

The relevance of coagulation in cardiovascular disease: what do the biomarkers tell us?

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Summary

Several haemostatic factors have been associated with incident arterial cardiovascular disease in prospective studies and meta-analyses. Plasma fibrinogen shows a strong and consistent association with risk; however, this may reflect its inflammatory marker status, and causality remains to be proven. The common haemostatic gene polymorphisms for factor II, factor V and the von Willebrand factor: Factor VIII (non-O blood group) show significant associations with coronary

heart disease (CHD) risk, consistent with potential causality. Increased D-dimer and t-PA antigen levels are associated with CHD risk, suggesting roles for coagulation activation and endothelial disturbance. There is little evidence for associations with CVD with other haemostatic factors.

Keywords

Arterial thrombosis, epidemiological studies, coagulation factors

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Introduction

Much cardiovascular disease (CVD) morbidity and mortality is due to atherothrombosis, the main pathological process which underlies coronary heart disease (CHD), stroke and peripheral arterial disease (PAD). Over the past 50 years, increasing pathological, experimental, and clinical trial evidence supports the hypothesis that arterial thrombosis is "haemostasis in the wrong place" (1). More recently, prospective epidemiological studies of several circulating biomarkers of haemostasis, and also of inflammation which is associated with haemostasis and thrombosis both in the arterial wall and systemically (2), have shown associations with risk of a first, incident CVD or CHD event. These associations are summarised in ► Table 1.

Over the last 30 years, our coagulation laboratory has collaborated with prospective studies of CVD risk worldwide, assaying circulating biomarkers of haemostasis and inflammation in over 100,000 participants in over 20 studies. We have also participated in systematic reviews and meta-analyses of such studies. In this review, we discuss the most recent studies, and what they tell us about the contributions of haemostasis (especially coagulation) to arterial CVD. We do not discuss in detail their associations with venous thromboembolism (VTE), because it is generally accepted that all known coagulation proteases, are associated with risk of VTE (3).

Study design and analysis

Prospective studies of first arterial CVD events provide the most sound study design when assessing the associations of biomarkers with risk of CVD (4). Case-control studies are quicker and cheaper, can study many factors simultaneously, and require smaller sample sizes. However, they do not involve as accurate a time sequence, and do not capture first, fatal episodes of CVD. However, they have been valuable in studies of VTE, where first episodes have a lower mortality (5). Systematic reviews, including meta-analyses where appropriate, provide the most reliable estimates of the associations of biomarkers with risk of CVD.

Studies should address pre-analytical variables, including timing of blood sampling, sample handling, centrifugation and storage; and a sampling protocol which minimises both activation of haemostatic factors, and their degradation, *in vitro*. The European Concerted Action in Thrombosis and Disabilities (ECAT) manual contains useful advice (6). Citrated plasma is the preferred anticoagulant. Several haemostatic factors have been assayed in stored serum in some studies, including von Willebrand factor (VWF), tissue plasminogen activator (t-PA) and D-dimer antigens (7); however, plasma is preferred due to possible influence of clotting *in vitro*. Fibrinogen can also be assayed in dipotassium edetate (EDTA) anticoagulated samples by immunological methods (8). In a prospective study, there was longitudinal stability of fibri-

nogen, factor VII, protein C, protein S, D-dimer, t-PA and plasminogen activator inhibitor type 1 (PAI-1) levels when stored at -70°C over periods ranging from 7-59 months (9). Study design and data analysis are reviewed in detail in standard texts (10).

Inter- and intra-assay variability of assays should be considered in statistical analyses (6). Coagulation activation *in vitro* can result in individual high outlier results, especially for some coagulation activation markers such as fibrinopeptide A (FpA), prothrombin fragment F1+2, and thrombin-antithrombin (TAT) complexes (11). Variation in levels of coagulation activation markers between study centres may be seen in multicentre studies (e.g. [12]), presumably due to subtle variations in procedure between centres.

Finally, population studies of CVD utilise venous blood sampling and hence systemic levels of coagulation factors or activation markers. This may be insensitive to local activation of coagulation in arterial thrombosis, for example following arterial plaque rupture.

