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Prothrombotic factors and the risk of myocardial infarction and ischaemic stroke in young women;

differences, similarities and implications

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Promotores: Prof. dr. A. Algra
Prof. dr. F.R. Rosendaal

Overige leden: Prof. dr. H. ten Cate
Prof. dr. J.W. Jukema
Dr. M.J.H. Wermer

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CONTENTS

1 General introduction

Introduction

This thesis discusses the role of coagulation proteins in the aetiology of cardiovascular disease, especially myocardial infarction and ischaemic stroke. The underlying question in each chapter is not only the effect of the protein under study, but also whether this effect differs for these two cardiovascular diseases. Ultimately, this thesis will discuss the implications of the findings for future research and treatment of myocardial infarction and ischaemic stroke. As an introduction to this thesis, this chapter provides background information on myocardial infarction and ischaemic stroke, the coagulation system, the RATIO study and an overview of the following chapters.

Myocardial infarction

Mechanism Myocardial infarction occurs when the flow of oxygen-rich blood to the downstream tissue is restricted and the oxygen demands can no longer be met, leading to tissue death. A reduced blood flow can be caused by stenosis due to an atherosclerotic lesion or a 'plaque' . This can cause angina but often the blood supply is sufficient to sustain the downstream myocardium when oxygen demands are low. However, these atherosclerotic plaques may erode or rupture exposing the content of the lesion directly to the blood. This content is highly thrombogenic and activates blood coagulation, thrombus formation and thereby suddenly reduces or even completely blocks blood flow, resulting in infarction of the myocardium.

Epidemiology The average age-adjusted incidence of myocardial infarction in the Netherlands is 2.3 per 1000 person-years (2.9 per 1000 person-years for men and 1.7 per 1000 person-years for women.[1] With age as the largest determinant of the incidence, these incidences strongly differ per age category. For example, myocardial infarction occurs 0.4 times per 1000 person- years in young white men aged 30-39 and up to 13.7 per 1000 person-years for men aged 70-79. For women these two figures are 0.1 and 6.9 per 1000 person-years. This sex disparity is also present in the average age of onset for myocardial infarction in data from the United States of America which is 64.5 years for men and 70.3 for women.[2] Data from the United Kingdom and the United States of America paint a similar picture, although the incidences may be more pronounced than in the Netherlands.[1,3] The case fatality rate of patients suffering from myocardial infarction is approximately 15% within one year after the event.[2] Classical cardiovascular

risk factors include, but are not limited to, age, sex, hypertension, smoking, unhealthy diet, abnormal blood lipid levels, diabetes, and abdominal obesity.[2]

Ischaemic stroke

Mechanism Stroke is a cardiovascular disease of the brain in which a rapid decline of brain function occurs and of which the aetiology can be roughly divided in cerebral bleeding and cerebral ischaemia. In the United States of America, 10% of all strokes constitute an intracerebral haemorrhage, 3% a subarachnoid bleeding and 87% an ischaemic stroke.[2] Ischaemic stroke, like myocardial infarction, occurs when blood flow is markedly reduced with ischaemia as a result. The location of the infarcted cerebral tissue is dependent on the artery with reduced blood flow, resulting in a plethora of phenotypes. Several systems have been proposed to classify ischaemic strokes, such as the Oxford Community Stroke Project classification and the TOAST criteria.[4,5] The latter, proposed in 1993, has become widely used in both the clinic and research and classifies ischaemic strokes into five categories based on clinical presentation and further investigation. The five categories are defined by the location or presumed cause of the ischaemic stroke: large-artery atherosclerosis, cardio-embolism, small-vessel occlusion, stroke of other determined aetiology and stroke of undetermined aetiology. Such a classification is helpful in public health planning, diagnosis, subsequent treatment choices, but also in research by reducing the heterogeneity of the studied disease and its underlying causes.

Epidemiology The average age-adjusted incidence of stroke in the Netherlands is 1.9 per 1000 person-years.[6] As with myocardial infarction the incidence of ischaemic stroke rises sharply with age, although there is no clear sex disparity.[2,3,6] The 30 days case fatality of ischaemic stroke ranges between 8% and 12% and increases with age.[3] Functional recovery after stroke occurs in 50-70% of stroke survivors, whilst it leaves 15-30% permanently disabled. Major risk factors for ischaemic stroke are for example, in addition to classic atherogenic risk factors, atrial fibrillation, and carotid stenosis.[2]

Coagulation system

Traditionally, the biological system involved in haemostasis is divided in two parts, the primary haemostatic system focused around blood platelets and the secondary haemostatic system focused around the formation of fibrin.[7] This latter system comprises a series of activation steps leading to clot formation. Each of these activation

steps involved a zymogen (inactive enzyme precursor) being activated by a serine protease. Once activated, this protein can activate the next zymogen ultimately leading to the conversion of fibrinogen to fibrin by thrombin. This 'waterfall' or 'cascade' model was simultaneously proposed by Davie and Ratnoff in the journal Science and McFarlane in the journal Nature (see figure 1).[8,9] Both showed coagulation factor XII as the starting point of their cascade, but the mechanism was not clear. MacFarlane noted that 'contact' of FXII to 'foreign surfaces' activates this protein and hypothesised that the unfolding of the protein was the underlying mechanism. Later, when the complexity of the secondary coagulation system became more apparent a distinction was made between the intrinsic, extrinsic and common pathway.[7] The intrinsic coagulation pathway, also known as the contact activation pathway, was long considered of lesser importance in thrombosis and haemostasis, since persons who are deficient in the proteins of this part of the coagulation cascade have a mild or absent bleeding phenotype.[10] However, recent evidence suggests that these proteins are actively involved in the amplification of the thrombotic response and even pathologic thrombus formation.[11] Part of this knowledge is summarised in the so-called flywheel model (figure 1, panel C): small amounts of thrombin are formed by activated coagulation factor X with coagulation factor V as a cofactor. Factor X can be activated by two routes, i.e. after tissue damage driven tissue factor release or factor XI activation. This small amount of thrombin can then activate more factor XI, amplifying the thrombotic response which leads to the formation of fibrin monomers which are crosslinked by coagulation factor XIII to result in a stabilised haemostatic plug.[12–16] Additionally, it has become clear that the coagulation system is not an isolated system and has several links to other biological processes such as primary haemostasis, fibrinolysis, atherosclerosis, and inflammation.[10,17–22]

The RATIO study

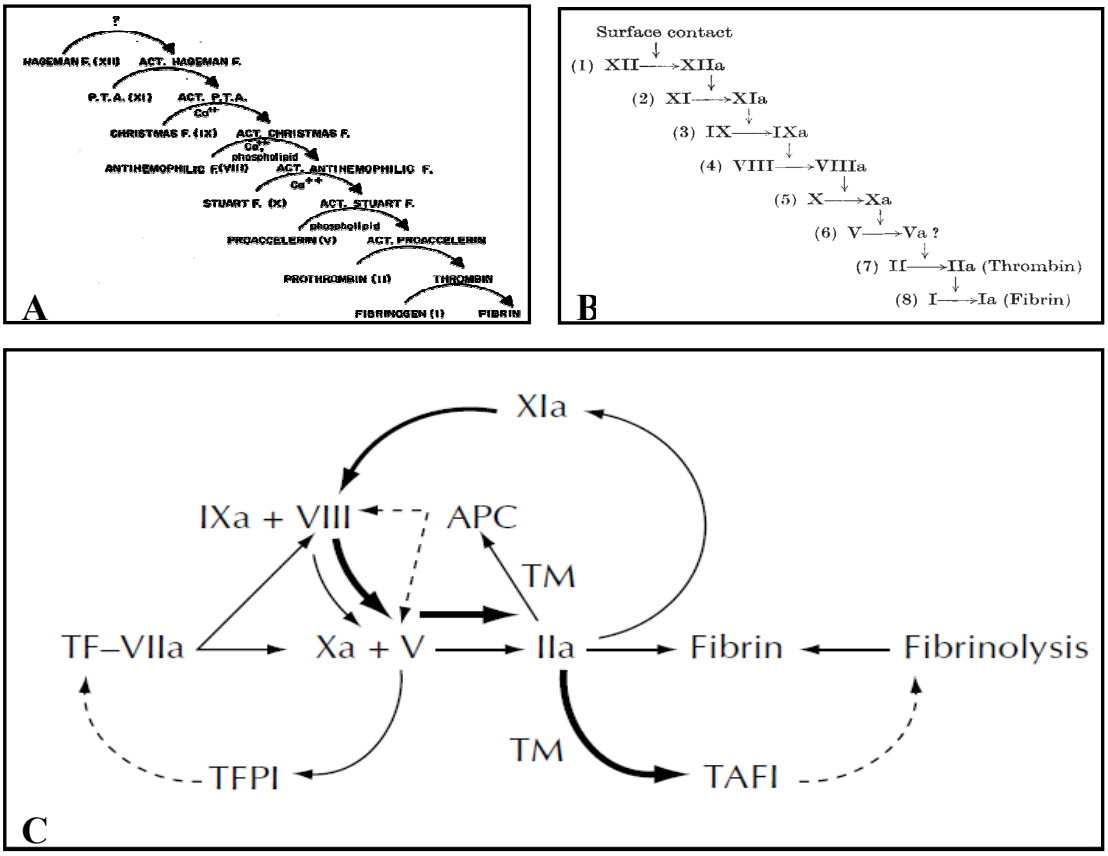
Motivation To investigate the role of thrombotic factors in the aetiology of both myocardial infarction and ischaemic stroke the studies presented in this thesis are embedded within the RATIO (Risk of Arterial Thrombosis In relation to Oral contraceptives) study.[23–25] There are several reasons why the RATIO study provides a good opportunity to answer our research questions. First of all, the RATIO study only included women under 50 years. The implication of this choice is that the prevalence of comorbidities such as hypertension, hypercholesterolaemia, and diabetes, is kept to a

minimum. Therefore, the effects of prothrombotic risk factor are easier to identify in the young than in the elderly where the abundance of comorbidities could obscure these effects. There is a drawback to the use of this age category; the incidence of myocardial infarction and ischaemic stroke is low rendering well-powered prospective cohort study unfeasible. However, the RATIO study uses a population-based case-control study design which allows the calculation of odds ratios as estimates of rate ratios with sufficient power.[26] Secondly, because the RATIO study was initially designed with a focus on the risk of myocardial infarction and ischaemic stroke in relation to oral contraceptive use, detailed information on oral contraceptive use is available. Previous studies have shown that oral contraceptives use changes the expression and activity of certain coagulation factors and can synergistically increase the risk conferred by other prothrombotic risk factors.[27–29] Therefore, oral contraceptive use might be an important player in the risk of myocardial infarction and ischaemic stroke in young women and the RATIO study provides a unique opportunity to investigate this topic. Thirdly, the RATIO study allows us to investigate two acute forms of arterial thrombosis, i.e. myocardial infarction and ischaemic stroke, within one study. This means that the results regarding these two diseases can be compared more easily than when they are derived from different studies with different designs and limitations. The incidences of myocardial infarction and ischaemic stroke are not only low, but also similar: approximately 0.14 per 1000 women under the age of 50 per year experience a myocardial infarction, whereas the incidence of ischaemic stroke is 0.12 or 0.14 per 1000 person-years, depending on the ICD codes used[1,6] This implies that the estimates obtained in the RATIO study can be compared directly without scaling effects.

Study design Eligible patients were women aged 18–50 years who were admitted for a first myocardial infarction or ischaemic stroke to one of the 16 participating centres (eight academic centres and eight large, non-academic hospitals) between 1990 and 1995. Myocardial infarction was confirmed by symptoms, elevated concentrations of cardiac enzymes, and changes seen on electrocardiography; ischaemic stroke without an overt cardioembolic source was confirmed by medical history, physical examination, and CT or MRI scans, which were assessed by experienced neurologists at the participating centres. Exclusion criteria were transient ischaemic attack that lasted less than 24 hours, haemorrhagic stroke, cerebral sinus venous thrombosis, carotid artery dissection,

aphasia or cognitive impairment that prevented completion of the study questionnaire, or not speaking Dutch. It is important to note that women with an overt cardioembolic source were excluded, although it cannot be ruled out that a small percentage of the participants indeed suffered from a cardiac embolic stroke.

Figure 1. Graphic representations of the coagulation system

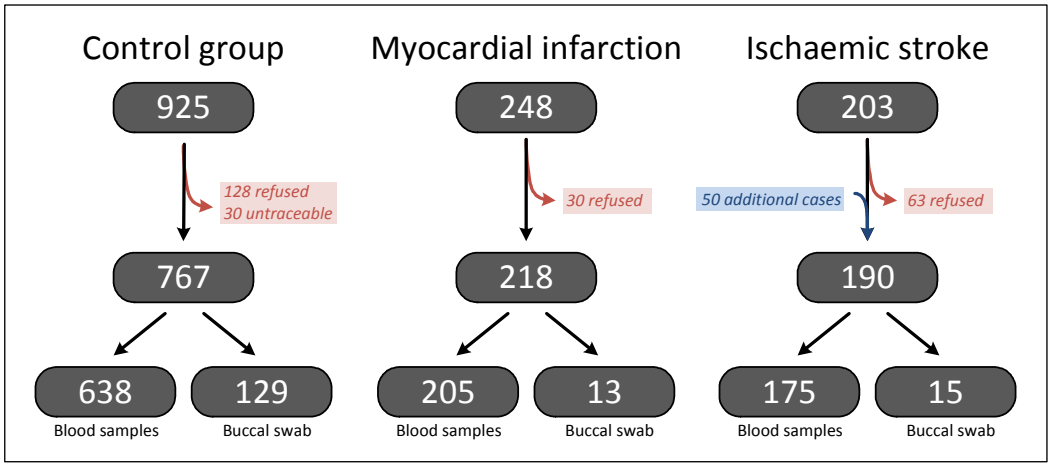


Panel A (Davie and Ratnoff, Science 1964) and panel B (MacFarlane, Nature 1964) picture the waterfall or cascade model of coagulation which has long been the basis of thrombosis and haemostasis research. New insights lead to several additions and revisions. For example, Bouma and Meijers proposed a fly wheel model (Current opinions in haematology, 2000) which includes the important role of coagulation factor XI in both amplification of the thrombin formation and the downregulation of fibrinolysis (Panel C).[8,9,13]

Control subjects were recruited by random-digit dialling, a technique based on the generation of a random set of telephone numbers that does not use existing telephone number databases. Each number was dialled at least seven times or until contact was made. Individuals were included as controls when they were women, aged 18–50 years, free of coronary, cerebral or peripheral arterial disease and did not meet the same exclusion criteria that were used for patient recruitment. Controls were frequency-matched to patients for age, residence area, and index date of the event (defined as mid-year of the same year as the event). Since control subjects were sampled from a dynamic population and matched on person-time, the distribution of exposure in the control group can be regarded as the distribution of person-time in the source population and the odds ratios calculated in the RATIO study can therefore be interpreted as measures of rate ratios.[26] The matching procedure was chosen to ensure an optimal number of controls in each of the stratification categories given the number of cases. In this first phase of the RATIO study, women were asked to complete an extensive, structured and standardised questionnaire which was focussed on classic cardiovascular risk factors, family history and oral contraceptive use. The questions referred to the period before the index date (the date of myocardial infarction or ischaemic stroke for patients and mid-year of the same year for controls). A total of 248 women with myocardial infarction and 203 women with ischaemic stroke and 925 control subjects participated in this first phase of the RATIO study.

In the second phase of the RATIO study all participating women were requested to donate either blood or a buccal swab for analyses. Not all women participated in this second phase. From the initial 248 women with myocardial infarction 30 refused to participate, leaving 218 cases. From the initial 203 women with ischaemic stroke, six had died, 10 refused to participate, 44 could not be traced and blood sampling failed in three, leaving 140 cases. From the initial 925 controls 128 refused to participate and 30 could not be traced, leaving 767 controls (see figure 2). To counteract this loss of statistical power in the ischaemic stroke group, an additional 50 women who presented with an ischaemic stroke at the University Medical Center Utrecht were additionally recruited between 1996 and 2001 using the same in- and exclusion criteria.

Figure 2. Flowchart of RATIO study participants



Blood samples were taken by venipuncture into tubes containing 0.106 M tri-sodium citrate for coagulation assays. Plasma was obtained by centrifugation of whole anticoagulated blood at 2000 G for 10 min and stored at –80°C. EDTA-anticoagulated blood was used for DNA extraction. If participants were adverse to venepuncture, a buccal swab was offered as an alternative for DNA extraction. In total, blood samples were available from 205 cases with myocardial infarction, 175 cases with ischaemic stroke and 638 control subjects. Blood was collected after a median of 69 months (range 38 - 117) for myocardial infarction and 95 months for ischaemic stroke cases (range 23-146) thereby ensuring blood was sampled after the acute phase to minimise the risk of revere causation, but increasing the likelihood of changes over time resulting in attenuation bias.

With these numbers, the RATIO study has an approximate statistical power of 82% in the myocardial infarction analyses to detect an odds ratio of 1.65 with an exposure prevalence of 25% in controls under an alpha of 5%. The statistical power, with the same parameters, in the ischaemic stroke analyses is lower, i.e. 78%, given the lower number of ischaemic stroke cases. Similarly, a doubling in risk, given a 10% exposure prevalence in controls under an alpha of 5%, can be detected with 82% power in the ischaemic stroke analyses and 86% power in the myocardial infarction analyses. These figures, however, are approximations of the true power of the RATIO study due to the frequency-matched character of the study which is not accounted for in these calculations. The

matching procedure needs to be taken into account in the logistic regression analyses by including the matching variables (i.e. age, area of residence and index year) as covariates. In logistic regression analyses, this generally leads to a loss of precision, especially when the covariates are weak predictors of the outcome.[30,31]

Outline of this thesis

The research presented in this thesis is focused on the role of thrombotic factors in the aetiology of myocardial infarction and ischaemic stroke and whether this role differs between these two diseases.

As a first exploration of a difference between these two cardiovascular diseases, the relation between a positive family history of either myocardial infarction, stroke (of all types) or a combination thereof and the risk of myocardial infarction and ischaemic stroke is investigated in ***chapter 2***.

The intrinsic coagulation proteins are dealt with subsequently: the relation between activation of these proteins and myocardial infarction and ischaemic stroke is studied in ***chapter 3***, followed by the relationship of the antigen levels of these proteins and the diseases of interest in ***chapters 4 and 5***. The role of the non-enzymatic protein of the intrinsic coagulation cascade, i.e. high-molecular weight kininogen, is interrogated in ***chapter 6.*** This co-factor is important in the binding of the intrinsic coagulation proteins to negatively charged surfaces, and therefore promotes the activation of the intrinsic coagulation proteins.

The following section uses a genetic approach to determine whether proteins from the common pathway for coagulation are involved in myocardial infarction and ischaemic stroke. First, ***Chapter 7*** discusses a particular form of instrumental variable analysis also known as Mendelian Randomisation, which uses genetic variation as a proxy for phenotypic changes that might lead to disease. Although this technique is not formally used in the following chapters, its line of reasoning is used in ***Chapter 8***. This chapter presents results on the causal role of fibrinogen levels in the aetiology of myocardial infarction and ischaemic stroke based on the associations between genetic variation on one hand and disease and fibrinogen levels on the other. ***Chapter 9*** focuses on the question whether the risk of myocardial infarction is affected by genetic variation in the

gene coding for coagulation XIII of which previous RATIO investigators showed there was an increase in risk of ischaemic stroke.

Besides an increase in coagulation propensity a decreased fibrinolytic propensity might also increase the risk of myocardial infarction and ischaemic stroke. ***Chapter 10*** addresses the results of our analyses on fibrinolysis capacity as a risk factor for myocardial infarction and ischaemic stroke as measured by clot lysis time. Other thrombotic conditions are the topic of the two following chapters. ***Chapter 11*** investigates the role of Von Willibrand Factor and its natural counterpart ADAMTS13. ***Chapter 12*** investigates the markers of the anti-phospholipid syndrome and their effect on the risk of myocardial infarction and ischaemic stroke.

Finally, chapter 13 summarises the findings of the study presented in this thesis and discusses their similarities, differences and implications for our understanding of the etiologic mechanisms, for future research and for treatment of myocardial infarction and ischaemic stroke.

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2

Family history differs between young women with myocardial infarction and ischaemic stroke

Bob Siegerink, Frits R Rosendaal and Ale Algra

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Abstract

Introduction The phrases 'cardiovascular disease' and 'arterial thrombosis' might suggest that myocardial infarction (MI) and ischaemic stroke (IS) share a common aetiology and risk factors. This would imply that the family history of one disease would be predictive of occurrence of the other. A positive family history may help in risk stratification, especially in the young. This study assesses the specific predictive value of a positive family history of MI and stroke (of all types) for specific outcomes.

Methods The RATIO study is a population-based case-control study including women with MI (N=248), IS (N=203) and 925 healthy controls, frequency-matched on age, year of event and area of residence. Odds ratios (OR) and 95% confidence intervals (CI) were calculated as measures of rate ratios for a positive family history of MI or stroke (affected first-degree relative <60 years) with logistic regression analyses.

Results The risk of MI was almost fourfold increased in women with a family history positive for myocardial infarction (OR 3.70, 95%CI 2.68-5.10), whereas the risk of IS was, if anything, only slightly elevated (1.25, 0.83-1.87). A family history of stroke was associated with a twofold increased MI risk (2.00, 1.29-3.12), whereas the IS risk was again not clearly associated (1.37, 0.79-2.40).

Discussion In young women, a positive family history of cardiovascular disease is a predictor for MI but not for IS. The differences between the associations of family history of myocardial infarction and stroke might be caused by the heterogeneous nature of stroke. These findings support the notion that MI and IS have different risk factor profiles and therefore different aetiologies.

Introduction

The term 'arterial thrombosis' is used to denote disorders resulting from obstructive arterial thrombi. The reduction in blood flow can result in acute ischaemia and may present clinically as myocardial infarction or ischaemic stroke. The more common term 'cardiovascular disease' is used even more broadly and can include, but is not restricted to, myocardial infarction, angina, stroke, peripheral arterial disease but also venous thrombosis and even hypertension and diabetes .[1–4] The use of one term for all forms of cardiovascular disease is common, but can be misleading in aetiologic studies since these diseases could have, despite several common risk factors, different causal mechanisms.[4–6]

Risk stratification is used to identify those at high risk of developing a disease, and consequently to consider preventive measures. Family history can be used as such a tool: it can determine whether disease clusters in families and acts as a proxy for both genetic and environmental risk factors. The advantages of such an aggregate measure in a clinical setting is that family history is obtained easily and likely to be reported accurately.[7,8] However, the predictive value of a positive family history as a risk factor for cardiovascular diseases may differ per disease and patient group.

Previous research in different patient populations has shown that a positive family history of myocardial infarction is associated with an approximate doubling of the risk of myocardial infarction.[4,8] The risk of ischaemic stroke associated with a positive family of ischaemic stroke has also been studied extensively; a systematic review of 53 heterogeneous studies indicated that a positive family history of stroke is associated with a small increase in stroke risk whereas these associations are likely to differ for the several subtypes of stroke.[9–13] The addition of family history to a predictive model improved the prognostic value of some models, but not all.[14–16] This is not surprising since improvements of prediction models can only be achieved by including variables with a strong association.[17] One of the factors that could determine the value of family history is the age of the patient group. Since the incidence of cardiovascular disease rises sharply with increasing age, mainly due to the accumulation of traditional risk factors and a shift in the distribution of ischaemic stroke subtypes, an association between a positive family history and MI or IS is therefore likely to be stronger when studies are focused on early onset of disease and potentially more useful in risk stratification in these age categories.[15]

As both environmental and genetic risk factors for MI and IS might differ in their effects, the versus one of IS may differ too. Such differences would have direct consequences for the use of family history in risk stratification. In this study we first aim to determine the association of a family history of arterial thrombosis defined as a history of myocardial infarction or stroke (of all types) and the risk of MI and IS. Additionally, we impact of a family history of MI will also determine whether a positive family history of one of these diseases is equally prevalent in patients suffering from the other disease.

Table 1. Characteristics of participants

	Myocardial infarction N=248		Ischaemic stroke N=203		Control N=925	
Age (mean, SD)	42.9	(6.1)	39.4	(8.4)	38.1	(8.3)
Caucasian ethnicity	234	(94%)	188	(93%)	864	(93%)
History of *						
Hypertension	59	(24%)	48	(24%)	55	(6%)
Diabetes	15	(6%)	11	(5%)	13	(1%)
Hypercholesterolaemia	28	(11%)	10	(5%)	24	(3%)
Oral contraceptives use *	99	(40%)	100	(49%)	340	(37%)
Smoking *	208	(84%)	126	(62%)	395	(43%)
Family members affected by myocardial infarction before the age of 60 years						
Father	73	(29%)	40	(20%)	138	(15%)
Mother	39	(16%)	9	(4%)	40	(4%)
Brother	38	(15%)	12	(6%)	41	(4%)
Sister	16	(6%)	5	(2%)	12	(1%)
Family members affected by stroke before the age of 60 years						
Father	14	(6%)	14	(7%)	36	(4%)
Mother	18	(7%)	10	(5%)	37	(4%)
Brother	7	(3%)	2	(1%)	10	(1%)
Sister	9	(4%)	4	(2%)	7	(1%)

** in the year prior to event (cases) or index year(controls).*
SD = standard deviation, percentages might not add due to rounding.

Methods

Study design & participants The RATIO (Risk of Arterial thrombosis in relation to oral contraceptives) study is a nationwide multicenter case-control study designed to search for risk factors for myocardial infarction , ischaemic stroke and peripheral arterial disease in young women as has been described earlier.[18–20] The current study focuses on the two acute forms, myocardial infarction and ischaemic stroke. In short, women between the ages 18 and 50 who were diagnosed with a form of arterial thrombosis in the 16 participating hospitals were asked to participate. Diagnosis of myocardial infarction was based on the presence of symptoms, elevated cardiac-enzyme levels, and electrocardiographic changes (N=248), whereas ischaemic stroke was diagnosed on the basis of medical history, neurological examination, and CT or MRI scan by experienced neurologists in the participating centres. (N=203) The control group comprised women not suffering from myocardial infarction, ischaemic stroke or peripheral arterial disease who agreed to participate after they were approached by random digit dialling (N=925). The control group was frequency-matched with the case groups on age (in five year categories), year of event and area of residence. Al participants were asked to fill in a standardised questionnaire on several topics such as demographic characteristics, medical history among which family history and oral contraceptive use in index year. The study protocol was approved by the ethics committees of all participating hospitals and oral informed consent was obtained from all participants.

Definitions A positive first degree relative family history of arterial thrombosis was defined as having a first degree relative (parent, sibling) with either a 'myocardial infarction' or a 'stroke' before the age of 60. We use the more generic term 'stroke' instead of ischaemic stroke deliberately because we cannot distinguish haemorrhagic from ischaemic stroke with the questions used in the questionnaire. For clarity and emphasis, we will not use abbreviations to refer to the family history. We also determined the risks associated with a family history positive for myocardial infarction or stroke separately. Additionally, a positive parental history was defined in a similar manner, but restricted to affected parents. To account for a variation in the number of siblings and to investigate a graded dose-dependent association we defined the percentage of affected first degree relative and categorised these as 0% (no affected sibling) >0-20%, 20-40% and 40% and up. Graded associations in parental family history were determined by investigating the number of affected parents.

Table 2. Risk of myocardial infarction and ischaemic stroke due to a family history of arterial thrombosis

History of arterial thrombosis in first degree relatives	control	Myocardial infarction			Ischaemic stroke		
	N (%)	N (%)	OR*	95%CI	N (%)	OR*	95%CI
Negative family history of AT	655(74%)	98 (43%)	1	[ref]	119 (66%)	1	[ref]
Positive family history of AT	229(26%)	130 (57%)	3.45	(2.51-4.75)	62 (34%)	1.35	0.91-1.99
Parental history of arterial thrombosis							
No parent affected	693(78%)	132 (54%)	1	[ref]	131 (70%)	1	[ref]
Any parent affected	201(22%(	114 (56%)	2.87	2.09-3.94	56 (30%)	1.27	0.85-1.89
One parent affected	180(20%)	95 (39%)	2.62	1.88-3.65	49 (26%)	1.29	0.85-1.96
Both parents affected	21(2%)	19 (8%)	5.38	2.64-11.0	7 (4%)	1.14	0.40-3.27
AT in father	155(17%)	80 (33%)	2.24	1.59-3.14	47 (25%)	1.32	0.86-2.03
AT in mother	67(7%)	53 (22%)	3.42	2.24-5.22	16 (9%)	1.03	0.54-1.97

AT = Arterial Thrombosis, OR = Odds Ratio, CI = Confidence Intervals. Percentages might not add due to rounding.
**Odds ratios are calculated with the control group as reference and adjusted for stratification factors (i.e. area of residence, year of event and age (continuous.)). A family history of AT is defined as myocardial infarction or stroke before the age of 60 in a first degree relative*

Statistical analyses We calculated odds ratios and corresponding 95% confidence intervals (OR, 95%CI) from logistic models as measures of rate ratios. All models included age, index year and area of residence to account for the matching strategy.

Results

Traditional risk factors were more present in both case groups than in the control group (see table 1). Data on family history were missing in some participants resulting in 884 control women, 228 MI cases and 181 IS cases available for the analyses. Data from missing individuals (20 MI cases, 22 IS cases and 41 controls) were considered as missing at random.

Family history of arterial thrombosis A positive history of arterial thrombosis in a first degree relative was more prevalent in patients with MI and to a lesser extent in IS patients than in controls (table 2). The risk of MI was increased in women with a positive family history of arterial thrombosis (OR 3.45, 95%CI 2.51 - 4.75), whereas the risk of IS was not substantially affected (OR 1.35, 95%CI 0.91 - 1.99). A similar pattern was observed when the analyses were restricted to parental history of arterial thrombosis.

Family history of myocardial infarction A positive family history of myocardial infarction increased the risk of MI almost 4-fold (OR 3.70, 95%CI 2.68 - 5.10), whereas the risk of IS remained unchanged (OR 1.25, 95%CI 0.83 -1.87) (table 3). The risk of IS was only increased when >40% of first degree relatives had been affected with myocardial infarction. A similar pattern was observed for the analyses restricted to parental history of myocardial infarction: the risk of MI increased 3-fold (OR 3.00, 95%CI 2.17 - 4.15), and the risk of IS remained unaffected (OR 1.21, 95%CI 0.80 - 1.85).

Family history of stroke A positive family history of stroke (both ischaemic and haemorrhagic) was associated with a doubling of MI risk (OR 2.00, 95%CI 1.29 - 3.12), whereas the risk of IS was only marginally increased (OR 1.37, 95% CI 0.79 - 2.40) (table 3). With more than 40% of their first degree relatives affected by stroke the risks were highest (MI OR 8.44, 95%CI 1.74 - 41; IS OR 6.78, 95%CI 1.15 - 40).

Discussion

A positive family history of arterial thrombosis is associated with an increased risk of MI and not clearly associated with IS risk. Furthermore, we found that a positive family history

of myocardial infarction was more frequent in cases who suffered from MI than in IS cases. A positive family history of stroke was associated with a moderate increase in risk of both MI and IS and the difference between MI and IS was less clear in these analyses. This was especially so in the analyses of parental family history, where most associations were less pronounced. So, our results show that a family history of neither myocardial infarction nor stroke are strong predictors of IS, whereas both are associated with MI indicating that the use of a combined family history as a predictor of disease is limited.

Our results are in line with a recent study on family clustering of coronary and cerebral events; myocardial infarction tends to cluster more in families compared with ischaemic stroke.[21] This is perhaps a demonstration of the heterogeneous nature of ischaemic stroke, as is hypothesised by the auhors: myocardial infarction may be primarily caused by atherosclerotic plaque instability and subsequent rupture, where thromboembolisms and small vessel disease also determines ischaemic stroke risk. The OXVASC study also investigated the relation between the two diseases and showed there was sex-specific familial clustering across different vascular beds.[22] The RATIO study only included women and therefore lacks the data to confirm this sex-differential heritability.

Our study has some limitations. The information on family history was self-reported and was not validated. However, previous studies indicated that questionnaires are highly accurate on family history, especially among women and the young.[7] Women who had missing data regarding their family history were excluded from our analyses. Upon closer examination of these data (not shown) the women with incomplete data were slightly more likely to rapport classical risk factors, although groups were too small to perform formal statistics. This means that, if anything, our point estimates are an underestimation of the true effects. Our questionnaire can also be a source of misclassification bias: the questions did not differentiate between ischaemic or haemorrhagic stroke. This could have affected our results if only a family history of ischaemic stroke affects risk in family members. However, ischaemic stroke makes up the bulk of all strokes, especially in stroke under 60 years of age, minimising the effect of this non-differential misclassification.[4]

Myocardial infarction is thought to manifest at an earlier age than ischaemic stroke.[4,23] This could mean that the affected parents of an ischaemic stroke patient are older than parents of a patient suffering from a myocardial infarction which could result in a differential chance of being ‘exposed’ for the two case groups introducing bias. However, since our

study is an age-matched case-control study with subjects under 50 years of age, and a positive family history defined as a event before the age of 60, this effect is minimised. Nonetheless, our restriction to young patients results in a lower prevalence of a positive family history resulting in a lower power for our analyses.

Conclusion Our results indicate that a family history of the cardiovascular diseases myocardial infarction and stroke is a predictor of MI but not for IS, limiting the potential use of a these family histories for risk stratification. Additionally the difference in associations of these aggregate measures between the MI and IS analyses raises the question whether these two diseases are different diseases and should preferably be treated as such in aetiologic research.

Table 2. Risk of myocardial infarction and ischaemic stroke due to a family history of myocardial infarction and ischaemic stroke

	Control	Myocardial infarction			Ischaemic stroke		
	N (%)	N (%)	OR	95%CI	N (%)	OR	95%CI
History of MI in first degree relatives							
FDR MI -	702(78%)	114 (49%)	1	[ref]	131 (71%)	1	[ref]
FDR MI +	194(22%)	121 (51%)	3.70	2.68-5.10	54 (29%)	1.25	0.83-1.87
FDR MI >0-20%	86(10%)	47 (20%)	3.04	1.97-4.68	22 (12%)	1.15	0.69-2.06
FDR MI >20-40%	89(10%)	52 (22%)	3.81	2.49-5.83	23 (12%)	1.19	0.70-2.12
FDR MI >40%	19(2%)	22 (9%)	6.28	3.11-13	9 (5%)	1.93	0.72-4.73
Parental history of MI							
No parent affected	734(81%)	146 (59%)	1	[ref]	143 (75%)	1	[ref]
Any parent affected	169(19%)	100 (41%)	3.00	2.17-4.15	48 (25%)	1.21	0.80-1.85
One parent affected	160(18%)	88 (36%)	2.79	1.99-3.90	47 (25%)	1.27	0.83-1.94
Both parents affected	9(1%)	12 (5%)	7.36	2.78-19.5	1 (1%)	0.36	0.03-3.82
MI in father	138(15%)	73 (30%)	2.30	1.62-3.28	40 (21%)	1.26	0.80-1.97
MI in mother	40(4%)	39 (16%)	4.34	2.62-7.17	9 (5%)	0.82	0.35-1.92
History of stroke in first degree relatives							
FDR stroke -	814(91%)	189 (83%)	1	[ref]	166 (87%)	1	[ref]
FDR stroke +	76(9%)	40 (17%)	2.00	1.29-3.12	24 (13%)	1.37	0.79-2.40
FDR stroke >0-20%	41(5%)	11 (6%)	1.97	1.12-3.45	11 (6%)	1.14	0.53-2.44
FDR stroke >20-40%	32(4%)	10 (5%)	1.53	0.72-3.21	10 (5%)	1.30	0.55-3.07
FDR stroke >40%	3(0.5%)	5 (2%)	8.44	1.74-41	3 (2%)	6.78	1.15-40
Parental history of stroke							
No parent affected	834(92%)	217 (88%)	1	[ref]	174 (89%)	1	[ref]
Any parent affected	58(8%)	29 (12%)	1.52	0.93-2.49	22 (11%)	1.35	0.75-2.41
One parent affected	63(7%)	26 (11%)	1.45	0.87-2.42	20 (10%)	1.36	0.74-2.48
Both parents affected	5(1%)	3 (1%)	2.67	0.54-13.2	2 (1%)	1.22	0.17-9.04
Stroke in father	36(4%)	14 (6%)	1.35	0.68-2.69	14 (7%)	1.53	0.72-3.25
Stroke in mother	37(4%)	18 (7%)	1.77	0.97-3.28	10 (5%)	1.13	0.50-2.51

FDR = first degree relative, OR = Odds Ratio, CI = Confidence Intervals. Percentages might not add due to rounding. Odds ratios are calculated with the control group as reference and adjusted for stratification factors (i.e. area of residence, year of event and age (continuous.)) A family history of MI is defined as myocardial infarction before the age of 60 in a first degree relative or parent, a family history of stroke is defined as a stroke before the age of 60 in a first degree relative or parent.

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3

Intrinsic coagulation activation and the risk of myocardial infarction and ischaemic stroke in young women

Bob Siegerink, Jose WP Govers-Riemslag, Frits R Rosendaal, Hugo ten Cate and Ale Algra

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Abstract

Introduction Classically, intrinsic coagulation proteins are thought to have a minor role in haemostasis. Recently, these proteins, especially FXII, were implicated as possible key players in the aetiology of thrombosis. This study aims to determine the risks of myocardial infarction and ischaemic stroke conferred by increased activation of intrinsic coagulation proteins in young women and the effect of oral contraceptive use on this association.

Methods To do so, intrinsic coagulation protein activation was determined in the RATIO study, a population based case control study including young women (18-50 years) with myocardial infarction (MI; N=205), ischaemic stroke (IS; N=175) and 638 healthy controls. Activated protein-inhibitor complexes were determined as measures of protein activation. These complexes consist of activated proteins bound to C1-esterase inhibitor (FXIIa:C1-inh, FXIa:C1-inh, Kallikrein-C1-inh) or antitrypsin inhibitor (FXIa:AT-inh). Odds Ratios (OR) and corresponding confidence intervals (95%CI) were calculated with logistic regression.

Results High levels of protein activation (>90th percentile of controls) showed an increased risk of IS: FXIIa:C1-inh (OR 2.1; 95%CI 1.3-3.5), FXIa:C1-inh (2.8; 1.6-4.7), FXIa:AT-inh (2.3; 1.4-4.0) Kallikrein:C1-inh (4.3; 2.6-7.2). If anything, MI risk was only increased by Kallikrein:C1-inh (1.5; 0.9-2.5). Oral contraceptive use further increased the risks.

Discussion High levels of activated proteins of the intrinsic coagulation system are associated with IS, but not with MI. This contradicts similar analyses among men in the Northwick Park Heart Study. Together with the finding that oral contraceptive use further increases the risks, the question whether the role of intrinsic coagulation proteins in the aetiology of ischaemic stroke thrombosis is sex-specific is raised.

