Atherosclerosis is the underlying cause of ≈50% of all deaths in developed countries.1 An important reason for this chronic inflammatory disorder to become life threatening is thrombus formation on ruptured atherosclerotic plaques, resulting in myocardial infarction, peripheral artery disease, and stroke. Primary treatment of these disorders is with antiplatelet (eg, aspirin and clopidogrel) and anticoagulant drugs (eg, warfarin, heparins, factor Xa, and thrombin inhibitors).2 These drugs are undeniably good in terms of effectiveness but have serious side effects of which the most pronounced is with antiplatelet therapy.3 To overcome these bleeding problems, coagulation factor XI has been proposed as alternative target for anticoagulation4−7 because factor XI is associated with thrombosis, whereas it has a relatively minor role during normal hemostasis. This means that normal clot formation is still preserved in the absence of factor XI, while thrombosis is prevented. For example, inhibition of factor XI protects rodents and primates from experimentally induced thrombosis in both arteries and veins.8−12 Another argument for factor XI as antithrombotic target is the epidemiological evidence that patients with factor XI deficiency have a lower incidence of deep vein thrombosis and ischemic stroke.13,14

Till date, little is known about the role of factor XI inhibition in atherosclerosis and subsequent atherothrombosis. This is a important extension because atherosclerotic plaques contain many components and cells with a thrombogenic potential beyond the coagulation cascade, such as oxidized low-density lipoproteins, lysophosphatidic acid, and macrophages.15,16 In addition, tissue factor (TF) and collagen are important determinants of thrombus formation on ruptured plaques.17 The question is whether factor XI inhibition is capable to overcome this prothrombotic environment. Because coagulation is not dependent on factor XI when high concentrations of TF are present,17,18 we questioned whether factor XI inhibition was still effective to prevent atherothrombosis.
Furthermore, atherothrombotic disorders, such as myocardial infarction and stroke, are diseases of the elderly and occur in patients with pronounced atherosclerotic vessels. In contrast, many animal experiments have been performed in young (4–8 weeks) mice with healthy nonatherosclerotic vessels. In this study, we investigated the antithrombotic potential of factor XI antisense oligonucleotides in a model of plaque rupture, which is sensitive to both antplatelet and antithrombotic therapy, in older (18–20 weeks) mice with pronounced atherosclerotic vessels.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Factor XI Inhibition Affects Thrombus Formation on Ruptured Plaques
The role of factor XI in mouse models has only been established in artificially damaged healthy vessels. Here, we studied the role of factor XI in thrombus formation after acute rupture of an atherosclerotic plaque in diseased vessels. Factor XI inhibition was accomplished by injection of factor XI antisense oligonucleotides (50 mg/kg) in Apoe−/− mice fed with a high-fat diet to produce sufficient atherosclerosis.

Three injections with factor XI antisense oligonucleotides significantly reduced plasma factor XI activity levels to 20% (Figure 1A). Because the factor XI levels in the control group showed some variation, we normalized these levels for prothrombin. As depicted in Figure 1B, prothrombin levels were not significantly different between groups. Subsequently, we calculated the mean factor XI/prothrombin ratio for both controls (0.36) and factor XI antisense (0.13)–treated animals. By dividing these ratios, we found that factor XI levels were 2.8× lower in the plasma of factor XI antisense–treated animals than in the control group, corresponding to a 64% reduction of factor XI plasma levels.

Initially, in vivo thrombus formation after plaque rupture occurred similarly in both control Apoe−/− mice and Apoe−/− mice pretreated with factor XI antisense oligonucleotides (Figure 2A and 2B; Movies I and II in the online-only Data Supplement). However, pretreatment with factor XI antisense oligonucleotides reduced thrombus propagation and fibrin deposition, an effect that became significant 5 minutes after plaque rupture and continued for 10 minutes (Figure 2A and 2B). Furthermore, the platelet aggregates seemed to be less stable than the thrombi formed in animals treated with non–sense oligonucleotides as depicted by the number of platelets shed from the initial thrombus (Figure 2C). This indicates that inhibition of factor XI results in less stable clots, which should be a local event because systemic dimer levels were not different between groups (data not shown). Treatment with factor XI antisense oligonucleotides is safe in mice because the bleeding time was unaffected by the oligonucleotides (Figure 2D).

