Coagulation Factor Xa Stimulates Interleukin-8 Release in Endothelial Cells and Mononuclear Leukocytes

Implications in Acute Myocardial Infarction

Gabi Busch, Isabell Seitz, Birgit Steppich, Sibylle Hess, Robert Eckl, Albert Schömig, Ilka Ott

Objective—In acute myocardial infarction (AMI), proinflammatory plasma C-reactive protein values are strongly associated with postinfarction morbidity and mortality. So far, the cause of these inflammatory changes is not well understood. Therefore, we sought to investigate the relationship between the activation of coagulation and subsequent systemic inflammatory changes in AMI.

Methods and Results—Factor Xa (FXa) bound to tissue factor pathway inhibitor and prothrombin fragments F1+2 (F1+2) were used as a measure for activated coagulation. To assess systemic inflammatory changes, plasma interleukin (IL)-6 and IL-8 concentrations were analyzed by immunoassay. Blood samples were taken from 21 patients with AMI and 20 patients with stable angina pectoris. In AMI, tissue factor pathway inhibitor FXa but not F1+2 plasma levels were associated with circulating IL-8 (P=0.01). In vitro experiments revealed that FXa stimulated IL-8 and monocyte chemoattractant protein-1 release and RNA expression in endothelial cells and mononuclear leukocytes by activation of protease-activated receptor-1.

Conclusion—Our data suggest that coagulation FXa may contribute to proinflammatory changes in AMI by stimulation of IL-8 release. Therapeutic inhibition of the proinflammatory effects of FXa may improve the clinical course in AMI.

Key Words: coagulation • myocardial infarction • inflammation • cytokines

The main initiator of the extrinsic coagulation cascade is tissue factor (TF), the receptor and cofactor for plasma coagulation factor VII/VIIa. Under physiological conditions TF is mainly expressed at extravascular sites. However, TF is activated coagulation factors may enhance inflammatory responses and deteriorate infarct size.8

The endogenous Kunitz-type inhibitor tissue factor pathway inhibitor (TFPI)-1 inhibits initiation of TF-induced blood coagulation. TFPI binds and inactivates factor Xa (FXa). The TFPI–FXa complex then binds and inactivates FVIIa. Increased levels of the TFPI–FXa complex may reflect both increased FXa generation and increased TFPI concentrations.9 In addition to the full length TFPI, most of the plasma TFPI circulates in truncated forms that are bound to plasma lipoproteins. These truncated forms lack their C-terminal domains and exhibit reduced affinity for vascular wall proteolysis.

Binding of the serine protease FVII to TF results in generation of the coagulation protease FXa and subsequently thrombin, both known to induce cell signaling. FXa shows dose-dependent induction of intracellular calcium transients in endothelial cells that is active-site–dependent and –independent of thrombin.10 Potential pathophysiological responses to FXa include stimulation of proliferation and production of proinflammatory cytokines and prothrombotic TF.11 Genetic studies and receptor desensitization experiments indicate that signaling by FXa is mediated by the protease-activated receptors (PAR)-1 and PAR-2.12,13 Although PAR-2 does not seem to be activated by thrombin, the cellular effects of thrombin are mediated by PAR-1, PAR-3, and PAR-4. After enzymatic cleavage of PAR receptors, thrombin enhances cytokine release and adhesion molecule expression as well as chemotactic and proliferative responses.11

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Once activated, the inflammatory and coagulation pathways interact with one another. Inflammatory mediators induce TF expression on the surface of circulating monocytes and possibly endothelial cells. Conversely, serine proteases are capable of interacting with PARs on the surface of monocytes and endothelial cells, leading to activation and additional inflammation.

Recent evidence has demonstrated that inflammation plays an important role in the pathophysiology of acute coronary syndromes. A number of studies showed that levels of circulating C-reactive protein are associated with an increased risk of cardiovascular events. In experimental studies, increased C-reactive protein levels aggravate infarct size. The importance of systemic inflammatory changes for extension of the infarcted myocardium was further confirmed in clinical studies demonstrating that the inflammatory response is predictive for the amount of salvaged myocardium.

Moreover, inflammatory markers such as IL-6, IL-8, or MCP-1 are elevated in AMI and are predictive for recurrent plaque instability. In addition to inflammatory markers, activation of the coagulation cascade may also serve as a marker for an unfavorable outcome in acute coronary syndromes.

The interplay between activation of coagulation and systemic inflammatory changes in AMI is poorly understood. The aim of this study, therefore, was to investigate the relationship between systemic inflammatory changes and activation of the coagulation cascade in AMI to analyze the underlying mechanism in vitro.

