

Biomarkers for arterial and venous thrombotic disorders

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Keywords

Genetics, single nucleotide polymorphisms, thrombotic disorders, haemostasis

Summary

The haemostatic system maintains the blood in a fluid state, but allows rapid clot formation at sites of vascular injury to prevent excessive bleeding. Unbalances within the haemostatic system can lead to thrombosis. In spite of successful research our understanding of the disease pathogenesis is still incomplete. There is great hope that genetic, genomic, and epigenetic discoveries will enhance the diagnostic capability, and improve the treatment options. During the preceding 20 years, the identification of polymorphisms and the elucidation of their role in arterial and venous thromboses became an important area of research. Today, a large body of data is available regarding associations of single nucleotide polymorphisms (SNPs) in candidate genes with plasma concentrations and e.g. the risk of ischaemic stroke or myocardial infarction. However, the results for individual polymorphisms and genes are often controversial. It is now well established that besides acquired also hereditary risk factors influence the occurrence of thrombotic events, and environmental factors may add to this risk. Currently available statistical methods are only able to identify combined risk genotypes if very large patient collectives (>10 000 cases) are tested, and appro-

appropriate algorithms to evaluate the data have yet to be developed. Further research is needed to understand the functional effects of genetic variants in genes of blood coagulation proteins that are critical to the pathogenesis of arterial and venous thrombotic disorders. In this review genetic variants in selected genes of the haemostatic system and their relevance for arterial and venous thrombosis will be discussed.

Schlüsselwörter

Genetik, Einzelnukleotidpolymorphismen, thrombotische Erkrankungen, Hämostase

Zusammenfassung

Das Gerinnungssystem stellt sicher, dass das fließende Blut im Falle von Verletzungen sehr rasch ein Gerinnsel bilden kann, Wunden verschließt und damit einen Blutverlust großen Ausmaßes verhindert. Dies wird durch ein feinst ausgewogenes Gleichgewicht zwischen pro- und antikoagulatorischer Aktivität gewährleistet. Jedwede Verschiebung der Balance kann zu Thrombosen oder Blutungen führen. Trotz umfangreicher Forschungsaktivitäten sind die pathogenetischen Ursachen dafür nicht gut verstanden. Es besteht die Hoffnung, dass genetische, genomische und epigenetische Erkenntnisse das Wissen vertiefen, die Diagnostik schärfen und schließlich die Therapie verbessern werden. Im Laufe der vergangenen

20 Jahre wurden zahlreiche genetischen Varianten identifiziert und hinsichtlich ihrer Bedeutung für arterielle und venöse Thrombosen wissenschaftlich untersucht. Heute liegen uns umfangreiche Daten zur Assoziation verschiedenster polymorpher Varianten von zahlreichen Kandidatengenen mit den Plasmakonzentrationen der entsprechenden Proteine und z. B. dem Risiko für Schlaganfall und Herzinfarkt vor. Allerdings sind die publizierten Ergebnisse zur Rolle verschiedener Polymorphismen und ihrem Beitrag zum Auftreten von Thrombosen oft kontrovers. Das mag zum Teil mit dem Einfluss externer Faktoren auf das Thromboserisiko zusammenhängen, es liegt aber wahrscheinlich auch an den statistischen Analyseverfahren und der erforderlichen großen Zahl der Patienten. Zur Erreichung signifikanter Ergebnisse benötigt man sehr große Kohorten (>10 000 Fälle), die oft sehr heterogen sind. Ohne Zweifel bedarf es neuer Algorithmen, um die immer umfangreicheren Daten sinnvoll auszuwerten und die verschiedenen funktionellen Effekte, die für die Pathogenese arterieller und venöser Thrombosen verantwortlich sind, zu verstehen.

In diesem Übersichtsbeitrag werden verschiedene Varianten in ausgewählten Genen des Hämostasesystems vorgestellt und ihre Relevanz für arterielle und venöse Thrombosen kritisch diskutiert.

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Biomarker für arterielle und venöse thrombotische Erkrankungen

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Genetic variations (single nucleotide polymorphisms, SNPs) and athero-thrombotic disorders

Arterial and venous thrombotic disorders with the clinical manifestations myocardial infarction (MI), stroke, peripheral arterial disease and venous thromboembolism (VTE) – e.g. pulmonary embolism and deep vein thrombosis of the lower limbs – are major causes of morbidity and mortality. Age, sex, systolic blood pressure, hypertension, total and high-density lipoprotein cholesterol levels, smoking, and diabetes as well as N-terminal fragment of pro-hormone B-type natriuretic peptide levels, chronic kidney disease, leukocyte count, C-reactive protein levels, homocysteine levels, uric acid levels, coronary artery calcium [CAC] scores, carotid intima-media thickness, peripheral arterial disease, and pulse wave velocity are known important risk factors for arterial thrombosis. Inflammation is associated with development and progression of atherosclerosis (1, 2). Furthermore, interactions between lipoproteins, monocyte-derived macrophages, T-lymphocytes, and normal cellular elements of the vessel wall are relevant for the development of atherosclerotic lesions. However, the ultimate event leading to myocardial infarction (MI) is the occlusive coronary thrombus formation superimposed on an atherosclerotic plaque. Thus, haemostatic proteins contribute to the pathogenesis of vascular diseases.

The haemostatic system maintains the blood in a fluid state, but allows rapid clot formation at sites of vascular injury to prevent excessive bleeding. Unbalances within the haemostatic system can lead to thrombosis, which can occur in arteries, veins, or in the chambers of the heart. Factors contributing to thrombosis at these sites include endothelial injury, reduced blood flow, and hypercoagulability of the blood. Vessel wall injury and stasis are particularly important in the pathogenesis of arterial thrombosis, whereas hypercoagulability and stasis are the main contributors to venous thrombosis. However, evidence is increasing that both systems are relevant for

venous as well as arterial thrombotic events.

Despite of successful research our understanding of disease pathogenesis still needs to be improved. There is great hope that genetic, genomic, and epigenetic discoveries will enhance the diagnostic capability, and revolutionize the treatment modalities. Today we know that hereditary and acquired risk factors influence the risk for thrombosis, and environmental factors add to this risk. While hereditary risk factors for venous thrombosis are well established, the genetic risk factors for arterial thrombosis are less well defined. During the last 20 years, the identification of genetic variations and the elucidation of their role in arterial and venous thrombosis became an important area of research. Even though a large body of data is available for associations of single nucleotide variations (SNPs) in candidate genes with e.g. the risk of ischaemic stroke or myocardial infarction, the results for individual polymorphisms and genes are often controversial (3). In 2007 Knowles et al. tested 21 SNPs in coagulation factor and platelet glycoprotein genes (platelet glycoprotein Ia/IIa (*ITGA2*), glycoprotein Ibá (*GP1BA*), glycoprotein IIb (*ITGA2B*), glycoprotein IIIa (*ITGB3*), glycoprotein IIIb/platelet glycoprotein IV (*CD36*), plasminogen activator inhibitor I (*PAI1*), thrombomodulin (*THBD*), coagulation factor III/tissue factor (*F3*), and tissue plasminogen activator (*PLAT*)) and evaluated the association with myocardial infarction (MI) in the ADVANCE study population (4). The authors did not find significant associations of the chosen polymorphisms with acute MI.

