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Chapter 7

The role of ADAMTS13 in acute myocardial infarction: cause or consequence?

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Submitted
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ABSTRACT

Background: ADAMTS13 is a metalloprotease that cleaves von Willebrand factor (VWF), thereby reducing its prothrombotic properties. There is considerable evidence that VWF levels increase and ADAMTS13 levels decrease in STEMI patients. It is unclear if this contributes to no reflow, infarct size and intramyocardial hemorrhage (IMH).

Objectives: The aim of this study was to determine the role of ADAMTS13 in patients with acute myocardial infarction; and to investigate the benefits of recombinant (r)ADAMTS13 in a porcine model of myocardial ischemia-reperfusion with dual antiplatelet therapy and heparin.

Methods: In 49 consecutive PCI-treated STEMI patients, blood samples were collected directly after and up to 7 days following PCI. Cardiac magnetic resonance was performed 4-6 days after PCI to determine infarct size and IMH. In 23 Yorkshire swine, the circumflex coronary artery was occluded for 75 minutes by a balloon catheter. rADAMTS13 or vehicle was administered intracoronary following reperfusion. Myocardial injury and infarct characteristics were assessed using cardiac enzymes, ECG, and histopathology.

Results: In patients with IMH, VWF activity and VWF antigen were significantly elevated directly after PCI and for all subsequent measurements, and ADAMTS13 activity significantly decreased at 4 and 7 days following PCI; in comparison to patients without IMH. VWF activity and ADAMTS13 activity were not related to infarct size. For rADAMTS13 treated animals no differences in myocardial infarct size, IMH, or formation of microthrombi were witnessed in comparison to controls.

Conclusions: No correlation was witnessed between VWF/ADAMTS13 and infarct size in patients; and intracoronary administration of rADAMTS13 did not decrease infarct size or IMH in a porcine model of myocardial ischemia-reperfusion. These data dispute the imbalance in ADAMTS13 and VWF as the cause of no reflow. Restoring the imbalance between ADAMTS13 and VWF most likely will not be beneficial in STEMI patients already treated with standard antiplatelet and anticoagulant therapy.
INTRODUCTION

In up to 40% of STEMI patients, imperfections in myocardial blood flow are observed despite reperfusion at the epicardial level.1 This phenomenon, also referred to as no reflow, is occasionally observed angiographically directly after PCI. In a majority of cases, it is established several days later by cardiac magnetic resonance (CMR) as a hypoenhanced area within the hyperenhanced infarcted myocardium.2, 3 Both angiographic no reflow and CMR–defined no reflow are related to increased cardiac failure and death.4-6 Various mechanisms for this phenomenon have been proposed including tissue edema, distal embolization of atherosclerotic debris, and also local microthrombi causing occlusion of capillaries leading to larger infarct size and worse outcome.7 Recently, it was shown that CMR-defined no reflow actually contains intramyocardial hemorrhage (IMH) and complete microvascular destruction.8-10 Von Willebrand factor (VWF) is an important actor in primary hemostasis. It attracts platelets to damaged endothelium, causing platelet adhesion; and it thereby initiates and stabilizes platelet aggregation. VWF is released from endothelium during vessel injury in the form of ultra-large VWF multimers which contain several platelet binding sites and are therefore more prothrombotic. ADAMTS13, a disintegrin and metalloprotease with a thrombospondin type 1 repeats-13, is a metalloprotease that cleaves VWF, reducing the size of VWF multimers and diminishing its prothrombotic features.11 In STEMI patients, VWF levels are increased,12, 13 but it is not known whether this is related to infarct size and occurrence of no reflow and IMH. In parallel with the increase in VWF, ADAMTS13 decreases in STEMI patients.12, 13 Recently it was shown that ADAMTS13 knock-out mice developed larger myocardial infarctions after coronary occlusion and showed decreased left ventricular function as compared to wild-type mice. Also, treatment with rADAMTS13 reduced infarct size in wild type mice.14-16 However, the potential beneficial effects of rADAMTS13 on infarct size and infarct characteristics have never been tested in a large animal model of myocardial ischemia-reperfusion.

In the present study, the relationship between ADAMTS13 and VWF levels and CMR-derived infarct size and occurrence of no reflow and IMH was determined prospectively in STEMI patients. Also, in a porcine model of myocardial ischemia-reperfusion, closely resembling the clinical scenario of AMI treated with primary PCI, intracoronary infusion of rADAMTS13 was tested for its effects on infarct size, formation of microthrombi and IMH.

METHODS

Patient study
A total of 60 consecutive patients presenting with their first STEMI and successfully treated by primary PCI were prospectively included as described earlier.17 For the present analysis, only patients who underwent CMR at day 4-6 were selected (n=
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46). The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the ethics committee of the VU University Medical Center. Upon arrival, daily administration of 100mg acetylsalicylic acid and 10mg prasugrel was started. Bivalirudin was administered as a single bolus, followed by 0.25 mg/kg intravenously during 4 hours. Blood was withdrawn directly after (T0) and at 1, 4, and 7 days (T1, T4 and T7) after PCI. VWF activity, VWF antigen, ADAMTS13 activity, fibrinogen, and D Dimer were measured. Further details on methods such as exclusion criteria, and measurement of ST-resolution can be found in the supplementary material.

