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Role of plasma kallikrein in diabetes and metabolism

Edward P. Feener*, Qunfang Zhou, and Ward Fickweiler

Research Division, Joslin Diabetes Center, Department of Medicine, Harvard Medical School

Summary

Plasma kallikrein (PK) is a serine protease generated from plasma prekallikrein, an abundant circulating zymogen expressed by the *Klkb1* gene. The physiological actions of PK have been primarily attributed to its production of bradykinin and activation of coagulation factor XII, which promotes inflammation and the intrinsic coagulation pathway. Recent genetic, molecular, and pharmacological studies of PK have provided further insight into its role in physiology and disease. Genetic analyses have revealed common *Klkb1* variants that are association with blood metabolite levels, hypertension, and coagulation. Characterization of animal models with *Klkb1* deficiency and PK inhibition have demonstrated effects on inflammation, vascular function, blood pressure regulation, thrombosis, hemostasis, and metabolism. These reports have also identified a host of PK substrates and interactions, which suggest an expanded physiological role for this protease beyond the bradykinin system and coagulation. The review summarizes the mechanisms that contribute to PK activation and its emerging role in diabetes and metabolism.

Keywords

Plasma kallikrein; diabetes; *Klkb1*; contact activation pathway

Plasma Kallikrein

Plasma kallikrein (PK; EC 3.4.21.34) is serine protease with well-characterized effects in innate inflammation and the intrinsic coagulation cascade (1). This protease is generated from zymogen activation of plasma prekallikrein (PPK); an abundant 20–50 ug/ml (~0.5 μ M) circulating 86–88 kDa glycoprotein. PPK is the product of the *Klkb1* gene, which is highly expressed in liver > pancreas > kidney, and to a much lesser extent in a variety of other organs, including brain, heart, spleen, thymus, testis, and intestine (2;3). Cleavage of PPK at Arg371 results in disulfide-linked light and heavy chains, which reveals its catalytic activity and exposes protein-binding domains. The catalytic site is located in the C-terminal light chain and exhibits cleavage selectively toward Arg-X and Lys-X sites with a preference for dibasic sequences (4). The N-terminal heavy chain includes four apple-like domains that mediate the binding of PK to high molecular weight kininogen (HK) a primary cofactor and substrate (5), as well as other potential protein interactions, such as extracellular matrix (6;7).

*Correspondence: Edward P. Feener, PhD, Joslin Diabetes Center, One Joslin Place, Boston, Massachusetts 02215; USA, TEL: (617)309-2599, FAX: (617)309-2637, edward.feener@joslin.harvard.edu.

The majority of PK's physiological actions have been attributed to cleavage of its two primary substrates and cofactors, namely FXII and HK. Conversion of FXII to FXIIa leads to activation of FXI and the intrinsic coagulation cascade resultant in fibrin production and thrombus stabilization. Cleavage of HK releases the nonapeptide bradykinin, which is the ligand for the G protein-coupled B2 receptor (B2R). Subsequent cleavage of bradykinin by carboxypeptidases generates des-Arg⁹-bradykinin, which binds and activates the B1 receptor (B1R). Activation of B2R and B1R by bradykinin and des-Arg⁹-bradykinin, respectively, have been implicated in nearly all the effects of the plasma kallikrein kinin system (plasma KKS) on inflammation, vascular function, blood pressure regulation, and nociceptive responses (8).

Although the mechanisms that contribute to the zymogen activation of PPK *in vivo* are not fully understood, factor XII (FXII) deficiency reduces basal bradykinin levels by ~50% and blocks bradykinin production generated by contact activation (9); demonstrating the physiological importance of FXII in PPK activation. Interactions of FXII with an activating surface results in its conversion to a serine protease (FXIIa), which mediates cleavage of PPK to PK leading to positive feedback cleavage of FXII to FXIIa (10). This amplification process can rapidly, locally, and robustly increase plasma contact system activation. PK and FXIIa bind to extracellular matrix proteins, including collagen and laminin (6;7;11;12), which may contribute to retention and amplification of PK activation in affected tissue. The recruitment of inflammatory cells (13) and the release of polyphosphate (14) from activated platelets further support contact system activation. The catalytic activities of PK and FXIIa are inhibited by abundant circulating serine protease inhibitors, including C1-inhibitor (C1-INH), alpha 2 macroglobulin (α2M), and protein C inhibitor. C1-inhibitor deficiency can lead to uncontrolled PK activity, which is a clinically significant cause of hereditary angioedema (15).

