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Antigen levels of coagulation FXI and the risk of myocardial infarction and ischaemic stroke in young women

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Abstract

Introduction FXI activation is associated with an increase in risk of ischaemic stroke. However, it is not known whether this is caused by an increase in the inactive precursor or a higher rate of protein activation. Therefore we set out to determine whether FXI antigen levels alter the risk of myocardial infarction and ischaemic stroke in young women. Additionally, we investigated to what extent measures of FXI presence, activation and activity are related.

Methods The RATIO study is a nationwide case-control study including young women with myocardial infarction (N=205), ischaemic stroke (N=175), and healthy matched controls (N=638). FXI:ag levels were measured with a commercially available ELISA based assay and expressed as percentage of pooled normal plasma. Odds Ratios (OR) and corresponding 95% confidence interval (CI) were calculated as measures of rate ratios and adjusted for potential confounders. In the control group, we calculated correlation coefficients (r) between the several measurements of FXI to examine relationship between FXI antigen and activity assays and activation state.

Results High levels of FXI:ag were marginally associated with an increase in risk of myocardial infarction (OR 1.55, 95%CI 0.89 – 2.69), whereas the risk of ischaemic stroke was substantially increased (OR 2.65, 1.51 – 4.88). Oral contraceptive use further increased this risk. Levels of FXI:ag did not correlate with the measures of FXI activation (FXIa:C1-inh: r -0.05, p=0.22 and FXIa:AT-inh: r -0.07, p=0.08).

Conclusion Higher antigen levels of FXI increase the risk of ischaemic stroke, whereas the risk of myocardial infarction is only affected marginally. These effects do not depend on FXI activation. Therefore, new antithrombotic drugs could target FXI through protein synthesis as well as activation to lower the risk of ischaemic stroke.

Introduction

Cardiovascular disease is a major cause of morbidity and mortality in high income countries.[1] Antithrombotic treatments (e.g. antiplatelet therapies, or inhibitors of coagulation such as heparin, vitamin K antagonist or direct factor inhibitors) can be used as secondary prevention measure to lower the thrombotic potential of a patient. However, this also increases the risk of severe bleeding. Therefore, treatment strategies should target groups with the best benefit-risk ratio and new treatments could focus on either increasing the efficacy or reducing the impact of side effects: new anticoagulants could therefore be targeted at reducing the bleeding risk. Animal studies suggest that coagulation factor XI might be a target for the prevention of cardiovascular diseases with a low bleeding risk.[2,3]

FXI deficiency (i.e. FXI activity <15%) is uncommon in the general population, except among Ashkenazi Jews. Patients experience a mild bleeding disorder and have a reduced risk of deep venous thrombosis, ischaemic stroke, but not myocardial infarction.[4–6] Increased levels of FXI are associated with increased risks of deep venous thrombosis.[7] The risk of myocardial infarction in men is also increased by high FXI activity, especially in the young.[8]

The protein structure of FXI shows strong homology with kallikrein; both serine proteases have apple domains which play an important role in the binding of these proteins to other proteins.[9] FXI is a dimer with two identical subunits.[10] Each subunit comprises a catalytic domain and four apple domains. A disulfide bond links the fourth apple domain of the two subunits. Activation of FXI is a stepwise process in which each catalytic domain is activated separately; a conformational change is induced by the activation of the catalytic domain, thereby exposing several exosites on the apple domains that can bind other proteins such as platelets, thrombin, FIX, heparin and glycoprotein Ib.[11] FXI is activated by FXIIa, but perhaps also by thrombin resulting in a positive feedback loop (or flywheel model) in the coagulation system.[12,13] High molecular weight kininogen (HMWK) is a co-factor in the activation of FXI by FXII, but other co-factors have been identified: activated coagulation factor V and platelet derived polyphosphates act as cofactors in the activation of FXI by thrombin. [14,15] After activation of FXI, FXIa is quickly inhibited by its inhibitors such as the antitrypsin inhibitor and C1-inhibitor which are present in the bloodstream in large quantities, making the formation of FXIa the rate limiting step for the formation of the

protein-inhibitor complexes FXIa:AT-inh and FXIa:C1-inh.[16] The level of these protein-inhibitor complexes can be regarded to reflect the activation state of the protein of interest; this activation state can be thought of the 'background activation of FXI' and is therefore not directly linked to the amount of FXIa that is generated during the acute activation of the coagulation system and the subsequent formation of a blood clot. High levels of these complexes might therefore be more indicative of a procoagulant state rather than direct evidence of the causal role of FXIa.

Different key properties of FXI can be measured from citrated plasma with different assays which, due to their nature, do not necessarily have to correlate**. FXI presence** can be determined and quantified by a simple ELISA based assay, in which FXI proteins are captured and detected by antigens targeted towards FXI. The results of FXI antigen levels (FXI:ag) are normally expressed as percentage of a reference sample. **FXI coagulant activity** (FXI:C) is normally measured by a clotting assay in which a patients plasma is 1:1 mixed with reference plasma after which the clotting potential of this mixture is determined and expressed as percentage of clotting potential of the reference plasma**. FXI activation** can be determined by measuring the FXIa protein-inhibitor complexes by an ELISA based assay. The results of the measurement of these FXIa protein-inhibitor assays (e.g. FXIa:AT-inh) are expressed as percentage of a fully activated reference plasma.[17] Presence, as measured by an antigen assay, and coagulant activity, as measured by an activity assay, are closely related: in a complete deficiency both are zero, and activity increases linearly with increasing antigen levels. However, when the protein is produced but not functioning properly, there may be discrepancies, which may be extreme (e.g. no activity and normal antigen levels when the protein is fully defect).

