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A Mutation in the Thrombomodulin Gene, ^{127}G to A Coding for Ala25Thr, and the Risk of Myocardial Infarction in Men

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Summary

Thrombomodulin is an endothelial cell surface receptor that transforms the procoagulant thrombin into an anticoagulant. A mutation in the thrombomodulin gene is a potential risk factor for venous and arterial thrombosis.

We screened a region within the coding sequence of the thrombomodulin gene by single-strand conformation polymorphism analysis (SSCP) in a pilot study of 104 patients with myocardial infarction and 104 age, sex and race matched controls. We identified a ^{127}G to A mutation in the gene, which predicts an Ala25Thr substitution, in 2 out of 104 patients (1 man and 1 woman) with myocardial infarction but in no controls. We assessed the risk of myocardial infarction associated with the mutation in a larger "Study of Myocardial Infarctions Leiden" (SMILE). Among 560 men with a first myocardial infarction before the age of 70, 12 were carriers of the Ala25Thr substitution. In a control group of 646 men, frequency-matched for age, seven were carriers of the Ala25Thr substitution. The allelic frequencies were 1.07% among patients and 0.54% among controls suggesting risk associated with the mutation [odds ratio (OR) 2.0, 95% confidence interval (CI) 0.8-5.1]. In patients aged below 50, the predicted risk was almost seven times increased (OR 6.5, CI 0.8-54.2). In the presence of additional risk factors, such as smoking and a metabolic risk factor, the predicted risk increased to 9-fold (OR 8.8, CI 1.8-42.2) and 4-fold (OR 4.4, CI 0.9-21.3), respectively.

While not conclusive, these results strongly suggest that the Ala25Thr substitution is a risk factor for myocardial infarction, especially in young men, and when in the presence of additional risk factors.

Introduction

Thrombomodulin is an endothelial cell membrane proteoglycan receptor for thrombin. Once bound to thrombomodulin thrombin loses most of its procoagulant properties, such as its ability to coagulate fibrinogen, to activate factor V and platelets (1, 2). Mechanisms that counteract coagulation are stimulated. The thrombin-thrombomodulin complex rapidly activates protein C, which with protein S as cofactor then cleaves and inactivates the co-factors Va and VIIIa, with inhibition of thrombin generation as a result. Besides this mechanism, thrombomodulin is known to stimulate the inhibition of thrombin by antithrombin (3, 4). Another function of thrombomodulin is to activate thrombin activatable fibrinolysis inhibitor (TAFI; also known as plasma carboxypeptidase B or carboxypeptidase U) which inhibits the degradation of the fibrin network by plasmin (5).

Anticoagulant mechanisms such as that involving protein C/thrombomodulin are of crucial importance in the prevention of thrombosis. Individuals with mutations in genes that encode protein C, protein S, antithrombin and factor V Leiden, have an increased tendency toward venous thrombosis (6-11). Carriers of mutations in genes coding for other clotting (co)-factors, such as fibrinogen and prothrombin, may also be at increased risk for venous (12, 13) and for arterial disease (14-16).

Mutation in the thrombomodulin gene is also a potential risk factor for venous and arterial thrombosis. This has not been extensively investigated, due to an inability to select individuals with an abnormal phenotype because the protein is integral to the endothelial cell membrane.

To date, a few variations in the thrombomodulin gene have been described (17-20). The risk of venous and arterial thrombosis associated with these mutations has been assessed for some of these in small groups of patients with varying results (20-25). While certain of these mutations are associated with thrombosis, raising the possibility of causality, none of the clinical studies have been large and none of the mutations have been characterized in detail in *in vitro* test systems. Further evidence is therefore required to confirm or refute the role of thrombomodulin mutations in thrombosis.

In this report, we present such evidence. In a pilot study of 104 patients with myocardial infarction we have screened the 5' and the coding regions of the thrombomodulin gene by single-strand conformation polymorphism analysis (SSCP) and have identified a mutation. In a large case-control "Study of Myocardial Infarctions Leiden" (SMILE) we evaluated the risk of this particular mutation for myocardial infarction in men.

