The extrinsic coagulation pathway in coronary artery disease and endotoxemia
Moons, A.H.M.

Citation for published version (APA):
Inhibition of the tissue factor pathway of coagulation by recombinant nematode anticoagulant protein c2 in patients undergoing elective coronary stent implantation: effects on hemostatic and inflammatory parameters

Arno H.M. Moons¹, Nick R. Bijsterveld¹, Joost C.M. Meijers², Jan G.P. Tijssen¹, Tom van der Poll³, Harry R. Büller², Ron J.G. Peters¹

¹Department of Cardiology, ²Vascular Medicine, and ³Laboratory of Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, The Netherlands
Chapter 5

Abstract

Background Exposure of tissue factor (TF) to the circulation during coronary stent implantation initiates coagulation activation and may contribute to the risk of thrombotic complications. In this study, we investigated whether inhibition of TF-factor VIIa by recombinant Nematode Anticoagulant Protein c2 (rNAPc2) is able to suppress hemostatic and inflammatory activity in patients undergoing elective intracoronary stenting.

Methods In a randomized, double blind design 102 patients received either placebo or rNAPc2 (biological half-life >50 hours) at doses of 3.5, 5.0, 7.5, and 10.0 μg/kg as a single subcutaneous administration 2-6 hours before angioplasty. All patients also received aspirin, clopidogrel, and unfractionated heparin (activated clotting time >250 seconds during angioplasty). Serial blood samples were collected before and after the intervention.

Results At 30 hours after stenting, all rNAPc2 treatment groups but not the placebo group demonstrated a reduction from baseline of prothrombin fragment F_{1+2} and D-dimer plasma levels (to 23% and 12% below baseline values at the highest dose, respectively), which were significantly lower in three rNAPc2 groups compared to placebo (p≤0.03). TF plasma levels were initially reduced in all rNAPc2 groups and returned to baseline values 18 hours after stent implantation. In the placebo group only, these three markers increased to above baseline values. Levels of P-selectin, antithrombin III, and interleukin-8 were not or only slightly affected by the intervention or by rNAPc2, whereas a significant 2.8 to 4.1 fold increase of C-reactive protein plasma levels was found in all patient groups after the procedure.

Conclusion In contrast to the inflammatory response, coagulation activation after elective coronary stent implantation, that is observed in spite of the use of multiple antithrombotic drugs, can be attenuated by inhibition of the TF-factor VIIa complex using rNAPc2. Inhibition of the TF mediated pathway of coagulation may be an important target to prevent thrombotic complications after coronary stenting.
**Introduction**

Intracoronary stents are thrombogenic, presenting a risk of thrombotic occlusion within the first weeks after placement\(^1^,\(^2\). Current treatment to prevent stent thrombosis consists of clopidogrel plus aspirin. The addition of glycoprotein (GP) I\(\text{Ib}/\text{IIa}\) receptor antagonists further reduces the risk of stent thrombosis\(^3^,\(^4\). This suggests that platelet aggregation plays an important role in thrombotic complications after intracoronary stenting. However, increased thrombin generation is also observed after percutaneous coronary intervention (PCI) with or without stent implantation\(^5^,\(^6\). As a significant component of atherosclerotic plaques\(^7\), tissue factor (TF) is thought to be a trigger of thrombosis following plaque rupture, both spontaneously and during PCI\(^8\). Inhibition of the extrinsic (TF-dependent) coagulation pathway markedly attenuates fibrin and thrombus formation after arterial balloon injury in animal models\(^9^,\(^10\). Tissue factor or the TF-factor VIIa complex have also been shown to stimulate acute inflammatory responses\(^11^,\(^12\), which may contribute to complications after PCI such as restenosis.