Genetic Influences

A limited number of rare genetic Klkb1 mutations, including for example nonsense variants, have been identified as causes of PPK deficiency (16). Affected individuals are often first identified based on prolonged activated partial thromboplastin time (APTT). The contribution of PPK to APTT has recently been confirmed using Klkb1 deficient mice, which exhibit a 3–5 fold prolongation in APTT (7;17). Recent genome-wide association studies (GWAS) have identified additional common SNPs in the Klkb1 locus that are associated with levels of circulating metabolites and complex metabolic diseases (Table 1). While most of these Klkb1 single nucleotide polymorphisms (SNPs) are in non-coding regions, at least one common variant (minor allele frequency, 37%) in exon 5 results in an amino acid substitution Asn124Ser (18). This variant in the heavy chain apple 2 domain influences HK binding, which is critical for PK function (19). Moreover, this report identified a family with a second variant, Gly104Arg, in the apple 2 domain and showed that the combination of Ser124 and Arg104 resulted in severe deficiency in PK activity and prolongation in APTT. GWAS on a European cohort in the Prevention of Renal and End-Stage Disease (PREVEND) study identified strong associations of the Klkb1 SNP (rs3733402) encoding the Asn124Ser variant with proteolytic products of pre-pro-endothelin-1 (CT-pro-ET-1, $P=4.46E-52$) and pre-pro-adrenomedullin (MR-pro-ADM,

$P=1.23E-122$) (20). In addition, this report demonstrated PK cleavage of pre-pro-ET-1 and -ADM precursors, which encode potent vasoactive factors involved in blood pressure regulation. GWAS of 815 Hispanic children also revealed an association of free insulin-like growth factor 1 (IGF-1) with rs3733402 (21), however the physiological connection between *Klkbl* and IGF-1 is unknown. GWAS on a European population revealed a common variant (rs4253252, 40%) in the *Klkbl* locus is associated with serum bradykinin levels and hypertension (22). This study showed that the minor allele (T) is associated with lower concentration of serum des-Arg9 bradykinin compared with the major allele (G), suggesting genetic factors influence PK activity in a large fraction of the human population. GWAS on a Finnish cohort of 8,330 individuals revealed an SNP (rs4241816) located in a *Klkbl* intron that is associated with serum histidine levels and the histidine to valine ratio (23). Although the molecular basis for this SNP association is not yet available, histidine metabolism by histidine decarboxylase generates histamine, which is a proinflammatory mediator that may interact with *Klkbl* pathways. Candidate gene case control studies have identified an association between rs3087505 in the 3' UTR of *Klkbl* and venous thrombosis (24;25). Recent reports have shown that PPK deficiency in mice provides protection against venous thrombosis induced by $FeCl_3$ (17;26), which provides experimental evidence consistent with the clinical findings. Analysis of a cohort of 2544 individuals from the British Women's Heart and Health using a high-density cardiovascular-focused genotyping array reveal SNP associations for FXII, HK and *Klkbl* (rs4253304) with APTT (27). In addition to the potential influence of genetic variants in the *Klkbl* locus, functional variants in other contact system components, including FXII, HK, and C1-INH, could alter PPK activation or function. While a limited number of mutations and epistatic variant interactions in contact system components may be detected with prominent phenotypic effects, such as prolongation of APTT or susceptibility to angioedema, recent observations from large population-based genetic studies have revealed that *Klkbl* variants with high minor allele frequencies are associated with quantitative metabolic traits and complex diseases.

Role of Plasma Kallikrein in diabetes

The plasma KKS has been implicated in a variety of coagulation, vascular, and metabolic abnormalities in diabetes mellitus. However, most of the physiological effects of the KKS have been examined using bradykinin receptor-targeted approaches. While these studies provide important understanding of the actions and regulation of the bradykinin system, the contributions of PK-derived bradykinin in these experimental models are usually not available. Tissue kallikrein (*Klk1*, EC: 3.4.21.35) is also a major source of bradykinin receptor agonists. Tissue kallikrein cleaves low molecular weight kininogen to liberate Lys-bradykinin (kallidin) that can be metabolized to Lys-desArg9-bradykinin, which are B2R and B1R agonists, respectively. Thus, bradykinin receptor functions are associated with both PK and tissue kallikrein systems, which have different regulatory mechanisms and could have markedly different contributions to bradykinin peptide action among various tissues and diseases, including diabetes. Measurements of PK levels/activity and the direct measurement of PK's actions have provided insight into its *in vivo* functions, which include but are not limited to the bradykinin system. The recent availability of selective PK inhibitors and *Klkbl* deficient mice have created opportunities to further investigate the

actions of PK on coagulation, vascular function, blood pressure regulation, metabolism, and wound healing (Figure 1). These approaches are beginning to characterize the specific actions of PK, apart from the bradykinin system, in diabetes and its complications.