Today, next-generation DNA sequencing is used for genetic association studies in complex diseases. This technology represents a powerful tool to identify genetic variants that confer disease risk within populations. Recently, a genetic Risk Score (GRS) for MI could be established and an association with MI could be demonstrated for 35 SNPs in the following 12 genes of the coagulation system: factor V (F5), protein S (PROS1), factor XI (F11), integrin alpha 2 (*ITGA2*, platelet glycoprotein Ia), factor XII (F12), factor XIIIa (F13A1), plasminogen activator inhibitor-1 (*SERPINE1*), tissue plasminogen activator (*PLAT*), von

Willebrand factor (VWF), thrombomodulin (THBD), endothelial protein C receptor (PROCR), and factor IX (F9). The GRS differed significantly between cases and controls, and subjects in the highest quintile had a 2.69-fold increased risk for MI compared with those in the lowest quintile (5).

In a study on patients with DVT a molecular-barcode based technique on a next-generation DNA sequencing platform was used to sequence the coding areas of 186 haemostatic/proinflammatory genes (6). With this approach, known disease-associated variants as well as novel potentially deleterious variants in disease-associated genes were identified. Interestingly, a number of rare missense mutations in known anticoagulant genes were found. Furthermore, an association of DVT with the variant rs6050 in the fibrinogen alpha gene (FGA) was observed. The discovery of rare missense mutations in anticoagulant genes and the association of rs6050 with idiopathic DVT suggests that ‘unrecognized’ anticoagulant deficiencies and mutations for which currently used biochemical assays are not sensitive, could represent risk factors of DVT or even cause the disease.

Regarding the role of genetic variants in stroke the analyses of six cohorts gave controversial results. No single locus of significance (approximately $p < 10^7$) could be identified (7). This is in contrast to a recent meta-analysis which reported that three biomarkers (C-reactive protein, P-selectin and homocysteine) could differentiate between ischaemic stroke and healthy control subjects. These discrepant results show that intelligent, appropriately powered, multi-disciplinary studies incorporating knowledge from clinical medicine, epidemiology, genetics, and molecular biology are required to fully characterize the genomic contributors to vascular diseases.

The strategy to examine one biological system, e.g. the haemostatic system, and specifically focus on DNA sequence changes in genes known to impact on haemostasis and thrombosis, and concentrate on specific combinations of variants and their influence on disease predisposition might eventually lead to success (8). Elevated circulating levels of haemostatic factors, such as fibrinogen, plasminogen

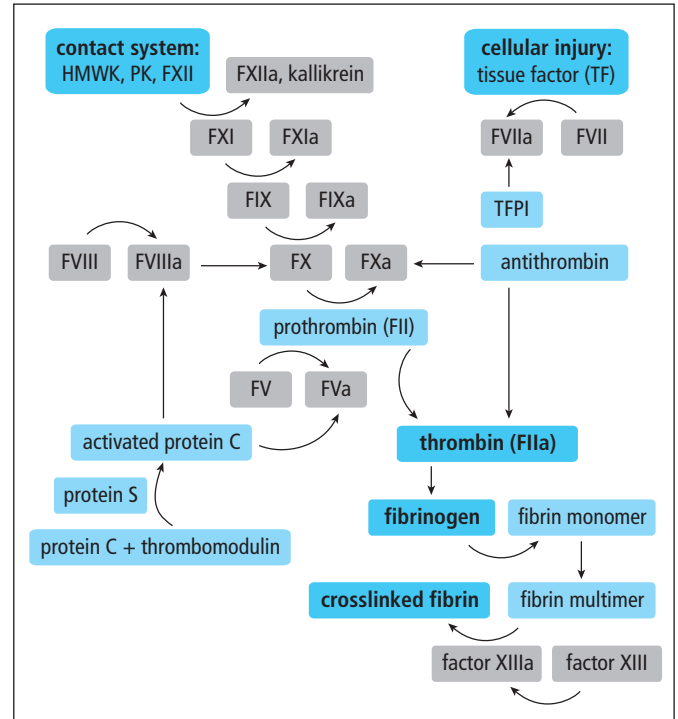
activator inhibitor (PAI-1), von Willebrand factor (VWF), tissue plasminogen activator (tPA), factor VII (FVII), and D-dimer could already be linked to the development of atherothrombosis, and have been established as risk markers for coronary heart disease (CHD), stroke and other cardiovascular disease (CVD) events.

The haemostatic system, gene polymorphisms, and thrombotic diseases

Coagulation can be initiated via two pathways. At negatively charged surfaces the intrinsic system comprising factors XII, prekallikrein, high-molecular-weight kininogen, factors XI, IX, and VIII is activated, while the extrinsic pathway is initiated by activation of factor VII in the presence of tissue factor. Both pathways converge at a common stage involving factors X, V, and II. As a result thrombin is formed, which converts fibrinogen (factor I) to fibrin. (► Fig. 1).

The crosslinking of fibrin by activated factor XIII increases clot stability and its resistance to fibrinolysis. Today, we know that the intrinsic and extrinsic coagulation systems are intertwined and the *in vivo* activation of the coagulation cascade is complex. It has been shown that activated platelets promote plasma clotting, and this process is dependent on blood coagulation factor XII. Upon activation, platelets release linear inorganic polymers of 60–100 phosphate residues (polyphosphates) that bind to and possibly activate factor XII. The interaction of polyphosphates from platelets with FXII was claimed to link platelet activation (primary haemostasis) and fibrin production (secondary haemostasis) (9–11). However, this could not be verified in a recent study which found platelet-derived polyphosphates to be only a weak activator of FXII leading to a generation of <10% FXIIa compared to kaolin (12). Polyphosphates released by platelets may be too short to activate FXII. The relative importance of polyP in the process of thrombus formation is highly interesting but will require further investigation. Particularly, as targeting polyphosphate-mediated

Fig. 1
Coagulation cascade



factor XII activation would offer an exciting new possibility for a safe anticoagulation with minimal therapy-associated bleeding (10).

During the preceding three decades, great effort has been made to understand the molecular genetic mechanism of the haemostatic system. The DNA sequences of all coagulation factors have been elucidated and many genetic alterations affecting the function of coagulation factors have been identified. Now it is known that the majority of DNA sequence changes that influence haemostatic and thrombotic phenotypes are either single-nucleotide mutations/variants resulting in missense, nonsense or splice site mutations, or small microinsertions and micro-deletions which disrupt the reading frame. For many of these variants, sequence data, phenotypic annotations and original literature citations are available from the Human Gene Mutation Database (HGMD®). Since the 1990s it is clear that besides mutations and polymorphisms in coding regions of genes, genetic variations in regulatory regions of coagulation factor genes, particularly in the promoter regions, have an important impact on blood coagulation because of their effect on the concentration of the proteins

(13, 14). For a number of coagulation factors reproducible data exist regarding the contribution of SNPs to the variability of their plasma concentration (► Tab. 1) and subsequently a possible effect on the clinical phenotype. Recently it was shown that a large percentage of the genetic variance of antithrombin (AT), protein C and protein S (PS) plasma levels is mediated by single nucleotide variations (15).

Today, the importance of still other alterations has been recognised. It was found that copy number variations (CNVs) – defined as DNA sequences greater than 1 kb, which vary in individual genomes – seem to play a role in haemostatic diseases (16). Full gene gains are equivalent to a duplication of the gene and hence, have the potential to result in an increase in protein levels. Thus, CNVs may represent an additional heritable element of risk for either bleeding or thrombotic disorders. At present, our understanding of the impact of copy number variations with regard to disease association is still fairly limited. On the basis of recent studies, 45 of 109 (41.3%) gene members of the haemostatic system have already been found to exhibit copy number variations (e.g. F10, F5, F7, F8, F13, F12, HMWK) (8).

