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Epidemiology of arterial thrombosis
Gene-environment interaction, risk factors for coronary heart disease

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Coronary Heart Disease is a complex multifactorial disorder, and in recent years it has become clear that as well as plasma lipids, elevated levels of certain proteins within the coagulation pathway contribute independently to IHD risk, for example, high plasma levels of factor VII, PAI-1 and fibrinogen. Recently, assays have become available to examine the importance of other coagulation factors, and levels of activated factor XII (FXIIa) have been identified as a potential risk factor. For all of these proteins, the genes coding them have been studied and functional polymorphisms detected that are associated with plasma levels and in some cases with risk of IHD. In this review we focus on work from our laboratory that has explored these genetic determinants and in particular the way genetic and environmental factors interact.

Επιδημιολογία της αρτηριακής θρόμβωσης. Αλληλεπίδραση γονιδίων-περιβάλλοντος, παράγοντες κινδύνου για στεφανιαία νόσο

Περίληψη στο τέλος του άρθρου

Key words

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Factor XII
Fibrinogen
Genetic polymorphism
PAI-1

1. INTRODUCTION

Although the risk factors for ischemic heart disease (IHD) have not yet been fully defined, studies have shown that factors such as smoking, diet, diabetes mellitus, hypertension, dyslipidaemia and gender are all associated with an increased risk.[1] The Northwick Park Heart Study was one of the first to investigate the role of the coagulation pathway in this area,[2] but now many other studies have confirmed the importance of the coagulation system in IHD.[3,8] The best established predictor of risk is higher levels of plasma fibrinogen, but elevated factor VII (FVII) and plasminogen activator inhibitor-1 (PAI-1) have also been implicated.

The critical role of genes is in coding for structural proteins and enzymes which enable the cell, organ or organism to maintain homeostasis in the face of the environmental challenges experienced. Within a population, genetic variation will mean that individuals will have different ability to maintain homeostasis when faced with a specific environmental challenge. The clinical features of any disorder with a late age of onset can therefore be thought of as being caused by the failure of the individual to maintain homeostasis, and this is particularly true for the disorder of IHD. Thus, for an individual in the general population, the level of a coagulation risk factor in the blood such as fibrinogen or FXIIa is due to the individual's genetically-determined ability to maintain homeostasis in response to the environment being experienced. The current epidemic of IHD, being seen in Western societies, is thus mainly due to an in-

ability, in some individuals, to maintain optimum blood levels of these risk-factor components, in the light of the environment experienced as a result of "affluent" lifestyle changes, such as changes in dietary fat intake and in the proportion of individual's smoking cigarettes.

In this review we will illustrate these points briefly for FVII and PAI-1 and focus mainly on fibrinogen and FXII.

Factor VII (FVII) is a vitamin K-dependent protein, that is important in the initiation of tissue factor-induced coagulation.[9] In the presence of tissue factor, native FVII is converted to its activated form, FVIIa. The tissue factor-FVIIa complex cleaves the factors IX and X to their active forms and causes thrombin generation and fibrin clot production. In prospective studies in middle aged men, FVII coagulant activity (FVIIc) has been reported in some studies as a risk factor for ischemic heart disease.[2,4] FVIIc levels, in normal individuals, are influenced by several environmental factors, including dietary fat intake, increasing age, obesity, diabetes, plasma triglyceride levels, fasting insulin levels and menopause.[2,4,5,10]

Several genetic polymorphisms of the FVII gene have been reported that are strongly associated with plasma FVIIc levels. Firstly, a substitution of the arginine residue at position 353 by glutamine (designed R353Q) was described.[11] Individuals who carry a Q allele have 20–25% lower levels of FVIIc and FVII antigen (FVIIag). Subsequently, an insertion of a decanucleotide (CCTATATCCT) in the promoter region (-322) of the FVII gene was described and also shown to be associated with lower levels of plasma FVIIc levels in healthy individuals.[12,13] There is strong allelic association between the two polymorphisms, with the 10 bp promoter insertion and the Q353 polymorphism almost always occurring together.[13] There is some data to suggest that the Q353 allele is protective from IHD.[14]

