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

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Abstract

Introduction High levels of activated intrinsic coagulation proteins increase the risk of ischaemic stroke but not myocardial infarction in young women. This study aims to determine whether the antigen levels of coagulation factor XII (FXII) and prekallikrein (PK) are risk factors for both myocardial infarction and ischaemic stroke.

Methods The RATIO study included young women (<50 years) with myocardial infarction (N=205), ischaemic stroke (N=175) and 638 healthy frequency-matched controls. Antigen levels of FXII and prekallikrein (PK) were measured and expressed as percentage of pooled normal plasmas. Odds ratios (OR) and corresponding 95% confidence intervals (95%CI), adjusted for matching factors, were calculated for high levels (≥ 90th percentile of controls) as measures of rate ratios.

Results Traditional risk factors were more common in cases than in healthy controls. Antigen levels for FXII and prekallikrein related poorly with the levels of the activated form of the protein as measured by protein-inhibitor complexes. If anything, high levels of FXII increased the risk of MI moderately and but not of IS (OR 1.6, 95% CI 0.9-2.7 for MI and OR 1.1, 0.6-2.1 for IS). PK was not associated with an increased risk (MI 1.3, 0.8-2.15; IS 0.7, 0.4-1.4).

Conclusion The lack of a strong correlation between antigen level and activated protein-inhibitor complexes, the low risk for IS conferred by high FXII and PK antigen levels, as well as a lack of attenuation after adjustments suggest that our previous result could be driven by a higher activation rate of the intrinsic coagulation proteins, instead of the protein level itself.

Introduction

The intrinsic coagulation system has long been regarded to play only a minor role in blood haemostasis.[1] Activation of these proteins was thought to be mainly an in vitro artefact, caused by the negative surface provided by for example glass or kaolin.[2,3] Recent biochemical and animal studies, however, implicated the intrinsic coagulation system in several mechanisms relevant for thrombus formation.[4–9]

Coagulation factor XII, also known as Hageman factor, is a serine protease of which the activation occurs in two steps yielding α-FXIIa and subsequently β-FXIIa (or FXII fragment, or Hageman fragment), each with different functions.[1] Transcription of FXII may be influenced by female hormones due to an estrogen receptive element in the promoter region of F12, the gene encoding FXII.[10] Negatively charged surfaces such as platelet derived polyphosphates act as a scaffold on which FXII and cofactors can co-localize and be activated.[2,11–17] The polyphosphate mechanism, which is not unlike the activation of FXII by bacteria, provides the link between primary and secondary haemostatic processes.[18–21] Activation of FXII and subsequently prekallikrein can lead to several distinct actions under which clot propagation, bradykinin formation, complement activation, neutrophil aggregation and promotion of fibrinolysis through activation of plasminogen.[1,3,16,22–25] Differentiation between these actions might be caused by the size of the negatively charged surface on which the activation reactions occur, as well the different actions of α-FXIIa and β-FXIIa.[3,26–28] Also, activated FXII can bind to fibrinogen, thereby altering the clot structure which is another pathway by which the intrinsic coagulation system is involved in the mechanisms underlying thrombotic diseases.[29]

Prekallikrein, the zymogen form of kallikrein, is also a serine protease with 4 apple domains similar to FXI (58% homology).[1] FXII can convert prekallikrein to kallikrein (α-FXIIa when bound to a surface and β-FXIIa in the fluid phase), where high molecular weight kininogen (HMWK) is a co-factor by providing a site on negatively charged surfaces. Kallikrein can activate FXII amplifying the activation cascade, and together they can convert plasminogen into plasmin providing a link to the fibrinolytic system.[22,25,30–32]

Deficiency of FXII or prekallikrein is rare and without overt bleeding diathesis.[33] Somewhat paradoxically, John Hageman, the first patient identified with this trait died of a massive pulmonary embolus after he sustained pelvic fractures.[34,35] An Austrian epidemiological study studied the association between FXII activity and overall survival; the highest risks

were found in the categories with its highest and lowest levels, resulting in a U-shaped curve.[36] FXIIa-alpha (a subform of activated FXII) in patients with acute coronary syndrome upon admission was an predictor for all cause mortality, especially in patients with low troponin levels (<0.05ng/mL).[37] Clinical studies on the effect of plasma kallikrein are scarce. A quartile analyses (high vs low) of the amidolytic activity of prekallikrein measured with a chromogenic substrate, was associated with a 5-fold increase in myocardial infarction risk.[38] A case-control study found elevated levels of HMWK and normal levels of prekallikrein in patients with deep vein thrombosis.[19] This is in contrast with results from the Northwick Park Heart study, that implicated low levels of FXIIa protein-inhibitor complexes as a risk factor for coronary heart disease and stroke, whereas low levels of kallikrein protein-inhibitor complexes only seemed to be related to an increase in stroke risk.[39]