A novel specific inhibitor of the extrinsic coagulation pathway is recombinant nematode anticoagulant protein c2 (rNAPc2), an 85 amino acid protein that was originally isolated from the hematophagous hookworm *Ancylostoma caninum*\(^13\). Recombinant NAPc2 inhibits the complex TF-factor VIIa by a mechanism of action that requires initial binding to zymogen or activated factor X before formation of the final quaternary inhibitory complex with TF-factor VIIa\(^14\). The formation of a complex between rNAPc2 and factor X results in a biologic half-life of more than 50 hours. Because rNAPc2 binds to factor X at a site distinct from its catalytic center, it does not require activated factor X for its biological effect, which implies that rNAPc2 can form a complex with circulating factor X and rapidly inhibit TF-factor VIIa following a thrombogenic challenge. Recombinant NAPc2 has been shown to be very effective in reducing the incidence of acute deep venous thrombosis, without hemostatic compromise, when administered prophylactically in patients undergoing knee arthroplasty\(^15\).

We previously investigated the safety and pharmacodynamics of escalating doses of rNAPc2 in patients undergoing elective PCI with or without stent implantation. That study showed in patients receiving rNAPc2, dosed up to 7.5 \(\mu\)g/kg, in combination with aspirin, clopidogrel and unfractionated heparin, significant inhibition of thrombin generation without increasing bleeding complications compared to placebo treated patients. Here, we present in detail the findings on coagulation and inflammation in the subgroup of patients that underwent intracoronary stenting.
Chapter 5

Methods

Patients

In 5 centers, 154 patients with a history of stable angina, underwent elective PCI of 1 or 2 lesions of >50% diameter stenosis, and were randomized to one of four doses of rNAPc2 or placebo. Exclusion criteria were: total occlusion, unstable angina or myocardial infarction within 14 days, renal or hepatic insufficiency, and treatment with or intended use of antithrombotic or antiplatelet agents other than heparin, aspirin and clopidogrel during study period. In the present analysis, we included only those patients who underwent stent implantation. The study was carried out according to the principles of the Declaration of Helsinki and approved by the institutional review boards. Written informed consent was obtained from all patients.

Study design

Following randomization, patients received a single subcutaneous dose of rNAPc2 or placebo 2-6 hours prior to PCI. After access to the femoral artery, unfractionated heparin (UFH) was administered as intravenous bolus injections to reach an activated clotting time of >250 seconds throughout the intervention. Four doses of rNAPc2 were used: 3.5, 5.0, 7.5, and 10.0 μg/kg bodyweight. All patients received aspirin (at least 80 mg daily) throughout the study. A loading dose of 300 mg clopidogrel was administered either within 1 day before or immediately after the intervention, followed by 75 mg daily for at least 3 weeks. The follow up period was 2 weeks after PCI.

Blood collection and assays

Blood samples were taken from each patient before the administration of study drug, after access to the femoral artery but before the angioplasty, at 2 hours after the last bolus administration of UFH, and at 24 and at 36 hours after study drug administration. Blood was collected both in citrated and EDTA containing vacutainer tubes, and centrifuged at 4 °C for 15 minutes at 1600g immediately after collection. Then, separated plasma was transferred to a clean tube and spun for another 5 minutes at the same temperature and speed. Plasma samples were stored at -70 °C until assayed. Enzyme-linked immunosorbent assays (ELISA’s) were used to quantify the following markers: prothrombin activation fragment F$_{1\ 2}$ (F$_{1\ 2}$, Dade Behring, Marburg, Germany), fibrin degradation products (D-dimer, Roche Diagnostics, Mannheim, Germany), soluble TF (American Diagnostica, Greenwich, CT, USA), P-selectin (R&D Systems, Minneapolis, MN, USA), and IL-8 (Central Laboratory of the Netherlands Red Cross Blood Transfusion, Amsterdam, The Netherlands). Levels of
antithrombin (AT) III activity were determined by a chromogenic activity assay and calibrated using Standard Human Plasma (Dade Behring, Marburg, Germany). An immunoturbidimetric assay was used to quantify C-reactive protein (CRP) plasma levels (Roche Diagnostics, Mannheim, Germany). All plasma markers were analysed until the last sampling time point at 36 hours except for soluble TF and P-selectin, which were analysed until 24 hours after study drug administration due to limited plasma samples.