Using and interpreting associations of biomarkers with risk of CVD

Associations may be of clinical use in prediction of CVD events. For example, a persistently elevated level of D-dimer has been shown to be associated with an increased risk of recurrent VTE (13), and proposed as part of a risk factor score for evaluating continued anticoagulant use (14). When using biomarkers for risk prediction, we seek complementary information to clinical risk data. However, to date the additional clinical value of adding haemostatic or inflammatory variables to standard risk scores for predicting risk of arterial CVD appears small, even for the most studied variables, fibrinogen and C-reactive protein (CRP) (15). It is possible that future studies using multiple biomarkers may increase their predictive value (16).

These associations might also indicate a potential pathophysiological role for haemostatic or inflammatory biomarkers in CVD. However, even after adjustment for CVD risk factors, the associations of biomarkers with CVD risk may reflect residual confounding by other CVD risk factors which are not measured routinely, such as social deprivation (17). In the Scottish Heart Health Study, social deprivation increased both CVD risk and fibrinogen levels independently of smoking; and fibrinogen level did not increase CVD risk prediction independently of smoking, social deprivation and other risk factors (17).

Furthermore, such associations might also reflect asymptomatic arterial disease, so that biomarkers are markers of disease. Proof of causality therefore remains to be established by randomised controlled trials of lowering the levels of individual biomarkers, as has been established for blood pressure and cholesterol. This is rarely possible, but an alternative approach is Mendelian randomisation studies. These use the random allocation at conception of different functional genotypes, which are common in the general population, and which confer a lifelong difference in mean circulating levels of a phenotype. The demonstration of an association between functional genotypes and CVD risk (which should not be confounded by environmental factors) pro-

Table 1: Summary of associations of haemostatic factors and risk of coronary heart disease in recent meta-analyses of prospective studies in general populations (or genetic studies). Odds ratios (95%CI) for CHD are shown per 1 SD higher baseline level of circulating haemostatic factors (following adjustment for age, sex and conventional CHD risk factors); and per-allele CHD risk for genetic analyses. Full details of the numerous individual study results are available in the references in the right-hand column.

Factor	OR (95%CI)	Cases (n)	Studies (n)	Reference
Fibrinogen	1.78 (1.69–1.86)	7,213	31	18
II G20120A	1.31 (1.12–1.52)	11,625	40	39
F V Leiden (V G1691A)	1.17 (1.08–1.28)	15,704	60	39
VWF antigen	1.16 (1.10–1.22)	6,556	15	7
D-dimer	1.23 (1.16–1.32)	6,799	18	7
t-PA antigen	1.13 (1.06–1.21)	5,494	13	7

vides provisional evidence for a causal relationship of the phenotype to CVD.

Fibrinogen and other inflammatory markers

Fibrinogen is the major circulating clotting factor by mass, and may potentially promote atherothrombosis by several mechanisms. It infiltrates the arterial wall, is an important cofactor in platelet aggregation, the precursor of fibrin, and a major determinant of plasma and blood viscosity. Plasma fibrinogen has been associated with CVD risk in many prospective studies. The Fibrinogen Studies Collaboration meta-analysis (18) observed that an increase of one standard deviation (0.65 g/l) is associated with an odds ratio (OR) for CHD of 1.78 (95% confidence interval [CI] 1.69–1.86); for stroke of 1.60 (1.48–1.73), for other vascular events of 1.93 (1.71–2.19) and for non-vascular mortality of 1.58 (1.52–1.66) (► Table 1). Some prospective studies have also reported significant associations of plasma fibrinogen level with incident PAD (19, 20).

Plasma fibrinogen levels are associated with several CVD risk factors, and also with blood levels of other inflammatory markers including plasma viscosity, erythrocyte sedimentation rate (ESR), white cell count, albumin (low) and C-reactive protein (CRP) (21), each of which has also been associated with risk of CHD or CVD in meta-analyses of prospective studies (22, 23). The Emerging Risk Factors Collaboration recently studied the potential clinical use of assessing fibrinogen, CRP, white cell count or albumin in prediction of first CVD events (15). Each of these inflammatory markers showed similar, small improvements in reclassification of estimated risk. The C-index quantifies the degree to which the addition of a marker to a standard cardiovascular score including classical risk factors can predict the order of CVD events. Addition of fibrinogen increased the C-index by 0.0027. The net reclassification index (NRI) quantifies the resulting improvement in the

