (P = 0.56).

Confocal microscopy showed that undifferentiated CD105+ cells that were not present in T3, appeared infiltrating the T6 coronary thrombi (*Figure 3A*). Immunohistochemical analysis confirmed the presence of undifferentiated CD34+ and CD105+ cells in aged

occlusive thrombi. Thus, CD34 and CD105 cells represented ~1% (0.4 and 0.9%, respectively) of the thrombus in T6 thrombi (*Figure 3B*).

Using H&E stain, we have not detected cholesterol clefts in the thrombi (Supplementary material online, *Figure S1*). With a specific lipid staining (Herhxeimer), positive signals were detected in 20%

of the thrombi, with similar degree of presence in the T3 and T6 groups.

Onset-of-pain-to-percutaneous coronary intervention time influences profilin-1 expression pattern in coronary thrombi

The protein signature of thrombi was resolved by two-dimensional (Supplementary material online, *Figure S5A*). Cytoskeleton-related proteins and haemoglobin (specific marker of erythrocytes) were consistently identified (Supplementary material online, *Table S3*). T3 and T6 showed significant differences in the cell cytoskeleton proteome. Specifically, beta-actin (Swiss Prot number P02675, spots 1 and 2), tropomyosin-3 (Swiss Prot number P06753, cluster 3), and tropomyosin-4 (Swiss Prot number P67936, spot 4) were decreased in T6, whereas haemoglobin (Swiss Prot number P68871, spot 5) did not change (Supplementary material online, *Figure S5B* and *C*). Among differential proteins of the cytoskeleton-associated proteome, we identified Pfn-1 (Swiss Prot number P07737; *Figure 4A*) as a single spot with a molecular weight of 15.8 kDa, a pI of 8.8, a Mascot score of 80 and a sequence coverage of 49 (Supplementary material online, *Figure S6*). Profilin-1 content in coronary thrombi was inversely associated with onset-of-pain-to-PCI elapsed time

(*Figure 4B*). It was possible to differentiate the two clusters of patients (T3 and T6) based on thrombus Pfn-1 content (χ^2 distribution $P = 0.001$). T6 had a four-fold lower level of Pfn-1 than T3 ($P = 0.0004$; *Figure 4C*). The same pattern was seen when Pfn-1 content was normalized by actin, marker of thrombus cellularity ($P = 0.02$).

The presence of Pfn-1 in the coronary thrombi was validated by western blot (*Figure 4D*). In addition, we generated *in vitro* normal healthy plasma clots with and without platelets (PRP or PFP, respectively) in order to evidence whether Pfn-1 was present in platelet and in plasma. We observed that Pfn-1 was only present in clots rich in platelets and not in PFP clots, indicating its presence in the platelets compartment in fresh clots (*Figure 4D*).