### Methods

**Patient Selection**

The study group comprised 21 patients with AMI based on a history of prolonged ischemic chest pain and significant ST-segment elevation. From onset of symptoms to the first blood drawing was 2 to 8 hours. Twenty patients with stable angina undergoing elective stenting were included in the control group. Patients with interfering noncardiac diseases were excluded. The study was approved by the institutional ethics committee for human subjects. Informed consent was obtained from all patients.

In both groups, peri-interventional therapy consisted of abciximab (0.25 mg/kg bolus followed by continuous infusion, 10 μg/min) plus boluses of weight-adjusted heparin and 500 mg aspirin IV. Postinterventional therapy consisted of 250 ticlopidine bid and 100 mg aspirin bid throughout the study. Peripheral blood samples were obtained after application of heparin in the emergency room but before stenting, as well as 24 and 96 hours after stenting. All samples were put on ice and processed immediately.

**Immunoonnassays**

Concentrations of TFPI-Xa, F1+2, IL-6, IL-8, and MCP-1 were determined by immunoonnassays (Immunobind TFPI-Xa, American Diagnostica; Enzymost F1+2 micro Behring Diagnostica; and IL-6, IL-8, and MCP-1 Quantikine, R&D Systems). Detection limits were 0.1 nmol/L for TFPI-Xa, 0.04 nmol/L for F1+2, 7 pg/mL for IL-6, 31.2 pg/mL for IL-8, and 5 pg/mL for MCP-1. Intra-assay variabilities for the lower assay range were <10%.

**RNA Preparation and Quantitative Polymerase Chain Reaction**

Total RNA was extracted from HUVECs according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). One μg of total RNA was reverse transcribed using Omniscript Reverse Transcriptase (Omniscript RT Kit, Qiagen). Quantitative polymerase chain reaction (PCR) of 150 ng cDNA was performed in a final volume of 25 μL. The PCR mixture contained 1× TaqMan Universal PCR Master Mix, 900 nmol/L of each primer, and 250 nmol/L probe corresponding to 1× Assay-on-Demand (Applied Biosystems). Assay-on-Demand containing primers and 6-carboxy-fluorescein (FAM)–labeled probes for IL-6 (Hs00174313_m1), IL-8 (Hs00174103_m1), PAR-1 (Hs0169258_ml), PAR-2 (Hs00173741_m1), and GAPDH (Hs99999905_ml) were purchased from Applied Biosystems. The PCR protocol included 2 minutes at 50°C and 10 minutes at 95°C for enzyme activation, then 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Real-time fluorescence detection was performed and analyzed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Fold increase was calculated by normalization on GAPDH.

**Other Methods**

Serum creatine kinase concentrations were determined in the clinical chemistry laboratory. No endotoxin contamination of leukocyte suspensions or buffers was detected (E-toxate, Sigma).

**Statistical Analysis**

The Kolmogorov–Smirnov test showed that the coagulation variables were not normally distributed. Differences between >2 matched samples were tested by Friedman test followed by Wilcoxon on matched-pairs signed-ranks test, and differences between the study group and the control group were tested by the Mann–Whitney–Wilcoxon rank sum test. Correlations of parameters were calculated using linear regression analysis. P<0.05 in the 2-tailed test was regarded as significant.

**Results**

**Clinical and Angiographic Data**

The study group did not differ significantly from the control group with respect to age, sex distribution, risk factor profile, medication, and target vessels (Table 1, available online at http://atvb.ahajournals.org)

**TFPI-Xa, F1+2, IL-6, and IL-8 Plasma Concentrations in AMI**

Plasma concentrations of TFPI-Xa were significantly increased in patients with AMI compared with the control group (Figure 1A) and significantly decreased thereafter (Figure I, available online at http://atvb.ahajournals.org).
Thrombin generation in vivo, assessed by prothrombin fragment F1+2, was elevated compared with the control group and increased over time even further (Figure I). In patients after elective stenting, no changes in plasma concentrations of prothrombin fragment F1+2 over time were observed (data not shown). Compared with the control group, in AMI, plasma levels of the proinflammatory cytokines IL-6 and IL-8 were significantly elevated (Figure 1B and 1C) and increased further up to 24 hours (Figure I), whereas no significant changes were observed in the control group. When we investigated the relationship of initial TFPI-Xa and F1+2 concentrations with IL-6 and IL-8 levels, we found an association of TFPI-Xa with IL-8 on admission (Figure 2). No correlation of TFPI-Xa with initial IL-6 levels was found ($P_{0.08}$, $R_{0.74}$). Yet subsequent IL-6 plasma concentrations were associated with the initial TFPI-Xa levels (Table II, available online at http://atvb.ahajournals.org). Contrary to the association of TFPI-Xa with proinflammatory cytokines, we did not detect an association between prothrombin fragment FI+2 and IL-6 or IL-8. Likewise, in the control group, no association between markers of coagulation or inflammatory responses was found (Table II).