predicted 10-year risk categories of low (<10%), intermediate (10–20%) and high (>20%); addition of fibrinogen increased the NRI by 0.83%. From this it was estimated that among 100,000 adults aged 40 years or over, addition of fibrinogen or CRP in those at intermediate risk (for whom statin therapy is recommended in several guidelines) could help prevent about 30 additional events over 10 years. The combination of fibrinogen and CRP did not increase risk prediction. Interestingly, fibrinogen or CRP improved CVD risk prediction in men, but not women. Limitations of this analysis include the non-measurement in most studies of other emerging CVD risk factors such as measures of social deprivation, which are associated with fibrinogen and other inflammatory markers, as well as with CVD risk (17). Hence, assaying fibrinogen does not add to CVD prediction beyond risk scores which include measures of social deprivation, such as those currently used in the United Kingdom (17).

Because the association of plasma fibrinogen with risk of CVD appears similar to that of other circulating inflammatory markers, it may simply reflect its inflammatory response behaviour, rather than a causal role in atherothrombosis. Drugs which lower plasma fibrinogen levels (such as some fibrates and ticlopidine) also reduce CVD risk; however they may lower risk by other mechanisms (such as lipid reduction and platelet aggregation inhibition). Selective reduction in plasma fibrinogen by defibrinogenating enzymes

(ancrod, batroxobin) reduces risk of postoperative VTE (24), but has shown inconclusive results in acute ischaemic stroke and increases the risk of bleeding (24, 25). Mendelian randomisation studies of functional genotypes for plasma fibrinogen have generally shown no association with CVD, including a recent large meta-analysis (26). Hence, the causality of plasma fibrinogen level in CVD remains to be proven.

Nevertheless, the role of fibrinogen and fibrin in arterial CVD remains under active investigation. Infusion of human fibrinogen in mice reduced time to occlusion for both FeCl₃-induced carotid artery and saphenous vein thrombosis, and increased clot fibrin content, clot elastic strength, network density, and resistance of the clot to lysis (27). Hence, acute elevation of fibrinogen level might increase risk of thrombosis, which would be consistent with the reductions in both postoperative VTE and acute stroke brain ischaemia in randomised controlled trials of ancrod (24). There is also current interest in fibrinogen variants, such as fibrinogen γ' , a variant which arises from altered splicing of the γ' -chain mRNA. Increased γ' fibrinogen levels have been associated with increased risks of CHD and stroke, but paradoxically with decreased risk of VTE (for review, see [28]). Finally, fibrin clot structure has been associated with increased risks of both arterial CVD and VTE, as recently reviewed by Ariens ([28]; and in this issue).