RATIO study Results from the RATIO study showed that the presence of protein-inhibitor complexes indicative of an increased state of activation of the intrinsic coagulation proteins is associated with ischaemic stroke. Women with high levels of these complexes of factor XI, XII and kallikreine (i.e. above the ≥90th percentile of controls) have a 2-5 fold increase in risk of ischaemic stroke, but not myocardial infarction.[40] It is, however, unclear to what extent these measures of protein activation are increased due to a higher activation rate of the proteins or a higher availability of the zymogen form. This study aims to determine to what extent the risk of myocardial infarction and ischaemic stroke in young women are affected by antigen levels of FXII and PK.

Methods

Study design & participants We used data from the RATIO study, a nationwide population-based case-control study focused on the identification of risk factors for myocardial infarction and ischaemic stroke in young women. [41–43] Two hundred-and-forty-eight women under 50 years and diagnosed with myocardial infarction as well as 203 young women with ischaemic stroke were recruited for the first phase of the study. Healthy women were requested to participate in the study as control, yielding 925 control subjects frequency-matched on age, area of residence and index year (year of event for cases and corresponding date for controls). The second phase of the study included the collection of biologic samples (blood and buccal swabs for DNA extraction). An additional 50 ischaemic stroke cases were recruited to increase the power of the study finally yielding blood

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%)
FXII:ag Mean (SD)	127 (44)	125 (41)	126 (40)
PK:ag† Mean (SD)	135 (32)	128 (34)	130 (32)

N = number, SD = standard deviation, FXII:ag = antigen level of coagulation factor XII, PK:ag = antigen level of prekallikrein. in year prior to event. Levels are expressed as percentage of normal pooled plasma.*

samples from 205 myocardial infarction cases, 175 ischaemic stroke cases and 638 healthy controls available for measurement of antigen levels of FXII, FXI and PK.

Measurements Antigen levels of FXII and PK were measured with polyclonal antibody sandwich ELISA assays, which are commercially available from Cedarlane (Cedarlane inc., Burlington, Ontario, Canada). These polyclonal antibody kits use purified coating IgG antibodies targeted against FXII (CL20055K-C) or PK (CL20090K-C) incubated overnight at 2-8°. These kits also provide purified o-phenylenediamine-based detection antibodies (CL20055K-D for FXII and CL20067K-D for PK) of which light absorbance can be measured at 490 NM. Signal strengths were converted to levels expressed as percentage of normal pooled plasma. Each sample was diluted in duplo and the lab technician was unaware of the case or control status of the measured blood samples.

Protein-inhibitor complexes of FXIIa and kallikrein were determined with a C1-esterase inhibitor assay (FXIIa:C1-inh andKAL:C1-inh). These complexes were measured by an ELISA, as described earlier.[39] In short, for the FXIIa:C1-inh ELISA we used mAB KOK 12 which is specific for complexed C1-esterase inhibitor as antigen and mAb F3 which recognises FXII as well as α-FXIIa and β-FXIIa subsequently as conjugate. The KAL:C1-inh assay uses the same antigen, but uses mAb K15 which is directed against prekallikrein and kallikrein as conjugate.[44,45] 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; activation was performed by adding an equal volume of 0.2 mg mL^{-1} dextran sulphate (Mr 500 000; Sigma Chemical Co., St Louis, MO, USA) in the FXIIa:C1-inh and KAL:C1-inh assay. Activation was stopped by adding three volumes of phosphate-buffered saline (PBS) containing 0.1 mg mL–1 soybean trypsin inhibitor (Sigma Chemical Co.) and 0.05% (w/v) polybrene (Sigma Chemical Co.).