Profilin-1 distribution in coronary thrombi

To elucidate the distribution of Pfn-1 within the coronary thrombus, serial thrombus sections were analysed by confocal microscopy (*Figure 5*). In T3, Pfn-1 expression was highly abundant within the thrombus. Moreover, co-staining for platelets (CD61) and nucleated cells (Hoechst stain for nuclei) revealed that Pfn-1 co-localized with platelets (CD61) and leucocytes in *in vivo* thrombi (*Figure 5A* and *B*). T6 thrombus showed less Pfn-1 staining and reduced platelet
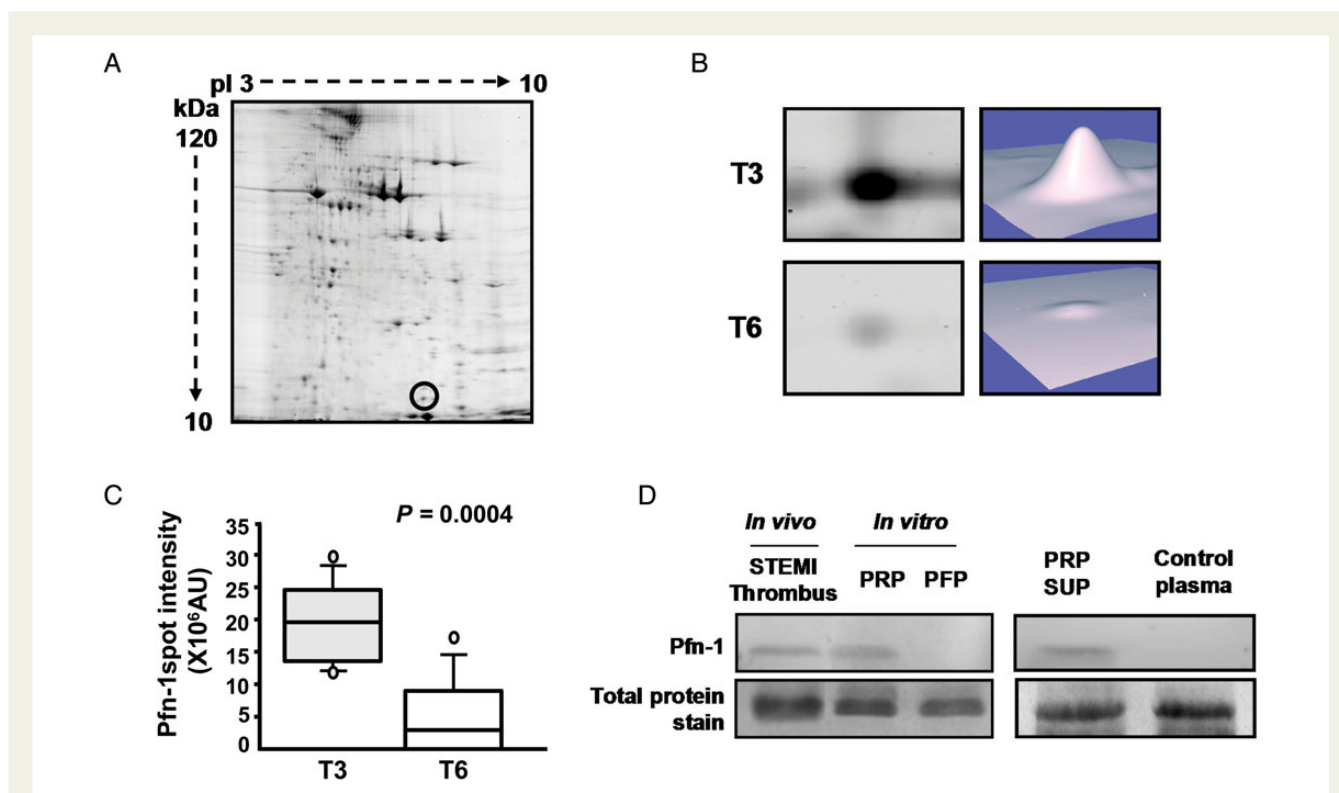