Tab. 1 Polymorphisms in genes of the haemostatic system: association with plasma concentrations

gene	polymorphism	rs number	functional consequences	references
fibrinogen	-455G>A Thr331Ala	rs1800790	increased fibrinogen plasma levels	125
		rs6050	modestly lower plasma levels	126
FXIII	Val35Leu	rs5985	increased FXIII activity, decreased clot stability, resistance to fibrinolysis	127
PAI-1	-675(4G/5G)	rs34857375	lower PAI-1 levels	31, 128–130
FXII	-4C>T	rs1801020	association with lower FXII plasma levels	39,40,44
FVII	Arg413Gln -401G>T -402G>A 323 0/10bp insertion	rs6046	associated with lower FVII activity	131
		rs510335	associated with lower FVII activity	55
		rs510317	associated with higher FVII activity and antigen	55
		rs5742910	associated with lower FVII activity	132
FV Leiden	506Arg>Gln	rs6025	causes APC resistance, impacts on inactivation of FV	64,133,134
prothrombin	20210G>A	rs1799963	associated with increased prothrombin plasma levels	72,73,135
von Willebrand factor	-2709C>T -2661A>G -2527G>A Thr789Ala Ala1381Thr	rs7964777	associated with VWF antigen levels	81–83,136
		rs7954855		
		rs7965413		
		rs1063856		
		rs216311		
FVIII	Asp1260Glu	rs1800291	associated with FVIII activity	90,137
protein C	-1654C/A -1641 G/T 1386T>C, 5'-flanking	rs2069901	association of CG haplotype with protein C levels	92,94,138,139
protein C receptor (PROCR)	Ser219Gly 4054T>C	rs867186 rs2069948	associated with PROCR levels	94,95,98
GPIa (ITGA2)	Glu534Lys	rs1801106	influences GPIa activity, affects expression density	102,104,140
P-selectin	Thr715Pro	rs6133	associated with soluble P-selectin levels	141–143

The relationship between haemostasis and thrombosis and atherothrombotic cardiovascular disease has been extensively studied in the past decades. Several haemostatic variables were identified as cardiovascular risk markers. The concentrations of fibrinogen, factor VII and VIII, von Willebrand factor, plasminogen activator inhibitor-1 (PAI-1), and tissue plasminogen activator have been associated with coronary-heart disease. Twin and sibling studies have shown that genetic variations in genes coding for coagulation proteins influence the risk of cardiovascular disease and ischaemic stroke (17, 18). Twin studies could also show that genetic factors have a major effect on plasma concentrations of haemostatic proteins. They contribute to approximately 41–75% of the variation in concentrations of fibrinogen, factor VII, factor VIII, PAI-1, tissue plasminogen activator, factor XIII A-subunit and B-subunit, and von Willebrand factor.

Factor XIII activity showed higher (82%) and factor XIIa lower (38%) heritability (19). In 2004, Peetz et al. presented data on the heritability of proteins of the fibrinolytic system in healthy twins. Heritability was 76% for thrombin activatable fibrinolysis inhibitor (TAFI), 43% for tissue plasminogen activator and 44% for plasminogen activator inhibitor-1 (PAI-1). Interestingly, the PAI-1 4G/5G polymorphism explained 56.4% of the calculated heritability (20). The genetic influence on D-dimer and TAFI plasma levels (33% for D-dimer, 71% TAFI) persisted in elderly sib-pairs, and concentrations of haemostatic variables showed a substantial genetic variation in the elderly (21). However, one has to consider that in diseases with a complex genetic basis, phenotypes can be modified by the combined action of several genetic variants. Presumably, known hereditary factors will confer disease predisposition along and together with novel mutations

which have not yet been investigated. A single variant may not have a strong enough effect to be regarded as an independent risk factor.

In the following chapters known genetic variants in selected genes of the haemostatic system and their relevance for thrombotic disorders will be discussed.

Fibrinogen (factor I), factor XIII and fibrinolysis

Fibrinogen

Fibrinogen is a 340-kDa glycoprotein consisting of three nonidentical polypeptide chains (α , β , and γ) linked by disulfide bridges. The three genes encoding fibrinogen B β (FGB), A α (FGA), and γ (FGG) are located on human chromosome 4. Each gene is separately transcribed and translated to the A α , B β , and γ -polypeptides, which assemble to form fibrinogen. Fibrin-

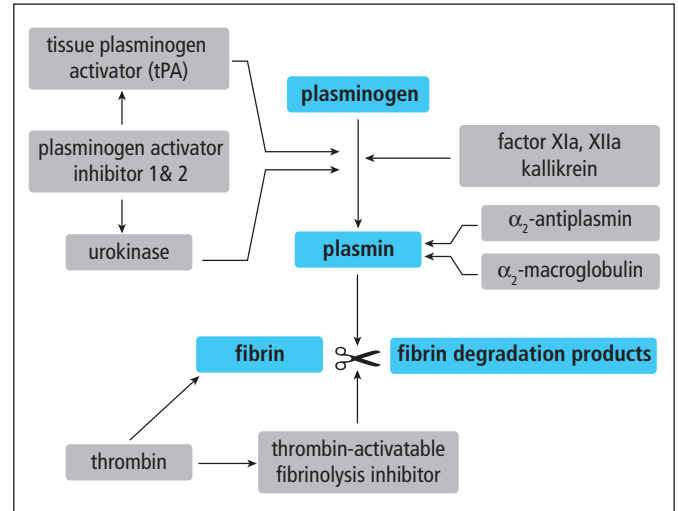
nogen levels are upregulated during acute phase reactions via the activation of the IL-6 responsive elements in the promoter region of all three fibrinogen chains (22–24).

High levels of fibrinogen have been associated with the development of arterial thrombosis. Prospective studies such as the Northwick Park Heart Study, the Gothenburg study, and the PROCAM study have related elevated fibrinogen levels to MI and stroke (25, 26). It has been shown that genetic variants contribute to the variability of fibrinogen plasma levels (27). The two most frequently studied variants are the rs1800790 polymorphism (–455G>A), located in the promoter region of the fibrinogen beta-chain (FGB) and the rs6050 polymorphism (312Thr>Ala) in the fibrinogen alpha-chain (FGA). rs1800790 leads to increased plasma fibrinogen levels, while rs6050 is associated with lower plasma fibrinogen levels. The FGA rs6050 single-nucleotide polymorphism (SNP) indicates a decreased risk whereas the FGB rs1800790 seems to increase the risk of stroke. Interestingly, an association between poststroke mortality and the rs6050 variant in patients with atrial fibrillation had been observed while the risk of myocardial infarction is not altered by either variant (28, 29). Recently, FGB rs1800790 has also been shown to be important in DVT. Thus, although the general risk for thrombotic diseases does not seem to be significantly affected by genetic variants of fibrinogen, they may play a role in subgroups of patients.

Fibrinogen is cleaved by thrombin leading to the generation of fibrin monomers which have to polymerize and be cross-linked by factor XIII. This crosslinking of the fibrin polymers is essential for clot stability and its resistance to fibrinolysis (30). The fibrin structure then influences the enzymatic lysis of the fibrin network which is linked to thrombotic diseases (31).

Biochemical studies identified intermolecular interactions that control fibrin structure and showed that the molecular mechanisms that control the fibrin structure also control clot stability (32). FXIII catalyses the formation of covalent $\epsilon(\gamma$ -glutamyl)-lysyl bonds between the γ and α

Fig. 2
The fibrinolytic system



chains of fibrin and plays an important role in this process.