Statistical analyses Participants' characteristics are summarised as means and corresponding standard deviation (SD) or median and the cut-off values for the first and third quartile (Q1-Q3). Linear regression was used to calculate the levels of FXII:ag and PK:ag in relation to cardiovascular risk factors. Logistic regression models were used to obtain Odds Ratios (OR and corresponding 95% confidence intervals (95%CI) as measures of rate ratios. Three models were used: model 1 includes 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 putative confounders; model 3 also includes high levels (i.e. >90th percentile) of FXIIa:C1-inh or kal:C1-inh to determine whether the results were mediated by a state of increased protein activation. For each protein we assessed whether low levels (<10th percentile of controls) and high levels (≥90th percentile of controls) were associated with altered risk of myocardial infarction and ischaemic stroke. Quartile analyses were performed to investigate the shape of the association; the cut-offs for these quartile analyses were based on the 25th, 50th and 75th percentile of controls. To investigate the relation between the antigen levels and measures of protein activation (levels of FXIIa:C1-inh and KAL:C1-inh) we calculated Spearman's rank correlation coefficient as well as odds ratios to determine the relation between high levels. Participants with unsuccessful measurements of FXII:ag and PK:ag were excluded from analyses when appropriate.

Results

As expected, classical risk factors were more common in the two case groups than in the control group (table 1). FXII:ag measurements were available for 195 myocardial infarction

Table 2. Levels of FXII:ag and PK:ag in relation to cardiovascular risk factors

	Hypertension	Diabetes	Hypercholesterolaemia	OC use	smoking	age change/year
FXII:ag	1 % (-12% to 15%)	-13 % (-40% to 13%)	22% (4% to 41%)	12% (5% to 19%)	-5% (-13% to 2%)	-0.3% (-0.7% to 0.1)
PK:ag	-1% (-11% to 10%)	-5% (-24% to 14%)	17% (2% to 31%)	4% (-2% to 9%)	2% (-4% to 9%)	0.3% (-0.1% to 0.6%)

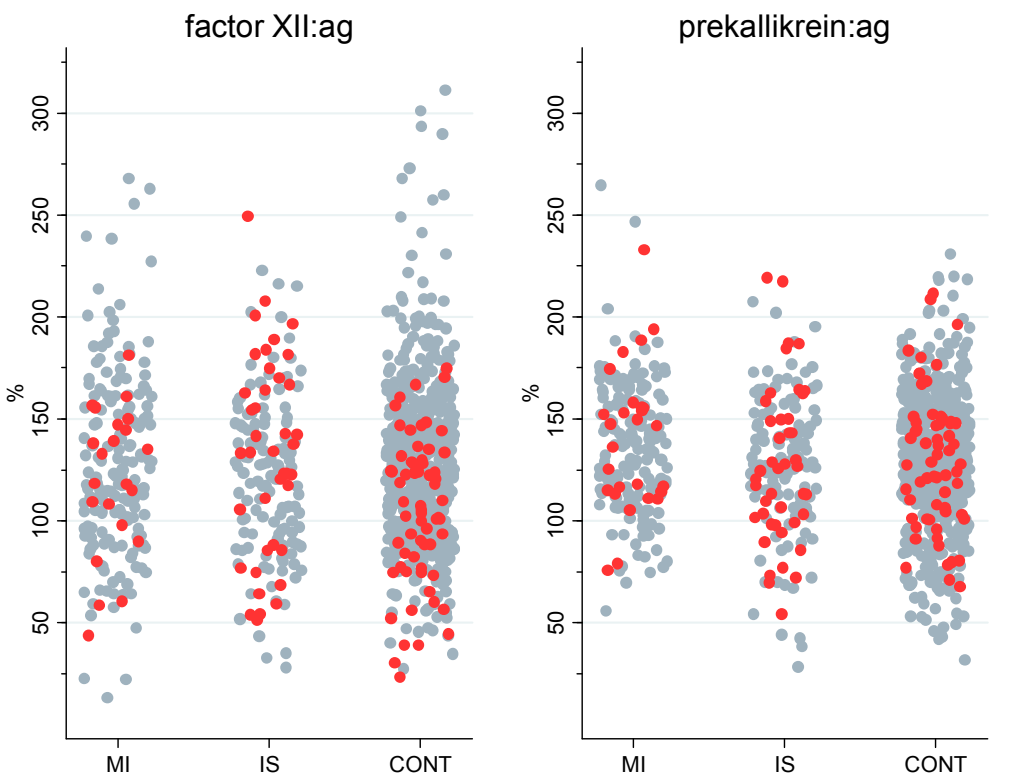
Differences are expressed as absolute changes in levels as expressed as percentage of pooled normal plasma. FXII:ag = antigen level of coagulation factor XII, PK:ag = antigen level of prekallikrein

cases, 163 ischaemic stroke cases and 617 controls. PK:ag measurements were available for 194 myocardial infarction cases, 163 ischaemic stroke cases and 616 controls. Mean FXII:ag levels were equal amongst all three groups, PK:ag levels were slightly increased in myocardial infarction cases compared with controls (mean difference 6%, 95%CI 1% to 11%).