Methods

Patients and Controls

Pilot study

The patients and controls have been described in a previous report of screening of the thrombomodulin gene promoter region (25). The patient group consisted of 104 unselected individuals admitted to hospital who had confirmed myocardial infarction using WHO criteria. Controls were matched on a 1:1 ba-

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sis for sex, age and race using patients attending the outpatient department of Charing Cross Hospital, London, UK, for blood tests. The only criteria used for exclusion of controls was that they had had venous or arterial thrombosis. This was ascertained by questionnaire and signed consent was given after the study had been fully explained. The study was approved by the Ethics Committees of West Middlesex and Charing Cross Hospitals.

SMILE

The Study of Myocardial Infarctions Leiden (SMILE) is a large population-based case-control study. Men aged 70 years or less, with a first myocardial infarction between January 1990 and January 1996 were eligible for this study. Potential cases were identified from computerized discharge records of an academic and a general hospital, both in Leiden, the Netherlands. Two of the following three characteristics had to be identifiable in the discharge report or hospital care record to confirm acute myocardial infarction: typical chest pain, electrocardiographical changes indicative of evolving myocardial infarction or a transient rise in cardiac enzymes to more than twice the upper limit of normal.

As a control group, men with an orthopaedic intervention between January 1990 and May 1996 were invited. The orthopaedic intervention varied from one person to another, for example a plaster cast for a ruptured hamstring or a hip replacement. These controls were identified via the Leiden Anticoagulation Clinic and had received prophylactic anticoagulation for a few weeks or months after the orthopaedic event. In the Leiden region it is routine procedure to prescribe anticoagulant treatment to every person who is (partly) immobilized, temporarily or for a longer period. The controls did not have a history of myocardial infarction and had not used anticoagulants for at least 6 months prior to participation in this study. The controls were frequency matched to cases on 10-year age groups.

Both patients and controls were born in the Netherlands and were living in the Leiden region. Excluded were men with renal disease (n = 10), severe (neuro)psychiatric problems (n = 28), or a life expectancy less than one year (n = 16). The response among the remaining patients and controls was 84.3% (560) and 77.0% (646), respectively. The study protocol was approved by the Ethics Committees of both hospitals.

All individuals completed a questionnaire concerning the presence of cardiovascular risk factors such as smoking habits and alcohol consumption. For patients all questions referred to the period prior to their myocardial infarction. The Quetelet index was derived by dividing weight (kilograms) by squared height (m^2). Individuals were considered obese if their Quetelet index exceeded 30 kg/m^2. Medication use and history of diabetes were ascertained by interview with controls and retrieved from discharge letters for patients. A person was classified as hypertensive or hypercholesterolemic when he was prescribed specific medications for these conditions. The variables obesity, diabetes, hypertension and hypercholesterolemia were grouped together as "metabolic risk factors" (16). Note that the prevalence of "metabolic risk factors" is based upon drugs prescribed for such conditions and is therefore almost certainly an underestimate. This implies that the actual associations between "metabolic risk factors" and the Ala25Thr substitution will be larger than the estimates given in Results.

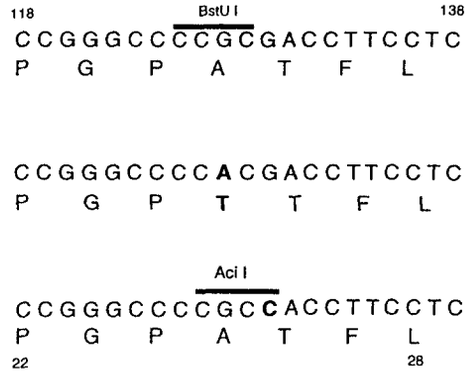

Fig. 1 Representation of thrombomodulin gene base changes (upper line, numbered in normal sequence), amino acid sequences (single letter, lower line, numbered in c) and restriction enzyme cutting sites (recognition sequences indicated by bars), described in Methods and Results. a) Normal sequence, b) mutation at G127A with amino acid substitution Ala25Thr (A25T), which abolished a Bstu I site, c) silent nucleotide mutation G129C which introduces an AciI site