Cardiovascular magnetic resonance imaging
CMR was performed between 4 and 6 days after PCI using a 1.5 Tesla MR-scanner (Avanto, Siemens, Erlangen, Germany). IMH was identified on T2w-images as hypointense areas within the hyperintense signal of infarct-related edema. Cine images were analyzed by tracing the endocardial and epicardial myocardial borders in both end-diastolic and end-systolic phases, providing myocardial volumes and ejection fraction. Quantification of infarct size was performed on short axis late gadolinium enhanced (LGE) images. Further details on acquisition and analysis and definitions of CMR parameters are specified in the supplementary material.

Porcine ischemia-reperfusion model
Approval was obtained from the local Animal Ethics Committee. A full description of animal experimental procedures, including continuous twelve lead electrocardiograms and transthoracic echocardiography, can be found in the supplementary material. In brief, 23 female Yorkshire swine were included. Twelve animals received vehicle and eleven were given rADAMTS13. An over-the-wire balloon was placed in the proximal left circumflex artery and inflated for 75 minutes. During coronary occlusion, animals received a bolus of 5000 IU of unfractionated heparin and the same amount after deflation of the balloon. After reperfusion, 300 mg acetylsalicylic acid and 300 mg clopidogrel were administered. All animals were given daily doses of 80 mg acetylsalicylic acid and 75 mg clopidogrel until their planned sacrifice seven days after ischemia-reperfusion. Recombinant ADAMTS13 (rADAMTS13) (400 U/kg bodyweight = 320 μl/kg bodyweight, Baxter Innovations Vienna, Austria) or a comparable amount of vehicle were administered intracoronary in one single bolus fifteen minutes after reperfusion by an investigator blinded for treatment.

Histopathological analysis
Detailed information on histopathological methods can be found in the supplementary material. In brief, on the seventh day after experimental AMI, animals were sedated and killed. Total infarct size and IMH were determined macroscopically and microscopically (figure 1). Anti-CD31 staining was performed to detect (micro-
thrombi and (micro-)vessels. For both treatment arms, microthrombi and the number of vessels and thrombi per mm² were calculated. To assess inflammatory involvement, myocardial tissue was stained for MPO and CD45.\textsuperscript{18, 19}

**Laboratory measurements performed in the patient and porcine study**

Laboratory measurements are described in detail in the supplementary material. VWF factor activity (Innovance VWF Ac) was determined on a Behring Coagulation System according to protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany). ADAMTS13 activity was determined as described earlier.\textsuperscript{20} VWF antigen and VWF propeptide levels were measured by ELISA using commercial antibodies (DAKO, Denmark and Sanquin, The Netherlands, respectively). D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-Dimer, Siemens Healthcare Diagnostics). Fibrinogen concentration was derived from the change in optical signal during prothrombin time determination. Fibrinogen antigen was determined by ELISA using antibodies from DAKO (Glostrup, Denmark). The ability of recombinant ADAMTS13 to cleave
porcine VWF was determined *in vitro* by measuring porcine VWF activity in plasma of animals before and after addition of rADAMTS13.

**Statistical analysis**

Categorical data are presented as frequencies (percentage) and continuous data as mean ± standard error (SE) or median with interquartile range (IQR). For the patient study, missing values for coagulation parameters at specific time points were imputed using multiple imputation. The imputation model included age, sex, presence of IMH, CMR-derived infarct size, and all coagulation parameters and 20 datasets were created. AUC’s for levels of d-dimer, VWF activity, VWF antigen, VWF propeptide and ADAMTS13 were determined using blood measurements taken at T0, T1, T4 and T7 (separately for each of the imputed datasets). Mean AUC’s of patients with and patients without IMH were compared using independent samples t-tests. Independent Student's T-tests were used to compare means of coagulation parameters between groups at the separate time points. To determine the correlation between continuous variables, Pearson’s R was calculated. Estimated mean differences and p-values of the tests were pooled over the imputations using the standard pooling procedures for multiple imputed datasets available in SPSS.

For the porcine study, differences between treatment groups were compared with Mann-Whitney U tests for unpaired and Wilcoxon signed rank tests for paired non-parametric analysis. Pearson’s Chi square test was performed on categorical variables, and ANOVA was used for regression. Plots of means were drawn using GraphPad Prism (GraphPad Software 6.00, San Diego, California, USA). Statistical analyses were performed using SPSS software package (IBM SPSS Statistics 22.0, Chicago, IL, USA).

**RESULTS**

**General characteristics of the patient study**

Clinical demographics and CMR parameters of the 49 patients are shown in table 1.