Introduction

Arterial thrombosis occurs when an artery is occluded by a thrombus. Classical risk factors are diabetes, hypercholesterolemia, hypertension, smoking, obesity and oral contraceptive use. The most common manifestations are peripheral arterial disease, myocardial infarction and ischaemic stroke. Arterial thrombosis is common among the elderly and the most common cause of death in high income countries.[1]

A thrombus is formed upon activation of the coagulation system. Historically, this system has been characterised by two separate activating pathways, i.e. the extrinsic and intrinsic. The intrinsic coagulation pathway consists of the serine proteases coagulation factor XII (FXII), coagulation factor XI (FXI) and prekallikrein (PK). It further includes one non-enzymatic protein, i.e. High Molecular Weight Kininogen (HMWK).[2]

FXII has the capacity of autoactivation upon binding on negatively charged surfaces.[3,4] After activation, activated FXII (FXIIa) activates both FXI into activated factor XI (FXIa) and PK into its active form kallikrein (KAL). HMWK acts as a co-factor in both steps, during which the potent vasodilator bradykinin is released. FXIa further activates coagulation factor IX which forms together with cofactor VIIIa, Ca^{2+} ions and phospholipids the tenase complex, thereby activating the common pathway of the coagulation cascade.[5] A subform of FXIIa (also known as βFXIIa) is also known to activate coagulation factor VII, initiating the common pathway by priming the extrinsic pathway.[6] The intrinsic coagulation proteins are also involved in other biologic processes such as fibrinolysis (activation of plasminogen by FXIIa, FXIa, and KAL), vasoconstriction, inflammation and blood pressure control (KAL and bradykinin). Upon activation, the serine proteases of the intrinsic coagulation pathway are quickly bound by an inhibitor such as the C1-esterase inhibitory forming a protein-inhibitor complex which lacks the serine protease function.[7,8] An excess of these inhibitors is readily available in plasma, so the availability of activated intrinsic coagulation proteins forms the limiting step in the formation of these protein-inhibitor complexes. This makes these protein-inhibitor complexes a good measure of the level of activated proteins.

FXI deficiency causes mild bleeding in patients, whereas FXII, KAL and HMWK deficiencies do not.[2] These observations led to the traditional view that the intrinsic coagulation proteins do not play a major role in haemostasis, and are not likely to be a risk factor for thrombosis. However, recent evidence, both laboratory and clinical, indicates that these proteins may play a role in thrombus formation. Murine studies with FXII and FXI knockout

mice show that initiation of clot forming is not FXII dependent, but that propagation of clot formation is. [9,10] FXI deficient patients showed apart from their mild bleeding diathesis also a decreased risk of ischaemic stroke and venouos thrombosis, but this decrease is not observed for myocardial infarction.[11–13] In middle aged men, high levels of FXI and low levels of FXII increased the risk of MI.[14] An Austrian record-linkage study suggested that low FXII levels were associated with reduced overall death rates.[15] In the Northwick Park Heart Study, which included middle-aged men, low levels of activation from selected intrinsic coagulation proteins caused an increase in risk for both myocardial infarction and ischaemic stroke.[16] Since estrogens could have an impact on transcription levels of coagulation proteins, especially FXII due to an oestrogen receptive element in the promoter of the F12 gene, the relation between (intrinsic) coagulation proteins and the risk of arterial thrombosis is potentially different in females.[4,17]

It is unknown to what extent intrinsic coagulation proteins are involved in the pathophysiologic processes that lead to the different forms of arterial thrombosis, especially in young women. This patient group is of particular interest because of their use of oestrogen containing medication (i.e. oral contraceptives). We therefore set out to determine whether high levels of activation of the intrinsic coagulation system are associated with myocardial infarction or ischaemic stroke in young women and whether oral contraceptive use further increases this risk.

Methods

Study design & participants The Risk of Arterial Thrombosis In relation to Oral contraceptives (RATIO) study is a multicenter population-based case-control study. The study consists of three substudies, including patients with confirmed myocardial infarction, ischaemic stroke or peripheral arterial disease. One control group was frequency-matched to all three case groups. The study was initiated to evaluate the risk of arterial thrombosis due to the changing composition of oral contraceptive pills (1990 – 1995).[18–20] Blood and DNA were collected during the second phase of the study (1998 – 2002). Informed consent was obtained from all participants and the study was approved by the medical ethics committees of the participating hospitals. For the current study we report data from the myocardial infarction and ischaemic stroke substudies.

Patient selection has been described in detail previously.[18,19,21] In short, all women aged 18 to 50 years old who presented with a first event of myocardial infarction or ischaemic

stroke to one of the sixteen participating hospitals in the Netherlands between 1990 and 1995 were eligible and approached for study participation. A standardised questionnaire on patient characteristics and possible cardiovascular risk factors such as (familial) medical history, use of oral contraceptives and smoking habits was filled in by both cases and controls. Some of these questions were targeted to the year prior of diagnosis (cases) or the matched index year (controls). All participants were reapproached to donate blood or buccal swab for DNA analyses during the second phase of the study.

Measurements We determined protein-inhibitor complexes of the serine proteases of the intrinsic coagulation system as a measure of enzyme activation in citrated plasma. The inhibitor could either be a C1-esterase inhibitor for FXIIa, FXIa of kallikrein (FXIIa:C1-inh, FXIa:C1-inh, KAL:C1-inh) or antitrypsin inhibitor (FXIa:AT-inh). These complexes were measured by an ELISA, as described earlier.16 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.22,23 The FXIa protein-inhibitor assays both use the XI-5 mAB

Table 1. Characteristics of the RATIO participants

	Myocardial infarction N=205	Ischaemic stroke N=175	Control N=638
Age (mean)	43	39	39
Caucasian ethnicity	195 (95%)	167 (97%)	602 (94%)
History of *			
Hypertension	53 (26%)	50 (29%)	40 (6%)
Diabetes	10 (5%)	7 (4%)	10 (2%)
Hypercholesterolaemia	21 (10%)	14 (8%)	19 (3%)
Oral contraceptives use *	81 (40%)	92 (53%)	213 (33%)
Smoking *	169 (82%)	101 (58%)	270 (42%)
FXIIa:C1-inh, median (Q1-Q3)	0.23 (0.13-0.33)	0.28 (0.15-0.45)	0.24 (0.12-0.33)
FXIa:C1-inh, median (Q1-Q3)	0.23 (0.14-0.60)	0.37 (0.18-0.76)	0.23 (0.15-0.52)
FXIa:AT-inh, median (Q1-Q3)	0.24 (0.20-0.29)	0.29 (0.22-0.42)	0.25 (0.21-0.31)
KAL:C1-inh, median (Q1-Q3)	0.05 (0.03-0.13)	0.11 (0.03-0.27)	0.04 (0.03-0.12)

** in the year prior to event/index year, # levels are expressed as a proportion of fully activated normal pooled plasma. AT-inh, alpha 1-antitrypsin inhibitor; C1-inh, C1-esterase inhibitor; FXII, coagulation factor XII; FXI, coagulation factor XI; KAL, kallikrein; Q1-Q3, range of first and third quartile.*

as antigen, which recognises both the native and activated FXI form of FXI.24 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.25,26 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).

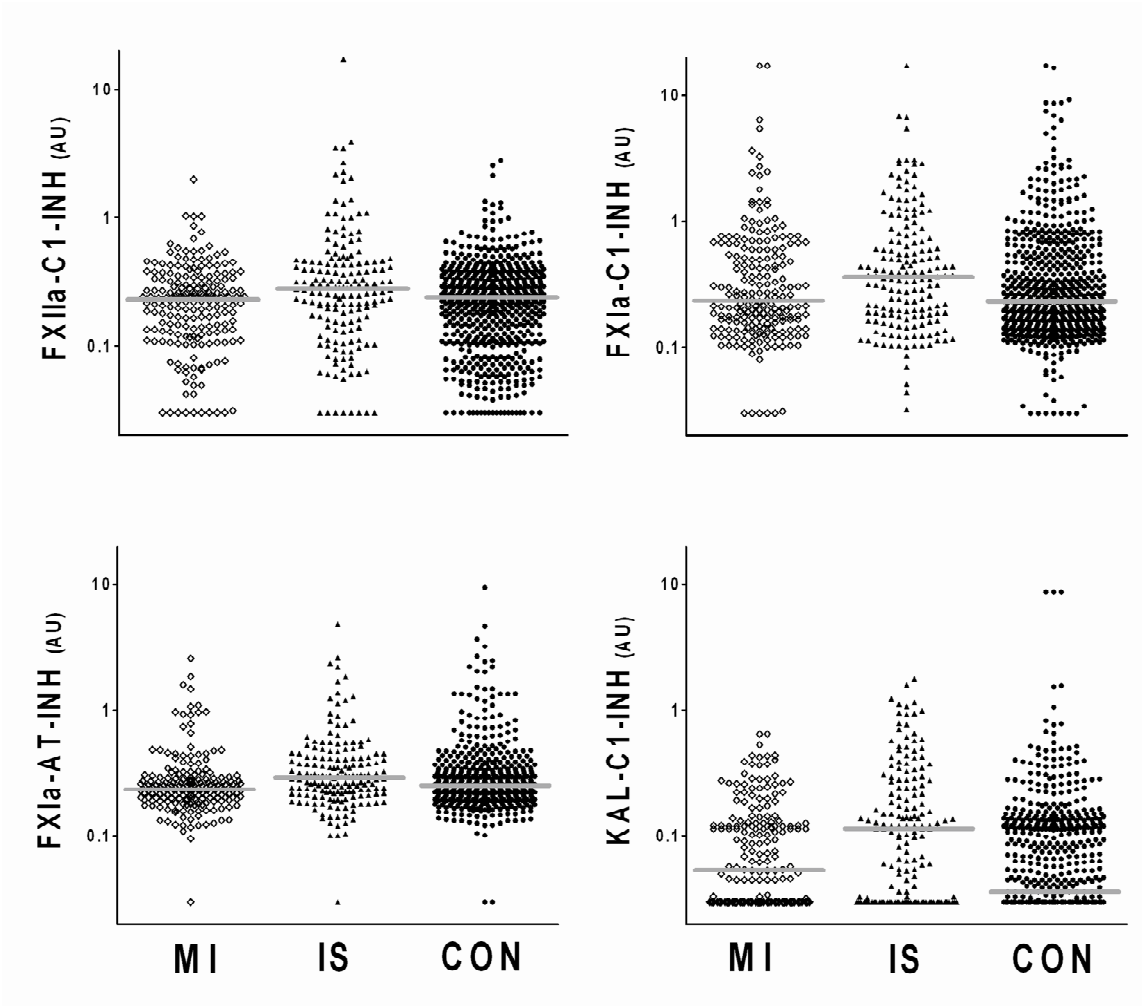
Results were expressed as a proportion of fully activated normal pooled plasma; activation was performed by adding an equal volume of 0.2 mg mL^{-1} dextran sulphate (Mr 500 000; Sigma Chemical Co., St Louis, MO, USA) in the FXIIa:C1-inh and KAL:C1-inh assay. Normal pooled plasma for the FXIa protein-inhibitor assays was fully activated by adding kaolin (final concentration 5 mg mL^{-1}). 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.). Kaolin was removed by centrifuging the reaction mixture for 5 min at 13 000 x g. No signal was detected in FXII-, FXI- or kallikrein-deficient plasmas as a control for the specific ELISA.

Statistical analyses With the 90th percentile of the controls as a predefined cut-off point, we applied a logistic regression model to obtain odds ratios as measures for rate ratios associated with high levels of activation of the intrinsic coagulation factors. All odds ratios were adjusted for the frequency matching factors area of residence, year of event and age. Further adjustments were made for potential confounders (diabetes, hypertension and hypercholesterolemia and smoking) in a fully adjusted model. To assess the intrinsic coagulation protein system as a whole, a dummy variable was created which counted the number of proteins with high activation levels. Interaction of all intrinsic coagulation proteins with OC use in the year prior to the event was assessed by comparing the risk in those with either or both exposures with those with no exposures.

Results

Table 1 displays the baseline characteristics of the study participants. As expected, cases had more cardiovascular risk factors such as smoking, hypercholesterolemia, diabetes and hypertension than controls. The median levels of activated protein-inhibitor complexes of the intrinsic coagulation proteins are displayed in table 1. Figure 1 shows the distribution of the four protein-inhibitor complexes for cases and controls separately.

Figure 1. Levels of activated intrinsic coagulation proteins



Individual levels of protein activation are expressed as a proportion of fully activated normal pooled plasma. Medians are displayed with horizontal bars. Patients with Myocardial infarction are depicted with open squares (◇), ischaemic stroke patients with triangles (◢) and controls with circles (●).

AT-inh, alpha 1-antitrypsin inhibitor; AU, arbitrary units; C1-inh, C1-esterase inhibitor; FXII, coagulation factor XII; FXI, coagulation factor XI; KAL, kallikrein; MI, myocardial infarction; IS, ischaemic stroke; CON, controls.

Table 2. Risk of arterial thrombosis and high levels of activated intrinsic coagulation factors

FXIIa:C1-inh	≤ 90th percentile	> 90th percentile	OR (95% CI)	OR fully adjusted (95% CI)
Control	568 (90%)	63 (10%)	*1 [ref]*	*1 [ref]*
Myocardial infarction	182 (90%)	21 (10%)	0.82 (0.46-1.47)	0.74 (0.38-1.46)
Ischaemic stroke	130 (75%)	39 (23%)	2.10 (1.27-3.48)	1.87 (1.07-3.26)
FXIa:C1-inh				
Control	568 (90%)	63 (10%)	*1 [ref]*	*1 [ref]*
Myocardial infarction	184 (91%)	19 (9%)	0.96 (0.54-1.71)	1.13 (0.60-2.15)
Ischaemic stroke	133 (79%)	35 (21%)	2.77 (1.63-4.73)	2.92 (1.63-5.22)
FXIa:AT-inh				
Control	568 (90%)	63 (10%)	*1 [ref]*	*1 [ref]*
Myocardial infarction	185 (91%)	18 (9%)	0.94 (0.53-1.68)	0.94 (0.49-1.82)
Ischaemic stroke	134 (80%)	34 (20%)	2.33 (1.37-3.96)	2.18 (1.22-3.87)
KAL:C1-inh				
Control	570 (90%)	63 (10%)	*1 [ref]*	*1 [ref]*
Myocardial infarction	174 (85%)	30 (15%)	1.50 (0.91-2.47)	2.12 (1.18-3.81)
Ischaemic stroke	123 (72%)	47 (28%)	4.34 (2.62-7.18)	5.14 (2.93-9.00)

Odds ratios are obtained from logistic regression and are adjusted for the stratification factors age, area of residence and year of event. Odds ratios depicted as 'fully adjusted' are additionally adjusted for diabetes, hypertension and hypercholesterolaemia and smoking.
AT-inh, alpha 1-antitrypsin inhibitor; C1-inh, C1-esterase inhibitor; FXII, coagulation factor XII; FXI, coagulation factor XI; KAL, kallikrein; OR, odds ratio; CI, confidence interval; ref, reference.

The relative risks for both myocardial infarction and ischaemic stroke conferred by high levels of activated intrinsic coagulation protein levels, as well as the corresponding cut-offs, are displayed in table 2. By our definition, 10% of controls have high levels of protein-inhibitor complexes. No association between myocardial infarction and FXIIa:C1-inh was found (10% of cases exposed, Odds Ratio 0.82; 95% Confidence Interval 0.46-1.47). Also FXIa:C1-inh (9%, OR 0.96; 95% CI 0.54-1.71) and FXIa:AT-inh (9%, OR 0.94; 95% CI 0.53-1.68) were not associated with myocardial infarction. KAL:C1-inh slightly increased the risk of myocardial infarction (15%, OR 1.50; 95% CI 0.91-2.47). In ischaemic stroke patients, high levels of FXIIa:C1-inh were more frequent than in controls (23% exposed, OR 2.10; 95% CI 1.27-3.48). Also high levels of FXIa:C1-inh (21%, OR 2.77; 95% CI 1.63-4.73) and FXIa:AT-inh (20%, OR 2.33; 95% CI 1.37-3.96) were more frequent. KAL:C1-inh conferred a fourfold increase in ischaemic stroke (28%, OR 4.34; 95% CI 2.62-7.18). Further adjustments for hypertension, diabetes, hypercholesterolemia and smoking did not change the overall pattern, as is displayed in table 2 ('fully adjusted' odds ratios).

The use of an oral contraceptive in the year prior to the event roughly doubled the risk of both myocardial infarction (OR 2.3; 95% CI 1.6-3.4) and ischaemic stroke (OR 2.8; 95% CI 1.8-4.2).[16, 17] The risk of ischaemic stroke conferred by a combination of high levels of intrinsic coagulation protein activation (i.e. >p90) and prior oral contraceptive use was higher than could be expected by the separate effects as can be seen in table 3. The risk of myocardial infarction was not further increased by the combination of high levels of intrinsic coagulation protein activation and oral contraceptives, except for KAL:C1-inh. The combination of high levels of KAL:C1-inh and oral contraceptives use when compared with women who had neither risk factor increased the risk of myocardial infarction six fold (OR 6.1; 95% CI 2.5- 15). This combined effect was also present in the ischaemic stroke analysis: women with both high levels of KAL:C1-inh and oral contraceptive use had a 17-fold increase in ischaemic stroke risk (OR 17; 95% CI 7.4 – 41). Further adjustments for potential confounders (hypertension, diabetes, hypercholesterolemia and smoking) did not change the overall pattern, as is displayed in table 3 ('fully adjusted' odds ratios).

Table 4 shows the odds ratios per number of proteins with high levels of activation. For each additional high level of protein-inhibitor complexes the risk of ischaemic stroke is increased 2-fold (OR 2.11; 95%CI 1.31-3.38) whereas the risk for myocardial infarction was not affected (OR 1.14; 95%CI 0.75-1.73). When compared to those without a high level of intrinsic coagulation protein activation, people with two or more high levels had a fourfold increase in risk for ischaemic stroke (OR 4.39; 95%CI 2.44-7.90), but no such association was found between multiple high levels and myocardial infarction (OR 1.30; 95% CI 0.66-2.57).

In total, 19 myocardial infarction and 21 ischaemic stroke patients were on anticoagulants. The results of the analyses when restricted to those patients who did not take oral anticoagulant therapy at the time of blood drawing did not change. Therefore, oral anticoagulant therapy does not change our results. The associations of FXIa:C1-inh, FXIa:AT-inh and KAL:C1-inh persist in ischaemic stroke patients whose blood was sampled years after the event (data not shown). The association of FXIIa:C1-inh appeared to attenuate with increasing time between event and blood drawing. However, absence of association cannot be established due to the broad confidence intervals.

Table 3. Risk of arterial thrombosis due to high levels of activated intrinsic coagulation protein and prior oral contraceptives

	> 90th percentile	OC use	# controls	Myocardial infarction			Ischaemic stroke		
				# MI cases	OR (95% CI)	OR fully adjusted (95% CI)	# IS cases	OR (95% CI)	OR fully adjusted (95% CI)
FXIIa:C1-inh	-	-	376	107	*1 [ref]*	*1 [ref]*	62	*1 [ref]*	*1 [ref]*
	-	+	192	75	2.4 (1.6-3.5)	2.0 (1.3-3.2)	68	2.8 (1.8-4.5)	2.7 (1.6-4.5)
	+	-	47	16	0.9 (0.4-1.7)	0.8 (0.4-1.7)	18	1.8 (0.9-3.6)	1.6 (0.7-3.3)
	+	+	16	5	2.4 (0.7 -	1.8 (0.5-7.2)	21	8.7 (3.7-21)	8.1 (3.1-21)
FXIa:C1-inh	-	-	382	111	*1 [ref]*	*1 [ref]*	60	*1 [ref]*	*1 [ref]*
	-	+	186	73	2.5 (1.7 -	2.2 (1.4-3.5)	73	3.2 (2.0-5.1)	3.4 (2.0-5.7)
	+	-	40	12	1.1 (0.5 -	1.5 (0.7-3.2)	20	3.8 (1.9-7.7)	4.7 (2.1-10)
	+	+	23	7	1.8 (0.7 -	1.5 (0.5-4.4)	15	6.1 (2.5-14)	5.5 (2.2-14)
FXIa:AT-inh	-	-	383	112	*1 [ref]*	*1 [ref]*	65	*1 [ref]*	*1 [ref]*
	-	+	185	73	2.5 (1.6 -	2.2 (1.4-3.5)	69	3.1 (1.9-4.9)	3.1 (1.9-5.3)
	+	-	39	11	1.0 (0.5 -	1.2 (0.5-2.8)	15	3.2 (1.5-6.6)	3.3 (1.4-7.3)
	+	+	24	7	1.9 (0.7 -	1.3 (0.4-3.8)	19	4.8 (2.1-11)	4.1 (1.7-9.8)
KAL:C1-inh	-	-	377	106	*1 [ref]*	*1 [ref]*	62	*1 [ref]*	*1 [ref]*
	-	+	193	68	2.2 (1.5- 3.3)	1.9 (1.2-3.0)	61	2.7 (1.7-4.4)	2.6 (1.5-4.5)
	+	-	46	17	1.2 (0.6 -	1.6 (0.8-3.4)	19	3.7 (1.9-7.4)	4.2 (1.9-9.0)
	+	+	17	13	6.1 (2.5 - 15)	6.8(2.5-18)	28	17 (7.4-41)	23 (9.2-59)

Relative risks are stratified to oral contraceptive use prior to the event or index year (-/+), high levels of protein activation (+/-) or both (+/+) with the -/- category as reference. Odds ratios are obtained from logistic regression and are adjusted for the stratification factors age, area of residence and year of event. 'Fully adjusted' odds ratios are additionally adjusted for diabetes, hypertension and hypercholesterolemia and smoking.
AT-inh, alpha 1-antitrypsin inhibitor; C1-inh, C1-esterase inhibitor; FXII, coagulation factor XII; FXI, coagulation factor XI; KAL, kallikrein; OC, oral contraceptive; OR, odds ratio; CI, confidence interval; ref, reference.

Discussion

Our study shows that high levels of activated proteins of the intrinsic coagulation system are associated with ischaemic stroke, and not or to a lesser extent with myocardial infarction, in young women. In general, high levels increased the risk of ischaemic stroke 2.5 fold whereas the risk of myocardial infarction was hardly affected. The increased risk of ischaemic stroke was further increased by oral contraceptive use. When the intrinsic coagulation proteins were assessed jointly, high levels were more frequent in cases with ischaemic stroke than in myocardial infarction cases.

The rate-limiting step in the formation of the protein-inhibitor complexes is the availability of activated coagulation proteins, since the inhibitors are present in excess in plasma. The cause of the elevated complexes is not clear. It could either be a relative increase in which the absolute levels of zymogens remain stable with an increased activation rate or it could be an absolute increase of zymogens with a stable activation rate. Our study has some limitations; because of the case-control study design blood was collected after the event in the case groups. This might have led to reverse causation, a process in which a consequence of an event is mistaken for the cause of the event. Because blood sampling in the RATIO study was several months after the

Table 4. Risk of arterial thrombosis with increasing numbers of high levels of activated intrinsic coagulation proteins

# of high levels	Controls	Myocardial infarction			Ischaemic stroke		
	N	N	OR (95%CI)	OR $_{\text{fully adjusted}}$ (95%CI)	N	OR (95%CI)	OR $_{\text{fully adjusted}}$ (95%CI)
0	454 (72%)	137 (69%)	*1 [ref]*	*1 [ref]*	84 (50%)	*1 [ref]*	*1 [ref]*
1	117 (19%)	44 (23%)	1.14 (0.75-1.73)	1.16 (0.72-1.88)	44 (26%)	2.11 (1.31-3.38)	2.10 (1.125-3.53)
2	42 (6.5%)	16 (7.5%)	1.27 (0.67-2.43)	1.42 (0.68-3.00)	17 (11%)	3.00 (1.50-6.00)	3.57 (1.72-7.43)
3	13 (2%)	2 (1%)	0.44 (0.08-2.33)	0.58 (0.10-3.47)	15 (8%)	4.67 (1.88-12)	5.41 (2.00-14)
4	3 (0.5%)	1 (0.5%)	1.10 (0.08-15)	3.84 (0.29-51)	7 (5%)	12.6 (2.7-58)	8.09 (1.5-42)

Odds ratios are obtained from logistic regression and are adjusted for the stratification factors age, area of residence and year of event. Odds ratios depicted as 'fully adjusted' are additionally adjusted for diabetes, hypertension and hypercholesterolemia and smoking.

OR, odds ratio; CI, confidence intervals; ref, reference

event, we can safely rule out the possibility that our findings directly reflect the transient effects of the acute phase which lasts days to weeks. However, slow subsiding effects which are induced by ischaemic stroke could explain some of the relation between FXIIa:C1-inh and ischaemic stroke. Non-transient effects (chronic effects) can still be the cause of reverse causation in our study and can only be ruled out in a prospective study. Furthermore, the necessity of a blood sample after the event implies that our results are only valid for non-fatal arterial thrombosis. Although our study can be considered to be of reasonable size, or even large due to the rare nature of cardiovascular disease in young women, the RATIO study lacks power to detect small effects. Lack of precision, which is reflected in the wide confidence intervals, hampers definite interpretation of some of our results. This is most clear when the intrinsic coagulation system is assessed as a whole; only few patients and controls were positive on three or all four assays, making it difficult to interpret the odds ratios for these strata. However, the pattern is clear: the risk increase found for ischaemic stroke was higher for each additionally elevated activated protein-inhibitor complex, whereas this pattern was absent for myocardial infarction. This observation suggests that the pathogenic role of the intrinsic coagulation proteins is not similar for all manifestations of arterial thrombosis.

Our results indicated that the risks conferred by high levels of intrinsic coagulation proteins were further increased by oral contraceptive use. This raises the question whether women who want to start with oral contraceptive use should be screened. The highest risk, after adjustment for potential confounders, was found in women who both had high levels of activated prekallikrein (>p90 of controls) and used oral contraceptives: a 23 fold increase in risk compared with women with neither risk factor (fully adjusted model). Even with this relative risk a total of ~15 000 women have to be screened for their kallikrein activation levels before the start of oral contraceptives to prevent one event. If the 10% of these women with the highest kallikrein activation levels do not start to use oral contraceptives, one ischaemic stroke case will be prevented per year. Although a formal cost-benefit analyses is likely to show a slightly different number, these calculations readily demonstrate that screening is not desirable, mainly due to the low incidence of ischaemic stroke in this population. This is even more clear when one considers the beneficial effects of oral contraceptive use in this population, such as a reduction of pregnancy-associated morbidity and mortality and ovarian and endometrial cancer.

A case control study nested in the second Northwick Park Heart Study (NPHS II) also assessed the relationship between the protein-inhibitor complexes of intrinsic coagulation proteins and the risk of coronary heart disease (CHD, N=231) and stroke (N=56 of which 12 haemorrhagic). CHD was defined as definite MI (fatal and nonfatal), possible MI (fatal), angina or coronary angiographic findings requiring intervention and ECG changes at 5 years of follow up. Strokes were diagnosed and categorised on the basis of clinical presentation, computed tomography, lumbar puncture, and autopsy findings. CHD risk was decreased in the second tertile of FXIIa:C1-inh; if anything, the third tertile also showed a decrease in risk, but to a lesser extent, resulting in a U-shaped relation. Other protein-inhibitor complexes (FXI-C1-inh, FXI-AT-inh, KAL:C1-inh) did not alter CHD risk. A similar U-shaped pattern was observed for the relation between KAL:C1-inh complexes and stroke. These results are essentially similar after reanalysis with the 90th percentile as a cut-off (analyses done in collaboration with the NPHS II investigators; results not shown here). There are several possible explanations for the discrepant findings in the RATIO and the NPHS II analyses. First, the differences might be due to chance. Second, the NPHS II is a nested case control study, so blood draw was before the event. Although reverse causation in the RATIO study is not a likely explanation for our results, due to the timing of blood draw and the different effects for myocardial infarction and ischaemic stroke, it cannot be ruled out. Third, the case definitions in the RATIO study were more stringent than those in the NPHS. In the NPHS ischaemic and hemorrhagic strokes were combined and CHD also included possible MI and angina. Fourth, the largest difference between the studies is found in the sex and age of the participants: NPHS included only middle-aged men, whereas the RATIO study included only relatively young women. This difference is of particular interest because FXII transcription is oestrogen sensitive, due to an oestrogen responsive element in the promoter region of the F12 gene.[3] Furthermore, a recent murine study suggests that oestrogens influence the transcription of several coagulation proteins.[15]

The mechanisms by which these proteins are involved in the pathogenesis of ischaemic stroke in young women still have to be established. Perhaps such an increased activity of these proteins results in an imbalance of several systems in which the intrinsic coagulation proteins are involved (e.g. coagulation, fibrinolysis and inflammation). Ultimately, this imbalance then leads to a hypercoagulable state which increases the risk of ischaemic stroke. According to this hypothesis, some event, such as a plaque rupture or changes in

blood flow, acts as a trigger of the start of coagulation. The extent of the following thrombus formation is dependent on the tendency to clot: patients suffering from a hypercoaguable state are more likely to form an occlusive thrombus with clinical effects than those without such a predisposition. This mechanism predicts that a dose-dependent effect should be observed: the greater the imbalance, the larger the tendency to coagulate, the greater the risk of ischaemic stroke. Not all forms of arterial thrombosis need to be subject to this hypothesised mechanism. Possibly, exposure of the highly thrombogenic surface after rupture of a coronary plaque inevitably will start clot formation, whether the patient is more prone to coagulation or not. Although the formation of a thrombus in itself is involved in the aetiology of both myocardial infarction and ischaemic stroke, we postulate that the tendency to start a clot is an important risk factor for ischaemic stroke but not for myocardial infarction.

Conclusion These results indicate that increased levels of activated intrinsic coagulation proteins are associated with ischaemic stroke, but not myocardial infarction, in young women. These risks are further increased by oral contraceptive use. These results differ from an earlier study in middle aged men (NPHS II). This raises two questions: why are the effects of intrinsic coagulation proteins different for young myocardial infarction and ischaemic stroke, and are these effects sex specific? Answers to these questions could come from both basic and epidemiologic research. Basic research is needed to further unravel the function of activated intrinsic coagulation proteins, and perhaps identify different mechanisms for different forms of arterial thrombosis. Epidemiologic research could also provide more insight in the potentially different mechanisms for MI and IS, for example by specifying the associations per stroke subtype. Furthermore, epidemiologic research could provide more insight on the sex-specific effects, by including both men and women. A prospective study design could also diminish the risk of reverse causation, but the low incidence of myocardial infarction and ischaemic stroke in the young makes such a study unfeasible

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4

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

Bob Siegerink, Frits R Rosendaal and Ale Algra

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

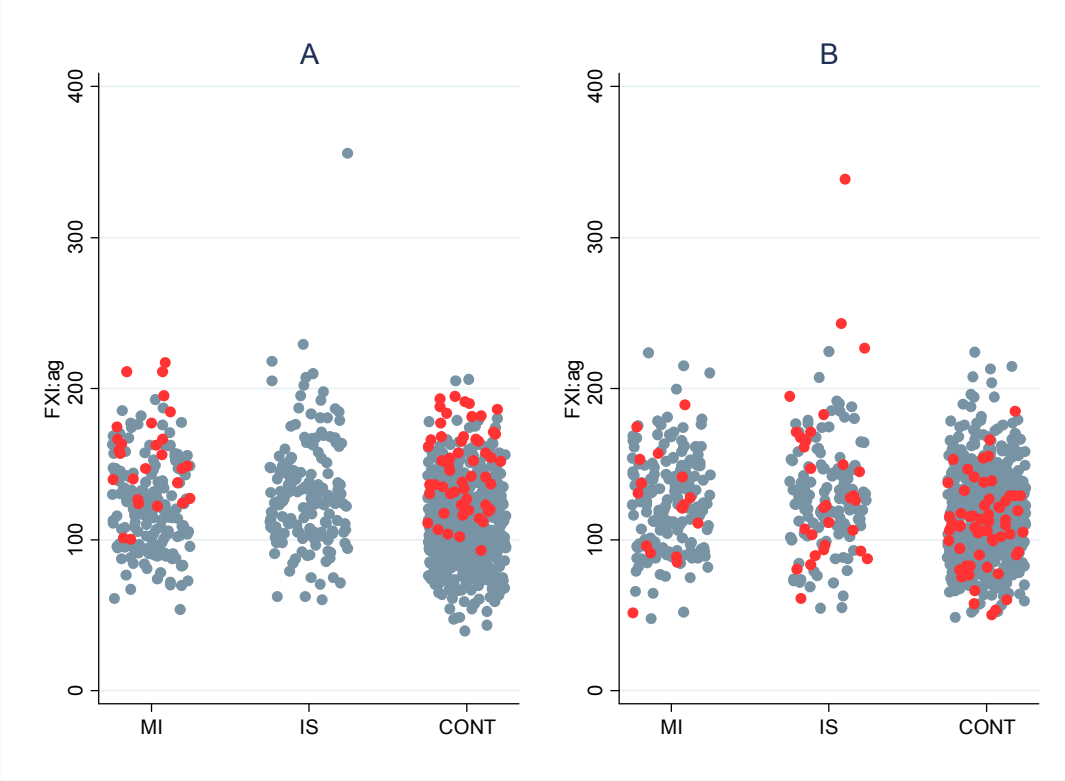
(version 18.0, IBM SPSS statistics, Chicago, Ill, USA) or Stata (version 11.2, Statacorp, College Station, Tx, USA).

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.

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5

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

Bob Siegerink, Ale Algra and Frits R Rosendaal

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%)

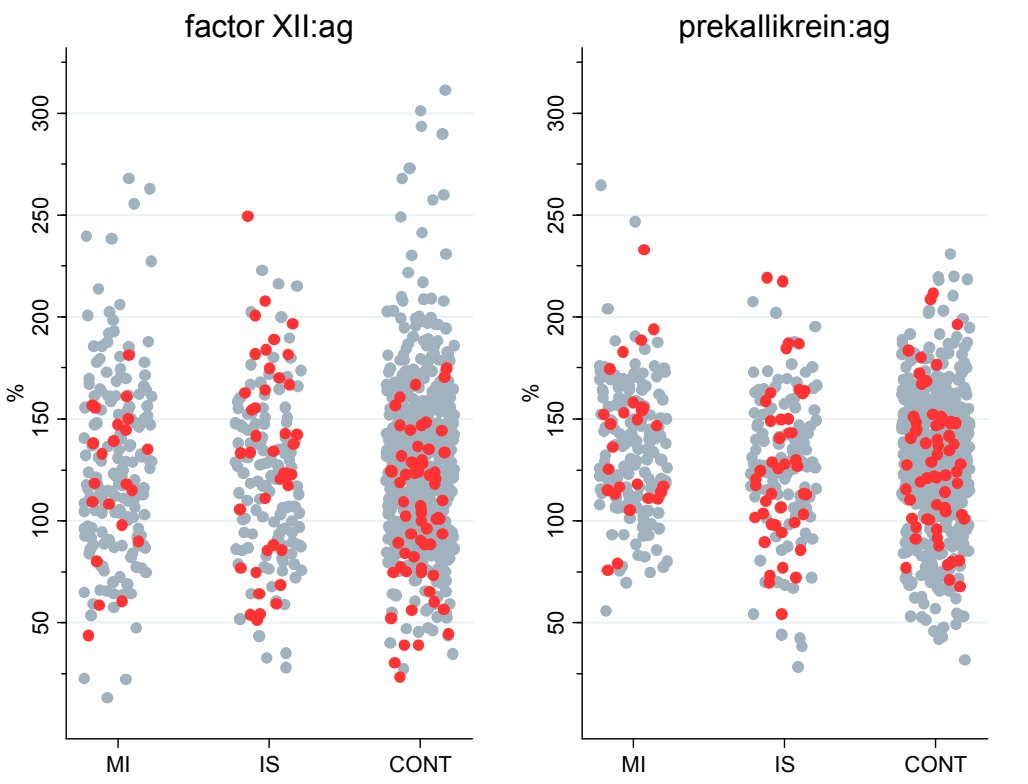
Differences are expressed as absolute changes in levels as expressed as percentage of pooled normal plasma. FXII:ag = antigen level of coagulation factor XII, PK:ag = antigen level of prekallikrein

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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6

High Molecular Weight Kininogen and the risk of myocardial infarction and ischaemic stroke in young women: the RATIO case-control study

Bob Siegerink, Frits R Rosendaal and Ale Algra

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Abstract

Introduction High molecular weight kininogen (HMWK) deficiency is associated with an increased aPTT, but not with a bleeding diathesis. HMWK is a cofactor of the intrinsic coagulation system and as such a pivotal element in the activation of these proteins. Additionally, it is involved in other biologic processes such as inflammation. It is unknown whether HMWK levels affects the risk of myocardial infarction (MI) and ischaemic stroke (IS).

Methods HMWK levels were measured in the RATIO case-control study, which includes young women with MI (N=205), IS (N=175) and 638 healthy controls. Antigen levels of HMWK were measured with a polyclonal ELISA-based assay and expressed as percentage of pooled normal plasma. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated by means of logistic regression as measures of rate ratios.

Results Mean HWMK levels were similar in MI cases and controls (117%) and slightly higher in IS cases (121%). High levels of HWMK (i.e. ≥90th percentile of controls) were associated with an increase in risk of IS ($OR_{adjusted}$ 1.82, 95%CI 1.00-3.29), whereas the risk of MI was not affected ($OR_{adjusted}$1.05, 0.57-1.91). If anything, low levels (≤10th percentile) were associated with a small increase in risk of both MI and IS.

Conclusions High levels of HWMK increase the risk of IS in young women, whereas its role is less clear for MI.