Blood Collection and DNA Analysis

Pilot study

Blood samples were collected on initial presentation of the patients at the hospital Accident and Emergency Department, prior to confirmation of diagnosis. Genomic amplification, sequencing and SSCP of the thrombomodulin gene were essentially as described previously (25). Following the identification of mutations in the promoter region of the thrombomodulin gene (25) we continued to screen for mutations in the coding region of the gene. This report is only concerned with the screening of a fragment which codes for the first 47 amino acids and 53 bases 5' to the coding region. Primers used for amplification prior to SSCP were TM5A (sense): 5'-TGT CGC AGT GCC CGC GCT TT-3'; TM5B (antisense): 5'-TCG CAG ATC TGA CTG GCA TT-3'. These primers resulted in an amplification product of 211 bp. Following an initial denaturation at 94° C for 4 min, the amplification protocol was 32 cycles of: denaturation at 94° C for 30 s, annealing at 63° C for 30 s, extension at 72° C for 30 s. SSCP was continued for 800vh (Phast System, Pharmacia).

SMILE

A morning fasting blood sample was drawn from the antecubital vein in two Sarstedt Monovette® tubes containing 0.106 mM trisodium citrate. Blood in the citrated tubes was centrifuged for 10 min at 3000 × g at room temperature. Genomic DNA was extracted from the white blood cells by a salting-out method (26). The DNA was stored at 4° C. Amplification of a fragment of the thrombomodulin gene was performed using polymerase chain reaction (PCR) with 1 mg genomic DNA and 0.2 units thermostable Taq DNA polymerase (Perkin Elmer, New Jersey, USA) in a Barnstead thermocycler (Biomed GmbH, Germany). The nucleotide sequence of the primers used were respectively: TM5A (sense, as in pilot study): 5'-TGT CGC AGT GCC CGC GCT TT-3' and TM5C (antisense): 5'-GCT GGT GTT GTT GTC TCC CGT AA-3'. The initial cycle consisted of a denaturation step at 95° C for 4 min; this was followed by 33 cycles of 94° C for 40 s, 60° C for 40 s and 71° C for 2 min. This results in a fragment of 406 bp coding for a part of the 5' untranslated region, the signal peptide and amino acids one through 100 of the N-terminal lectin-like module of the thrombomodulin gene (numbering according to the amino acid sequence of the mature protein) (27). Ten µl of the PCR reactions were digested with 3.5 units BstU I (Biolabs, New England) for 2 h at 60° C. DNA fragments were separated by electrophoresis on a 2% agarose gel. The common G allele coding for Ala25 gave fragments of 190 bp and 18 bp. The rare A allele coding for Thr25 gave a relevant fragment of 208 bp long.

To distinguish the ^{127}G to A mutation from a possible GCG to GCC polymorphism two nucleotides downstream (17), we used AciI (Biolabs, New England) digestion of the Thr-encoding fragments. Ten µl of the PCR reactions were digested with 1.7 units of AciI overnight at 37° C. In the presence of the mutation without the polymorphism, the relevant AciI fragment is 98 bp. Without the mutation but in the presence of the polymorphism, this results in 58 and 40 bp fragments. All positive samples were directly sequenced for final confirmation of the mutation (automated cycle sequencing, Model 373, Applied Biosystems Inc, with the support of the Advanced Biotechnology Centre, Charing Cross Hospital).

All patients and controls were tested for the presence or absence of the factor V Leiden and prothrombin 20210A mutations, the results of which have been published elsewhere (15).

Statistical Analysis

Means are presented with standard deviation (s.d.). An odds ratio (OR) was calculated as a measure of relative risk. This odds ratio estimates the risk of a

myocardial infarction in the presence of the Ala25Thr mutation relative to its absence, the reference category. A 95% confidence interval (CI) was calculated according to the method of Woolf (28). Multiple logistic regression was performed to adjust for age. All computations were carried out using the SPSS for Windows Version 7 0 statistical package.

Results

Pilot Study

A mutation was identified by SSCP, ^{127}G to A predicting Ala25Thr, in 2 of the 104 patients with myocardial infarction but in none of the controls, see Figs. 1 and 2. Unlike most of the patients with mutations in the previous promoter region study (25), both patients were Caucasian, 1 man (51 years), and 1 woman (66 years). No other sequence variation was identified by SSCP in this region.

SMILE

The characteristics of patients and controls participating in SMILE are shown in Table 1. The mean age of the 560 patients was 56.2 (s.d. 9.0) years compared to 57.3 (s.d. 10.8) years in 646 controls. Smoking was confirmed as a cardiovascular risk factor in this study, being present in 62.3% of the patients compared to 33.3% in controls.