Statistical analysis
Descriptive statistics included mean values and standard deviation (SD)/standard errors of the mean (SEM), or median values and interquartile ranges (IQR), for outcomes with or without a normal distribution, respectively. Due to wide inter-individual variations, plasma levels of each biochemical marker were analyzed as a percentage of their baseline value. A p-value <0.05 was considered significant.

Inter-group comparisons between each rNAPc2 dose group and the placebo group were tested for all study measurements. Comparison of baseline and procedural characteristics was by χ² analysis for categorical variables and either the unpaired Student’s t test or Mann-Whitney U test for continuous variables with or without a normal distribution, respectively. All biochemical markers were analyzed by repeated measurement analysis. The treatment groups were compared for each time point separately by means of one-way analyses of variance (ANOVA) and subsequent pairwise comparisons with placebo, using Dunnett’s multiple comparison test.

To study the effect of intracoronary stenting on marker plasma levels, intra-group comparisons between the measurements immediately pre-procedure (at femoral access) and either at 24 and at 36 hours were performed by paired Student’s t-tests.

Results
Patients
Out of 154 patients, 110 received a stent. Of these 110 patients, 8 patients were excluded because PCI was performed 24 hours after study drug administration (n=1), a diagnosis of unstable angina prior to PCI (n=1), glycoprotein IIb/IIIa receptor inhibitor or UFH administration after the procedure (n=5), or the first dose of clopidogrel was administered 1 day after PCI (n=1). Baseline and procedural characteristics of the remaining 102 patients are shown in Table 1. The mean time between study drug administration and access to the femoral artery was 3.4 ± 1.4 hours.
Table 1. Baseline and Procedural characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (N=20)</th>
<th>3.5 (N=28)</th>
<th>5.0 (N=16)</th>
<th>7.5 (N=21)</th>
<th>10.0 (N=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y): mean ± SD</td>
<td>59 ± 8</td>
<td>60 ± 9</td>
<td>59 ± 13</td>
<td>61 ± 8</td>
<td>58 ± 10</td>
</tr>
<tr>
<td>Male gender</td>
<td>70%</td>
<td>93%‡</td>
<td>94%</td>
<td>67%</td>
<td>82%</td>
</tr>
<tr>
<td>Previous MI: N (%)</td>
<td>4 (20)</td>
<td>8 (29)</td>
<td>4 (25)</td>
<td>5 (24)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Previous PCI: N (%)</td>
<td>2 (10)</td>
<td>7 (25)</td>
<td>0 (0)</td>
<td>6 (29)</td>
<td>5 (29)</td>
</tr>
<tr>
<td>Previous CABG: N (%)</td>
<td>0 (0)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td>3 (14)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1-vessel disease: N (%)</td>
<td>12 (60)</td>
<td>15 (54)</td>
<td>4 (25)‡</td>
<td>13 (62)</td>
<td>9 (53)</td>
</tr>
<tr>
<td>2-vessel disease: N (%)</td>
<td>5 (25)</td>
<td>9 (32)</td>
<td>10 (63)‡</td>
<td>5 (24)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>3-vessel disease: N (%)</td>
<td>3 (15)</td>
<td>4 (14)</td>
<td>2 (12)</td>
<td>3 (14)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²): mean ± SD</td>
<td>28 ± 4</td>
<td>28 ± 3</td>
<td>28 ± 3</td>
<td>26 ± 3</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Hypertension: N (%)</td>
<td>4 (20)</td>
<td>6 (21)</td>
<td>3 (19)</td>
<td>4 (19)</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Hypercholesterolemia: N (%)</td>
<td>6 (30)</td>
<td>11 (39)</td>
<td>5 (31)</td>
<td>7 (33)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>Diabetes: N (%)</td>
<td>2 (10)</td>
<td>1 (4)</td>
<td>0 (0)</td>
<td>3 (14)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Duration PCI (min): median (IQR)‡</td>
<td>13 (7-44)</td>
<td>25 (13-44)</td>
<td>22 (13-39)</td>
<td>11 (6-57)</td>
<td>25 (7-40)</td>
</tr>
<tr>
<td>Number of lesions per PCI</td>
<td>1.4</td>
<td>1.3</td>
<td>1.7</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>PCI site:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- RCA: N (%)</td>
<td>7 (35)</td>
<td>10 (36)</td>
<td>10 (63)‡</td>
<td>14 (67)‡</td>
<td>6 (35)</td>
</tr>
<tr>
<td>- LAD: N (%)</td>
<td>12 (60)</td>
<td>15 (54)</td>
<td>9 (56)</td>
<td>6 (29)‡</td>
<td>10 (59)</td>
</tr>
<tr>
<td>- LCx: N (%)</td>
<td>5 (25)</td>
<td>7 (25)</td>
<td>4 (25)</td>
<td>5 (24)</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Start clop. administration prior study drug: N (%)</td>
<td>5 (25)</td>
<td>3 (11)</td>
<td>8 (50)</td>
<td>5 (24)</td>
<td>3 (18)</td>
</tr>
</tbody>
</table>