Factor XIII circulates in plasma as a heterotetramer consisting of two catalytic A-subunits and two carrier protein B-subunits (A2B2). Monocytes and megakaryocytes synthesize FXIII, and FXIII has also been identified in platelets. The inactive protein is converted to an active transglutaminase (FXIIIa) in the final phase of coagulation by thrombin and Ca^{2+} . FXIIIa protects newly formed fibrin from fibrinolysis, primarily by cross-linking $\alpha(2)$ -plasmin inhibitor to fibrin. In the F13 gene a common G>T variation at nt163 (rs5985), leading to a valine (Val) to leucine (Leu) substitution at amino acid position 35 (just three amino acids away from the thrombin activation site in exon 2 of the FXIII A-subunit gene), was described (33). Due to the close proximity of the thrombin activation site, this polymorphism seems to impair FXIII activation and contributes to thrombotic disorders (34). In vitro, the 35Leu allele confers an increased catalytic activity to FXIII, but decreases clot stability and resistance to fibrinolysis by alterations of the clot structure (35). A lower prevalence of the 35Leu allele has been found in subjects with MI or deep vein thrombosis compared to healthy controls (36, 37). In spite of some positive reports, the importance of the FXIII 35Leu genetic variant for premature coronary artery disease (CAD) and thrombotic events remains controversial. There was no effect of FXIII 35Leu on

long-term clinical outcome defined as a composite of cardiovascular death, recurrent MI and revascularisation. However, factor XIII 35Leu lead to a stepwise decrease in the rate of fibrinolysis with a significant gene-dose-effect in patients (38). The role of the F13 rs5985 polymorphism in ischaemic stroke is currently controversial. Our group found no differences in genotype distribution between patients with ischaemic stroke and healthy controls. This suggests that an association of the FXIII Val35Leu polymorphism with a decreased risk of ischaemic stroke or an increased risk of intracerebral haemorrhage is unlikely (39). The results were recently confirmed by Shemirani et al. (40) who also found no association between the risk of acute ischaemic stroke and FXIIIa 35Leu carriership in either gender. In contrast to that Elbaz et al suggested a protective effect of the Leu allele against thrombotic cerebral artery occlusion in a case – control study in patients with brain infarction. Adjustment for traditional vascular risk factors did not modify these findings (41). Meta-analyses of published data suggest that this polymorphism provides a moderate protection against coronary artery disease and venous thromboembolism. However, no evidence for an association with ischaemic stroke was found (42). Further studies are required to explore other FXIII polymorphism on the risk of thrombotic diseases.

The fibrinolytic system, PAI-1

In mammalian blood the fibrinolytic system is essential for the dissolution of blood clots and the maintenance of a patent vascular system. Abnormalities in the fibrinolytic system have been implicated in the pathogenesis of atherosclerotic diseases such as myocardial infarction and stroke (43). Fibrinolysis is initiated by specific interactions between fibrin and plasmin, generated from the inactive proenzyme plasminogen. Two plasminogen activators exist in blood: tissue-type plasminogen activator (t-PA) and urokinase. Inhibition of fibrinolysis can occur at the level of plasminogen activation by a plasminogen activator inhibitor or at the level of plasmin by alpha-2 antiplasmin.

The plasminogen activator inhibitor (PAI)-1 is the key regulator of the fibrinolytic system and has the greatest inhibitory effect. It is important in various physiological processes including fibrinolysis, tissue repair, blood coagulation, thrombolysis, ovulation, embryogenesis, angiogenesis, and cell adhesion and migration. PAI-1 has been studied in different settings with thrombotic pathophysiology, such as coronary artery disease and ischaemic stroke. Controversial results have been published which may in part be due to differences in study designs or presence of confounders. PAI-1 is synthesized locally in the vascular wall (in endothelial and smooth muscle cells). High levels of PAI-1 have been reported in patients with a history of stroke (29, 44). Genetic variants in PAI-1 affect PAI-1 levels, and gene polymorphisms have been associated with myocardial infarction and stroke (45). The promoter region of the PAI-1 gene contains a common single guanine nucleotide insertion/deletion polymorphism (4G/5G) situated 675 bp from the transcription start site (rs34857375). The 5G allele contains a repressor site and binds an enhancer and a repressor, while the 4G site binds only an enhancer. Thus, carriers of the 5G genotype A show a lower transcription of PAI-1 (46). Elevation of PAI-1 and reduction in tPA levels were observed in stroke patients. However, in some studies the 4G/4G genotype conferred a reduced risk of stroke, possibly due to a more stable clot and less

likelihood of embolic events. Other studies suggested an important contribution of PAI-1 in cerebrovascular pathology and speculated that rs34857375 in the PAI-1 gene may protect against destabilization of the atherosclerotic plaque, or it may inhibit neurotoxicity of tissue plasminogen activator in the brain (47–50)

In contrast, a recently published meta-analysis comprising 41 studies including 12461 cases and 14993 controls reported the PAI-1 4G allele as weak risk factor for MI in Caucasian, Asian or African populations with an odds ratio at 1.16 (51). Data on the association of PAI-1 and tPA levels with stroke as a function of PAI-1 4G/5G genotypes is controversial. A nested case-control study in two independent Swedish cohorts also found an association of the 4G allele with an increased risk of future ischemic stroke (52). Besides rs34857375 the PAI-1 gene comprises other polymorphisms, and in 2011 Jankun et al. reported an association of two missense mutations located in the central hydrophobic core of PAI-1 with PAI-1 deficiency due to a slower PAI-1 secretion (53). Thus, even though there are many studies that suggest a contribution of PAI-1 to the risk of vascular events, the exact mechanisms as well as the disease entity are unclear.

Recently, for the factor VII activating protease (FSAP), a circulating serine protease with high homology to fibrinolytic enzymes, a role in the regulation of coagulation and fibrinolysis has been suggested. However, up to now there is just a large number of studies which have investigated the linkage of single nucleotide polymorphisms (SNP) in the FSAP gene (HABP2) with various diseases. Some SNPs have been linked to carotid stenosis, stroke as well as thrombosis in the elderly, and plaque calcification. Therefore, a role for FSAP in the regulation of the haemostatic system may indeed exist (54)

SNPs in other coagulation factor genes and their role in atherothrombosis

Factor XII

In vitro contact activation of the haemostatic system requires the presence of factor

XII, prekallikrein, and high molecular weight kininogen. Based on these results it has been proposed that a conformational change in factor XII is a key event in the activation process of this molecule (55, 56).

Zito et al. showed that FXII and FXIIa levels are highly variable in the population (57). Even though it is known that FXII levels are influenced by hypertriglyceridaemia, smoking, or estrogens, this data suggested that the FXII variability is genetically determined. In 1998, a polymorphism in the F12 gene (46C>T, -4C>T, rs1801020) was identified by Kanaji et al. (58). This polymorphism destroys a Kozak consensus sequence, resulting in lower translation efficiency and a decrease in FXII plasma levels. In carriers of the T-allele FXII and FXIIa levels are significantly lower. The variant is frequent in Orientals and may be responsible for the lower plasma FXII activity found in this population. According to a recent genome wide linkage screen, the F12 gene represents a quantitative trait locus (QTL) that influences FXII levels. Eight of 26 polymorphisms were highly significantly associated with FXII levels. However, after correction for multiple testing only rs1801020 had a direct effect on the quantitative trait (59).