Overall 19 individuals carried the Ala25Thr thrombomodulin substitution. All mutations identified by digestion with BstU I were investigated further using AciI digestion and confirmed by direct sequencing. The mutation was found in 12 patients (2.1%) and 7 controls (1.1%), leading to an odds ratio of 2.0 (CI 0.8-5.1) (Table 2). Adjustment for age did not affect this result. Thus, the A allele frequency was 1.1% (CI 0.5-1.7%) among patients and 0.5% (CI 0.1-0.9%) among controls. No homozygous carriers were found. Based on a supposed A allele frequency of 1%, the frequency of homozygotes is expected to be about 1 in 10,000 individuals, which explains their absence in our study population. One patient, with a myocardial infarction at the age of 44 years, carried the Ala25Thr thrombomodulin substitution as well as the factor V Leiden mutation. One control subject carried the Ala25Thr substitution as well as the prothrombin 2021A allele. After exclusion of persons with either the factor V Leiden or the prothrombin mutation, the odds ratio increased to 2.2 (CI 0.8-6.0).

Dividing the group into two age categories, 50 years or above, and below 50 years of age, resulted in different predicted risks for myocardial infarction in the presence of the Ala25Thr substitution, Table 2. In

Table 1 Characteristics of patients* and controls in the "Study of Myocardial Infarctions Leiden"

	560 patients	646 controls
Current smokers (%)	349 (62 3)	215 (33.3)
Alcohol users (%)	450 (80.4)	561 (86.8)
Obesity (%)‡	96 (17.2)	106 (16.4)
Diabetes (%)	26 (4.6)	22 (3.4)
Hypertension (%)§	106 (18.9)	107 (16.6)
Hypercholesterolemia (%)§	12 (2.1)	11 (1.7)

* Data refer to the period prior to myocardial infarction
‡ Obesity is present as the Quetelet index exceeds 30 kg/m^2.
For two individuals length and weight were not available.
§ A person was classified as hypertensive or hypercholesterolemic if he was taking specific medications

Table 2 Distribution of amino acid substitution in patients and controls

Genotype	Patients N (%)	Controls N (%)	Odds ratio (95% CI)
Ala25	548 (97 9)	639 (98 9)	1 0†
Ala25Thr	12 (2 1)	7 (1.1)	2 0 (0.8 - 5.1)
Total	560	646	
>= 50 years			
Ala25	400 (98 5)	480 (98.8)	1.0†
Ala25Thr	6 (1.5)	6 (1 2)	1.2 (0.4 - 3.8)
Total	406	486	
< 50 years			
Ala25	148 (96 1)	159 (99.4)	1.0†
Ala25Thr	6 (3.9)	1 (0.6)	6.5 (0.8 - 54.2)
Total	154	160	