‡p value vs placebo <0.05. ‡Period between first and last balloon inflation.
MI=myocardial infarction. PCI=percutaneous coronary intervention. CABG=coronary artery bypass grafting. RCA=right coronary artery. LAD=left anterior descending artery. LCx=left circumflex artery. Clop.=clopidogrel.

Hemostatic markers
$F_{1-2}$
As shown in Figure 1, $F_{1-2}$ plasma levels decreased in all rNAPc2 dose groups starting from study drug administration and continuing during the intervention. In the placebo group, these levels only decreased during the procedure, coincident with UFH administration. After PCI, there was continued suppression of $F_{1-2}$ plasma levels in all rNAPc2 dose groups, that differed significantly from the placebo group at 24 hours, and for three dose groups even at 36 hours following a single subcutaneous administration.
$F_{1-2}$ plasma levels remained below pre-treatment levels in all rNAPc2 groups but not in the placebo group. At 24 hours, levels in the 4 rNAPc2 dose groups showed a decrease of 15% to 31%. The 24% and 23% reduction in the two highest dose groups (i.e. 7.5 and 10.0 µg/kg, respectively) at 36 hours was statistically significant compared to their pre-treatment values.
Figure 1. Mean changes (± SEM) of prothrombin activation fragment 1+2 (F1+2) plasma levels for the placebo and rNAPc2 dose groups, expressed as % of baseline.

rNAPc2 = recombinant Nematode Anticoagulant Protein c2. PCI = percutaneous coronary intervention. Statistical significant differences (p<0.05) were found between the following rNAPc2 dose groups and placebo: *3.5 - 10.0 vs placebo; **3.5, 7.5 and 10.0 vs placebo.

Figure 2. Mean changes (± SEM) of D-dimer plasma levels for the placebo and rNAPc2 dose groups, expressed as % of baseline.

rNAPc2 = recombinant Nematode Anticoagulant Protein c2. PCI = percutaneous coronary intervention. Statistical significant differences (p<0.05) were found between the following rNAPc2 dose groups and placebo: *3.5, 7.5, and 10.0 vs placebo; **3.5, 7.5 and 10.0 vs placebo.
D-dimer
D-dimer plasma levels significantly increased after PCI in the placebo group only, whereas both at 24 and at 36 hours, levels were significantly suppressed in three rNAPc2 dose groups compared to placebo (Figure 2). The rNAPc2 doses 7.5 and 10.0 mg/kg, demonstrated a 10% and 12% decrease, respectively, at 36 hours (p<0.05).

Soluble TF
From study drug administration until shortly after the intervention, all four rNAPc2 dose levels showed a reduction of soluble TF plasma levels varying between 9% and 16% , while these levels remained unchanged in the placebo group (Figure 3). After PCI at 24 hours, soluble TF levels returned to their baseline values in the rNAPc2 dose groups, whereas a significant increase was observed in the placebo group.

Figure 3. Mean changes (± SEM) of tissue factor (TF) plasma levels for the placebo and rNAPc2 dose groups, expressed as % of baseline.

rNAPc2=recombinant Nematode Anticoagulant Protein c2. PCI=percutaneous coronary intervention. Statistically significant differences (p<0.05) were found between the following rNAPc2 dose groups and placebo: *5.0 vs placebo; **5.0 and 7.5 vs placebo; ***3.5 vs placebo.