The role of the polymorphism for the development and progression of coronary artery disease has been debated for more than 10 years. In Viennese patients the homozygous F12 -4TT genotype was less frequent in patients with an acute coronary syndrome (ACS) than in patients with stable CAD (60). However, in a large retrospective study performed in Vienna we observed gradually increasing hazard ratios for vascular mortality with decreasing FXII levels down to 10% (from 1.0 in the >100% FXII to 1.5 in the 80–90% to 4.7 in the 10–20% FXII group). No significant increase in all-cause mortality was observed in subjects with FXII levels between 0–10% (61). Bach et al. (62) found no association of the F12 genotype with any clinical phenotype but could confirm the association of FXII levels with coronary risk. Recently, the paradigm that plasma elements contributing to thrombosis are primarily those involved in haemostasis, has been challenged. Deficiencies of factor XII and factor XI were not associated with abnormal

haemostasis in mice, rather, the lack of these factors impaired the formation of occlusive thrombi in arterial injury models (63).

To bring light into the long discussion of an association between low factor XII levels or F12 gene variants and thrombotic outcomes a recent review and meta-analysis searched MEDLINE, EMBASE, and HuGE Navigator for an association between F12 -4C>T and venous thromboembolism as well as myocardial infarction. Sixteen candidate gene studies (4386 cases, 40,089 controls) were analyzed. Only a very weak association with myocardial infarction for the TT + CT versus CC genotype (Odds ratio 1.13) was found. Overall, the evidence for an association between F12 -4C>T and venous thromboembolism and myocardial infarction was weak (64). Furthermore, the F12 -4C>T variant does not play a role in the development of ischaemic stroke, except probably in patients with atherothrombotic stroke (65). However, FXII levels seem to be important for the development of acute ischaemic stroke (AIS).

It could be shown that the selective inhibition of FXII-mediated FXI activation improved the outcome of experimental AIS in mice. Antibody treated mice displayed less ischaemic damage, manifested as reduced cerebral infarction and fibrin deposition (thrombosis), increased cortical reperfusion, and improved neurological behaviour. Importantly, the antibody-anti-coagulated mice had no detectable bleeding complications. This suggests that FXII-mediated FXI activation contributes to the development of experimental AIS (66). While several studies in patients with venous thrombosis provide evidence that the -4C/T genotype does not or only marginally contribute to the risk of DVT (67, 68), another publication found an association of the -4TT variant with DVT (69). These controversial findings suggest that FXII may only be important in combination with other factors. A possible candidate seems to be protein S. Low PS activity in association with the homozygous F12 -4CC conferred a high thrombotic risk (70). Thus, further studies regarding the role of SNPs in the F12 gene will be necessary before a final conclusion can be drawn.

Factor VII

The extrinsic coagulation pathway is initiated by activation of factor VII. Coagulation factor VII (FVII) is a vitamin K-dependent coagulation factor circulating in the blood as inactive zymogen. In the Northwick Park Heart Study, elevated FVII coagulant activity has been associated with fatal ischaemic heart disease. However, the results could not be fully confirmed in the prospective PROCAM study (71, 72). FVII activity is linked with plasma triglyceride concentrations which may explain the association between hypertriglyceridaemia and arterial thrombotic disease (73). The gene for FVII contains five polymorphic sites, which account for ~30% of the variation in FVII plasma levels (74, 75). Polymorphisms in the F7 gene contribute to the development of arterial thrombosis (76). Already in 1998 Iacoviello et al. reported a protective effect of the F7 353Q allele (rs 6046) for the development of MI (77). Girelli et al. described similar results for the 353R>Q polymorphism and the decanucleotide polymorphism at nt(-323) (78). In 2006, Campo et al. published that FVII and TF antigen levels at admission are independent predictors of mortality and reinfarction in patients with acute MI (75). The levels partially corresponded to polymorphisms in the F7 gene. In 2012, an investigation on the transcriptional regulation of F7 indicated that the epigenetic regulation of the F7 promoter through methylation affects FVIIa plasma concentrations and is associated with coronary artery disease (79). Our group presented evidence that the FVII -402A (rs510317) allele in the promoter region which confers increased transcriptional activity and higher FVIIa influences the risk of early ischemic cerebrovascular events, whereas the 353R>Q (rs6046), the -401G>T (rs510335), and the -323ins/del (rs5742910) sequence variations, which are associated with lower FVII activity have no effect (80). The hypothesis that individuals with the -323ins allele have an increased risk of primary intracerebral haemorrhage (PICH) could not be confirmed by Greisenegger et al. (81).

Factor V

The intrinsic and the extrinsic coagulation system converge at a common stage involving factors X, V, and II. Coagulation factor V (FV) acts as cofactor of FXa and plays an important role in the regulation of the coagulation process. FV is synthesized in the liver and is found in plasma as inactive pro-factor. It is also detectable in α -granules of platelets. To develop its procoagulatory activity, FV has to be activated either by thrombin or FXa by limited proteolysis (82). Activated FV (FVa) is inactivated by cleavage at Arg506, Arg306 and Arg679 by activated protein C (APC). In 1993 Dahlbäck et al. described a family with resistance to activated protein C (83). The resistance was shown to be caused by a point mutation in the F5 gene at nt1691. The mutation leads to an Arg>Gln amino acid exchange at amino acid position 506 (FV R506Q, FV Leiden, rs6025) (84), is frequent in the Caucasian population (~5%) and represents one of the most important risk factors for inherited venous thrombosis. For arterial thrombosis and myocardial infarction (MI) the data are less clear. While a large meta-analysis reported an odds ratio of ~1.3 for MI in carriers of the FV 506Q allele (85) another meta-analysis published by Wu and Tsongalis (86) in unselected patients with CAD found no association with factor V Leiden. They concluded that screening for the FV 506R>Q polymorphism in unselected patients at risk for MI is not indicated. However, it still cannot be excluded that the FV 506Q mutation has a role in selected patient groups. Rosendaal et al. (87) observed that the FV R506Q mutation represents a risk factor in young women with MI before the age of 45 years. In addition, a high prevalence of the FV 506Q allele in patients with MI without signs of coronary atheromatosis was reported (88). In these patients vasospasms followed by coronary thrombosis in FV Leiden carriers seemed to trigger the development of MI. The analysis of the FV R506Q genotype could help with risk assessment in this patient subgroup. The contribution of the FV Leiden mutation to the risk for ischemic stroke in adults is also unclear. Wu and Tsongalis (86) found an increased risk for cerebrovascular disease

for carriers of the FV 506Q allele. In contrast, Juul et al. (85) saw no association between FV R506Q and adult ischaemic stroke in patients from the Copenhagen City Heart Study and from meta-analysis of available data, but the statistical power was too low to rule out a 20% risk increase. According to Lalouschek et al. (89) FV Leiden confers a statistically significantly increased risk of stroke in female smokers (OR 8.8; $p = 0.004$). No interaction between the mutation, smoking and risk of stroke was observed in men. While there is very good evidence for an increased risk of venous thrombosis in carriers of the FV Leiden mutation due to the impaired inactivation by activated protein C, a general role of this mutation in patients with cardiovascular or cerebrovascular thrombosis has not yet been proven. It appears that the mutation is only relevant in selected patient populations, e.g. those with vasospasms, patients with MI or stroke at young age (< 55 years old) or without significant coronary stenosis. Recently, it was reported that FV Leiden represents a risk factor for peripheral arterial disease in patients < 45 years. The increased frequency of FV Leiden in young patients with PAD suggests that underlying minor vessel alterations become clinically relevant in the presence of FV Leiden while in older patients the vessel alterations exceed the added effect of thrombophilia. Interestingly, the authors also found a contribution of polymorphisms in platelet glycoprotein Ia (ITGA2 807T, 837T, 873A) leading to increased levels of glycoprotein Ia-IIa (a2b1 integrin) to the development of PAD (90). However, it has to be pointed out that even though factor V Leiden seems to be a risk factor in certain patient groups, testing may not serve any clinical use.

