# Novedades en trombofilia

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## Resumen del simposio

El simposio, diseñado para hablar de novedades en trombofilia, incluye exclusivamente ponentes extranjeros. No significa que las novedades en este campo sólo procedan de fuera de España. Afortunadamente, nuestro país también tiene importantes grupos de investigación con relevantes aportaciones que contribuyen a conocer mejor la trombofilia. Sin embargo, es quizá más sencillo que estos conocimientos se difundan con mayor facilidad en nuestro entorno, mientras que en términos generales, el acceso a las novedades generadas en el resto del mundo sólo son accesibles tras su publicación, con un retraso que ciertamente es preocupante. De hecho, en los últimos 15 años, la presencia de ponentes extranjeros en nuestro congreso ha ido reduciéndose, en parte por la dificultad de contar con relevantes ponentes. Con este simposio hemos pretendido hablar de temas novedosos en trombofilia que no se han tratado en congresos anteriores, de mano de ponentes de relevancia internacional.

El doctor Thomas Renné (Institute of Clinical Biochemistry and Pathobiochemistry, University of Würzburg, en Alemania) es un joven y brillante científico particularmente interesado en los mecanismos de formación del trombo y las respuestas inflamatorias locales implicados en la regulación de la permeabilidad endotelial. Sus trabajos, publicados en revistas como el *J Exp Med* (2005 y 2006), *J Immunol* o, muy recientemente, *J Thromb Haemost*, han modificado radicalmente la visión de la ruta intrínseca de la coagulación que desde 1964 habían definido Macfarlane, Davie y Ratnoff. Particular importancia tienen los resultados obtenidos por su grupo sobre el papel del FXII en hemostasia e inflamación. Sus estudios con modelos animales deficientes en FXII, junto a resultados similares en pacientes con déficit de esta molécula, coinciden en la ausencia de sangrado espontáneo o excesivo asociado con la deficiencia de FXII, lo que sugiere que esta molécula no es imprescindible (incluso se atreve a decir que es completamente prescindible) en hemostasia normal y no está implicada en la formación de fibrina. Sin embargo, el papel de esta molécula parece crucial en la hemostasia patológica, ya que la deficiencia de FXII alivia de forma extraordinariamente significativa las consecuencias de la isquemia en patología trombótica arterial, tanto infarto cerebral como infarto de miocardio. Estos últimos resultados, además del potencial terapéutico que implican y que debe explorarse, plantean una interesante controversia: la hemostasia normal, la diseñada para responder a una rotura endotelial que evite la hemorragia podría ser diferente a la presente en condiciones patológicas y, por tanto, el papel de ciertas moléculas difiere en unas condiciones u otras. Quizá, sea el efecto secundario (a veces poco conocido por poco explorado) que moléculas de la hemostasia puedan tener en otros sistemas. Así, destacamos también los estudios del doctor Renné sobre el papel que el FXII pudiera tener en el sistema kalikreina-kinina que culmina en la generación de bradikinina, un potente mediador inflamatorio. Sus resultados demuestran la conexión del FXII con una patología tan alejada de la hemostasia como el angioedema hereditario. En resumen, unos resultados apasionantes, novedosos y, en cierta medida, conflictivos. De hecho, los trabajos del doctor Renné han sido intensamente discutidos y debatidos en el XXI Congreso de la Sociedad Internacional de Trombosis y Hemostasia (ISTH), recientemente celebrado en Ginebra (julio 2007). Es por tanto una gran oportunidad y privilegio contar con la presencia del doctor Renné en nuestro congreso.

La doctora A. Yaël Nossent (Department of Thrombosis and Hemostasis, Leiden University Medical Center) es otra joven investigadora que explora nuevos mecanismos implicados en el riesgo trombótico. En su ponencia nos hablará de sus recientes e interesantes resultados publicados en el *J Thromb Haemost*, relacionados con los mecanismos que controlan los niveles de factor von Willebrand y factor VIII, y su implicación en el riesgo trombótico. Su trabajo describe brillantemente los diferentes elementos o procesos que pueden

afectar los niveles circulantes de proteínas hemostáticas de relevancia como el FvW o el FVIII, y muestra su original forma de identificar nuevos mecanismos (y por tanto nuevas moléculas candidatas) a desempeñar un papel relevante tanto en hemostasia como en el riesgo trombótico. Además del grupo ABO, evalúan el efecto de haplotipos en el gen que codifica el FVIII y, finalmente, estudian variaciones en una molécula que regula la liberación de FvW (el receptor de la arginina vasopresina 2). La validación de la relevancia clínica de estas alteraciones genéticas ha sido realizada en estudios caso-control de alto reconocimiento internacional como el LETS para trombosis venosa, el SMILE para infarto de miocardio y el RATIO para mujeres con anticonceptivos orales. Se trata de un trabajo excepcional que, como el resto de investigaciones realizadas por los expertos de Leiden, es siempre interesante conocer.

La doctora Norma Maugeri (Clinical Cardiovascular Biology Research Centre, University Vita-Salute San Raffaele, en Milán) cambia un tanto el rumbo del simposio al tratar elementos celulares de la hemostasia, plaquetas y especialmente neutrófilos, y su papel en el riesgo trombótico. Lo hará hablando de una patología con elevado riesgo trombótico, como los síndromes mieloproliferativos, en el que desgraciadamente se conoce poco el mecanismo implicado. En su ponencia, la doctora Maugeri nos mostrará sus trabajos en los que ha evaluado el papel de la selectina P y su receptor (PSGL-1) en la formación de agregados mixtos de plaquetas y leucocitos (y su control por parte de la hidroxiurea en los pacientes con policitemia vera y trombocitemia esencial tratados con este fármaco), así como de las reacciones posteriores a la activación de neutrófilos con la liberación de sustancias protrombóticas como la elastasa y catepsina G, la síntesis de metabolitos del ácido araquidónico con alto poder protrombótico o la expresión de factor tisular, procesos todos ellos, que condicionan un claro estado protrombótico.