‡ Reference category

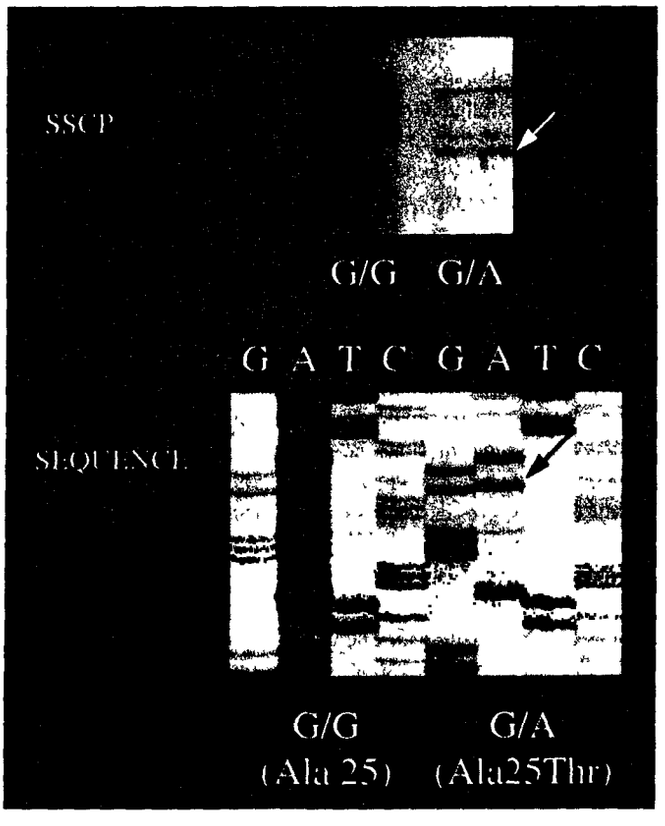

Fig. 2 SSCP and sequencing audioradiograph of Ala25Thr (G to A) mutation compared to normal. Mutation detection by SSCP (upper) and direct sequencing (lower). The SSCP results demonstrating mobility shifts caused by the G127A mutation and detected with primers TM5A and TM5B (see Methods) are illustrated (abnormal band arrowed), while the addition A nucleotide is arrowed in the sequencing gel

Cardiovascular risk factor	Amino acid	Number of patients (%*)	Number of controls (%*)	Odds ratio (95% CI) †
Non - Smoking	Ala25	207 (98 1)	426 (98 8)	1
	Ala25Thr	4 (1 9)	5 (1 2)	1 7 (0 4- 6 2)
Smoking	Ala25	341 (97 7)	213 (99 1)	3 3 (2 6 - 4 2)
	Ala25Thr	8 (2 3)	2 (0.9)	8 8 (1 8 - 42 2)
Absence of metabolic risk factor	Ala25	350 (98 6)	444 (98 9)	1
	Ala25Thr	5 (1 4)	5 (1 1)	1 3 (0 4 - 4 4)
Presence of metabolic factor	Ala25	198 (96.6)	195 (99 0)	1 3 (1 0 - 1 7)
	Ala25Thr	7 (3 4)	2 (1 0)	4 4 (0 9 - 21 3)

Table 3 Risk effect of cardiovascular risk factors without and with the Ala25Thr substitution

* percentages are calculated within each stratum of cardiovascular risk factor

† odds ratios adjusted for age, with 95% confidence interval Reference categories are non-carrier non-smokers, and respectively non-carriers with an absence of a metabolic risk factor

the group 50 years or above (total n = 892) the odds ratio became 1.2 (CI 0.4-3.8), which was unchanged after correction for age, indicating at most a mildly increased risk associated with the substitution. However, among 314 men aged less than 50, six patients and one control carried the thrombomodulin substitution. This gives an odds ratio of 6.5 (CI 0.8-54.2), an indication of a strong effect on the risk of myocardial infarction by the substitution. When we contrasted the frequency of carriership among patients aged under 50 and all controls, to limit the possibility of a coincidentally low frequency among young controls, the unadjusted odds ratio became 3.7 (CI 1.2-11.2) and 4.2 (CI 1.1-15.9) after age adjustment.

Among smokers with the Ala25Thr substitution the odds ratio increased about nine-fold compared to non-smokers without the mutation (OR 8.8, CI 1.8-42.2), Table 3. In comparison, smokers without the abnormality had a relative risk of about three (Table 3). A similar indication of a synergistic effect was found for the presence of one or more metabolic risk factors together with the substitution. The single effect was small without the concomitant presence of the Ala25Thr substitution, whereas a marked increase in predicted risk was found when one or more metabolic risk factors were present in combination with the Ala25Thr substitution (OR 4.4, CI 0.9 - 21.3), although the confidence intervals were wide.

Discussion

We have in the pilot study identified a ^{127}G to A mutation leading to the Ala25Thr substitution in the coding region of the thrombomodulin gene in two patients with myocardial infarction. This mutation was also independently discovered by a Swedish group. They reported this particular mutation in a case study concerning a 42-year-old Swedish woman with venous thrombosis in the sinus sagittalis and her 16-year-old daughter, the latter who had not suffered from any thrombotic event (19). On the basis of the present large, case-control study SMILE, it appears that there is an approximate two-fold increased risk of myocardial infarction associated with the presence of this Ala25Thr substitution. When the mutation is present together with smoking or a metabolic risk factor, the risk appears to be greatly increased, respectively nine- or four-fold. The apparent risk associated with the Ala25Thr substitution is most pronounced (~7-fold) in men under the age of 50 years. While the odds ratio for the mutation conferring risk consistently exceeded unity, the results are cautiously interpreted here as suggestive of increased risk, because of the associated wide confidence limits. These wide limits most probably arise primarily from the low population prevalence of the mutation, which was unknown at the inception of the study.