Figure 4 Impact of the time of ischaemia on profilin-1 levels in coronary thrombi. (*A*) Representative Two-dimensional electrophoresis image of coronary thrombus, proteins are separated according to their isoelectric point (pH range 3−10) and molecular mass range (10−120 kDa) in 12% SDS−PAGE gels. Profilin-1 spot is marked with a circle. (*B*) Three-dimensional images of profilin-1 spot identified in coronary thrombus comparing <3 h and >6 h of ischaemia. (*C*) Box plot diagram [median (inter-quartile range)] shows relative intensity of profilin-1 content in two-dimensional electrophoresis analysis of coronary thrombus comparing 3 and 6 h of ischaemia, spot quantification was performed by PD-Quest 8.0. (*D*) Profilin-1 western blot representative image of coronary thrombus of ST-segment elevation myocardial infarction patients, *in vitro* generated clot of platelet-rich plasma, platelet-free plasma, supernatant of PRP clot, and control plasma.
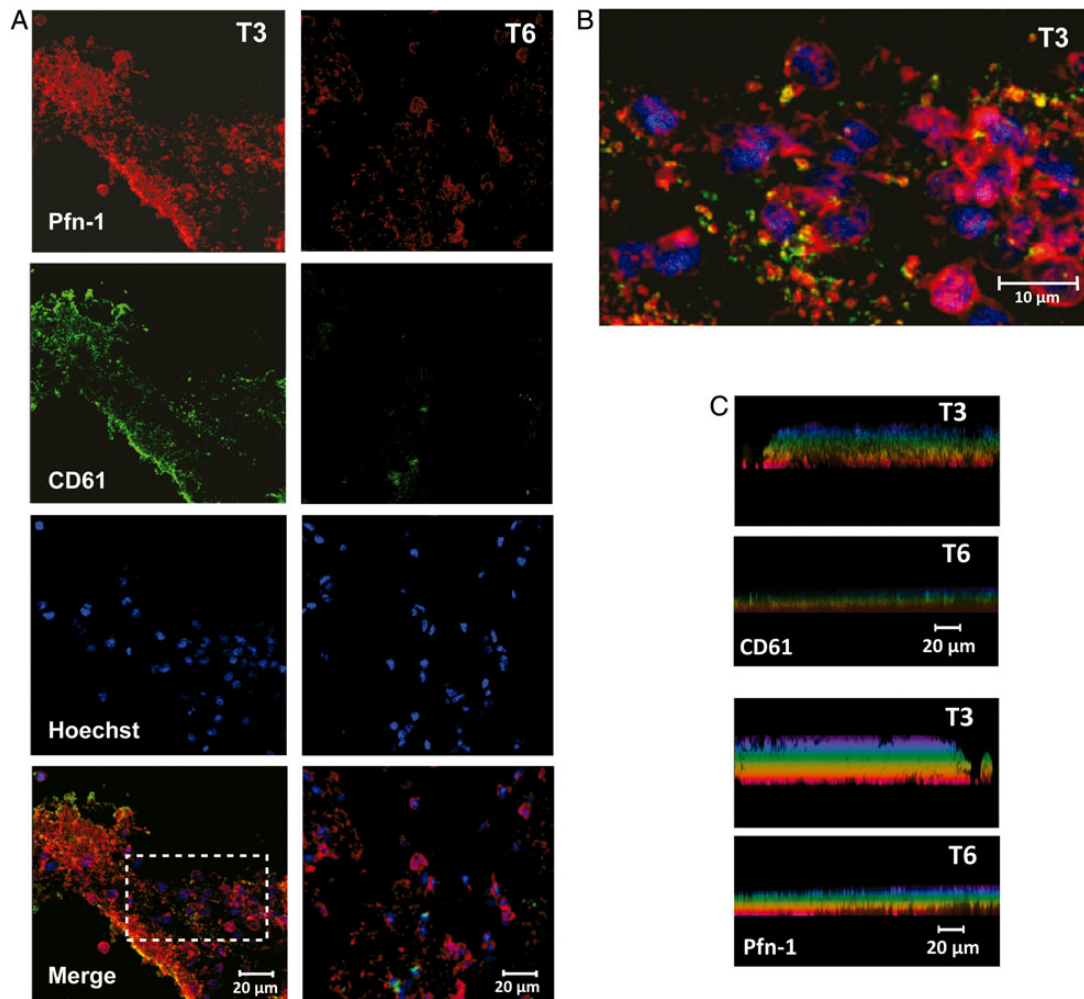
Figure 5 Immunofluorescence detection of profilin-1, platelet, and nucleated cells in coronary thrombi in relation to ischaemia time. (*A*) Representative confocal immunofluorescence analysis of Pfn-1 (red), platelet (green, CD61), and nucleated cells (blue, Hoechst) in permeabilized thrombus section comparing short (T3) and long (T6) time of ischaemia. Scale bar = 20 μm. (*B*) Cropped region of A with factor 4. Scale bar = 10 μm. (*C*) Three-dimensional rotation projections created from stack series of selected thrombi (0.2 μm distance). The maxim projection showing the z-value by colour was created and thereafter a 90° rotation animation was performed creating a projection every 2°.

content (CD61). Interestingly in T6, Pfn-1 was found associated with the thrombus matrix proteins indicating a possible release from platelets (*Figure 5A*).