**Effect of FVIIa and FXa on IL-8 and IL-6 RNA Levels and Release in HUVECs and Mononuclear Leukocytes**

Because plasma TFPI-Xa levels reflect activation of the coagulation cascade upstream of thrombin, we analyzed the effects of FVIIa and FXa on cytokine expression in mononuclear leukocytes and endothelial cells (HUVECs) that are in contact with the circulating blood and, therefore, may contribute to the observed inflammatory changes. In HUVECs, dose-dependent stimulation with FXa revealed a significant increase in the release of the proinflammatory cytokines IL-8, MCP-1, and IL-6. Furthermore, 2 hours after stimulation with FXa, mRNA expression was only observed for IL-8 and MCP-1 but not for IL-6 (Figure 3B, 3D, and 3F). The cells were serum-starved to exclude additional effects of contaminating serum proteases. In particular, activation of prothrombin by FXa could interfere with the observed effects of FXa. Specificity of the observed effect of FXa was investigated using the synthetic FXa inhibitor MC53255 (Figure 4). Dose-dependent inhibition of IL-8 release in endothelial cells by MC53255 occurred with an IC50 of 230 nmol/L (data not shown). In contrast, the observed IL-8 release was not due to thrombin generation, because in the presence of the thrombin inhibitor lepirudin FXa, induced IL-8 release remained unchanged (Figure 4). Similar results were obtained in mononuclear leukocytes. FXa induced the release of MCP-1, IL-8, and IL-6 protein; however, RNA levels were only induced of IL-8 and IL-6 (data not shown).

FXa is known to stimulate cells by activation of PARs. To further investigate the different responses of FXa in mononuclear leukocytes and endothelial cells, we measured PAR-1 and PAR-2 RNA levels by quantitative PCR. Analysis revealed a 4-fold increased expression of PAR-1 in HUVECs.
compared with mononuclear cells (40,000 and 10,000 molecules per 150 ng RNA). PAR-2 expression was low in HUVECs (200 molecules per 150 ng RNA), and only minimal amounts of PAR-2 mRNA were detected in mononuclear cells (8 molecules per 150 ng RNA). It is known that FXa stimulates cells by activation of PAR-1 and PAR-2. We therefore measured the effects of PAR-1 and PAR-2 activation on cytokine release in endothelial cells using the agonistic peptides SFLLRN and SLIGKV.

In endothelial cells, FXa induced a 1.8- to 2.2-fold increase in IL-8 and MCP-1 secretion (Figure IIA and IIC, available online at http://atvb.ahajournals.org). Similarly, addition of SFLLRN induced a 2.7- to 4.9-fold increase in IL-8 and MCP-1 release, and SLIGKV stimulated cytokine release 1.6-fold (Figure IIB and IID). In mononuclear leukocytes, stimulation with SFLLRN induced a 4- to 10-fold increase in IL-8 and MCP-1, whereas PAR-2 agonists did not show any effect (data not shown). The peptide YFLLRNP that inhibits mainly PAR-2 activation had no effect on IL-8 release, whereas inhibition with anti–PAR-1 antibody APAP2 abolished the effect of FXa on cytokine release in endothelial cells (Figure 4). Similar results were found in mononuclear cells where FXa induced increase in IL-8 from 2 ± 0.2 pg/mL to 22 ± 0.5 pg/mL was decreased to 4.5 ± 0.9 pg/mL in the presence of APAP2 or to 2 ± 0.6 pg/mL in the presence of MC53255. No significant changes by FXa were found in the presence of hirudin (1.3 ± 0.2 pg/mL) or PAR-2 inhibition (1.2 ± 0.3 pg/mL), whereas inhibitors alone showed no effect on cytokine release. These results suggest that mainly activation of PAR-1 mediates IL-8 and MCP-1 release in response to FXa. Moreover, in mononuclear cells, cytokine release was only induced by activation of PAR-1. This may reflect the very low PAR-2 receptor expression on monocytes and suggests that PAR-1 activation is sufficient to mediate the proinflammatory effect of FXa.