AT III
Recombinant NAPc2 did not affect AT III activity, showing similar changes among all dose groups as observed in the placebo group (Figure 4). A decrease of ± 6% was found during the procedure when all patients also received at least one intravenous bolus of UFH, and levels subsequently recovered to baseline values after the intervention at 36 hours.
rNAPc2 during coronary stent implantation

**Figure 4.** Mean changes (± SEM) of antithrombin (AT) III activity for all the placebo and rNAPc2 dose groups, expressed as % of baseline.

\( \text{rNAPc2}= \text{recombinant Nematode Anticoagulant Protein c2. PCI}= \text{percutaneous coronary intervention.} \)

**P-selectin**

Changes in platelet activation, as measured by plasma levels of P-selectin, were comparable between placebo and rNAPc2 dose groups (Figure 5). Except for the 5.0 \( \mu \text{g/kg} \) dose group, showing a significant decrease after PCI, all other patient groups demonstrated a minor decrease varying between 4% and 9% at 24 hours.

**Figure 5.** Mean changes (± SEM) of P-selectin plasma levels for the placebo and rNAPc2 dose groups, expressed as % of baseline.

\( \text{rNAPc2}= \text{recombinant Nematode Anticoagulant Protein c2. PCI}= \text{percutaneous coronary intervention.} \)
Inflammatory markers

The inflammatory response following PCI was studied by plasma levels of IL-8 and CRP. All measurements of IL-8 plasma levels resulted in low values just above its ELISA detection limit. Levels did not change after PCI in either patient group and were comparable between each rNAPc2 dose group and placebo (data not shown). In contrast, a significant 2.8 to 4.1 fold increase of CRP plasma levels was observed in all patient groups after the intervention, without significant differences between placebo and each rNAPc2 dose level (Figure 6).

![Figure 6. Mean changes (± SEM) of C-reactive protein (CRP) plasma levels for the placebo and rNAPc2 dose groups, expressed as % of baseline.](image)

rNAPc2=recombinant Nematode Anticoagulant Protein c2. PCI=percutaneous coronary intervention.

Discussion

The TF-factor VIIa complex plays a pivotal role in initiating the thrombotic response to vascular injury, which makes this enzymatic complex an attractive target for an antithrombotic strategy. However, there is little information about specific inhibition of TF in humans with arterial thrombotic diseases. We found in patients undergoing elective coronary stent implantation, that rNAPc2 in addition to aspirin, clopidogrel and UFH, significantly inhibited thrombin generation and reduced plasma levels of D-dimer and TF compared to placebo, without affecting the increased levels of CRP.

Large clinical trials have demonstrated the beneficial effects of platelet inhibition by GP IIb/IIIa receptor antagonists in combination with aspirin and a thienopyridine
derivative after urgent and elective coronary stenting\textsuperscript{3,4}. Still, approximately 5% of these patients experienced a major coronary event (i.e. death or myocardial infarction) with the majority of these events occurring in the first days following stent implantation\textsuperscript{3,4}. Besides platelet activation, the generation of thrombin likely plays an important role, and tissue factor may be a potent initiator of this process. In a study by Mizuno et al., a significant increase of thrombin generation was shown in patients with stable and unstable coronary disease at 24 hours after stenting, despite a 24 hours treatment with UFH on top of aspirin and ticlopidine\textsuperscript{6}. They also found significantly elevated plasma levels of TF and fibrinolytic markers such as plasminogen activator inhibitor-1 and tissue plasminogen activator. These results correspond with our observations in the placebo group, that received UFH during the intervention combined with aspirin and clopidogrel. After a temporary decrease at the time of PCI, increasing thrombin generation was observed as measured by $F_{\text{1-2}}$ plasma levels, to levels above pre-procedural up to 30 hours after stent implantation. This response was accompanied by elevated D-dimer plasma levels and increasing levels of circulating TF to values that also exceeded pre-PCI levels. In contrast, thrombin generation was significantly suppressed in the rNAPc2 treatment groups compared to placebo, which persisted to the last measurement at 36 hours after drug administration. This was associated with markedly reduced plasma levels of D-dimer. These data suggest that the activation of coagulation observed in the placebo group following the intervention can be effectively attenuated by rNAPc2.