In confocal 3D projections, Pfn-1 appeared within the entire mass of T3; on the contrary, Pfn-1 was mainly localized at the thrombus surface in T6 (*Figure 5C*), possibly as a marker of newly incoming platelets.

Plasma profilin-1 levels in STEMI patients are related to onset-of-pain-to-percutaneous coronary intervention time

Because Pfn-1 content in T6 thrombus was significantly reduced with respect to T3, we searched for its tentative release into the circulation. Levels of Pfn-1 in coronary artery blood taken from the culprit STEMI-thrombus site and in the peripheral blood were measured by ELISA. Profilin-1 levels were significantly higher both in coronary and systemic blood in T6 patients compared with T3 patients ($P = 0.01$ and $P = 0.0002$, respectively; *Figure 6A*).

Coronary and peripheral Pfn-1 levels were similar in T3 patients, while patients with >6 h from onset-of-pain-to-PCI showed significantly higher peripheral blood Pfn-1 levels than coronary blood ($P = 0.04$; *Figure 6A*). Profilin-1 levels at the culprit coronary site directly correlated with those in the peripheral blood ($R^2 = 0.797$, $P \leq 0.0001$; *Figure 6B*).

As shown in *Figure 6C*, ROC curve analysis showed that Pfn-1 levels had a predictive value for thrombus age (elapsed time from onset-of-pain-to-PCI), with a cut-off value of ~320 pg/mL, 69% of sensitivity and 76% of specificity. Levels of Pfn-1 >320 pg/mL indicate >6 h of ischaemia.

We have evaluated changes in P-selectin levels in these samples, to test for platelet activation in these time periods. Levels of platelet
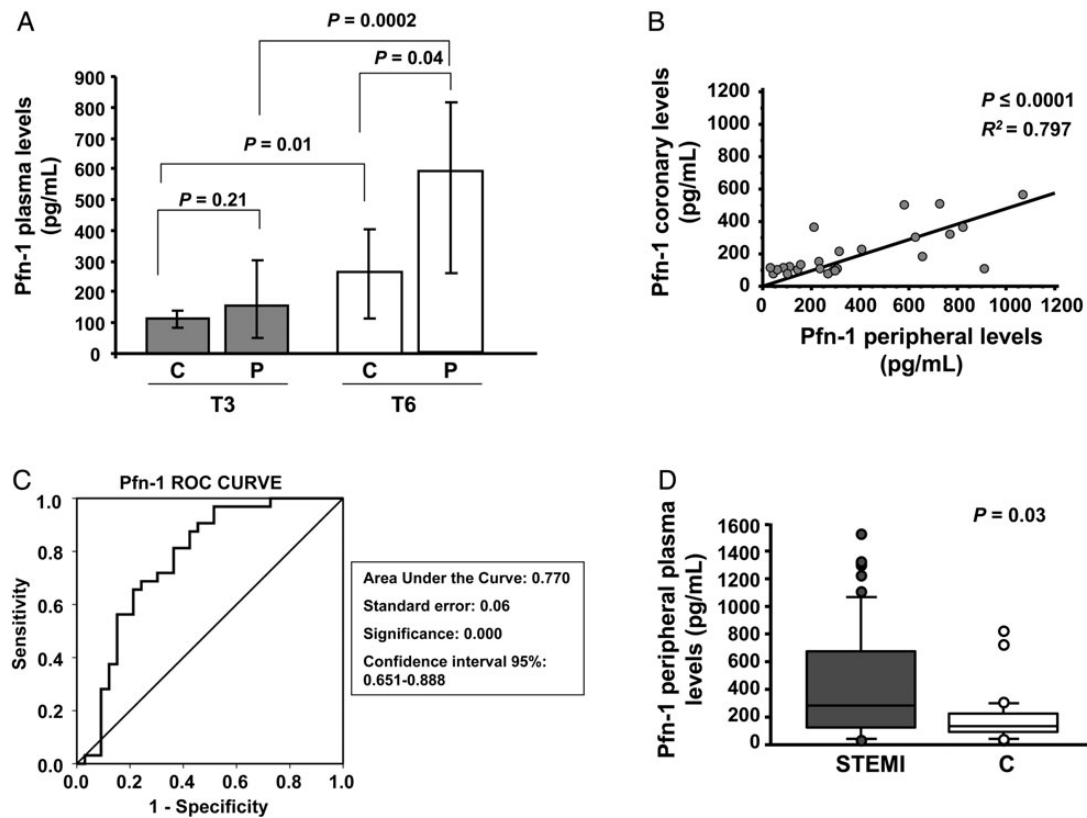
Figure 6 Profilin-1 levels in coronary and peripheral plasma: results from the ELISA assay. (*A*) Bar diagram [median (inter-quartile range)] of Pfn-1 levels in coronary (*C*) and peripheral (*P*) plasma from ST-segment elevation myocardial infarction patients comparing <3 h (T3) and >6 h (T6) of ischaemia. (*B*) Linear regression between coronary and peripheral plasma levels of profilin-1. (*C*) Receiver-operating characteristic curve of profilin-1 predicting ischaemia time. (*D*) Box plot diagrams [median (inter-quartile range)] of profilin-1 levels in the peripheral plasma from ST-segment elevation myocardial infarction patients were compared with the control group.