A history of hypertension and smoking status did not substantially affect the levels of both FXII:ag and PK:ag, as can be seen in table 2. Women previously diagnosed with diabetes showed a decrease in FXII:ag, whereas diagnosed hypercholesterolaemia was associated with an increase of both FXII:ag and PK:ag levels. Oral contraceptive use was associated with an increase of FXII:ag, also after adjustment for age. As can be seen in table 3, antigen levels of FXII were slightly negatively related with FXII activation levels. This inverse relationship was most pronounced for extreme levels (i.e. ≥90th percentile of controls): no control subject had both high antigen levels and high activation levels. Antigen levels of PK were not associated with KAL:C1-inh levels. However, PK:ag was moderately associated with FXI:ag levels (Pearsons 's correlation coefficient 0.31, 95%CI 0.24 - 0.38, Spearman's correlation coefficient 0.32, p<0.001). The same pattern arises from figure 1, in which all the antigen levels of all participants are depicted; high levels of protein activation are marked in red.

Levels of FXII:ag did not clearly affect the risk of myocardial infarction or ischaemic stroke: high levels of FXII:ag (i.e. ≥90th percentile of control group) did not increase the risk of myocardial infarction (OR 1.18, 95% 0.62-2.25) nor the risk of ischaemic stroke (OR 0.99, 95%CI 0.48-2.01) (see table 4). Low levels of FXII:ag were associated with a mildly elevated

Figure 1. Levels of FXII and PK per case group



Antigen levels of coagulation factor XII and prekallikrein per case group, expressed as percentage of pooled normal plasma. Patients with high levels of activated factor FXII (for FXII:ag analyses) or kallikrein (for PK:ag analyses) are indicated in red. MI, myocardial infarction; IS, ischaemic stroke; cont: control group.

Table 3. Correlation between antigen levels and activated protein-inihibitor complexes in the RATIO control group

A	FXII:ag	FXIIa:C1-inh
FXII:ag	NA	r: -0.07 (p=0.08)
FXIIa:C1-inh	OR: - (p=0.01)	NA

B	PK:ag	KAL:C1-inh
PK:ag	NA	r: -0.03 (p=0.42)
KAL:C1-inh	OR: 1.23 (p=0.63)	NA

Panel A: relation between FXII and log transformed levels of activated FXII, Panel B: relation between antigen levels of PK and log transformed levels of activated kallikreine. Associations are expressed as Spearman's non parametric correlation coefficient (r) for continuous values and as an odds ratio (OR) for high levels. No odds ratio could be calculated for the FXII measurements, because no control subject had both high antigen levels and high activation levels.

risk of both myocardial infarction (OR 1.46, 95%CI 0.77-2.75) and ischaemic stroke (OR 1.49, 95%CI 0.77-2.87) by about 50 percent, but there was no pattern of increasing risk with increasing levels in the quartile analyses (table 5). Adjustment for FXIIa:C1-inh levels did not change the results. PK:ag levels showed a different picture for myocardial infarction risk compared with ischaemic stroke risk: increased levels were mildly associated with the risk of myocardial infarction (OR 1.54, 95%CI 0.82-2.89), but not at all with the risk of ischaemic stroke. Low levels were associated with decreased risk of myocardial infarction (OR 0.60, 95%CI 0.28-1.28), but, if anything, increased the risk of ischaemic stroke (OR 1.32, 95%CI 0.66-2.65). The quartile analyses showed that women in the highest quartile had a twofold increase in myocardial infarction risk compared with the lowest quartile although a clear pattern of risk with level was absent. Again, adjustment for KAL:C1-inh levels did not affect the estimates.

Discussion

Our results indicate that levels of FXII:ag do not have a clear impact on the risk of myocardial infarction or ischaemic stroke in young women. PK:ag levels are, if anything, related to the risk of myocardial infarction, but not ischaemic stroke. The antigen levels of FXII and PK levels are not positively associated with the presence of protein-inhibitor complexes indicative of a state of increased protein activation, which previously were shown to be related to a 2- to 5-fold increase in ischaemic stroke risk, but not myocardial infarction risk.