Introduction

Cardiovascular disease is a major cause of morbidity and mortality in high income countries.[1] Arterial thrombosis is a major contributor to this burden; peripheral arterial disease, myocardial infarction (MI) and ischaemic stroke (IS) all occur when a thrombus is lodged in an artery which subsequently reduces the blood flow to downstream tissue. As multicausal cardiovascular diseases, MI and IS share several risk factors. These include, but are not restricted to, classical risk factors such as hypertension, hypercholesterolemia, obesity, smoking and diabetes.[1] Other factors related to primary and secondary haemostasis are also known to be associated with an increase in risk for both MI and IS.[1–6]

An emerging body of evidence suggests that the intrinsic coagulation proteins are involved in the aetiology of cardiovascular diseases. This is in contrast with prior beliefs: despite a relation with the aPTT, deficiencies of coagulation Factor XII and prekallikreine are not related with a bleeding diathesis, whereas coagulation factor XI (FXI) deficiency can result in a mild bleeding phenotype. Therefore, these proteins were regarded as unimportant in haemostasis.[7] Murine and other laboratory studies indicate that these proteins indeed are not involved in regular haemostasis, but are involved in pathological processes leading to thrombosis.[8–12] Clinical studies also suggest that the intrinsic coagulation proteins are involved in the development of human disease.[13–18] Striking results come from studies on FXI deficiency which is relatively common among Ashkenazi Jews. This trait confers a reduced risk of ischaemic stroke and deep venous thrombosis, but not of myocardial infarction.[19–21] A similar pattern is observed in other studies: evidence of an increased state of activation of the intrinsic coagulation proteins was associated with ischaemic stroke, but not myocardial infarction.[22] This contrasts with middle-aged men in whom the same activation markers were associated with both stroke and coronary heart disease.[23]

High molecular weight kininogeen (HMWK) is a cofactor of FXI and prekallikrein; both enzymes are noncovalently bound to HMWK in the circulation through one of the 4 apple domains present in these structurally homologous proteins.[24–27] HMWK also interacts with platelets: it can bind to platelets through the glycoprotein Ib-V-IX receptor complex on platelets, providing a link between primary and secondary haemostasis.[28] Additionally, HWMK may interact with other surfaces such as endothelium and neutrophils, which confirms the pleiotropic role of HWMK in biological systems.[29] The activated intrinsic coagulation system has several actions such as coagulation activation through FXI

activation by FXIIa and the formation of bradykinin through HMWK splicing by kallikrein, which is formed from its precursor prekallikrein by FXIIa.[25,27] Several recent studies indicate that certain circumstances would favor one of these actions. Misfolded proteins can activate FXII which leads to kallikrein formation without activation of coagulation.[30] Also, HMWK can be activated by prekallikrein leading through FXII independent bradykinin formation.[25] Both mechanisms need a negatively charged surface to facilitate protein-protein interactions and HMWK is thought to facilitate this binding, promoting the activation of the intrinsic coagulation system.[27,29,31] These surfaces include well-established activators such as kaolin and glass, but also nucleic acids and polyphosphates released from activated platelets.[31,32] Studies focused on the role of polyphosphates indicate that the size of the negatively charged surface could be dictating the different mechanisms following contact activation.[33]

HMWK is part of this intrinsic coagulation system, but is also involved in other biological systems related to cardiovascular diseases.[34] Cleavage of HMWK by kallikrein leads to the formation of vasoactive substance bradykinin and cleaved kininogen, which are involved in the innate inflammatory response, blood pressure control, brain edema, the renin-angiotensin system and endothelial cell differentiation.[35–38] HMWK has been sparsely studied in a clinical setting. It was found to be 10% higher in patients suffering from venous thrombosis than in blood donors, suggesting that it is associated with an increased clotting potential.[14] The relation with myocardial infarction was also studied; two small studies showed that HMWK levels were lower in patients than healthy controls.[39,40] However, sample collection in these small studies occurred within hours after the disease onset. Since HWMK levels may be affected by the acute phase these results cannot be easily interpreted.

Previous results from the RATIO study showed that increased levels of activated protein-inhibitor complexes are associated with ischaemic stroke but not myocardial infarction.[22] Of the antigen levels of these proteins, only high levels of FXI were clearly associated with an increase in ischaemic stroke. This study is designed to determined whether HMWK, as co-factor for the activation steps in this system, is also linked to the risk of myocardial infarction and ischaemic stroke in young women.

Material and Methods

Study design & participants The RATIO study is a population-based case-control study, originally set up to investigate the association between the use of oral contraceptives and incident arterial thrombosis. Details of this study have been published before.[2,41,42] In short, women under the age of 50 years who where diagnosed using standard criteria with myocardial infarction, ischaemic stroke or peripheral arterial disease in one of the 16 participating centers were eligible to participate. Healthy controls, frequency-matched on age (5 years categories), area of residence and index date, were contacted via random digit dialing. This analysis focuses on myocardial infarction and ischaemic stroke. During the first phase of RATIO we included 248 cases with myocardial infarction, 203 cases with ischaemic stroke and 925 frequency-matched controls. During the second phase of the study, in which all participants were recontacted to provide either a blood sample or buccal swabs for DNA extraction. Ultimately, blood samples of 205 myocardial infarction cases, 175 ischaemic stroke cases and 638 healthy controls were available. The study was approved by institutional review committees of the participating hospitals and all subjects gave informed consent.

Measurements HMWK levels were measured with a sandwich ELISA based assay using a polyclonal, commercially available antibody kit optimised to reduce signal to noise ratio (CEDARLANCE inc., Burlington, Ontario, Canada). This kit uses a purified coating antibody

Table 1. Characteristics of 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%)
HMWK †	Mean (SD)	117%	(21%)	121%	(25%)	117%	(22%)
	Median (Q1-Q3)	115%	(103% -131%)	120%	(102%-140%)	115%	(103%-131%)

Abbreviations: HMWK = High molecular weight kininogen, SD = standard deviation, Q1 = 1st quartile, Q3 = 3rd quartile. in the year prior to event (cases) or index year(controls). † levels are expressed as percentage of HMWK levels in normal pooled plasma percentages might not add due to rounding.*

Table 2. Levels of HMWK in relation to baseline characteristics in the control group

HMWK levels in control group*	
10th percentile	92%
25th percentile	103%
50th percentile (median)	115%
75th percentile	131%
90th percentile	145%

Risk factor	HMWK levels* Mean (SD)	difference (95% CI)
Hypertension -	117% (21%)	[ref]
Hypertension +	118% (20%)	1 %(-6% to 8%)
Diabetes -	117% (21%)	[ref]
Diabetes +	131% (23%)	14% (1% to 27%)
Hypercholesterolaemia-	117% (21%)	[ref]
Hypercholesterolaemia+	126% (21%)	8% (-1% to 18%)
Oral contraceptive use -	118% (22%)	[ref]
Oral contraceptive use +	116% (19%)	-2% (-6% to 1%)
Smoking -	118% (20%)	[ref]
Smoking +	116% (21%)	-2% (-6% to 2%)
Age†		0.4% (0.2% to 0.6%)

Abbreviations: HMWK = High molecular weight kininogen, SD = standard deviation, 95% CI = 95% confidence interval. levels are expressed as percentage of HMWK levels in normal pooled plasma. Percentages might not add due to rounding.† difference is expressed in the increase of HMWK levels per year.*

targeted against HMWK, incubated overnight at 2-8° C (CL20027K-C), in combination with a purified peroxidase labeled detection antibody (CL20027K-D). Being an o-phenylenediamine based antibody kit, we measured light absorbance at 490 NM and signal strengths were converted to HMWK levels expressed as percentage of normal pooled plasma. Data on medical history, oral contraceptive use and other patient characteristics were obtained by questionnaire and reflect the year prior to the event (for the cases) or the frequency-matched index date (controls) unless stated otherwise.

Statistical analyses Odds Ratios (ORs) as measures of rate ratios and corresponding 95% confidence intervals (95%CIs) were calculated by means of logistic regression; all models included age (continuous), area of residence and index date (categorical) to account for the

frequency-matching procedure. Traditional risk factors, i.e. hypertension, diabetes, smoking and hypercholesterolaemia were additionally included in subsequent models as putative confounders. To determine the risk associated with extreme levels of HWMK the 10th and 90th percentile of the control group were used as cutoff values for low and high levels (i.e. ≤p10 & ≥p90). Quartile analyses, with categories based on the 25th, 50th and 75th percentile of controls were used to investigate possible dose response associations.

Results

As expected, traditional risk factors such as hypertension, diabetes and hypercholesterolemia were more frequent in the cases than the control group (Table 1). The mean HMWK levels in the control group (117%) and MI case group (117%, mean difference 0%, 95%CI -4 %to 3%) were similar, whereas the levels were slightly increased in the IS case group (121%, mean difference 4%, 95%CI 0% to 8%). Table 2 shows HMWK levels in relation with classical risk factors in the control group. The levels were higher in those suffering from diabetes (mean difference 14%, 95%CI 1% to 27%) and hypercholesterolemia (mean difference 8%, 95%CI -1% to 18%), whereas hypertension, previous OC use and previous smoking habits were not associated with substantial changes in HMWK levels.

The risk of myocardial infarction was not affected by high levels (≥p90) of HWMK (unadjusted OR 1.26, 95%CI 0.75-2.10; adjusted OR 1.05, 95%CI 0.57-1.91) (table 3). There was an association with the risk of ischaemic stroke, which remained after adjustment for all confounders (adjusted OR 1.82, 95%CI 1.00-3.29). Low levels of HMWK were weakly associated both with the risk of myocardial infarction and ischaemic stroke (MI: adjusted OR 1.39, 95%CI 0.74-2.61; IS: 1.49, 95%CI 0.77-2.89). Table 4 shows that there was no clear pattern of increasing risk with increasing levels for myocardial infarction, while the risk of ischaemic stroke was best represented by a U-curve. A post-hoc analysis with 5 categories can be found in table 5.

Interaction analyses with oral contraceptive use in the year prior to the event are shown in table 5. As expected, the risk in the group of women who were only exposed to oral contraceptive use but had normal HWMK levels (i.e. the -/+ group) was about two-threefold increased for both myocardial infarction and ischaemic stroke when compared with women with neither risk factor. The risks associated with high HWMK levels and no OC use were similar to the risks observed in the primary analyses: after adjustment, the

Table 4. HWMK levels categorised in quartiles in relation to the risk of myocardial infarction and ischaemic stroke

	Control		Myocardial infarction						Ischaemic stroke					
	N	%	N	%	OR_1	95%CI	OR_2	95%CI	N	%	OR_1	95%CI	OR_2	95%CI
Q1	154	0.25	53	0.27	1	[ref]	1	[ref]	41	0.25	1	[ref]	1	[ref]
Q2	146	0.24	39	0.20	0.75	(0.46-1.24)	0.77	(0.44-1.37)	26	0.16	0.57	(0.32-1.03)	0.51	(0.27-0.97)
Q3	157	0.25	55	0.28	0.82	(0.51-1.31)	0.97	(0.56-1.67)	39	0.24	0.67	(0.38-1.17)	0.71	(0.38-1.30)
Q4	161	0.26	47	0.24	0.72	(0.44-1.17)	0.75	(0.42-1.33)	57	0.35	1.01	(0.60-1.71)	1.00	(0.56-1.78)

See table 3 for description

Table 3. High and low levels of HWMK and the risk of myocardial infarction and ischaemic stroke

		Control		Myocardial infarction						Ischaemic stroke					
		N	%	N	%	OR_1	(95%CI)	OR_2	(95%CI)	N	%	OR_1	(95%CI)	OR_2	(95%CI)
High	**<p90**	554	0.90	169	0.87	1	[ref]	1	[ref]	137	0.83	1	[ref]	1	[ref]
	≥p90	64	0.10	25	0.13	1.26	(0.75-2.10)	1.05	(0.57-1.91)	29	0.17	1.69	(0.99-2.89)	1.82	(1.00-3.29)
Low	**>p10**	557	0.90	170	0.88	1	[ref]	1	[ref]	143	0.88	1	[ref]	1	[ref]
	≤p10	61	0.10	24	0.12	1.69	(0.98-2.89)	1.39	(0.74-2.61)	20	0.12	1.62	(0.88-2.99)	1.49	(0.77-2.89)

Abbreviations: OR = odds ratio, 95% CI = 95% confidence interval, ref = reference group, p10 = 10^{th} percentile of control group, p90 = 90^{th} percentile of control group. OR_1 = odds ratios adjusted for stratification factors (i.e. age, area of residence and index year). OR_2 = odds ratios additionally adjusted for potential confounders (i.e. hypertension, diabetes and hypercholesterolemia). Percentages might not add due to rounding.

Table 5. Interaction analyses with OC use

			Control		Myocardial infarction						Ischaemic stroke					
	≥p90	**OC**	N	%	N	%	OR_1	95%CI	OR_2	95%CI	N	%	OR_1	95%CI	OR_2	95%CI
High HMWK	-	-	366	0.59	104	0.54	1	[ref]	1	[ref]	61	0.31	1	[ref]	1	[ref]
	-	+	188	0.30	65	0.34	2.06	(1.37-3.09)	1.75	(1.11-2.77)	73	0.38	2.87	(1.80-4.56)	2.87	(1.73-4.78)
	+	-	47	0.07	14	0.07	0.94	(0.49-1.82)	0.85	(0.40-1.81)	18	0.09	1.89	(0.97-3.70)	2.16	(1.01-4.62)
	+	+	17	0.03	11	0.06	4.90	(2.04-12)	2.90	(1.01-8.26)	11	0.06	4.34	(1.71-11)	3.89	(1.47-10)
	≤p10	**OC**														
Low HMWK	-	-	368	0.60	108	0.56	1	[ref]	1	[ref]	72	0.44	1	[ref]	1	[ref]
	-	+	189	0.31	62	0.32	2.03	(1.35-3.06)	1.64	(1.03-2.61)	71	0.44	2.61	(1.66-4.14)	2.46	(1.47-4.07)
	+	-	45	0.07	10	0.05	1.12	(0.53-2.41)	0.85	(0.35-2.08)	8	0.04	1.35	(0.55-3.33)	1.06	(0.39-2.83)
	+	+	16	0.03	14	0.07	6.55	(2.77-16)	4.53	(1.73-12)	13	0.08	6.24	(2.43-16)	6.70	(2.44-18)

Abbreviations: OC = oral contraceptive use. See table 3 for further description.

risk for myocardial infarction was not affected, whereas the risk for ischaemic stroke remained doubled. The risk associated with the combination of OC use and low HMWK was increased 4.5 fold for myocardial infarction and 6.5 fold for ischaemic stroke.

Discussion

This study indicates that extreme levels of HMWK are associated with an increased risk of ischaemic stroke, whereas the risk of myocardial infarction is only mildly increased with low levels of HMWK. Analysis by HMWK levels suggested a U-shaped curve for ischaemic stroke. The increase in risk was pronounced higher in women with oral contraceptive use.

When these results are compared with our previous results on the intrinsic coagulation proteins, some similarities can be seen: high levels of HMWK, which could result in more activation of the intrinsic coagulation system, are related to an increased risk of ischaemic stroke, whereas the risk of myocardial infarction is not affected. However, the U-shaped risk curve observed for HMWK levels and the risk of ischaemic stroke does not readily comply with this notion. Moreover, due to different roles of HWMK we cannot draw strong conclusions on whether this increase in ischaemic stroke only reflects the activation rate of the intrinsic coagulation proteins.

Our study is the first to investigate the role of HWMK levels in plasma in relation to both myocardial infarction and ischaemic stroke. Our results are not in line with previous studies which focused on myocardial infarction.[39,40] However, since the blood samples in these small studies were collected in the acute phase, their results are subject to reverse causation and should therefore not be interpreted causally. We did not identify prior

TABLE 6. HWMK levels categorised in quintiles in relation to the risk of myocardial infarction and ischaemic stroke

	Control		Myocardial infarction						Ischaemic stroke					
	N	%	N	%	OR_1	95%CI	OR_2	95%CI	N	%	OR_1	95%CI	OR_2	95%CI
Q1	123	0.20	43	0.26	1.33	(0.77-2.29)	1.19	(0.63-2.23)	34	0.21	1.53	(0.81-2.90)	1.51	(0.76-3.00)
Q2	119	0.19	37	0.22	1.02	(0.58-1.78)	1.03	(0.54-1.94)	21	0.13	0.88	(0.44-1.75)	0.74	(0.35-1.57)
Q3	119	0.19	36	0.22	1	[ref]	1	[ref]	26	0.16	1	[ref]	1	[ref]
Q4	128	0.21	37	0.22	0.85	(0.49-1.49)	0.88	(0.47-1.66)	30	0.18	0.90	(0.47-1.72)	0.87	(0.43-1.75)
Q5	129	0.21	41	0.25	0.98	(0.57-1.70)	0.92	(0.49-1.74)	52	0.32	1.85	(1.02-3.39)	1.85	(0.96-3.57)

See table 3 for description

studies which focused on the relation of plasma levels of HMWK and ischaemic stroke. However, some studies on tissue kallikrein levels as well as the bradykinin-receptor indicate that bradykinin is involved in stroke aetiology through the exacerbation of stroke related brain oedema.[38,43,44] Although the biological mechanisms that were investigated as well as the outcome in these studies are not directly comparable to our study, they do suggest that HMWK and especially activation of bradykinin plays a role in the pathophysiology of ischaemic stroke. Unfortunately, our current observational epidemiological study cannot discriminate between the possible mechanisms by which HWMK exerts its risk increasing action and further study is needed to elucidate the underlying mechanisms.

Remarkable results come from the interaction analyses. The analysis of high HMWK levels in combination with oral contraceptive use showed that the observed effect on ischaemic stroke was present for both users and non-users. All other analyses (i.e. low HMWK levels and the risk of ischaemic stroke, low HMWK levels and the risk of myocardial infarction, high HMWK levels and the risk of myocardial infarction) suggested an increase in risk only for women who were used oral contraceptives. This difference could explain in part why there is a difference in the risks conferred by high HWMK levels between myocardial infarction (OR 1.05) and ischaemic stroke (OR 1.82). The value of this observation, however, is unclear; although it is unlikely that bias could be the sole explanation for this observation, there is also no strong direct biological evidence why certain HMWK concentrations would only be harmful in combination with oral contraceptive use.

Our case-control study has some limitations. A major problem in case-control studies in particular is the possibility of reverse causation where cause and effect are mistaken for each other. It is unclear whether HMWK increases or decreases after a major event and could therefore be considered an acute phase reactant which could lead to reverse causation. Our blood samples have been drawn a minimal of 23 months after the event, i.e., after the acute phase, which renders reverse causation unlikely. Survival bias may also play a role in case-control studies. In this study design, blood samples could only be drawn after the event. Therefore, patients who died shortly after the initial diagnosis were not included. This selection of survivors could influence our results only if the etiologic role of HMWK is different between fatal and non-fatal disease. We believe that HMWK levels are not likely to have a large effect on case fatality rate. If such an effect exists, we hypothesise that high HMWK levels lead to an increase in case fatality rate, since several studies

indicated that bradykinin is associated with worse stroke outcome.[38,43,44] Therefore, the results as presented are at most an underestimation of the true effect. Another problem could be confounding; a mechanism in which an observed effect is not caused by the exposure of interest but by a third factor related to the exposure of interest. In our study, strong cardiovascular risk factors (i.e. hypertension, diabetes, hypercholesterolemia, age) were not or only slightly associated with HMWK levels. Therefore we added these risk factors as putative confounders in our fully adjusted model of which the results did not differ substantially from the unadjusted model. We cannot rule out that our analyses are still confounded to some extent, but we expect that the observed effects cannot be completely explained by confounding mechanisms.

Conclusion Our study shows that both high and low levels of HMWK are associated with increased risk of ischaemic stroke in young women. The risk of low HWMK is only present within oral contraceptive users, whereas the risk of increased HWMK was also present in women who did not use oral contraceptives. HWMK does not seem to have a major impact on the risk of myocardial infarction. Further research is needed to determine the exact underlying mechanism and the role of oral contraceptive use in the causal relation between HWMK levels and the risk of ischaemic stroke.

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7

Mendelian randomisation: use of genetics to enable causal inference in observational studies

Marion Verduijn, Bob Siegerink, Kitty J Jager, Carmine Zoccali and Friedo W Dekker

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Abstract

The aim of aetiologic studies in epidemiology is to investigate whether factors are causally related to diseases and therefore become a potential target for therapeutic interventions. Mendelian randomisation enables estimation of causal relationships in observational studies with genetic variants as instrumental variables. An instrumental variable is a variable that can be considered to mimic the coin toss in a randomised study. Given the random assignment of alleles in gamete formation, the use of genetic variants is an alternative method to control for confounding. This chapter describes the approach of Mendelian randomisation, its underlying rationale and its assumptions.

Introduction

While a randomised controlled trial (RCT) is an unbeatable standard in intervention studies, RCTs might be inappropriate or even impossible to conduct when studying the effect of factors potentially involved in the aetiology of disease.[1] Observational study designs are the obvious means for studying these types of research questions, each with their own strengths and weaknesses.[2,3] Despite the valuable contribution of observational studies in understanding the aetiology of diseases, these studies are limited for making causal inference. Exposures that seemed to affect the risk of disease in observational studies turned out later to be non-causal (i.e. only a risk marker), as no or reverse effects of interventions on the presumed cause of the disease was shown in later RCTs. A likely explanation for the initially suggested relationships is 'residual confounding', caused by an incomplete or a lack of measurement of relevant identified or unidentified confounding factors. Moreover, the findings could be due to 'reverse causation', being the (subclinical) presence of disease influencing the presence of the exposure under investigation, rather than vice versa.[4] The subclinical presence of cancer, for instance, might cause leanness, instead of resulting from it, although leanness might be observed prior to cancer diagnosis (Figure 1). Observational studies, however, enable the estimation of causal relationships when an 'instrumental variable' is available that mimics the coin toss in a randomised study reducing confounding.[5] The use of 'genetic variants' as an instrumental variable in observational research is an example hereof. This approach is known as Mendelian randomisation.[6–8]

This chapter describes the approach of Mendelian randomisation used to make causal inference in observational studies, and its underlying rationale and assumptions. All concepts in italics are briefly defined in the glossary.

Example 1: Are low serum cholesterol levels a causal risk factor for cancer? The concept of Mendelian randomisation was originally suggested by Katan in 1986 in the debate on the hypothesis that low serum cholesterol levels (exposure) directly increase the risk of cancer (outcome).[9] In order to investigate whether the association between low serum cholesterol levels and cancer is causal, Katan suggested making use of the data of the apolipoprotein E (ApoE) gene. This gene is known to affect serum cholesterol levels, with the E2 variant being associated with lifelong lower serum cholesterol levels. Katan

hypothesised that, if the low serum cholesterol is a causal risk factor for cancer, an increased risk of cancer should be observed in individuals carrying the ApoE2 variant.

Rationale The main rationale in Mendelian randomisation is that, if an exposure is causally related to an outcome, a genetic variant, which is associated with the exposure, should have a similar relation to the outcome as the supposedly causal exposure itself. In contrast, if the genetic variant turns out to be not related to outcome, a causal role of the genetic product (i.e. the exposure) is less likely. In theory, the exposure of interest in Mendelian randomisation studies can be any property, but in general, protein levels as measured in blood are studied. In the study of Katan, the genetic variant ApoE2 was suggested for its association with the lower levels of serum cholesterol. Persons carrying the ApoE2 variant are lifelong exposed to lower cholesterol levels; if low cholesterol is indeed a causal risk factor for cancer, an increased risk of developing cancer would be expected in the carriers of the ApoE2 variant. A recent publication, however, clearly showed that carriers of the ApoE2 variant are not more susceptible to develop or die from cancer, ruling out low cholesterol as a causal risk factor for cancer.[10]

The use of a genetic variant as an instrumental variable for causal reasoning, whether it is a *single-nucleotide polymorphism* (SNP), a *haplotype* or a deletion, is directly based on the independent assortment of *alleles*. According to Mendel's second law (Gregor Mendel, 1822–1884), an individual's *genotype* is randomly assigned from his/her parental genotypes at gamete formation. In this respect, an observational study investigating the effect of a genetic variant has important similarities with an RCT studying a treatment effect. In Figure 2, both study designs are shown. The randomisation of treatment in an ideal RCT guarantees that all differences in patient characteristics are due to chance, so no confounding by indication is present. The differences observed during follow-up can be regarded as the likely sole effect of the treatment that was allocated in a randomised manner. Similarly, the random assortment of alleles guarantees that, when comparing patients according to a genetic variant, the differences in patient characteristics are due to chance except for the differences that result from the genetic variant. As such, confounding is eliminated. Moreover, since the genetic makeup is fixed at conception, a genetic variant cannot be influenced by the (subclinical) presence of disease, thereby excluding the possibility of reverse causation.

Figure 1. An association between an exposure and an outcome in observational studies might be due to (residual) confounding and/or reverse causation.

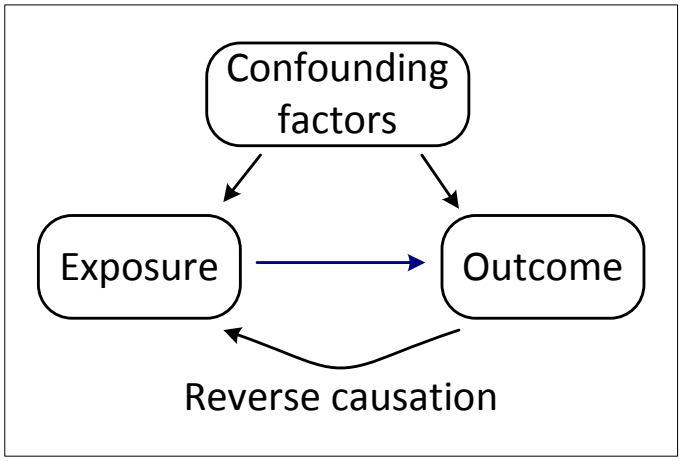
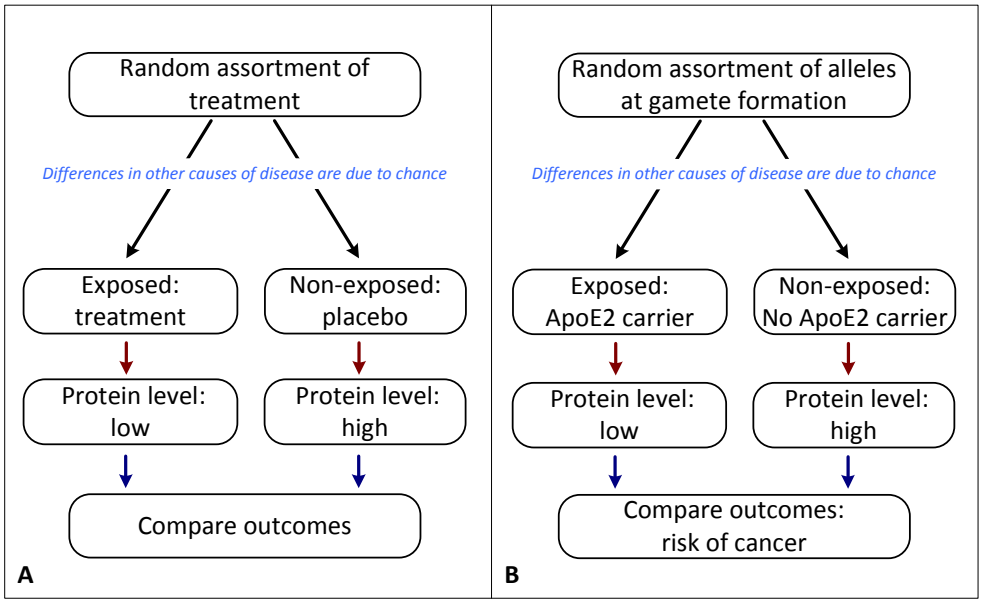


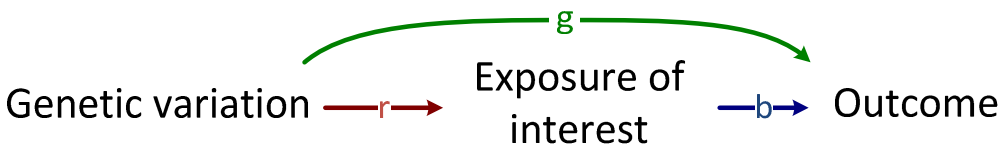
Figure 2. Parallel between RCT and Mendelian randomisation



Parallel between randomised control trials (panel A) and Mendelian randomisation (panel B), with in the diagram of Mendelian randomisation the original idea of Katan as an example.

Framework The framework of a Mendelian randomisation study is summarised in Figure 3 together with the text that describe the associations as examined in the studies used as examples in this chapter. Mendelian randomisation in its most basic form is to study the causal relationship between the exposure levels and the outcome (**blue arrow**) with a genetic variant known for influencing the exposure levels under investigation (**red arrow**) as an instrumental variable, by estimating the association between the genetic variant and the outcome (**green arrow**). In a more sophisticated approach, a quantitative analysis of the three associations is done. Given the observed association in the data between the genetic variant and the outcome and between the exposure level and the outcome, the expected associations can be calculated under the assumption that the exposure levels are causally related to the outcome. Under this assumption, the association between the genetic variant and the outcome (**green arrow**) is expected to be equal to the association between exposure levels and outcome (**blue arrow)** if a genetic variant would explain 100% of the variance in the exposure levels (**red arrow**). This relation between the exposure of interest and its proxy is also known as the instrument strength and can be

Figure 3. Framework of a Mendelian randomisation study; the table describes the associations as examined in the studies used as examples



Example	Association		
	blue arrow	red arrow	green arrow
Katan [9,10]	cholesterol levels – cancer	ApoE genotype – cholesterol levels	ApoE genotype – cancer
Zacho [15]	CRP levels – ischaemic heart disease Cholesterol levels – ischaemic heart disease	CRP genotype – CRP levels ApoE genotype – cholesterol levels	CRP genotype – ischaemic heart disease ApoE genotype – ischaemic heart disease

*The **blue arrow** (also denoted by a small letter b) represents the association between the exposure of interest and the outcome of the study. To determine whether this association indeed is a causal one, Mendelian randomisation uses the associations represented by the **red arrow**(r) and **green arrow**(g).*

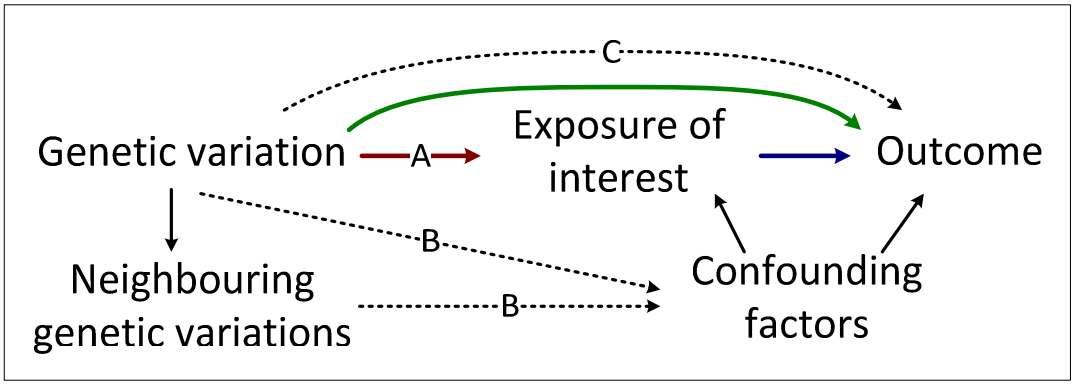
estimated for example with the F statistic.[11] A much more realistic scenario is that the genetic variant explains does not explain 100% of the variance in the exposure levels. In this case, the expected associations can be calculated in two stage regression analyses.[6] If the observed and expected associations between the genetic variant and the outcome and between the exposure level and the outcome are not similar (in direction and magnitude), this indicates that the observed association between the exposure levels and the outcome could be due to residual confounding and/or reverse causation. For more detailed methods for calculating the expected associations please refer to Lawler et al.[6] The use of this formal way of Mendelian randomisation analyses does require the presence of a strong instrument or, which is most often the case, a large study to circumvent the lack of a strong association between the exposure of interest and its instrument to prevent weak instrument bias.[11]

Assumptions Figure 4 depicts the three assumptions that are underlying a Mendelian randomisation study. The first assumption is that there is a robust association between the genetic variant and the exposure levels, i.e. the chosen instrument must not be weak. In order to ensure this, the association should preferably be demonstrated in independent (healthy) populations and confirmed in own data. More robust associations between genotype and exposure levels might be obtained with haplotypes or a genetic score as the instrumental variable instead of a single genetic variant (Figure 4, presence of association A). Also, additional variables which are not on the causal path can be used to minimise the variance of the exposure of interest to be explained by its instrument.[11,12] Where this first assumption can be evaluated, the other assumptions are about the absence of *pleiotropic* effects of the genetic variant which in theory cannot be verified empirically.

The second assumption is that the genetic variant is not associated with factors that confound the association between the exposure levels and outcome. The association between a genetic variant and a confounding factor can be direct, namely if the confounding factor itself is regulated by the genetic variant, or indirect when neighbouring variants (i.e. variants that are in linkage disequilibrium with the variant used as instrumental variable) regulate one or more confounding factors (Figure 4, absence of association B).

In the third assumption, the genetic variant is assumed to be independent of the outcome given the exposure levels and its confounding factors. This means that the genetic variant

Figure 4. Assumptions made in a Mendelian randomisation study



Three assumption may not be violated in order to yield an unbiased result from Mendelian randomisation analyses, denoted by a capital letter: ***A)*** *presence of a robust association between genetic variant and exposure (no weak instrument)* ***B)*** *absence of association, direct and indirect, between genetic variant and confounding factors (no pleiotropy / no confounding such as population stratification nor canalisation)* ***C)*** *absence of other pathways between genetic variants and outcome (no pleiotropy)*

is assumed to be exclusively related to the outcome via the exposure levels and that there are no other pathways to the outcome. Otherwise, the observed association between the genetic variant and the outcome also includes the impact of the variant on the outcome via other pathways. As such, this association does not prove that the particular exposure levels under investigation are causally related with the outcome (Figure 4, absence of association C). Knowledge of the function of the genetic variant and its neighbouring variants is needed to evaluate whether

the assumptions are likely to hold in a particular Mendelian randomisation study. When these assumptions hold, Mendelian randomisation can be used to test the null hypothesis that the exposure levels of interest are not associated with the outcome. The assumption that all associations are linear and unaffected by statistical interactions is needed when aiming at a precise estimate of the size of the causal effect. Although several approaches can be used to determine whether the assumptions are likely to hold, some of the assumptions can never be checked empirically, because they are based on the absence of a effect. Nonetheless, if thoroughly investigated and critically evaluated, Mendelian randomisation can be a valuable technique in causal inference.[13]

Some additional issues are relevant to mention in the context of Mendelian randomisation studies, as they may in part be underlying causes invalidating the assumptions. The first issue is ‘population stratification’, which is related to weak instrument bias and occurs

when the allele frequencies of the genetic variant and the distribution of the exposure levels (or outcome) vary substantially between the different subgroups in the study population.[6] In that case, an association is induced between the genetic variant and the exposure levels (or outcome) at the population level. This phenomenon can be regarded as confounding by ethnicity. Population stratification is unlikely to be a problem in practice, except in extreme situations,[14] and can be overcome by studying populations that are homogeneous with respect to ethnicity. A second issue is the possibility that a lifelong genetic (or environmental) exposure has induced developmental compensation via alternative pathways. This is known as '*canalisation*' and may invalidate the estimation of associations like those in Figure 3. In this perspective, it is worthy to note that Mendelian randomisation studies and RCTs are different in this respect: in RCTs, the random allocation treatment is normally done in adulthood, after the developmental period, while the random allocation of alleles takes place at conception, allowing the possibility of *canalisation*. It is, however, unclear how important this issue is in practice.[6]

Finally, as for all genetic association studies, relatively large sample sizes are required for Mendelian randomisation studies. The sample size highly depends on both the frequency of the genetic variation used as instrument, and the expected effect size of the association of interest between the genetic variant, the exposure and outcome. Conventional sample size calculations can be performed in order to get an approximation of the required sample size. Studies that are underpowered might be unable to observe small (but existing) effects, incorrectly suggesting that an association between an exposure and outcome is not causal. This encourages enlarged sample sizes through collaboration between research groups and by the setting up of well-defined cohorts. In addition, the evidence of the different studies can be combined in meta-analyses.

Example 2: Is CRP a causal risk factor for ischaemic vascular disease? The study of Zacho and colleagues shows an interesting application of the Mendelian randomisation approach. They aimed to test whether the observed association between the elevated levels of C-reactive protein (CRP) and increased risk of ischaemic vascular disease (i.e. ischaemic heart disease and ischaemic brain disease) is a causal association.[15] So, the exposure levels under investigation were CRP levels, and the outcome was ischaemic vascular disease. As the instrumental variable, they used four polymorphisms in the CRP gene that partially affect plasma CRP levels and combined them to one variable with nine genotype combinations. They observed that the risk of ischaemic heart disease was increased by a factor of 2.2

[95% confidence interval (CI) 1.6–2.9] and the risk of ischaemic brain disease was 60% increased [95%CI 1.1-2.5] in persons with CRP levels >3 mg/L as compared with persons with CRP levels <1 mg/L (adjusted for age, sex and statin use); this is the **blue arrow** in the framework of Figure 3 and 4. Moreover, the data confirmed that the CRP levels are regulated by the CRP genotype (**red arrow**): a difference of up to 64% in CRP levels among the genotype combinations. With these findings, an expected ischaemic heart disease relative risk of 1.32 (1.26–1.39) can be calculated for the genotype combination that was related to the highest CRP levels. However, no increased risk for ischaemic heart disease was observed for any of the CRP genotype combinations (**green arrow**). The results for ischaemic brain disease follow the same pattern. These findings suggests that the observed increased risk of ischaemic vascular disease associated to elevated CRP levels does not reflect a causal relationship, and the CRP is a mere risk marker for this outcome. In the same study, a proof of principle of the Mendelian randomisation approach was given, with the ApoE genotype as the instrumental variable for examining the causality of the association between cholesterol levels and ischaemic heart disease.[15] The increased risk for the outcome was expected across the ApoE genotypes [up to 1.12 (1.06– 1.17)] and was also observed [up to 1.35 (1.12–1.61)]. These results suggest that the association between cholesterol levels and ischaemic heart disease is indeed a causal one, although the difference in magnitude of effect could suggest the presence of residual confounding.

Conclusion Mendelian randomisation enables to study the hypothesis that an observed association between the exposure levels, as determined by the genetic variant used as the instrumental variable, and the outcome is causal. As such, it is a unique approach for providing more insights into potential causal relationships in aetiologic research using observational data, both as a formal analyses as well as a line of thought in causal inference.

Glossary

Alleles:	variant forms of a gene at a locus; a single allele is inherited from each parent.
Canalisation:	developmental compensation in response to disruptive influences on normal development from genetic and environmental forces.
Gene:	stretch of DNA which encodes for a particular protein.
Genetic variants:	variation in genes, including single-nucleotide polymorphisms, and insertion or deletions of stretches of DNA. Genotype: genetic makeup of an individual with regard to genetic variants.
Linkage:	tendency of DNA to be co-inherited due to their close physical proximity; linkage disequilibrium (LD) is a measure of linkage and indicates whether a non-random association between two alleles at different loci is present
Locus:	physical location of a gene or other genetic marker
Haplotypes:	combinations of SNPs; usually, a limited number of haplotypes can be used to cover most of the genetic variation within a population due to linkage
Pleiotropy:	phenomenon in which a genetic variant has multiple distinct phenotypic effects
Population stratification:	a form of confounding by race due to differences in allele frequencies in subgroups in a population
SNP:	or Single-Nucleotide Polymorphism. A genetic variant in which one specific nucleotide in the DNA is altered
Instrumental variable:	a variable in non-experimental data that can be considered to mimic the coin toss in a randomised study
Residual confounding:	confounding remaining after incomplete adjustment for confounders due to lack of measurement of relevant identified or unidentified confounding factors
Reverse causation:	(subclinical) presence of disease, or its effects, alters the exposure under investigation, rather than vice versa, i.e. cause and consequence are switched

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8

Genetic variation in fibrinogen; its relation to fibrinogen levels and the risk of myocardial infarction and ischaemic stroke

Bob Siegerink, Frits R Rosendaal and Ale Algra

J Thromb Haemost. 2009; 7:385-90

Abstract

Introduction Confounding by common causes and reverse causation have been proposed as explanations of the association of high fibrinogen levels and cardiovascular disease. Genetic variants, such as plasma levels, can alter fibrinogen characteristics and are not subject to these problems.

