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# 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

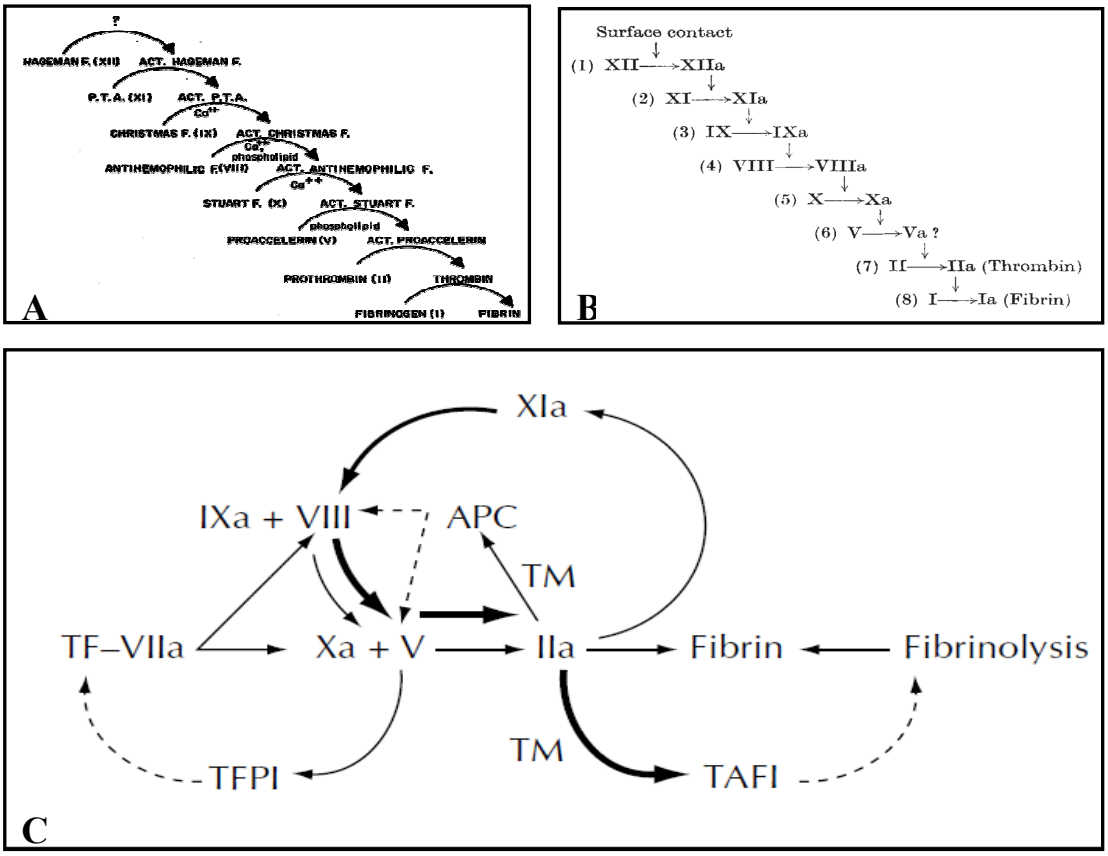
**Motivation** To investigate the role of thrombotic factors in the aetiology of both myocardial infarction and ischaemic stroke the studies presented in this thesis are embedded within the RATIO (Risk of Arterial Thrombosis In relation to Oral contraceptives) study.[23–25] There are several reasons 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**

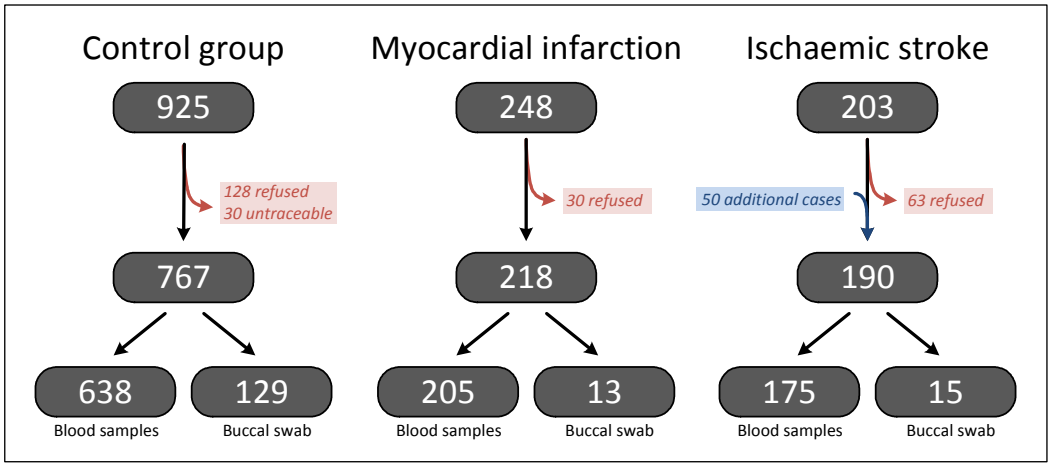



*Panel A (Davie and Ratnoff, Science 1964) and panel B (MacFarlane, Nature 1964) picture the waterfall or cascade model of coagulation which has long been the basis of thrombosis and haemostasis research. New insights lead to several additions and revisions. For example, Bouma and Meijers proposed a 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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