**IMH associated with higher VWF activity and lower ADAMTS13 activity**

CMR-defined IMH was found in 19 patients (39%). VWF activity was significantly elevated directly after PCI at T0 in patients with IMH in comparison to patients without IMH (IMH vs. no IMH; mean± SE 230±18% vs. 192±10%, p= 0.048). This difference persisted at all subsequent measurements (T1, T4, T7), and sustained when comparing AUCs (p= 0.021, see also figure 2a). VWF antigen showed a comparable pattern, with significant differences at T0 (IMH vs. no IMH: 192.6±11.6% vs. 161.5±9.5%, p= 0.040) and for all subsequent measurements separately and combined, after calculation of the AUCs (p= 0.020, see also figure 2b). ADAMTS13 activity was significantly decreased at 4 and 7 days after PCI in patients with IMH (IMH vs. no IMH; T4: 79.5±3.0% vs. 92.3±4.5%, p= 0.037; T7: 82.6±3.4% vs.
Table 1. Clinical demographics, angiographic characteristics and functional parameters in the patient study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>STEMI patients (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>47 (78%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59±9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>85±14</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27±4</td>
</tr>
<tr>
<td>CAD risk factors</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>42 (70%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>8 (13%)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>46 (77%)</td>
</tr>
<tr>
<td>Family history</td>
<td>40 (67%)</td>
</tr>
<tr>
<td>Time to reperfusion (hours)</td>
<td>2.7±1.4</td>
</tr>
<tr>
<td>CK-MB peak (U/L)</td>
<td>164±170</td>
</tr>
<tr>
<td>Infarct-related artery</td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>30 (50%)</td>
</tr>
<tr>
<td>LCx</td>
<td>5 (8%)</td>
</tr>
<tr>
<td>RCA</td>
<td>25 (42%)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>Thienopyridine</td>
<td>60 (100%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>53 (88%)</td>
</tr>
<tr>
<td>Glycoprotein IIb/IIIa inhibitor</td>
<td>16 (27%)</td>
</tr>
<tr>
<td>Heparin</td>
<td>19 (32%)</td>
</tr>
<tr>
<td>Bivalirudin</td>
<td>58 (97%)</td>
</tr>
<tr>
<td>TIMI 3 flow grade post-PCI</td>
<td>57 (95%)</td>
</tr>
<tr>
<td>Incomplete (≤70%) ST-segment resolution post-PCI</td>
<td>28 (48%)</td>
</tr>
</tbody>
</table>

Data are N or mean±SD. BMI = body mass index; CAD = coronary artery disease; CMR = cardiovascular magnetic resonance; LV = left ventricle; LVEDV = left ventricular end-diastolic volume; LVEF = left ventricular ejection fraction; LVEF = left ventricular end-systolic volume; CK-MB = creatin kinase - myocardial band; PCI = percutaneous coronary intervention.
93.8±3.6%, p= 0.033), as well as when using the AUC (p= 0.049, see also figure 3). Fibrinogen and d-dimer levels were not significantly higher in patients with IMH except for day 7 (data not shown).

No strong correlation between VWF activity, ADAMTS13 activity and infarct size
Mean infarct size (as percentage of the LV) in STEMI patients was 17.5±12.3% and did not correlate substantially to ADAMTS13 activity measured directly after PCI (R= -0.162, p= 0.265) or to any other subsequent measurement. VWF levels correlated statistically significant with infarct size, but only at T4 and the correlation was weak (R= 0.298, p= 0.037).

**Figure 2. VWF activity and antigen in STEMI patients treated with PCI.** A) VWF activity (%) and B) VWF antigen (%) measured directly after PCI (T0) and at 1, 4 and 7 days following PCI (T1, T4, T7). VWF activity and antigen were significantly higher in patients with IMH compared to patients without IMH (p= 0.021 for AUC of T0 to T7 VWF activity and p= 0.020 for VWF antigen).
General characteristics of the porcine study
In the present study 23 animals were included (median age 83 days, IQR 79-91; median weight 30 kg, IQR 27-34). Twelve animals received vehicle and eleven were given rADAMTS13. Two animals from the vehicle arm died within 24 hours after the intervention. In ex vivo experiments, when adding increasing concentrations of rADAMTS13 to porcine plasma, a corresponding decrease in VWF activity was measured (figure 4). In the porcine model, at t=75 minutes after reperfusion VWF activity, ADAMTS13 activity and fibrinogen decreased in comparison to baseline values. As all animals received a substantial amount of intravenous fluids, albumin levels at baseline and after release of the balloon were measured, showing clear evidence of dilution (percentage decrease albumin at t=75 min: 18%, p< 0.001, no differences between treatment groups). This percentage was used to correct measurements of all coagulation assays performed at t=75 minutes.

<table>
<thead>
<tr>
<th>ADAMTS13 activity (% Mean±SE)</th>
<th>No IMH (n=30)</th>
<th>IMH (n=19)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>71.8±3.3</td>
<td>68.4±4.0</td>
<td>0.521</td>
</tr>
<tr>
<td>T1</td>
<td>84.3±3.2</td>
<td>79.6±2.7</td>
<td>0.302</td>
</tr>
<tr>
<td>T4</td>
<td>92.3±4.5</td>
<td>79.5±3.0</td>
<td>0.037</td>
</tr>
<tr>
<td>T7</td>
<td>93.8±3.6</td>
<td>82.6±3.4</td>
<td>0.033</td>
</tr>
<tr>
<td>AUC T0 – T7</td>
<td>622.2±23.8</td>
<td>555.9±19.2</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Figure 3. ADAMTS13 activity in STEMI patients treated with PCI. ADAMTS13 activity (%) measured directly after PCI (T0) and at 1, 4 and 7 days following PCI (T1, T4, T7). ADAMTS13 activity was significantly higher in patients with IMH compared to patients without IMH at T4, T7 and overall (AUC).
VWF and ADAMTS13 levels in experimental myocardial ischemia-reperfusion