FXI:C has previously been studied in the myocardial infarction subset of the RATIO study, a nationwide population-based case-control study on myocardial infarction, ischaemic stroke and peripheral arterial disease in young Dutch women. FXI:C was not strongly associated with myocardial infarction in a quartile analysis, with odds ratios of 1.1 for the second quartile, 1.0 for the third, and only marginally increased in the highest quartile (OR 1.6, 95% confidence interval 0.8-3.2), all relative to the lowest quartile of FXI:C level.[18] In the same study, levels of FXIa:C1-inh and FXIa:AT-inh were not related to myocardial infarction.[19] However, ischaemic stroke risk was 2- to 3-fold increased in those with high levels of FXI activation (i.e. >90th percentile of controls). These results raised the question whether FXI:C and FXIa:AT-inh or FXI-C1-inh are truly measures of increases in activity or

activation of FXI, or whether they reflect an increase in the presence of the inactive precursor, or zymogen, form of FXI, as measured by FXI:ag. Therefore, we set out to determine FXI:ag levels and the associated risks of myocardial infarction and ischaemic stroke. Additionally, we compared the different measures of FXI to see whether these truly reflect different properties of FXI.

Methods

Study design & participants The RATIO study is a population-based case-control study set up to investigate the association between the use of oral contraceptives and arterial thrombosis (i.e. myocardial infarction, ischaemic stroke and peripheral arterial disease), as has been described previously.[20–22] Briefly, young women (aged 18-50) who where diagnosed with myocardial infarction, ischaemic stroke or peripheral arterial disease in one of the 16 participating centres were eligible to participate. Healthy controls were approached via random digit dialling and frequency-matched on age category, area of residence and index date. This analysis focuses on myocardial infarction and ischaemic stroke. In total, 248 cases with myocardial infarction, 203 cases with ischaemic stroke and 925 frequency-matched controls were included in the first phase of the RATIO study. All participants were subsequently requested to provide either a blood sample or buccal

Table 1. Characteristics of RATIO participants

		Myocardial infarction N=205		Ischaemic stroke N=175		Control N=638	
Mean age		43	(6.1)	39	(7.9)	39	(7.9)
Caucasian ethnicity		195	(95%)	168	(95%)	602	(94%)
History of *							
Hypertension (N, %)		53	(26%)	50	(29%)	40	(6%)
Diabetes (N, %)		10	(5%)	7	(4%)	10	(2%)
Hypercholesterolaemia (N, %)		21	(10%)	14	(8%)	19	(3%)
Oral contraceptives use (N, %) *		81	(40%)	92	(53%)	213	(33%)
Smoking (N, %) *		169	(82%)	105	(60%)	270	(42%)
FXI:ag†	Mean (SD)	126	(44)	132	(29)	115	(26)
	Median (Q1-Q3)	125	(107-145)	128	(110-150)	112	(96 - 133)

SD = standard deviation, Q1 = 1st quartile, Q3 = 3rd quartile
** in year prior to event*
† Antigen levels of FXI (FXI:ag) are expressed as percentage of pooled normal plasma

swabs for DNA extraction. During this phase 168 women refused to participate (30 MI cases, 10 IS cases and 128 control women) and 83 women were untraceable, died or had blood samples of low quality (53 IS cases and 30 control women). To counteract the loss of participants in the ischaemic stroke case group, an additional 50 cases were recruited yielding blood samples from 203 myocardial infarction cases, 175 ischaemic stroke cases and 638 healthy controls available for measurement of FXI:ag.

Measurements FXI:ag levels were measured with a sandwich ELISA based assay. This commercially available kit (CEDARLANE inc., Burlington, Ontario, Canada) uses polyclonal purified coating IgG antibodies targeted against FXI (CL20250K-C) which were incubated overnight at 2-8° C. Patient samples, together with reference samples, were incubated for one hour after which FXI:ag levels could be determined by the purified peroxidase labeled detection antibody (CL20250K-C). Being an o-phenylenediamine based antibody kit, we measured light absorbance at 490 NM and signal strengths were converted to FXI:ag levels expressed as percentage of a reference sample for which pooled normal plasma was used. The lab technician was unaware of case status of the plasma samples measured.