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of both tissue- (t-PA) and urokinase type plasminogen activator.[15] Deficient fibrinolytic activity, resulting from increased plasma PAI-1 levels, might predispose to thrombotic events.[6] PAI-1 is also implicated in IHD; patients with angina pectoris or those with a history of myocardial infarction have higher PAI-1 activity.[8] A strong positive correlation has been observed between plasma PAI-1 levels and BMI, and WHR, and with fasting insulin levels and VLDL-Tg, all these factors being related to the insulin resistance syndrome.[18,24]

There is accumulating evidence for the genetic control of circulating PAI-1. A common PAI-1 polymorphism is the insertion (5G) or deletion (4G) of a G at position -675 of the promoter region.[18,19] Studies with the 4G/5G polymorphism have shown higher plasma PAI-1 levels in 4G/4G subjects, in MI patients, non-insulin-dependent diabetics than in healthy control subjects.[47,50]

2. FIBRINOGEN

The fibrinogen molecule comprises of two subunits, each composed of three polypeptide chains (Aα, Bβ and γ). Factors associated with high fibrinogen levels include obesity, diabetes, age and smoking.[2,4,10] Fibrinogen is an acute phase protein,[25] and its plasma level is raised following infection or injury, and because of its sensitivity to environmental factors, the within-individual variation of fibrinogen levels is high. The rate-limiting step in the production of fibrinogen is the Bβ-chain synthesis,[26] that is responsive to cytokines, mainly IL-6.[27] Several prospective studies have shown a direct association between plasma fibrinogen concentration and the subsequent incidence of arterial thrombosis as revealed by MI.[2,4] In men in the Northwick Park Heart Study (NPHS), an elevation of one standard deviation in fibrinogen (about 0.6 g/L) was associated with an 84% increase in the risk of cardiovascular disease within the next 5 years.[2]

The extent to which genetic factors may determine plasma fibrinogen level is unclear, and published estimates range between 0.5–0.30.[28,29] Recently, complex segregation analysis has suggested that there is a major gene determining fibrinogen levels but with strong evidence for environmental factors such as age and gender interacting with the major gene effect in determining levels.[30] The obvious candidate for this major gene is the fibrinogen gene cluster itself, but no studies have yet addressed this possibility, and other genes may also be important.

3. VARIABILITY AT THE FIBRINOGEN LOCUS AND PLASMA FIBRINOGEN LEVELS

The three fibrinogen genes are in a cluster of less than 50 kb on the long arm of chromosome 3, each chain being synthesized as a separate mRNA, with the levels of all three mRNAs being co-ordinately controlled. The rate-limiting step in the production of the mature fibrinogen molecule in the human hepatoma cell-line HepG2 is the synthesis of the Bβ-polypeptide chain,[26] which in turn is influenced by the amount of its mRNA available. It is therefore likely that an alteration in the level of synthesis of the Bβ-chain may have an effect on

the amount of fibrinogen secreted by the liver. The structure of the beta-gene promoter has been well studied, and the region from -150 base pairs to the start of transcription has homology with other acute phase genes such as alpha-1-antitrypsin. This region has been reported to contain all the information required to act as promoter in HepG2 cells and has been shown to bind proteins from a HepG2 cell nuclear extract.[27]

The sequence from -89 to -76 contains a conserved liver-specific transcription element which binds hepatic nuclear factor I (HNFI), and deletion mapping shows that just upstream lies an interleukin 6 (IL-6) responsive element, which has been identified in other genes as the motif CTGGGA.[27] It is therefore possible that sequence changes in this region of the gene may have a direct effect on the rate of transcription and thus on plasma fibrinogen levels.