activation in the STEMI population are high and as expected P-selectin levels were higher than normal (18–40 ng/mL; Supplementary material online, *Figure S7A*). However, P-selectin levels were not influenced by onset-of-pain-to-PCI ischaemic time and there was no significant correlation between P-selectin values and Pfn-1 levels in STEMI patients (Supplementary material online, *Figure S7B*).

All the STEMI patients showed higher hsC-reactive protein levels when compared with controls ($P < 0.001$; Kruskal–Wallis Test; Supplementary material online, *Table S4*). However, no correlation was detected between hsC-reactive protein plasma levels and the time of ischaemia ($R^2 = 0.015$, $P = 0.156$; Spearman correlation; *Figure 7A*). As expected, because they measure different functions, there was no correlation with Pfn-1 plasma levels ($R^2 = 0.117$, $P = 0.23$).

Clopidogrel administration (60% of STEMI patients) did not influence plasma Pfn-1 levels neither in T3 nor T6 patients [Pfn-1 levels, −/+ clopidogrel; T3: 95 (33–267)/164 (80–301) pg/mL $P = 0.21$; T6: 649 (405–860)/598 (317–792) pg/mL $P = 0.91$].

By multiple lineal regression analysis, including those variables statistically significant for Pfn-1 levels in the bivariate analysis (ischaemia time, age, and gender), showed that only time of ischaemia and patient age remained as independent factors for Pfn-1 levels, with an R^2

of 0.38. Unstandardized coefficients gave a beta-value of 0.531 ($P < 0.001$) for ischaemia-time and 0.014 ($P < 0.001$) for patient age.

STEMI patients showed a two-fold increase in plasma Pfn-1 levels compared with the control population [median (IQR) (pg/mL); STEMI: 287 (128–653) vs. C: 129 (90–225), $P = 0.03$; *Figure 6D*]. Analysis by the ANCOVA test showed no interference of GPIIb/IIIa antagonists and clopidogrel when included as co-variables for differences in Pfn-1 plasma levels.

Patients with onset-of-pain-to-PCI time between 3 and 6 h ($n = 21$) were included in the validation studies. Profilin-1 levels showed significant differences between the different ischaemia time groups (T3 vs. T6 $P = 0.0002$; T3–6 vs. T6 $P = 0.0001$; Mann–Whitney test; Supplementary material online, *Table S4*). Indeed, Pfn-1 levels were positively and significantly correlated with the time of ischaemia ($R^2 = 0.166$, $P < 0.001$; *Figure 7B*).