FXI:C levels were measured by a one-stage clotting assay with factor XI-deficient plasma, respectively, and automated activated partial thromboplastin time (APTT; Organon Teknika, Boxtel, the Netherlands) on a STA (Diagnostica Stago, Boehringer Mannheim).[18]

FXI activation was measured as a C1-esterase inhibitor FXIa:C1-inh and or anti-trypsin inhibitor FXIa:AT-inh) complex. These complexes were measured by an ELISA, as described earlier.[17,19] The FXIa protein-inhibitor assays both use the XI-5 mAB as antigen, which recognises both the native and activated form of FXI.[16] R11 mAB which binds to native, complexed and inactive C1-inhibitor, was used as conjugate for the FXIa:C1-inh assay; mAb AT-15, which is directed against complexed AT, was used as conjugate in the FXIa:AT-inh assay.[23,24] All conjugates where biotinylated with EZLink N-hydroxysuccinimide ester-biotin according to instructions from the manufacturer (Pierce, Rockford, IL, USA). Absorbance was read at 450 nm on an EL 808 Ultra microplate reader (Bio-tek Instruments Inc.,Winooski, VT, USA). Results were expressed as a proportion of fully activated normal pooled plasma, activated by adding kaolin (final concentration 5 mg mL^{-1}).

Statistical analyses Characteristics of the RATIO participants are summarised as mean and corresponding standard deviation (SD) or median or the cut-off values for the first and third quartile (Q1-Q3) as appropriate. Logistic regression models were used to obtain Odds

Ratios (OR) and corresponding 95% confidence intervals (95%CI) as measures of rate ratios associated with high levels of FXI:ag (i.e. ≥90th percentile of FXI:ag level in controls). These ORs were obtained for three models. Model 1 included the variables area of residence, year of event and age (on a continuous scale) to account for the frequency matching procedure. Model 2 additionally included smoking behaviour, diagnosis of hypertension, diabetes and hypercholesterolaemia as potential confounders. Model 3 included FXIa:AT-inh measurements, to asses whether the associations found in model 2 truly reflect the effect of high FXI:ag or whether the observed effect, in part or in whole, can be explained by increased FXI activation. Quartile analyses were performed to investigate potential dose response relationships; the cut-offs for these quartile analyses were based on the 25th, 50th and 75th percentile of controls. To answer our second research question, we determined the Pearson's correlations coefficient and the more conservative Spearman's Rank correlation coefficient between FXI:ag, FXI:C and FXIa:AT-inh levels in the control group. Because FXI:AT-inh and FXI:C1-inh are not normally distributed, all levels were first logtransformed for these analyses. Additionally, we analysed the relationship between high levels (i.e. ≥ 90th percentile) of FXI:ag, FXI:C and measures of FXI activation by calculating an odds ratio as measure of association. All analyses were performed with SPSS statistics

Table 2. Levels of FXI:ag in relation to cardiovascular risk factors

cardiovascular risk factor *	FXI:ag levels in control group†	Mean difference (95% confidence interval)	
		Crude	Adjusted for age
Hypertension -	115	ref	Ref
Hypertension +	117	2 (-6 to 11)	1 (-8 to 9)
Diabetes -	115	ref	Ref
Diabetes +	118	3 (-13 to 20)	-2 (-17 to 14)
Hypercholesterolaemia -	115	ref	Ref
Hypercholesterolaemia+	117	12 (0 to 24)	-7 (-18 to 5)
Oral contraceptives use-	118	Ref	Ref
Oral contraceptives use+	110	-8 (-12 to -4)	-1 (6 to 3)
Smoking -	117	ref	Ref
Smoking +	115	-2 (-7 to 4)	-3 (-8 to 2)

** self reported history - measured in the year prior to the event*
† Antigen levels of FXI (FXI:ag) are expressed as percentage of pooled normal plasma

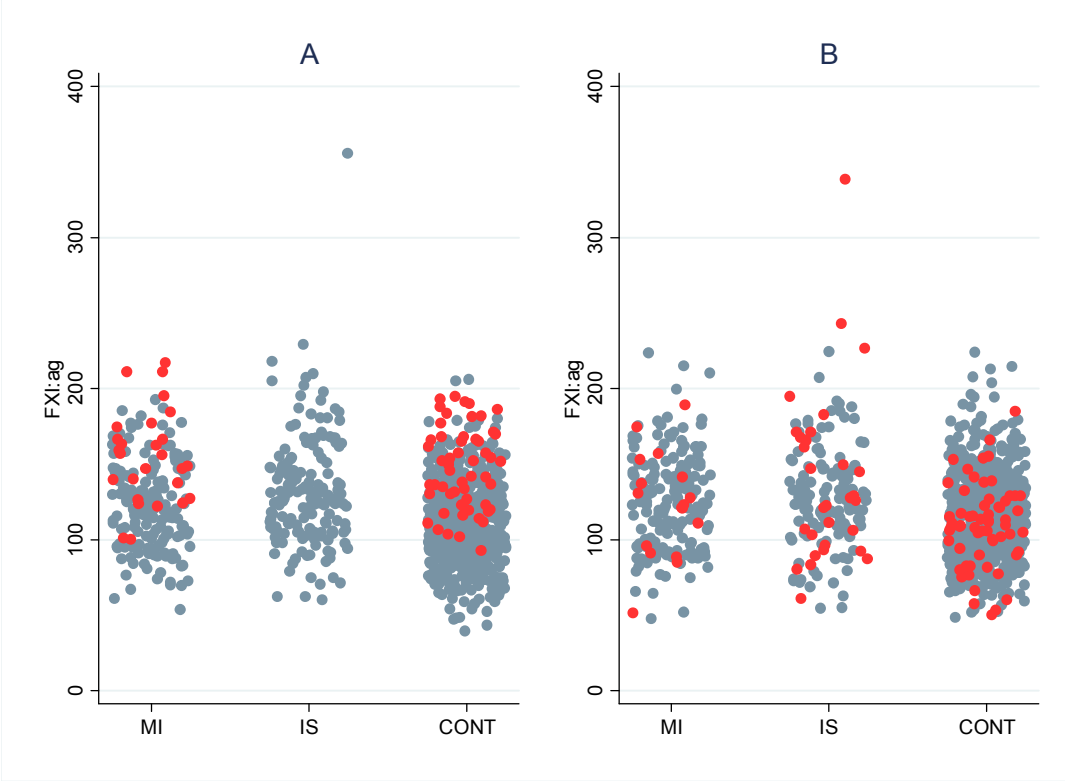
(version 18.0, IBM SPSS statistics, Chicago, Ill, USA) or Stata (version 11.2, Statacorp, College Station, Tx, USA).