Recently, an Italian group performed a cost-effectiveness study regarding genetic testing for factor V Leiden and the prothrombin variant in females with a previous thrombosis or family history who requested prescription of oral contraception. They came to the conclusion that in women with a previous VTE, the common testing strategy is cost-ineffective and leads to an overall loss of quality-adjusted life years. They suggest that there is an urgent need to better monitor the indications for

which tests for factor V Leiden and factor II are prescribed (91).

Prothrombin (FII)

FII is a vitamin K-dependent glycoprotein of about 70 kDa and represents the proenzyme of thrombin. Defects in the prothrombin gene can lead to inherited prothrombin deficiencies, which are associated with increased risk of bleeding. In 1996, Poort et al. (92) identified a single nucleotide exchange (G>A transition) in the 3'-untranslated region of the prothrombin gene at position 20210, (rs1799963) which leads to ~25% increase in plasma thrombin activity (93, 94). The mutation, located 20 nt downstream of the poly A signal, increases the post-translational 3' end processing efficiency and causes a higher transcription rate (95). An association between the 20210A allele and the risk for venous thrombosis was described. Reports about an influence of the 20210A allele on the development of arterial thrombosis and MI are contradictory. A meta-analysis performed by Franco et al. in 1999 (96) revealed a statistically significant association of the prothrombin 20210A variant with the development of MI (OR 2.5). A more recent meta-analysis testing the association of factor V Leiden, the prothrombin 20210G>A, and the MTHFR 677C>T (TT genotype) mutations with myocardial infarction, ischaemic stroke, and peripheral vascular disease showed only a modest association of the factor V Leiden mutation (OR 1.21), the PT 20210G>A mutation (OR 1.32), and MTHFR TT mutation (OR 1.20) with arterial ischaemic events. Subgroup analyses of younger patients (<55 years old) and of women revealed slightly stronger associations (97). We investigated the prevalence of the prothrombin mutation in 468 patients with acute stroke or transient ischaemic attack (TIA) before the age of 60 years. We found that the frequency of the F2 20210G>A mutation was significantly higher in male patients compared with controls (6% versus 1%; adjusted OR 6.1). Our data indicate an increased risk of stroke/TIA at young age in men who have the F2 20210G>A mutation (89). The benefit of testing patients with arterial thrombotic diseases for factor V

Leiden (FV) 1691G>A and/or F2 20210G>A polymorphisms is still disputed. Recent data suggests that the F2 20210G>A polymorphism represents a relevant risk factor for MI in about 5% of aged subjects (55–80 years old). Thus, genetic screening may have to be considered in older subjects in whom other major risk factors, such as hypertension and atherosclerosis, are also present (98).

Von Willebrand factor and factor VIII

For an efficient initiation and activation of the haemostatic system, von Willebrand factor and factor VIII are highly important. FVIII is a large glycoprotein which is synthesized in the liver. It is elevated in acute phase conditions like stress, inflammation, etc. The F8 gene is located on the X-chromosome, and mutations within the gene cause haemophilia A. In plasma, FVIII is bound to von Willebrand factor, a large plasma glycoprotein synthesized in endothelial cells and megakaryocytes. VWF mediates platelet adhesion to damaged vascular subendothelium and subsequent platelet aggregation. VWF also acts as stabiliser of FVIII in the circulation. Elevated plasma levels of FVIII and VWF have been associated with an increased risk for MI and stroke (99). In the prospective ARIC study Folsom et al. found out that individuals within the fourth quartile of FVIII levels (median: 171%) have an almost twofold increased risk to suffer from ischaemic stroke) (100).

It is well established that quantitative deficiencies by of von Willebrand factor (VWF) are associated with abnormal haemostasis that can manifest in bleeding or thrombotic complications. Consequently, many studies have attempted to elucidate the mechanisms underlying the regulation of VWF plasma levels. It has been known for many years that the blood group exerts a major influence on plasma levels of the von Willebrand factor (VWF) – factor VIII (FVIII) complex. Individuals who are carriers of blood group 0 have significantly lower VWF and F VIII levels than carriers of blood groups A, B or AB. Therefore, several investigators have studied the association between ABO blood

group and the risk of developing bleeding or thrombotic events (101, 102). Polymorphisms within the ABO blood-group locus have been shown to contribute to the wide population variability of VWF plasma levels. However, there is no doubt that high FVIII and VWF levels are also influenced by other genetic variations. Several association studies have been performed to assess the effect of genetic variants in the VWF gene (VWF) on VWF antigen and activity levels, and on the risk of arterial thrombosis. The studies differed in design, population and endpoint, and were often underpowered. Thus, it is still unclear how VWF levels are genetically modulated and if the genetic variants are causally related to the occurrence of arterial thrombosis.

The VWF gene is large, consisting of 178 kb and 52 exons. It is located on the short arm of chromosome 12, and encodes a protein of 2813 amino acids with several functional domains. A number of studies tested the VWF promoter region for an association with the VWF plasma concentration. The findings were controversial. While some studies showed a significant association of variations within the promoter region with VWF antigen (VWF:Ag) levels (103) other studies did not support these findings (104, 105). Possibly, the discrepancies can be explained by the heterogeneity of the study populations, regarding blood group, age, medical history and ethnicity (106).

Several polymorphisms were identified in the coding regions of the VWF gene. For rs1063856 a strong association with VWF:Ag levels and VWF:RCO activity has been found which is independent of the disease state (107). Interestingly, until today no association between VWF gene loci and CVD endpoints could be established, not even in GWAS studies (108–110). These findings raise the question whether variations in the VWF gene are relevant for the occurrence of arterial thrombosis at all. However, a review of the current literature regarding associations between genetic variations in the VWF gene and the risk of arterial thrombosis shows that VWF cannot be excluded as causal mediator of arterial thrombotic events and further research into the rela-

tionship between VWF and arterial thrombosis is necessary and justified (111).

Other genes may be involved in the regulation of VWF and FVIII levels. Recently, an association of SNPs in *STXBP5*, *STX2*, *TC2N* and *CLEC4M* genes with VWF levels and of *SCARA5* and *STAB2* genes with FVIII levels has been reported. Collectively, these genes explain ~10% of the variability of VWF and FVIII levels (110). Syntaxin 2 (*STX2*) is a binding substrate for syntaxin binding protein 5 (*STXBP5*) and a member of the Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor (SNARE) protein family. SNARE proteins drive vesicle exocytosis, a process involved in the regulation of numerous secretory events, such as exocytosis from Weibel palade bodies (WPB). WPBs and alpha-granules release large amounts of VWF after endothelial cell activation which is important for VWF levels in plasma. Moreover, these storage granules secrete not only VWF molecules but also other substances, such as P-selectin. Hence, these SNARE genes may have a direct effect on the risk of CVD (112).

Von Willebrand factor is a multimeric plasma glycoprotein (GP) which acts as a bridge between the injured subendothelium and the platelet receptors. The multimeric structure of VWF allows it to support multiple interactions with platelets and endothelial components under high shear stress. A specific VWF-cleaving protease (ADAMTS-13) physiologically down-regulates the multimeric size of VWF and prevents unwanted platelet thrombus formation. The proteolysis by the metalloprotease ADAMTS 13 converts hyper-reactive and thrombogenic ultralarge VWF into smaller and less adhesive plasma forms. ADAMTS 13 can directly interact with VWF under static and flowing conditions. Positional cloning approaches identified ADAMTS-13 as the gene responsible for familial TTP. These findings indicated that VWF proteolysis by ADAMTS-13 is a key regulatory pathway for haemostasis (113).