APPT in diabetes

Analysis of coagulation parameters in a cohort of 6,185 outpatients revealed APTT shortening in subjects with impaired fasting plasma glucose (FPG) concentrations (5.6–6.9 mmol/L) and in diabetic individuals (>7.0 mmol/L) compared with euglycemic participants (28). In contrast, prothrombin time (PT) and fibrinogen levels were similar among these groups. Zhao et al confirmed this inverse correlation of APTT with FPG concentration in a population of 1,300 patients in China and also showed that APTT inversely correlates with HbA1c, with diabetic subjects (HbA1c 6.5) displaying shorter APTT compared with subjects with HbA1c 5.7–6.4 and the APTT for both of these groups were shorter compared with euglycemic subjects (HbA1c 5.6)(29). PT was not significantly different in these groups characterized according to either FPG or HbA1c. These population-based studies have provided evidence for intrinsic pathway activation in diabetic patients. However, the mechanisms contributing to APTT shortening in diabetes are not fully understood. Patrassi et al reported that both prekallikrein and factor XII activities are increased in diabetes (30). An analysis of a limited number of patients with diabetes indicated increased PPK concentration compared with nondiabetic subjects (31). This study also showed a negative correlation between PPK and APTT, suggesting that prekallikrein contributes to APTT shortening in diabetes. In a cohort study of 47 diabetic patients and 20 control subjects, PK activity was significantly higher in subjects with diabetes compared with controls (1.04 ± 0.04 versus 0.69 ± 0.04 U/ml, $p < 0.001$)(32). A recent study showed that PPK is increased in plasma of diabetic rats compared with controls (33). APTT and tail bleeding time are shortened in diabetic rats (7;34), and these coagulation times were normalized by systemic administration of a small molecule PK inhibitor (7). Taken together, these reports indicate that diabetes is associated with increased intrinsic pathway activity, which are mediated in part by PK.

Cerebral hemostasis in diabetes

Although diabetes is considered a hypercoagulable state (35), there is a paradoxical increase in hematoma volumes following intracerebral hemorrhage (ICH) and an elevated risk of hemorrhage during thrombolytic therapy in hyperglycemia and diabetes (36). Liu et al have shown that diabetic rodents exhibit increased hematoma expansion in an experimental model of ICH induced by intracerebral injection of autologous blood (7). This impairment in cerebral hemostasis in diabetes was ameliorated by PK inhibition and *Klkbl* deficiency, and mimicked by intracerebral injection of PK (7). The report demonstrated that PK blocked collagen-induced platelet aggregation. PK also inhibited platelet activation induced by collagen-related peptide, a glycoprotein VI selective agonist, but not by other platelet activators, including thrombin, ADP, and convulxin. These findings suggest that PK interferes with collagen-mediated activation of platelet glycoprotein VI receptors, which is an early step in platelet aggregation and hemostasis in damaged blood vessels. The nature of the interaction between PK and collagen is not fully understood, however this report showed that PPK did not block collagen-induced platelet aggregation and that the inhibition of

collagen-stimulated by PK was retained following covalent inactivation of its catalytic site (7). These findings suggest that PPK activation to PK reveals its collagen-binding domain, which is functionally separate from its catalytic site. This appears consistent with a previous report showing that collagen-bound PK retained its catalytic activity (6). Taken together these findings suggest that PK binding to exposed subendothelial collagen interferes with platelet activation and may contribute to local retention of PK activity.