We measured soluble TF in the first 24 hours only. Up to that time point a persistent suppression of plasma TF in the rNAPc2 treated patient groups was found. The source of plasma TF is not exactly known. Pathology studies have shown that simple balloon friction abrades the endothelium and that angioplasty results in plaque fracturing and dissection\textsuperscript{17,18}. Subsequently, procoagulant TF containing microparticles present in atherosclerotic lesions\textsuperscript{19} may be released into the circulation during the dilation procedure, thereby increasing levels of plasma TF with procoagulant activity, which has recently been suggested by Giesen et al.\textsuperscript{20} However, there are no available data about the relation between TF-positive microparticles and circulating coagulation markers. Therefore, it remains speculative that the suppression of thrombin generation observed in the rNAPc2 treatment groups is partly due to inhibition of plasma TF activity.

The effect of rNAPc2 on $F_{\text{1-2}}$, D-dimer and TF plasma levels did not show a clear dose-response. Possibly, the suppression of these markers was already maximal at the lowest dose (3.5 $\mu$g/kg).
Chapter 5

Antithrombin III plasma activity decreased slightly during the intervention, corresponding with the use of UFH. A slightly enhanced clearance of AT III from the circulation and the formation of thrombin-antithrombin complexes during the dilation procedures, both induced after binding of AT III to UFH, may have contributed to the observed decreased activity, and rNAPc2 did not affect this response.

Platelet activation, as measured by plasma levels of P-selectin in the first 24 hours, was not changed after PCI in the placebo group under concomitant treatment with aspirin, clopidogrel and UFH. Except for the 5.0 μg/kg dose showing a significant decrease, other rNAPc2 dose levels did not affect this marker. The trend to more clopidogrel use prior PCI observed in the 5.0 μg/kg dose group (Table 1), have contributed to the more pronounced suppression of P-selectin levels in this group compared to the other patient groups. Apparently, the dual antiplatelet treatment of aspirin and clopidogrel or ticlopidine, results in a marked inhibition of systemic platelet activation after stenting, which has been observed earlier in patients with stable and unstable angina. This was also studied by Mizuno et al. who measured plasma levels of platelet activation markers (i.e. β-thromboglobulin and platelet factor 4) in patients undergoing stent implantation and receiving aspirin, ticlopidin and UFH for 24 hours. They found no significant changes at 24 hours after stenting.

Interleukin-8 plasma levels were not affected by the intervention or by rNAPc2. However, levels of the acute phase reactant CRP, a sensitive but nonspecific marker of inflammation, significantly increased after PCI in all patient groups. Other studies did also find a significant increase in CRP plasma levels after stent implantation both in patients with stable and unstable angina. An association has been found between elevated CRP levels after coronary stenting and late clinical or angiographic restenosis. In addition, increased pre-procedural CRP levels are a prognostic indicator for early complications and for late clinical restenosis after PCI. Continuous anticoagulant treatment with UFH or the direct thrombin inhibitor argatroban after coronary angioplasty did not influence the intervention induced inflammatory response. We also observed no inhibitory effect of rNAPc2 on this response. This argues against a role of TF in the inflammatory response following arterial injury.
In conclusion, we observed that the activation of coagulation after coronary stent implantation can effectively be attenuated through inhibition of the TF-factor VIIa complex by rNAPc2. Thus, the TF mediated pathway of coagulation may contribute to thrombotic complications after stenting. We found no apparent influence of rNAPc2 on the inflammatory response following PCI.

Acknowledgements
We gratefully acknowledge George P. Vlasuk, PhD, and William E. Rote, PhD, of Corvas International, for their support of the study and their critical review of the manuscript. We also thank the personnel of the diagnostic laboratory of Vascular Medicine for their excellent technical assistance.
References