Discussion

Major findings of our study are as follows: (1) In patients with AMI, elevated levels of TFPI-Xa but not F1+2 are associated with increased plasma concentrations of IL-8. (2) In vitro experiments revealed that FXa stimulates IL-8 and MCP-1 transcription in endothelial cells and mononuclear leukocytes. (3) According to the expression of PAR-1 and PAR-2, PAR-1 and PAR-2 agonists induce IL-8 and MCP-1 release in endothelial cells, whereas only PAR-1 agonists stimulated cytokine release in mononuclear cells.

Elevated TFPI-FXa and prothrombin fragments F1+2 plasma levels indicate activation of the coagulation cascade in AMI. Under physiological conditions, an inverse relationship between TFPI-FXa and F1+2 suggests that TFPI-FXa regulates prothrombinase activity in vivo. Under conditions associated with activation of the coagulation cascade, however, increased TFPI-Xa plasma levels occur. Activation of coagulation as measured by TFPI-FXa but not by F1+2 is associated with plasma concentrations of the proinflammatory cytokine IL-8 in AMI. Furthermore, subsequent IL-6 levels in the course of AMI are associated with initial TFPI-FXa concentrations. These results give rise to the assumption of a proinflammatory role of FXa in AMI that is independent of thrombin. Although thrombin exerts proinflammatory effects similar to FXa in vitro (data not shown), the effects of thrombin may be diminished after heparin treatment in vivo. That FXa only enhances cytokine release at concentrations higher than those in circulating blood may reflect that proinflammatory effects of FXa occur within the myocardial ischemic microcirculation. In addition, stress-related steroid release and adrenergic receptor stimulation may alter cytokine release in AMI.

Activated vascular endothelial cells are known to contribute to the development of inflammatory responses by secreting proinflammatory cytokines and chemokines including IL-8. IL-8 has been associated with postischemic reperfusion injury and increased risk of systemic inflammatory response syndrome. Because proinflammatory changes in acute coronary syndromes appear to be a determinant of prognosis, understanding the causes of inflammation may facilitate the development of new beneficial therapeutic strategies. So far, antiinflammatory treatments displayed no unequivocal benefit in patients with AMI. There is some evidence, however, that treatment with FXa inhibitors proves to be superior to thrombin inhibitors. In particular, treatment with low-molecular-weight heparins, which possess
additional anti-FXa activity as compared with unfractionated heparin, has been shown to decrease inflammatory changes in vitro and in vivo. Our study identifies the protease FXa as an inflammatory mediator in AMI using mechanisms other than thrombin. Thus, FXa inhibitors in AMI may exhibit antiinflammatory as well as anticoagulant applications. Experimental studies have shown that anticoagulant treatment not only diminishes activation of coagulation but also inhibits inflammation, and the studies support the concept of an interplay between activation of coagulation and cytokine release in vivo. Genetic studies have shown that PARs are required for FXa signaling. In endothelial cells, the actions of FXa are mediated by PAR-1 and PAR-2. The association of TFPI-FXa but not prothrombin fragment F1+2 with IL-8 in patients with AMI suggests that FXa but not thrombin contributes to the acute proinflammatory changes in AMI. Further evidence for the importance of PAR-1 and PAR-2 activation for cytokine release in endothelial cells and PAR-1 activation in mononuclear cells is derived from in vitro experiments with agonistic peptides. Yet in these experiments, the role of PAR-2 may be underestimated because, in contrast to PAR-1, PAR-2 expression is enhanced after stimulation with cytokines, and PAR-2 receptors are upregulated in atherosclerotic vessels.

Differences in the patterns of RNA and protein expression in mononuclear cells and HUVECs might reflect cell type–specific activation patterns. They may also occur as a result of the isolation procedure in mononuclear cells or due to different culture conditions.

In patients with AMI, endothelial and leukocyte activation occurs within the reperfused myocardium and might contribute to systemic inflammatory changes. Because intracoronary thrombus formation precedes the development of myocardial infarction, activated coagulation factors may alter endothelial as well as leukocyte activation. Stimulation of PAR-1 and PAR-2 within the infarct area might, therefore, link generation of FXa to inflammatory cytokine release. This may be the underlying mechanism for the observed association of circulating TFPI-FXa and IL-8 in AMI. Anticoagulant therapy using anti-VIIa or anti-FXa strategies may be superior to thrombin inhibition, because they block generation of thrombin and abolish activation of PARs. Thereby, they possess additional antiinflammatory actions as compared with thrombin inhibitors or heparin alone.