To investigate whether the observed changes in plasma Pfn-1 levels in STEMI patients might be influenced by the high cardiovascular risk background of these patients, a subset of control individuals with high-cardiovascular risk (C-RF) was analysed. We found that within this control group, there were no significant changes in Pfn-1 plasma levels by the presence of cardiovascular risk factors [median (IQR) (pg/mL); C-RF: 98 (73–136) vs. C-NoRF: 185 (114–241), $P = 0.07$].
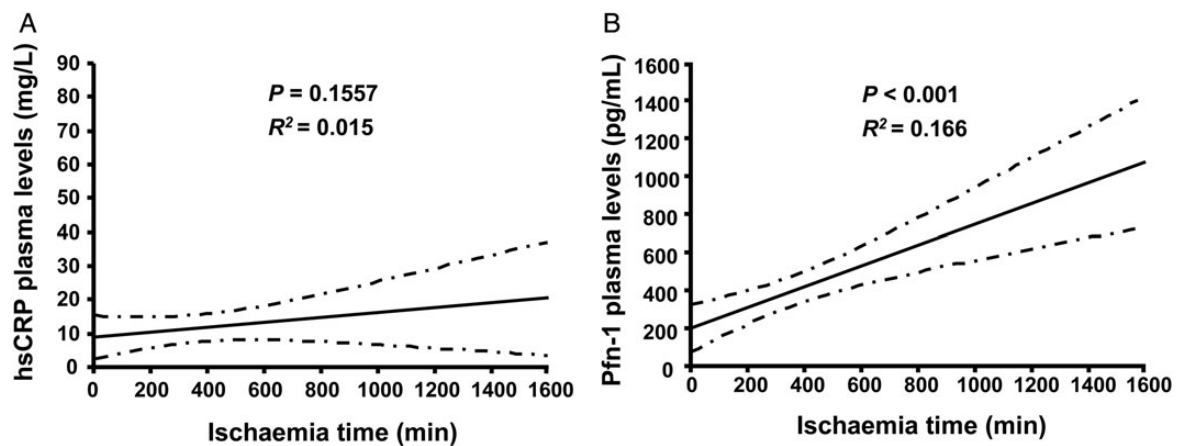
Figure 7 Profilin-1 and hsC-reactive protein plasma levels: results from the ELISA assay. (*A*) Linear regression between hsC-reactive protein plasma levels and ischaemia time (Spearman correlation). (*B*) Linear regression between profilin-1 plasma levels and ischaemia time (Spearman correlation).
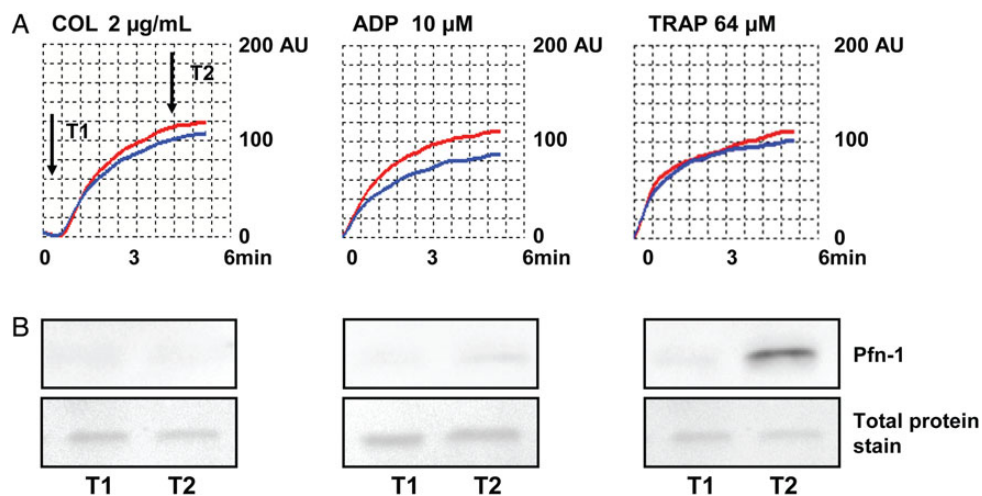

Figure 8 Profilin-1 release upon platelet aggregation. (*A*) Representative image of the aggregation curve on the Multiplate Analyzer using collagen (2 µ/mL), adenosine diphosphate (10 µM), and thrombin receptor-activating peptide-6 (64 µM) in citrate whole blood. (*B*) Western blot for profilin-1 of human plasma at t_1 and t_2 from blood treated with collagen, adenosine diphosphate and thrombin receptor-activating peptide.