In the FVIII gene the SNP 92714C>G (rs1800291), a non-synonymous SNP encoding the B-domain substitution D1241E (Asp1260Glu), has been found associated with FVIII levels (114). Each C-allele in-

creases the FVIII:C level by 14.3 IU per dL. Recently, the role of copy number variations (CNV) within the F8 gene for levels of FVIII activity and the risk of VTE was investigated in 179 VTE patients and 176 healthy individuals (115). The authors confirmed the association of FVIII levels and VTE, and they could show that CNV in the F8 gene were more frequent in patients and in individuals with high FVIII levels. Thus, the presence of CNV will have to be evaluated and their importance for vascular diseases has to be studied in large cohorts.

The protein C anticoagulant pathway

The protein C (PC) anticoagulant pathway is very central in the control of thrombosis. Protein C is activated on the endothelium by the thrombin-thrombomodulin-endothelial protein C receptor (EPCR) complex. In the presence of the cofactor protein S, activated protein C (APC) proteolytically inactivates coagulation factors VIIIa and Va, thereby inhibiting clot formation. Mutations and polymorphisms of PROC (protein C), PROCR (protein C receptor), and PROS1 (coding for protein S) are associated with risk of familial venous thrombotic disease. Within the promoter region of PROC two polymorphisms, -1654 C/A and -1641 G/T, had been reported to be associated with lower plasma levels (116) and lower transcriptional efficiency of protein C (117). Recently, the PROC rs2069901 variant allele was shown to be more frequent in individuals with lower protein C levels. However, the majority of the inter-individual variation in protein C variation cannot be explained by common polymorphisms of PROC. No evidence for association between PROC or PROS1 genotype and risk of incident CVD or death was found in a study by Reiner et al. (118). In a genome-wide association study (GWAS) it was shown that two SNPs, rs867186 (Ser219Gly) and rs6060278 in the protein C receptor (PROCR) explain about 20% of the variance of plasma PC levels (119). The endothelial PC receptor (EPCR) is mainly expressed on endothelial cells of large vessels. By binding to PC, EPCR accelerates

the rate of PC activation. Once PC is activated (Activated PC, APC), EPCR mediates its antiapoptotic effect on endothelial cells. High levels of plasma sEPCR can be found in patients with e.g. CAD. Several studies demonstrated that sEPCR levels are strongly genetically controlled (120–122). The rs867186 single nucleotide polymorphism in the PROC2 gene (g.6936A_G, c.4600A_G), resulting in a serine-to glycine substitution at codon 219 in the membrane spanning domain of EPCR, explains between 56% and 87% of the variations in sEPCR levels (120). However, in a recent prospective study no effect of high sEPCR levels could be found in patients with CAD. Currently, the physiological role of sEPCR is still unclear. Theoretically, elevated plasma sEPCR levels could increase thrombotic risk via inhibition of PC and APC. Alternatively, high sEPCR could lead to reduced protein C activation. While the effect of Ser219Gly EPCR polymorphism on sEPCR levels has been confirmed in several studies, the association with future vascular or thrombotic events is still controversial (123).

SNPs in platelet glycoproteins: GPIa, P-selectin

Platelet glycoprotein Ia (GPIa)

The role of platelets in thrombotic complications of disrupted plaques is well characterized (124). It is known that occlusive thrombi are initiated by plaque rupture and adhesion of platelets to subendothelial surface. Following endothelium denudement collagen, one of the major proteins of subendothelial vasculature, gets exposed and mediates platelet adhesion and aggregation. Recent advances have led to the identification of two prominent collagen receptors, glycoprotein Ia/IIa (GPIa/IIa or integrin $\alpha_2\beta_1$) and glycoprotein VI (GPVI). Several studies reported that genetic factors in platelet receptors involved in platelet adhesion and activation can influence platelet reactivity and/or their interaction with endothelium, collagen, leukocytes and platelets. The genetic variability of platelet receptors has been related to the risk of arterial thrombosis (125). A polymorphism in the GPIa gene

(ITGA2) at nt 807C>T (rs 1801106, Glu534Lys) has been related to expression density and activity of GPIa (► Fig. 3). The higher surface density of GPIa is augmenting platelet deposition onto collagen under shear stress (126). A four-fold variation in concentrations of GPIa/IIa correlated with significant differences in platelet adhesion to collagen. However, the importance of the 807T variant is controversial. While some investigations suggest a correlation between the 807T allele and the risk of thrombotic events others do not (127, 128). Different frequencies of the rs1801106 polymorphism among various ethnic groups may explain the contradictory results. In the Chinese Han ethnic population, the prevalence of carriers of the 807T allele was markedly higher in patients with unstable angina pectoris (UAP) (129). In this population, the lag time to achieve a 30 % platelet aggregation was significantly longer in CC genotype than in TC genotype carriers, although there was no significant difference in maximal platelet aggregation among healthy subjects with either genotype. Possibly, the platelet membrane GPIa 807T allele confers a rapid initiation of collagen-induced platelet aggregation. In diabetic patients a higher prevalence of the 807T allele supports the generation of more reactive platelets which presumably cause a

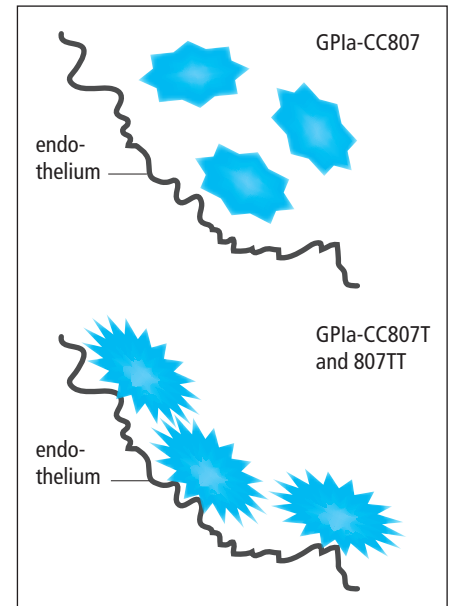


Fig. 3 Expression density of GPIa-IIa on platelets (courtesy of Andreas Greinacher)

persistently increased release of pro-atherogenic TGF-beta and platelet-derived growth factor presumably accelerating the atherothrombotic disease (130). A study on young female patients revealed an association between the GPIa 807C>T polymorphism and ischaemic stroke (131), indicating the possibility of gene – sex hormone interactions. A contribution of GPIa

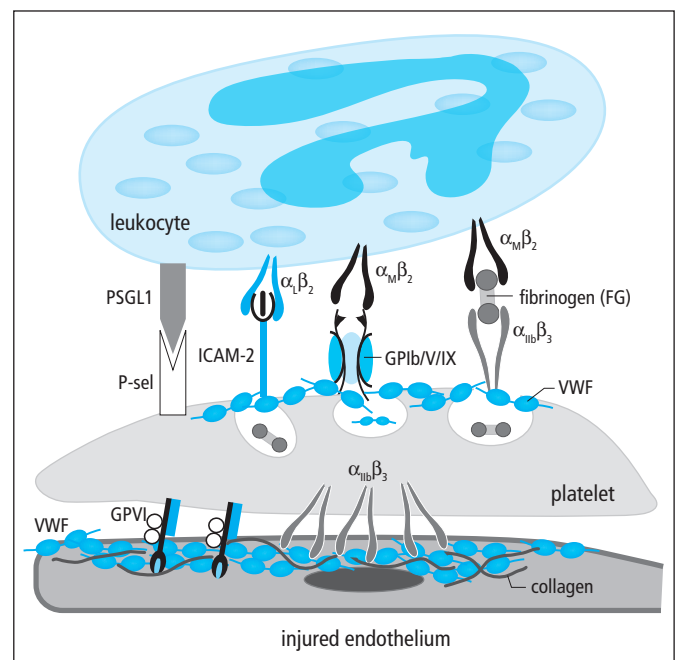


Fig. 4 Interaction of platelets with injured endothelium; modified from Ghasemzadeh M & Hosseini E (150); reprinted with kind permission from Elsevier

807C>T to stroke risk in patient subgroups cannot be ruled out, even though a meta-analysis evaluating candidate genes for ischaemic stroke did not consider the modest association between GPIa 807C>T and ischemic stroke as positive result (132).