La doctora Ida Martinelli (A. Bianchi Bonomi Hemophilia and Thrombosis Center, IRCCS Maggiore Hospital, de la Universidad de Milán) forma parte desde hace muchos años del prestigioso grupo del doctor P.M. Mannucci, uno de los más veteranos y potentes en Europa en el campo de la hipercoagulabilidad. En concreto, las aportaciones de la doctora Martinelli destacan especialmente en el abordaje del papel que los estados de hipercoagulabilidad pueden desempeñar en la aparición de problemas trombóticos durante el embarazo y puerperio, habiendo liderado estudios clínicos que no sólo han definido el cometido que tienen diversos polimorfismos que afectan a moléculas de la hemostasia en estas patologías, sino que también han servido para sugerir pautas de comportamiento en la práctica clínica. Como hitos más destacados citaremos su estudio del papel que representan el factor V Leiden (FVL) y la mutación G20210A de la protrombina (G20210A) como factores de riesgo de muerte fetal (N Engl J Med 2000; 343: 1015-8), o el estudio que llevó a su grupo a recomendar profilaxis antitrombótica al menos en el puerperio para las mujeres con un estado hipercoagulable particularmente severo (FVL en homocigosis, dobles heterocigotas de FVL y G20210A; Thromb Haemost 2001; 86: 800-3). Asimismo, son frecuentes sus comentarios, sugerencias o participación en foros en las principales revistas que abordan esta área, especialmente en *J Thromb Haemost*. Esta ponencia, probablemente la más "aplicada" de las cuatro, intenta ser una puesta al día de, por un lado, los hechos (papel que desempeñan los diversos factores de riesgo) y, por otro, las acciones (práctica clínica diaria según el perfil trombofílico de la paciente) dentro de un espacio en el que, tal vez, las alteraciones hemostáticas no han tenido tradicionalmente el espacio que merecen.

# MECHANISM UNDERLYING ELEVATED LEVELS OF VON WILLEBRAND FACTOR AND FACTOR VIII AND THE RISK OF THROMBOSIS

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## Introduction

Elevated levels of von Willebrand factor (VWF) and coagulation factor VIII (FVIII) are important risk factors for the development of thrombosis. Epidemiologic studies have shown that high levels of VWF and especially FVIII increase the risk of venous thrombosis[1]. VWF is the carrier protein of FVIII and plasma levels of both proteins generally fluctuate together. Levels of both VWF and FVIII are highly dependent on ABO blood group. Individuals with non-O blood groups have higher levels of VWF and FVIII than individuals with blood group O. ABO blood group can explain up to 30% of total variability of VWF and FVIII in the population. Even though high plasma levels of VWF and FVIII cluster within families, only few genetic variations were found to be associated with VWF and FVIII levels, besides ABO blood group. Therefore, the mechanisms that underlie the substantial inter-individual variations in VWF and FVIII levels in the general population are still poorly understood[1].

In general, increased plasma levels of a protein can be caused either by increased synthesis, increased secretion or release or decreased clearance of the protein in question. We have investigated the influence of these three parameters on plasma levels of VWF and FVIII and the risk of thrombosis. Our research is based on several large population-based case control studies on venous thrombosis, the Leiden Thrombophilia Study (LETS)[2] in both men and women, and on arterial thrombosis, the Study of Myocardial Infarctions Leiden (SMILE)[3] in men and the Risk of Arterial Thrombosis in Relation to Oral Contraceptives Study (RATIO)[4] in younger women.

First we have studied the effects of several haplotypes in the gene encoding FVIII[5,6]. Variations in the FVIII gene may influence both protein synthesis and protein function and could therefore alter plasma levels and the risk of thrombosis. Second, we have used a marker for the VWF secretion to estimate secretion and clearance rates of VWF[7]. We looked at the influence of VWF secretion and clearance separately on plasma levels of VWF and FVIII and on the risk of thrombosis. Finally, we have studied variations in the gene encoding the arginine vasopressin 2 receptor, which is involved in the regulated release of VWF[8].

## FVIII gene variations

In 2003, Machiah *et al.* first reported a single nucleotide polymorphism (SNP), c94901g, in the X-chromosomal FVIII gene, which causes an amino acid change in the B-domain of FVIII, Asp1241Glu (D1241E)[9]. This amino acid change was associated with a decrease in FVIII activity (FVIII:C). Machiah *et al.* found that in the GAIT study, this polymorphism accounts for approximately 5% of the total variation in FVIII:C. More recently, Scanavini *et al.*[10] reported that 1241E was associated with an 11% reduction in FVIII:C in 145 healthy women and 150 women with venous thrombosis.

It is unclear by which mechanism the 1241E variant would influence levels of FVIII. According to data from SeattleSNPs (http://pga.gs.washington.edu), the SNP encoding 1241E is present in at least three different haplotypes, here referred to as HT1, HT3 and HT5, of which possibly only one is responsible for the reported effects on FVIII levels. We investigated the effects of these three haplotypes, that all carry the 1241E allele, on levels of FVIII and on the risk of venous and arterial thrombosis in the LETS, SMILE and RATIO[5,6].

## FVIII haplotypes and levels of FVIII

Effects on levels were studied in two control groups. The first group consisted of all healthy male control subjects from the LETS and the SMILE, the second consisted of all healthy female control subjects from the LETS and the RATIO. For D1241E in all the controls combined, lower levels of FVIII:C were observed in the absence of the 1241D encoding allele. Heterozygous carriers and homozygous wildtype carriers had similar levels of FVIII.

In the male control group (Table 1), hemizygous carriers of the 1241E encoding alleles had lower levels of FVIII. In this group, FVIII:C was reduced by 6%. Looking at the three separate haplotypes, HT1, HT3 and HT5, the reduction disappeared for carriers of HT3. For carriers of HT5, a reduction in FVIII:C was observed, however with wide confidence intervals. Also, correction for age made the reduction much smaller while confidence intervals remained wide. For HT1 however, the 6% reduction in FVIII:C remained.