Results

Traditional risk factors, such as smoking and oral contraceptive use, were as expected more common among the two case groups than among controls (table 1). The FXI:ag levels per case group are displayed in figure 1. The mean level of FXI:ag was 115% in the control group, 126% in the myocardial infarction group (mean difference 11%, 95%CI 6% to 15%) and 132% in the ischaemic stroke case group (mean difference 16%, 95%CI 12% to 21%). FXI:ag levels increased with increasing age (increase of 0.9% FXI:ag per year, 95%CI 0.7 to 1.2%). Table 2 shows the FXI:ag levels in the control group in relation to traditional risk factors: oral contraceptive use was associated with a reduced FXI:ag level (mean difference -8%, 95%CI -12% to -4%), which was mainly explained by a difference in age for oral contraceptive users and non-users (mean difference after adjustment for age (-1%, 95%CI -6% to 3%). Hypercholesterolemia was associated with an increase (mean difference 12%, 95%CI 0% to 24%).

The risks associated with high levels of FXI:ag are displayed in table 3: high levels of FXI:ag (i.e. ≥90th percentile of controls) increased the risk of myocardial infarction slightly (OR 1.55, 95%CI 0.89-2.69), whereas the risk of ischaemic stroke was 2.5 fold increased (adjusted OR 2.65, 95%CI 1.51-4.66). Quartile analyses showed that the risk of ischaemic stroke increased in a dose-dependent way (increasing risks with increasing levels), whereas such a relationship was not present for the effect on the risk of myocardial infarction (see table 4). The inclusion of FXIa:AT-inh in the regression models, both as continuous variable and dichotomised with the 90th percentile of controls as cut-off value, did not change these results. The addition of FXIa:C1-inh instead of FXI-AT-inh resulted in similar results (data not shown). Table 5 shows the correlations between the several measures of FXI from the control group: FXI:ag was, as expected, strongly correlated to FXI:C (Pearson's correlation coefficient 0.68, 95%CI 0.61-0.70, Spearman's correlation coefficient 0.68, p<0.001). Not surprisingly, the two measures of FXI activation, FXIa:C1-inh and FXIa:AT-inh were also correlated (Pearson's correlation coefficient on log transformed data 0.61, 95%CI 0.56-0.66, Spearman's Rank correlation coefficient = 0.24, p<0.001). In our risk analyses we focussed on extreme levels of FXI. Therefore, we determined whether people with high FXI:ag were also more likely to have high levels of FXI activity and activation.

Figure 1. FXI:ag levels per case group in relation to high levels of FXI:C and FXIa:AT-inh



MI, myocardial infarction; IS, ischaemic stroke; cont: control group. FXI:ag levels per case group, expressed as percentage of pooled normal plasma. Subjects with high levels of FXI:C (panel A, data not available for ischaemic stroke) and FXIa:AT-inh (panel B) are marked in red.

Table 3. High levels of FXI:ag and the risk of myocardial infarction and ischaemic stroke

		Control		Case groups							
	FXI:ag	**N**	**prop**	**N**	**prop**	**OR_1**	**(95%CI)**	**OR_2**	**(95%CI)**	**OR_3**	**(95%CI)**
MI	**<p90**	557	0.90	158	0.81	1	[ref]	1	[ref]	1	[ref]
	≥p90	61	0.10	36	0.19	1.61	(1.00-2.61)	1.55	(0.86-2.69)	1.49	(0.85-2.61)
IS	**<p90**	557	0.90	122	0.75	1	[ref]	1	[ref]	1	[ref]
	≥p90	61	0.10	41	0.25	2.91	(1.75-4.83)	2.65	(1.51-4.66)	2.64	(1.49-4.67)

MI, myocardial infarction; IS, ischaemic stroke; p90, 90th percentile of control group; p10, 10th percentile of control group; ref, reference group;95%CI, 95% confidence interval; N, number; OR, odds ratio; prop, proportion. Model 1: adjusted for stratification factors (i.e. age, index year, area of residence). Model 2: additionally adjusted for hypertension, diabetes, hypercholesterolaemia, smoking. Model 3: additionally adjusted for FXIa:AT-inh as measure of FXI activation. The addition of FXIa:C1-inh as a measure of FXI activation resulted in similar results (data not shown).