Reduced Thrombus Formation Is Partly Because of a Less Severe Inflammatory Response
To study the thrombi and plaques after the rupturing procedure in more detail, we poststained the carotid arteries and subjected these to immunohistological analysis. Thrombus phenotype characteristics were determined by staining the sections for fibrin, MAC-3 (macrophage infiltration), Ly-6G (neutrophil recruitment), and with Sirus red (collagen) and hematoxylin and eosin. In the arteries of control Apoe−/− mice, thrombi contained many erythrocytes as a major component, which is visible in control animals and not in the factor XI antisense–treated animals.

Finally, to confirm that the thrombi are formed on a ruptured atherosclerotic plaque, we visualized the fibrous cap with subsequent thrombus formation was only visible in control animals and not in the factor XI antisense–treated animals.

The (innate) immune system contributes to the initiation and amplification of venous thrombosis in mice. Especially, monocytes and neutrophils seem to be responsible for...
thrombus formation. Therefore, we stained the carotid arteries for the presence of macrophages and neutrophils to investigate whether these cells are also involved during thrombus formation on ruptured atherosclerotic plaques. As shown in Figure 3F and 3G, there was a higher degree of macrophage infiltration in thrombi from placebo-treated animals than in factor XI antisense–treated animals (18.5±1.1% versus 11.4±2.6 of thrombus area; n=4 per group; P=0.05). However, we did not observe any neutrophil infiltration in the thrombi from both control animals and factor XI antisense–treated animals (Figure 3H).

**Discussion**

The results of our study demonstrate that initial clot formation on atherosclerotic plaques is not impaired in mice treated with factor XI antisense drugs. Platelet adhesion and aggregation were not significantly different between treatment groups, which imply that primary hemostasis is not influenced by factor XI inhibition. However, because clots were significantly smaller after 5 to 10 minutes in the factor XI antisense group when compared with controls, this shows that secondary hemostasis is impaired by factor XI inhibition.
**Figure 3.** The role of factor XI in coagulation and inflammation. **A**, Images of the carotid artery stained with hematoxylin and eosin, used to visualize the extent of atherosclerotic plaque burden and erythrocyte infiltration. The arrow indicates a ruptured plaque. **B**, There was a significant difference in thrombus size between factor XI (FXI) antisense and placebo-treated animals. **C**, Sections were stained with an antifibrinogen antibody to study thrombus formation. The arrow indicates the formed thrombus, with the fibrin threads in brown. **D**, Fibrin deposition was significantly lower in thrombi from factor XI antisense-treated animals when compared with placebo. **E**, The fibrous cap was visualized with a collagen staining (Sirius red) both in bright-field (top) as underpolarized light (bottom), the arrow indicates positive staining. **F**, Macrophage infiltration at the ruptured site was analyzed using MAC-3 staining. The arrow indicates positive staining; macrophages are in brown. **G**, Macrophage infiltration was not significantly different between the 2 treatment groups. **H**, Finally, the sections were stained for neutrophils using a Ly-6G antibody; however, no positive staining was observed. Error bars represent mean±SEM; *P*<0.05. Representative lower (bar, 200 μm) and higher (bar, 50 μm) magnification images are given.
This is the first study that focused on factor XI inhibition in diseased atherosclerotic vessels of older animals. Because atherosclerotic plaques contain a variety of prothrombotic substances, including TF, it is important to study the process of clot formation in ruptured plaques. We know from in vitro and in vivo studies that thrombus formation is probably less dependent on factor XI when there is a high concentration of TF. Furthermore, several other thrombogenic substances, such as oxidized low-density lipoproteins and macrophages, are exposed to the bloodstream after plaque rupture. Despite these prothrombotic conditions, factor XI inhibition resulted in smaller thrombi, which suggests that factor XI antisense may be used in patients having atherosclerosis-related disorders, such as myocardial infarction and stroke. Because the plaque rupture model is known to be dependent on TF, these data indicate that, at least in mice, TF is a limiting factor in the in vivo coagulant activity, making the whole system dependent on factor XI. This observation on factor XI provides excellent possibilities for the secondary prevention of cardiovascular patients. Furthermore, previous experiments with this model have shown that this model relies on collagen exposure and that thrombus formation is inhibited by glycoprotein VI inhibition and ADP receptor blockage, as well as inhibition of thrombin. Hence, we are able to conclude that thrombus formation on these ruptured plaques relies on both platelet activation (via glycoprotein VI and P2Y12) and coagulation.