Median baseline VWF activity for all swine was 150% (IQR 127-172). After correction for dilution, there was a trend towards increased VWF activity directly following the intervention (median 165%, IQR 123–183, p=0.056). ADAMTS13 activity did not change after the induced AMI (baseline vs. T=75 min: 18%, IQR 15-4 vs. 18, IQR 16–24, p=0.57). Following intracoronary infusion of rADAMTS13 at 3 hours after AMI, ADAMTS13 activity increased significantly to 324% (IQR 117-384, p=0.003 in comparison to baseline, p< 0.001 versus control group that had a median level of 15%, IQR 11-19). ADAMTS13 activity remained increased in the rADAMTS13 treated group on all following measurements (p< 0.001 for repeated measures ANOVA in comparison to control group). There was no difference in VWF activity after infusion between both treatment groups for all following measurements (p=0.99 repeated measures ANOVA) (figures 5 and 6).

Induction of myocardial infarction had no direct influence on fibrinogen levels (baseline vs. T=75 min: 3.3 g/L, IQR 3.0-3.5 vs. 3.3, IQR 2.7-3.7; p=0.82). However, fibrinogen levels were increased at 1 and 7 days after induction of ischemia-reperfusion (6.9, IQR 5.7- 7.5 and 4.8, IQR 4.0-5.6 respectively; p< 0.001 versus baseline for both) compared to baseline levels (figure 7). There were no differences in fibrinogen measurements throughout the experiment between treated animals and controls (p= 0.80 Repeated Measures ANOVA). There were no differences in red blood cell count and platelet count between rADAMTS13 and vehicle treated animals throughout the study (data not shown).

Figure 4. Cleavage of porcine VWF by rADAMTS13. In vitro studies show that adding rADAMTS13 to porcine plasma results in a dose-dependent decrease in VWF activity.
No effects of rADAMTS13 on ST-segment resolution, infarct size and cardiac function

ST-segment resolution and cardiac enzymes were similar between treatment groups (see also the supplementary material). Microscopic infarct size as measured by PTAH staining was comparable between rADAMTS13 treated group (6.7%, IQR 5.0-9.4) and control group (8.2%, IQR 2.4-10.2; p=0.75). Macroscopic estimation of infarct size from photographed cross sections of the heart showed no differences between rADAMTS13 treated animals and controls (rADAMTS13 vs. controls: 25.1%, IQR 20.6-26.6 vs. 22.2%, 20.0-29.3; p= 0.97). rADAMTS13 treated animals had an increased WMSI at 60 minutes after reperfusion (p= 0.02 for delta 2-1, see also Supplementary table 1). No other differences were observed between first and final echocardiogram for all echocardiography parameters, when comparing rADAMTS13 treated animals and controls (see also the results section and Supplementary table 1).

No effects of intracoronary rADAMTS13 infusion on IMH or formation of microthrombi

Histopathological findings showed that the core of all infarctions contained hemorrhage. The size of IMH as a percentage of LV was similar between treatment groups (rADAMTS13 vs. controls: 2.4%, IQR 1.2-3.7 vs. 2.1%, IQR 0.8-4.8, p= 0.97). Same results were found for IMH as percentage of infarct size (rADAMTS13 vs. controls: 37.0%, IQR 27.3-43.7 vs. 30.1%, IQR 19.0-48.3, p= 0.56).
The total number of microthrombi in the infarct tissue did not differ between groups (rADAMTS13 vs. controls: 19.0 per section, IQR 17.0-27.5 vs. 21.5 per section, IQR 17.5-28.5; p=0.65).

Figure 5. ADAMTS13 activity in the porcine ischemia-reperfusion model. ADAMTS13 activity (%) measured before and after 75 minutes of balloon occlusion (t=75 min) of the left circumflex artery (median and IQR). rADAMTS13 treated animals demonstrated elevated ADAMTS13 activity levels compared to controls (P< 0.001).
Leukocytes as detected with CD45 and MPO staining were found more often in the border zone (97.5, IQR 63.8 – 135.8) in comparison to infarct tissue (17.8, IQR 11.1 – 28.9, p< 0.001). There was no difference between rADAMTS13-treated and control animals with regard to the presence of leukocytes in the infarcted tissue, the border zone or the non-affected myocardium.

**Figure 6. VWF activity in the porcine ischemia-reperfusion model.** VWF activity (%) measured before and after 75 minutes of balloon occlusion (t=75 min) of the left circumflex artery (median and IQR). rADAMTS13 treated animals had similar VWF activity levels compared to controls directly after reperfusion (t=75 min). P= 0.99 for rADAMTS13.

Leukocytes as detected with CD45 and MPO staining were found more often in the border zone (97.5, IQR 63.8 – 135.8) in comparison to infarct tissue (17.8, IQR 11.1 – 28.9, p< 0.001). There was no difference between rADAMTS13-treated and control animals with regard to the presence of leukocytes in the infarcted tissue, the border zone or the non-affected myocardium.