Table 1. **FVIII:C (IU/dL) in all male control subjects from the LETS and SMILE combined**

| *Male controls* | *Geno-/haplotype (N)* | *Mean FVIII:C (SD)* | Δ *(CI95)* | Δ* *(CI95)* |
|---|---|---|---|---|
| D1241E | D (701) | 121.1 (32.7) | – | – |
| – | E (145) | 114.7 (35.3) | 6.4 (0.4 to 12.3) | 6.0 (0.2 to 11.7) |
| HT1 | HTx (726) | 120.9 (32.8) | – | – |
| – | HT1 (118) | 115.0 (35.5) | 5.9 (–0.5 to 12.4) | 6.3 (0.1 to 12.5) |
| HT3 | HTx (829) | 120.1 (33.2) | – | – |
| – | HT3 (15) | 121.3 (37.4) | –1.3 (–18.3 to 15.7) | –2.6 (–19.0 to 13.8) |
| HT5 | HTx (835) | 120.2 (33.2) | – | – |
| – | HT5 (10) | 107.6 (32.3) | 12.6 (–8.2 to 33.3) | 5.3 (–14.7 to 25.4) |

*Δ is the difference between the means given with the corresponding CI95. Δ* is the difference between the mean distances to the regression line after linear regression adjusting for age. HTx signifies all haplotypes but the one given.*

In the female control group, no clear effect of the 1241E encoding alleles on levels of FVIII was observed. Also the separate haplotypes showed no consistent effects.

### *FVIII haplotypes and the risk of venous thrombosis*

In men alone we found a protective effect of the 1241E encoding alleles against venous thrombosis. Carriers of 1241E had an odds ratio (OR) of 0.5 (95% confidence interval [CI95] 0.3-0.9). After stratification for haplotypes this reduction in risk remained for HT1, for which the OR was 0.4 (CI95 0.2-0.8).

After adjustment for FVIII:C with logistic regression, the OR for HT1 in men was 0.5 (CI95 0.2-1.0). The reduction in FVIII levels accounted only for part of the reduction of the risk of venous thrombosis, but HT1 seems to influence risk in a manner independent of levels as well. HT3 and HT5 were too rare to allow meaningful risk estimates.

In women, virtually no effect on the risk of venous thrombosis was observed. For both hetero- and homozygous carriers of 1241E, the OR was approximately 1. Similar ORs were observed for heterozygous carriers of HT1 and HT5 and homozygous carriers of HT1. In heterozygous carriers of HT3, the risk appeared increased (OR 2.3), however, the confidence interval was wide (0.7-7.6).

### *FVIII haplotypes and the risk of arterial thrombosis*

In contrast to what we found for venous thrombosis, neither the 1241E allele nor HT1 were associated with the risk of myocardial infarction in men. In the SMILE, the OR for 1241E was 1.0 (CI95 0.8-1.4) and for HT1 the OR was 0.9 (0.7-1.3). For HT5, an increase in risk was observed, with an OR of 2.7 (CI95 1.1-6.6) in hemizygous carriers of HT5. HT3 was rare and the risk estimate had a wide confidence interval.

In the RATIO, no clear effect of 1241E on the risk of myocardial infarction in young women was observed, although the results were compatible with a graded reduced risk, with an OR of 0.9 (CI95 0.6-1.3) for heterozygous 1241E carriers and an OR of 0.5 (CI95 0.2-1.5) for 1241E homozygotes. This risk reduction was most pronounced for HT1 carriers: for heterozygous women the OR was 0.8 (CI95 0.6-1.2) and for homozygous women the OR was 0.2 (0.02-1.2). In accordance with the increase in risk observed in hemizygous men for HT5, the risk of myocardial infarction appeared to be increased slightly in women heterozygous for HT5, with an OR of 1.5 (CI95 0.7-3.2).

In conclusion, FVIII haplotypes that carry the 1241E allele are associated with levels of FVIII and with the risk of both venous and arterial thrombosis. However, results were different for the three haplotypes, which indicates that D1241E is not the functional variation, but that it is linked to one or more other variations that cause the observed associations. Our finding that D1241E is probably not a functional variation itself was recently confirmed by Viel *et al.*[11].

## VWF propeptide

VWF is synthesized by endothelial cells and megakaryocytes. The primary translation product under-

goes a number of post-translational modifications, including endoproteolytic cleavage of a propeptide. Both mature, highly polymerized VWF and the propeptide are targeted to Weibel-Palade bodies (WPb), endothelial cell-specific storage organelles. The contents of WPb are released into the circulation only after exposure of the endothelium to specific stimuli[12].

Upon stimulation, mature VWF and propeptide are released in equimolar amounts. Once in the blood, mature VWF and its propeptide become completely dissociated and have a different life span. The VWF propeptide is cleared from the circulation at a much faster rate than mature VWF, with a half-life of approximately 2 hours[13]. Mature VWF has a half-life between 10 and 12 hours.

Considering that the VWF propeptide is secreted in equimolar amounts with the mature VWF and assuming that the inter-individual variation in the half-life of the propeptide is relatively small, plasma levels of VWF propeptide reflect the rate of VWF secretion at steady state[13-15]. Measuring concentrations of VWF propeptide and mature VWF at steady state then allows an estimation of the clearance rate of mature VWF[7].

To investigate the contribution of VWF secretion and clearance to VWF and FVIII levels, we have measured VWF propeptide levels as a measure of the VWF secretion rate in the LETS and estimated VWF half-life as a measure of VWF clearance. Using these variables, we studied the influence of VWF secretion and clearance on levels of VWF and FVIII. We also evaluated the contribution of both secretion and clearance of VWF to the risk of venous thrombosis[7].

### *Levels of VWF propeptide in healthy LETS control subjects*

Higher levels of VWF propeptide indicate increased VWF secretion. As expected, in the healthy control subjects from the LETS, high levels of VWF propeptide were associated with elevated levels of both VWF and FVIII. Mean VWF:Ag and FVIII antigen (FVIII:Ag) levels were 1.52 and 1.41 IU/mL respectively for the highest quartile of VWF propeptide (>1.26 U/mL) versus 0.93 and 0.84 IU/mL for the lowest VWF propeptide quartile (<0.94 U/mL). Regression analysis showed a linear association of the VWF propeptide with VWF:Ag (Pearson's correlation coefficient [R] = 0.61, regression coefficient [β] = 0.95 [CI95 0.81 to 1.09]), FVIII:Ag (R = 0.57, β = 0.91 [CI95 0.76 to 1.06]) and FVIII:C (R = 0.51, β = 0.61 [CI95 0.49 to 0.72]).