Table 4. Dose response analyses for levels of FXII, FXI and PK and the risk of myocardial infarction and ischaemic stroke.

	Control		Myocardial infarction						Ischaemic stroke					
	N	prop	N	prop	OR_1	95%CI	OR_2	95%CI	N	prop	OR_1	95%CI	OR_2	95%CI
Q1	147	0.24	28	0.14	1	[ref]	1	[ref]	19	0.12	1	[ref]	1	[ref]
Q2	160	0.26	39	0.20	1.17	(0.67-2.05)	1.12	(0.60-2.05)	24	0.15	1.04	(0.52-2.08)	0.76	(0.36-1.64)
Q3	156	0.25	43	0.22	1.12	(0.64-1.95)	0.98	(0.64-1.95)	51	0.31	2.38	(1.27-4.45)	2.03	(1.04-3.96)
Q4	154	0.25	84	0.43	2.01	(1.20-3.36)	1.74	(0.97-3.13)	69	0.42	3.14	(1.70-5.83)	2.50	(1.30-4.82)

MI, myocardial infarction; IS, ischaemic stroke; p90, 90th percentile of control group; p10, 10th percentile of control group; ref, reference group;95%CI, 95% confidence interval; N, number; OR, odds ratio; prop, proportion. Proportions might not addup to one due to rounding. Model 1: adjusted for stratification factors (i.e. age, index year, area of residence). Model 2: additionally adjusted for hypertension, diabetes, hypercholesterolaemia, smoking.

Table 5. Association between different measures of FXI in the control group

	FXI:ag	FXI:C	FXI:C1-inh	FXI:AT-inh
FXI:ag	NA	r: 0.68 (<0.00)	r: -0.04 (p=038)	r: -0.07 (p=0.08)
FXI:C	OR: 11 (p<0.00)	NA	r: -0.01 (p=0.70)	r: 0.02 (p=0.54)
FXI:C1-inh	OR: 0.60 (p=0.33)	OR 0.66 (p=0.43)	NA	r: 0.24 (p<0.00)
FXI:AT-inh	OR: 0.28(p=0.06)	OR: 1.07 (p=0.89)	OR: 41 (<0.00)	NA

This table shows the Spearman's rank correlation coefficient (r) in the upper right part of the table and the odds ratio (dichotomised at 90th percentile of controls) in the lower left corner. NA, not applicable; OR, odds ratio

Table 6. Interaction analyses of extreme levels of FXI:ag and the risk of myocardial infarction and ischaemic stroke

		Control		Myocardial infarction				Ischaemic stroke			
P90	**OC**	**N**	**prop**	**N**	**prop**	OR_2	**(95%CI)**	**N**	**prop**	OR_2	**(95%CI)**
-	-	366	0.59	95	0.49	1	[ref]	61	0.37	1	[ref]
-	+	191	0.31	63	0.32	1.82	(1.12-2.95)	61	0.37	2.26	(1.33-3.81)
+	-	47	0.08	23	0.12	1.47	(0.75-2.88)	18	0.11	1.85	(0.87-3.93)
+	+	14	0.02	13	0.07	3.08	(1.18-8.02)	23	0.14	8.70	(3.55-21)

See table 4. Additional adjustments for measures of FXIa:AT-inh or FXIa:C1-inh did not alter these results (results not shown).

While controls with high FXI:ag were more likely to have high levels of FXI:C than those with normal FXI:ag; they were not more likely to have high levels of FXI activation. This can also be appreciated from figure 1: the women with high levels of FXIa:AT-inh are depicted in red and do not cluster in the top region of FXI:ag levels.

To investigate the interrelated effects of high levels of FXI:ag and oral contraceptives on risk, women were categorised according to the combination of their risk factors yielding 4 categories: exposed to OC use (-/+), to high FXI:ag levels (≥90th percentile) (+/-), none (-/-) or both (+/+). For myocardial infarction, the risk for those who were exposed to both risk factors was 3-fold increased compared with the risk of those exposed to neither risk factor (table 6). This corresponds with what would be expected based on the effects of the two risk factors separately (only OC use: OR 1.82, 95%CI 1.12-2.95 and only high FXI:ag: OR 1.47, 95%CI 0.75-2.88). For ischaemic stroke the joint presence of oral contraceptive use and high FXI:ag was associated with a 9-fold increased risk, which was substantially higher than expected based on only oral contraceptive use (OR 2.26, 95%CI1.33-3.81) and only high FXI:ag (OR 1.85, 95CI% 0.87-3.93), again all relative to women with neither risk factor.