P-selectin

Another important platelet glycoprotein is P-selectin which is expressed on the surface of thrombin stimulated platelets (133, 134). In unstimulated resting platelets P-selectin is localized on the membrane of α -granules. It is translocated to the platelet membrane following platelet activation (135, 136). Elevated levels of plasma P-selectin are found in thrombotic consumptive disorders like disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, and heparin-induced thrombocytopenia (137, 138). The plasma P-selectin concentration represents a predictive marker for future cardiovascular events such as myocardial infarction and stroke (139). As shown (► Fig. 4), P-selectin is important for the interaction of leukocytes with the vessel wall and in the early steps of leukocyte recruitment at the sites of inflammation (140). The P-selectin gene spans > 50 kb and contains 17 exons. It is highly polymorphic and several non-synonymous polymorphisms and polymorphisms in the upstream regulatory regions have been identified. Four non-synonymous polymorphisms (S290N – rs6131, D562N – rs6127, L599V – rs6133 and T715P – rs6136) have been studied in more detail (141, 142). It was found that 715Pro is associated with lower soluble P-selectin levels in plasma (sP-sel). 715Pro has also been reported to be less prevalent in myocardial infarct patients than in controls in the ECTIM study. The protective effect of 715Pro was confirmed in a large population with both genders in the United Kingdom (143) and in the ECTIM study (144). The latter group showed that the occurrence of 290Asn and 562Asn on the same allele confers a high risk for myocardial infarction. In 2004, Bugert et al. reported a higher frequency of the 715P allele among CHD patients with hypercholesterolaemia compared to patients with normal cholesterol levels and described a novel

SNP in exon 5 (V168M – rs6125) (145). While there is general agreement about the association of 715Pro with P-selectin levels the association with CHD and ischaemic stroke is still disputed. Volcik et al. did not find differences in the allele frequency between patients and healthy individuals in the prospective cohort of the Atherosclerosis Risk in Communities (ARIC) Study (146). However, in 2007 these authors specified their results by showing that the effect of 715Pro depends on the simultaneous presence of the 290N genotype (147). In combination with 290N the 715P variant was associated with increased risk of incident CHD in whites. This study demonstrated very clearly that the determination of haplotypes is often more informative than the analysis of a single SNP. In 2009, data on the role of 715Pro and three SNPs in the promoter region of P-selectin for the risk of ischemic stroke were reported by Wei et al. (148). The authors found no association between P-selectin gene polymorphisms and ischaemic stroke or any subtype of ischaemic stroke in the Chinese population. Their data is in good agreement with our results published in 2007. We saw no significant interaction of the 715Pro variant with vascular risk factors, stroke severity, or stroke etiology and no association with risk or clinical characteristics of ischaemic stroke (149). However, we found a trend towards an association in patients with diabetes. Therefore, further studies are needed to explore a possible interaction between environmental factors and P-selectin gene polymorphisms and haplotypes in ischaemic stroke, particularly in ethnically different populations.

Epigenetic modifications

The complete platelet transcriptome and proteome as well as many important protein-protein interactions that are critical for platelet function under normal and pathological conditions such as cardiovascular disease (CVD) have been well explored. However, only recently, epigenetic processes such as DNA methylation and histone modification have received attention. Epigenetic marks are erased in early embryogenesis and reset during development. Genes modulating the epigenetic status of

the chromatin are systematically activated in the placenta. In contrast, tissue-specific differences exist in regard to transcriptional alterations. Gene pathways are commonly altered in organ-dependent manner (151). Furthermore, environmental influences can lead to changes in the epigenome that alter the individual's susceptibility to disease. In 2009, results on epigenetic modifications in serotonin (5-HT) released from platelets were published. Peripheral 5-HT is actively taken up by platelets and stored in the dense granules. As a result, 5-HT is widely distributed in the body through the blood flow. High 5-HT levels have been proposed to be predictive of coronary artery diseases, especially in young people. Platelet 5-HT is degraded by monoamine oxidase and high plasma levels of the degradation product 5-hydroxyindolacetic acid have been shown to be correlated to cardiovascular risk. The correlation was modulated by smoking. It was found that cigarette smoke induced an increase of nucleic acid demethylase activity and reduced the methylation of the monoamine oxidase gene promoter, leading to a more active transcription of the gene and hence a greater protein concentration. The report showed that smoking induces an important epigenetic modification (152). Preliminary evidence obtained for the G-protein cluster suggests that changes in DNA methylation can alter platelet activity (153). However, much more work is needed to clarify the role of imprinting for platelet function.

Currently, information regarding epigenetic modifications of haemostatic proteins is extremely scarce which is not surprising as methylation marks are tissue-specific and it is uncertain whether leukocyte DNA reflects the correct epigenetic signature of coagulation factors. Only the F7 promoter methylation pattern has been studied and related to F7 promoter polymorphisms. It was shown that plasma FVIIa was inversely related to methylation of the F7 gene promoter. A higher FVIIa paralleled the lower methylation in the F7 gene promoter. Variation in methylation was associated with the different –402G>A genotypes. As a higher methylation was found in the CAD – free group it was speculated that the epigenetic regulation

through methylation of the F7 promoter is associated with CAD by affecting plasma FVIIa concentrations (15).

Conclusions and clinical consequences

Arterial thrombosis, with MI and stroke as severe complications, represents the most frequent cause of death in the western world. Although there is no doubt that genetic factors contribute significantly to the prothrombotic state, data on mutations and polymorphisms in candidate genes are still inconclusive. However, one has to be aware that there may be reporting or publication bias for certain observations. Furthermore, the study populations may differ significantly between different studies. Therefore, all results have to be viewed critically.

While some laboratory parameters correlate consistently with diseases (e.g. fibrinogen levels with MI), and polymorphisms in certain genes are associated with plasma levels of the respective proteins [e.g., fibrinogen -455G>A polymorphism with plasma fibrinogen levels], positive correlations between genotype and disease phenotype can often not be established.

Up to now our knowledge regarding polymorphisms in coagulation factor genes predisposing to arterial thrombosis is insufficient. This is not surprising as the pathogenesis of arterial thrombosis is a complex process in which environmental and genetic factors interact. The clinical manifestation of MI or stroke represents the common endpoint of several different processes, and a single gene variation will contribute only a small percentage to the total risk and will not reflect these complex events. Well-defined and homogenous subgroups of patients may help as they may share a common "pathogenetic" genotype. For example, in the patient subgroup with MI caused by vasospastic angina with subsequent coronary thrombosis, the FV 506R>Q mutation is a highly significant risk factor while the contribution of the FV 506R>Q mutation to the relative risk for MI in unselected patients is modest. The simultaneous testing of numerous polymorphisms or the genome wide association

analysis could represent suitable approaches to identify genetic risk profiles for thrombosis. Unfortunately, currently available statistical methods are unable to reliably identify combined risk genotypes. Thus, appropriate algorithms to evaluate the data produced by new genotyping technologies have to be developed, and this remains a demanding task for the years to come.

Conflict of interest

The author declares that she has no conflict of interest.

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