FXII:ag and PK:ag levels were influenced by the presence of cardiovascular risk factors, although for some risk factors the prevalence was so low in our control group that no definite conclusions can be drawn. Only smoking and oral contraceptive use were sufficiently prevalent in sufficient numbers to draw conclusions: smoking did not affect FXII:ag or PK:ag levels substantially. Oral contraceptive use increased FXII:ag levels by 12%, which is in accordance with the presence of an estrogen receptive element in the promoter region of F12.[10]

Previous research has indicated that increased levels of FXII activation, as measured by FXIIa:C1-inh, were associated with an increased risk of ischaemic (about twofold) whereas high KAL:C1-inh levels were associated with a twofold increased risk of myocardial infarction and a fivefold increased risk of ischaemic stroke.[40] Combined with the current results, we conclude that this higher level of activation is not caused by a higher level of

Table 4. Extreme levels of FXII, FXI and PK and the risk of myocardial infarction and ischaemic stroke

		Control		Myocardial infarction								Ischaemic stroke							
		N	%	N	%	OR	(95%CI)	OR_1	(95%CI)	OR_2	(95%CI)	N	%	OR	(95%CI)	OR_1	(95%CI)	OR_2	(95%CI)
FXII:ag	High <p90	556	0.90	169	0.87	1	[ref]	1	[ref]	1	[ref]	147	0.90	1	[ref]	1	[ref]	1	[ref]
	≥p90	61	0.10	25	0.13	1.57	(0.92-2.68)	1.18	(0.62-2.24)	1.18	(0.62-2.25)	16	0.10	1.03	(0.55-1.93)	1.03	(0.52-2.05)	0.99	(0.48-2.01)
	Low >p10	557	0.90	170	0.88	1	[ref]	1	[ref]	1	[ref]	140	0.86	1	[ref]	1	[ref]	1	[ref]
	≤p10	60	0.10	24	0.12	1.19	(0.69-2.03)	1.46	(0.77-2.75)	1.54	(0.81-2.93)	23	0.14	1.27	(0.70-2.29)	1.49	(0.77-2.87)	1.36	(0.82-2.26)
PK:ag	High <p90	555	0.90	170	0.86	1	[ref]	1	[ref]	1	[ref]	149	0.91	1	[ref]	1	[ref]	1	[ref]
	≥p90	62	0.10	24	0.14	1.29	(0.76-2.18)	1.54	(0.82-2.89)	1.43	(0.75-2.69)	14	0.09	0.67	(0.34-1.30)	0.90	(0.44-1.85)	0.91	(0.44-1.88)
	Low >p10	558	0.91	182	0.94	1	[ref]	1	[ref]	1	[ref]	147	0.90	1	[ref]	1	[ref]	1	[ref]
	≤p10	58	0.09	12	0.06	0.69	(0.35-1.33)	0.60	(0.28-1.28)	0.61	(0.28-1.30)	16	0.10	1.28	(0.68-2.43)	1.32	(0.66-2.65)	1.33	(0.66-2.67)

All odds ratios are adjusted for stratification factors (i.e. age, area of residence and index year). OR_1 is additionally adjusted for hypertension, diabetes and hypercholesterolaemia. OR_2 is additionally adjusted for high levels of activated factor FXII (for FXII:ag analyses) or kallikrein (for PK:ag analyses). OR = odds ratio, N = number, ref = reference category, FXII:ag = antigen level of coagulation factor XII, PK:ag = antigen level of prekallikrein.

protein presence. This leaves the question how an increase in protein-inhibitor complexes can be interpreted. Perhaps these increased levels reflect a more general notion of an activated coagulation system. Or, although not likely, perhaps it can be explained by an increase in activation rate of the zymogen, for example by a gain of function mutation in F12 or KLKB1, the genes encoding FXII and prekallikrein.

Our study has some limitations. The collection of blood samples in the RATIO study was, dictated by the use of a case-control design, after the event. This harbours the possibility of reverse causation, a situation in which an effect of the disease is mistaken for the cause of the disease. This is, however, especially a problem when blood is drawn in the acute phase of the disease. In our study blood was drawn at a minimum of 23 months after the event, minimising the possibility of reverse causation. Also, our case-control study only included survivors of myocardial infarction and ischaemic stroke. This selection will only affect the external validity of our study if FXII:ag and PK:ag levels affect the case fatality rate without having a major effect on nonfatal diseases, a scenario that we deem unlikely.