This study aims to determine the fibrinogen plasma levels for genotypic variants in fibrinogen-alpha (FGA Thr312Ala, rs6050) and fibrinogen-beta (FGB -455G/A, rs1800790) and whether these variants are associated with myocardial infarction and ischaemic stroke in young women.

Methods Fibrinogen genotypes were determined in a population-based case-control study including women aged 18-50 years; 218 cases with myocardial infarction, 190 cases with ischaemic stroke and 767 healthy controls. Fibrinogen levels were determined in the control population. Blood samples were available for 205 cases with myocardial infarction, 175 ischaemic stroke cases and 638 patients.

Results The FGB -455G/A variant increased plasma fibrinogen levels, whereas the FGA Thr312Ala variant lowered plasma fibrinogen levels, albeit to a modest extent. The risk of ischaemic stroke was altered when the homozygote minor allele was compared with the homozygote major allele. The FGA Thr312Ala single-nucleotide polymorphism (SNP) was associated with a decrease in risk [odds ratio (OR) 0.43; 95% confidence interval (CI) 0.21 - 0.87], whereas the FGB -455G/A SNP might have increased the risk (OR 1.76; 95% CI 0.7 - 4.03). The risk of myocardial infarction was not altered for either SNP (FGA Thr312Ala, OR 0.98, 95% CI 0.40 - 2.40; FGB -455G/A, OR 0.98, 95% CI 0.40 - 2.40).

Discussion With the genetic variations as markers of plasma fibrinogen levels alterations, thereby ruling out confounding and reverse causation, our results suggest that plasma fibrinogen levels play a more pronounced role as risk factor for ischaemic stroke than for myocardial infarction.

Introduction

Fibrinogen is an acute phase coagulation factor produced by hepatocytes and is activated by thrombin to form fibrin monomers and polymers. It is covalently cross-linked by coagulation Factor XIIIa to form a fibrin mesh of which is the main constituent of blood clots. Fibrinogen consists of three pairs of polypeptide chains, denoted (Aα / Bβ / γ), which are linked in the central domain. All three genes for these polypeptide chains are located in the fibrinogen module located on the short arm of chromosome 4.[1,2]

High fibrinogen levels have been associated with cardiovascular diseases in large studies such as the Framingham Study and Northwick Park Heart study and further in meta analyses such as the one by the Fibrinogen Studies Collaboration.[3–5] Results from the RATIO study also showed high fibrinogen to be associated with myocardial infarction (3.5-4.5 g/L vs. <2.5 g/L OR 2.1; 95% confidence interval 1.2 to 3.6).[6]

However, the acute phase property of fibrinogen together with the associations with environmental risk factors of cardiovascular disease (e.g. smoking / oral contraceptive (OC) use) make it difficult to determine whether fibrinogen is either a cause or a mere marker of increased.[7–9] This question can be addressed by means of Mendelian randomisation, a process which uses genetic variation a as marker for lifelong exposure to altered protein levels, in this case an increase of fibrinogen.[10,11] With these genetic variants as markers for fibrinogen levels confounding by extraneous factors and reverse causation are virtually excluded as possible explanations of the association. The genetic marker might also be in linkage disequilibrium with another variation causing variation in the plasma fibrinogen level. Common genetic variants in the fibrinogen module have been associated with increased fibrinogen plasma level and therefore can be used as markers of lifelong increased levels of plasma fibrinogen.[12–14] This current study uses the fibrinogen-Aα (FGA) Thr312Ala (rs6050) single- nucleotide polymorphism (SNP) as a marker of decreased plasma fibrinogen levels, and fibrinogen-Bβ (FGB -455G/A) (rs1800790) as a marker for an increase in level.

In spite of the associations between FGA Thr312 as well as FGB -455A and increased levels of plasma fibrinogen, no association was found for these SNPs or associated haplotypes with myocardial infarction and ischaemic stroke in the Cardiovascular Health Study.[14] Similar results were obtained earlier in the Copenhagen City Study, the SMILE study, the Rotterdam study and a cross sectional analysis of the CARDIA cohort; the genetic variations

are associated with increased plasma fibrinogen level, but not with cardiovascular disease.[12,13,15,16] Another study, however, found an increase in myocardial infarction risk for carriers of the Bcl I β-chain fibrinogen polymorphism (rs209502) which is also a marker of increased plasma fibrinogen levels.[17] This SNP is almost completely in linkage disequilibrium with the FGB - 455G/A SNP (|D'| = 0.96 in the ECTIM study).[18]

The FGA Thr312Ala SNP has also been associated with a decreased risk of pulmonary embolism as well with a lower risk of post-stroke mortality in patients with atrial fibrillation but not of deep venous thrombosis. These decreases in risk are not likely to be caused by increases in plasma fibrinogen level, but through the changes in the mechanical properties of the clot by the genetic variant. The FGA Thr312Ala variant alters clot stability by affecting FXIII cross-linking making carriers of the FGA Thr312 less prone to embolisation.[19,20] This study assessed the risk implications of genetic variations in the fibrinogen module on myocardial infarction and ischaemic stroke in young women. We estimated the risks for carriers of the genetic variants FGA Thr312Ala and FGB - 455G/A as markers for altered fibrinogen levels for myocardial infarction and ischaemic stroke.

Methods

Study design & participants The Risk of Arterial Thrombosis In relation to Oral Contraceptives (RATIO) study is a multicenter population-based case-control study. It consists of three sub-studies which included cases with myocardial infarction, ischaemic stroke and peripheral arterial disease and frequency-matched controls. The study was initiated to evaluate the risk of myocardial infarction and ischaemic stroke due to oral contraceptives of different generations (1990 - 1995).[21–23] Blood and buccal swabs were collected during the second phase of the study in order to study prothrombotic conditions of the coagulation system (1998 - 2002). Informed consent was obtained from all participants and the study was approved by the medical ethics committees of the participating hospitals. For the current study we used data from the myocardial infarction and ischaemic stroke sub-studies.

Patient selection has been described in detail previously. [21–24] In summary, we included women aged 18 to 50 years old who presented with myocardial infarction or ischaemic stroke to one of the sixteen participating hospitals in the Netherlands between 1990 and 1995. Myocardial infarction was diagnosed by the presence of clinical symptoms, elevated cardiac enzyme levels and electrocardiographic changes. Clinical symptoms of ischaemic

stroke were confirmed by either computed tomography (CT) or magnetic resonance imaging (MRI). Controls were approached by random digit dialing, and matched on age, area of residence and year of event. A standardised questionnaire on (familial) medical history, use of oral contraceptives, smoking habits and participant characteristics was filled in by all participants. Some of these questions were targeted at the year prior to the event for cases. Controls were asked to answer these same questions for a particular year (index year) to ensure comparability. Blood or buccal swaps were collected for DNA analysis from 218 myocardial infarction cases, 190 ischaemic stroke cases and 767 controls.

Measurements and definitions DNA was isolated from 10 ml EDTA-augmented blood or buccal swabs and amplified by polymerase chain reaction. Two SNPs in fibrinogen Aα (Thr312Ala, rs6050) and fibrinogen Bβ (-455G/A, rs1800790) were genotyped with the 5' nuclease / Taqman Assay.[25] Polymerase chain reactions based on fluorescent allele specific oligonucleotide probes (assay-by-design / assay-on-demand; Applied Biosystems, Foster city, USA) were performed on a PE9700 thermal cycler (Biozym, Hessisch Oldendorf,

Table 1. Characteristics of RATIO participants

	Myocardial infarction N=218	Ischaemic stroke N=190	Control N=767
Mean Age (SD)	43 (6)	39 (8)	39 (8)
Caucasian ethnicity	207 (95%)	182 (96%)	732 (94%)
History of *			
Hypertension	55 (25%)	55 (29%)	47 (6%)
Diabetes	11 (5%)	8 (4%)	10 (1%)
Hypercholesterolaemia	23 (11%)	15 (8%)	22 (33%)
Oral contraceptives use *	86 (39%)	98 (52%)	272 (35%)
Smoking *	181 (83%)	115 (61%)	319 (42%)
alcohol use			
never	83 (38%)	89 (47%)	227 (30%)
0-15 glasses per week	128 (59%)	84 (44%)	500 (65%)
>15 glasses per week	7 (3%)	2 (1%)	32 (4%)

All data are with respect to the year of event (cases) or index date (controls). Data are missing on ethnicity for one ischaemic stroke (IS) case and four controls, on history of hypertension for four controls, on history of diabetes for one myocardial infarction (MI) case and three controls, on hyperlipidaemia for one MI case and five controls, on oral contraceptive use for six controls, on smoking for six controls, and on alcohol use for one IS case and six controls. SD, standard deviation.

Germany) and fluorescence endpoint reading for allelic discrimination was performed on a ABI 7900 HT (Applied Biosystems). Primers and probe sequence are available upon request. During genotyping, technicians were unaware of outcome status. Fibrinogen in the control population was determined according to Von Clauss using the reagents of Behringwerke AG (Marburg, Germany).

Statistical analyses Logistic regression was used to obtain odds ratios as measures of rate ratios and accompanying 95% confidence intervals (95% CI) for the relation between genetic variants in the fibrinogen gene and myocardial infarction ischaemic stroke. Odds ratios were adjusted for the three stratification variables age (as a continuous variable), area of residence and index event. Differences in fibrinogen plasma levels and corresponding 95% CI were calculated per genotype. Expected relative risks corresponding the observed fibrinogen differences were based on hazard ratios from a meta analysis.[5] For all analyses, the homozygote carriers of the major allele were used as the reference group. Women with missing data were excluded from analysis when appropriate.

Results

Study participants Table 1 shows the baseline characteristics of the study participants. Known risk factors, such as hypertension, diabetes and hyperlipidaemia, were more prevalent in both case groups than in controls. A larger proportion of patients than of controls were smokers. The overall call rate of the genotyping was 97% for the two SNPs, and the success rate was irrespective of case - control status. The allele frequencies of the variants were 0.31 for FGA Thr312Ala and 0.19 for FGB - 455G/A. The likelihood of departure from Hardy - Weinberg equilibrium (HWE) was determined in the control population, and was P = 0.043 for FGA Thr312Ala and P = 0.84 for FGB - 455G/A.

Genetic variants and fibrinogen plasma levels Plasma fibrinogen levels were available for 602 control women. The mean fibrinogen level in this population was 3.20 g/L. The plasma fibrinogen levels per genotype as well as the associated expected relative risks are shown in Table 2. L Levels were lower in carriers of the FGA - 455G/A variant (3.15 g/L for carriers vs 3.24 g/L for non-carriers, 95% CI of difference -0.20 to 0.02 g/L). Levels were higher for carriers of the FGB Thr312Ala variant (3.32 g/L for carriers vs 3.13 g/L for non-carriers, 95% CI of difference 0.07 to 0.30 g /L).

Table 2. Mean fibrinogen plasma levels per genotype

	N	fibrinogen level	difference	95% CI of difference	expected RR	
FGA Thr312Ala SNP (rs6050)					MI	IS
Thr / Thr	319	3.24 g/L	ref	-	-	-
Thr / Ala	237	3.14 g/L	-0.06	-0.21 to 0.01 g/L	0.90 (0.80-1.01)	0.90 (0.81-1.01)
Ala / Ala	46	3.20 g/L	-0.04	-0.25 to 0.17 g/L	0.96 (0.76-1.20)	0.96 (0.77-1.19)
FGB -455G/A SNP (rs1800790)						
G / G	397	3.13 g/L	ref	-		
A / G	184	3.30 g/L	0.17 g/L	0.05 to 0.28 g/L	1.20 (1.01-1.35)	1.19 (1.05-1.33)
A / A	21	3.50 g/L	0.37 g/L	0.06 to 0.67 g/L	1.49 (1.06-2.05)	1.46 (1.04-1.98)

Fibrinogen was measured in 602 control women. The expected relative risk (RR) was calculated on the basis of the hazard ratio (HR) per increase of fibrinogen plasma level of 1 g L)1 for (non)-fatal coronary heart disease [i.e. HR 2.93; 95% confidence interval (CI) 2.59 - 3.31] and (non)-fatal stroke (i.e. HR 2.77; 95% CI 2.17 - 3.53) for ages 40 - 59 years reported by the Fibrinogen Studies Collaboration.[5] MI, myocardial infarction; IS, ischemic stroke; RR, relative risk; OR, odds ratio; Ref, reference; FGA, fibrinogen-Aα; FGB, fibrinogen-Bβ.

Genotype analyses The genotype distribution among case groups and controls, aswell as the risk per genotype with the homozygote major allele genotype as reference, are shown in Table 3. After adjustment for the stratification variables, no effect on myocardial risk was observed for the FGA Thr312Ala variant (OR 0.82; 95% CI 0.49 - 1.39) but a decreased risk was found for ischaemic stroke (OR 0.43; 95% CI 0.21 - 0.87) when the homozygote minor allele genotype was compared with the homozygote major allele genotype. The homozygote carriers of the FGB -455G/A variant also showed no altered risk of myocardial infarction (OR 0.98; 95% CI 0.40 - 2.40). If anything, the risk of ischaemic stroke was increased (OR 1.76; 95% CI 0.77 - 4.03). No substantial effect on myocardial infarction or ischaemic stroke was observed for heterozygotes when compared with those with the homozygote major alleles for the FGA Thr312Ala SNP or the FGB -455G/A SNP.

The results of this per-genotype analysis are consistent with the risks when one assumes a recessive inheritance pattern: homozygous minor allele genotype of the FGA Thr312Ala SNP, when compared with the homozygous major allele genotype combined with the heterozygote genotype, lowered the risk of ischaemic stroke (OR 0.43; 95% CI 0.21 - 0.85), but not of myocardial infarction (OR 0.91; 95% CI 0.55 - 1.53). The homozygous minor allele genotype of the FGB -455G/A SNP tended to elevate the risk of ischaemic stroke (OR 1.84; 95% CI 0.81 - 4.19) but not of myocardial infarction (OR 0.94; 95% CI 0.39 - 2.29) when compared with the homozygous major allele and heterozygote genotype combined. A dominant inheritance pattern diluted all effects; no substantial effect of the FGA Thr312Ala

Table 3. Genotype distribution and the relative risk of MI and IS

		Genotype distribution						OR (95%CI)			
FGA Thr312Ala SNP (rs6050)		**MI**		**IS**		**Control**		**MI**		**IS**	
Homozygote major allele	Thr/Thr	121	56%	97	53%	370	49%	1	[ref]	1	[ref]
Heterozygote	Thr/Ala	81	33%	74	40%	295	39%	0.76	0.54 - 1.07	1.00	0.69 - 1.46
Homozygote minor allele	Ala/Ala	24	11%	12	7%	89	12%	0.82	0.49 - 1.39	0.43	0.21 - 0.87
FGB -455G/A SNP (rs1800790)											
Homozygote major allele	G/G	135	64%	122	67%	483	66	1	[ref]	1	[ref]
Heterozygote	A/G	70	33%	60	27%	221	30%	1.15	0.80 - 1.62	0.85	0.57 - 1.27
Homozygote minor allele	A/A	7	3%	10	5%	29	4%	0.98	0.40 - 2.40	1.76	0.77 - 4.03

Percentages might not add up to 100%, because of rounding. Single-nucleotide polymorphism (SNP) determination was either unsuccessful or ambiguous in seven control women, one myocardial infarction (MI) case and seven ischaemic stroke (IS) cases for the fibrinogen-Aα (FGA) Thr312Ala SNP. The fibrinogen-Bβ (FGB) -455G/A SNP determination did not succeed in 34 controls, six MI cases and eight IS cases. ORs were calculated per genotype, with the homozygote major allele genotype as reference, and are adjusted for matching factors (i.e. age, area of residence, and year of event).

SNP was observed on myocardial infarction (OR 0.77; 95% CI 0.56 - 1.07) or on ischaemic stroke (OR 0.86; 95% CI 0.60 - 1.22). The FGB -455G/A SNP also showed no effect on either myocardial infarction (OR 1.13; 95% CI 1.13 - 1.58) or ischaemic stroke (OR 0.94; 95% CI 0.64 - 1.37).

Smoking and oral contraceptive use OC use in the year of the event increased the crude risk of myocardial infarction and ischaemic stroke two-fold to three-fold (OR 2.3, 95% CI1.6 - 3.3, for myocardial infarction; OR 2.6, 95% CI 1.7 - 3.9, for ischaemic stroke). Smoking in the year prior to the event also increased arterial thrombotic risk (OR 7.1, 95% CI 4.7 - 10.7, for myocardial infarction; OR 2.2, 95% CI 1.6 - 3.3, for ischaemic stroke). These effects showed no marked interaction with the effects of the SNPs (not shown).

Discussion

We used two genetic variants in the fibrinogen module as markers of decreased (FGA Thr312Ala) and increased (FGB -455G/A) plasma fibrinogen levels. We found that the effects of these SNPs on fibrinogen levels in our control population were according to the literature, albeit to a modest extent. Both genetic variant SNPs as markers of altered fibrinogen levels also altered the risk the risk of ischaemic stroke in the expected direction; FGA Thr312Ala decreased the risk twofold (OR 0.43; 95% CI 0.21 - 0.87), and FGB -455G/A possibly increased this risk (OR 1.76; 95% CI 0.77 - 4.03). Neither SNP affected the risk of myocardial infarction. These altered risks were greatest in the per-genotype analysis, with similar results being obtained, assuming a recessive inheritance pattern. Smoking and the use of OCs, both established risk factors for arterial thrombosis and related to increased

fibrinogen, increased the risk of both myocardial infarction and ischaemic stroke, but showed no marked interaction with the FGA Thr312Ala and FGB -455G/A SNPs, possibly because of lack of power.

We used a Mendelian randomisation line of thought to rule out any confounding by environmental factors and to ascertain whether the role of fibrinogen is a cause or a consequence. For this process, it is necessary to establish the relationship between genetic variants and plasma fibrinogen level, either from the literature or in the control population; both the FGA Thr312Ala and FGB -455G/A SNPs have been found to alter fibrinogen plasma levels. [12,13,15,19,26,27] We showed similar effects of the genetic variants in our control population, albeit to a modest extent. The expected increase in risk based on the increase in fibrinogen levels associated with these genetic variants is 4 - 49%, which is lower than the increase in risk that we established. These calculations are based on the hazard ratios from the meta-analysis by the Fibrinogen Studies Collaboration, focusing on coronary heart disease and stroke in the age group 40 - 59 years.[5] This meta-analysis also includes studies with participants aged 18 - 90 years, with an emphasis on the elderly. The effect of fibrinogen on the risk of arterial thrombosis is larger in the young, which might explain why the effect was larger in the young women in our study.

This fair-sized population-based case - control study for young women with myocardial infarction or ischaemic stroke provides a unique opportunity to determine (genetic) risk factors for this specific group that may be indicative of risk factors in older age categories. Nonetheless, our subgroup analyses (i.e. smoking and OC use analyses) had limited numbers for some subgroups. For this reason, we chose to use a dominant inheritance pattern. The dilution of effect that is observed in the risk analysis with a dominant inheritance pattern could therefore also affect these subgroup analyses. However, numbers were limited and strong conclusions cannot be drawn. We can therefore only conclude that there is no evidence for a strong interaction between the environmental factors and genetic variants.

The control population can be considered not to be in HWE because a P-value of 0.043 was calculated for the FGA Thr312Ala SNP. As our controls were sampled from the entire Netherlands through random digit dialing, we consider this slight deviation of the HWE to be probably due to chance. We chose to study two SNPs in the fibrinogen module that are known to be associated with altered fibrinogen levels. It is possible that these SNPs are

causal for this change in plasma level, but they could also be in linkage with other genetic traits that influence fibrinogen levels (e.g. Bcl I β-chain fibrinogen polymorphism). These other traits could potentially affect the plasma fibrinogen levels, still leaving our SNPs as proper markers of lifelong exposure to altered fibrinogen levels. The used SNPs could also be in linkage with genetic variations that result in functionally different fibrinogen and alter the risk via a mechanism other then altered plasma fibrinogen levels. This might be the case for the FGA Thr312Ala SNP. It has been negatively associated with venous thromboembolism, pulmonary embolism and poststroke mortality in patients with atrial fibrillation and ischaemic stroke.[19,20,27,28] This increase in risk might be caused by the changed characteristics of the fibrin fibers, which make the clot more prone to embolisation.[20] This increase in risk is in contrast with our results. But as the stroke cases included in our study are primarily of non-cardiac origin, we feel that the FGA Thr312Ala variation is a marker not of decreased disposition to embolisation but primarily of lower fibrinogen plasma levels. Nevertheless, conclusions based on the FGA Thr312 results should be drawn cautiously. The large individual-participant meta-analysis by the Fibrinogen Studies Collaboration showed a similar increase in risk for all major cardiovascular diseases, including non-fatal ischaemic stroke and myocardial infarction. The difference between our results (different effects of fibrinogen on the risks of myocardial infarction and ischaemic stroke) and those of the Fibrinogen Studies Collaboration (similar effects of fibrinogen on the risks of those diseases) might be due to confounding and reverse causation, problems that are resolved by the use of Mendelian randomisation in our study. Earlier studies with the same approach also showed an association of the FGB -455G/A SNP and increased plasma fibrinogen levels, but failed to show an association with myocardial infarction or ischaemic stroke.[13–15,27,29] These studies mainly included middle-aged to old subjects, in contrast to our young female population: the older age, and hence more developed stage of atherosclerosis, of the previously studied populations might explain why no direct relationship between the genetic variants and arterial thrombosis were found. If it is present at all, we hypothesize that our cases have minimal late stage atherosclerosis, because of their young age; only 11% of the myocardial infarction cases and 8% of the ischaemic stroke cases were reported to be diagnosed with hyperlipidaemia at the time of the event. Fibrinogen levels and other coagulation factors might therefore play a more pronounced role in arterial thrombosis in the young than in the old, either as a direct causal factor for thrombosis or a strong

cofactor in thrombosis initiated by subclinical atherosclerosis. This may be even more the case for the development of ischaemic stroke than for myocardial infarction.

Because of the use of a detailed questionnaire in the first phase of this case-control study, we were able to include only survivors of myocardial infarction and ischaemic stroke; fatal events could not be included, possibly leading to a survivor bias. Even if there is a difference between the aetiologies of fatal and non-fatal arterial thrombosis, we hypothesize that the effect of fibrinogen, or any other risk factor, is more pronounced in the fatal events. Therefore, if our study is affected by this survivor bias, this bias is likely to have led to an underestimation of the true effect of fibrinogen levels.

Conclusion In conclusion, this study showed an association between the FGA Thr312Ala SNP and a decreased risk of ischaemic stroke. If anything, the FGB -455G/A SNP was associated with an increase in risk. The direction of the effects is in accordance with the effects of the two SNPs on plasma fibrinogen levels. Neither SNP affected the risk of myocardial infarction. The FGA Thr312Ala SNP could also be a marker of altered clot characteristics, and the effect of the FGB -455G/A SNP has not been established firmly, which makes the interpretation of the increased risk associated with this variant difficult. Taking survivor bias into account, as well as the non-cardiac origin of the ischaemic stroke included in this study and the fact that the SNPs used in this study are proper markers of altered fibrinogen levels, we conclude that fibrinogen probably plays a causal role in the development of ischaemic stroke in young women.

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9

Genetic variants of coagulation factor XIII and the risk of myocardial infarction in young women

Bob Siegerink, Ale Algra and Frits R Rosendaal

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Abstract

Introduction Coagulation factor XIII is involved in the crosslinking of fibrin molecules and determines the architecture of the fibrin mesh. Genetic variation in FXIII could lead to differences in clot structure and density. Previous research indicated that genetic variation in genes coding for FXIII increased the risk of ischaemic stroke (IS) nine-fold (204Phe carriers: OR 9.1; 5.5-15). This study aims to determine whether genetic variation in genes coding for FXIII confers a similar increase in risk of myocardial infarction (MI) as with IS.

Methods We determined the four genetic variants in the MI arm (N=218) and control group (N=767) of the RATIO study, a population-based case-control study into risk factors for myocardial infarction and ischaemic stroke in young women (18-50 years). Odds ratios (OR) with 95% confidence intervals were calculated as measures of rate ratios assuming a dominant inheritance pattern.

Results FXIIIA Pro564Leu moderately increased the risk of MI by 40% (564Leu carriers: OR 1.4; 1.0-1.9). This increase in risk was confined to heterozygotes. The other SNPs did not alter MI risk (FXIIIA 34Leu carriers: OR 1.1; 0.8-1.5, FXIIIA 204Phe carriers: OR 0.8; 0.4-1.7 and FXIIIB 95Arg carriers: OR 0.8; 0.5-1.2).

Discussion FXIII SNPs do not play a major role in the aetiology of myocardial infarction whereas they do in IS. This pattern is consistent with earlier results from the RATIO study: prothrombotic defects are important risk factors for IS rather than for MI.

Introduction

Several proteins are involved in thrombus formation. Coagulation factor XIII is, upon activation by thrombin, responsible for the crosslinking of fibrin monomers.[1] The importance of FXIII and its role in haemostasis is demonstrated by the bleeding diathesis of FXIII deficient patients.[2] The protein consists of four chains which are encoded on different chromosomes: two B-chains (encoded on chromosome 1q31 - q32.1) which have no enzymatic activity and serve as carriers of the A-chains, and two A-chains (chromosome 6p25 - p24) that consist of the protransgluteminase which is involved in the crosslinking. This process determines clot structure and clot permeability and resistance to shear stress and fibrinolysis, factors that are of possible importance in pathologic mechanisms underlying thrombotic disease.[3–6] Genetic variants in one of the two genes which encode for coagulation factor XIII (*F13A1* and *F13B*) have been shown to be associated with an altered risk of both arterial and venous thrombosis thrombosis.[7–9] We previously showed that one of these four genetic variants, the *F13A1 204Phe* SNP, was associated with a 9-fold increased risk of ischaemic stroke in young women. Oral contraceptives use further increased this risk (odds ratio 20; 95% confidence interval 9-46).[10]

Since ischaemic stroke (IS) and myocardial infarction (MI) are both manifestations of acute forms of arterial thrombosis, we hypothesised that the risk of MI is also increased by these genetic variants of FXIII. We therefore set out to assess the relationship between these four SNPs in the FXIII genes and the risk of MI in the RATIO study.

Table1. Characteristics of 218 women with a first myocardial infarction and 767 control women.

		Patients N= 218		Control women N=767	
Age	Mean, SD	42.9	6.0	38.6	8.0
Caucasian Ethnicity	N, (%)	207	(95)	723	(94)
History of Hypertension	N, (%)	55	(25)	47	(6)
Diabetes	N, (%)	11	(5)	10	(1)
Hyperlipidaemia	N, (%)	23	(11)	22	(3)
Oral Contraceptive use	N, (%)	86	(39)	272	(36)
Smoking	N, (%)	181	(83)	319	(42)

All variables are measured within respect to the index date for controls and the year of event for cases

Methods

Study design & participants The Risk of Arterial Thrombosis In relation to Oral contraceptives (RATIO) study is a multicenter, population-based case-control study. The study consists of 3 arms, on MI, IS and peripheral arterial disease, of which details have been published earlier.[11–13] In short, for this study we included 218 women aged 18 to 50 years who were hospitalised for a confirmed first MI in one of 16 participating hospitals. Random digit dialling yielded 767 women aged 18 to 50 years who served as controls; the control group was frequency-matched to the patients for age (in five-year categories), residence, and index year. Furthermore, the women did not have a history of coronary heart disease, cerebrovascular event, or peripheral vascular disease. All participants filled in a questionnaire on possible risk factors and medical history focused on cardiovascular. diseases and provided DNA. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Measurements A total of 4 genetic variants was genotyped with the 5' nuclease/TaqMan assay: Val34Leu (rs5985), Tyr204Phe (rs3024477), and Pro564Leu (rs5982) variants in the FXIII subunit A gene (*F13A1*) and the His95Arg variant (rs6003) in the FXIII subunit B gene (*F13B*). Primer sequences, probe sequences, and restriction enzymes used are available on request. Laboratory technicians were unaware of case-control status and other patient characteristics.

Statistical Analysis The effect of the genetic variation in the genes encoding for FXIII on the risk of MI was assessed by the calculation of odds ratios as measures of rate ratios with the corresponding 95% confidence intervals with logistic regression. Odds ratios were calculated per genotype and according to dominant inheritance pattern and were adjusted for the stratification factors age (on a continuous scale), area of residence and index year.

Results

The baseline characteristics of patients and controls are displayed in table 1. As expected, patients reported more cardiovascular risk factors than controls. Genotype distributions and corresponding odds ratios are shown in table 2. The overall call rate was 97.8% (range, 96.1% - 98.7%). No deviation from Hardy-Weinberg equilibrium was found in the control women for any of the genotypes. Only the *F13A1* 564Leu variant of the *F13A1* gene was associated with an increased risk of 40% (OR 1.40; 95% confidence interval 1.01 – 1.93) in the dominant inheritance pattern analysis. The increase in risk for the *F13A1* Pro564Leu

Table 2. Genotype distribution amongst cases and controls and their corresponding risks for myocardial infarction

Gene variant		Allele frequency	Myocardial infarction patients N=218			Control women N=767			Odds Ratio	
			AA	AB	BB	AA	AB	BB	OR	95% CI
F13 A1	Val34Leu (rs5985)	0.25	124 (57)	80 (37)	14 (6)	419 (56)	283 (38)	45 (6)	1.07	0.78 - 1.48
F13 A1	Tyr204Phe (rs3024477)	0.03	208 (95)	10 (5)	0 (0)	711 (94)	42 (6)	1 (0)	0.82	0.39 - 1.71
F13 A1	Pro564Leu (rs5982)	0.21	119 (55)	91 (42)	7 (3)	466 (62)	251 (33)	34 (5)	1.40	1.01 - 1.93
F13 B	His95Arg (rs6003)	0.09	185 (86)	28 (13)	3 (1)	609 (83)	112 (15)	9 (1)	0.79	0.50 - 1.24

AA = major allele homozygote (non-carrier), AB = heterozygote (carrier), BB = minor allele homozygote (carrier). Allele frequency in control population. Odds ratios are calculated for carriers of the gene variant (i.e. dominant inheritance pattern is assumed) and are adjusted for the stratification factors age, calendar year of the index event, and area of residence.

variant was confined to the heterozygous carriers (1.46; 1.05 – 2.03): the homozygous carriers of the minor allele had the same risk of disease as the homozygous carriers of the major allele (0.96; 0.40 - 2.28). The variants *F13A1* Val34Leu, *F13A1* Tyr204Phe and *F13B* His95Arg SNPs did not affect the risk of MI in both the dominant and the per genotype analyses.

Discussion

The *F13A1*564Leu variant is the only variant that affected the risk of MI in our study. This SNP has been associated with both a lower FXIII plasma level and an increase FXIII activity.[14,15] This, together with the lack of dose response, does not add to a plausible biologic mechanism which explains the increase in risk of MI in heterozygotes, nor does the absence of an effect in homozygotes. This suggests that the minor increase in risk we observed for the heterozygous genotype is a false positive finding.

An earlier study on FXIII SNPs suggested an increase risk of IS for *F13A1* 34Leu and *F13A1* 204Phe, but not for MI.[7] However, due to the limited number of cases (68 MI cases and 36 IS cases) no definite conclusions could be drawn. The modest protective effect of *F13A1* 34Leu variant on MI could not be replicated, probably due to lack of power.[8] Earlier results from the RATIO study showed a nine-fold increased risk of ischaemic stroke for carriers of the *F13A1* 204Phe whereas carriers of the *F13B* 95Arg variant had a 1.7 fold increase in the risk of myocardial infarction.[10]

Conclusion Although myocardial infarction and ischaemic stroke are both acute manifestations of arterial thrombosis, SNPs in the FXIII genes have different effects. Even

though the exact underlying causal mechanism cannot be established from these data, these differences in effects suggest that FXIII has a different role in the aetiology of MI and IS. Further study into the differences between the aetiology of myocardial infarction and ischaemic stroke is warranted.

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10 Clot lysis time and the risk of myocardial infarction and ischaemic stroke in young women

Bob Siegerink,Mirjam E Meltzer, Phillip G de Groot, Ale Algra, Ton Lisman and Frits R Rosendaal

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Abstract

Introduction Reduced overall fibrinolytic capacity increases the risk of myocardial infarction (MI), as demonstrated previously in studies which included primarily men. We aimed to determine the influence of altered fibrinolysis on the risk of MI and ischaemic stroke (IS) in young women.

Methods The RATIO study is a population-based case-control study including young women with MI (n=205), IS (N=175) and 638 matched healthy controls. Fibrinolytic potential was determined with a tissue factor/tissue plasminogen activator induced clot-lysis assay. Odds ratios (OR) and corresponding confidence intervals adjusted for cardiovascular risk factors were obtained with logistic regression as measures of rate ratios. Clot-lysis time (CLT) was divided into tertiles based on the control group (T1-T3), with the middle tertile (T2) as reference.

Results *Hypofibrinolysis* (prolonged CLT) was associated with an increase in risk of MI (T3 vs T2, OR 2.8;95%CI 1.7-4.7). *Hyperfibrinolysis* (decreased CLT) only a small effect (T1 vs T2, 1.6; 0.9-2.9). *Hypofibrinolysis* did not affect the risk of IS significantly (T3 vs T2, 1.5; 0.7-3.0), whereas *hyperfibrinolysis* increased this risk substantially (T1 vs T2, 4.1; 2.1-8.0). Oral contraceptive use and smoking further increased these risks.

Discussion *Hypofibrinolysis* increases the risk for MI in young women, a finding similar to previous studies. Counter-intuitively, *hyperfibrinolysis* increased the risk of IS fourfold, which suggests that MI and IS have different aetiologies.

Introduction

Cardiovascular disease places a large burden on western societies, both on the healthcare systems as well as on the quality of life. The two major forms of arterial thrombosis are myocardial infarction and ischaemic stroke.[1] Arterial thrombosis is most prevalent in the elderly; risk factors, such as diabetes, hypertension and hypercholesterolaemia are also more common with progressing age. Because the prevalence of these 'traditional' risk factors is less pronounced in the young, new risk factors can more easily be identified in young participants.[1]

An increased capacity to form blood clots leading to a procoagulant state may increase the risk of arterial thrombosis.[2] A procoagulant state may not solely be the result of an increased propensity to initiate or propagate clot formation, but can also result from a decreased ability to dissolve these newly formed clots.[3] The main fibrinolytic factor is plasmin, which degrades fibrin and thereby dissolves the clot. The zymogen of plasmin, plasminogen, can be activated by tissue plasminogen activator (tPA) or urokinase.[4] Plasmin itself can be inhibited directly by α2-antiplasmin. Furthermore, plasminogen activator inhibitor 1 (PAI-1) decreases the formation of plasmin by direct inhibition of tPA and urokinase. Thrombin Activatable Fibrinolysis Inhibitor (TAFI) also hampers fibrinolysis by altering the fibrin structure and thereby reducing binding and activation of plasminogen. Individual factors of the fibrinolytic system, especially PAI-1 and TAFI, have been linked to myocardial infarction and ischaemic stroke although results have been inconclusive and contradictory as was reviewed by Meltzer et al.[5]

Besides individual factors, clot lysis assays can be used to asses the fibrinolytic capacity. Some studies, but not all, indicate an association between the euglobin clot lysis time or dilute whole blood clot lysis time and myocardial infarction. This association might be most pronounced in patients with minimal atherosclerosis.[5,6] Studies on ischaemic stroke are limited.[7,8] However, these global tests in these studies do not reflect all the appropriate components of the fibrinolytic system. A previously described test takes all primary components of the fibrinolytic system (plasminogen, alpha2-antiplasmin, PAI-1 and TAFI) into account and therefore reflects the true global plasma fibrinolytic potential better.[9,10] The main determinant of this test is the level of PAI-1, followed by plasminogen TAFI, prothrombin, and α2-antiplasmin levels.[10] Two studies indicate that a prolonged clot lysis

time (CLT) is associated with venous thrombosis, especially in combination with other risk factors such as oral contraceptives.[11,12]

Hypofibrinolysis is also associated with different cardiovascular diseses: prolonged CLT (fourth quartile vs. first quartile) was associated with a twofold increase in risk of myocardial infarction for men aged <50 years, whereas no such association was found for men aged ≥50.[12,13] Another case-control study with 330 young patients with coronary heart disease, ischaemic stroke (including transient ischaemic attack) and peripheral arterial disease also showed an increased risk for the combined endpoint arterial thrombosis with increasing clot lysis time.[14] The inclusion of both sexes (37% men) and the broad case definitions make it difficult to draw conclusions for some subanalyses, such as an interaction analysis focussing on oral contraceptive use and smoking. We therefore set out to determine whether abnormal fibrinolysis, both hyper- and hypofibrinolysis, is associated with both myocardial infarction and ischaemic stroke in young women and whether other risk factors influence this risk.

Methods

Study design & participants The RATIO (Risk of Arterial thrombosis in relation to oral contraceptives) study is a multicenter case-control study set up to identify risk factors for arterial thrombosis in young women, and has been described in detail earlier.[15–17] In short, young women (18-50 years) diagnosed with a form of arterial thrombosis in the 16 participating hospitals including eight academic medical centres in the Netherlands, were approached to participate. Diagnosis of myocardial infarction was based on the presence of symptoms, elevated cardiac-enzyme levels, and electrocardiographic changes, whereas ischaemic stroke was diagnosed on the basis of medical history, neurological examination, and CT or MRI scan by experienced neurologists in the participating centres. Women without a history of arterial thrombosis were approached by random digit dialling and frequency-matched with the case groups on age (in five year categories), year of event and area of residence. All participants were asked to fill out a standardised questionnaire on several topics such as demographic characteristics, medical history among which oral contraceptive use in the year prior to event or comparable time frame for healthy controls.