**DISCUSSION**

There is ample proof of the association between VWF/ADAMTS13 levels and myocardial infarction, and rADAMTS13 has been effective in reducing myocardial injury in rodent models. In the present study, VWF/ADAMTS13 levels were assessed in 49 STEMI patients and IMH was determined using CMR. Furthermore, recombinant ADAMTS13 was administered with the aim to limit myocardial injury in a porcine myocardial ischemia-reperfusion model. The main findings are: 1) IMH was associated with higher VWF activity and (eventually) lower ADAMTS13 activity; 2) Intracoronary administration of rADAMTS13 did not reduce infarct size in a porcine myocardial ischemia-reperfusion model on a background of dual anti-platelet therapy and heparin.
VWF and ADAMTS13 levels in patients with IMH: cause or consequence?
STEMI patients with IMH had higher VWF activity immediately after PCI and in all subsequent measurements. Directly after PCI, there were no differences in ADAMTS13 activity between patients with and without IMH. Lower ADAMTS13 activity was found in patients with IMH at 4 and 7 days following STEMI. These findings are in line with Zhao et al. who showed that VWF levels in STEMI patients were higher immediately after PCI in patients with no reflow, whereas ADAMTS13 levels did not differ between patients with and without no reflow, until the seventh day after PCI.22
The present findings that VWF levels are increased in patients with IMH and ADAMTS13 levels decreased are novel and counter-intuitive at first. However, a similar pattern has been shown in patients after hip surgery, and also patients with subarachnoid bleeding display increased levels of VWF.23-25 VWF is believed to be an acute phase protein, and its levels increase in various inflammatory diseases.26-28 The changes observed in the present patient study are most likely the consequence of intramyocardial bleeding rather than the cause. Possibly, the rise in VWF following acute myocardial infarction is an epiphenomenon or a necessary compensation for IMH. VWF and ADAMTS13 may nevertheless serve as diagnostic markers to detect patients at risk of developing IMH. Especially, given that no reflow is detected angiographically in a minority of cases 3 and that CMR, although more effective in detecting no reflow, 2,3 cannot be performed directly in acute STEMI patients. Other frequently used markers of coagulation, d-dimer and fibrinogen, were similar in both patient groups.

No effect of rADAMTS13 in reducing myocardial injury
Treatment with rADAMTS13 reduced infarct size in a murine stroke model by 30% without signs of increased bleeding.29 Furthermore, rADAMTS13 exerted antithrombotic effects in a model of vessel damage in which ADAMTS13 decreased occlusion time of mesenteric arterioles after FeCl3-induced thrombus formation.30 In open chest models myocardial infarction models, a therapeutic benefit of rADAMTS13 was shown in both ADAMTS13 knock out mice and wild type mice treated with rADAMTS13.14-16 In all murine AMI studies, rADAMTS13 led to a decrease in neutrophil accumulation in the infarcted myocardium. Interestingly, there was no evidence of any antithrombotic effect of rADAMTS13. For instance, there was no reduction of thrombotic occlusions in myocardial microvessels. Therefore, its therapeutic properties were deemed to be anti-inflammatory rather than antithrombotic.14-16 The inflammatory response may have been caused by the use of an open chest model, unlike the closed chest approach in our study. Possibly, a beneficial effect of rADAMTS13 was not found in our study due to the fact that the model used was not inflammatory or thrombotic enough. VWF activity did not increase substantially after the infarction, and fibrinogen did not increase
until 24 hours. However, the model implemented in the present analysis has been established and proven effective in detecting treatment effect in myocardial injury models. Furthermore, the medical regime used in our study was according to the ESC guidelines for STEMI patients, mimicking the treatment of patients with AMI realistically. In the rodent studies, no antiplatelet agent, heparin, or any other anticoagulant was applied besides rADAMTS13.

**High dosage and lack of efficacy of rADAMTS13**

ADAMTS13 levels increased more than 15-fold immediately after rADAMTS13 administration, and remained elevated throughout the following 7 days. It seems likely therefore that enough rADAMTS13 was applied. Perhaps an overdose of rADAMTS13 may have caused a potential direct effect on myocardial performance. Even though the difference in WMSI was the only statistically significant finding, all echocardiographic parameters showed a decrease between the first and the second echocardiogram for the rADAMTS13 group, followed by a recovery from the second to the third (final) echocardiography (Supplementary table 1). This may indicate a negative effect of rADAMTS13 on cardiac function that would not have occurred with lower dosages. However, treatment did not lead to an increased bleeding tendency or increased IMH.

**No reflow and IMH**

An important finding in this study was provided by results from anti-CD31 staining. Previously, it has been postulated that in no reflow, distal embolization of atherosclerotic debris and local microthrombi cause occlusion of capillaries. In contrast with this assumption, we witnessed no microthrombi and very few microvessels in the core of the infarction. Instead, obstruction of the microvasculature was more evident in the border zone, whereas the core of the infarction contained IMH. These results are in line with previous animal studies, and recent reports suggesting that the pathophysiology behind no reflow is IMH rather than microvascular obstruction. These insights in the pathophysiology behind no reflow may explain why few therapeutic regimens have been effective in reducing the morbidity and mortality caused by no reflow. This may also explain why IMH size was similar in rADAMTS13 treated animals versus controls, and why rADAMTS13 was not beneficial in the porcine study: because of the lack of a causal relationship between ADAMTS13 levels and IMH.