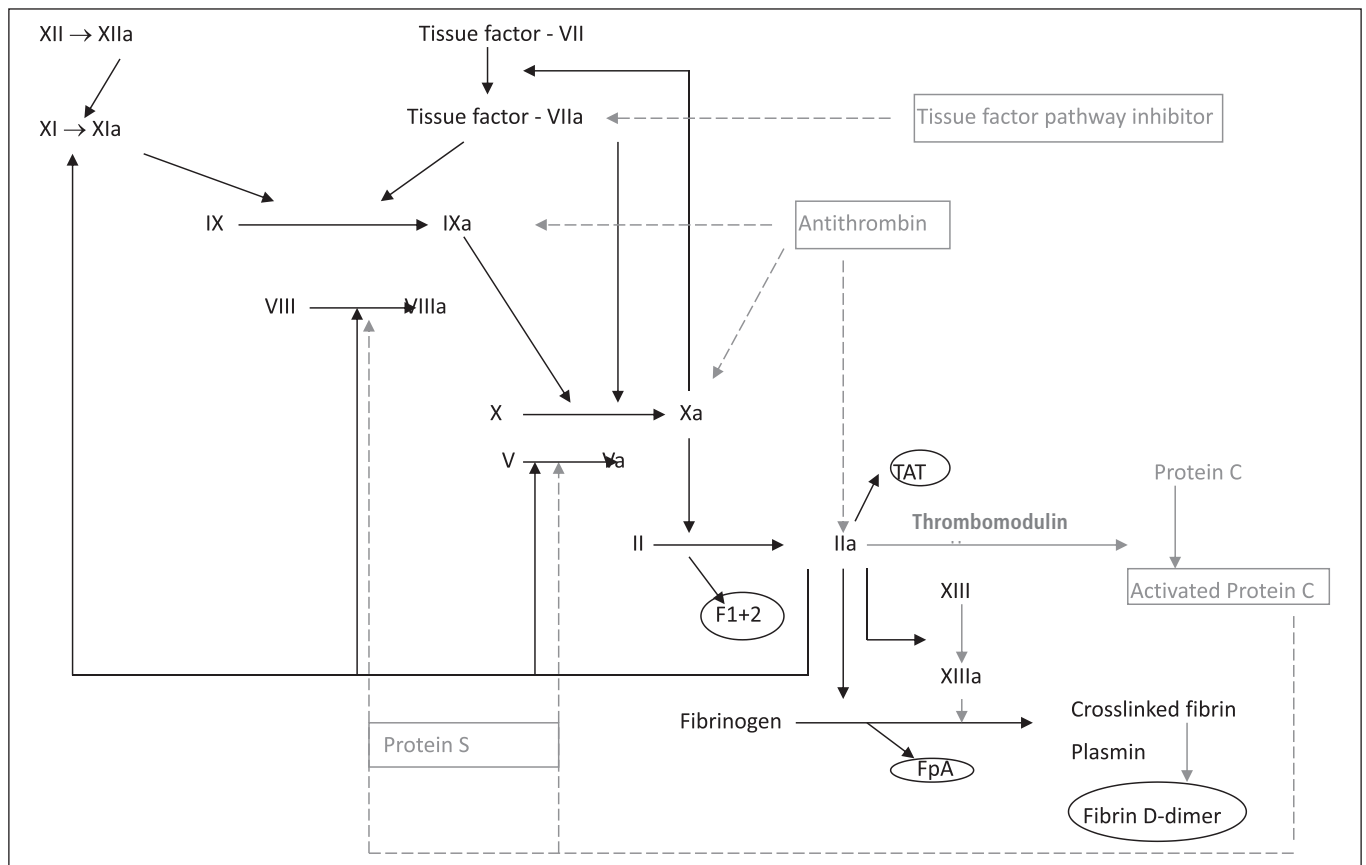


Figure 1: Outline of blood coagulation factors, inhibitors and activation markers. Inhibitory pathways are indicated in grey boxes and broken lines. Activation markers are indicated in ovals.

Recent evidence suggests that the associations of fibrinogen and other „downstream“ inflammatory markers with arterial CVD may result from upregulation of „upstream“ promoters of inflammation, notably the key proinflammatory cytokine, interleukin-6 (IL-6). These cytokines promote activation of inflammation, hence are “upstream” of “downstream” markers of activated inflammation, such as fibrinogen, CRP and white cell count. Several prospective studies and a recent updated meta-analysis (29) have shown associations of circulating IL-6 levels with CHD, and in the Caerphilly prospective study this association was calculated to explain the associations with CHD of several downstream inflammatory markers known to be regulated by IL-6, including fibrinogen, CRP, plasma viscosity and white cell count (30). A potential causal role for IL-6 level is suggested by a recent meta-analysis of general population studies, in which not only IL-6 levels, but also an associated functional mutation in the IL-6 receptor gene (rs 8192284), which affects circulating IL-6 levels, were associated with CHD risk (31). This positive Mendelian randomisation study contrasts with the lack of association of functional genotypes for fibrinogen (26) or for CRP (32) with risk of CVD. The potential causal role for IL-6 in arterial CVD could be further investigated by studies of IL-6 antagonists (31). These might be particularly appropriate in high-risk persons such as those with diabetes, in whom a recent large study suggests that IL-6 levels were independently associated with macrovascular complications, in contrast to fibrinogen or CRP levels (33).