Platelet aggregation: profilin-1 secretion is thrombin dependent

We further investigated several agonists for platelet activation to identify the triggers of Pfn-1 release from platelets. Platelet aggregation was induced by collagen (2 µg/mL), ADP (10 µM), and TRAP-6 (64 µM) in whole blood collected in citrate (*Figure 8A*). Supernatants of the platelet aggregation test were taken before the initiation of aggregation (t_1; 10 s after agonist addition) and when the aggregation curve reached plateau (t_2; 4 min after agonist addition). Secreted

Pfn-1 levels in both conditions were analysed by western blot. While aggregation induced by collagen did not cause Pfn-1 release, ADP showed a negligible amount of Pfn-1 release after 4 min. However, samples induced with TRAP, a thrombin mimetic, showed abundant secretion of Pfn-1 (*Figure 8B*).

Discussion

In the last few years, mechanical reperfusion has proved to be a superior treatment strategy compared with systemic thrombolysis

in STEMI patients. Manual thrombus aspiration leads to better myocardial reperfusion and lower risk of distal embolization improving late clinical outcome.[27–29] Thrombus aspiration from coronary arteries has also facilitated to investigate the composition of *in vivo* human coronary thrombi.

The changes in thrombus structure in relation to onset-of-pain-to-PCI elapsed time were analysed comparing thrombi of <3 h to thrombi of >6 h of evolution. These two groups of thrombi were retrieved from patients presenting similar characteristics in demographics, risk factors and in antithrombotic treatment.

Here, we demonstrate that the onset-of-pain-to-PCI elapsed time significantly influences the cellular and protein composition of the coronary thrombi. We cannot conclude from our work how plaque type has affected thrombus characteristics. However, Kramer *et al.* suggested that coronary thrombi in fatal erosions are in later stages of maturation when compared with ruptures.[15]

In this study, we have seen that platelet deposition and the formed fibrin mass changes rapidly within the first hours in agreement with previously reported data.[16] Further, there is a dynamic change with time in the infiltration of leucocytes subtypes. While neutrophils and monocytes were already present at 3 h of thrombus evolution, T-lymphocytes and B-lymphocytes appeared in thrombus of longer evolution. Similarly, these aged occlusive thrombi (T6) showed infiltration of CD105$^+$ progenitors and CD34$^+$cells that were not present in <3 h occlusive thrombi.

Together with the detected changes in cellularity, by applying proteomic approaches, we have detected a significant presence of Pfn-1 in the coronary thrombi. Interestingly, we have found that Pfn-1 content in thrombi is inversely related to ischaemic thrombus age. A significantly lower content of Pfn-1 is found in T6 thrombi (>6 h pain-to-PCI). Profilin-1 is an actin-binding protein that has been associated with atherosclerosis and smooth muscle cell proliferation,[24,25,30,31] regulation of the microfilament system and signalling pathways in mesenchymal cells.[32,33] Formerly known as an inhibitor of actin polymerization, it has been shown that it also promotes actin depolymerization.[34] In nucleated cells, Pfn-1 interacts with a multitude of ligands including various phosphoinositides and proteins containing proline-rich motifs that are involved in actin cytoskeletal regulation, endocytosis, and gene transcription, so playing an important role in processes such as cell motility, development, signalling, and membrane trafficking.[35] In platelets, Pfn-1 has been associated with shape change cytoskeleton proteins changes.[36–39]

Our data by confocal microscopy suggest that Pfn-1 is released by fully activated and aggregated platelets that were recruited in the initial stages of thrombosis on the culprit plaque because intrathrombus Pfn-1 levels significantly decrease with thrombus ageing.