Measurements Citrated plasma used for these measurements was not thawed previously. Clot lysis time was assessed by measuring the changes in plasma turbidity during tissue-factor induced clot formation and subsequent lysis by exogenous t-PA.[9] In short, 50 µl of

mixture containing phospholipid vesicles (40% L-α-dioleoylphosphatidylcholine, 40% L-α-dioleoylphosphatidylethanolamine and 20% L-α-dioleoylphosphatidylserine in a final concentration of 10 µmol/l), t-PA (final concentration 56 ng/ml), tissue factor (final dilution 1/1000) and $CaCl_2$ (final concentration 17 mmol/L) diluted in HEPES buffer [25mmol/L HEPES (N-2-hydroxytethylpiperazine-N'2ethanesulfonic acid) 137mmol/L NaCl, 3,5 mmol/L KCl, 3 mmol/L $CaCl_2$, 0,1% bovine serum albumin, pH 7,4] was added to 50 µl of citrated plasma, all in a 96-well microtiter plate. After thorough mixing, the plate was placed in a Spectramax 340 kinetic microplate reader at 37º C (molecular devices corporation, Menlo Park, CA, USA). Optical density (OD) was measured every 20 seconds at 405 nm, resulting in a clot-lysis turbidity profile. Clot-lysis time is defined as the time from the midpoint of the clear to the maximal turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing clot lysis. The laboratory technician

Table 1. Characteristics of participants stratified by case and control status

	Myocardial infarction N=205	Ischaemic stroke N=175	Control N=638
Mean age[1]	42	39	39
Caucasian ethnicity	195 (95%)	167 (97%)	602 (94%)
History of [2]			
Hypertension	53 (26%)	50 (29%)	40 (6%)
Diabetes	10 (5%)	7 (4%)	10 (2%)
hypercholesterolaemia	21 (10%)	14 (8%)	19 (3%)
Oral contraceptives use [2]	81 (40%)	92 (53%)	231 (33%)
Smoking [2]	169 (82%)	105 (60%)	270 (42%)
Median BMI [3] *(Q1 - Q3) ka/m²*	24.6 (22.4-27.7)	23.3 (21.3-27.0)	22.8 (21.0-25.1)
Median triglycerides [3] *(Q1 - Q3) mmol/L*	1.68 (1.13-2.71)	NA	1.24 (0.88-1.84)
Clot-lysis time[3,] *minutes*			
Median (Q1 - Q3)	70.7 (60.8- 4.0)	60.6 (52.7-72.0)	61.9 (56.1- 69.7)
Mean (SD)	75.2 (25.0)	68.1 (36.3)	64.4 (14.0)

[1] at moment of event (cases) or index date (controls).[2] in the year prior to event (cases) or index year (controls). [3] at time of blood draw. SD = standard deviation, Q1 - Q3= 25th and 75th percentile, CLT = Clot-lysis time, NA = not applicable. Clot-lysis time measurements were unavailable or missing for 8 controls, 3 cases in the myocardial infarction group, and 10 patients in the ischaemic stroke group.

Table 2. Hypo and hyperfibrinolysis and the risks of myocardial infarction and ischaemic stroke

		MYOCARDIAL INFARCTION					ISCHAEMIC STROKE					CONTROLS	
	CLT (min)	N	%	Odds ratio (95% confidence interval) model 1	Odds ratio (95% confidence interval) model 2	Odds ratio (95% confidence interval) model 3	N	%	Odds ratio (95% confidence interval) model 1	Odds ratio (95% confidence interval) model 2	Odds ratio (95% confidence interval) model 3	N	%
T1	0-58	36	18%	1.18 (0.69-1.95)	1.60 (0.88-2.92)	1.59 (0.87-2.90)	69	42%	2.10 (1.28-3.49)	4.07 (2.07-8.04)		207	33%
T2	58-66	37	18%	1 [REF]	1 [REF]	1 [REF]	34	21%	1 [REF]	1 [REF]	NA	209	33%
T3	66-301	129	64%	3.15 (2.04-4.86)	2.82 (1.69-4.72)	2.84 (1.69-4.76)	62	38%	2.07 (1.24-3.48)	1.50 (0.74-3.03)		214	34%

NA = not applicable, CLT = Clot-lysis time, REF = reference group, T1 = 1st tertile of clot-lysis time, or hyperfibrinolysis. T2 = 2nd tertile of clot-lysis time, or normofibrinolysis. T3 = 3rd tertile of clot-lysis time, or hypofibrinolysis. All tertiles are based on the clot-lysis time of the control group. Odds ratios are calculated with the middle tertile as reference. Regression model 1 includes stratification factors age, year of event and area of residence as covariates. Model 2 includes the covariates of model 1 plus smoking, body mass index, hypertension, diabetes and hypercholesterolaemia. Model 3 includes the covariates of model 2 plus log transformed triglycerides levels.

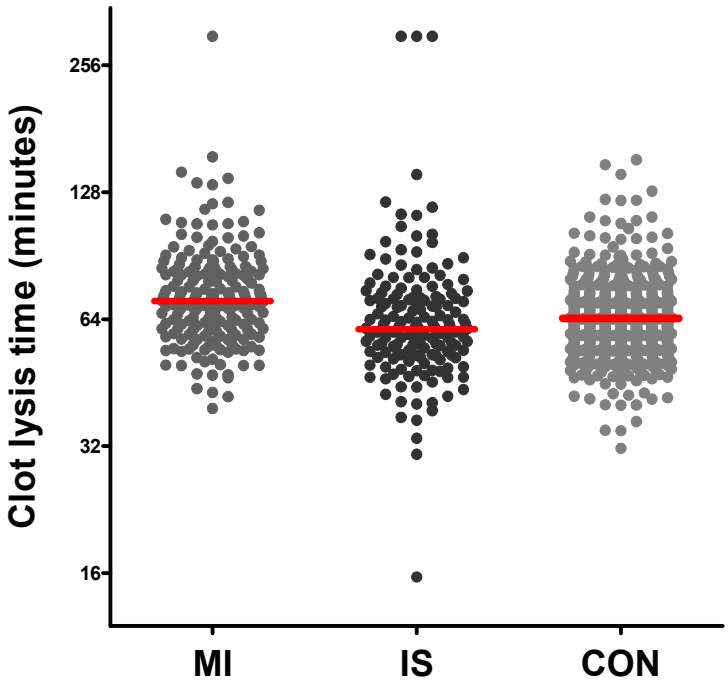
was unaware of the case-control status of the samples.

Statistical analyses To assess clot lysis time in relation to arterial thrombosis we calculated the mean difference and corresponding 95% confidence interval (CI) of clot lysis time in the control and case groups with the t-test for independent samples. To further study the effect of both *hypo-* and *hyperfibrinolysis* clot lysis time was divided into three categories based on the tertiles of the control group. Logistic regression models were used to obtain odds ratios and corresponding 95% CIs as measures of rate ratios with *normofibrinolysis,* defined as the middle tertile as the reference category. The covariates in these models included at least the stratification factors age (on a continuous scale), index year and area of residence. Furthermore, smoking status, body mass index, hypertension, diabetes and hypercholesterolemia were considered as confounders and included in subsequent models. Since triglyceride levels could also affect clot lysis time, we also additionally included log transformed trigyceride levels in the model.[11,13] Triglyceride data were only available for the myocardial infarction and control groups.

Results

As expected, known cardiovascular risk factors such as smoking and diabetes were more prevalent in the two case groups than in the controls (table 1). Since age was a matching variable, the three groups were similar with

Figure 1. Clot lysis time per case group



MI = Myocardial infarction, IS = Ischaemic stroke, CON = controls
The clot lysis time of all participants are shown, stratified per case group. The horizontal lines represent the mean clot lysis time for each case group.

re

spect to age. Clot-lysis time was higher among myocardial infarction cases than in controls (mean difference 10.8 minutes, 95% CI 7.2 to 14.5). CLT in ischaemic stroke cases was shorter (mean difference 3.7 minutes, 95% CI -2.0 to 9.4). Figure 1 shows the clot-lysis time for each participant with the horizontal lines indicating the mean, stratified per case group.

With the middle category as reference, hypofibrinolysis (or the third tertile and longest CLT) was associated with a threefold increase in risk of myocardial infarction (OR 3.15, 95% CI 2.04 - 4.86; table 2). Adjustment for confounders, including triglyceride levels, decreased this risk slightly (OR 2.84, 95% CI 1.69 - 4.76). *Hyperfibrinolysis*, or the first tertile and shortest CLT, was not clearly associated with myocardial infarction. The effects were more pronounced in women younger than 40 years (table 3). *Hypofibrinolysis* (T3) increased the risk of ischaemic stroke about twofold (OR 2.07, 95% CI 1.24-3.48), but this risk greatly decreased after adjustment for confounders (OR 1.50, 95% CI 0.74 - 3.05). *Hyperfibrinolysis* (T1) also increased the risk of ischaemic stroke (OR 4.07, 95% CI 2.07 - 8.03) after

adjustment. The risks were most pronounced in the young (see table 3). Upon exclusion of oral anticoagulant users (16 ischaemic stroke cases and 19 myocardial infarction cases) these results were largely the same (data not shown).

Oral contraceptive use within the subgroup of participants with *normofibrinolysis* increased the risk of both myocardial infarction and ischaemic stroke (table 4) about two- to fourfold (T2/- OC vs. T2/+ OC OR 2.4, 95% CI 1.0 - 5.6 for myocardial infarction and OR 3.7, 95% CI 1.3 - 11 for ischaemic stroke). This was also true for smoking (T2/- smoking vs. T2/+ smoking OR 3.3, 95 %CI 1.4 - 7.7 for myocardial infarction and OR 1.9, 95% CI 0.7 - 5.3 for ischaemic stroke). The risk of smoking was highest in women with abnormal clot lysis time when compared with women with *normofibrinolysis* and who did not smoke (T3/+ smoking OR 14, 95% CI 6.2-31 for myocardial infarction and T1/+ smoking OR 7.2, 95% CI 2.7 - 19 for ischaemic stroke).

Discussion

Our study indicates that *hypo*fibrinolysis increases the risk of myocardial infarction in young women. Counter intuitively, we found that *hyper*fibrinolysis was associated with an increased risk of ischaemic stroke. Furthermore, the use of oral contraceptives increased the risk in all strata of CLT.

A decreased fibrinolytic capacity is a plausible causal risk factor for thrombosic events and the results for myocardial infarction are in correspondence with current knowledge and the results in earlier studies.[13,14] Our results, unexpectedly, indicate also that *hyperfibrinolysis* increases the risk of ischaemic stroke. This finding could be due to chance, but also raises the question whether the observed association reflects a causal mechanism in which an altered fibrinolytic capacity plays a role in the aetiology of ischaemic stroke or whether it can be explained by other mechanisms. For instance, *hyperfibrinolysis* in patients suffering from atrial fibrillation could increase the risk of cardioembolic stroke as theoretically an increased potential to dissolve blood clots can lead to instable clots and subsequent embolisation. However, the RATIO study excluded patients with an an overt cardiac source and therefore we feel that this mechanism cannot explain our results.

Our counter-intuitive findings on hyperfibrinolysis and ischaemic stroke are not necessarily in contrast with previous findings. The 4G/5G polymorphism of the PAI-1 promotor, which is associated with low levels of circulating PAI-1 (with carriers of the 5G allele having lower

Table 3. Risks of myocardial infarction and ischaemic stroke; younger vs older

	MYOCARDIAL INFARCTION		ISCHAEMIC STROKE	
	Odds ratio (95% confidence interval) Model 2		Odds ratio (95% confidence interval) Model 2	
	<40 years	≥ 40 years	<40 years	≥ 40 years
T1	3.8 (1.2 - 12)	1.0 (0.5 - 2.4)	7.7 (2.4 - 24)	2.8 (1.1 - 7.2)
T2	1 [REF]	1 [REF]	1 [REF]	1 [REF]
T3	7.8 (2.5 - 24)	2.3 (1.2 - 4.2)	2.8 (0.8 - 10)	1.0 (0.4 - 2.5)

T1 = 1st tertile of clot-lysis time, or hyperfibrinolysis. T2 = 2nd tertile of clot-lysis time, or normofibrinolysis. T3 = 3rd tertile of clot-lysis time, or hypofibrinolysis. Tertiles are based on clot-lysis time in control group. Odds ratios and corresponding confidence intervals are calculated with the middle category as reference and with smoking, body mass index, hypertension, diabetes and hypercholesterolaemia as covariables ('model 2').

Table 4. Interaction analyses; oral contraceptive use and smoking behaviour

	TERTILE	OC USE	CONTROLS	MYOCARDIAL INFARCTION		ISCHAEMIC STROKE	
				#	OR (95%CI)	#	OR (95%CI)
ORAL CONTRACEPTIVE	**T1**	-	119	15	1.5 (0.6 - 3.5)	31	6.2 (2.4 - 16)
	T1	+	88	21	3.8 (1.6 - 8.9)	38	9.1 (3.4 - 25)
	T2	-	127	19	1 [REF]	17	1 [REF]
	T2	+	82	18	2.4 (1.0 - 5.6)	17	3.7 (1.3 - 11)
	T3	-	174	88	3.1 (1.6 - 6.1)	31	1.5 (0.6 - 3.8)
	T3	+	40	41	7.4 (3.3 - 17)	31	7.0 (2.4 - 21)
		SMOKING					
SMOKING	**T1**	-	129	7	0.7 (0.2 - 1.9)	34	3.0 (1.2 - 7.8)
	T1	+	78	29	6.5 (2.7 - 16)	35	7.2 (2.7 - 19)
	T2	-	110	9	1 [REF]	13	1 [REF]
	T2	+	99	28	3.3 (1.4 - 7.7)	21	1.9 (0.7 - 5.3)
	T3	-	122	20	1.3 (0.5 - 3.3)	22	0.8 (0.3 - 2.3)
	T3	+	92	109	14 (6.2- 31)	40	4.8 (1.8 - 13)

See table 3 for description. All presented ORs are from model 2;smoking was only included in the oral contraceptive use interaction analyses.

levels), has been suggested to be associated with an increased risk of ischaemic stroke.[18,19] PAI-1 is one of the main determinants of CLT and is known to have functions, other than decreasing fibrinolytic potential, which could explain our findings.[10] One of these functions is the so-called tPA-serpin axis which is involved in neuronal damage after cerebral ischaemia.[20] Animal studies suggest that endogenous tPA or rtPA used as treatment after ischaemic stroke could lead to an N-Methyl-D-aspartic acid-mediated Ca2+ influx which enhances neuronal damage. PAI-1, a serpin which inhibits tPA, could counteract this mechanism. Therefore, low levels of PAI-1, reflected by hyperfibrinolysis in our study, could lead to neuronal damage. In addition, high PAI-1 has been shown to be related to a decreased tendency of plaque rupture.[20] Although atherosclerosis is not abundantly present in these young women, low levels of PAI-1 could be related to increased tendency of plaque rupture and subsequent thrombus formation and then increase the risk of ischaemic stroke of atherosclerotic origin. So, the observed association between low CLT and increased risk of ischaemic stroke in our study does not necessarily reflect a causal role of increased fibrinolytic tendency but can also indicate other causal mechanisms that include low levels of PAI-1. Given this uncertainty, we can still conclude that our results suggest a clear difference in the aetiology of myocardial infarction and ischaemic stroke.

Our study has some limitations and strengths: we cannot stratify our results according to the subtype of stroke (e.g. TOAST criteria) which hampers the interpretation of the results.[21] It is possible that our results reflect the causal mechanism of only one stroke subtype, which would imply that the effect for that subtype would even be stronger. Furthermore, due to the case-control design of this study it cannot be ruled out that the differences in clot-lysis time between the case groups are a consequence of the disease or disease treatment, instead of a cause (i.e. 'reverse causation'). A major source of this bias is the use of blood samples taken during the acute phase of the disease, directly after the event. However, since blood was drawn after the acute phase (mean 82 months, at least 23 months after the event), it is unlikely that the results are explained by this phenomenon. Other sources of this bias, such as changes in cardiovascular risk factors after the event, would have led to an underestimation of the true effect and are not likely to exert a different effect between patients suffering from myocardial infarction and ischaemic stroke. Also, treatment initiated after the event could have introduced a bias: since antihypertensive drugs and statins might increase clot lysis and hence decrease clot lysis time, the effect of clot lysis time on myocardial infarction might be underestimated

whereas the effect on ischaemic stroke might be overestimated.[22] Because treatment strategies largely overlap for these two diseases it is not likely that our main finding, the difference in effect of clot lysis time between myocardial infarction cases and ischaemic stroke cases, is to be explained by this potential source of bias. Our study only includes patients that survived a first event which may affect the results. Therefore our results only apply to those who survived their first event. If the effect of clot lysis time leads to a more severe forms of disease, our results are an underestimation of the true effect.

The secondary analyses regarding oral contraceptive and smoking yield six strata and this results in small numbers of participants per stratum, as is reflected by the wide confidence intervals. Therefore, these analyses provide some idea into the associations with the combination of risk factors, but do not allow strong conclusions on the presence of interactions. Increased levels of triglycerides could confound the relation between clot-lysis time and arterial thrombosis.[13] Unfortunately, triglyceride levels were not available for the ischaemic stroke analyses. However, adjustment for triglyceride levels in the myocardial infarction analyses only minimally changed the point estimates. Therefore, it is unlikely that effects observed in the ischaemic stroke analyses can be attributed entirely to triglycerides levels.

Conclusion We found that in young women hypofibrinolysis increased the risk of myocardial infarction whereas hyperfibrinolysis increased the risk of ischaemic stroke. Although the results obtained from the ischaemic stroke analyses are not easily interpreted and causal conclusions on the mechanism by which hyperfibrinolysis increases the risk of ischaemic stroke cannot be drawn, these results indicate that myocardial infarction and ischaemic stroke may have different causal mechanisms.

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11

High VWF, low ADAMTS13, and oral contraceptives increase the risk of myocardial infarctions and ischaemic stroke in young women

Helena M Andersson,Bob Siegerink, Brenda M Luken, James TB Crawley,Ale Algra, David M Lane, and Frits R Rosendaal

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Abstract

Introduction VWF and ADAMTS13 are major determinants of platelet adhesion after vessel injury. In the present study, we aimed to determine whether VWF or ADAMTS13 plasma antigen levels influence the risks of ischaemic stroke (IS) or myocardial infarction (MI) in young women and how these risks are affected by oral contraceptive use.

Methods VWF and ADAMTS13 plasma antigen levels were measured in a frequency-matched case-control study young (18-49 years) women including 205 MI patients, 175 IS patients and 638 controls.

Results Increasing levels of VWF and decreasing levels of ADAMTS13 were associated with the risk of IS and MI in a dose-dependent manner. Having both high VWF and low ADAMTS13 resulted in an odds ratio (OR) of 6.9 (95% confidence interval [95% CI], 2.0-23.0) for IS and 11.3 (95% CI 3.6-35.2) for MI. Use of oral contraceptives increased the risk of IS and MI associated with high VWF (OR = 12; 95% CI 5.5-26.2 and OR = 7.5, 95% CI 3.6-15.7, respectively) and the risk of IS associated with low ADAMTS13 (OR = 5.8, 95% CI 2.7-12.4).

Discussion We conclude that high VWF and low ADAMTS13 plasma levels both increase the risk of IS and MI. The risks associated with high VWF or low ADAMTS13 levels are further increased by the use of oral contraceptives.

Introduction

VWF is a plasma glycoprotein synthesised by endothelial cells and megakaryocytes[1]; it is a major determinant of platelet adhesion after vessel injury and consequently of clot formation and circulates in plasma at approximately 10 μg/mL as multimers with a molecular weight(MW)ranging from 500-20 000 kDa.[1,2] High-MW forms of VWF are stored in Weibel-Palade bodies in endothelial cells and also in platelet α-granules.[3,4] These VWF stores are secreted into plasma after endothelium or platelet activation.[5,6] The multimeric composition of plasma VWF is regulated by ADAMTS13 (a disintegrin and metalloproteinase with the thrombospondin type I repeat 13), which preferentially cleaves the large VWF multimers into smaller, less prothrombotic variants. ADAMTS13 is a metalloproteinase synthesised mainly by hepatocytes, but also by endothelial cells and megakaryocytes.[7–9] It circulates in plasma at a concentration of approximately 1 μg/ mL.[10] The clinical importance of VWF and ADAMTS13 in regulating haemostasis is highlighted by the bleeding and thrombotic diseases associated with their respective deficiencies. VWD is characterised by a qualitative or quantitative deficiency in VWF resulting in impaired platelet tethering. This is the most common inherited bleeding disorder in humans.[11] Conversely, thrombotic thrombocytopenic purpura, which is characterised by a deficiency in ADAMTS13, results in the presence of hyperreactive plasma VWF that leads to thrombotic microangiopathy.[12]

Cardiovascular disease (CVD) is a leading cause of disability and death in both developed and developing countries, and is predicted to affect an increasing number of people worldwide over the next decades.[13]Ischaemic stroke (IS) and myocardial infarction (MI) are among the most common CVD manifestations. Prospective studies investigating the risk of CVD associated with increased VWF antigen levels have been performed, generally in individuals over the age of 45 years. Some prospective studies have investigated the risk of coronary heart disease, atrial fibrillation, or IS and did not find an association with VWF antigen levels[14–17]; however, other studies investigating the risk of CVD, angina pectoris, hemorrhagic stroke, or IS did find this association.[17–20] Several case-control studies have been carried out, and, although some controversy exists, many report an association of high VWF antigen levels with both IS[21–23]and MI.[24–27]However, in certain case-control studies, blood sampling was performed during the acute phase of the thrombotic event. This makes it difficult to determine whether the observed increase in VWF levels are a cause or a consequence of the event, because it has been shown that VWF plasma levels

are increased during the acute phase after stroke or MI, possibly as a consequence of damage to the endothelium.[28,29] This may even be the case in prospective studies, in which VWF levels could be the result of vessel wall damage that predisposes to disease.

A small number of studies have investigated the association of ADAMTS13 with IS and MI, with conflicting outcomes. Studies investigating ADAMTS13 antigen levels during the acute phase (0-14 days) of MI found decreased levels of ADAMTS13.[26,29]It is, however, difficult to establish whether this was a cause or a consequence of the event. Other studies evaluating the association between ADAMTS13 antigen levels and MI between 1 and 9 months after the event found an association with either low ADAMTS13 [25,30]or high ADAMTS13[24] plasma levels. Only 1 study looked at the association between ADAMTS13 antigen levels and a subgroup of IS patients, and found no association.[30]

In the present study, we have reinvestigated whether plasma VWF and ADAMTS13 levels are associated with the risk of IS and MI in the previously published RATIO case-control study.[31–33] This study included only young women (18-50 years), so the findings were presumably less complicated by age-related deterioration of the vasculature. Blood was taken well after the acute phase of the event, meaning that VWF and ADAMTS13 antigen levels should have returned to those before the event, ensuring they were not influenced by acute-phase post event endothelial damage. We also investigated the combined effect of both VWF and ADAMTS13 levels on the risks of IS and MI, and how the risk was affected by use of oral contraceptives.

Methods

Study design and participants Study design and details of the RATIO case-control study have been extensively described elsewhere.[31–33] Briefly, women between 18 and 49 years of age with a first event of IS (N = 175) or MI (N = 205) were included. IS was diagnosed based on clinical symptoms, neurologic examination, and computed tomography or magnetic resonance imaging. Exclusion criteria were haemorrhagic stroke, transient ischaemic attack, venous sinus thrombosis, carotid artery dissection, history of cardiovascular or cerebrovascular disease, severe illness, cognitive impairment, and aphasia. MI was diagnosed based on clinical symptoms, elevated cardiac- enzyme levels, and electrocardiographic changes. Blood samples were collected after a median of 95 months (range, 23-146 months) for IS and 69 months (range, 38-117 months) for MI, ensuring that plasma proteins should not have been influenced by any acute-phase protein

response to the thrombotic event. The control group (N = 638) was frequency-matched based on age, area of residence, and index year. Plasma samples from 167 IS patients, 202 MI patients, and 626 patients were available for analysis in the present study. The presence of conventional risk factors for CVD, such as hypertension, hypercholesterolaemia, diabetes, and smoking, and the use of oral contraceptives were based on the year before the event/index year. Our study protocol was approved by the ethical committees of the participating hospitals and informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Measurements VWF plasma antigen levels were determined through an in-house ELISA. A polyclonal rabbit anti-VWFAb (3.1 µg/mL; Dako) was immobilised in a 96-well Maxisorp microplate (Nunc) in 50mM carbonate buffer, pH 9.6, at 4°C overnight. Washing steps were performed in triplicate with 300 µL/ well of PBS with 0.1% Tween-20 (PBST) between each step. All steps were carried out at room temperature on a shaker. Wells were blocked with 2.5% BSA(Sigma-Aldrich) in PBSTfor 2 hours. A standard curve of 0-125 ng/mL was made with normal control plasma (Technoclone), and samples were diluted 1:320 in 1% BSA in PBST and added to the wells in triplicate. Bound VWF was detected with 1.1 µg/mL of HRP-conjugated polyclonal Ab against VWF (Dako) for 1 hour. The plate was developed with 100 µL/well of o-phenylenediamine dihydrochloride (Sigma-Aldrich) for 3 minutes, and the reaction was stopped with 50 µL/well of 2.5M H2SO4 and the absorbance read at 492 nm. The inter- and intra-assay coefficients of variation were 6.4% and 6.3%, respectively. ADAMTS13 plasma antigen levels were determined with an in-house ELISA as described previously.[24] A polyclonal rabbit Ab (5 µg/mL,depleted of anti–TSP2-4 Abs) against ADAMTS13 was immobilised in a 96-well Maxisorp microplate (Nunc) in 50mM carbonate buffer, pH 9.6, at 4°C overnight. Washing steps were performed in triplicate with 300 µL/well of PBST between each step. All incubations were carried out at room temperature on a shaker. Wells were blocked with 2.5% BSA in PBS for2 hours. A standard curve of 0-108 ng/mL was made with normal control plasma (Technoclone) and samples were diluted 1:25 in 1% BSA in PBS and added to the wells in triplicate. Bound ADAMTS13 was detected with 0.2 µg/mL of biotinylated polyclonal Ab against the TSP2-4 domains of ADAMTS13 for 2 hours. Wells were incubated with streptavidin-HRP (GE Healthcare) diluted 1:1000 for 1 hour and the plate was developed with 100 µL/well of o-phenylenediamine dihydrochloride (Sigma-Aldrich) for 10 minutes. The reaction was stopped with 50 µL/well

Table 1. Baseline characteristics of participants of the RATIO case-control study

	Myocardial infarction (n=205)	Ischaemic stroke (n=175)	Control (n=638)
Age, median	43	39	39
Caucasian ethnicity	195 (95%)	167 (95%)	602 (94%)
Oral contraceptive use*	81 (40%)	92 (53%)	213 (33%)
Hypercholesterolemia*	21 (10%)	14 (8%)	19 (3%)
Hypertension*	53 (26%)	50 (29%)	40 (6%)
Diabetes*	10 (5%)	7 (4%)	10 (2%)
Smoking*	169 (82%)	101 (58%)	270 (42%)

** in the year prior to event / index year*

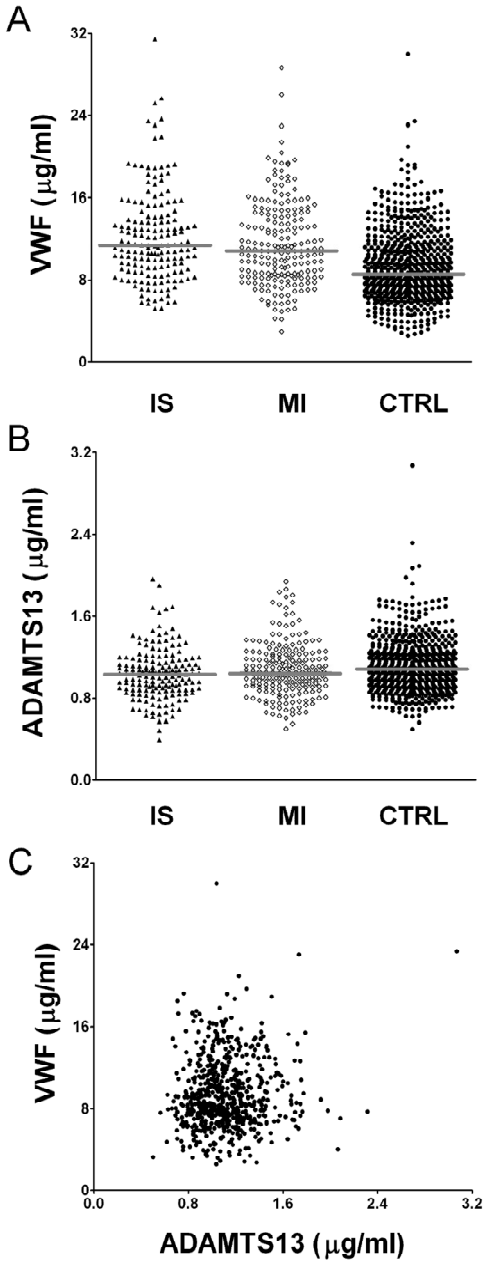
of 2.5M H2SO4 and the absorbance was read at 492 nm. Inter- and intra-assay coefficients of variation were 6.9% and 7.3%, respectively.

Statistical analyses Odds ratios (ORs) and corresponding 95% confidence intervals (95% CI) were calculated as measures of rate ratios for MI and IS by unconditional logistic regression. The lowest quartile and > 90th percentile (p90, VWF analyses), or the highest quartile and < 10th percentile (p10, ADAMTS13 analyses) of the control group was used as a reference category. Adjustments for stratification variables (i.e. age, index year, and area of residence) were made in the first model, OR_1. Additional adjustments for potential confounders (hypercholesterolemia, hypertension, diabetes, and smoking) were included in the second model, OR_2. To assess the risk of IS and MI associated with the presence of a combination of multiple risk factors (eg, VWF, ADAMTS13, and oral contraceptives), dummy variables were created. To calculate the joint risk conferred by these risk factors, > p90 was used as a cut-off for VWF and < p10 as a cut-off for ADAMTS13. The joint category, representing no exposure to any of these risks, was used as a reference category. All statistical analyses were performed using SPSS Version 19 (IBM SPSS statistics, Chicago, Ill, USA).

Results

Decreased levels of ADAMTS13 conferred an increased risk for both IS and MI (Table 2). An OR_1 of 3.1 (95% CI 1.8-5.5) for IS and an OR_1 of 1.8 (95% CI 1.1-3.0) for MI was conferred by the lowest quartile (Q1) ofADAMTS13. After adjustment, the OR_2 for IS did not change, although the OR_2 for MI decreased. The OR_1 values for high VWF for IS and MI were 3.2

Figure 1. Distribution of VWF and ADAMTS13 per casegroup



VWF and ADAMTS13 plasma levels in controls (●), ischaemic stroke patients (▲) and myocardial infarction patients (◊). A) VWF plasma levels. B) ADAMTS13 plasma levels. C) VWF plasma levels of controls plotted against ADAMTS13 plasma levels. No significant correlation between VWF and ADAMTS13 antigen levels was observed (Pearson r 0.07). IS, ischaemic stroke; MI, myocardial infarction; CTRL, controls.

Table 2. Risk of ischaemic stroke and myocardial infarction in relation to VWF and ADAMTS13 plasma levels

			Ischaemic stroke			Myocardial infarction		
		Controls, n	Cases, n	OR_1 (95% CI)	OR_2 (95% CI)	Cases, n	OR_1 (95% CI)	OR_2 (95% CI)
VWF	Q1	156	13	1 [ref]	1 [ref]	20	1 [ref]	1 [ref]
	Q2	157	22	1.4 (0.7-3.1)	1.6 (0.7-3.6)	35	1.6 (0.9-3.0)	1.7 (0.9-3.4)
	Q3	157	50	3.8 (1.9-7.6)	4.2 (2.0-9.0)	60	2.5 (1.4-4.5)	3.1 (1.6-5.9)
	Q4	156	82	5.9 (3.0-11.7)	6.7 (3.2-13.8)	87	3.7 (2.1-6.5)	4.2 (2.2-8.0)
ADAMTS13	Q4	156	61	3.1 (1.8-5.5)	3.1 (1.6-5.8)	63	1.8 (1.1-3.0)	1.4 (0.8-2.4)
	Q3	157	45	2.4 (1.4-4.4)	3.0 (1.6-5.7)	50	1.2 (0.8-2.0)	0.9 (0.5-1.6)
	Q2	157	33	1.5 (0.8-2.7)	1.7 (0.9-3.3)	44	1.0 (0.6-1.7)	0.8 (0.5-1.5)
	Q1	156	28	1 [ref]	1 [ref]	45	1 [ref]	1 [ref]

ORs were calculated by logistic regression and were adjusted for age, year of event/index year and area of residence. OR_2 were also adjusted for hypercholesterolemia, hypertension, diabetes and smoking. Q denotes quartile; Ref denotes reference; 95%CI denotes the 95% confidence interval.

Table 3. Risk of ischaemic stroke and myocardial infarction following combination of two risk factors

Exposures			Ischaemic stroke			Myocardial infarction		
		Cont, n	Cases, n	OR_1 (95% CI)	OR_2 (95% CI)	Cases, n	OR_1 (95% CI)	OR_2 (95% CI)
VWF <p90	ADAMTS13 >p10							
-	-	507	107	1 (ref)	1 (ref)	130	1 (ref)	1 (ref)
-	+	57	20	2.2 (1.2-4.1)	2.0 (1.1-3.8)	21	1.6 (0.9-2.8)	1.4 (0.8-2.5)
+	-	57	31	3.2 (1.8-5.5)	3.1 (1.7-5.5)	39	2.6 (1.6-4.2)	2.6 (1.6-4.3)
+	+	5	9	6.9 (2.0-23.0)	5.8 (1.7-20.2)	12	11.3 (3.6-35)	9.8 (3.1-31)
VWF <p90	OC use							
-	-	372	65	1 (ref)	1 (ref)	92	1 (ref)	1 (ref)
-	+	192	62	2.2 (1.3-3.5)	2.0 (1.2-3.3)	59	2.2 (1.5-3.4)	2.1 (1.4-3.3)
+	-	45	14	1.8 (0.9-3.8)	1.6 (0.8-3.5)	29	2.7 (1.5-4.7)	2.8 (1.5-5.0)
+	+	17	26	12.0 (5.5-26)	11.4 (5.2-25)	22	7.5 (3.6-16)	7.1 (3.3-15)
ADAMTS13 >p10	OC use							
-	-	384	69	1 (ref)	1 (ref)	103	1 (ref)	1 (ref)
-	+	180	69	2.6 (1.7-4.3)	2.6 (1.6-4.1)	66	2.6 (1.7-3.9)	2.5 (1.6-3.8)
+	-	33	10	2.0 (0.9-4.6)	1.8 (0.8-4.3)	18	2.5 (1.3-4.9)	2.3 (1.1-4.5)
+	+	29	19	5.8 (2.7-12.4)	5.1 (2.4-11.2)	15	3.0 (1.4-6.2)	2.7 (1.3-5.7)

See table 2 for description

(95% CI 1.9-5.3) and 3.0 (95% CI 1.9-4.7), respectively, and the OR_1 values for low ADAMTS13 were 2.3 (95% CI 1.3-4.0) and 2.0 (1.2-3.2), respectively. Further adjustment (OR_2) did not change the estimates appreciably. Quartile analyses of ADAMTS13 levels showed no clear dose response for MI. Because VWF and ADAMTS13 are functionally linked to each other in vivo, we investigated whether having both high VWF levels and low ADAMTS13 levels further increases the risk of IS and MI. The second row in table 3 shows the OR_1 and OR_2 for IS and MI conferred by low ADAMTS13:Ag levels only, and the third row shows the OR_1 and OR_2 conferred by high VWF levels only. These results were similar to those obtained before stratification, suggesting that VWF and ADAMTS13 each act as risk factors. More importantly, table 3 shows that the joint effect of high VWF and low ADAMTS13 levels increased the risk of IS and MI, resulting in an OR_1 of 6.9 (95% CI 2.0-23.0) and 11.3 (95% CI 3.6-35.2), respectively (row 4). These associations were also seen after further adjustment.

The use of oral contraceptives is known to be a risk factor for IS and MI, associated with an approximately 2-fold increased risk in young women.[31,32] We therefore evaluated whether this risk was further increased by the concomitant presence of high VWF or low ADAMTS13. Strikingly, the interaction analyses (Table 3) showed that high levels of VWF plasma levels and oral contraceptive use yielded an OR_1 of 12.0 (95% CI 5.5-26.2) for IS and an OR_1 of 7.5 (95% CI 3.6-15.7) for MI. Similar results were obtained after adjustment for potential confounders (see OR2). Women on oral contraceptives with high levels of VWF were therefore >5 times more likely to develop IS and >3 times more likely to develop MI than women on oral contraceptives with lower VWF antigen levels. Joint analysis of oral contraceptives use and ADAMTS13 Ag levels (Table 3) showed that the risk of IS and MI related to low ADAMTS13:Ag levels in women on oral contraceptives was 5.8 (95% CI 2.7-12.4) and 3.0 (95% CI 1.4-6.2), respectively. The OR_2 decreased slightly after adjustment. A joint effect of low ADAMTS13:Ag levels and use of oral contraceptives was therefore observed for IS but not for MI.

Discussion

In the present study, we measured plasma VWF and ADAMTS13 antigen levels in 167 young women with IS, 202 young women with MI, and in 626 frequency-matched healthy controls. Quartile analysis showed a concentration-dependent association of VWF and ADAMTS13 levels with the risk of IS and MI. The highest quartile of VWF conferred an OR_2

of 6.7 (95% CI 3.2-13.8) for IS and 4.2 (95% CI 2.2-8.0) for MI. The lowest quartile of ADAMTS13 conferred an OR_2 of 3.1 (95% CI 1.6-5.8) for IS and 1.4 (95% CI 0.8-2.4) for MI, a difference in strength which suggests that ADAMTS13 has a differential effect on MI and IS. The risks of IS and MI were also evident in both OR models when using the 90th percentile and the 10th percentile as cut-off values for VWF and ADAMTS13. Interestingly, as can be seen from the interaction analyses, risks of IS and MI were elevated both by high VWF and low ADAMTS13, even when mutually adjusted, which raises an important point when considering the relationship between VWF and ADAMTS13 plasma levels. Although ADAMTS13 regulates VWF multimeric size through proteolysis, we and others have found no association between ADAMTS13 antigen levels and VWF antigen levels.[24,25,30] Whereas for ADAMTS13 it is reasonable to assume that antigen and activity levels are essentially synonymous in healthy volunteers and in patients with CVD, this is not the case when comparing VWF antigen and function. [34,35] One hypothesis is that the influence of ADAMTS13 concentration on the risk of IS and MI is manifest through the reduction of the multimeric sise of circulating VWF or the modulation of VWF function locally at the site of vessel damage.