Conclusion Antigen levels of coagulation factor XII and prekallikrein are not associated with a major effect on the risk of either myocardial infarction or ischaemic stroke. Previous research showed that the presence of protein-inhibitor complexes of these proteins was associated ischaemic stroke risk, but not with myocardial infarction; the antigen levels do not correlate with these measures of protein activation. We conclude that the previously observed effect is not caused by an increased availability of the protein, and that an increased activation rate may explain these previous findings. Additional research is needed to determine the causal implications of these observations.

Table 6. interaction analyses for extreme levels of FXII and PK in combination with oral contraceptive use

			Control		Myocardial infarction						Ischaemic stroke					
	≥p90	OC	N	prop	N	prop	OR	(95%CI)	OR_1	(95%CI)	N	prop	OR	(95%CI)	OR_1	(95%CI)
FXII:ag	-	-	385	0.62	107	0.55	1	[ref]	1	[ref]	70	0.43	1	[ref]	1	[ref]
	-	+	171	0.28	62	0.32	2.24	(1.49-3.38)	1.85	(1.16-2.93)	77	0.47	3.17	(2.01-5.01)	3.26	(1.97-5.39)
	+	-	28	0.05	11	0.06	1.39	(0.63-3.04)	0.95	(0.37-2.45)	9	0.06	1.97	(0.83-4.68)	2.36	(0.92-6.06)
	+	+	33	0.05	14	0.07	2.98	1.40-(6.32)	2.1	(0.89-4.94)	7	0.04	1.55	(0.60-3.97)	1.34	(0.47-3.81)
PK:ag	-	-	371	0.60	108	0.56	1	[ref]	1	[ref]	73	0.45	1	[ref]	1	[ref]
	-	+	184	0.30	62	0.32	2.02	(1.34-3.03)	1.69	(1.06-2.67)	76	0.47	2.60	(1.65-4.08)	2.54	(1.55-4.17)
	+	-	41	0.07	10	0.05	0.80	(0.38-1.69)	1.04	(0.45-2.42)	6	0.04	0.50	(0.19-1.29)	0.58	(0.19-1.74)
	+	+	21	0.03	14	0.07	4.24	(1.92-9.38)	4.31	(1.59-11.7)	8	0.05	2.36	(0.89-6.25)	3.00	(1.10-8.19)
	≤p10															
FXII:ag	-	-	385	0.62	107	0.55	1	[ref]	1	[ref]	70	0.43	1	[ref]	1	[ref]
	-	+	171	0.28	62	0.32	2.27	(1.52-3.60)	1.85	(1.17-2.92)	77	0.47	2.41	(1.52-3.82)	2.32	(1.40-3.84)
	+	-	28	0.05	11	0.06	1.19	(0.61-2.31)	1.36	(0.62-2.97)	9	0.06	0.81	(0.35-1.89)	0.81	(0.30-2.17)
	+	+	33	0.05	14	0.07	3.14	(1.19-8.26)	3.45	(1.15-10.3)	7	0.04	6.15	(2.39-15.9)	7.31	(2.69-19.9)
PK:ag	-	-	371	0.60	108	0.56	1	[ref]	1	[ref]	73	0.45	1	[ref]	1	[ref]
	-	+	184	0.30	62	0.32	2.07	(1.39-3.08)	1.74	(1.11-2.72)	76	0.47	2.54	(1.62-3.99)	2.56	(1.56-4.21)
	+	-	41	0.07	10	0.05	0.36	(0.12-1.05)	0.33	(0.11-1.06)	6	0.04	0.90	(0.35-2.31)	1.03	(0.37-2.87)
	+	+	21	0.03	14	0.07	2.82	(1.07-7.41)	1.93	(0.64-5.84)	8	0.05	5.64	(2.08-15.3)	4.80	(1.64-14.0)

All odds ratios are adjusted for stratification factors (i.e. age, area of residence and index year). OR_1 is additionally adjusted for hypertension, diabetes and hypercholesterolaemia. OR = odds ratio, N = number, ref = reference category, OC=oral contraceptive use in the year prior to index year, FXII:ag = antigen level of coagulation factor XII, PK:ag = antigen level of prekallikrein.

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