Our study provides evidence for activation of coagulation in AMI and identifies a mechanism that shows how coagulation FXa may enhance systemic IL-8 concentrations. In vitro, FXa induces release of the proinflammatory mediators IL-6, IL-8, and MCP-1 in endothelial and mononuclear cells. Extending previous studies, we identified transcriptional activation of IL-8 and MCP-1. The mechanisms of IL-6 release warrant further investigation.

In studies investigating the cellular effects of separate coagulation factors, there is always concern about the activation of interfering proteases. Therefore, we performed in vitro experiments in the absence of serum. Because stimulation of IL-8 release by FXa was abolished only in the presence of a FXa inhibitor but not a thrombin inhibitor, specificity of the observed effect is suggested. As inflammatory responses contribute to the clinical outcome in patients with AMI, inhibition of the coagulation cascade upstream of thrombin might permit an improved outcome by inhibition of proinflammatory mediator release through activation of PARs.

**Acknowledgments**

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**References**


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Supplementary Figure I: Circulating cytokines and coagulation parameters before and after stenting in acute myocardial infarction.

Serial changes in plasma IL-8 (A), IL-6 (B), TFPI-FXa (C) or F1+2 (D) concentrations in patients with acute myocardial infarction. Values are expressed as mean±SEM. *P<0.05 compared to the values before stenting.
Supplementary Figure II: Effect of FXa, PAR-1 and PAR-2 activation on IL-8 and MCP-1 release in HUVEC. HUVEC were stimulated with FXa (A, C), PAR-1 agonistic peptide SFLLRN (closed circles) or PAR-2 agonistic peptide SLIGKV (open circles) as indicated (B, D). After incubation for 18 hours IL-8 (A,B) or MCP-1 (D,C) release was analyzed in cellular supernatants by immunoassay. Shown are mean±SEM of 4 independent experiments. * indicates p<0.05 compared to unstimulated values.
# SUPPLEMENTARY TABLE I

## Baseline Characteristics of Study Patients

<table>
<thead>
<tr>
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<th>Stable Angina (n=20)</th>
<th>Acute Myocardial Infarction (n=21)</th>
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<tr>
<td>gender (F/M)</td>
<td>4/16</td>
<td>4/17</td>
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<td>5 (23)</td>
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<td>Hypercholesterolemia, n (%)</td>
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<tr>
<td>Systemic hypertension, n (%)</td>
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<td>14 (61)</td>
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<td>Diabetes mellitus, n (%)</td>
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<td>10 (48)</td>
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<tr>
<td>1-vessel disease</td>
<td>6 (30)</td>
<td>9 (43)</td>
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<tr>
<td>Target vessel:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>8 (40)</td>
<td>12 (57)</td>
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<tr>
<td>LCx</td>
<td>7 (35)</td>
<td>4 (19)</td>
<td>0.2</td>
</tr>
<tr>
<td>RCA</td>
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<td>5 (24)</td>
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<tr>
<td>peak CK, U/L (range)</td>
<td>&lt;80</td>
<td>1277 (230-5962)</td>
<td>&lt;0.01</td>
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CK indicates creatine kinase. LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; RCA, right coronary artery.
SUPPLEMENTARY TABLE II

Correlation between IL-6 and IL-8 with TFPI-FXa (A) or F1+2 (B): Shown are the p values of the correlations that were calculated using linear regression analysis.

### A

<table>
<thead>
<tr>
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<th>TFPI-FXa before stenting</th>
<th>TFPI-FXa 24 hrs after stenting</th>
<th>TFPI-FXa 96 hours after stenting</th>
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<td>n.s</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-8 96 hours after stenting</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-6 before stenting</td>
<td>n.s.</td>
<td>n.s</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-6 24 hours after stenting</td>
<td>0.01</td>
<td>n.s</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-6 96 hours after stenting</td>
<td>n.s</td>
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n.s. not significant

### B

<table>
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<th>F1+2 96 hours after stenting</th>
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<td>n.s</td>
<td>n.s.</td>
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<tr>
<td>IL-8 24 hours after stenting</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s.</td>
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<tr>
<td>IL-8 96 hours after stenting</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s.</td>
</tr>
<tr>
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<td>n.s</td>
<td>n.s.</td>
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<tr>
<td>IL-6 96 hours after stenting</td>
<td>n.s</td>
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n.s. not significant