The case-control study SMILE is unique in that it has examined the relation between a mutation in the thrombomodulin gene and the risk of myocardial infarction in a large number of patients and controls. Only two relatively small studies regarding the association of other mutations in the thrombomodulin gene and the risk of arterial thrombosis have been reported. In these two studies the importance of the previously published dimorphism Ala455Val was investigated (21). Among 97 Swedish patients with premature acute myocardial infarction the allele coding for Ala455 was slightly over-represented, suggesting it as a risk factor for myocardial infarction (24). These results were not confirmed, however, by our study among 104 patients with myocardial infarction and control subjects matched for age, sex and race (25). Selection and origin of patients might explain the different results between these two studies. Three novel thrombomodulin gene promoter mutations were identified in the latter study, but the study was too small to be able to estimate the risks for myocardial infarction (25).

SMILE has suggested that the risk associated with the Ala25Thr substitution for myocardial infarction is higher in men than other common mutations in the genes encoding proteins of the coagulation cascade, such as the 20210 G to A mutation in the prothrombin gene or the factor V Leiden mutation 15. The risk of myocardial infarction associated with the Ala25Thr substitution seems to be confined to men below 50 years of age and is approximately sevenfold elevated. This finding is in accordance with the risk of the Arg353Gln polymorphism in the gene of clotting factor VII which is also more pronounced in men under the age of 50 and our recent reports on the effect of factor V Leiden mutation and factor II 20201A variant in young women (14, 16, 29). Thus, again a genetic risk factor for myocardial infarction seems to be most evident in the young.

When we consider the predicted risk of myocardial infarction associated with the Ala25Thr substitution in the presence of other cardiovascular risk factors, there appears to be a synergistic effect. Its prob-

able risk in combination with the presence of a metabolic risk factor is approximately fourfold elevated compared with non-carriers without a metabolic risk factor More striking is the risk associated with the Ala25Thr substitution and smoking the risk is highly increased, i e approximately ninefold It therefore seems that this mutation exerts most effects in combination with other risk factors Synergy has been found previously for factor V Leiden and factor II 20210A and the same cardiovascular risk factors in women (14) and men (15)

SMILE included only men, born in the Netherlands The predicted risk associated with the Ala25Thr substitution with respect to myocardial infarction among women or other populations could be dissimilar The Ala25Thr substitution was, however, found in one woman in our pilot study Another point to be made is that we only included surviving patients in our study, an inevitable feature of virtually all case-control studies Thus, we cannot completely exclude the possible selection on genotype among survivors of myocardial infarction, men who died could have had a different frequency of the mutation from survivors, leading to a biased risk estimate An argument against the existence of such bias, is that surviving a myocardial infarction is dependent on many other factors, such as time lag between onset of symptoms of myocardial infarction and start of medical interventions e g thrombolytic therapy (30, 31) It does not seem likely that the Ala25Thr substitution would play a major role in this

Thrombomodulin is comprised of 5 functional domains (27) Alterations in any one of these domains could conceivably have an effect on the anticoagulant and antifibrinolytic functions of thrombomodulin Indeed, mutation in the thrombomodulin gene could result in dysfunctional thrombomodulin or alter the expression level of the protein on the endothelium (22) In addition, the susceptibility of thrombomodulin to proteolytic degradation by proteases circulating in the blood might be changed as a result of a different molecular structure (24) The Ala25Thr substitution is located in the N-terminal lectin-like module of thrombomodulin This domain has been implicated in the constitutive endocytic routing of thrombomodulin from the cellular membrane and a substitution in this region could affect this function (32) However, at present it cannot be excluded that the G to A mutation causing the Ala25Thr substitution is in linkage disequilibrium with a functional variation elsewhere

In conclusion, the results of the two independent studies presented here strongly suggest that the ^{127}G to A mutation in the thrombomodulin gene resulting in the Ala25Thr mutation increases the risk of myocardial infarction among men The increased risk appears most evident when in the presence of another cardiovascular risk factor such as smoking or a metabolic risk factor The results need to be confirmed by other large studies Future research should be aimed at the risk in women, the search for and risk of other mutations in thrombomodulin, their interaction with other risk factors, and the clarification of functional aspects of these mutations

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