Factor II, factor V, factor X, and APC resistance

There are few reported prospective studies of circulating levels of factors II (prothrombin), V or X and risk of arterial CVD. The ARIC study observed no associations after adjustment for major risk factors (34). The prothrombin G20120A mutation and factor V G1691A (Leiden) mutation are each associated with the phenotype of activated protein C (APC) resistance. In prospective population studies, the risk of VTE is increased in heterozygotes about two- to four-fold (35, 36). In case-control studies, the combination of both mutations increases the risk of VTE about 20-fold (37), perhaps due to a supra-additive effect on APC resistance. Meta-analyses have shown that each mutation also increases the risks of CHD and stroke (38, 39), although the relative risk is an order of magnitude lower than the relative risk of VTE (about 1.3 compared to about 3). The combination of both mutations increases the risk of CHD about six-fold (35). Two prospective studies report associations of the APC resistance phenotype with risk of CHD (40) or stroke (41); however another study reported no association with CHD (42).

Tissue factor (factor III)

Experimental studies suggest that tissue factor-driven thrombin generation and inflammation may be important in atherothrom-

bosis (43). Measurement of circulating levels of tissue factor in humans are problematic. However, a recent genome-wide association study of circulating fibrin D-dimer levels, which are associated with increased risk of CHD (7), identified a tissue factor locus as a genetic determinant of D-dimer levels (44). This is consistent with the hypothesis that tissue factor expression may be one determinant of thrombin and fibrin turnover in man. The association of tissue factor related genotypes with arterial CVD would therefore be of interest.

Factor VII

The first Northwick Park Heart Study reported an association between FVIIc levels and fatal CHD, which persisted after 30 years of follow-up (45). However, the second Northwick Park Study did not find this association (46) and other prospective studies have not shown significant associations between VIIc levels and incident CHD, stroke or PAD (20, 40, 42, 47–53). Heterogeneity of results may result partly from differences in FVII assays (54). A meta-analysis of the functional 10976A mutation in the FVII gene showed no significant association with CHD risk (39), which is consistent with the overall results of prospective studies of VII levels, and is evidence against a causal role for FVII in CHD. FVII levels do not appear to be associated with risk of VTE (3).

Factor VIII : VWF complex

Several prospective studies have reported associations of VWF antigen (VWF:Ag) levels with risk of first CHD event. A recent updated meta-analysis reported an adjusted OR for CHD, for a 1 standard deviation increase in VWF level, of 1.16 (95% CI 1.10, 1.22) (7) (► Table 1). Other studies have reported associations of VWF:Ag with incident stroke (52, 55, 56) or PAD (20). VWF levels can also be assayed as ristocetin-induced platelet cofactor (VWF:RiCof); and VWF is the carrier protein for factor VIII in the circulation. In the Caerphilly Heart Study, we assayed VWF:Ag, VWF:RiCof and FVIIIc (57). All three assays were closely correlated, and all showed significant associations with risk of CHD. Other reports of FVIIIc have confirmed associations with incident CHD (40, 58, 59) or stroke (40); however, at present there is insufficient data on FVIIIc for meta-analysis.

These data suggest that increased circulating levels of the VWF:FVIII complex are associated with increased risk of arterial CVD; as they are with risk of VTE (60). The strength of association is small, hence levels are unlikely to be clinically useful in risk prediction. However, the association suggests potential pathophysiological significance for the VWF:VIII complex in arterial CVD, either as a marker of endothelial damage (VWF:Ag), promotion of platelet adhesion and aggregation at sites of arterial injury (VWF:RiCof), or thrombin generation (VIIIc) (57). The dose-dependent association of haemophilia A with decreased risk of CHD (61,62) constitutes Mendelian randomisation evidence for potential causality of VIIIc levels. Likewise, the reduced risk of

CHD in persons with von Willebrand's disease, who have low VWF levels or function (63) constitutes Mendelian randomisation evidence for potential causality of VWF levels. Because these bleeding disorders have little or no protective effect on atherosclerosis (64), it is likely that the reduced risk of CHD is mediated by lower thrombotic tendency.