Discussion

Our results indicate that high antigen levels of FXI increase the risk of ischaemic stroke in young women. The risk increases gradually with increasing FXI:ag levels and is markedly increased in oral contraceptive users. The effect on the risk of myocardial infarction is only small, and in line with our previous report on FXI:C. Adjustment for FXI inhibitor-complexes, which are indicative of a heightened state of protein activation, did not affect the relative risks. This indicates that both high FXI antigen levels, as well the process leading to an increased FXI activation, increase the risk of ischaemic stroke.

In order to answer the question whether the different measurements of FXI reflect a property, we compared the different measurements in the control group. FXI:ag and FXIa:AT-inh are not clearly associated, as can be seen in table 4. However, FXI:ag and FXI:C are positively associated, as has been reported before.[25] This is reflected in the odds ratio of 11, which is substantially lower than the odds ratio of 41 for the relation between

FXIa:AT-inh and FXIa:C1-inh, the two measures designed to measure the same FXI property. The lack of a strong association between FXI:ag and FXI:C explains why these measures of FXI have a similar relationship with the risk of myocardial infarction.

Our quartile analyses indicate that high FXI:ag levels increase the risk of myocardial infarction about 70%, which is in line with our previous report on FXI:C.18 Reanalyses of the FXI:C data showed that extreme levels of FXI:C (i.e. ≥ 90th percentile of controls) were associated with 30% increase in risk (OR 1.27, 95%CI 0.77-2.09) which was attenuated after adjustment for confounders (OR 0.94, 95%CI 0.53-1.67). Such a decrease is not found for the FXI:ag analyses, which could point to residual confounding or indeed a differential effect. But the broad confidence intervals, as well as the lack of a dose response relationship make it difficult to determine whether these risk estimates truly differ. Additionally, prekallikrein, another intrinsic coagulation protein, is homologous to FXI, also complicating causal inference from these associations of polyclonal measurements of FXI. In short, although our data do not allow strong conclusions on the differences or similarities of FXI:ag and FXI:C, we can conclude that the increase in risk of myocardial infarction associated with these FXI measurements is only small. Together with the observation that FXI activation, as measured by protein-inhibitor complexes, is not related to the risk of myocardial infarction (FXIa:C1-inh OR 0.94, 95%CI 0.49-1.82, FXIa:AT-inh OR 0.94, 95%CI 0.49-1.82), we conclude that FXI is not related to the risk of myocardial infarction in young women. Contrastingly, based on the analyses of FXI:ag and FXIa protein-inhibitor complexes, we conclude that FXI is associated with ischaemic stroke risk.

Previous studies in men have shown different results: a case-control study including 560 men with myocardial infarction and 646 control subjects showed that FXI:C levels were associated with an increased risk of myocardial infarction, especially in the young (highest vs lowest quintile analyses, adjusted for confounders OR 2.5, 95%CI1.2-5.1 and when restricted to men <50 years OR 19, 95% 2 - 182).[8] Data from the NPHS-II showed that neither FXIa:AT-inh nor FXIa:C1-inh were associated with myocardial infarction or stroke in middle aged men.[19] However, this study also included haemorrhagic stroke which hampers causal inference on the relation with ischaemic stroke. Other studies showed that FXI, as measured with several assays, was associated with ischaemic stroke incidence, stroke subtype and severity.[25–27] A striking observation comes from a small study which compared middle-aged women suffering from coronary artery disease with controls.[28] This showed that FXI:C levels were higher in patients than in controls, but that this effect could have been driven by the presence of hypercholesterolaemia. A case-control study into the role of FXI:C on ischaemic stroke risk even suggested that there might be a synergistic effect between FXI:C levels and dyslipidaemia, something that could indicate a role of FXI in the

coagulation-inflammation-atherosclerosis triad.[29–31] This specific interplay of FXI and dyslipidaemia is, however, not likely to play a role in our study since we included young women with a low hypercholesterolaemic burden. It might, however, be important in explaining the differences between our results and the results of other studies.

Besides the possible link to dyslipidaemia, differences in the aetiology of myocardial infarction and ischaemic stroke could explain the differences between our risk estimates for the two diseases. FXI is first and most of all a coagulation factor and is a well-established risk factor for deep venous thrombosis in genetic studies and studies using pre-event and post-event blood measurement of FXI.[7,32–34] Furthermore, FXI deficient patients have a reduced risk of ischaemic stroke and venous thrombosis but not myocardial infarction,[4–6] which leads to the idea that ischaemic stroke in young women, in contrast to myocardial infarction, can be regarded as a thrombotic disease caused by a hypercoagulable state.

Since FXI can theoretically be targeted by new drugs to reduce its thrombotic potential without an increased risk of severe bleeding, FXI targeted treatments might be successful in primary or secondary prevention of ischaemic stroke.[2] Several animal studies corroborate this idea: FXI antisense oligonucleotides counteract FXI transcription, and reduce antigen levels of FXI which reduces clotting propensity without a major increase in bleeding risk in mice and cynomolgus monkeys.[35,36] A non-peptide serine protease inhibitor has been shown to be a selective irreversible inhibitor of FXIa. This compound, identified as BMS-262084, has achieved antithrombotic efficacy in both a venous and arterial thrombosis rabbit model with a minimal increase in bleeding tendency.[37] Our results indicate that the risks associated with high levels of FXI:ag are independent of a heightened state of protein activation as measured by protein-inhibitor complexes. These complexes could only reflect an increased procoagulant activity, with no direct causal effect for high levels of FXIa:C1-inh or FXIa:AT-inh. This implies that the new FXI-targeted compounds, which target different properties of FXI, might very well differ in their safety and efficacy.