We observed no differences in bleeding time between factor XI antisense and nonsense oligonucleotide–treated animals. This does not imply that (complete) factor XI inhibition in humans is safe. Patients with a severe factor XI deficiency may have a bleeding diathesis, whereas factor XI knockout mice do not have an increased bleeding tendency. This indicates that there is a discrepancy between mice and humans about factor XI levels and bleeding.

Because of the half-life of factor XI (≈48 hours), it takes 2 to 3 days before an antithrombotic effect is observed in mice and men. In humans, because of multiple factors, it will probably take 1 to 3 weeks before antithrombotic protection can be achieved. These factors include the time needed for the factor XI antisense oligonucleotides to distribute effectively to hepatocytes and clearance of the existing factor XI in circulation, which is dependent on its half-life. Nevertheless, this is a potential disadvantage of the drug. However, a treatment strategy incorporating heparin to bridge these first weeks before factor XI levels are reduced is probably a good solution. Most likely, the primary therapeutic use of factor XI antisense oligonucleotides will be for prevention and treatment of thrombosis in conditions, such as atrial fibrillation and venous thromboembolism. Obviously, this has to be established in clinical trials.

Interestingly, clots were not only smaller but also more unstable in factor XI antisense–treated animals. Because we observed limited amounts of fibrin, this would suggest that the instability is because of insufficient thrombin generation and fibrin formation, rather than to the disintegration of preformed rigid fibrin clots. Reduced thrombus stability in factor XI–deficient mice has been described before; however, the explanation for this is not entirely clear. One possibility is that there is reduced activation of thrombin activatable fibrinolysis inhibitor, which protects the clot from degradation by plasmin. This might cause enhanced fibrinolysis with less stable clots. An argument against this theory is that α2-antiplasmin levels were not significantly different between factor XI antisense– and nonsense–treated animals.

Because mice with an atherosclerotic phenotype do not develop spontaneous thrombosis, an artificial trigger is necessary. Common methods, such as ferric chloride or Rose Bengal, produce large thrombi, which fully occlude the lumen of the vessel. However, thrombi formed on ruptured plaques in humans hardly ever cause full occlusion of the vessel. In addition, these methods do not expose the content of the plaque core with all its thrombogenic substances to the bloodstream. In our model, plaque rupture was induced with an ultrasound wave, which induced thrombus formation without affecting blood flow. The formed thrombi never fully occluded the vessel but did expose the plaque core to the bloodstream. Therefore, we think that this is an excellent model to study the dynamic process of thrombus formation on ruptured plaques. However, it is still a mouse model with an artificial trigger, so our model has to be translated to humans with some caution.

Inhibition of factor XI activation attenuates inflammation, and indeed we found that macrophage infiltration was lower in thrombi from factor XI antisense–treated animals. This was only observed in thrombi formed on the ruptured plaques and not in the underlying plaque itself. Because factor XI activity might be involved in pathways that affect leukocyte function and trafficking, this observation could merely reflect a decrease in circulating leukocytes. At present, there is no known mechanistic link between factor XI activation and leukocyte function although there is evidence that factor Xla has direct cellular signaling functions through various receptors. Furthermore, factor XI is, together with factor XII and prekallikrein, a part of the contact system that constitutes a link between coagulation and inflammation, and inflammatory cells, such as activated neutrophils, are proposed to trigger coagulation. Therefore, inhibiting factor XI might suppress inflammatory pathways, which are responsible for pathological thrombus formation on atherosclerotic plaques.