At the general population level, the major genetic influence on VWF and VIIIc levels is non-O ABO blood group. Approximately half of Western populations have non-O blood group, and approximately 30% higher mean circulating levels of VWF and VIIIc. This is due to decreased clearance of circulating VWF, on which non-O proteoglycans are expressed, which reduce clearance of VWF from the circulation by the reticulo-endothelial system (60). Several studies and meta-analyses (65–67) have reported associations between non-O blood group and risk of CHD, stroke, PAD or VTE. In the most recent meta-analyses, the association was strongest for VTE, with an OR of 2.09 (95% CI 1.83, 2.38) (66). The association was weaker for CHD, with an OR of 1.28 (1.17–1.40); and for stroke, with an OR of 1.17 (1.01–1.35) (67). While these findings again constitute Mendelian randomisation evidence for potential causality of VWF (and VIII), other genetic determinants of VWF level have not been associated with increased risks of CHD (68) or stroke (69), possibly due to their weaker effects on circulating VWF levels.

Factor IX

As discussed above for factor VIII, the lower risk of CHD in patients and carriers of haemophilias (61, 62) is consistent with a potential causal role for FIX in CHD. Three recent prospective studies report associations between circulating FIX levels and risk of CHD (34, 42, 70) although these became statistically non-significant after adjustment for classical risk factors. Results from two case-control studies are conflicting (71, 72). Further studies are required.

Factors X, XI, XII and activated partial thromboplastin time (aPTT)

There are few studies of the associations of CHD with FXI levels (34, 72–74) or FXII levels (34, 51, 72). Homozygosity of the T allele of the 46C-T polymorphism in the F12 gene has been associated with risk of CHD (75) and of ischaemic stroke (76). A recent genome-wide association study of the aPTT (77) identified associations with F11, F12, KNG1, HRG and ABO genes, which collectively account for about 29% of variance in aPTT. Of these, two (the proxy SNP for O blood group, and the PROCRA/EDM2 locus) were associated with coronary artery disease, indicating a potential role for intrinsic system factors in CHD. In the endoplasmic reticulum (ER), misfolded proteins are retrotranslocated to the cytosol and degraded by the proteasome in a process known as ER-associated degradation (ERAD). EDM2 belongs to a family of proteins involved in ERAD of glycoproteins.

Factor XIII

A recent meta-analysis of 5,346 CHD cases and 7,053 controls (78) showed a significant inverse association with the Val 34 Leu SNP (OR 0.82, 95% CI 0.73–0.94). A similar association was reported for VTE risk (79). The mechanisms, and relationships to circulating XIII levels, are unclear.

Coagulation inhibitors

Overall there is little evidence that low levels of antithrombin, protein C or protein S increase the risk of arterial disease (80). This reflects both the lack of prospective studies, and the rarity of major deficiencies of these proteins.

Coagulation activation markers

Fibrin D-dimer, a marker of fibrin formation and lysis, is the most widely used circulating marker of activated coagulation. It is used routinely in diagnosis of clinically suspected VTE; and has been advocated in assessment of risk of recurrent VTE (14). Several prospective studies have reported associations of D-dimer levels with risk of CHD. In the most recent meta-analysis (7), the adjusted OR for CHD for a one standard deviation increase in D-dimer levels was 1.23 (95% CI 1.16–1.32). Interestingly, D-dimer levels were inversely associated with several conventional risk factors for CHD; and also inversely associated with t-PA antigen levels (see next section). This inverse association could arise because t-PA antigen levels largely reflect circulating t-PA/PAI-1 complexes, and higher PAI-1 levels reduce endogenous fibrinolysis and hence D-dimer levels. Some prospective studies have also associated D-dimer levels with increased risk of stroke (56, 81) and PAD (81). A systematic review has also associated D-dimer levels with increased risk of arterial thrombotic events in patients with PAD (82).

As with VWF levels, the associations of D-dimer with risk of CVD in healthy persons appear small, hence assay of levels appears unlikely to add significantly to clinical risk prediction. However, the associations are consistent with a potential role for coagulation activation in pathogenesis of arterial CVD. In a randomised controlled trial of low-dose warfarin in prevention of CHD, the efficacy of warfarin was associated with intensity of the INR, which in turn was associated with reducing plasma levels of D-dimer (83). The association of D-dimer levels with risk of first VTE (3) as well as recurrent VTE (13) suggests that D-dimer level may be a marker of both arterial and venous thrombogenicity.