Vascular permeability and inflammation

Increased vascular permeability is a hallmark of diabetic vascular complications. The pathological increase in vascular permeability in diabetes contributes to both microvascular and macrovascular diabetic complications. In diabetic retinopathy the breakdown of blood retinal barrier function is a primary cause of diabetic macular edema (DME). This sight threatening disease is a leading cause of vision loss in working age adults in developed countries (37). Proteomic and pharmacological studies have suggested that increased plasma KKS component levels and activities contribute to retinal vascular hyperpermeability in diabetes (recently reviewed elsewhere (38)). Intravitreal injection of PK induces retinal vascular permeability, thickening, leukostasis, and hemorrhage (33;39). Systemic administration of a small molecule PK inhibitor decreased retinal vascular permeability induced by hypertension (40) and by diabetes (33). These findings are consistent with the well-characterized effects of the plasma KKS on vascular permeability, inflammation, and edema (41).

Glucose and Energy Homeostasis

The effects of bradykinin receptors in diabetes have received considerable investigation (42–45), however much less is known regarding the role of PK in glucose and energy homeostasis. Recent reports have shown that blood glucose levels were not altered by *Klkbl* deficiency in mice compared with wild-type controls (7) and by systemic PK inhibition in rats (33). While these findings indicate that PK is not required for maintenance of blood glucose homeostasis, information on the potential influences of PPK deficiency or hyperactivity on specific glucose regulatory mechanisms in diabetes and conditions of metabolic stress are not yet available.

Although HK and FXII are the most extensively characterized PK substrates, *in vitro* experiments have revealed that PK exhibits proteolytic activity towards a number of peptides and proteins (4;20;46–49). Recent studies have suggested that PK can cleave and inactivate both glucagon like peptide-1 (GLP-1) (50) and neuropeptide Y (NPY) (51). These findings have suggested that PK may exert effects on metabolism and diabetes that extend beyond bradykinin receptors. Dipeptidyl peptidase-4 (DPP-4) is the major GLP-1 degrading enzyme in human plasma (52;53), which cleaves GLP-1 at Ala⁸-Glu⁹, near the N-terminus. In addition to this N-terminal cleavage, GLP-1 is also susceptible to cleavage at its C-terminus (50;54). GLP-1 analogs with C-terminal truncation GLP-1(7-34) displayed impaired potency compared with GLP-1(7-37)(55). A GLP-1 analogue containing a α -aminoisobutyric acid substitution at position 35 (taspoglutide) has been developed that is resistant to PK-mediated cleavage at the C-terminus and displays improved plasma stability (50). Interestingly, the T-Emerge 2 trial has recently reported the once-weekly efficacy of

tasoglutide for HbA1c reduction in type 2 diabetes (56), revealing a prolongation of GLP-1 agonist efficacy compared with exenatide. An analysis of neuropeptide Y (NPY) stability in human serum has provided further evidence for a role of PK in regulatory peptide metabolism (51). NPY is a 36 amino acid peptide involved in central and peripheral control of energy balance and blood pressure regulation. DPP-4 mediates the conversion of NPY (1-36) to NPY(1-36); resulting in loss of Y1 receptor binding while maintaining interactions with Y2 and Y5 receptors. Abid et al has shown that NPY(3-36) undergoes C-terminal cleaved by PK to generate NPY(3-35), which displays decreased binding to Y2 and Y5 receptors (51). These findings suggest that PK can influence regulatory peptide metabolism involved in glucose and energy homeostasis, however clinical significance remains to be determined.

Adipogenesis

PK-mediated activation of the plasminogen cascade has been implicated in adipocyte differentiation and adipogenesis during mammary gland involution via activation of plasmin-mediated cleavage of preadipocyte stromal matrix (57;58). Incubation of 3T3-L1 cells with an ecotin inhibitor selective for PK reduced cell differentiation to an adipocyte phenotype (57). Systemic administration of the ecotin PK inhibitor to neonatal mice reduced numbers of mature mammary gland adipocytes and stromal remodeling (57;58). PK mediates the conversion of plasminogen to plasmin *in vitro*, however this biochemical activity of PK appears markedly less potent compared with tPA (7;57;59). These reported physiological functions of PK on adipogenesis are largely based on responses of mice to pharmacological inhibition by a single type of PK inhibitor, namely a PK-specific ecotin variant. Recent reports have indicated that Klkb1 deficiency does not significantly affect body weight (7;17), however data on specific adipose depots in these mice are not yet available. Further characterization of Klkb1 deficient mice and additional interventions specifically targeting PK are needed to further evaluate its effects on adipogenesis, mammary gland involution, and other plasmin cascade-related processes, including wound healing (59).