We conclude that inhibition of factor XI prevents thrombosis on atherosclerotic plaques. The question remains whether factor XI inhibition influences the process of atherogenesis. Several clotting factors, including thrombin, contribute to atherosclerotic plaque formation. Therefore, it would be interesting to study the role of factor XI during this long-term process. Our current observations are that inhibition of factor XI prevents thrombosis on acutely ruptured atherosclerotic plaques without impairment of normal clot formation and indicates that targeting factor XI could be a safe alternative for the prevention of atherothrombotic disease.

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Disclosures
S. Bhanot and B.P. Monia are employees of ISIS Pharmaceuticals. The other authors report no conflicts.
Significance

This is the first study that establishes the importance of coagulation factor XI during atherothrombotic diseases. Current opinion assumes that atherothrombotic disorders, such as myocardial infarction and stroke, are mainly a platelet-driven event, accompanied by tissue factor–initiated coagulation. In this study, we show that thrombus formation on acutely ruptured atherosclerotic plaques is dependent on factor XI, and that the thrombotic burden can be decreased by inhibition of factor XI using factor XI antisense oligonucleotides. Furthermore, inhibition of factor XI is not associated with an increased bleeding tendency, which indicates that targeting factor XI could be a safe alternative for the treatment of atherothrombotic disease.
Factor XI Regulates Pathological Thrombus Formation on Acutely Ruptured Atherosclerotic Plaques

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Supplementary Methods

Immunohistochemistry

Carotid artery sections were fixed in 4% paraformaldehyde and subsequently embedded in paraffin. For immunostaining, paraffin sections were deparaffinized before endogenous peroxidase quenching and heat-induced epitope retrieval (HIER) (HIER citrate buffer pH 6, Thermo Scientific). After blocking with Ultra V Block (Thermo Fischer Scientific, Fremont, CA, USA) slides were incubated with either rabbit anti-mouse fibrinogen IgG (1:100.000, Gentaur, Kampenhout, Belgium), monoclonal antibody Ly-6G (clone 1A8, BD Biosciences Pharmingen, San Jose, CA, USA) or rat anti mouse Mac-3 (clone M3/M4, BD Biosciences) overnight at 4°C. Staining was performed with anti-rabbit Horse Radish Peroxidase (HRP) labelled IgG (ImmunoLogic, Duiven, The Netherlands) followed by Bright DAB+ visualization (ImmunoLogic, Duiven, The Netherlands). Visualization was with vector red (Vector laboratories, Burlingame, CA, USA) for Ly-6G. Counterstaining was performed using hematoxylin and slides were cover-slipped with VectaMount (Vector Laboratories, Burlingame, CA, USA). Positive controls for Ly-6G and Mac-3 consisted of samples of mice spleen. Fibrin, neutrophil and macrophage content of the thrombus were quantified using ImageJ software.

Supplemental Movies 1-4

The first 3 minutes of thrombus formation after ultrasound treatment in ApoE⁻/⁻ mice injected with factor XI antisense are shown in supplementary movie 1 and the nonsense oligonucleotide treated animals are shown in supplementary movie 2. Thrombus formation 5 minutes after ultrasound treatment in ApoE⁻/⁻ mice treated with factor XI antisense (suppl. movie 3, duration 1 minute) or nonsense oligonucleotides (suppl. movie 4, duration 1 minute)
Material and Methods

Materials

Factor XI antisense and nonsense oligonucleotides were provided by ISIS Pharmaceuticals (Carlsbad, CA, United States)(1;2). Antisense therapy is based on base-pair hybridization through which antisense oligonucleotides selectively bind to their complementary mRNA target. This binding typically results in the selective and catalytic degradation of the targeted mRNA and leads to a corresponding reduction in target protein levels(3). Factor XI antisense oligonucleotides have been proposed as preventive strategy for thrombosis.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Leiden, the Netherlands). Human fibrinogen labeled with alexa fluor 546 was from Invitrogen (Leiden, the Netherlands). Prothrombin and factor XI levels were measured on an automated coagulation analyzer (Behring Coagulation System, BCS) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany). D-dimer levels were determined using a commercially available ELISA (Enzygnost, Siemens Healthcare Diagnostics, Marburg, Germany).