Our study has some limitations. Being a case-control study, blood samples were by definition collected after the event. Therefore, differences in levels of blood markers could be the consequence of the disease instead of the cause. Although we cannot rule out such a mechanism, we think that this possibility is very limited for several reasons. The blood samples were collected after the acute phase of the disease (minimally 23 months after the

event). Also, FXI:ag levels were not associated with the time interval between event and blood draw (data not shown). Our results indicate that high levels of FXI:ag more than double the risk of ischaemic stroke. Although this study excluded women with an overt cardiac source of their ischaemic stroke, we cannot distinguish the several other subtypes of ischaemic stroke.[38] Similarly, our results come from a study which only includes young women (under 50 years) and only investigated the risk of a first non-fatal ischaemic stroke. Therefore, additional research is needed to see whether FXI contributes evenly to the risk of the different subtypes, different patient populations and the risk of recurrence. The results of the research addressing these questions will help to identify a group of patients that could benefit optimally of targeting FXI as a possible new anticoagulation strategy.

Conclusion In short, high antigen levels of FXI increases the risk ischaemic stroke and affects the myocardial infarction risk only minimally. The effect is independent of an increased state of FXI activation, which can be regarded as a measure of a procoagulant state and as such is also related to an increased risk of ischaemic stroke. These findings suggest that transcription, activity and activation of FXI may be targets for the development of new antithrombotics for use as possible treatments in primary or secondary prevention of ischaemic stroke.

1. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation*. 2011;123:e18–e209.
2. Löwenberg EC, Meijers JCM, Monia BP, Levi M. Coagulation factor XI as a novel target for antithrombotic treatment. *J Thromb Haemost*. 2010;8:2349–57.
3. Gailani D, Renné T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *J Thromb Haemost*. 2007;5:1106–1112.
4. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood*. 2008;111:4113–7.
5. Salomon O, Steinberg DM, Dardik R, Rosenberg N, Zivelin A, Tamarin I, Ravid B, Berliner S, Seligsohn U. Inherited factor XI deficiency confers no protection against acute myocardial infarction. *J Thromb Haemost*. 2003;1:658–61.
6. Salomon O, Steinberg DM, Zucker M, Varon D, Zivelin A, Seligsohn U. Patients with severe factor XI deficiency have a reduced incidence of deep-vein thrombosis. *Thromb Haemost*. 2011;105:269–73.
7. Meijers JCM, Tekelenburg WLH, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. *New Eng J Med*. 2000;342:696–701.
8. Doggen CJM, Rosendaal FR, Meijers JCM. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. *Blood*. 2006;108:4045–51.
9. Kaplan AP, Silverberg M. The coagulation-kinin pathway of human plasma. *Blood*. 1987;70:1–15.
10. Gailani D, Smith SB. Structural and functional features of factor XI. *J Thromb Haemost*. 2009;7 Suppl 1:75–8.
11. Emsley J, McEwan PA, Gailani D. Structure and function of factor XI. *Blood*. 2010;115:2569–77.
12. Bouma BN, Meijers JC. Role of blood coagulation factor XI in downregulation of fibrinolysis. *Curr Opin Hematol*. 2000;7:266–72.
13. Pedicord D, Seiffert D, Blat Y. Feedback activation of factor XI by thrombin does not occur in plasma. *Proc Natl Acad Sci*. 2007;104:12855–12860.
14. Choi SH, Smith S a, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood*. 2011;118:6963–70.
15. Maas C, Meijers JCM, Marquart JA, Bakhtiari K, Weeterings C, de Groot PG, Urbanus RT. Activated factor V is a cofactor for the activation of factor XI by thrombin in plasma. *Proc Natl Acad Sci*. 2010;107:9083–7.
16. Wuillemin W a, Minnema M, Meijers JC, Roem D, Eerenberg a J, Nuijens JH, ten Cate H, Hack CE. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. *Blood*. 1995;85:1517–26.
17. Govers-Riemslag JWP, Smid M, Cooper JA, Bauer KA, Rosenberg RD, Hack CE, Hamulyak K, Spronk HMH, Miller GJ, ten Cate H. The plasma kallikrein-kinin system and risk of cardiovascular disease in men. *J Thromb Haemost*. 2007;5:1896–903.
18. Tanis BC, Algra A, van der Graaf Y, Helmerhorst FM, Rosendaal FR. Procoagulant factors and the risk of myocardial infarction in young women. *Eur J Haematol*. 2006;77:67–73.
19. Siegerink B, Govers-Riemslag JWP, Rosendaal FR, Ten Cate H, Algra A. Intrinsic coagulation activation and the risk of arterial thrombosis in young women: results from the Risk of Arterial Thrombosis in relation to Oral contraceptives (RATIO) case-control study. *Circulation*. 2010;122:1854–61.
20. Kemmeren JM, Tanis BC, van den Bosch MAAJ, Bollen ELEM, Helmerhorst FM, van der Graaf Y, Rosendaal FR, Algra A. Risk of Arterial

Thrombosis in Relation to Oral Contraceptives (RATIO) study: oral contraceptives and the risk of ischemic stroke. *Stroke*. 2002;33:1202–8.