### *VWF half-life in healthy LETS control subjects*

We used steady state kinetics to estimate the half-life of VWF, which ranged from 4.6 to 20.1 hours in the LETS[7]. To estimate the half-life of the mature VWF, two important assumptions were made. First, we assume that VWF propeptide half-life is relatively invariable (2 hours) for the whole LETS population, compared to the half-life of mature VWF and that the clearance of both polypeptides follows first-order kinetics. Second, we assume that both mature VWF and VWF propeptide levels were at steady state at the time of blood draw. When these conditions are met, steady state kinetics enable us to estimate VWF half-life for each individual:

$$\text{Concentration}_{\text{Steady state}} = \frac{\text{Infusion Rate}}{\text{Total Body Clearance}}$$

In case of VWF, the infusion rate is the rate of secretion. To calculate the half-life, we use the following formula, in which Total Body Clearance is the apparent volume of distribution ($V_D$) times ln2 divided by the half-life.

$$\text{Secretion Rate} = \text{Concentration}_{\text{Steady state}} * \frac{V_D * \ln 2}{\text{Half-life}}$$

If Secretion Rate and $V_D$ are equal for VWF and the VWF propeptide, that leads to the following:

$$\frac{[\text{VWF}]_{\text{Steady state}}}{\text{VWF half-life}} = \frac{[\text{Propeptide}]_{\text{Steady state}}}{\text{Propeptide half-life}} = \frac{[\text{Propeptide}]_{\text{Steady state}}}{2 \text{ hours}}$$

After converting all VWF propeptide and mature VWF concentrations in the LETS from (international) units to molar concentrations, the VWF half-life could be estimated for each individual. Here we refer to these estimates as 'VWF half-life', but it should be noted that these are just estimates calculated from the VWF/propeptide ratio after making two important assumptions.

Longer VWF half-life, indicating decreased VWF clearance, was associated with elevated levels of both VWF and FVIII. Mean VWF:Ag and FVIII:Ag levels were 1.56 IU/ml and 1.28 IU/dL respectively for the highest quartile of VWF half-life (>12.8 hours) versus 0.84 IU/mL and 0.87 IU/dL for the lowest half-life quartile (<9.2 hours). Again, regression analysis showed a linear association of the VWF half-life with VWF:Ag (R = 0.71, β = 0.10 [CI95 0.09 to 0.11]), FVIII:Ag (R = 0.38, β = 0.05 [CI95 0.04 to 0.07]) and FVIII:C (R = 0.41, β = 0.04 [CI95 0.03 to 0.05]).

Both secretion and clearance of VWF are determinants of levels of VWF and FVIII. However, secretion

Table 2. **VWF propeptide quartiles and VWF half-life quartiles and the risk of venous thrombosis in the LETS**

| *Quartiles of VWF propeptide* (range)* | *Cases* | *Controls* | *Odds ratio (CI95)* |
|---|---|---|---|
| 1 (0.44-0.94) | 54 | 80 | 1 |
| 2 (0.94-1.06) | 41 | 71 | 0.9 (0.5-1.4) |
| 3 (1.06-1.26) | 80 | 79 | 1.5 (0.9-2.4) |
| 4 (1.26-2.03) | 126 | 71 | 2.6 (1.7-4.1) |
| *Quartiles of VWF half-life* (range)* | *Cases* | *Controls* | *Odds ratio (CI95)* |
| 1 (4.6-9.2) | 47 | 75 | 1 |
| 2 (9.2-11.0) | 91 | 75 | 1.9 (1.2-3.1) |
| 3 (11.0-12.8) | 97 | 76 | 2.0 (1.3-3.3) |
| 4 (12.8-20.1) | 66 | 75 | 1.4 (0.9-2.3) |

**Quartiles based on half-lifes in controls only.*

and clearance rates of VWF did not influence each other. Linear regression showed no association between levels of VWF propeptide and VWF half-life (R = 0.096, β = –1.089 [CI95 –2.372 to 0.195]).

### Levels of VWF propeptide and VWF half-life and ABO blood group

VWF and FVIII levels are strongly influenced by ABO blood group. Healthy control subjects from the LETS with blood group O had lower levels of VWF and FVIII than those with non-O blood groups, namely 1.06 and 0.94 IU/mL VWF and FVIII respectively in individuals with blood group O vs. 1.33 and 1.19 IU/mL VWF and FVIII in individuals with non-O blood groups. The half-life of VWF was also influenced by ABO blood group. Non-O individuals had a longer mean half-life of approximately 12 hours compared to O individuals, whose mean half-life was approximately 10 hours. In contrast, levels of VWF propeptide were similar in both O and non-O controls, 1.08 and 1.13 U/mL respectively.

We hypothesized that the lack of association between ABO blood group and VWF propeptide levels can be explained by the absence of ABO antigens on the VWF propeptide. Therefore, we have studied whether or not ABO antigens are present on VWF propeptide using an ELISA based method[7,16]. In both blood group A pooled plasma, normal blood group B plasma and in the plasma of a patient with blood group A and acquired VWD, we could measure normal amounts of VWF propeptide. A secondary antibody against mature VWF showed that no VWF had bound the primary anti-VWF propeptide antibody in either plasma sample (data not shown). Even though both the blood group A pooled plasma and the acquired VWD plasma samples came from blood group A individuals only, no A antigen was present on the VWF propeptide in either sample. Also, no B antigen was present on VWF propeptide in the blood group B plasma pool, indicating that ABO antigens are expressed only on mature VWF and not on the VWF propeptide. Both during these experiments and in the past, under similar conditions, the presence of A and B antigens could easily be demonstrated on mature VWF[16].