Animal experiments

Four-week-old male Apoe<sup>-/-</sup> mice on C57Bl/6 background were obtained from Charles River (Maastricht, the Netherlands). Plaque rupture and measurement of acute thrombus formation was performed as described earlier(4). In short, Apoe<sup>-/-</sup> mice were fed a Western-type diet with 0.25% cholesterol (Arie Blok, Woerden, the Netherlands) for 18-20 weeks. Then, mice received an intraperitoneal injection (50 mg/kg) of either factor XI antisense or nonsense oligonucleotides every 3-4 days for a total of 10 days (3 injections). The surgical procedure was performed 3 days after the final injection and proceeded as follows. After the mice were anesthetized (subcutaneous injection of ketamin and xylazin (0.1 mg/g and 0.02 mg/g body weight, respectively)), the carotids were dissected free from surrounding tissue and the animal was injected intravenously with CFSE-labeled platelets obtained from a donor mouse with the same genetic background(5). A subset of mice was also injected with fluorescently labelled human fibrinogen. Using intravital fluorescence microscopy, a plaque was selected and rupture was induced by ultrasound application using a VibraCell VCX130 processor (Sonics, Newtown, CT, United States). Thrombus formation was recorded as soon as possible by capturing fluorescent images for at least 10 minutes. Images were analyzed by selecting a region of interest (ROI) representing the site of thrombus formation, total pixel intensity was
calculated and corrected for background. To quantify thrombus size after specific time intervals, fluorescent images were processed using ImageJ software. Within the image of a carotid artery, two similar ROIs were defined, one representing the background and one representing the thrombus. Subsequently, a threshold level was set by eliminating all pixels with intensity lower than 99.0% of the pixels of the background ROI. Intensities of all pixels in the thrombus ROI were then integrated.

**Collection of mouse plasma samples**

Blood samples were collected by cardiac puncture under anaesthesia. Blood was quickly withdrawn from the heart using a 1-mL plastic syringe (citrate-rinsed) with a 27-G needle and collected into a final ratio of 9 parts of whole blood to one part of 3.2% sodium citrate. Blood samples were immediately mixed by tapping and inverting the tube 5 times to ensure proper anticoagulation and then centrifuged for 15 minutes at 600g at room temperature. Plasma was stored at -80°C until assayed.

**Tail vein bleeding assay**

A mouse tail bleeding assay was used as described(6). Bleeding was assessed by determining the time until bleeding stopped with a maximum recording time of 30 minutes.

**Histology and Immunohistochemistry**

Mice were perfused with 25 ml of 0.9% NaCl (2 ml/min) followed by 15 ml of 4% paraformaldehyde in phosphate-buffered saline (0.5 ml/min). Arteries were removed and fixed for 48-72 hours in 4% paraformaldehyde, embedded in paraffin (Leica EG1160, Wetzlar, Germany) and cut into 5 µm sections using a microtome (Leica E2235, Wetzlar, Germany).

Carotid arteries were histomorphologically visualized using hematoxylin (Merck, KGaA, Darmstadt, Germany) and eosin (Sigma Aldrich, St. Louis, MO, USA) stains according to standard protocols. Tissue specimen were stained with antibodies against fibrinogen (rabbit anti mouse fibrinogen IgG, Gentaur, Kampenhout, Belgium), neutrophils (monoclonal antibody Ly-6G, clone 1A8, BD Biosciences Pharmingen, San Jose, CA, USA) and macrophages (rat anti mouse Mac-3, clone M3/M4, BD Biosciences). Sirius red staining (Polysciences Inc, Warrington, PA, USA) determined the relative collagen content in the atherosclerotic lesions. **Both bright-field as polarized light was used to visualize collagen content.**
Morphometric analysis using ImageJ analysis software was performed on the various 5 µm sections of the carotid arteries at the site of the thrombus. Additional information regarding staining is available in the supplement.

**Statistical analysis**
Statistical comparisons were made using non-parametric Mann-Whitney U tests. $P$ values less than 0.05 were considered significant. Data were analyzed using SPSS software package for Windows, Version 19.0. Graphics were constructed using GraphPad Prism, Version 5 for Windows (GraphPad Software).
References