Joint analysis showed that having both high VWF and low ADAMTS13 increased the ORs 2- to 3-fold for IS and 4- to 7-fold for MI compared with having only one risk factor. The joint effect of high VWF and low ADAMTS13 was higher for MI (OR_2 9.8) than for IS (OR_2 5.8). However, because of the low number of individuals in this category the CIs were quite wide. Results from the joint analysis suggest that both VWF and ADAMTS13 are risk factors for IS and MI. In addition, the joint effect of high VWF and low ADAMTS13 supports previous findings suggesting that the ratio between VWF and ADAMTS13 in the same individual might be important.[36–38]

Our finding that the joint risk of having both high VWF and low ADAMTS13 is higher than that of the individual risk factor is in agreement with a former study investigating the joint risk of VWF and ADAMTS13 in a broad group including a combination of patients with coronary heart disease, IS, and peripheral arterial disease.[30] Our study differs from the previous one because it only includes young women and involves cases that are more precise in phenotype (either IS or MI) and therefore also more precise in the respective effect estimates. Other case-control studies have investigated the individual risks conferred by VWF or ADAMTS13 antigen levels. Our results showing that VWF is a risk factor for IS and MI are in agreement with previous studies. [21–27] Our results showing that low

ADAMTS13 levels are associated with MI is in agreement with the findings from the GLAMIS case-control study.[25] Others did not find an association between low ADAMTS13 and IS and MI, whereas one group found a positive association between ADAMTS13 plasma levels and MI.[24,26,30] The reason for the discrepancy with the latter finding is not known. Whereas our study shows an association of low ADAMTS13 plasma levels with IS and MI, CIs were sometimes wide after stratification. Because the association between low ADAMTS13 and the risk of IS and MI is relatively weak, it is possible that previous studies have failed to show an association because of a lack of power.

Our results are consistent with recent in vivo studies in mice. It has been shown that VWF+/- and VWF-/- mice have a reduction in infarct volume of approximately 40% and 50%-60%, respectively, compared with wild-type (WT) mice after the induction of IS.[39–41] The susceptibility of VWF-/- mice to IS was, however, restored after reconstitution of plasma VWF.[40,41] These studies suggest that the lack of VWF or low VWF levels protects against IS, and that VWF plasma levels (and not VWF from endothelial cells or platelets) are important in the development of IS in mice. [40,41] In contrast, it has been found that ADAMTS13-/- mice were susceptible to larger infarctions than WT mice.[39,42] Interestingly, infusion of ADAMTS13 into WT mice after ischaemic occlusion reduced the infarct volume by approximately 30%.[39] Mice deficient in both ADAMTS13 and VWF had a phenotype similar to mice deficient in only VWF, showing that the action of ADAMTS13 is dependent on the presence of VWF.[39]

By stratifying data according to oral contraceptive use, we showed that high VWF or low ADAMTS13 is associated with the risk of MI irrespective of oral contraceptive use; however, CIs were wide after stratification due to a loss in power, because only a small number of cases (14 and 10, respectively) were present in these strata. Joint analysis showed that patients with high levels of VWF who were on oral contraceptives had an approximately 11-fold higher risk of developing IS and an approximately 7-fold higher risk of developing MI than individuals with neither risk factor. Individuals with low ADAMTS13 using oral contraceptives were approximately 5 times more likely to develop IS than individuals with none of the risk factors. However, the risk of MI in patients with low ADAMTS13 antigen levels was not increased by the use of oral contraceptives. These interaction analyses raise the question whether women starting with oral contraceptive use should be screened for abnormal levels of VWF or ADAMTS13. We argue that such a

screening strategy is not likely to be cost-effective. We performed some calculations using population statistics and the combination of high VWF and oral contraceptive use which yielded the highest increase in risk. With an annual incidence of myocardial infarction of 14 per 100 000 women aged 18-50 per year,[43] approximately 2700 women with high VWF levels should refrain from starting oral contraceptive use annually in order to prevent one case of myocardial infarction. However, since 'high VWF' in our study was defined as ≥90th percentile of the general population, ten times as many women should be screened to identify these women. Similarly, approximately 14 000 women need to be screened to prevent one case of ischaemic stroke annually.[44] Although this is not a formal cost-effectiveness analysis, these numbers indicate that such a screening program is not warranted. The main reason for this, perhaps counterintuitive result given the high relative risks, is that for young women the incidence of myocardial infarction and ischaemic stroke, or any other disease for that matter, is very low. Therefore, it is not likely that any genetic or plasma marker based screening program used to determine which women could 'safely' start using oral contraceptives would ever be cost effective.[45,46]

Our study has some limitations because of its design. Blood was drawn after the event and only in cases that were not fatal. Because blood was drawn long after the event (median 69 months), it is unlikely that the antigen levels measured were influenced by the acute phase of the event. We cannot, however, exclude any chronic effect or account for potential reductions in exposure to environmental or behavioural risks after the event. Whereas VWF levels may be a result of vessel wall damage, this can even be the case in prospective studies. Association of genetic variations of VWF and ADAMTS13 with IS and MI might provide a means of excluding confounding and reverse causation. Preliminary support for associations between single nucleotide polymorphisms of VWF and ADAMTS13 and the risk of CVD has been provided by a few studies.[47–49] However, large prospective studies evaluating single nucleotide polymorphisms will be required to establish the roles of VWF and ADAMTS13 in arterial thrombotic disease.

Conclusion The present study is the first, to our knowledge, to examine the risk of IS and MI conferred by the combination of both high VWF and low ADAMTS13 antigen levels in young women. We believe that analysis in a young population is of interest because they have been exposed for a shorter time to cardiovascular risk factors than older individuals. Similarly, atherosclerosis is likely to play a smaller role in young individuals. We have shown here that in young women, high VWF and low ADAMTS13 plasma levels are both

associated with an increased risk of IS and MI, and that the use of oral contraceptives can further increase these risks.

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Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women

Rolf T Urbanus , Bob Siegerink, Mark Roest, Frits R Rosendaal, Phillip G de Groot, Ale Algra

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Abstract

Background Myocardial infarction and ischaemic stroke are major clinical manifestations of the antiphospholipid syndrome, which is an autoimmune disease found mostly in young women. Although the presence of circulating antiphospholipid antibodies in individuals who have a thrombotic event is a prerequisite for the diagnosis of the antiphospholipid syndrome, the risk of myocardial infarction and ischaemic stroke associated with antiphospholipid antibodies in the general population is unclear.

Methods We used the RATIO (Risk of Arterial Thrombosis In relation to Oral contraceptives), a large multicentre population-based case-control study including women aged under 50 years who were admitted to hospital at 16 centres with first myocardial infarction or ischaemic stroke and frequency-matched controls to measure markers of the antiphospholipid syndrome. Odds ratios and corresponding 95% confidence intervals were calculated as measures of rate ratios.

Results Blood samples of 175 patients with ischaemic stroke, 203 patients with myocardial infarction, and 628 healthy controls were used in the present study. Lupus anticoagulant was found in 30 (17%) patients with ischaemic stroke, six (3%) patients with myocardial infarction, and four (0.7%) in the control group. The odds ratio for myocardial infarction was 5.3 (95% CI 1.4–20.8), which increased to 21.6 (1.9–242.0) in women who used oral contraceptives and 33.7 (6.0–189.0) in those who smoked. The odds ratio for ischaemic stroke was 43.1 (12.2–152.0), which increased to 201.0 (22.1–1828.0) in women who used oral contraceptives and 87.0 (14.5–523.0) in those who smoked. In women who had anti-β2-glycoprotein I antibodies, the risk of ischaemic stroke was 2.3 (1.4–3.7), but the risk of myocardial infarction was not increased (0.9, 0.5–1.6). Neither anticardiolipin nor antiprothrombin antibodies affected the risk of myocardial infarction or ischaemic stroke.

Conclusion Our results suggest that lupus anticoagulant is a major risk factor for arterial thrombotic events in young women, and the presence of other cardiovascular risk factors increases the risk even further.

Introduction

Cardiovascular diseases are the second most common cause of death in developed countries. Although the incidence of myocardial infarction and ischaemic stroke in individuals younger than 50 years is low, the burden of disease in young people is large. Myocardial infarction and ischaemic stroke are usually associated with several risk factors for cardiovascular disease, such as smoking, hypertension, diabetes, obesity, hyperlipidaemia, or a family history of cardiovascular disease.[1]

The antiphospholipid syndrome is an acquired risk factor for thrombotic diseases. The antiphospholipid syndrome is more prevalent in young women than in the general population.[2] It is characterised by vascular thrombosis or complications during pregnancy and a repeatedly positive test for antiphospholipid antibodies.[3] There are several subpopulations of antiphospholipid antibodies; the coagulation inhibitor lupus anticoagulant and antibodies against the phospholipid cardiolipin or the plasma proteins β2-glycoprotein I and prothrombin are the antibody subpopulations most commonly tested for.

Owing to its non-specific clinical symptoms, diagnosis of the antiphospholipid syndrome is dependent on the detection of antiphospholipid antibodies. Data on the risk of a first thrombotic event in the presence of antiphospholipid antibodies are inconclusive[4] because most studies were done in selected populations of patients. Several studies have been done to assess the thrombotic risk associated with antiphospholipid antibodies in the general population.[5–11] Although there is a general consensus from the results of these studies that the presence of antiphospholipid antibodies is independently associated with increased risk of thrombotic diseases, there is still debate on the value of measuring the concentrations of different antiphospholipid antibody subpopulations in the general population.[12]

We investigated whether the presence of specific antiphospholipid antibody subpopulations (lupus anticoagulant, anticardiolipin, or anti-β2-glycoprotein I and antiprothrombin antibodies) affects the risk of myocardial infarction or ischaemic stroke in young women. Furthermore, we studied the effect of smoking, hyperlipidaemia, diabetes, use of oral contraceptives,[13,14] the factor V G1691A mutation (factor V Leiden),[15,16] the

prothrombin G20210A mutation,[16–18] and the factor XIII 204Phe allele[19,20] on the relation between antiphospholipid antibodies and myocardial infarction and ischaemic stroke.

Methods

Study design & participants The Risk of Arterial Thrombosis In relation to Oral contraceptives (RATIO) study is a multicentre, population-based, case-control study focussed on risk factors of myocardial infarction, ischaemic stroke and peripheral arterial disease in young women. Details of the study have been published previously. [13,14,21] The current study focuses on the two acute forms of arterial thrombosis i.e., myocardial infarction and ischaemic stroke. Eligible patients were recruited under all women under 50 presenting with one these two diseases at one of the 16 participating centres. Myocardial infarction was confirmed by symptoms, elevated concentrations of cardiac enzymes, and changes seen on electrocardiograph; ischaemic stroke without an overt cardioembolic source was confirmed by medical history, physical examination, and CT or MRI scans, which were assessed by experienced neurologists at the participating centres.[13,14] Exclusion criteria were transient ischaemic attack that lasted less than 24 h, haemorrhagic stroke, cerebral sinus venous thrombosis, carotid artery dissection, history of cardiovascular or cerebrovascular disease, aphasia or cognitive impairment that prevented completion of the questionnaire, or not speaking Dutch. Controls were approached by random digit dialling and frequency-matched to the cases on age, residence area, and index date of the event. The study was approved by the local medical ethics committee of all participating hospitals. Informed consent was obtained from all patients and controls, in accordance with the declaration of Helsinki.

Measurements All patients and controls completed a standardised, structured questionnaire on several topics such as their use of oral contraceptives, smoking status, alcohol intake, weight, height, family and medical history. All antiphospholipid antibody tests were done at a central laboratory. All samples were measured masked to case or control status. Presence of lupus anticoagulant was detected with dilute Russell's viper venom time (dRVVT) reagents (LA-screen and LA-confirm; Gradipore, Australia). This assay is based on a modification of the common pathway of coagulation. Plasma samples were thawed for the first time and diluted 1:1 (vol:vol) with pooled normal plasma from 173 healthy volunteers. Coagulation was initiated by adding an equal volume of LA-screen reagent to the mixed plasma, and coagulation times were recorded. In the event of a

prolonged coagulation time (LA-screen time >99th percentile of time recorded for 40 healthy volunteers), LA-confirm assays were done. Normalised ratios for LA-screen and LA-confirm coagulation times (ratios/c) were calculated with the following equation:

$$RATIO_{S/C} = \frac{LA_S / LA_{S\,normal}}{LA_C / LA_{C\,normal}}$$

where $LA_{s\ (normal)}$ is the mean LA-screen coagulation time of 40 healthy volunteers, and $LA_{c\ (normal)}$ is the mean LA-confirm coagulation time of 40 healthy volunteers. Samples were deemed positive for lupus anticoagulant when the $ratio_{s/c}$ was 1.15 or higher, on the basis of the 99th percentile of the value recorded for 40 healthy volunteers. This measurement is in accordance with current recommendations for testing for lupus anticoagulant.[3] Human plasma-derived β2-glycoprotein I was purified as previously described.[22] β2-glycoprotein I-coated 96-well microtitre plates (Nunc MaxiSorp U96; Nunc, Wiesbaden, Germany) were washed and incubated for 1 h with plasma samples diluted 1:100 in PBST (phosphate-buffered saline [10 mM phosphate, 140 mM NaCl, pH 7.35] with 0.1% Tween-20) at ambient temperature. After washing, plates were incubated with horseradish peroxidase-labelled goat anti-human-IgG antibodies (Southern Biotech, Birmingham, AL, USA) and developed with Amplex Red reagent (Invitrogen, Paisley, UK). Fluorescence was measured

Table 1. Characteristics of RATIO participants

	Myocardial infarction N=205	Ischaemic stroke N=175	Control N=638
Age (mean)	43	39	39
Caucasian ethnicity	195 (95%)	167 (97%)	602 (94%)
History of *			
Hypertension	53 (26%)	50 (29%)	40 (6%)
Diabetes	10 (5%)	7 (4%)	10 (2%)
Hypercholesterolaemia	21 (10%)	14 (8%)	19 (3%)
Oral contraceptives use *	81 (40%)	92 (53%)	213 (33%)
Smoking *	169 (82%)	101 (58%)	270 (42%)

All data are in respect to the year of event (cases) or index date (controls). Data on ethnicity are missing for 1 ischaemic stroke case & 4 controls, on history of hypertension for 4 controls, on history of Diabetes for 1 myocardial infarction case & 3 controls, on hyperlipidaemia for 1 myocardial infarction case & 5 controls, on oral contraceptive use for 6 controls, on smoking for 6 controls and on alcohol use for 1 ischaemic stroke case & 6 controls.

in a FluoSTAR OPTIMA reader (BMG Labtech, Offenburg, Germany). PBST was used for all washing procedures. Values are reported as percentage of a positive control. Cut-off was set at the 90th, 95th, or 99th percentile of the value recorded for the control group. IgG anticardiolipin antibody concentrations were measured in plasma samples with a commercially available kit (Corgenix, Broomfield, CO, USA) in accordance with the instructions of the manufacturer. Measurements are reported as concentrations of anticardiolipin IgG (GPL). Cut-off was set at the 90^{th}, 95^{th}, or 99^{th} percentile of the concentration recorded for the control group. Concentrations of IgG antiprothrombin antibodies were measured as described previously[23] and values were reported as a percentage of the value of a positive control group. Cut-off was set at the 90^{th}, 95^{th}, or 99^{th} percentile of the control group.

Statistical analyses Rate ratios of myocardial infarction and stroke associated with antiphospholipid antibodies were estimated with odds ratios and 95% CIs with unconditional logistic regression. ORs were adjusted for the stratifying variables: age (continuous variable), residence area (four categories), and index year (six categories). Furthermore, we calculated the risk of myocardial infarction and ischaemic stroke in the presence of combinations of antiphospholipid antibody subtypes. To assess the contribution of cardiovascular risk factors to the risk of myocardial infarction and ischaemic stroke in patients with antiphospholipid antibodies, we calculated ORs to compare exposure to a cardiovascular risk factor, antiphospholipid antibodies, or both with the absence of both.

Results

Blood samples from 203 cases with myocardial infarction, 175 cases with ischaemic stroke and 628 control women were available for the current analyses. Table 1 shows the characteristics of the study population. As expected, patients with myocardial infarction or ischaemic stroke had a higher prevalence of cardiovascular risk factors, such as hypertension, diabetes, hyperlipidaemia, oral contraceptive use, and smoking, than did the controls. All oral contraceptives were combinations of an oestrogen (ethinylestradiol) and a progestin (levonorgestrel, desorgestrel, lynestrenol, or cyproterone acetate).

We analysed the effect of different antiphospholipid antibody subpopulations on the risk of myocardial infarction or ischaemic stroke after adjustment for the matching variables (table 2). Lupus anticoagulant was found in six (3%) patients with myocardial infarction and

Table 2. Antiphospholipid syndrome markers and the risk of myocardial infarction and ischaemic stroke

				Odds Ratio (95% CI)[§]	
	MI	**IS**	**Controls**	**Myocardial Infarction**	**Ischaemic stroke**
Lupus anticoagulant					
(N)	202	175	627		
$Ratio_{s/c} \geq 1.15$	6	30	4	5.3 (1.4 – 20.8)	43.1 (12.2 – 152)
$Ratio_{s/c} \geq 1.20$	5	19	2	11.7 (2.1 – 65)	48.3 (8.2 – 284)
$Ratio_{s/c} \geq 1.30$	4	10	0	NA	NA
Anticardiolipin IgG, cut-off (GPL)					
(N)	201	169	623		
≥14.6[*]	26	12	62	1.6 (0.9 – 2.6)	0.8 (0.4 – 1.7)
≥16.9[†]	16	8	31	1.8 (0.9 – 3.4)	0.9 (0.4 – 2.2)
≥24.5[‡]	2	6	6	1.5 (0.3 – 8.4)	1.9 (0.5 – 7.6)
Anti-β_2-Glycoprotein I IgG, cut-off (% of positive control)					
(N)	203	175	628		
>27.9[*]	18	39	62	0.9 (0.5 – 1.6)	2.3 (1.4 – 3.7)
>37.7[†]	11	24	31	1.2 (0.6 – 2.6)	2.8 (1.5 – 5.3)
>74.3[‡]	3	4	6	2.2 (0.5 – 10.3)	1.7 (0.4 – 7.9)
Anti-prothrombin IgG, cut-off (% of positive control)					
(N)	203	175	628		
>31.8[*]	14	21	62	0.7 (0.4 – 1.2)	1.3 (0.7 – 2.4)
>47.4[†]	7	13	31	0.8 (0.3 – 1.9)	1.8 (0.8 – 4.2)
>85.9[‡]	3	4	6	1.8 (0.4 – 7.4)	2.6 (0.6 – 11.1)

MI denotes myocardial infarction; IS denotes ischaemic stroke; CI denotes confidence interval; NA denotes not applicable; ref denotes reference category. All odds ratios are adjusted for age, residence area and index year. value corresponding to the 90th percentile of controls. † value corresponding to the 95th percentile of controls. ‡ value corresponding to the 99th percentile of controls.*

30 (17%) patients with ischaemic stroke, whereas only four (0.6%) controls had lupus anticoagulant. The OR for myocardial infarction was 5.3 (95% CI 1.4–20.8) and the OR for ischaemic stroke was 43.1 (12.2–152.0) in women with lupus anticoagulant compared with women without. 23 patients with myocardial infarction and 22 patients with ischaemic stroke received vitamin K antagonists; after these patients were excluded, the OR for myocardial infarction was 4.6 (1.1–19.5) and the OR for ischaemic stroke was 45.7 (12.4–169.0). When we used a more stringent cut-off for lupus anticoagulant ($ratio_{s/c}$ ≥1.20), the ORs for myocardial infarction and ischaemic stroke were higher. None of the control women had lupus anticoagulant with the $ratio_{s/c}$ ≥1.30 as the cut-off. Neither anticardiolipin nor antiprothrombin antibodies affected the risk of myocardial infarction or ischaemic stroke. In women with anti-β2-glycoprotein I antibodies, the risk of ischaemic stroke was 2.3 (1.4–3.7) compared with women without anti-β2-glycoprotein I antibodies; however, anti-β2-glycoprotein I antibodies did not affect the risk of myocardial infarction. The presence of lupus anticoagulant and any additional antiphospholipid antibody

Table 3. Risk of myocardial infarction or ischaemic stroke in relation to the presence of lupus anticoagulant and the presence or absence of cardiovascular risk factors

	No lupus anticoagulant					Lupus anticoagulant*				
	Distribution, N			Odds Ratio (95% CI)		Distribution, N			Odds Ratio (95% CI)	
	MI	IS	CONT	MI	IS	MI	IS	CONT	MI	IS
Oral contraceptives										
No	120	71	409	1 [ref]	1 [ref]	2	12	3	3.5 (0.5 – 22.6)	33.6 (6.8 – 167)
Yes	76	74	208	2.3 (1.6 – 3.4)	2.9 (1.8 – 4.6)	4	18	1	21.6 (1.9 – 242)	201 (22.1 – 1828)
Smoking										
No	36	57	354	1 [ref]	1 [ref]	0	13	2	NA	47.2 (8.1 – 276)
Yes	160	88	264	6.4 (4.2 – 9.7)	2.2 (1.5 – 3.4)	6	17	2	33.7 (6.0 – 189)	87 (14.5 – 523)
Factor V G1691A										
No	184	126	587	1 [ref]	1 [ref]	6	24	3	6.6 (1.5 – 29)	57 (13 – 251)
Yes	12	11	33	1.1 (0.5 – 2.2)	1.9 (0.9 – 4.2)	0	1	1	NA	11 (0.5 – 225)
Prothrombin G20210A										
No	192	139	605	1 [ref]	1 [ref]	6	27	4	5.3 (1.4 – 21)	42 (12 – 153)
Yes	4	3	15	0.7 (0.2 – 2.5)	0.7 (0.2 – 2.8)	0	1	0	NA	NA
FXIII 204Phe allele										
No	186	91	582	1 [ref]	1 [ref]	6	17	3	6.5 (1.5 – 28)	51.8 (9.9 – 270)
Yes	10	44	35	0.9 (0.4 – 1.9)	8.8 (4.9 – 5.9)	0	8	1	NA	81.4 (8.9 – 739)

MI denotes myocardial infarction; IS denotes ischaemic stroke; CI denotes confidence interval; NA denotes not applicable; ref denotes reference category. All odds ratios are adjusted for age, residence area and index year.

subpopulation did not affect the risk of myocardial infarction or ischaemic stroke, compared with the risk in patients with only lupus anticoagulant. Adjustment for hypertension, diabetes, and hyperlipidaemia did not affect the relative risks for myocardial infarction or ischaemic stroke in women with antiphospholipid antibodies.

We assessed the joint effect of additional cardiovascular risk factors in women with lupus anticoagulant compared with women without lupus anticoagulant and each cardiovascular risk factor (table 3). In women without lupus anticoagulant, the risk of myocardial infarction was 2.3 (95% CI, 1.6–3.4) in users of oral contraceptives and 6.4 (4.2–9.7) in smokers. In women with lupus anticoagulant, the OR for myocardial infarction for those who used oral contraceptives was 21.6 (1.9–242.0) and for those who smoked was 33.7 (6.0–189.0). The factor V G1691A variant, the prothrombin G20210A variant, and the factor XIII 204Phe variant did not affect the risk of myocardial infarction.

In women without lupus anticoagulant, the OR for ischaemic stroke was 2.9 (95% CI 1.8–4.6) in users of oral contraceptives, 2.2 (1.5–3.4) in smokers, and 8.8 (4.9–15.9) in women with the factor XIII 204Phe variant. In women with lupus anticoagulant, the OR for ischaemic stroke was 201 (22–1828) in users of oral contraceptives, 87 (14.5–523) in smokers, and 81 (8.9–739) in women with the factor XIII 204Phe variant. The factor V G1691A and prothrombin G20210A variants had no effect on the risk of ischaemic stroke.

Discussion

We found that lupus anticoagulants were associated with an increased risk of myocardial infarction (OR 5.3) and ischaemic stroke (OR 43.1) in women younger than 50 years. Increased concentrations of anti-β2-glycoprotein I antibodies were associated with an increased risk of ischaemic stroke (OR 2.3) but not myocardial infarction. Neither anticardiolipin nor antiprothrombin antibodies alone increased the risk of myocardial infarction or ischaemic stroke. There were no indications that the presence of more than one subpopulation of antiphospholipid antibodies affected the risk of myocardial infarction or ischaemic stroke. Additional cardiovascular risk factors, such as use of oral contraceptives, smoking, or the factor XIII 204Phe variant, increased the risk of myocardial infarction or ischaemic stroke further in women who had lupus anticoagulant. The frequency of the factor V G1691A or the prothrombin G20210A mutations was too low in women with lupus anticoagulant to ascertain whether they had an effect on the risk of myocardial infarction or ischaemic stroke.

The RATIO study compared the characteristics of a large group of women who had had myocardial infarction or ischaemic stroke with those of a large population-based control group obtained through random-digit dialling. Diagnoses and the location of all ischaemic strokes were verified with CT or MRI, which reduces the risk of misclassification. The random-digit dialling procedure and the high response rate in the control group, which was not informed about the risk factors assessed in this study, such as oral contraceptive use or smoking, minimises the risk of participation bias in the control group.

Our study has several limitations. All patients were survivors of a major arterial event who were admitted to hospital. If there is different survival rate for those with the antiphospholipid syndrome, our analyses which are limited to patients who survived potentially could lead to an over or underestimation of the true risk. This is, however, unlikely to have a major effect on our findings because ischaemic stroke and myocardial infarction are rarely fatal in young women.[24,25] Furthermore, we cannot completely exclude recall bias, although structured questionnaires were used and colour photographs of contraceptive preparations were provided to help women recall the oral contraceptive they had used. Data on hypertension, diabetes, and hyperlipidaemia at the time of the event were collected with standardised questionnaires completed several years later.

Correct handling of samples is important for the detection of lupus anticoagulant. Because plasma samples were obtained after only one centrifugation step, we cannot exclude the possibility that residual platelets were present in the sample. The handling of blood samples was standardised to exclude any possible effects of differences in sample handling between cases and controls. Another potential source of bias is anticoagulant treatment with vitamin K antagonists, which might interfere with the detection of lupus anticoagulant. Because lupus anticoagulants are phospholipid-dependent coagulation inhibitors, only longer than normal coagulation times that are corrected by the addition of excess phospholipids qualify as positive for lupus anticoagulant. Although all samples were mixed with normal pooled plasma to correct for possible deficiencies in coagulation factors, an international normalised ratio greater than 3·0 might not be corrected by phospholipids and might cause false-negative values for lupus anticoagulant rather than false-positive values. To exclude any effect of the use of vitamin K antagonists on the risk of myocardial infarction or ischaemic stroke, we repeated the analyses

without the results from the patients treated with anticoagulants. This resulted in a slight decrease in the risk of myocardial infarction, but the risk of ischaemic stroke was not affected. We do not have data on vitamin K antagonist use in the control population; however, because the controls were women without a history of cardiovascular disease, it is highly unlikely that any of these women were on anticoagulant drugs. All blood samples were obtained several years after the thrombotic event. We can therefore only assume that the presence of antiphospholipid antibodies is associated with the event. Other researchers have shown

the presence of antiphospholipid antibodies within hours after an arterial thrombotic event,[26] although the development of antibodies generally takes days. Therefore, our data indicate a causal relation between antiphospholipid antibodies and thrombosis. Because only one blood sample was taken for each patient, we cannot exclude the possibility that a proportion of the anticardiolipin antibodies are transient infection-related antibodies.

Two other studies compared the effects of antiphospholipid antibodies on the risk of ischaemic stroke[5] or myocardial infarction[6] in an unselected population of young women. In line with our results, each study reported an increased risk of an arterial thrombotic event associated with antiphospholipid antibodies, although the risks were attributed to different antiphospholipid antibody subpopulations. One of these studies[5] reported a two-fold increase in the risk of ischaemic stroke in the presence of lupus anticoagulant or anticardiolipin antibodies, whereas the presence of anticardiolipin antibodies was not associated with an increased risk of ischaemic stroke in our study. High titres of anticardiolipin antibodies (>40 GPL) were not detected in our study. Inclusion of patients with unconfirmed or cardioembolic strokes in this study might explain the low risk of stroke associated with lupus anticoagulant in this particular study. Another explanation is that the less specific aPTT-based lupus anticoagulant assay was used; this assay depends on the intrinsic pathway of coagulation and is therefore more sensitive for phospholipid independent coagulation inhibitors than is the dRVVT.[27]

One study in young women[6] reported a two-fold increased risk of myocardial infarction if anti-β2-glycoprotein I antibodies, but not anticardiolipin antibodies, were detected. The presence of anti-β2-glycoprotein I antibodies did not increase the risk of myocardial infarction in our study.

The lupus anticoagulant subpopulation of antiphospholipid antibodies is a heterogeneous pool of antibodies that have several antigenic targets. Insight into the mechanism behind the prothrombotic effects of antiphospholipid antibodies has increased steadily over the years. Antiphospholipid antibodies, particularly those with lupus anticoagulant activity, cause platelet activation and lead to a procoagulant endothelial phenotype.[28] Prothrombotic effects on the coagulation system, such as antiphospholipid antibody-related acquired activated protein C resistance,[29] are also seen in patients with the antiphospholipid syndrome. All of these prothrombotic effects of antiphospholipid antibodies might be responsible for the increased risk of myocardial infarction and ischaemic stroke described in this study. In view of the effects of antiphospholipid antibodies on the haemostatic system, it is not surprising that smoking, which causes endothelial dysfunction,[30] and oral contraceptive use, which affects the anticoagulant protein C axis,[31] increase the effect of lupus anticoagulants on the risk of ischaemic stroke or myocardial infarction. The reason why the effect of lupus anticoagulant on ischaemic stroke is more pronounced than its effect on myocardial infarction remains to be established, but might reflect a true difference in the aetiology of the two diseases. Although antiplatelet drugs are recommended over oral anticoagulants as secondary thromboprophylaxis for non-cardioembolic ischaemic stroke, the use of oral anticoagulants in patients with antiphospholipid syndrome, rather than treatment with antiplatelet drugs, is preferred by some experts.[32,33] Therefore, screening for lupus anticoagulant in young women with ischaemic stroke might be warranted.

Conclusions From the tested markers of the antiphospholipid syndrome, LAC proved to evoke the largest increase in risk for both myocardial infarction and ischaemic stroke. However, the was a striking difference between these two diseases in the magnitude of the effect. This, together with the observation that anti-β2-glycoprotein I antibodies were associated with ischaemic stroke but not with myocardial infarction, shows that the role of the antiphospholipid syndrome differs between these two diseases.

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13 Summary and general discussion

This thesis discusses the role of the coagulation system as a whole and its parts in the aetiology of both myocardial infarction and ischaemic stroke in young women. The underlying question of each chapter is whether 'hypercoagulability' has an equal effect on the occurrence of myocardial infarction and ischaemic stroke.

To investigate this, several prothrombotic factors were analysed in the RATIO study. The study design of RATIO, a frequency-matched population-based case-control design, implies some limitations all of which have been discussed extensively in the different chapters of this thesis. For example, no absolute risks can be calculated which makes external data necessary to provide a context in which we have to put the relative results of the presented analyses. Also, the case-control design implies that blood samples can only be collected after the event, making it difficult to definitely establish temporality between the presumed cause and the effect. Furthermore, other sources of bias (e.g. confounding, recall bias, survivor bias) play a role in the over- or underestimation of the causal effects of interest.

However, the mere presence of these threats to internal or external validity does not nullify the research done, but the effect as well as direction of the bias can. Therefore, these aspects should be discussed as is done in the discussion sections of each chapter. Only if it is unlikely that bias can explain the association, causal inferences can be made.[1] Knowledge derived from previous studies, both fundamental and clinical, should be taken into account during this process.[2] The aim of this chapter is to summarise the research presented in this thesis and review to what extent causal inferences can be made from the results. Additionally, this chapter compares the results from the myocardial infarction analyses and the ischaemic stroke analyses to discuss whether the prothrombotic factors have a different role in mechanisms underlying these two diseases.

Summary and discussion of causality

Chapter 2 is the only chapter in this thesis that does not directly touch a question of causality. In this chapter the role of a positive family history in the risk prediction for ischaemic stroke and myocardial infarction is discussed. Although not a formal prediction study aimed at developing a validated prediction model, lessons on the predictive value of family history can be learned from these results.[3,4] A positive family history of either myocardial infarction or stroke before the age of 60 in a first degree relative increases the risk of a myocardial infarction 3.5 fold, whereas the risk of developing an ischaemic stroke

remains virtually unchanged. But what can these data tell us from a causal point of view? Causal inference in the formal sense of the word is not possible since the exposure 'family history' cannot be regarded as a well defined intervention in a thought experiment. It does not adhere to both the positivity and the consistency conditions, even under conditional exchangeability, as formulated by Hernan and Robins.[5] At most, family history can be a regarded as a marker for a plethora of risk factors that cluster in families either through genetics or household effects. If myocardial infarction and ischaemic stroke were two similar diseases with the same aetiology, it is likely that such a marker will have similar associations. So, although no formal causal statements can be made, these data at least provide a clue that these diseases do not share a common aetiologic mechanism.

Chapters 3 through 6 focus on causality: they investigate the role of the intrinsic coagulation system and its effect on the risk of myocardial infarction and ischaemic stroke. This system includes coagulation FXI, FXII, prekallikreine and HMWK. People deficient in these proteins exhibit mild bleeding diatheses (FXI), or no increased bleeding at all (FXII, prekallikrein HMWK). Nonetheless, several studies suggest that the intrinsic coagulation system plays a pivotal role in pathologic thrombus formation.[6–15] The intrinsic coagulation system is also linked to several other biological mechanisms relevant in cardiovascular diseases, such as fibrinolysis and inflammation.[14,16–20] These proteins have several actions which complicates the unravelling of the causal mechanism, but does not have to complicate the analyses which determine whether these proteins are causal factors per se.

The results as presented in this thesis suggest that activation of FXI, as well as FXI antigen levels, increases the risk of ischaemic stroke, whereas the risk of myocardial infarction is not affected. The results on FXII and kallikrein are less clear: a heightened state of activation of these proteins is only increased in ischaemic stroke patients, whereas the antigen level does not seem to affect the ischaemic stroke risk. Prekallikrein, the zymogen form of kallikrein, is only marginally associated with an increase in risk of myocardial infarction, although this effect might be explained by the homology between prekallikrein and FXI and the use of polyclonal antibodies in the used assays. HMWK, the only non-enzymatic protein in the intrinsic coagulation cascade also only affects the risk of ischaemic stroke, albeit to a minor extent. In summary, these results in combination with other studies suggest a causal role for the intrinsic coagulation proteins especially for ischaemic stroke, although the mechanism is not known.

The differences between the protein-inhibitor analyses and the antigen analyses are not necessarily contradictory since the two measures, as intended by design, measure different protein characteristics. The discrepancy between FXII:C1-inh and FXII:ag data could for instance reflect difference in activation rate perhaps caused by a protein variant with altered functionality. More likely is that the levels of protein-inhibitor complexes reflect a more general notion of an activated coagulation system, either as a cause or a consequence of the disease. As said, the intrinsic coagulation proteins have several actions, which might also be the underlying reason for the differences between the results from the zymogen and activation analyses. Future studies, both laboratory and epidemiology, could help to further elucidate the underlying causal mechanism. The measurement of enzymatic active intrinsic coagulation proteins with specific antibodies can be useful for this purpose. Also, a genetic approach can be used to minimise the effect of confounding and reverse causation, could provide further insight in this matter.

Chapters 7 through 9 use a genetic approach to investigate the role of coagulation in the aetiology of myocardial infarction and ischaemic stroke. Genetic variation in a gene could result directly in a difference in protein functionality, as is the case for example with the Factor V_{Leiden} variation which causes the protein to be resistant to degradation by the natural anticoagulant activated protein C.[21–24] Additionally, genetic variation can also be used as marker for a certain property of the protein of interest. This approach, better known as Mendelian randomisation, is discussed in *chapter 7* and is a form of instrumental variable analysis where genetic variation is used as a proxy for a protein characteristic such as protein plasma level. When executed properly, this approach adheres to the three conditions of causal inference: 'conditional exchangeability' or even 'unconditional exchangeability' can be assumed from the second law of Mendel (i.e. the law of independent assortment). The 'positivity' condition also holds as a direct implication from this same idea that the genetic variation does not depend on the presence of other covariates. This way, the exposure is likely to vary in all strata of covariates and the 'positivity' condition therefore holds. These two conditions do, however, not hold in rare but important situations where 'population stratification' introduces bias. 'Consistency' holds when the genetic variant only affects the protein property of interest; pleiotropy, where the genetic variant has multiple independent causal or associated effects violates this condition. Although potentially a strong causal inference instrument, Mendelian randomisation, or any form of instrumental analyses for that matter, heavily depends on

assumptions that in principle cannot be verified empirically because these assumptions are based on the absence of certain associations. At best, these assumptions can be checked against known possible sources of assumption violation. Although this adds to the credibility of a Mendelian randomisation study, the 'absence of proof' indeed does not imply 'proof of absence' and a critical attitude is necessary to prevent erroneous conlusions.[25] Furthermore, a large number of observations are needed to provide sufficient power for formal instrumental variable analyses. Although the RATIO study is of reasonable size, especially when one considers the low incidence of myocardial infarction and ischaemic stroke in young women, it lacks the statistical power for formal Mendelian randomisation analyses.[26–29] So, instrumental variable analyses such as the Mendelian randomisation approach can hardly be called an 'epidemiologist dream' in the quest to understanding the causal mechanism of a disease. But the line of reasoning still holds some of its value and can be used in causal inference.[28] This is demonstrated in our study on genetic variation in the fibrinogen genes, fibrinogen levels and the risk of myocardial infarction and ischaemic stroke. An individual participant meta-analysis from the fibrinogen studies collaboration showed that high levels of fibrinogen are strongly associated with myocardial infarction and ischaemic stroke.[30] However, reverse causation by subclinical disease, confounding and the acute phase properties of fibrinogen could in part explain these associations. The two SNPs used in *chapter 8* were associated with a change in risk for ischaemic stroke, with the direction of the effects concordant with the effects of the SNPs on fibrinogen levels. Neither SNP affected the risk of myocardial infarction. However, these SNPs might also lead to pleiotropic effects so no strong conclusions can be drawn regarding the role of fibrinogen levels as causal factors of these two diseases. Several other studies, some with a Mendelian randomisation-like approach, drew similar conclusions: the role of fibrinogen levels on the risk of myocardial infarction is at best minimal, whereas the effect on ischaemic stroke risk is larger, although part of this role might be a qualitative rather than a quantitative effect.[31–37]

The analysis of 4 SNPs in the F13 genes presented in *chapter 9* showed no relationship with myocardial infarction, whereas results previously published by RATIO researchers show that these F13 SNPs are related to ischaemic stroke risk. However, the mechanism by which these SNPs affect the risk of ischaemic stroke is unclear. These SNPs might result in different forms of crosslinks, both quantitative and qualitative, in the fibrin mesh.[38] These alterations in clot structure are associated with thrombotic disease.[37,39–41] Nonetheless,

our results indicate that the effect of the genetic variants in the F13 genes differ for myocardial infarction and ischaemic stroke, as is the case in our analyses of the genetic variation in the fibrinogen genes. This, irrespective of their exact mechanism, shows that the role of fibrinogen and FXIII is different for these two diseases.