In studies of the ß-fibrinogen promoter, we detected a common G/A sequence variation at position -455, with the A being present in roughly 20% of alleles examined.[31] In more than eight independent studies from five different laboratories and with samples of more than 5,000 healthy individuals the A-455 allele has been consistently associated with higher fibrinogen levels,[32,36] with those with one or more copies of the A-455 allele having on average 0.28g/L higher fibrinogen levels than those with the genotype G/G (weighted average in healthy men). The magnitude of this genotype effect indicates that it is likely to be of biological significance in causing an elevated risk of thrombosis, and by extrapolation from the prospective data[2] of the relationship between fibrinogen and IHD risk (0.6 g/L associated with 84% greater risk), men with the A allele would be at 40% higher risk of a thrombotic event; this estimate is based on healthy middle-aged men from northern London, and may not be the same in other groups.

Several studies have reported that the A-raising effect on fibrinogen levels is greater in subjects with IHD than in healthy subjects.[31,34,36] Data from the Copenhagen City Heart Study[36] is presented in figure 1, and it can be seen that the A-raising effect was roughly threefold greater in women and twofold greater in men with IHD compared to those without, with the interaction between genotype and disease status being statistically significant in women (P=0.01). It has been proposed that this effect is due to the increased cytokine levels in patients with IHD,[37] with coronary artery disease acting as a persistent "acute phase" situation.

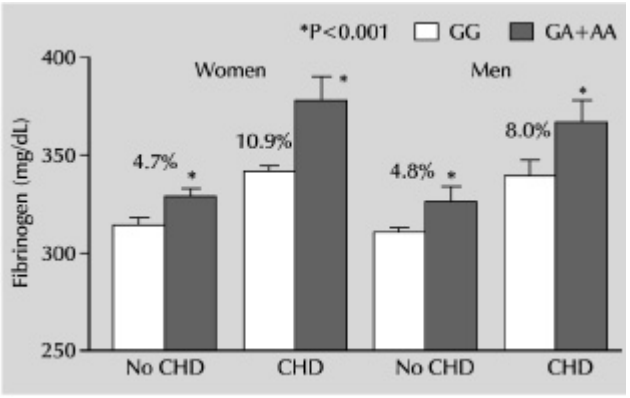

Figure 1. Histogram showing the relationship between G-445A genotype and plasma fibrinogen levels in men and in women with and without coronary heart disease (CHD) in the Copenhagen City Heart Study.[36]

Although the A-455 sequence is outside the region of the reported promoter sequence, it is possible that it is having a direct effect on transcription and preliminary studies have demonstrated binding of a hepatic nuclear protein to the G but not the A sequence.[38] However recently it has been found that the G-455 to A sequence change is in complete allelic association in all Caucasian populations studied to date with a C-148-T change located close to the consensus sequence of the IL6 element.[27] This raises the possibility that the G-A change is acting as a neutral marker for the C-T change, which is the functional change working through effects on transcription of the ß-fibrinogen gene that are mediated by IL6, i.e., the T-148 sequence close to the IL6-responsive element may increase the affinity of NF-IL6, leading to enhanced transcription of the ß-gene. Experiments are in progress to insert this fragment of the gene into the appropriate vector to test this hypothesis.

4. ACUTE PHASE STIMULATION OF FIBRINOGEN BY EXERCISE

It is possible that in the general population, some individuals may have a "plastic" genotype that responds easily to environmental factors, and that such individuals would experience a greater than average increase in plasma fibrinogen levels in response to a moderate environmental stimulus. These individuals would then be at greater than average risk of a thrombotic event at the peak of fibrinogen levels, and thus have a greater chance of developing IHD, and particularly myocardial infarction (MI). To investigate this inflammatory effect we have examined the impact of acute intensive exercise on plasma fibrinogen levels and the relationship of these