**Conclusions**

This is the first study to show that IMH as measured by CMR is associated with increased VWF levels and decreased ADAMTS13 levels in STEMI patients. Administration of recombinant ADAMTS13 was not effective in reducing infarct size and no reflow in a porcine model of myocardial ischemia-reperfusion.
Acknowledgments

We thank Klaas Walter Meyer and Paul Sinnige for their assistance during the animal experiments; and personnel of the department of Experimental Vascular Medicine of the AMC for their laboratory analyses. We thank Hanspeter Rottensteiner and Alexandra Schiviz from Baxter Innovations Vienna, Austria, for helpful discussions and critical reading of the manuscript.
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SUPPLEMENTARY MATERIAL

METHODS

Patient study
All patients gave written informed consent within 24 hours after PCI. Hemodynamically unstable patients and patients with three-vessel disease were excluded from the study. Other exclusion criteria were a previous myocardial infarction in the culprit coronary artery, previous coronary artery bypass graft (CABG) surgery, unsuccessful PCI (defined as TIMI 0 or 1 flow after procedure) and inability or refusal to give informed consent.

Continuous twelve leads electrocardiograms
Continuous twelve leads electrocardiograms were performed directly after successful PCI during 24 hours following reperfusion. ST-segment resolution was evaluated on a 12-lead electrocardiogram acquired pre-PCI and 1 hour after PCI. Sums of ST-segment elevation were measured 60 ms after the J point in leads I, aVL, and V1 to V6 for anterior and leads II, III, aVF, V5, and V6 for non-anterior acute myocardial infarctions, respectively. The percentage resolution of ST-segment elevation from before to after PCI was calculated and categorized as complete (≥70%), partial (30% to <70%), or no (<30%) ST-segment resolution. Incomplete reperfusion was defined as <70% ST-segment resolution on electrocardiography.1

Cardiovascular magnetic resonance imaging protocol
CMR was performed between 4 and 6 days after PCI and at 90 days after PCI using a 1.5 Tesla MR-scanner (Avanto, Siemens, Erlangen, Germany) and a dedicated phased array cardiac receiver coil. Functional imaging was performed using retrospectively ECG-gated steady-state free precession cine imaging with breath-holding. Standard 3 long axis orientations (4-, 3- and 2-chamber view) and short axis orientation with full LV coverage were obtained (typical parameters: voxel size ~1.6x1.9x5.0 mm, slice thickness 5.0 mm, slice gap 5.0 mm, TR/TE 3.2/1.6 ms, flip angle 75º, field of view 360x400 mm, temporal resolution <50 ms). Intramyocardial hemorrhage (IMH) was visualized using a segmented T2-weighted turbospinecho (T2w) sequence with fat suppression, at similar slice positions as the cine images. After administration of 0.2 mmol/kg Gd-DOTA (Dotarem, Guerbet, Villepinte, France), LGE images were acquired after 10-15 minutes, using a 2-dimensional segmented inversion-recovery gradient-echo pulse sequence, with individual correction of the inversion time to null the signal of normal myocardium (slice thickness 5.0 mm, slice gap 5.0 mm, field of view 360x400 mm, pixel size ~1.4x1.4 mm, TR 2x RR interval, typical inversion time 250-400ms). Cine and LGE images of each patient were matched by slice position.
Analysis and definitions of CMR parameters

All CMR analyses were performed using dedicated off-line software (QMassMR v7.5, Medis, Leiden, the Netherlands). Cine images were analyzed by tracing the endocardial and epicardial myocardial borders in both end-diastolic and endsystolic phases, providing myocardial volumes and ejection fraction. Quantification of infarct size was performed on short axis LGE images. IMH, representing no reflow, was identified on T2w-images as hypointense areas within the hyperintense signal of infarct-related edema. Volumes were converted to grams of tissue by correcting for density of myocardial tissue (i.e. 1.05 g/cm³). Analysis of IMH was performed independently from and blinded to the results of the other analyses. The total infarct size was standardized by dividing the infarct mass by the total left ventricular mass, to calculate mean infarct size (as percentage of the LV).