Potential mechanisms of PPK activation in diabetes

While pharmacological evidence for a role for PK in vascular dysfunction in diabetes is beginning to emerge (33;60), the mechanism(s) contributing to increased PK action remains poorly understood. PK activation in diabetes could be the result of both local activation at sites of vascular dysfunction and injury, including activated coagulation systems, as well as the effects of systemic factors that may increase circulating activity of the contact system (Figure 1). The mechanisms that contribute to increased contact system activity at local sites of inflammation and thrombosis have received considerable investigation and include activators of FXII (including heparin (13), polyphosphate (14), protein aggregates (61), exposed extracellular matrix (6;7;11;12)), activators of PK activity (carbonic anhydrase (60)), and increased local concentrations of contact system proteins (including vitreous fluid (62) and early atherosclerotic lesions (63)). These mechanisms may locally contribute to increased PK action in tissues affected by diabetic complications, which usually involve chronic worsening of vascular injury. Diabetes is also associated with increases in systemic

factors that have been implicated in contact system activation, such as AGE-modified proteins, platelet-derived microparticles, and activated endothelium. Although the effects of these factors on contact system activation in the diabetic milieu are not yet available, findings from other systems suggest that multiple points for contact system activation may contribute to PPK activation in diabetes and other metabolic disorders.

Advanced glycation endproducts

Hyperglycemia increases nonenzymatic glycation of protein on the primary amino groups of proteins leading to the formation of advanced glycation endproducts (AGEs). These posttranslational modifications have been extensively characterized and shown to contribute to inflammation and diabetic complications (reviewed in (64)). The effects of AGE-modified protein on vascular permeability and inflammation are blocked by administration of a soluble form of the receptor of advanced glycation endproducts (RAGE) (65). Maas et al (61) have reported that AGE-modified serum albumin can activate FXII leading to the generation of PK. These interesting findings could implicate a potential role for nonenzymatic glycation in PK activation in diabetes, however the physiological significance of endogenously-generated AGEs on the contact system is not yet available.

Microparticles

Microparticles (MPs) are small vesicles with a diameter in the range of 100 nm to 1 µm that are shed from stimulated and apoptotic cells. Circulating MPs are derived from platelets and a variety of cell types, including endothelial cells, monocytes, and erythrocytes. The surface of MP has procoagulant properties due to exposure of phosphatidylserine on the outer bilayer and the surface assembly of coagulation factors and complexes, potentially involving gC1qR(66). Van Der Meijden et al (67) has reported that platelet and erythrocyte MP from healthy individuals initiate thrombin generation via FXII, whereas thrombin generation on monocyte MP is mediated by tissue factor. This report also shows that platelet-, erythrocyte-, and monocyte-derived MPs, activate FXII in the presence of PPK and HK. These findings indicate that plasma MP can provide an activating surface for the contact system, which may contribute to the activation of circulating PPK. Aleman et al (68) reported that platelet MPs from healthy donors did not have tissue factor activity and exhibited a low level of contact-dependent thrombin generation compared with monocyte MPs, which exhibited tissue factor-dependent thrombin generation. In contrast, Nieuwland et al (69) has shown that thrombin generation by platelet MPs obtained from patients undergoing cardiopulmonary bypass surgery involved tissue factor and was FXII-independent. These findings demonstrate heterogeneity of MPs, which are influenced by disease and metabolic state (70). Increased levels of MPs have been identified in a number of chronic metabolic diseases, including diabetes (71;72), metabolic syndrome (73), obesity (74;75), and hypertension (76;77). Moreover, high levels of MP derived from platelets are implicated in diabetic vascular complications, including diabetic retinopathy and macrovascular disease (78–81). Since MP can activate the contact system and shorten APTT (67;82), elevated levels of MP in diabetes may also contribute to circulating PK activity.

Vascular Endothelium

PK activation can occur on the vascular endothelium via by both FXII-dependent and FXII-independent mechanisms. In the plasma, PPK is bound to HK, which exhibits high affinity binding to gC1qR expressed on endothelial cells (83). This complex in combination with cytokeratin 1 (CK1), FXII, urokinase plasminogen activator receptor (uPAR), facilitates the assembly and activation of the KKS (84). PPK activation on the endothelium can also occur via PRCP in the absence of FXII (85). The role of gC1qR in PK activation on the endothelium has been examined in experimental models, which demonstrated that an antibody that blocks HK binding to gC1qR blocks PK activation (86). Stimulation of angiotensin AT2 receptors on endothelial cells increases PK activity and resultant production of bradykinin (87;88). This mechanism has been implicated in contributing to the effects of AT1 receptor blockade on the activation of PK and increased bradykinin levels (89). Angiotensin II-induced hypertensive rats exhibit increased retinal vascular permeability that is ameliorated by systemic PK inhibition (40), revealing for a role of PK in angiotensin II action *in vivo*.