Chapter 10 through 12 do not focus on factors of the coagulation cascade, but rather investigate the role of other conditions related to an increased clotting propensity. VWF is not part of the coagulation cascade but is connected to prothrombotic processes in several ways. It is bound to FVIII in plasma, stored in platelets in α-granules and endothelial cells in Weibel Palade bodies and is released after activation which leads to platelet tethering, adhesion and activation.[42–44] Therefore, VWF might also be just a marker of endothelial dysfunction. However, most evidence, including some genetic studies, suggest that VWF is also a cause of thrombotic disease.[45–50] The results presented in *chapter 10* corroborate this notion and suggest that VWF levels indeed are a risk factor for both myocardial infarction (VWF:ag & VWF:act) and ischaemic stroke (VWF:ag).[51] Interestingly, low levels of ADAMTS13 antigen, a regulator of VWF size and therefore activity, also increased the risk of ischaemic stroke whereas the risk of myocardial infarction was only minimally affected. This difference might indicate that the relation of VWF and cardiac disease is be partly explained by endothelial dysfunction, but that the relation of VWF and ischaemic stroke indeed is a causal one. However, other studies that focus on the relation between ADAMTS13 and myocardial infarction are not conclusive on the size or even the direction of the association between the two, making it difficult to draw strong conclusions on the causal role of ADAMTS13 and its effect on VWF and the risk of myocardial infarction.[52–56] Nonetheless, observations on the role of the VWF / ADAMTS13 system and its relation to platelet activation have resulted in the idea that VWF might be used as a target in ischaemic stroke therapy.[57,58] Not surprisingly, recombinant ADAMTS13 is one of the new strategies that could prove to be useful in the treatment or prevention of ischaemic stroke.[59,60] Recent studies identifying a link between activated platelets and coagulation activation through the excretion of platelet derived polyphosphates, further emphasize the possible key properties of VWF in the aetiology of thrombotic disease.[61]

Another factor determining clotting propensity is fibrinolytic capacity. *Chapter 11* discusses the relation between fibrinolytic capacity and the risk of myocardial infarction and ischaemic stroke. Since atrial fibrillation is a major source of cardiac emboli causing ischaemic stroke, it is important to note that women with an overt cardiac source of their

ischaemic stroke were excluded from the RATIO study. The risk of myocardial infarction is increased in women with a diminished fibrinolytic capacity, or *hypofibrinolysis*. Interestingly, the results for ischaemic stroke were reversed; an increased fibrinolytic capacity, or *hyperfibrinolysis,* was associated with an increase in risk. This counterintuitive finding might be explained by the different effects of plasminogen activator inhibitor-1 additional to its effect on clot lysis, which has been shown to affect neurological cell death in mouse models.[62] Other research is inconclusive on this topic, leaving it difficult to draw strong conclusion on the true implications of these results.[63] This chapter also shows the difficulties in making causal inferences when the exposure of interest is a well defined, but composite measure. By definition, there are multiple ways to alter the value of a composite measure. Therefore, the use of composite measures violates the consistency assumption and causal inferences on the exact aetiologic mechanism cannot be made. Nonetheless, the results from our analysis do tell us something: although we cannot be sure on the underlying mechanisms, we can conclude that the proteins measured in this aggregate measure have a differential effect on myocardial infarction and ischaemic stroke.

Markers of the antiphospholipid syndrome are subject of research in *chapter 12.* This autoimmune syndrome is characterised by thrombotic events and the presence of antiphospholipid antibodies and lupus anticoagulant. This latter trait is marked by a paradoxically elongated aPTT, an in vitro artefact probably caused by the antiphospholipid antibodies. The results of the RATIO study show that women with lupus anticoagulant have a approximate 40-fold increase in risk of ischaemic stroke, whereas the risk for myocardial infarction is 5-fold increased. Although this increase in myocardial infarction risk is stronger than almost any other effect studied in this thesis, there is an unequivocal difference in effect between the two diseases. Of the antiphospholipid antibodies, the most important class is targeted towards β_2-glycoprotein I (also incorrectly referred to as apolipoprotein-H)[64], which has been identified to interact with several proteins of the coagulation cascade, including the intrinsic coagulation proteins FXI and FXII.[65–67] Anti β_2-glycoprotein I antibodies are associated with a doubling of ischaemic stroke risk, whereas no effect was observed for myocardial infarction. Although several functions of β_2-glycoprotein I, complexed with antibodies or not, have been identified that might explain the thrombotic events in the antiphospholipid syndrome, the exact mechanism still needs to be determined.[65,68–70] As is the case for the intrinsic coagulation proteins, this complicates

causal inference, but it does not nullify the notion that markers of such a prothrombotic condition are stronger related to ischaemic stroke risk than to myocardial infarction.

Similarities and differences: a direct comparison

When a comparison between two diseases is made, there are some major sources of bias that could hamper a correct interpretation. Firstly, to do so one has to rely on different analyses often from separate cohort and case-control studies. Such a comparison is hampered by differences in study design, data acquisition, data analyses and the underlying research questions in the separate studies. However, these problems of comparability are not present, or at least minimised, when the results come from one single study. This is the case for the analyses and results presented in this thesis, all of which are embedded within the RATIO study. This ensures that study design, control group, questionnaires and sample measurement, analyses and research questions were similar for the two case groups.

There might be an additional problem with such a direct comparison, even when the results for two diseases are compared within one single study: a difference in point estimates might be caused by a difference in background risk of the studied diseases. This could be interpreted as a real difference in causal effects of the exposure of interest, but is in fact a problem of interpretation of the point estimate used. This problem, which is in part similar to the mechanisms underlying so called paradoxes in recurrence research, is not present when the background risks of the different studies are similar.[71] Since the incidences of myocardial infarction and ischaemic stroke in young women in the Netherlands are low and similar (i.e. both are approximately 14 per 100 000 women per year),[26,27] this problem is not likely to affect a direct comparison of the myocardial infarction and ischaemic stroke results in the RATIO study.

So, there are no major sources of bias when the results from the RATIO study are used to compare the role of prothrombotic factors in the aetiology of myocardial infarction and ischaemic stroke. However, such a comparison must be non-selective, interpreted cautiously and if possible quantitatively. Therefore, the comparison will be made with the *relative odds ratio* (ROR) for all prothrombotic factors studied in the RATIO study to assess the difference in effects between the two diseases. Also, to evaluate the impact of the different prothrombotic conditions on the incidence of the diseases the *population attributable fraction* (also known as the population attributable risk) is calculated. This will

allow not only a comparison between diseases but also a comparison between prothrombotic factors.

ROR The results presented in this thesis, together with the publications from previous RATIO collaborators, yield a total of 30 prothrombotic factors that were studied for both myocardial infarction and ischaemic stroke.[21,24,72–84] They are listed in table 1 together with the effects on the risk of myocardial infarction and ischaemic stroke. To compare these odds ratios the relative odds ratio, or ROR, can be calculated, which is defined as

$$\mathrm{ROR} = \left(\frac{\mathrm{OR_{IS}}}{\mathrm{OR_{MI}}} \right)$$

, where OR_{IS} denote the adjusted odds ratio of the ischaemic stroke analyses and OR_{MI} represents the adjusted odds ratio of the myocardial infarction analyses. If the ROR >1 the effect of the prothrombotic factor is larger in the ischaemic stroke analyses, and conversely, if the ROR < 1 the effect is larger in the myocardial infarction analyses. If the ROR = 1 there is no difference in effect size. Normally, the variance of the natural logarithm of the ROR is the sum of the variances of the natural logarithm of the two separate odds ratios.[85] However, this measure overestimates the variance in these analyses since it does not take the single control group of the RATIO study into account: now, the part of the variance introduced by the control group is counted twice. As a result, all confidence intervals are too wide (see appendix). Because all confidence intervals of the ROR are affected in a similar fashion, and probably only to minor extent, it is not likely this overestimation of the variance will lead to erroneous conclusions.

Table 1 lists all 30 prothrombotic risk factors which were subject of research in the RATIO study, their effect estimates (i.e. OR_{MI} and OR_{IS}), the ROR and corresponding 95% confidence interval, in ascending order of the ROR. Twenty-one of the 30 prothrombotic conditions have a ROR >1, 13 >2, and 3 > 3. Is there a common mechanism to be found amongst the factors with the highest RORs? High levels of activated factor XII (ROR 2.8), PK (ROR 2.9) and factor XI (ROR 2.9) point towards a role of the intrinsic coagulation system. FXI can be activated by FXII, but also independent of FXII by thrombin in a positive feedback mechanism.[15,86] The effect of factor V_{Leiden} in combination with oral contraceptive use (ROR 5.89) points towards a role of the protein C system.[87] Protein C is a natural anticoagulant which inhibits coagulation factor FVIIIa and factor Va,[88] and thereby also

Table 1. Prothrombotic risk factors in the RATIO study, the effect on myocardial infarction and ischaemic stroke and their ROR

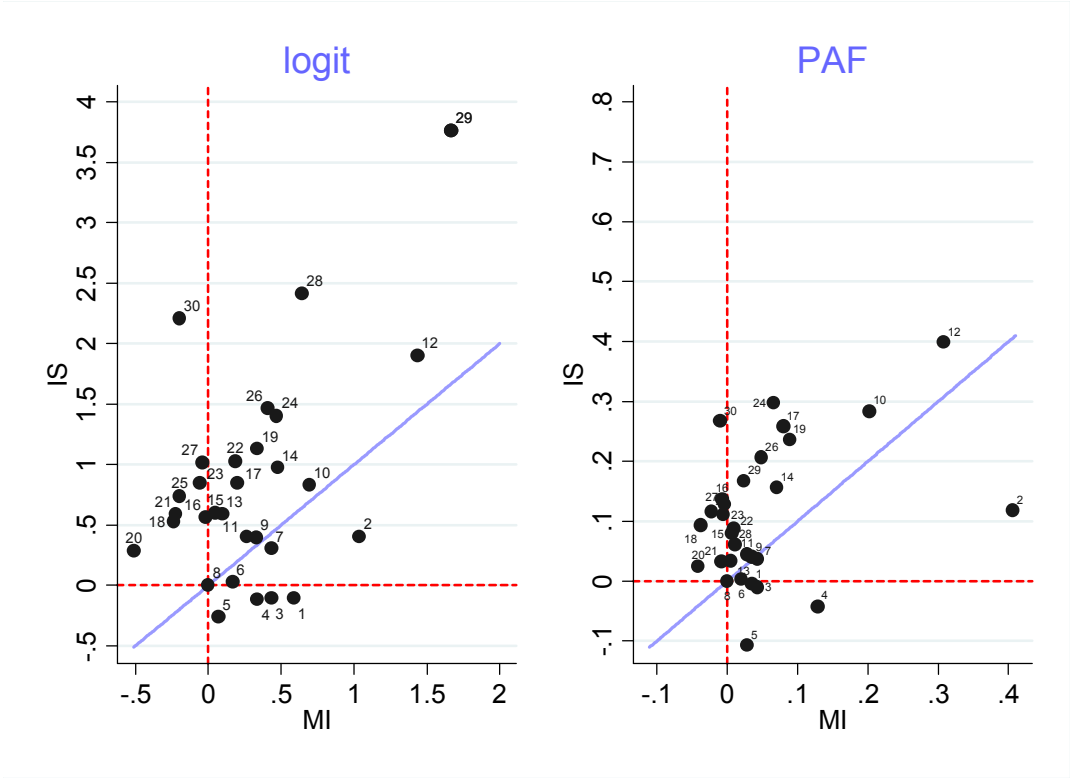
#	Thrombotic factor	ref	OR_{MI}	OR_{IS}	ROR	95%CI
1	Anticardiolipin antibodies, p95	[72]	1.80	0.90	0.50	0.17 - 1.45
2	hypofibrinolysis vs. normofibrinolysis	[78]	2.82	1.50	0.53	0.22 - 1.27
3	Prekallikrein:ag p90	[79]	1.54	0.90	0.58	0.23 - 1.52
4	F13A1 Pro564Leu, dominant	[73,74]	1.40	0.89	0.64	0.39 - 1.05
5	F13A1 Val34Leu, dominant	[73,74]	1.07	0.77	0.72	0.44 - 1.17
6	Factor XII:ag, p90	[79]	1.18	1.03	0.87	0.34 - 2.23
7	Factor XII:ag ,p10	[79]	1.54	1.36	0.88	0.39 - 2.00
8	Prothrombin G20210A, dominant	[24,75]	1.00	1.00	1.00	0.22 - 4.54
9	High molecular weight kininogen:ag, p10	[81]	1.39	1.49	1.07	0.43 - 2.67
10	Oral contraceptive use vs. non use	[21,84]	2.00	2.30	1.15	0.69 - 1.91
11	MTHFR TT snp, recessive	[24,82]	1.30	1.50	1.15	0.57 - 2.33
12	VWF:ag q4 vs q1	[80]	4.20	6.70	1.60	0.60 - 4.26
13	Factor V Leiden, dominant	[24,75]	1.10	1.80	1.64	0.65 - 4.11
14	Factor XI:ag, p90	[83]	1.61	2.65	1.65	0.79 - 3.44
15	High molecular weight kininogen:ag, p90	[81]	1.05	1.82	1.73	0.74 - 4.08
16	FGB -455 G/A, dominant	[77]	0.98	1.76	1.80	0.53 - 6.08
17	FGA 312Ala, dominant	[77]	1.22	2.33	1.90	0.79 - 4.61
18	F13B His95Arg, dominant	[73,74]	0.79	1.70	2.15	1.14 - 4.05
19	ADAMTS13:ag, q1 vs q4	[80]	1.40	3.10	2.21	0.93 - 5.27
20	prekallikrein:ag, 10	[79]	0.60	1.33	2.22	0.79 - 6.24
21	anti prothrombin antibodies, p95	[72]	0.80	1.80	2.25	0.63 - 8.03
22	anti-β2-glycoprotein antibodies, p95	[72]	1.20	2.80	2.33	0.92 - 5.93
23	Factor XI AT-INH, p90	[76]	0.94	2.33	2.48	1.13 - 5.41
24	Hyperfibrinolysis vs. normofibrinolysis	[78]	1.60	4.07	2.54	1.03 - 6.27
25	Factor XII C1-INH, p90	[76]	0.82	2.26	2.76	1.27 - 5.99
26	Kallikreine C1 INH, p90	[76]	1.50	4.34	2.89	1.42 - 5.89
27	Factor XI C1-INH, p90	[76]	0.96	2.76	2.89	1.31 - 6.34
28	FVL + OC + vs FVL- OC -	[24,75]	1.90	11.2	5.89	1.32 - 26.4
29	Lupus anticoagulant, ≥1.15	[72]	5.30	43.1	8.13	1.30 - 50.9
30	F13A1 Tyr204phe, dominant	[73,74]	0.82	9.10	11.1	4.52 - 27.2

 # = number, ref = reference, OR_{MI} = odds ratio from myocardial infarction analyses, OR_{IS} = odds ratio from ischaemic stroke analyses, 95%CI = 95% confidence interval, :ag = antigen levels, C1-INH = C1-inhibitor levels, AT-INH = antitrypsin-inhibitor levels, dominant = analyses based on dominant inheritance pattern

affects the thrombin - factor XI feedback mechanism in which factor V is a co-factor.[89] Lupus anticoagulant (ROR 8.1) is a marker for the antiphospholipid syndrome. Some have proposed a link between the antiphospholipid syndrome and coagulation factor XI/the intrinsic coagulation system, whereby anti-β2-glycoprotein antibodies might play a role in disrupting the activation of FXII and FXI.[6,90–92] The largest difference in effect was observed for a genetic variant of coagulation factor XIII (ROR 11.1), a protein which crosslinks fibrin monomers and thereby affects the clot structure. Although three of the four prothrombotic factors that have the largest difference in effect are linked to the intrinsic coagulation system, directly or indirectly, these links are not based on a full body of evidence. Nonetheless, from the numbers presented in table 1, a cautious conclusion can be drawn: the effect of prothrombotic factors on the risk of ischaemic stroke of non-cardiac origin is larger than the effect on myocardial infarction.

However, the use of the ROR as a measure of direct comparison entails a problem: being a single number denoting the ratio of two other numbers, the ROR is not dependent on the magnitude of its numerator and denominator. This way the ROR of two small effects could be similar to the ROR of two large effects. For example, exposure #12 (i.e. VWF with OR_{IS} 6.7 and OR_{MI} 4.2) and exposure #13 (Factor V_{Leiden} with OR_{IS} 1.8 and OR_{MI} 1.1) both have a ROR of approximately 1.6 whereas their effects and interpretation are quite different: high levels of VWF are a risk factor for both myocardial infarction and ischaemic stroke, whereas Factor V Leiden only imposes an increased risk of ischaemic stroke. Figure 1 circumvents this problem. Here, the left panel depicts the effects of the several prothrombotic factors plotted on a logit scale; the results from the ischaemic stroke analyses are plotted on the y-axis and the myocardial infarction results are plotted on the x-axis. The numbers in this graph denote the exposure of interest and correspond with the numbers in table 1. Points alongside the horizontal line represent factors that increase the risk of myocardial infarction but have no effect on the risk of ischaemic stroke. Conversely, points next to the vertical line represent factors which increase the risk of ischaemic stroke, whereas the risk of myocardial infarction is not affected. When exposures #12 and #13 are plotted in this figure, the aforementioned differences between these two exposures are immediately visible; differences which cannot be grasped by the ROR alone. Nonetheless, the ROR can be deduced from this figure: the distance of a line drawn perpendicular from the diagonal towards a single point represents the ROR of this point on a logarithmic scale. If prothrombotic factors were to play a similar role in the aetiology of myocardial infarction

Figure 1. Prothrombotic risk factors in the RATIO study and their effect on myocardial *infarction and ischaemic stroke*



MI = myocardial infarction, IS = ischaemic stroke, logit = log of the odds ratio, PAF = population attributable fraction. Each point 1 depicts the logit as a measure of effect (left panel) or the population attributable fraction (right panel) of a particular risk factor on the risk of myocardial infarction (x-axis) as well as the effect on the risk of ischaemic stroke (y-axis). The red dashed lines indicate the null effect for either myocardial infarction (vertical line) or ischaemic stroke (horizontal line). The blue diagonal line represents the theoretical line along which all points would cluster when the role of thrombotic factors is similar in the aetiology of myocardial infarction and ischaemic stroke.

and ischaemic stroke, all points, irrespective of the effect size, should cluster along a diagonal line. Since the incidences of myocardial infarction and ischaemic stroke in young women in the Netherlands are similar,[26,27] this diagonal should be the x=y diagonal which is depicted in blue.

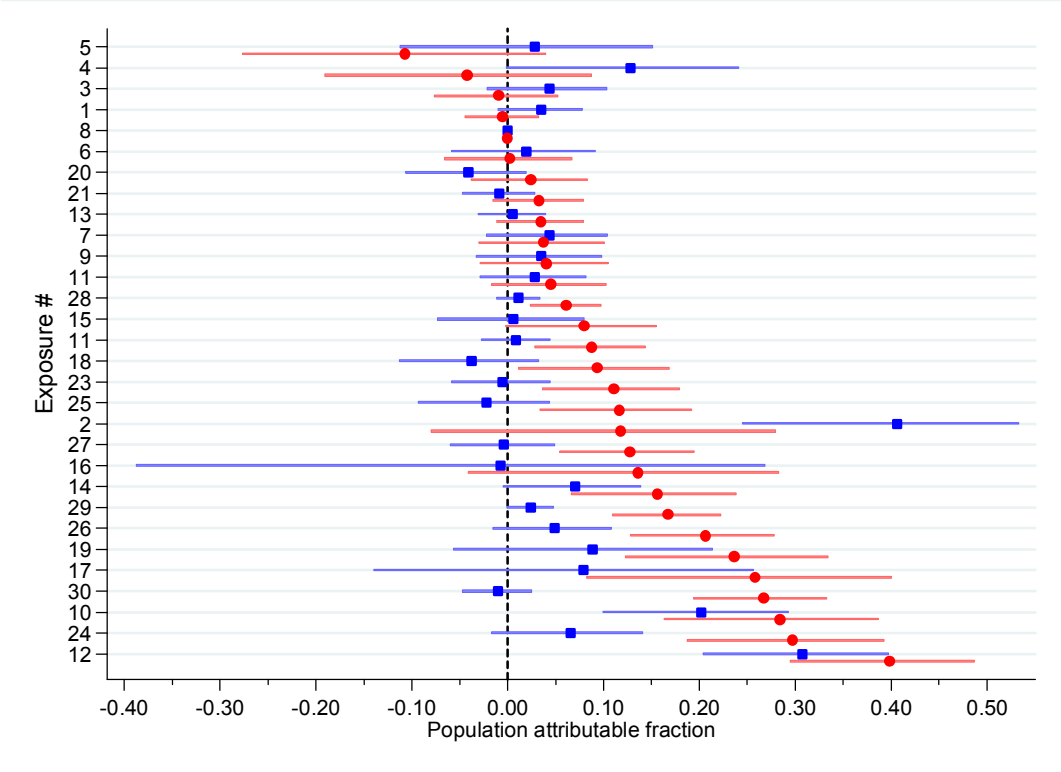
With the use of table 1 and the left panel of figure 1 the difference in effect on myocardial infarction and ischaemic stroke can be established for each prothrombotic factor. This kind of comparison is only possible when the same definition of exposure is used for the myocardial infarction analyses and the ischaemic analyses. However, with a listing of all studied prothrombotic risk factors, it is tempting to compare the different risk factors with each other with regard to their impact on the incidences of the two diseases. Still, the odds ratios and the ROR only tell part of the story; the impact of a risk factor is determined by its effect size and prevalence.

PAF For a proper quantitative comparison of the impact of these prothrombotic factors on myocardial infarction and ischaemic stroke the population attributable fraction can be used.[93] This number, also known as the aetiologic fraction, represents the fraction of disease incidence that is prevented in a population when the cause of the disease is removed. The population attributable fraction can be calculated by the formula

$$\text{population attributable fraction } = P_{cases}\left(\frac{OR-1}{OR}\right)$$

, where P_{cases} represents the proportion of exposed cases and OR represents the odds ratio of the risk factor of interest. Unlike other formulas that calculate the PAF, this formula yields valid estimates of the PAFs when confounding is present and therefore adjusted odds ratios are used.[94] The presented 95% confidence intervals are based on the variance calculated as proposed by Greenland, although these again might be slightly conservative since this method does not account for the use of a single control group in the RATIO study and therefore overestimates the true variance.[93,95] Since myocardial infarction and ischaemic stroke can be regarded as multicausal diseases, one has to keep in mind that the sum of this measure is not limited to 1.[93] The right panel of figure 1 depicts the calculated PAFs for myocardial infarction (x-axis) and ischaemic stroke (y-axis) comparable to the graph in the left panel of figure 1. To facilitate a side by side comparison of several factors, the PAFs and corresponding confidence intervals for all 30 prothrombotic factors are also

Figure 2. Prothrombotic risk factors in the RATIO study and their population attributable fractions for myocardial infarction and ischaemic stroke



Population attributable fractions from the myocardial infarction analyses are represented by blue squares where the ischaemic stroke results are denoted by red dots. The corresponding lines represent the corresponding 95% confidence intervals. These intervals underestimate the variance of the point estimates since they do not take the single control group used in the RATIO study into account. Exposure # refers to the exposure number as denoted in table 1 in which the exposures are ranked according to their relative odds ratio in ascending order.

plotted in figure 2. The PAFs based on the odds ratios derived from the myocardial infarction analyses are depicted in blue squares whereas the ischaemic stroke results are depicted in red circles.

Almost all points in the right panel of figure 1 show that the PAF derived from the ischaemic stroke analyses is higher than the PAF derived from the myocardial infarction results. Only 4 prothrombotic factors yield a PAF >0.1 in the myocardial infarction analyses, whereas 14 prothrombotic factors yield a PAF>0.1 in the ischaemic stroke analyses. Three points stand out, being exposure numbers 2, 4 and 5. Exposure 2, which represents the PAFs from the hypofibrinolysis vs normofibrinolysis analyses, points towards a large impact of this factor on the incidence of myocardial infarction with only a modest effect on ischaemic stroke incidence. As is discussed in chapter 10 and summarised above, the clot lysis time used to determine fibrinolytic capacity is mainly determined by PAI-1, which could be involved in other processes relevant for ischaemic stroke. However, it is also important to note that the RATIO study is focused on ischaemic stroke of non-cardiac origin, which might account for the modest impact of hypofibrinolysis. Exposure 4 and 5, i.e. two genetic variants in the F13A1 gene are the only two factors with a negative PAF for ischaemic stroke. However, the broad confidence intervals shown in figure 2 indicate that no strong conclusions may drawn from these PAFs; if anything the impact of these two genetic variants on both diseases is only modest.

The measures used in this direct comparison are approximations of the true underlying effect and are likely to be biased. But the question at hand is whether the differences between myocardial infarction and ischaemic stroke can be explained by these biases. Given the same control group, questionnaires measurements and data analyses such a scenario is not likely. We believe that the impact of bias is similar for the myocardial infarction and ischaemic stroke analyses and therefore cannot explain the observed contrast between these two diseases. With regard to residual confounding, the conclusion can even go further: the presence of a differential effect of residual confounding - that is, part of the observed difference can be explained by confounding that is present in one analyses but not in the other - also provides a clue that the causal mechanism underlying these two diseases are in fact different.

In short, with the RORs as measures of direct comparison of effect size and the PAFs facilitating the comparison of the impact on the incidence of the two diseases, one single picture arises: prothrombotic factors do play an important role in the aetiology of ischaemic stroke of non-cardiac origin in young women. The role of these factors in the aetiology of myocardial infarction is minimal if not absent.

Implications

Coagulation vs hypercoagulability To investigate the implications of these findings, the overall conclusion has to be put into the right perspective. Although there is a minimal effect of prothrombotic factors in the aetiology of myocardial infarction, this does not imply that blood coagulation does not play a role at all in this disease. In the research presented in this thesis the exposure was often chosen to represent 'hypercoagulability', a state with an increased clotting potential. So, an increased clotting potential is a cause of ischaemic stroke but not of myocardial infarction.

What can explain the involvement of coagulation processes in the mechanism underlying myocardial infarction while hypercoagulability does not play a role? Perhaps the answer to this question lies in the endothelial lining of the two vascular beds in relation to atherosclerotic lesions. The atherosclerotic burden of the women included in the RATIO is thought to be minimal: approximately 10% of the women included in both case groups were known with hypercholesterolaemia in the year prior to their event. Nonetheless, the subclinical atherosclerotic burden in the women suffering from myocardial infarction and ischaemic stroke women is higher than in other women and the related endothelial dysfunction could be part of the explanation of the observed differences between myocardial infarction and ischaemic stroke.

The endothelial lining of the coronary vessels affected by myocardial infarction is quite homogeneous and often a myocardial infarction is preceded by endothelial dysfunction, atherosclerotic lesions and subsequent rupture of a atherosclerotic plaque. The content of atherosclerotic plaques is highly thrombogenic, possibly to such an extent that variations in clotting potential do not affect the outcome of such a plaque rupture: a thrombus will inevitably form after plaque rupture and downstream tissue will be deprived of oxygen rich blood, irrespective of variations in clot propensity. To put it differently, hypercoagulability is not a trigger of the coagulation cascade in the aetiology of myocardial infarction. Or to refer to Virchows triad, myocardial infarction might be primarily caused by 'Phenomena

associated with irritation of the vessel and its vicinity' better known as endothelial injury or dysfunction.

Endothelial dysfunction, atherosclerotic lesions and plaque rupture can also be a cause of ischaemic stroke. For example, atherosclerotic plaques in the carotid arteries could rupture or erode resulting in coagulation initiation and embolising thrombi.[96] Although this scenario is already somewhat different from the scenario for myocardial infarction (i.e. differences in flow patterns, endothelial composition, lumen diameter and embolisation) a hypercoagulable state might not play a role in these forms of ischaemic stroke. However, ischaemic stroke is a heterogeneous multicausal disease and a considerable proportion of ischaemic strokes are thought to have a different causal mechanisms which include vasoconstriction, dissection, patent foramen ovale, inflammatory conditions and other vasculopathies.[96–101] The results from the RATIO study, as well as other studies, indicate that prothrombotic factors are likely to be also part of these different causal mechanisms: an increased clotting potential could in itself be a cause in the initiation of coagulation in the mechanism leading to of ischaemic stroke of non-cardiac origin. And in reference to Virchow's triad: as a multicausal disease, ischaemic stroke of non-cardiac origin might be caused by both endothelial injury as well as 'Phenomena of blood-coagulation' or hypercoagulability. When we also consider cardiac embolic stroke we can see that ischaemic stroke, being a heterogeneous disease, covers all three components of Virchow's triad.

So what can be said about the heterogeneity of ischaemic strokes included in the RATIO study? Several ischaemic stroke classification system have been proposed to deal with the heterogeneous nature of this disease.[97,102,103] Perhaps the most used system is the TOAST classification system, which categorises ischaemic stroke into 5 categories, each with their own causes and consequences: cardioembolism, large-artery atherosclerosis, small-vessel occlusion, stroke of other determined aetiology and stroke of undetermined aetiology.[97,103,104] Results from other studies indicate that the role of certain risk factors could very well be different in different categories.[35,39,46,105–111] The most intriguing category must be the 'stroke of undetermined origin', which comprises about one third of all strokes, a proportion that might be higher in the young.[39,101,103,112] This category comprises all stroke of which no overt cause is directly identified which makes it more difficult to directly target the underlying cause of the stroke to prevent recurrences of the disease.[110] Unfortunately, the RATIO study cannot be used to determine possible

differences between subtypes of ischaemic stroke since the necessary data are not available. New studies will help to further elucidate the role of thrombotic factors in young ischaemic stroke and help determine whether there is difference between the different subtypes.[113]

Future research Within these new studies there is range of topics that is of interest in the elucidation of the causal mechanisms of ischaemic stroke. The importance of the intrinsic coagulation proteins is slowly emerging and more insight in their diverse interactions and effects could further increase the knowledge of ischaemic stroke. As said in this chapter and shown in this thesis, murine, biochemical and clinical studies show that the intrinsic coagulation proteins can play a pivotal role in pathologic thrombus formation.[10,12,13,114–116] Additionally, several mechanisms have been shown to activate and modulate the several actions of the intrinsic coagulation proteins, of which the link to inflammatory processes and the activation route through platelet derived polyphosphates might be the most significant.[16,19,89,92,117–121] These new insights might even lead to new antithrombotic therapies: therapies targeting the intrinsic coagulation proteins, especially coagulation factor XI, could potentially be used in the secondary or even primary prevention of thrombotic disease.[58,120,122,123] Why FXI and not FXII? This choice starts with the observation that FXI deficient humans have a reduced risk of ischaemic stroke and deep venous thrombosis.[124,125] Also, FXII has a plethora of effects, also outside the realm of coagulation, whereas the role of FXI is more unambiguous and restricted to blood coagulation and the fibrinolytic system.[14,16,126–132] And finally, promising animal studies in which FXI transcription was minimised, for example with antisense oligonucleotides, have shown that this diminishes clot formation without a large increase in bleeding risk.[133–135] Inhibiting protein transcription lowers the antigen levels of the protein; compensatory mechanisms might increase protein activity by an increased activation rate nullifying this inhibition. In the RATIO study, FXI:ag levels were clearly and independently associated with ischaemic stroke whereas other antigen levels of the intrinsic coagulation proteins were not, leaving FXI as the most promising candidate to target.

It is likely that such a new therapy is first used in a setting in which hypercoagulation plays an important role in the aetiology of the targeted thrombotic disease, as is done with the newer anticoagulants dabigatran and rivaroxaban as treatments in the prevention of ischaemic stroke in atrial fibrillation patients.[136,137] Nonetheless, a FXI targeted drug might even be considered for testing as a secondary prevention measure for the recurrence of

myocardial infarction. This is not in contrast with the main conclusion of this thesis: the contrast used in this thesis is variation in thrombotic potential within a normal population. Treatment with an anticoagulant is not the counterfactual of 'hypercoagulability' since it reduces the thrombotic potential drastically beyond normal variation. This concept is demonstrated by the studies which tested anticoagulation treatments in the prevention of coronary disease; anticoagulation therapy does reduce the risk of coronary outcomesbut is inferior to dual anti-platelet therapy in the cost-benefit analyses which takes bleeding risks into account.[138] Therefore, if FXI targeted therapy indeed could reduce clotting potential with only minor increases in bleeding risks, such a therapy should be considered for the treatment of all thrombotic diseases, including those in which hypercoagulability is not a risk factor.

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The variance of a relative odds ratio

// Odds ratio

An odds ratio is a measure of association between two dichotomous variables. The OR can be calculated from a standard 2x2 table:

	Exp+	Exp-
Case	A	B
Control	C	D

$$OR = \frac{A \times D}{B \times C}$$

The variance of an OR is expressed as

$$\operatorname{var} \ln OR = \frac{1}{A} + \frac{1}{B} + \frac{1}{C} + \frac{1}{D}$$

// Relative odds ratio

A Relative Odds Ratio (ROR) can be used for example as a direct comparison of effects between two studies. The ROR is calculated as

Study 2	Exp+	Exp-
Case	A_2	B_2
Control	C_2	D_2

Study 1	Exp+	Exp-
Case	A_1	B_1
Control	C_1	D_1

$$ROR = \frac{OR_1}{OR_2} = \frac{\frac{A_1 \times D_1}{B_1 \times C_1}}{\frac{A_2 \times D_2}{B_2 \times C_2}}$$

where the two compared studies are denoted with 1 and 2, respectively. The variance of such a ROR is equal to the sum of the variances corresponding to the two ORs reflected in the ROR.[1] Or, more generally,

$$\operatorname{var} \ln ROR = \frac{1}{A_1} + \frac{1}{B_1} + \frac{1}{C_1} + \frac{1}{D_1} + \frac{1}{A_2} + \frac{1}{B_2} + \frac{1}{C_2} + \frac{1}{D_2}$$

// Relative odds ratio with a shared control group

A ROR can also be calculated in a single case-control study with multiple case-groups and a single control group. In such a study the ROR of a particular exposure can be calculated to investigate whether there is a difference in effect of the exposure between the two diseases. The ROR from such a single study, or $ROR_{\text{shared control}}$, can be calculated from a 2x3 table

	Exp+	Exp-
$Case_1$	A_1	B_1
$Case_2$	A_2	B_2
control	C	D

$$ROR_{shared\ control} = \frac{OR_1}{OR_2} = \frac{A_1 \times B_2}{B_1 \times A_2}$$

This can also been seen from the formulas for the ROR derived from two studies, because with a single control group $C_1 = C_2$ and $D_1 = D_2$. This has also implications for the variance of the $ROR_{shared\ control}$. This variance can now be calculated as

$$\text{var ln}\, ROR_{shared\ control} = \frac{1}{A_1} + \frac{1}{B_1} + \frac{1}{A_2} + \frac{1}{B_2} = \text{var ln}\, OR_1 + \text{var ln}\, OR_2 - \left(\frac{2}{C} + \frac{2}{D}\right)$$

Which implies that the variance of a $ROR_{shared\ control}$ calculated as if it where the variance of a ROR from two different studies would result in an overestimation of the true variance. The number of control subjects in case-control studies usually equals or outnumbers the number of cases in the case group. Therefore, the overestimation will be relatively small and is not likely to nullify the results.

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14 Nederlandse samenvatting

Cardiovasculaire ziekten

Cardiovasculaire ziekten hebben een grote invloed op de gezondheid in Nederland. Door de oorzaken van de verschillende presentaties van deze ziekten te onderzoeken is het mogelijk dat er aangrijpingspunten worden gevonden voor nieuwe preventie- en behandelingsstrategieën die de invloed van deze ziekten verminderen met een zo klein mogelijke kans op bijwerkingen. Hierbij is het van groot belang dat de verschillende cardiovasculaire ziekten een andere wijze van ontstaan kunnen hebben met directe gevolgen voor deze behandelingsstrategieën. Veel cardiovasculaire ziekten zijn het gevolg van trombose, waarbij een bloedstolsel ontstaat dat de bloeddoorstroming bemoeilijkt. Als zich een bloedstolsel vormt in een slagader spreekt men van arteriële trombose: zuurstofrijk bloed kan niet meer doorstromen naar de organen waardoor er een zuurstofcrisis ontstaat en het weefsel kan afsterven. Voorbeelden van arteriële trombose zijn het hart- en herseninfarct. Het is ook mogelijk dat een bloedstolsel ontstaat in een ader: zuurstofarm bloed kan dan niet meer ongehinderd terugstromen naar het hart en de longen, met stuwing ten gevolge. De meest voorkomende vorm van veneuze trombose, i.e. trombose van het been, heeft in de meeste gevallen niet een even sterk acuut en potentieel dodelijk effect als arteriële trombose. Echter, wanneer dit bloedstolsel in het been afbreekt, kan het in de longen of zelfs de hersenen terecht komen met mogelijk dodelijke afloop.