Porcine model

Approval was obtained from the local Animal Ethics Committee. 23 female Yorkshire swine were treated with 400mg amiodarone 7 days before intervention. Animals were fasted overnight and received a fentanyl plaster of 25 µg 24 hours before the beginning of surgery. At the start of the procedure, 400mg amiodarone and 5-15 mg metoprolol were given. Animals were premedicated with intramuscular administered ketamine hydrochloride (10-15 mg/kg bodyweight), midazolam (0.5-2 mg/kg bodyweight) and atropine (0.5 mg). Oxygen was provided through a mask and after intratracheal intubation with 45-50% O₂. Animals were ventilated with a tidal volume of 10 ml/kg bodyweight. Anesthesia was deepened with single intravenous (i.v.) administration of etomidate (15 to 20 mg), midazolam (0.5 mg/kg), and sufentanyl (100 µg). During the intervention, anesthesia was maintained using sevoflurane (inhalation of 1.2–1.8%), midazolam (0.5 mg/kg/hr i.v.), and sufentanyl (6-7 µg/kg/hr i.v.). To reduce the risk of infection, a closed chest model was used, amoxicillin/clavulanic acid (500/50 mg i.v.) was administered and the whole procedure was performed under sterile conditions. The right carotid artery was used for catheterization and an over-the-wire balloon was placed in the proximal left circumflex artery and inflated for 75 minutes. Total occlusion was confirmed angiographically and by continuous twelve leads electrocardiograms. During coronary occlusion, animals received a bolus of 5000 IU of unfractionated heparin and the same amount after deflation of the balloon. After reperfusion, 300 mg acetylsalicylic acid and 300 mg clopidogrel were administered. All animals were given daily doses of 80 mg acetylsalicylic acid and 75 mg clopidogrel until their planned sacrifice seven days after ischemia-reperfusion injury. Recombinant ADAMTS13 (rADAMTS13) (400 U/kg bodyweight = 320 µl/kg bodyweight, Baxter Innovations Vienna, Austria) or a comparable amount of vehicle, both containing the same buffer, were administered intracoronarily in one single bolus fifteen minutes after reperfusion by an investigator blinded for treatment. rADAMTS13 was manufactured using the expression of stable transfected Chinese hamster
ovary cell lines in serum-free medium, as described in detail previously.² The dosage was chosen based on the amount used in the cerebral infarction animal model.² Troponin T, (µg/L) was measured at 3, 6 and 24 hours after the balloon occlusion. Infarct size was calculated as the Area Under the receiver operator Curve (AUC) of Troponin T.³ Measurements of red blood cell count (10*12/L) and platelet count (10*9/L) were performed at baseline, as well as 24 hours and 7 days after the balloon occlusion. Albumin was measured from blood samples taken at baseline and immediately after the balloon occlusion (t=75 minutes). ADAMTS13 activity (%), VWF activity (%) and fibrinogen (g/L) were measured at baseline; 75 minutes, 3 hours, 24 hours and 7 days after the balloon occlusion.

**Continuous twelve leads electrocardiograms**
Continuous twelve leads electrocardiograms were performed during the intervention, and during a minimum of one hour following reperfusion, using the sum of ST-segment elevations for leads II, III and aVF. ST-segment resolution was calculated as the percentage of recovery at 30 and 60 minutes after reperfusion, in comparison to the ECG recorded at 70 minutes of balloon occlusion. ST-segment elevation was measured with a computerized recording of the ST-segment at 60 ms after the J point.¹ ST-segment resolution was considered complete when ≥70% decrease in ST-segment elevation was achieved, partial when 30-70% recovery occurred and absent in case of <30% normalization.¹

**Transthoracic echocardiography**
Two-dimensional echocardiography was performed at 60 minutes of balloon occlusion (first echocardiogram); 60 minutes after reperfusion (second echocardiogram); and seven days later on the day of termination (third echocardiogram). Wall motion score index (WMSI), left ventricular ejection fraction (LVEF), and fractional area change (FAC) were calculated. Echocardiography was performed by a blinded observer and results were validated by random sampling by a second, blinded observer.

**Histopathological analysis**
In the porcine model, the primary endpoint was infarct size as quantified by microscopic histopathology analysis. On the seventh day after experimental acute myocardial infarction, animals were sedated with 6 mg/kg tiletamine/zolazepam i.v. and 2 mg/kg xylazine i.v.. Animals were killed by an intravenous injection of 200 mg/kg bodyweight pentobarbital i.v.. Internal organs were inspected for macroscopic hemorrhage and the heart was excised. Myocardial infarction was clearly observed on gross macroscopic inspection (see also figure 1 in the manuscript). Using a wide margin, this area was cut into cross sections, photographed and measured. Measurements of cross sections and photographs were used for quantification of total left-ventricle area and wall thickness. Cross sections were then fixed in formaldehyde solution and embedded in paraffin. Afterwards, infarction and border
zone were cut into sections of 4 μm. Complete slides where scanned with a digital (microscopic) Mirax slide Scanner system (3DHISTECH, Budapest, Hungary). The actual scan resolution (effective pixel size in the sample plane) at 20x was 0.23μm. Infarcted tissue and IMH were detected with Phosphotungstic acid-hematoxylin (PTAH) staining as previously described. PTAH stains vital myocardium blue, infarcted tissue pink, hemorrhagic infarction (IMH) dark purple, and fibrosis red (figure 1). Total infarct size and IMH were determined macroscopically by planimetric tracing on photographs (figure 1), and microscopically by manually tracing the area stained by PTAH on the 4 μm sections, both using ImageJ software (Image J 1.46, National Institutes of Health, USA). Microscopic measurement of infarct size is presented as a percentage of total left ventricular mass. Given that microscopical infarct size was measured on fixed tissue, whereas total left ventricle area was calculated using measurements and photographs of the freshly cut heart, microscopical infarct analysis shows systematic underestimation. This is the result of shrinkage of the heart caused by fixation in formaldehyde solution, but this occurred similar for both rADAMTS13 as well as control group.