Future directions

The application of highly selective inhibitors and genetic approaches has provided new insight on the role of PK in physiology and disease. This work has confirmed PK's role in the intrinsic coagulation and bradykinin system, and has also revealed novel functions of PK that appear independent of these systems. Pharmacological interventions that inhibit PK may provide opportunities to control the proinflammatory effects of bradykinin peptides while preserving the physiological functions of tissue kallikrein that are mediated by bradykinin receptors, suggesting that PK inhibitors and B2R antagonists could have markedly different physiological and therapeutic effects. For example, selective PK inhibitors would not interfere with the physiological effects of tissue kallikrein-mediated B2R activation in the heart and kidney whereas inhibition PK would decrease the activation of both B1R and B2R at sites of contact system-mediated inflammation. In contrast, B2R antagonists would indiscriminately block both PK and tissue kallikrein actions, and would not inhibit B1R and bradykinin-independent effects of PK. An important challenge for the PK field is the development of strategies to monitor PK activity. Assessment of the activation state of PK *in vivo* is limited by the absence of specific assays and biomarkers that accurately quantify the activated pool of PK. This limitation is further complicated by the contributions of multiple mechanisms that modulate PK activity, the heterogeneous *in vivo* distribution of PK, which involves interactions with various cells types, structures, and protease inhibitors, and potential genetic influences. Since FXIIa is a primary physiological activator of PPK, information on FXII and contact system activation may provide insight, albeit indirect, on PK activation. Novel strategies, such as the use of nanobodies to assess FXIIa activation states (90), could provide exciting new opportunities to identify physiological and disease-related mechanisms that regulate PK activation. Information on the activation status of PK is critical for evaluating the effects of diabetes on PK activity and understanding the physiological mechanisms that modulate this pathway.

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Figure 1. Plasma Kallikrein (PK) activation and function

Multiple local and systemic factors have been implicated in contact system activation. The functions indicated with solid arrows are supported by clinical data.

Table 1

Genetic variants of Klkb1

SNP (position)	MAF ^I	Gene-region	Biochemical function and/or genetic association	References
Rs3733402 (187395028)	37.3	Exon 5	Asn124Ser-substitution in apple domain 2 (A2) of the PK heavy chain reduce the binding activity of A2 to HK	Katsuda et al. (2007)
			MR-pro-ADM (P=2.71E-36) and CT-pro-ET-1 (P=1.41E-68) plasma levels	Verweij et al. (2013)
			Serum-free IGF-1 levels (P=9.01E-08)	Comuzzie et al. (2012)
Rs4253238 (187385381)	37.1	2-kb upstream	Higher MR-pro-ADM (P=4.46E-52) and CT-pro-ET-1 (P=1.23E-122) plasma levels	Verweij et al. (2013)
Rs1912826 (187386534)	40.2	Intron	MR-pro-ADM (P=2.07E-51) and CT-pro-ET-1 (P=5.13E-121) plasma levels	Verweij et al. (2013)
Rs4253252 (187394452)	40.4	Intron	Serum bradykinin levels (P=6.6E-18) and hypertension (PKORA=1.7E-09; PTWINSUK=0.0495)	Suhre et al. (2011)
Rs4241816 (187389321)	40.2	Intron	Serum histidine levels (P=2.2E-11) and ratio of histidine to valine (P=5.6E-13)	Kettunen et al. (2012)
Rs3087505 (187416480)	7.3	3' UTR	Venous thromboembolism (P=0.02) and plasma FXI (P=0.03)	Austin et al. (2011)
			Venous thromboembolism (P=4.34E-07)	Heit et al. (2011)
Rs4253304 (187410565)	33.8	Intron	APTT (P=1.67E-07)	Gaunt et al. (2012)

^I Minor allele frequency (MAF); <http://www.ncbi.nlm.nih.gov/projects/SNP>