responses to G-455A genotype.[39] As expected, the fibrinogen-raising effect associated with the A allele was seen at baseline, but the effect was modest and non-significant in this small sample. However, the degree of rise at 2–3 days after an acute period of intense military exercise was strongly related to the presence of the A allele, with GG men showing an average 27% rise from baseline, the GA men a 37% rise, while the men homozygous for the A allele (representing 4% of the population based on the observed allele frequencies) had fibrinogen levels which by day 2 had risen by more than 100% compared to their untrained levels, and which were amongst the highest levels in the whole sample. These findings support an association of the A allele with greater fibrinogen level responsiveness after a cytokine-inducing event.

To investigate this further we have examined the impact of the -455G>A polymorphism on the rise in fibrinogen levels seen following cardiac surgery.[40] Sixty patients (mean age 61 years) undergoing cardiothoracic surgery for coronary bypass grafting were enrolled in the study. Blood was taken for analysis of plasma fibrinogen levels immediately prior to surgery and at 3, 12, 24, 60 and 120 hours post surgery. Fibrinogen levels rose significantly after cardiothoracic surgery, with peak levels achieved 60 hours post surgery. There were no significant differences between genotype groups in any baseline characteristic including gender, smoking status, type of disease or fibrinogen levels (GG group 3.01 g/dL compared to GA+AA 3.02 g/dL; P=ns). However, as shown in figure 2, there was a marked difference in the degree of rise in fibrinogen levels in carriers of the A allele compared with those without (375±13% vs 301±26%; P<0.02). These data support the hypothesis that gene-

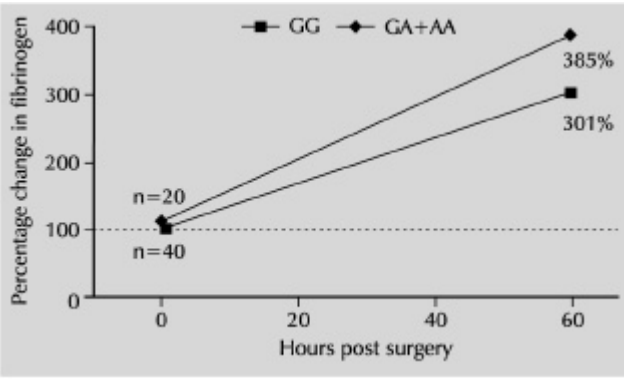

Figure 2. Graph showing baseline fibrinogen and fibrinogen 3 days post surgery in CABG patients with different FIBB G-455>A genotype.[40]

environment interactions are important in the determination of plasma fibrinogen levels and confirm previous studies, showing that possession of the A allele of the -455G>A ß-fibrinogen promoter polymorphism leads to an increased inflammatory response following exercise[39] and surgical trauma.[41] This greater rise in fibrinogen would expose patients to increased risk of venous thromboembolism or saphenous vein graft occlusion early after coronary bypass surgery, although no such adverse effects were noted in this small cohort of subjects. Thus this genetic test, which could easily be performed on a blood sample or a mouthwash sample taken at a pre-operative visit, may be useful to identify patients who would benefit from closer monitoring and/or prophylactic treatment to reduce risk of thrombosis.

Factor XII (FXII). Hereditary FXII deficiency is relatively asymptomatic and is usually detected by chance during *in vitro* testing as a result of prolonged activated partial thromboplastin times (APTT). Whether FXII is involved in the aetiology of IHD is not yet certain, however, it is known that FXII plays a major role in the extrinsic coagulation pathway.[42] FXII is a serine protease which is activated to activated factor XII (FXIIa) and is important as the first activating factor in the coagulation pathway. Activation of FXII occurs upon vessel wall injury where the circulation comes into contact with a variety of biological substances normally external to the circulation, and which are usually separated from the blood by the healthy vascular endothelium. FXIIa induces the activation of factor XI to factor XIa (FXIa), which then leads to a multistep process, finally resulting in the generation of thrombin, the cleavage of fibrinopeptides A and B from the full length fibrinogen molecule, and the generation of fibrin which forms the fibrin clot. FXIIa is also involved in the initiation of the coagulation cascade by cleaving prekallikrein to kallikrein which can in turn cleave the inactive zymogen FXII to yield alpha-FXIIa and beta-FXIIa. FXII is also involved in fibrinolysis, because kallikrein cleaves pro-urokinase to urokinase, which, in turn, activates plasminogen to plasmin which "dissolves" the fibrin clot. Taken together, this highlights FXII as a key factor for both the coagulation and prothrombotic processes.

