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

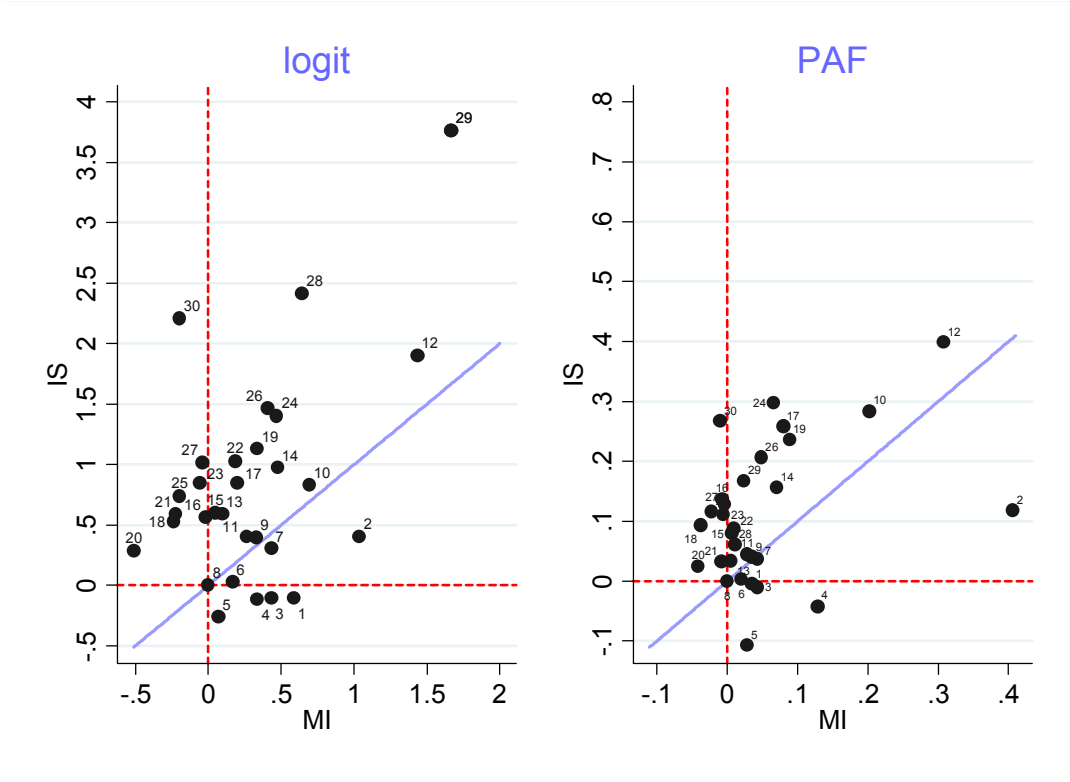
#	Thrombotic factor	ref	OR_{MI}	OR_{IS}	ROR	95%CI
1	Anticardiolipin antibodies, p95	[72]	1.80	0.90	0.50	0.17 - 1.45
2	hypofibrinolysis vs. normofibrinolysis	[78]	2.82	1.50	0.53	0.22 - 1.27
3	Prekallikrein:ag p90	[79]	1.54	0.90	0.58	0.23 - 1.52
4	F13A1 Pro564Leu, dominant	[73,74]	1.40	0.89	0.64	0.39 - 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.

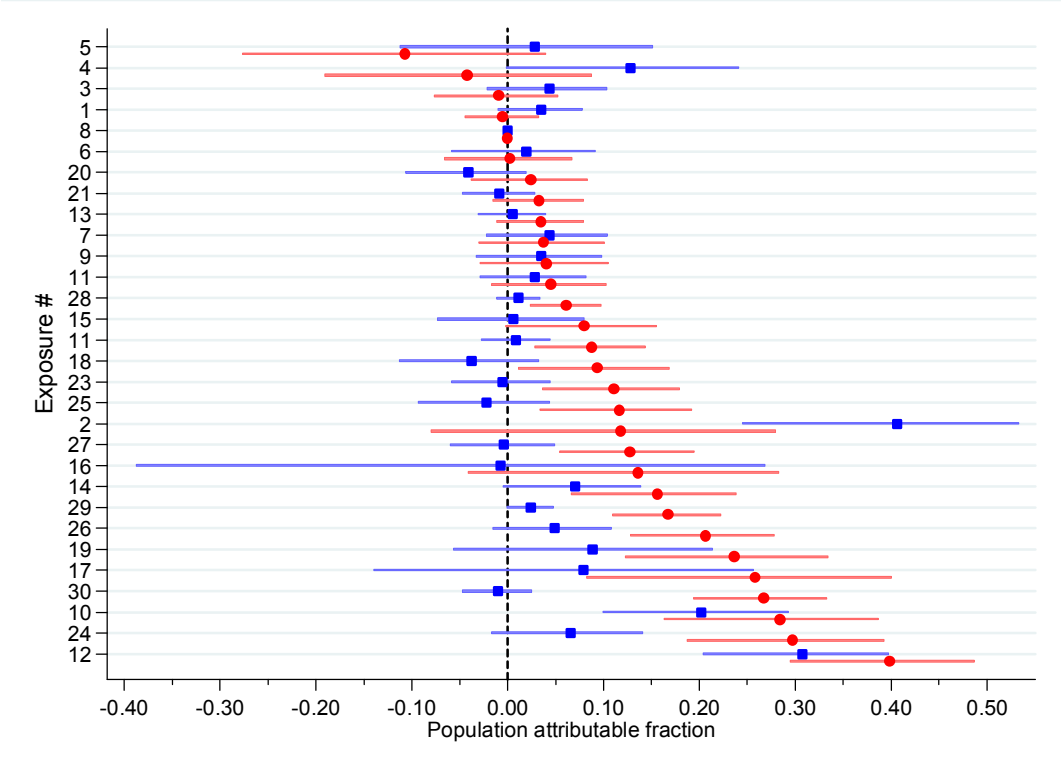
With the use of table 1 and the left panel of figure 1 the difference in effect on myocardial infarction and ischaemic stroke can be established for each prothrombotic factor. This kind of comparison is only possible when the same definition of exposure is used for the myocardial infarction analyses 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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