Anti-CD31 staining was performed to detect (micro-)thrombi and (micro-)vessels. CD31, also known as platelet endothelial adhesion molecule-1, is present on the surface of all platelets and endothelial cells. For both treatment arms, microthrombi and the number of vessels and thrombi per mm² were calculated. Due to logistic reasons, a random selection of 8 animals was chosen for counting microvessels and microthrombi. These were counted in both the infarct core as well as the border zone, each in four randomly selected circular sections (surface 31,416 μm²) per animal. Using section size and average number of counted microvessels and microthrombi per section, the number of vessels and thrombi per mm² was calculated. To assess inflammatory involvement in ischemia-reperfusion injury, myocardial tissue was stained for MPO, which is present in neutrophil granulocytes and for CD45, a leukocyte specific transmembrane glycoprotein. Per myocardium, three areas were chosen: infarcted tissue, border zone, and non-affected tissue (“normal”). Per area, three random microscopic photographs were taken with a 40x objective and assessed by two independent researchers. The mean of their quantification of respectively neutrophil granulocytes as stained with MPO and leukocytes as detected with CD45 was then compared between both treatment groups.

Laboratory measurements performed in the patient and porcine study

The assays Troponin T, RBC and platelet count in the porcine study were the only ones performed almost immediately on fresh blood samples. For all other assays measured in both studies, blood was collected in citrate tubes, centrifuged at 15 ºC for 20 minutes at 1700 g, and platelet poor plasma was then prepared and centrifuged at the same temperature for 15 minutes at 2000 g and frozen at -80 ºC before processing. VWF factor activity (Innovance VWF Ac) was determined on a Behring Coagulation System according to protocols from the manufacturer.
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(Siemens Healthcare Diagnostics, Marburg, Germany). ADAMTS13 activity was determined as described earlier. In this assay, barium-activated ADAMTS13 acts on urea-treated VWF concentrate (Haemate P) resulting in a decreased VWF activity. Normal human pooled plasma was used as standard. This material was calibrated to WHO 07/316 6th International Standard for VWF activity, and was arbitrarily assigned 100% for ADAMTS13 activity. VWF antigen and VWF propeptide levels were measured by ELISA using commercial antibodies (DAKO, Denmark and Sanquin, The Netherlands, respectively). D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-Dimer, Siemens Healthcare Diagnostics). Fibrinogen concentration was derived from the change in optical signal during prothrombin time determination. Fibrinogen antigen was determined by ELISA using antibodies from DAKO (Glostrup, Denmark). The ability of recombinant ADAMTS13 to cleave porcine VWF was determined in vitro by measuring porcine VWF activity in plasma of animals before and after addition of rADAMTS13, in increasing concentrations. For this purpose, baseline samples were used. Human pooled plasma underwent the same procedure for validation. In the test, porcine plasma was incubated with urea, and ADAMTS13 (in plasma or recombinant) was incubated with barium chloride after which VWF activity was determined as described above.

RESULTS

Porcine model

Cardiac enzymes
The AUC of cardiac enzyme troponin T was similar for both groups (rADAMTS13 vs. controls: 51.6 μg/L, IQR 42.1-77.3 vs. control group: 53.2, 39.6-86.8; p= 0.85).

Continuous twelve leads electrocardiograms
Thirty minutes after reperfusion, 1 pig in the rADAMTS13 group had 70% or more ST-segment recovery versus 2 animals in the control group (rADAMTS13 vs. controls: 55.8%, IQR 19.2-67.8 vs. 61.0, 53.2-68.3; p= 0.40). One hour after reperfusion, 9 animals had a recovery of ≥ 70% in both groups (rADAMTS13 vs. controls: 83.9%, IQR 71.7-96.1 vs. 72.8, 69.4-92.5; p= 0.22).

Echocardiographic analysis
Echocardiographic analysis was available for 18 animals (rADAMTS13 n=8 vs. control n=10) due to logistic reasons. Since 2 animals in the vehicle arm died within 24 hours after the intervention, final echocardiography was performed in 16 animals. Because of the small number of animals, per parameter deltas between all three measurements (echocardiograms 1, 2 and 3) were calculated per pig. See also supplementary table 1.
Echocardiographic results for rADAMST13 treated animals and controls after 1 week (delta 3-1), the response measured immediately after reperfusion (delta 2-1), and between second and final echocardiography (delta 3-2). Results are presented as median and IQR. *Considering the multiple testing for different diagnostic observations, this result (p= 0.02) was considered to be due to chance. There were no other significant p-values. WMSI = wall motion score index, LVEF = left ventricular ejection fraction, FAC = fractional area of change.

**Supplementary Table 1.** Echocardiographic results from the porcine ischemia-reperfusion model.

<table>
<thead>
<tr>
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<th>Delta 3-1</th>
<th>Delta 2-1</th>
<th>Delta 3-2</th>
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<td>WMSI(-)</td>
<td>LVEF(-)</td>
<td>FAC(%)</td>
</tr>
<tr>
<td></td>
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<td>[2.2, -0.32 to 0.00]</td>
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<tr>
<td>p value</td>
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Delta 3-1 to Delta 2-1, and between second and final echocardiography (Delta 3-2). Results are presented as median and IQR. Considering the multiple testing for different diagnostic observations, this result (p= 0.02) was considered to be due to chance. There were no other significant p-values.
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REFERENCES