Until recently, studies of FXII have been limited by an inability to measure its rate of activation *in vivo.* The advent of ELISA assays for FXIIa[43] has created an opportunity to examine the status of the contact system, showing that high FXIIa levels are associated with risk for IHD in healthy middle-aged men[44,46] and that ele-

vated levels of FXIIa are found in subjects with 3-vessel disease compared to those with no-vessel disease.[47] In the largest study reported to date, the relationship between FXIIa levels and risk of IHD has been examined in ~2000 healthy middle aged UK men taking part in the second Northwick Park Heart Study. Results showed that risk, as determined by the profile of conventional cardiovascular risk factors, including serum cholesterol, blood pressure and smoking habit, was found to be positively associated with FXIIa by ELISA.[46] Kohler et al,[47] in a study of 292 patients with a history of myocardial infarction, found the FXIIa concentration to be strongly associated with the extent of coronary artery stenosis, with mean FXIIa in healthy controls being 1.9 ng/mL, whereas the average value in patients was 2.5 ng/mL. Taken together these data strongly suggest that plasma FXIIa levels may serve as a marker of the severity of the atherosclerotic process, i.e. a marker of atherosclerotic burden which is seen as activation of the contact system because of atherosclerotic damage to the vessel wall.

We have recently taken this study further by examining the relationship between levels of FXIIa and IHD risk in prospective survey of these NPHS-II men.[48] Those with FXIIa levels in the lower and middle thirds of the population distribution had similar risks of IHD, while values in the upper third of the distribution (<2.08 ng/mL) were associated with a 96% increase (95% CI:1.20–3.20) in IHD risk (P=0.007), an effect which was independent of other measured risk factors. The present study is the first to show by prospective surveillance that whereas a low apparent FXIIa appears to afford no detectable protection against IHD, a high FXIIa concentration is a statistically significant predictor of a first event in middle-aged men, even when other established risk factors have been taken into account.

The origin of free FXIIa in normal human plasma, and the explanation for its increased concentration in men at high risk of IHD are uncertain. FXIIa levels are strongly associated with plasma triglyceride concentration, suggesting that circulating triglyceride-rich lipoproteins may have an important role in factor XII metabolism. *In vitro* studies have indicated that lipolysis of triglyceride-rich lipoproteins is associated with activation of the zymogen form of factor XII (FXII)[49] to its serine protease FXIIa, and that, when protected from its natural inhibitors, FXIIa activates factor VII both directly and indirectly by way of sequential activation of factor XI and factor IX.[50,52] It is now becoming increasingly recognised that inflammation is an important component of IHD risk,[53] and there is evidence to suggest that FXII is a negative acute phase protein.[54] Thus FXII levels would be expected to be lower in subjects in the inflammatory state, and this might be expected to reduce thrombotic risk. However, plasma apparent FXIIa concentration is raised in association with cigarette smoking, hypertension and hyperlipidemia, all known to predispose to atherothrombotic injury of the arterial endothelium.[55] The assay used here is specific for the activated form of FXII (FXIIa) and levels determined by the assay are dependent both on production rates from the liver and activation via the contact system. In this sample of men, levels of FXIIa were higher in smokers than non-smokers,[12] suggesting that the activation due to smoking-induced damage is more important than any down-regulation effect on liver synthesis.