21. Tanis BC, van den Bosch MAAJ, Kemmeren JM, Manger Cats VM, Helmerhorst FM, Algra A, van der Graaf Y, Rosendaal FR. Oral contraceptives and the risk of myocardial infarction. *New Eng J Med*. 2001;345:1787–93.

22. van den Bosch MAAJ, Kemmeren JM, Tanis BC, Mali WPTM, Helmerhorst FM, Rosendaal FR, Algra A, Van Der Graaf Y. The RATIO study: oral contraceptives and the risk of peripheral arterial disease in young women. *J Thromb Haemost*. 2003;1:439–44.

23. Nuijens JH, Huijbregts CC, van Mierlo GM, Hack CE. Inactivation of C-1 inhibitor by proteases: demonstration by a monoclonal antibody of a neodeterminant on inactivated, non-complexed C-1 inhibitor. *Immunology*. 1987;61:387–9.

24. Abbink JJ, Kamp AM, Swaak AJ, Hack CE. Production of monoclonal antibodies against inactivated alpha 1-antitrypsin. Cross-reactivity with complexed alpha 1-antitrypsin and application in an assay to determine inactivated and complexed alpha 1-antitrypsin in biological fluids. *J Immunol Methods*. 1991;143:197–208.

25. Yang DT, Flanders MM, Kim H, Rodgers GM. Elevated factor XI activity levels are associated with an increased odds ratio for cerebrovascular events. *Am J Clin Pathol*. 2006;126:411–5.

26. Undas A, Slowik A, Gissel M, Mann KG, Butenas S. Circulating activated factor XI and active tissue factor as predictors of worse prognosis in patients following ischemic cerebrovascular events. *Thromb Res*. 2011;128:e62–e66.

27. Undas A, Slowik A, Gissel M, Mann KG, Butenas S. Active tissue factor and activated factor XI in patients with acute ischemic cerebrovascular events. *Eur J Clin Inves*. 2012;42:123–9.

28. Berliner JI, Rybicki AC, Kaplan RC, Monrad ES, Freeman R, Billett HH. Elevated levels of Factor XI are associated with cardiovascular disease in women. *Thromb Res*. 2002;107:55–60.

29. Santamaría a, Oliver a, Borrell M, Belvís R, Martí-Fábregas J, Mateo J, Fontcuberta J. Higher risk of ischaemic stroke associated with factor XI levels in dyslipidaemic patients. *Int J Clin Pract*. 2007;61:1819–23.

30. Borissoff JI, Spronk HMH, ten Cate H. The Hemostatic System as a Modulator of Atherosclerosis. *New Eng J Med*. 2011;364:1746–60.

31. Borissoff JI, Heeneman S, Kilinç E, Kassák P, Van Oerle R, Winckers K, Govers-Riemslag JWP, Hamulyák K, Hackeng TM, Daemen MJAP, ten Cate H, Spronk HMH. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation*. 2010;122:821–30.

32. Cushman M, O'Meara ES, Folsom AR, Heckbert SR. Coagulation factors IX through XIII and the risk of future venous thrombosis: the Longitudinal Investigation of Thromboembolism Etiology. *Blood*. 2009;114:2878–83.

33. Bezemer ID, Bare LA, Doggen CJM, Arellano AR, Tong C, Rowland CM, Catanese J, Young BA, Reitsma PH, Devlin JJ, Rosendaal FR. Gene variants associated with deep vein thrombosis. *JAMA*. 2008;299:1306–14.

34. Li Y, Bezemer ID, Rowland CM, Tong CH, Arellano AR, Catanese JJ, Devlin JJ, Reitsma PH, Bare LA, Rosendaal FR. Genetic variants associated with deep vein thrombosis: the F11 locus. *J Thromb Haemost*. 2009;7:1802–8.

35. Zhang H, Löwenberg EC, Crosby JR, MacLeod a R, Zhao C, Gao D, Black C, Revenko AS, Meijers JCM, Stroes ES, Levi M, Monia BP. Inhibition of the intrinsic coagulation pathway factor XI by antisense oligonucleotides: a novel antithrombotic strategy with lowered bleeding risk. *Blood*. 2010;116:4684–92.

36. Younis HS, Crosby J, Huh J-I, Lee HS, Rime S, Monia B, Henry SP. Antisense inhibition of coagulation factor XI prolongs APTT without increased bleeding risk in cynomolgus monkeys. *Blood*. 2012;119:2401–8.

37. Wong PC, Crain EJ, Watson C a, Schumacher W a. A small-molecule factor XIa inhibitor produces antithrombotic efficacy with

minimal bleeding time prolongation in rabbits. *J Thromb Thrombolysis*. 2011;32:129–37.

38. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke*. 1993;24:35–41.