### Levels of VWF propeptide and VWF half-life in cases versus controls

Patients with venous thrombosis had a mean VWF propeptide level of 1.22 versus 1.11 U/mL in healthy controls. Stratification by ABO blood group did not influence this observation. In agreement with this observation, higher levels of VWF propeptide were associated with an increased risk of venous thrombosis. There was a clear dose-response relationship between VWF propeptide levels and thrombosis risk (Table 2). Individuals with VWF propeptide levels in the highest quartile had an increased risk of thrombosis (OR 2.6, CI95 1.7-4.1) compared to those in the lowest quartile.

In contrast to levels of VWF propeptide, mature VWF and FVIII, VWF half-life was similar in patients and controls (11.43 and 11.14 hrs, respectively). Stratification by ABO blood group did not influence this observation. Also, VWF half-life only marginally influenced thrombosis risk (OR 1.4, CI95 0.9-2.3 in the highest versus the lowest quartile) and no dose-response relationship was observed (Table 2).

In conclusion, both secretion and clearance of VWF are important determinants of levels of both VWF and FVIII. However, it appears that only increased VWF secretion increases the risk of venous thrombosis, whereas decreased VWF clearance does not or less so. Secretion and clearance rates of VWF do not influence each other. ABO blood group influences levels of VWF and FVIII via the clearance of VWF, rather than via VWF secretion. These findings are somewhat surprising, since ABO blood group does influence the risk of venous thrombosis and it was believed that this was achieved mainly via increased plasma levels of VWF and FVIII due to reduced clearance.

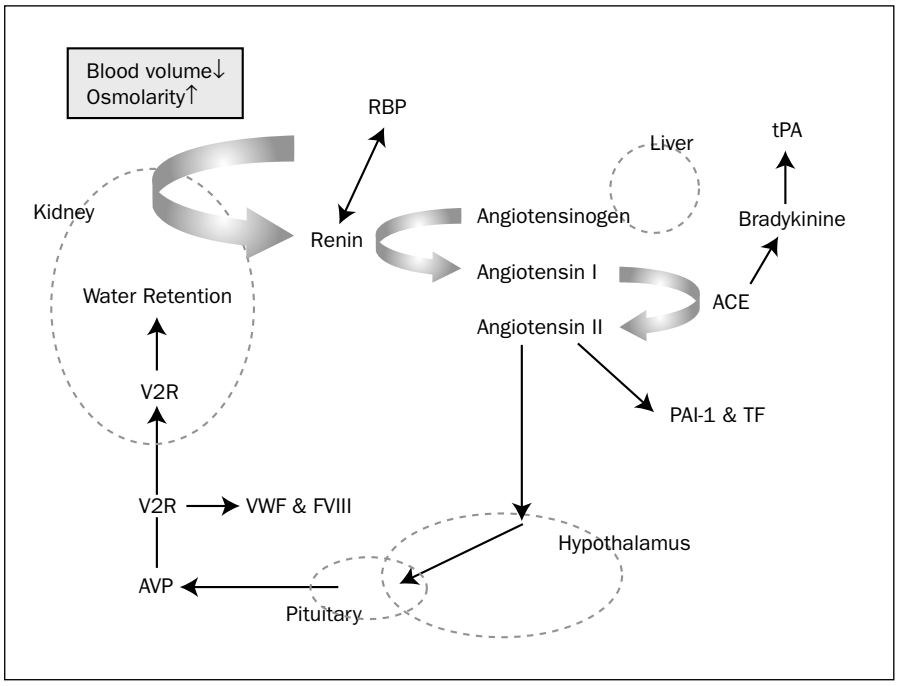


**Figura 1. EThe Renin Angiotensin System and the Vasopressin 2 Receptor. The green arrows indicate stimulation or activation, whereas the red arrows indicate inhibition or inactivation. Abbreviations used: RBP is renin binding protein; tPA is tissue-type plasminogen activator; ACE is angiotensin converting enzyme; PAI-1 is plasminogen activator inhibitor; TF is tissue factor; AVP is arginine vasopressin; V2R is vasopressin 2 receptor; VWF is von Willebrand factor; FVIII is coagulation factor VIII.**

## Arginine vasopressin 2 receptor

The main function of the vasopressin 2 receptor (V2R) is to maintain blood volume and pressure by stimulating water retention in the kidney[17]. Besides water retention, stimulation of the V2R also results in the release of Weibel Palade bodies (WPb) into the circulation, causing a sharp rise in the plasma levels of VWF and FVIII[18]. Changes in V2R functioning, caused by variations in the V2R encoding gene (AVPR2) may therefore influence levels of VWF and FVIII.

We investigated whether variations in AVPR2 could influence levels of VWF and FVIII and also the risk of venous thrombosis in the LETS. To study this possibility, several single nucleotide polymorphisms (SNPs) in AVPR2, namely G12E, S331S and L309L (2071126, rs5201 & rs5202 respectively), were genotyped in the LETS. Allelic distributions of these genotypes were used for association studies with thrombosis risk and levels VWF and FVIII[8].

### *AVPR2 SNPs and the risk of venous thrombosis*

The most common AVPR2 variation, L309L, had no effect on thrombosis risk. The other two SNPs were linked and consequently, showed similar effects on thrombosis risk. Because frequencies of the SNPs in the LETS were low, there were no women homozygous for the rare alleles. Heterozygous women showed no increase or decrease in risk compared to women homozygous for the common allele. However, men who were hemizygous for the rare alleles of these two SNPs were protected against thrombosis (OR 0, CI95s 0-1.1 and 0-0.7 for 12E and 331S respectively).

### *AVPR2 SNPs and levels of VWF and FVIII*

In men, clear associations with levels of both plasma proteins were observed for the two rare AVPR2 SNPs that also affect the risk of thrombosis, G12E and S331S. Male carriers of the rare alleles of these SNPs had higher levels of VWF and FVIII than carriers of the common alleles. These differences remained after adjustment for age.

The associations between G12E and S331S and levels in men were not present in women. However, there was a trend towards increased VWF and FVIII, levels in heterozygous carriers of the rare alleles of the two SNPs. These trends remained after adjustment for age.