5. FXII GENE VARIATION

Both rare mutations causing inherited deficiency of FXIIa and common polymorphisms within the gene for FXII have been identified.[56,57] One common polymorphism in FXII has been reported that is associated with differences in plasma levels of FXIIa. This is due to a sequence variation (C46>T) which alters the translation efficiency of FXII by creating a novel AUG methionine start-of-translation codon upstream of the correct AUG site. This has been demonstrated *in vitro* to result in less efficient translation of the T-mRNA compared to the C-mRNA,[57] either because of competition for translation or impairment of the consensus sequence for the translation-initiation scanning model.[58] The highest FXIIa levels occurred in individuals homozygous for the "C" allele which is more common in Caucasians (70%) than Japanese (20%). Recently a study in a UK group of MI survivors has confirmed that the T allele is strongly associated with levels of FXIIa[59] (fig. 3). We have now determined the association between this polymorphism and plasma FXIIa levels in the NPHS-II men, and explored the possibility that this genotype may be associated with differences in risk of IHD.[48]

The frequency of the T allele was 0.246 (95% CI: 0.24–0.26). As shown in figure 3, men homozygous for the T allele had levels of FXIIa that were significantly lower than those with the genotype CC, with the CT group having intermediate levels (median CC=2.0, CT=1.4, TT=0.8, by Kruskal-Wallis test P=0.004). This confirms the association originally reported in a small group of Japanese subjects,[57] and a small sample of patients from the UK.[59] Compared to those with

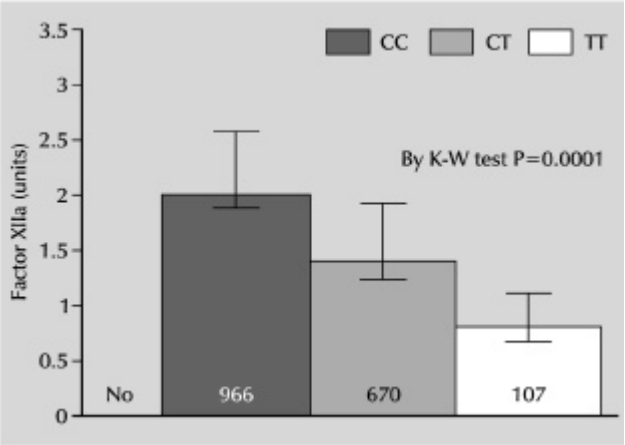

Figure 3. Histogram of median FXIIa levels in men with different FXII C46>T genotype.[48]

the genotype CC, men homozygous for the T allele also had lower levels of factor VII coagulant activity (25% lower, P=0.02) and fibrinopeptide A (25% lower, P=0.0001), confirming the overall lower coagulable state in these men. As expected from these results, the incidence of IHD in men with the genotype TT was less than in men with other genotypes (RR 0.41), but the difference was not statistically significant. In a multiple regression model, the effect on IHD risk associated with elevated FXIIa levels was essentially unchanged by adjusting for the FXII C/T genotype. These data indicate that there are as yet undiscovered genetic and environmental causes of elevated levels of FXIIa, and confirm the importance for IHD risk of the extrinsic system in the generation of a hyper-coagulable state in healthy middle aged men.

The FXII variant 46T allele is common, and 6% of men had the genotype TT. In this sample, less than 2% of men with this genotype had levels of FXIIa above 2.0 mg/dL (in the top tertile) and would thus be expected, as a group, to have a low IHD risk. Although this is a large prospective sample, to date only 139 IHD events have occurred, and there was only suggestive evidence that men with the genotype TT have a lower risk of IHD, and confirmation of this protective effect requires a larger study. However, it is most likely that any effect associated with the C46T genotype on IHD risk will be explained by the strong effect on FXIIa levels and thus genotype is unlikely to add significantly to risk prediction over and above measurement of FXIIa levels. However, the IHD risk associated with FXIIa levels >2.0 ng/mL was essentially unaltered by adjustment for the C46T genotype, or for other "classical" risk factors such as smoking, plasma triglyceride levels or BMI, suggesting that other genetic or environmental factors that contribute to the determination of individual levels of FXIIa are still to be identified.