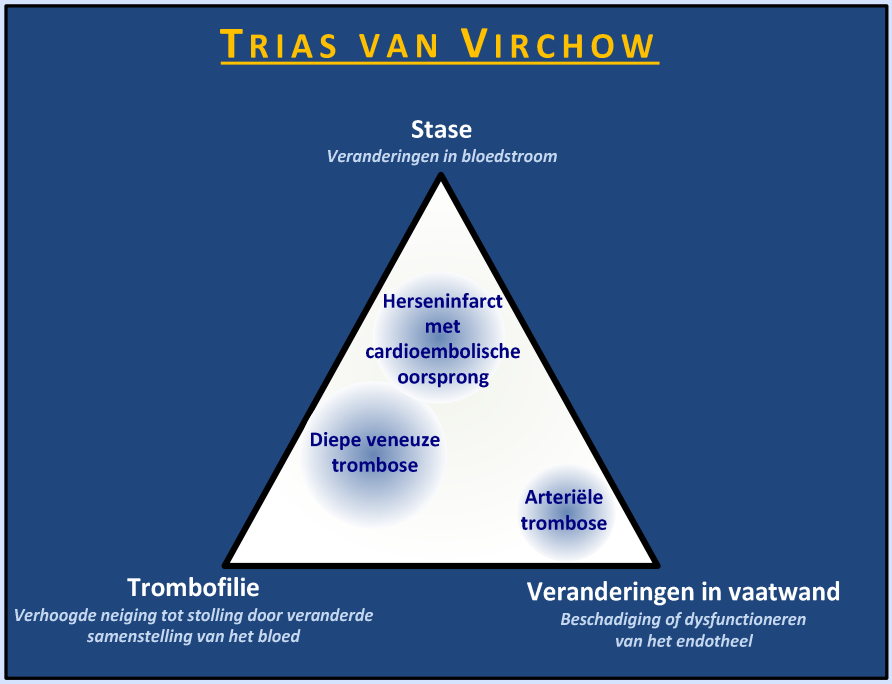
Trombose

Het is duidelijk dat het mechanisme dat leidt tot trombose een afwijking betreft: normaliter stolt het bloed alleen wanneer er sprake is van een beschadiging van een bloedvat, bijvoorbeeld bij een wondje opgelopen bij het scheren. Er zijn echter omstandigheden die de kans op een trombose vergroten. Al in 1865 heeft de Duitse patholoog Rudolph Virchow een drietal '*Formen der Trombose'* geformuleerd. Dit heeft uiteindelijk geleid tot de 'trias van Virchow', een concept dat gebruikt wordt om de oorzaken van trombose in te delen in drie categorieën, te weten I) veranderingen in de bloedstroom, II) verandering in de samenstelling van het bloed en III) veranderingen aan de vaatwand. Op het eerste gezicht lijken de verschillende manifestaties van trombose dan ook toe te schrijven aan één van de drie onderdelen van de trias van Virchow, zoals is weergegeven in figuur 1. Zo zijn veel, maar niet alle, bekende oorzaken van een trombosebeen terug te leiden tot verandering in de samenstelling van het bloed dat leidt

tot een verhoogde stollingsneiging. Dit gebeurt bijvoorbeeld door een genetische variatie zoals Factor V Leiden die aanwezig is bij ongeveer 5% van de Nederlandse bevolking. Bij een herseninfarct met een cardio-embolische oorsprong ligt de oorzaak veelal in boezemfibrileren, een conditie waarbij het hart niet op de juiste manier pompt, wat leidt tot abnormale wervelingen en lokale stilstand van het bloed. Hierdoor ontstaat vaak een bloedstolsel dat kan afbreken, en vervolgens meegevoerd wordt naar de hersenen. Bij arteriële trombose is de algemene opvatting dat de oorzaak primair ligt in atherosclerose, ofwel slagaderverkalking, wat leidt tot beschadigingen van de vaatwand waardoor op die plek een bloedstolsel kan ontstaan.

Trombotische ziekten zijn zogenaamde multi-causale ziekten, waarbij verschillende factoren samen leiden tot het ontstaan van een bloedstolsel: bijna geen enkele geïsoleerde risicofactor is een noodzakelijk voldoende oorzaak van trombose. Daarom is de voorstelling van verschillende typen van trombose met een eenduidig en makkelijk te classificeren ontstaansmechanisme, zoals weergegeven in figuur 1, een grove vereenvoudiging van de werkelijkheid. Toch heeft een dergelijke grafische weergave zin: het laat zien hoe in de klinische praktijk slechts op algemene kenmerken onderscheid gemaakt kan worden in diagnose en behandeling van trombose.

Er wordt vaak, zowel expliciet als impliciet, gesproken over hart- en herseninfarcten alsof het één ziekte betreft, te weten een vorm van arteriële trombose ten gevolge van slagaderverkalking. De huidige standaard behandeling van deze twee ziekten is dan ook grotendeels gelijk, maar voor beide ziekten niet optimaal. Als bij nader inzien deze twee ziekten toch verschillende ontstaansmechanismen blijken te hebben, kan het zijn dat nieuwe gedifferentieerde behandeling- en preventiestrategieën meer ziekte en sterfte kunnen voorkomen dan momenteel het geval is. Hoewel wordt aangenomen dat arteriële trombose zijn oorsprong vooral vindt in slagaderverkalking en de bijkomende vaatwandbeschadigingen, is het nog onduidelijk in hoeverre een verhoogde stollingsneiging ook een rol speelt in het ontstaan van deze multicausale ziekten. De centrale vraag van verschillende deelonderzoeken verricht binnen het RATIO onderzoek is dan ook of er een verschil is in de rol van een verhoogde stollingsneiging bij het ontstaan van hart- en herseninfarcten bij jonge vrouwen.



Figuur 1. Trias van Virchow

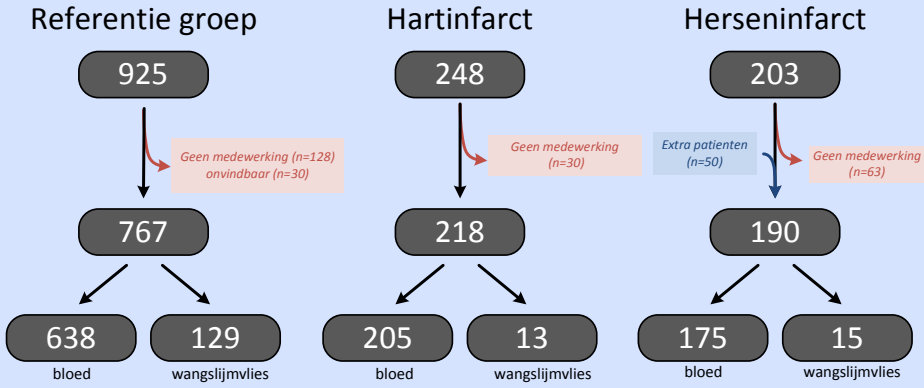
De trias van Virchow beschrijft de drie categorieën waarin de verschillende oorzaken van trombose vallen in te delen.

Het RATIO onderzoek

Het RATIO onderzoek is begonnen om de rol van orale anticonceptie, ook wel bekend als ‘de pil’, bij het ontstaan van arteriële trombose te onderzoeken: er werd aan vrouwen die een hart- of herseninfarct hadden doorgemaakt gevraagd of zij de pil hadden geslikt. De frequentie hiervan kan dan worden vergeleken met de frequentie in een referentiegroep, bestaande uit vrouwen van dezelfde leeftijd die verder vergelijkbaar waren met de vrouwen uit de algemene bevolking. Verschil in deze frequenties kan duiden op een causale rol van pilgebruik in het ontstaan van deze ziekten. De resultaten lieten dan ook zien dat onder vrouwen met een hart- dan wel herseninfarct pilgebruik vaker voorkwam dan onder de vrouwen van de referentiegroep. Dit verschil in frequentie kwam overeen met ongeveer een verdubbeling van het risico op deze ziekten. In een later stadium werd hen verzocht om een bloedmonster af te staan zodat onderzocht kon worden of de samenstelling van het bloed ook een oorzaak kan zijn van deze ziekten. Deze bloedafname bood de mogelijkheid om te onderzoeken of een verhoogde stollingsneiging ook vaker voorkomt onder vrouwen met een hart- of herseninfarct dan onder de vrouwen uit de referentiegroep (zie ook figuur 2). Het RATIO onderzoek is uniek omdat het zich richt op vrouwen onder de 50 jaar. Omdat leeftijd een sterke risicofactor is voor deze ziekten, is het mogelijk dat in onderzoek dat zich richt op oudere vrouwen het effect van hun hogere leeftijd de effecten van de te onderzoeken factoren overschaduwt. Door relatief jonge patiënten te onderzoeken, zal het effect van leeftijd beperkt blijven en is het mogelijk om bijzondere oorzaken van deze ziekten makkelijker te onderscheiden. Daarnaast geeft dit onderzoek de mogelijkheid om na te gaan of een verhoogde stollingsneiging en het gebruik van de pil elkaar beïnvloeden wat betreft het risico op zoelte, een fenomeen bekend uit onderzoek naar oorzaken van trombosebenen.

Het RATIO onderzoek is een zogenaamd observationeel onderzoek, een onderzoeksopzet waarbij, in tegenstelling tot bij een experiment, geen manipulatie van factoren die de ziekte kunnen beïnvloeden door de onderzoeker plaatsvindt. Een dergelijke onderzoeksopzet heeft enkele aspecten die, indien niet ondervangen, de conclusies kunnen invalideren: omdat de bloedmonsters worden verzameld na het hart- of herseninfarct is het mogelijk dat de bloedspiegels een gevolg zijn van de ziekte (zie ook figuur 2). Deze verwarring van oorzaak en gevolg, bekend als *reverse causation*, kan leiden tot foutieve conclusies over het ontstaansmechanisme. Tevens kan er in de studieopzet een systematische fout aanwezig zijn waardoor onvergelijkbaarheid optreedt tussen de

patiëntengroep en de referentiegroep, een fenomeen bekend als *bias*. Een voorbeeld hiervan is dat patiënten een vragenlijst anders invullen dan de controlepersonen of dat alleen gezondheidsbewuste mensen willen deelnemen aan de controlegroep. Daarnaast clusteren risicofactoren zich vaak binnen de patiëntengroep, waardoor het mogelijk is dat de effecten van twee risicofactoren met elkaar worden verward. Dit fenomeen staat bekend as *confounding*. De aanwezigheid van *bias* en *confounding* moet altijd overwogen worden bij observationeel onderzoek. Derhalve zijn er verschillende methoden ontwikkeld om bias en *confounding* tegen te gaan, en zo de kans op onjuiste conclusies te verkleinen.



Figuur 2. Stroomdiagram van RATIO deelnemers

De deelnemers van het RATIO onderzoek zijn in twee fasen benaderd: in de eerste fase beantwoorden zij een vragenlijst en in de tweede fase werd er een bloed- of wangslijmvliesmonster afgenomen.

Samenvatting van resultaten

Hoofdstuk 2 begint met de bespreking van een positieve familieanamnese als indicator van een verhoogd risico op het krijgen van een hart- of herseninfarct bij jonge vrouwen: als vrouwen een zij eerstegraads familielid (i.e. ouders, broers of zusters) hadden die een hartinfarct of een beroerte hadden gekregen voor hun zestigste levensjaar, was het risico op een hartinfarct bij hen zelf 3.5 maal verhoogd, terwijl het risico op een herseninfarct niet anders was dan het risico van de vrouwen die geen aangedane familieleden hadden. In de formele zin van het woord is het niet mogelijk om hier te spreken van een causaal verband: louter een positieve familieanamnese is niet direct een oorzaak van het krijgen van een ziekte. Wel is het mogelijk een 'familieanamnese' te zien als een indicator van de aanwezigheid van risicofactoren. Als ziekten clusteren in families kan dat erop wijzen dat er sprake is van een erfelijke aanleg, dan wel een door de familieleden gedeelde ongezonde levensstijl die leidt tot een hogere incidentie van die ziekte. Toch valt er een voorzichtige causale conclusie te trekken uit deze resultaten: als hart- en herseninfarcten precies dezelfde oorzaken hebben, zou ook de relatie tussen een positieve familieanamnese en het risico op deze twee ziekten ongeveer gelijk moeten zijn. Onze resultaten geven een eerste indicatie dat deze twee ziekten, ondanks gedeelde eigenschappen, verschillende oorzaken hebben.

Het onderzoek in hoofdstuk 3 tot 6 richt zich direct op een causaliteitsvraagstuk: zij beschrijven het onderzoek naar de eiwitten van het intrinsieke stollingssysteem als mogelijke oorzaken van zowel hart- als herseninfarcten bij jonge vrouwen. Deze eiwitten hebben de bijzondere eigenschap dat zij niet essentieel lijken te zijn voor een normale bloedstolling: mensen bij wie de functie van deze eiwitten drastisch verminderd is, vertonen slechts een milde neiging tot bloeden (in het geval van stollingsfactor XI) of hebben in het geheel geen verhoogde bloedingsneiging (in het geval van stollingsfactor XII, prekallikreine en co-factor hoog moleculair gewicht kininogeen, afgekort als HMWK). Daarnaast suggereren verschillende onderzoeken met diermodellen wel dat deze eiwitten een rol spelen in het ontstaan van een bloedstolsel.

Van deze eiwitten zijn verschillende eigenschappen onderzocht, grofweg te beschrijven als de **aanwezigheid**, de **activiteit** en de **activatie** van een eiwit. De aanwezigheid van een eiwit wordt doorgaans gemeten door het antigeenniveau te bepalen. De meting van deze antigeenniveaus wordt verricht met behulp van zogenaamde antilichamen, van origine

onderdelen van het immuunsysteem die andere eiwitten kunnen herkennen en binden. Door deze specifieke binding is het mogelijk om de hoeveelheid eiwit te kwantificeren, bijvoorbeeld uitgedrukt als percentage van een referentie plasma. De activiteit van een eiwit kan gemeten worden met behulp van een activiteitsmeting waarin het te onderzoeken plasma 1:1 wordt gemengd met een referentieplasma. De stollingstijd van dit mengsel na maximale activatie wordt uitgedrukt als percentage van de stollingstijd van het referentie plasma. De activatie van een eiwit kan op verschillende manieren worden gemeten. Hierbij wordt, omdat deze eiwitten in het bloed in het merendeel in inactieve vorm aanwezig zijn en pas werkbaar zijn na activatie, nagegaan of deze geactiveerde eiwitten, of sporen daarvan aanwezig zijn. De techniek die in dit proefschrift is gebruikt, is gebaseerd op antigeenniveaus, net als bij de bepaling van de aanwezigheid van een eiwit. Omdat de intrinsieke stollingseiwitten na activatie direct worden gekoppeld aan een remmer (inhibitor), kunnen antilichamen gericht tegen een dergelijk eiwit-inhibitor complex gemeten worden als mate van activatie. Normaliter wordt een stollingseiwit alleen geactiveerd in het geval van een bloeding. De eiwit-inhibitor complexen die gemeten zijn in dit proefschrift zijn geen maat voor deze tijdelijke eiwitactivatie, maar zijn indicatief voor een lage maar constante eiwitactivatie, hetgeen aangeeft dat er een proces op de achtergrond gaande is waarbij deze eiwitten worden geactiveerd.

In **hoofdstuk 3** wordt beschreven dat de activatie van de intrinsieke stollingseiwitten een verhoogd risico geeft op het krijgen van een herseninfarct. Dit effect was groter onder pilgebruikers dan onder niet-pilgebruiksters. Het risico op een hartinfarct blijft onveranderd bij een verhoogde maat van activatie. **Hoofdstuk 4** richt zich specifiek op stollingsfactor XI: een verhoogde aanwezigheid van dit eiwit verhoogt het herseninfarctrisico, terwijl het hartinfarctrisico opnieuw niet verandert. Dit effect is onafhankelijk van de activatiegraad van het eiwit, zoals gemeten door de eiwit-inhibitorcomplexen. Ook is dit effect onafhankelijk van de activiteit van stollingsfactor XI. **Hoofdstuk 5** is gericht op stollingsfactor XII. Uit het RATIO deelonderzoek blijkt dat een verhoogde aanwezigheid van stollingsfactor XII en prekallikreine geen sterke invloed heeft op het risico van zowel hart- en herseninfarcten. In **hoofdstuk 6** wordt beschreven dat vrouwen met een verhoogde aanwezigheid van HMWK wel een verhoogd risico hebben op het krijgen van een herseninfarct. Het hartinfarctrisico is echter vergelijkbaar met het risico van de vrouwen met niet verhoogde HMWK antigeenniveaus. Uit deze hoofdstukken valt in zijn algemeenheid te concluderen dat deze eiwitten, met name stollingsfactor XI, een

oorzakelijke rol spelen bij het ontwikkelen van herseninfarcten bij jonge vrouwen. Om het ontstaansmechanisme in meer detail te begrijpen is echter meer onderzoek nodig.

In de volgende hoofdstukken wordt een genetische aanpak gebruikt om de rol van het stollingssysteem bij het ontstaan van hart- en herseninfarcten te onderzoeken. In **hoofdstuk 7** wordt beschreven hoe genetische verschillen tussen mensen gebruikt kunnen worden om specifieke eigenschappen van een eiwit te onderzoeken teneinde problemen als *confounding* en het verwarren van oorzaak en gevolg te omzeilen. Bij deze methode wordt genetische variatie die geassocieerd is met bijvoorbeeld het antigeenniveau van een eiwit, en niet via een andere weg een effect heeft op het optreden van de ziekte, onderzocht op een relatie met ziekte. Deze methode wordt ook wel Mendeliaanse randomisatie genoemd, een naam die verwijst naar Gregor Mendel (1822-1884), één van de grondleggers van de moderne genetica, die stelde dat genetische variaties onafhankelijk van elkaar overerven. In het verlengde van deze waarneming ligt de basis van deze methode, namelijk dat genetische variatie in grote populaties niet samenhangt met mogelijk verstorende factoren zoals levensstijl, omgevingsfactoren en sociaal economische status. De gedachte is dat genetische variatie, analoog naar een gerandomiseerd geneesmiddelenonderzoek waarbij deelnemers willekeurig de nieuwe of de controlebehandeling krijgen toegewezen, gerandomiseerd zijn bij conceptie waardoor er geen *confounding* kan optreden.

Hoewel in potentie een krachtige methode, dienen een aantal nadelen van Mendeliaanse randomisatie genoemd te worden: Mendeliaanse randomisatie steunt sterk op enkele aannames die per definitie niet empirisch te controleren zijn, omdat zij uitgaan van de afwezigheid van enkele associaties. Bovendien vereist het getrapte karakter van de formele analyses behorende bij deze methode (dat wil zeggen: genetische variatie → antigeen niveau → risico op ziekte) grote aantallen deelnemers om fout-negatieve resultaten te voorkomen. Helaas heeft het RATIO onderzoek niet voldoende deelnemers om formele Mendeliaanse randomisatie analyses uit te voeren. Desalniettemin, de gedachten onderliggend aan deze formele analyses kunnen wel gebruikt worden bij het wegen van bewijs over causale vraagstukken. Dit gebeurt dan ook in **hoofdstuk 8**. Hierin wordt de rol van fibrinogeen bij het ontstaan van hart- en herseninfarcten onderzocht. Fibrinogeen is de voorloper van fibrine, het belangrijkste bestanddeel van bloedstolsels. En hoog fibrinogeenniveau is in eerdere onderzoeken geassocieerd met een verhoogd risico op zowel hart- als herseninfarcten, maar mogelijk waren deze resultaten vertekend door

confounding en *reverse causation*. In het RATIO onderzoek zijn 2 kleine genetische variaties, zogenaamde Single Nucleotide Polymorphisms (SNP's), in de fibrinogeengenen onderzocht op hun relatie met fibrinogeenniveaus alsmede het risico op hart- en herseninfarcten. Beide SNP's waren geassocieerd met een verandering van het herseninfarctrisico, terwijl het risico op hartinfarcten onveranderd blijft. De richting van het effect op het herseninfarctrisico was overeenkomstig met het effect op fibrinogeen niveaus: de SNP geassocieerd met hogere fibrinogeen niveaus geeft een hoger herseninfarctrisico, terwijl de SNP geassocieerd met lagere fibrinogeen niveaus een verlaging van het herseninfarct risico geeft. Van de onderzochte SNP's is bekend dat zij naast een kwantitatief effect mogelijk ook een kwalitatieve verandering van het fibrinogeen geven: de structuur van bloedstolsels is iets anders waardoor het risico op herseninfarcten ook verhoogd kan zijn. Het is dus niet mogelijk om het onderliggend mechanisme op grond van deze bevindingen precies te duiden. Desondanks is uit deze resultaten wel te concluderen, ondersteund door resultaten van andere onderzoeken, dat fibrinogeen een rol speelt bij het ontstaan van herseninfarcten maar niet van hartinfarcten.

De resultaten uit **hoofdstuk 9** hebben ook betrekking op de structuur van bloedstolsels. Nadat fibrinogeen wordt omgezet tot fibrine ontstaat er een netwerk van fibrinemoleculen. Stollingsfactor XIII speelt een belangrijke rol bij het ontstaan van dit netwerk, omdat dit eiwit de kruisverbindingen tussen fibrinemoleculen tot stand brengt. Een eerdere analyse van vier SNP's in de genen van FXIII hebben laten zien dat FXIII een rol speelt bij het ontstaan van herseninfarcten, met het hoogste risico voor vrouwen die ook orale contraceptiva gebruikten.15 Deze SNP's hebben mogelijk een effect op het ontstaan van de kruisverbindingen, zowel kwalitatief als kwantitatief, en verhogen daarmee het risico op trombose. Deze SNP's bleken echter geen verhoging te geven van het hartinfarctrisico, waaruit geconcludeerd kan worden dat de rol van FXIII verschilt bij mechanismen onderliggend aan hart- en herseninfarcten.

De volgende hoofdstukken richten zich niet zozeer op individuele factoren van het stollingsysteem, maar onderzoeken andere maten gerelateerd aan een verhoogde stollingsneiging. Zo wordt in **hoofdstuk 10** de rol van Von Willibrand Factor (VWF) besproken. VWF is gebonden aan FVIII in bloedplasma, en wordt opgeslagen in bloedplaatjes en in de cellen van de vaatwand. Na activatie wordt VWF uitgescheiden en ontvouwt het zich als een lange draad in het bloedvat waarop bloedplaatjes afremmen, binden en vervolgens activeren. Eenmaal geactiveerd kunnen deze bloedplaatjes

stollingsprocessen activeren of versnellen. Naast normale activatie kan VWF ook worden uitgescheiden door disfunctionerende cellen van de vaatwand, waarmee VWF naast een mogelijke oorzaak ook een gevolg kan zijn van vroege arteriosclerose. De lengte van VWF bepaalt in belangrijke mate de efficiëntie van zijn werking. Het eiwit ADAMTS13 reguleert de lengte, en daarmee de werkzaamheid van VWF: hoe minder ADAMTS13 hoe langer het VWF. Uit resultaten van het RATIO onderzoek blijkt dat hoge niveaus van VWF-antigeen het risico op zowel hart- als herseninfarcten verhoogt. Lage antigeenniveaus van ADAMTS13 verhogen echter alleen het herseninfarctrisico, waardoor de vraag opkomt of de associatie tussen VWF en hartinfarctrisico niet gedeeltelijk verklaard kan worden door beschadigingen van de vaatwand.

Hoofdstuk 11 onderzoekt de rol van het fibrinolytische systeem. De eiwitten in dit systeem voorkomen dat bloedstolsels ongebreideld blijven groeien. Een verlaagde capaciteit van dit systeem is daarmee mogelijk debet aan een verhoogde stolselvorming, hetgeen kan leiden tot trombose. Een verlaagde fibrinolytische capaciteit bleek dan ook het hartinfarctrisico bij jonge vrouwen te verhogen. De resultaten voor de analyses van het herseninfarct risico lieten echter het tegenovergestelde zien: een verhoogde fibrinolytische activiteit, theoretisch leidend tot een verminderde stolselvorming, verhoogt het herseninfarctrisico bij jonge vrouwen. Wat kan een dergelijk contra-intuïtief resultaat verklaren? Mogelijk ligt het antwoord bij PAI-1, één van de belangrijkste eiwitten binnen het fibrinolytische systeem: naast een groot effect op de fibrinolytische capaciteit beïnvloedt PAI-1 mogelijk ook de overlevingskansen van hersencellen bij een infarct. Omdat het niet mogelijk is om met deze test voor fibrinolytische capaciteit alleen het effect van bijvoorbeeld PAI-1 te onderzoeken, is het niet mogelijk om vergaande conclusies te trekken uit de resultaten. Maar ook ander onderzoeken geven een onduidelijk beeld. Extra onderzoek is derhalve nodig om antwoord te krijgen de precieze rol van het fibrinolysesysteem bij het ontstaan van een herseninfarct. Overigens worden de tegenstrijdige resultaten misschien wel veroorzaakt doordat er verschillende subtypen herseninfarct werden onderzocht. Nieuw onderzoek moet dan ook zo worden opgezet dat deze verschillende typen zo goed mogelijk te onderscheiden zijn. Ondanks de onduidelijkheden over de precieze interpretatie van de gebruikte test om de fibrinolytische capaciteit te meten, suggereren de resultaten dat de rol van het fibrinolytische systeem en zijn componenten anders is bij het ontstaan van een hartinfarct dan bij een herseninfarct.

In het volgende hoofdstuk wordt de relatie tussen het antifosfolipide syndroom en het risico op hart- en herseninfarcten onderzocht. Dit auto-immuunsyndroom wordt gekarakteriseerd door ziekten als gevolg van trombose, zoals hart en herseninfarcten maar ook zwangerschapscomplicaties en veneuze trombose. Er wordt echter alleen van het antifosfolipide syndroom gesproken als er naast een dergelijke ziekte ook nog antifosfolipiden antilichamen of aanwijzingen daarvan (vastgesteld met een positieve lupus anticoagulans test) herhaaldelijk worden aangetoond in het bloed van de patiënt. Alhoewel deze 'biomarkers' onderdeel zijn van de definitie van het syndroom was het niet bekend welke risicoverhoging de aanwezigheid van deze markers met zich meebrengt. De resultaten in **hoofdstuk 12** waren de eerste die een schatting gaven voor deze risicoverhoging: een positieve lupus anticoagulans test verhoogde het risico op een hartinfarct ongeveer 5 maal, terwijl het risico op een herseninfarct 40 maal werd verhoogd. Pilgebruik verhoogde deze risico's nog verder. Ook al is deze 5-voudige verhoging van het hartinfarctrisico vele malen groter dan de die van andere risicofactoren beschreven in dit proefschrift, er is opnieuw een opmerkelijk verschil in grootte van de hart- en herseninfarctrisicoverhoging. Van de overige biomarkers die getest zijn lijken antilichamen gericht tegen β2-glycoproteine I een directe rol te hebben in de pathofysiologie van dit syndroom. De aanwezigheid van β2-glycoproteine I antilichamen verdubbelt het herseninfarctrisico, maar leidt niet tot een verandering van het hartinfarctrisico. Ook al is het onderliggende mechanisme nog niet bekend het is wel duidelijk dat deze biomarkers een maat zijn voor een verhoogde stollingsneiging. Derhalve is ook uit deze resultaten te concluderen dat een verhoogde stollingneiging een sterk verhoogd risico geeft op een herseninfarct, terwijl het hartinfarctrisico veel minder sterk is aangedaan.

Verschillen en overeenkomsten

Samen met resultaten uit eerdere RATIO-deelonderzoeken geven de resultaten beschreven in dit proefschrift een overzicht van de rol van een verhoogde stollingsneiging in het ontstaan van hart- en herseninfarcten. Met een dergelijk overzicht valt dan ook makkelijker een antwoord te vinden op de vraag die eerder is gesteld: is er een verschil in de rol van een verhoogde stollingsneiging in het mechanisme dat leidt tot deze ziekten?

Om dat overzicht te geven, kan men gebruik maken van de populatie attributieve fractie: dit is de fractie van het aantal zieken in een onderzochte populatie dat voorkomen had kunnen worden als een bepaalde oorzaak van die ziekte in zijn geheel niet had bestaan. Dit

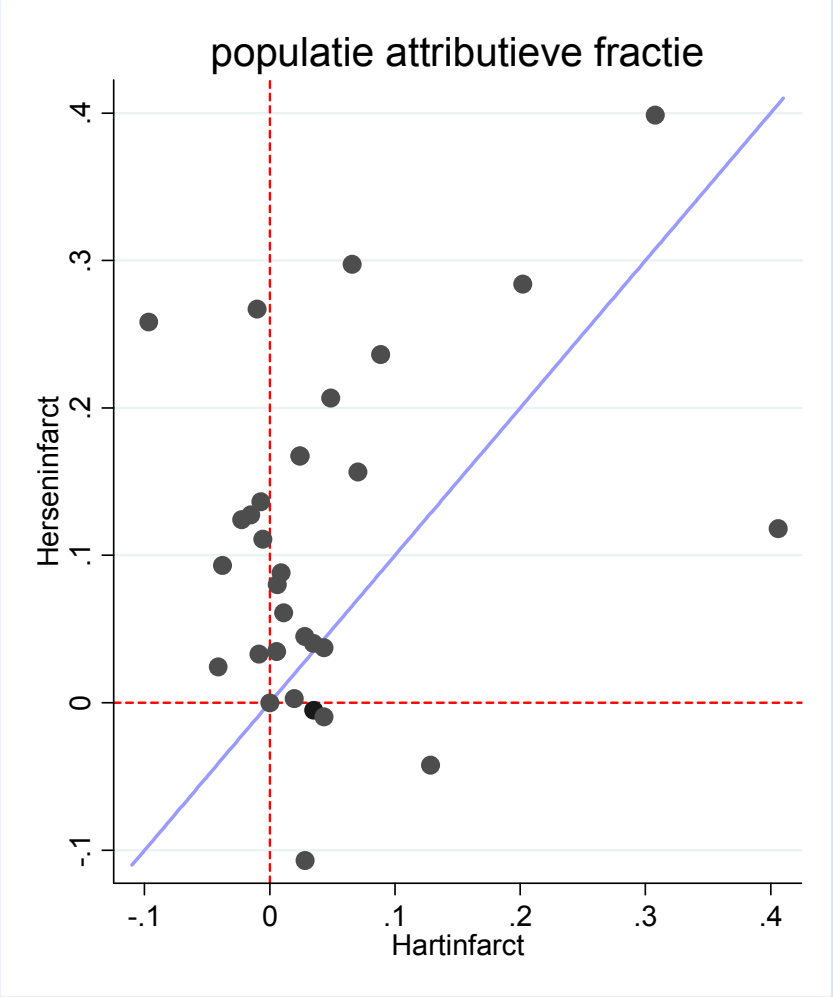
getal is een zuiver theoretisch getal en heeft geen directe implicaties voor het bepalen van de oorzakelijkheid van een bepaalde factor binnen één patiënt, maar dit getal is wel een goede maat voor het totale effect van een bepaalde factor op het vóórkomen van een bepaalde ziekte.

In figuur 3 staan deze populaties attributieve fracties weergegeven voor zowel hartinfarct (op de x-as) en herseninfarct (op de y-as). Als het effect van een verhoogde stollingsneiging voor beide ziekten gelijk was, zouden alle punten op de blauwe diagonale lijn moeten liggen. Dit is echter niet het geval: vele factoren hebben geen noemenswaardige invloed op het hartinfarctrisico terwijl ze wel het herseninfarctrisico wel degelijk vergroten en liggen daarmee rond de verticale rode lijn, de nullijn van de x-as. Er zijn geen factoren die een uitgesproken effect hebben op het hartinfarctrisico terwijl zij dat niet hebben op het herseninfarctrisico. Er zijn wel factoren die het risico op beide ziekten lijken te verhogen, maar het herseninfarct risico is bij deze factoren bijna altijd groter dan het hartinfarctrisico. Kortom, figuur 3 geeft antwoord op onze oorspronkelijke vraag: een verhoogde stollingsneiging is een onderdeel van het ontstaansmechanisme van herseninfarcten, en heeft geen belangrijke rol bij het ontstaan van hartinfarcten.

Implicaties

Betekenen deze resultaten nu dat de bloedstolling geen rol speelt bij de ontstaansmechanismen van hartinfarcten, maar wel bij herseninfarcten? Nee, beide ziekten zijn immers acute vormen van arteriële trombose waarbij een bloedstolsel ervoor zorgt dat de toevoer van zuurstofrijk bloed naar achterliggend weefsel vermindert. Deze resultaten willen wel zeggen dat een verhoogde stollingsneiging alleen onderdeel is van het ontstaansmechanisme van herseninfarcten en niet bij hartinfarcten. Misschien is het wel zo dat het ontstaan van hartinfarcten primair ligt in een abrupte beschadiging van de vaatwand; als de vaatwand eenmaal beschadigd raakt is de activatie van het stollingssysteem zo sterk dat er altijd een bloedstolsel gevormd zal worden, ongeacht of de patiënt in kwestie een iets verhoogde stollingsneiging heeft.

Zijn zogenoemde antistollingsmedicijnen dan alleen geschikt voor het behandelen en voorkomen van herseninfarcten? Dit zou wellicht zo zijn als deze medicijnen een verhoogde stollingsneiging weer zouden normaliseren. Dit is echter niet het geval: antistollingsmedicatie verlaagt de stollingsneiging in zeer sterke mate, zo sterk dat er gesproken kan worden van een verlaagde stollingsneiging, niet zelden gepaard gaand met



Figuur 3 Impact van een verhoogde stollingsneiging op het vóórkomen van hart- en herseninfarcten onder jonge vrouwen

Elke punt in deze grafiek geeft de impact van een bepaalde mate van verhoogde stollingsneiging op het vóórkomen van hart- en herseninfarcten onder jonge vrouwen. De blauwe diagonaal geeft de plaats aan waar de verschillende punten zouden staan als een verhoogde stollingsneiging een even grote impact op beide ziekten zou hebben; de grafiek laat echter zien dat een verhoogde stollingfactor vooral een grote impact heeft op het herseninfarctrisico.

ernstige bloedingscomplicaties. Door deze verlaagde stollingsneiging zal ook de stollingsreactie bij vaatwandbeschadigingen minder uitgesproken zijn: antistollingsmedicatie zal dus het risico op een hartinfarct verlagen, maar wel met bloedingscomplicaties tot mogelijk gevolg. De uitdaging bij het doseren van antistollingsmedicatie is dan ook om de stollingsneiging van een persoon wel te verlagen, zonder een al te grote kans op bloedingscomplicaties. Nieuwe vormen van antistollingsmedicatie zullen daarom gericht zijn op het normaliseren van de stollingsneiging om ziekte te voorkomen en ondertussen de kans op bloedingen niet of nauwelijks te verhogen. Het is daarmee mogelijk dat deze nieuwe antistollingsmedicatie de kans op een hartinfarct iets verlaagt, maar vanwege de verschillen in causaal mechanisme is het waarschijnlijk dat deze nieuwe medicatie een sterk effect zal hebben op het herseninfarctrisico.

In verschillende hoofdstukken van dit proefschrift staat beschreven dat de gevonden risico's sterker waren bij vrouwen die de orale anticonceptiva gebruikten. Dit is op zich geen nieuw verschijnsel: van pilgebruik is bekend dat het het risico op trombose ziekten ongeveer twee- tot driemaal verhoogt. Deze nadelige risicoverhoging weegt in de praktijk niet op tegen de tegen het voordeel van pilgebruik, vooral omdat het basisrisico van deze ziekten onder jonge vrouwen bijzonder laag is (zo treedt een herseninfarct op bij 14 per 100.000 vrouwen per jaar). Maar verscheidene onderzoeken laten zien dat het effect van pilgebruik synergie vertoont met andere risicofactoren: de risicoverhoging bij vrouwen die zowel de pil gebruiken als zijn blootgesteld aan een bepaalde tromboserisicofactor is dan hoger dan verwacht mag worden op basis van de afzonderlijke effecten. Bijvoorbeeld, het herseninfarctrisico is bij vrouwen die alleen blootgesteld zijn aan pilgebruik ongeveer twee tot driemaal verhoogd, vrouwen blootgesteld aan hoge niveaus van geactiveerd kallikreine hebben een 5-voudige verhoging van het risico op een herseninfarct, terwijl vrouwen blootgesteld aan beide risicofactoren zijn bootgesteld aan een 23-keer verhoogd risico hebben. Met een dergelijke synergie rijst de vraag of het mogelijk is om met een nieuwe screeningsstrategie bij vrouwen die de pil willen gaan gebruiken die vrouwen te identificeren bij wie deze synergie kan gaan optreden. Echter, vanwege het lage vóórkomen van herseninfarcten bij jonge vrouwen leidt dit tot meer dan 15.000 te screenen vrouwen per jaar. Van deze vrouwen moet 10% vervolgens niet starten met het gebruiken van de pil om één herseninfarct te voorkomen. Kortom, de aanwezigheid van synergie tussen het effect van een bepaalde risicofactor en het effect van pilgebruik op het

risico van hart- en vaatziekten zal niet snel klinische consequenties hebben in de vorm van een screeningsstrategie. Desalniettemin is dergelijke kennis nuttig: het kan inzicht geven in een specifiek mechanisme dat belangrijk is bij het ontstaan van de onderzochte ziekten.

Samenvattend kan worden gesteld dat niet alle vormen van arteriële trombose eenzelfde ontstaansmechanisme hebben: bij jonge vrouwen blijkt een verhoogde stollingsneiging een oorzaak te zijn van herseninfarcten, maar geen effect te hebben op het hartinfarctrisico. Deze resultaten roepen direct een andere vraag op: hebben alle herseninfarcten wel eenzelfde ontstaansmechanisme? Het antwoord op deze vraag is waarschijnlijk nee. Herseninfarct is een zeer heterogeen ziektebeeld waarbij het zeer goed mogelijk is dat een verhoogde stollingsneiging vooral invloed heeft op het risico van bepaalde typen herseninfarcten. Dit kan dan, analoog aan de hartinfarct-herseninfarctdifferentiatie, gevolg hebben voor de effectiviteit van nieuw te ontwikkelen preventie- en behandelingsstrategieën. Nieuw onderzoek, met oog voor de verschillende typen herseninfarcten is nodig om meer inzicht te krijgen in deze mogelijke verschillen.

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Curriculum Vitae

Bob Siegerink was born on December 29 1983 in Deventer and graduated from S.G. de Waerdenborch, 'Voorbereidend Wetenschappelijk Onderwijs', in Holten in 2002. He started his studies of the Biomedical Sciences at the Leiden University Medical Center in 2002 were he quickly gained interest in epidemiology. During his studies he performed research projects at the department of Clinical Epidemiology (supervisor dr D. Grootendorst) and the department of Clinical Genetics & Medical Decision Making (supervisors prof. dr C.J. van Asperen / dr. C. Jacobi). During his studies he was involved in undergraduate teaching as a student assistant at the department of Medical Statistics and the department of Physiology.

He graduated in 2008 under supervision of prof. dr A. Algra with his Master thesis, titled “Risk factors of arterial thrombosis”. He remained at the department of Clinical Epidemiology and Einthoven Laboratory for Experimental Vascular Medicine as a PhD-fellow to continue his work on the RATIO case-control study under supervision of prof. dr A. Algra and prof. dr F.R. Rosendaal. For this, he collaborated with several research groups on different projects of which he presented the results on several (inter)national meetings. He received several awards such as the Science prize of the Dutch Society of Thrombosis and Haemostasis and an Erasmus grant for a 3 month visit to the Deutches Krebs Forschungs Zentrum in Heidelberg (Germany) as a visiting scholar to work on the KAROLA follow-up study (supervisors prof. dr H. Brenner / dr L. Breitling).

Bob followed several courses on Epidemiology taught by several epidemiologists (Vandenbroucke, Rosendaal, Hernan, Rothman, Szklo and diStavola), many focussed on causal inference. With this knowledge he also contributed to several other research projects outside the field of thrombosis as an epidemiology consultant.

During his PhD-fellowship his interest in teaching further developed; he was involved in several undergraduate courses both as a teaching assistant and as an instructor. Together with prof. dr F. W. Dekker he developed several extra-curricular courses which have become part of the honours program of the Leiden University Medical Center.

Bob Siegerink currently holds a position as a research fellow at the department of Clinical Epidemiology, focussing on cardiovascular epidemiology and remains involved in the development and teaching of several courses on epidemiology.

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