We conclude that AVPR2 variations can influence both plasma levels of VWF and FVIII and the risk of venous thrombosis. We hypothesize that the AVPR2 G12E variant is a gain-of-function mutation that leads to a normally expressed, fully functional V2R with increased binding affinity for AVP. This increased affinity for AVP could lead to an increase in VWF secretion form WPb, which would explain the association with high plasma levels of VWF and FVIII. It was unexpected that the same AVPR2 variations are also associated with a decrease in thrombosis risk in men. An explanation for these apparently contrasting results may be found in the endocrine route that regulates blood pressure and vascular tone, the Renin Angiotensin System (RAS) which stimulates the secretion of AVP[19]. RAS has

strong pro-coagulant and anti-fibrinolytic properties[20,21]. A quick response of the V2R to low concentrations of AVP could down-regulate overall RAS activity and therefore decrease the risk of venous thrombosis even when VWF and FVIII levels are increased. An overview of RAS and the link to the V2R is given in Figure 1.

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# THE INTRINSIC PATHWAY OF COAGULATION: OLD FACTORS WITH NEW FUNCTIONS?

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Formation of fibrin is critical for limiting blood loss at a site of blood vessel injury (hemostasis), but may also contribute to vascular thrombosis. A complex group of plasma proteins interacts with blood vessel components to maintain blood in a fluid state within the circulatory system, while allowing localized clot formation when a vessel is injured, to stem blood loss. Disequilibrium of this "hemostatic" balance is thought to contribute to bleeding or thrombotic disorders. In 1964, Macfarlane in the United Kingdom, and Davie and Ratnoff in the United States, described a model for fibrin clot formation. In this model, commonly referred to as the coagulation cascade, formation of a clot proceeds through a series of sequential activation of plas-

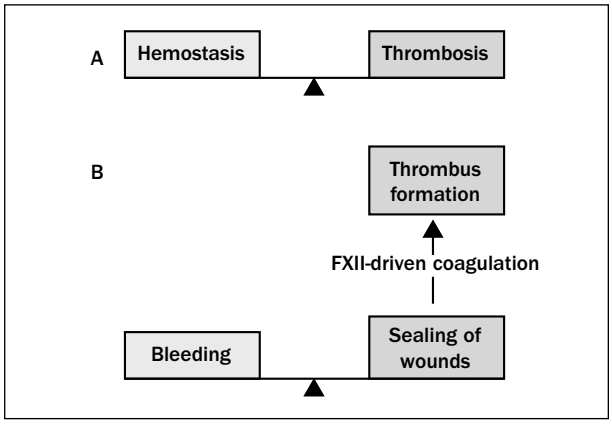


Figure 1. **A revised model of hemostasis and thrombosis. A: bleeding and thrombosis represent the two extremes of the classical blood coagulation balance. Conceptionally both disorders are considered to represent dysregulation of the normal hemostatic equilibrium. Deficiency of FXII severely impairs arterial thrombi formation but is not associated with excessive bleeding. B: in a novel revised model, FVIIa/TF-initiated thrombin formation triggers blood coagulation to stop bleeding from a wound site. Fibrin formed by this mechanism is sufficient to seal wounds and a defective extrinsic pathway impairs hemostasis resulting in bleeding. Formation of a stable three-dimensional, finally occlusive thrombus, however, involves additional thrombin generation, and requires the FXII-initiated pathway. FXII does not contribute to hemostasis at a wound site but is necessary for thrombus growth.**

ma proteases, and features two distinct pathways for initiating clot formation. One pathway, the extrinsic pathway, is triggered by tissue factor and the protease factor VII, while the second, the intrinsic pathway, is triggered by factor XII (FXII, Hageman factor). This model has been instrumental to many advances in hemostasis research, and is still the backbone of most clinical coagulation assays. However, it is clear that this model is not a complete description of clot formation in vivo. Deficiency of factor VII causes severe disruption of clot formation.

The most commonly used oral medications for treating thrombosis, the coumarins, inhibit coagulation through this pathway. In contrast, hereditary deficiency of factor XII (FXII), the protease that triggers the intrinsic pathway of coagulation in vitro, is not associated with spontaneous or excessive injury-related bleeding, indicating FXII is not required for hemostasis. Patients lacking FXII have undergone surgery with no apparent excess bleeding, despite abnormal results on in vitro clotting assays. Thus, factor XII does not appear to play a role in normal hemostasis, and subsequent revisions of the coagulation cascade usually exclude FXII. Indeed, it has become common knowledge that factor XII is not involved in fibrin formation. Additionally, FXII may initiate the kallikrein-kinin system, which culminates in the generation of the inflammatory mediator bradykinin.

We studied the contribution of the intrinsic pathway for thromboembolic diseases and for edema formation using genetically altered mice.

Hereditary angioedema (HAE) is characterized clinically by life-threatening swelling attacks. In contrast to the classic forms, HAE types I and II, which are due to deficiency of C1-inhibitor the pathomechanism of HAE type III is unknown. In a genome wide linkage analysis we found that HAE III is associated a missense mutation at position 1032 (C>A) in the FXII gene, which results in a threonine-to-lysine substitution at position 328 in the FXII protein. Mutated FXII has increased proteolytic activity and excessively generates bradykinin via the contact activation system in plasma. To characterize the pathomechanism of HAE type III we utilized edema-formation models in mice. Intravital confocal microscopy indicated that purified mutated FXII from patient plasma and recombinant expressed mutated protein severely increased microvascular leakage in wild-type mice. Infusion of human FXII into FXII-null mice restored mutated FXII-driven vascular leakage. Bradykinin B2 receptor deficient mice were resistant to FXII-induced edema formation. Treatment with bradykinin B2 receptor antagonists or FXIIa-antagonist PCK inhibited FXII-driven vascular leakage in wildtype mice.