Previous studies had shown that vascular smooth muscle cells express Pfn-1. However, Pfn-1 is found both in the supernatant and in the PRP clots (devoid of erythrocytes and leucocytes Supplementary material online, *Figure S1*) and it is not found neither in the supernatant nor in clots prepared with PFP (*Figure 4D*), suggesting that Pfn-1 is secreted by fully activated platelets. Indeed here we also show that Pfn-1 is released from platelets when they are activated with thrombin but not with collagen, having ADP a minor effect (*Figure 8*). Interestingly, a proteomic study of the secretoma of thrombin-activated platelets identified Pfn-1 as one of the components of platelet release.[40] All

together these data show that Pfn-1 is released from fully activated platelets (as seen with thrombin stimulation) in coronary thrombus where the main trigger for thrombus formation is tissue factor exposed by atherosclerotic plaques that leads to *in situ* thrombin formation.[41,42]

It is worth mentioning that, as shown in Supplementary material online, *Figure S6*, extracellular Pfn-1 can be found in the *in vitro* formed whole blood clots (generated by endogenous thrombin) and in leucocytes.

Because of the changes seen in T3 and T6 coronary thrombi, we searched for a tentative release of Pfn-1 in to the coronary blood. Indeed Pfn-1 was found in blood aspirated from the culprit lesion coronary, suggesting secretion of Pfn-1 upon platelet aggregation in the coronary thrombus mass. Our results show that Pfn-1 levels in plasma inversely correlate with Pfn-1 content thrombi and that the longer the thrombus occludes the coronary artery the higher Pfn-1 levels are found in the systemic circulation because platelets become depleted of Pfn-1. No differences were detected in Pfn-1 plasma levels between T3 patients and controls supporting that Pfn-1 release is strongly dependent on the elapsed time of ischaemia (Supplementary material online, *Table S4*; $P = 0.68$).

Receiver-operating characteristic analysis predicts a cut-off level of Pfn-1(320 pg/mL) in the peripheral circulation that indicated longer than 6 h of onset-of-pain-to-PCI of time of occlusion.

Interestingly, 85% of the patients that had major adverse cardiac events (MACE) within 2 weeks after PCI had plasma Pfn-1 levels above the median value of the entire STEMI-population. Moreover, the few cases of patients with *exitus* within the first 24 h after PCI had extremely high Pfn-1 plasma levels in their peripheral blood at 3 h (1400 pg/mL vs. 200 pg/mL average for the group).

Study limitations

For the correct interpretation of results, we should consider as a limitation of the study that the time-frame of the aspirated thrombus could be little wider than our recorded time (onset-of-the-pain-to-PCI), as previously seen in another study.[18,43] However, a time difference would still differentiate the two investigated groups. In addition, we could not exclude that sudden coronary thrombosis was preceded by a period of plaque instability and series of successive thrombotic events that may occur over days or weeks.

The study only includes patients who had an STEMI <12 h. Therefore, final occlusive thrombosis of the vessel leading to AMI code activation certainly occurred within the evaluated ranges. However, we have not assessed the rate of pre-infarction angina. Thus, we cannot rule out the fact that some degree of mural thrombosis may have preceded the last chest pain by hours or days. Although patients were pre-treated with clopidogrel just before angiography (60%) no effects associated with clopidogrel could be detected.

Conclusion

Older thrombus is an independent predictor of long-term mortality[18] and the possible use of a protein that indicates ongoing thrombosis and occlusion time may be a very convenient tool to guide clinical practice.

Here, we show that elapsed onset-of-pain-to-PCI time and hence ischaemic thrombus age has impact in the composition of STEMI thrombus. Specifically, we have found a higher presence of inflammatory innate and adaptive immunity cells, appearance of progenitor cells, reduction in platelets, increase in complex fibrin networks, and a decrease in Pfn-1 in the coronary thrombi of STEMI patients with longer onset-of-pain-to-PCI elapsed time. Because Pfn-1 is secreted from fully activated platelets in the thrombotic mass, it can be detected in the systemic circulation becoming a potential marker of ongoing thrombosis and of the elapsed time of ischaemia.

Supplementary material

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

Conflict of interest: M.S. is consultant at Abbott and Medtronic outside the submitted work.

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