The determinants of high D-dimer levels in the general population are unclear. Genetic factors include tissue factor related genes (44). Because D-dimer levels are influenced by fibrinolysis as well as activated coagulation, the associations of other, more selective coagulation activation markers with arterial CVD should be studied. There are limited reports for FpA, F1+2 and TAT complexes (11, 20, 40, 46, 47, 70, 84). In the largest study, we have re-

cently reported that levels of F1+2 and TAT complexes, as well as D-dimer, were associated with risk of CHD and stroke (70). There are also some reports on contact activation markers and CVD risk (85, 86).

Markers of fibrinolysis

The ARIC study observed no association between circulating levels of plasminogen or alpha-2-antiplasmin with arterial CVD (34).

Several prospective studies have reported associations of increased circulating levels of tissue plasminogen activator (t-PA) antigen with incident CHD events. In the most recent updated meta-analysis (7), the adjusted OR for CHD for a one standard deviation increase in t-PA antigen levels was 1.13 (95% CI 1.06–1.21) (► Table 1). Some prospective studies have also associated t-PA antigen levels with increased risk of stroke (56).

As discussed above, t-PA antigen levels largely reflect circulating t-PA/PAI-1 complexes, hence the associations of t-PA antigen with risk of arterial CVD events might reflect associations with PAI-1 levels. However, meta-analysis of the limited data (833 cases) on circulating PAI-1 levels and CHD risk in general populations showed little association: OR for top third compared to bottom third 0.98 (95% CI 0.53–1.81) (87). This is consistent with the weak association with CHD of a functional SNP for PAI-1 (-675G/5G) in a meta-analysis (39). Further prospective studies of PAI-1 are required. In addition, further prospective studies are required to determine whether reduction in PAI-1 levels increases endogenous fibrinolysis, as measured for example by increase in circulating levels of fibrin D-dimer (see previous section).

Global fibrinolytic activity was associated with CVD risk in the first NPHS (88).

Platelet markers

Neither platelet count, platelet aggregability (89–91), nor three functional polymorphisms for platelet aggregation (39) were associated with risk for CHD in prospective studies. A recent large study reports an association of platelet count with nonvascular mortality (92), which is also associated with other inflammatory markers including fibrinogen and CRP (15, 18). A recent meta-analysis of mean platelet volume (MPV) reports an association with myocardial infarction (93).

Conclusions

Plasma fibrinogen levels show the strongest and most consistent associations of any haemostatic factor with incident CHD and arterial CVD; however Mendelian randomisation studies do not support causality, and there is limited evidence that lowering levels reduces CVD risk. Studies of other inflammatory factors show similar associations with CVD risk, which might result from mutual associations with upregulation of pro-inflammatory cytokines

such as IL-6. However, further studies of fibrinogen variants, and of fibrin clot structure, are continuing.

The common haemostatic gene polymorphisms for factors II (G20120A), V (G1691A) and the VWF:FVIII complex (non-O blood group) show significant, but smaller associations with CHD risk, consistent with causal roles for these factors. VWF levels are also associated with CHD risk, and arterial thrombotic risk is lower in haemophilias and von Willebrand disease.

Increased D-dimer and t-PA antigen levels are associated with increased CHD risk, perhaps reflecting roles for, respectively, coagulation activation and endothelial disturbance in CHD.

At present, there is little evidence for association of platelet tests, or genotypes, with CHD.

In summary, what do those systematic reviews of prospective studies of circulating biomarkers tell us? Their results support a role for activation of blood coagulation in pathogenesis of CHD and stroke. Increased risk of arterial thrombosis appears the most likely mechanism, which would be consistent with the efficacy of antithrombotic drugs in primary and secondary prevention. There are two major applications of this information. First, future studies using multiple biomarkers may increase the accuracy of clinical risk prediction (16). For coagulation biomarkers, circulating levels of fibrinogen, VWF, D-dimer and t-PA antigen levels; and the genetic factors G20120A, G1691A, and non-O blood group; merit consideration. Second, these associations suggest possible targets for new antithrombotic drugs, such as reduction in fibrinogen, the F VIII:VWF complex, and D-dimer as a nonspecific marker of activated coagulation.

Conflicts of interest

None declared.

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