We analyzed FXII-null mice in arterial injury models. Intravital fluorescence microscopy and blood flow measurements in three distinct arterial beds revealed a severe defect in formation and stabilization of vessel occlusive thrombi induced by different methods of injuries. The importance of these findings for the pathology and treatment of acute occlusive diseases was further analyzed using models of ischemic stroke and myocardial ischemia/reperfusion injury. Following transient middle cerebral artery occlusion, the infarcted brain volume of FXII deficient and FXII inhibitor-treated mice was significantly less than in wild type controls, without an increase in infarct-associated hemorrhage. FXII-null and inhibitor treated mice were largely protected from ischemia/reperfusion injury and survival rate was significantly higher in myocardial infarction models. Targeting FXII reduced fibrin formation in ischemic vessels, and reconstitution of FXII deficient mice with human FXII restored fibrin deposition. Mice deficient in the FXII substrate factor XI were similarly protected from vessel-occluding fibrin formation in brain and heart, suggesting that FXII contributes to pathologic clotting through the intrinsic pathway. Despite the striking protective effect in these models, FXII deficient mice, like their human counterparts, do not have spontaneous or injury-related hemorrhage. Pharmacologic inhibition of FXII in wild type mice also provides protection from cerebral ischemia and ischemia reperfusion injury in the heart, without causing excessive bleeding at a surgical injury site.

The data suggest that FXII inhibition may offer a selective and safe strategy for preventing thromboembolic disease and edema formation in HAE type III. The study opens novel avenues for developing therapies for treating thromboembolic diseases that are associated with minimal risk of bleeding.

## Conclusion

For more than 50 years, FXII was considered to be unnecessary for coagulation in vivo, as severe deficiency of FXII is not associated with increased bleeding. The general concept that pathological thrombus formation represents dysregulation of the normal hemostatic mechanism is challenged by studies with FXII deficient mice, which have normal hemostasis, but are largely protected from arterial thrombosis. These findings suggest that fibrin formation and platelet activation differ in important respects in hemostasis and thrombosis, i.e. coagulation mechanisms responsible for pathological thrombosis are not an excessive physiological hemostatic reaction. If it can be demonstrated that the FXII-driven intrinsic pathway contributes to thrombosis in humans, targeting this protease may offer an effective form of antithrombotic therapy, without an associated increase in bleeding risk.

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# COMPLICATIONS OF PREGNANCY IN WOMEN WITH THROMBOPHILIA

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## Thrombophilia and VTE in pregnancy

Deficiencies of antithrombin, protein C and protein S. The majority of the studies on the association between deficiencies of the naturally occurring anticoagulant proteins and pregnancy-related VTE are family studies of small sample sizes, due to the rarity of these deficiencies. The risk of pregnancy-related VTE is 8- to 13-fold increased risk in carriers of the deficiencies (taken together). The risk of pregnancy-related VTE appears to be particularly high in carriers of antithrombin deficiency, with an annual incidence up to 30-40%, and lower in carriers of protein S deficiency, with an annual incidence up to 6-13%.

Factor V Leiden and G20210A prothrombin. The relative risk of pregnancy-related VTE in heterozygous carriers of factor V Leiden is increased 4- to 16-fold in studies with different designs. The largest case-control studies showed an approximately 10-fold increased risk of VTE, and the largest family studies a 2% incidence of pregnancy related VTE. The prothrombin mutation increases 3 to 15 times the risk of pregnancy-related VTE.

The risk in homozygous carriers of factor V Leiden is approximately 40-times higher than the risk of non carriers, with an annual incidence of 4 to 8%. Since homozygosity for factor V Leiden is rare, the point estimates were rather unstable and the corresponding confidence intervals wide. The relative risk of pregnancy-related VTE in double carriers of heterozygous factor V Leiden and prothrombin mutation appears to be lower than that reported for homozygous carriers of factor V Leiden, whereas there are no data on the risk in homozygous carriers of the prothrombin mutation.

Antiphospholipid antibodies. The presence of antiphospholipid antibodies (lupus anticoagulant and/or anticardiolipin antibodies) together with a history of either venous or arterial thrombosis, pregnancy losses and thrombocytopenia define the antiphospholipid syndrome. This acquired thrombophilic condition is associated with a 9-fold increased risk of VTE and with a high rate of recurrence during pregnancy. The strong association between antiphospholipid antibodies and pregnancy-related VTE claims for an anticoagulant prophylaxis throughout the whole gestational period in pregnant women with or without previous VTE.

## Thrombophilia and obstetrical complications

Since the early 1980s, when the antiphospholipid syndrome was recognized as an etiological cause for recurrent fetal loss, the pathogenic theory of placen-

tal thrombosis affecting the feto-maternal circulation and leading to fetal death has been put forward. In the last decade, following the discovery of the frequent mutations in factor V and prothrombin genes, the increased interest in the relationship between thrombophilia and VTE prompted out several studies dealing not only with pregnancy-related VTE, but also with various obstetrical complications that have as common determinant an impaired placental circulation. These complications are recurrent early fetal loss, late fetal loss, preeclampsia, placental abruption and intrauterine growth restriction. According to the WHO definition, fetal loss is defined early when it occurs before the 12th gestational week, late after the 20th gestational week and recurrent when 3 or more losses occur. However, these definitions are not strictly observed and vary in different studies. In general, early fetal loss occurs in 1 to 2% of pregnancies, late fetal loss in 1 every 200 pregnancies. The most widely investigated thrombophilic abnormality is heterozygous factor V Leiden. Although the majority of the studies showed a significant association between the mutation and various obstetrical complications, others found a trend or failed to find a relationship. Similar findings concern the association between the prothrombin mutation and obstetrical complications. Obstetrical complications other than fetal losses have been investigated; a case-control study on preterm infants with very low birth weight showed a 2-fold and a 3-fold increased risk in carriers of factor V Leiden or prothrombin mutation, respectively. Another case-control study investigated women with gestational hypertension, finding a 5-fold and 3-fold increased risk in the presence of either mutation. Data on homozygous factor V Leiden or combined coagulation abnormalities are limited but consistent in showing the association between these forms of thrombophilia and fetal loss. Moreover, fetal loss was associated with the presence of activated protein C resistance in the absence of factor V Leiden mutation, with an odds ratio of 2 to 7.