6. CONCLUSIONS

The fibrinogen-raising effect associated with the A-455 allele has now been demonstrated beyond any reasonable doubt, although the precise molecular mechanism of this effect remains to be elucidated. The magnitude of this effect indicates that it is likely to be of biological significance in causing an elevated risk of thrombosis. However, as described in this review, the magnitude of the effect is modulated (both up and down) by many environmental factors, and this means that in some subjects who have a particular life-style or environment this genotype may be making a significant contribution to risk, while in others it may be making essentially no contribution. As well as being an explanation as to why some studies fail to reproduce the IHD risk association reported by others, this also opens up the field for additional molecular experiments to determine the mechanisms of these interactions and to develop genotype-specific therapeutic approaches to reduce fibrinogen levels and thus reduce future risk of thrombosis. For the FXII C46T polymorphism further confirmatory studies are also required, as well as studies for both genes to explore whether similar genetic effects (and interactions for FIBB) occur in individuals of different ethnic origin, in both men and women, and in those with different forms of vascular disease. The observations to date suggest the possibility that individuals homozygous for the fibrinogen -455A allele or the FXII 46T allele may be at particular risk of a thrombotic event following an acute phase stimulus. Once identified, such individuals may benefit from risk factor reduction. Once the mechanisms controlling changes in plasma risk factors in response to personal environmental changes are better understood, it may also be possible to develop directed therapeutic strategies that will reduce risk in a genotype-specific manner, an approach which is not possible at present.

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ΠΕΡΙΛΗΨΗ

Επιδημιολογία της αρτηριακής θρόμβωσης. Αλληλεπίδραση γονιδίων-περιβάλλοντος, παράγοντες κινδύνου για στεφανιαία νόσο

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Η στεφανιαία καρδιοπάθεια (ΣΚΠ) είναι μια σύνθετη πολυπαραγοντική νόσος και τα τελευταία χρόνια έχει γίνει σαφές ότι, όπως και με τα λιπίδια του πλάσματος, η αύξηση των επιπέδων ορισμένων πρωτεϊνών της οδού της πήξης συμβάλλει ανεξάρτητα στον κίνδυνο της ΣΚΠ, όπως για παράδειγμα η αύξηση των επιπέδων πλάσματος του παράγοντα VII, του PAI-1 και του ινωδογόνου. Πρόσφατα, παρουσιάστηκαν μέθοδοι για την αξιολόγηση της σημασίας των άλλων παραγόντων κινδύνου και προέκυψαν ενδείξεις ότι τα επίπεδα του ενεργοποιημένου παράγοντα XII (FXIIa) είναι δυνητικά ένας παράγοντας κινδύνου. Για όλες αυτές τις πρωτεΐνες, μελετήθηκαν τα γονίδια που τις κωδικοποιούν, βρέθηκε δε ότι οι λειτουργικοί πολυμορφισμοί συνδέονται με τα επίπεδα του πλάσματος και, σε ορισμένες περιπτώσεις, με τον κίνδυνο ΣΚΠ. Στην ανασκόπηση αυτή εστιαζόμαστε σε εργασίες του δικού μας εργαστηρίου, που ερεύνησαν τους γενετικούς αυτούς καθοριστικούς παράγοντες και ιδιαίτερα τον τρόπο με τον οποίο οι γενετικοί και περιβαλλοντικοί παράγοντες αλληλεπιδρούν.

Λέξεις ευρετηρίου: Γενετικός πολυμορφισμός, Ινωδογόνο, Παράγοντας VII, Παράγοντας XII, PAI-1

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