In summary, a number of studies are available in the literature on the association between thrombophilia and fetal loss, but in many cases the results must be interpreted with caution because of the small sample size and selection and acquisition bias. In addition, the majority of these studies are retrospective case-controls or family studies. Therefore, if on one hand these studies consistently show an association between thrombophilia and fetal loss, in the absence of prospective observations the true risk of fetal loss in women with thrombophilia remains unknown. The only prospective study on thrombophilia and fetal loss showed a weak trend towards an association, with an odds ratio of 1.7 (95% CI 0.6-4.6) for deficiencies of antithrombin, protein C, protein S and factor V Leiden or prothrombin mutation considered together. Concerning the antiphospholipid antibodies, data are consistent in showing a strong association with obstetrical complication, e.g., fetal losses and preeclampsia. Several studies have shown that fetal loss rate in women with this acquired thrombophilic condition is up to 50-70% or even greater.

## TROMBOGENICIDAD DE LOS NEUTRÓFILOS EN SÍNDROMES MIELOPROLIFERATIVOS

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Los eventos tromboembólicos son la principal causa de mortalidad y morbilidad en los síndromes mieloproliferativos. A pesar de los años de intensa investigación clínica y de laboratorio dedicados al estudio de la causa de mayor trombogenicidad en estos pacientes, no se han podido identificar aún ni la patogénesis ni los factores de riesgo que lo inducen. Inicialmente se han identificado como factor de riesgo protrombótico la viscosidad (causada por el gran numero de eritrocitos) en policitemia vera (PV) y la trombocitosis observada en trombocitemia esencial (TE). Sin embargo, el número de plaquetas circulantes *per se* no se correlaciona con el riesgo de eventos tromboembólicos[1] mientras que tanto en PV como en ET, el número de glóbulos blancos parecería representar un papel importante. De hecho, algunos estudios epidemiológicos recientes han demostrado una directa asociación entre el número de leucocitos y el riesgo de trombosis e infarto de miocardio en pacientes con PV y TE[2,3]. Sin embargo existe una gran discrepancia acerca de si es solo el número de leucocitos circulantes o el estado de activación de las plaquetas y los leucocitos observados en estos pacientes[2-4]. De hecho, la trombofilia observada en ET y PV se ha asociado al incremento del estado de activación de los neutrófilos, a marcadores de daño endotelial[4-6] y al incremento de agregados mixtos de plaquetas y leucocitos en circulación[5-7] y, a su vez, la alta proporción de agregados mixtos de plaquetas y leucocitos se asocia significativamente a la trombocitosis y al estado de activación plaquetaria, en particular a la alta proporción de plaquetas que expresan P-selectina en estos pacientes[7].

Existe, en este momento, suficiente evidencia experimental y clínica que nos permita formular la hipótesis de que la adhesión celular entre plaquetas y leuco-

citos desempeñan un rol fundamental en los eventos tromboembólicos, observándose una estrecha relación entre la activación celular y adhesión de leucocitos y plaquetas en distintas patologías con alto riesgo tromboembólico[8,9].

Las plaquetas y los leucocitos pueden agregarse a través de diferentes vías adhesivas, pero cualquiera de ellas requeriría, como primer paso de la activación plaquetaria, con la consiguiente expresión de P-selectina[8-10].

La P-selectina es una molécula adhesiva producida por los megacariocitos y las células endoteliales, se almacena intracelularmente (gránulos alfa de las plaquetas y de Weibel Palade en endotelio) en condiciones de reposo y es rápidamente translocada sobre la superficie después de la activación celular.

La P-selectina es considerada el agonista de la activación leucocitaria por excelencia. Una vez expresada en la superficie celular, se une a su contrarreceptor P Selectin Granulocyte Ligand 1 (PSGL-1), el cual se expresa sobre la superficie leucocitaria en manera constitutiva[9], induciendo la transducción de la activación leucocitaria a través de un mecanismo descrito como *cross-talk*.

En condiciones fisiológicas, la función de la P-selectina es reclutar de los leucocitos en el sitio de inflamación e lesión vascular, permitiendo la migración leucocitaria desde el torrente sanguíneo hacia los tejidos.

La importancia del efecto de la P-selectina sobre los leucocitos, en particular sobre los neutrófilos adquirió particular importancia al observarse que la P-selectina expresada sobre la superficie de plaquetas activadas ejerce el mismo efecto estimulante que la presente en el endotelio inflamado[8-10]. En estas condiciones, los leucocitos circularían activados, expresando un fenotipo protrombótico. De hecho, la P-selectina induce o favorece lo siguiente[8, 9, 11, 12]:

- La desgranulación de los neutrófilos, con la consecuente liberación de sustancias protrombóticas como la elastasa y la catepsina G. Estas dos potentes serinproteasas son capaces de inducir la activación plaquetaria y endotelial a través de un mecanismo que no es bloqueado por la aspirina ni por las antiproteasas plasmáticas.
- La síntesis transcelular de metabolitos del ácido araquidónico con alto poder protrombótico, tales como el tromboxano A2 y el leucotriene C4.
- Reorganización del citoesqueleto y expresión de integrinas, las cuales actúan como receptor para el factor von Willebrand, la glicoproteína Ib plaquetaria, fibrinógeno, fibrina y colágeno, entre otros.
- La inmediata expresión de factor tisular biológicamente activo y la síntesis *ex novo* de la molécula.

La evidencia que los neutrófilos puedan producir, acumular y expresar factor tisular biológicamente activo[11,12], con la consecuente capacidad para producir trombina al ser estimulados por plaquetas activadas en circulación, sitúa a los granulocitos en una posición central del cuadro general de la trombosis.

Las capacidad potencialmente trombogénica de los neutrófilos activados por la P-selectina, que inicialmente se han verificado con modelos *in vitro*, fueron corroborados en un estudio piloto donde se estudiaron pacientes con policitemia vera y trombocitemia esencial[13].

En estos pacientes, se observó trombocitosis, leucocitosis, un marcado aumento de plaquetas activadas y de agregados mixtos de plaquetas y granulocitos en circulación. Todos los parámetros se relacionaron con la activación de los neutrófilos circulates en estos pacientes: completa degranulación, fibrinógeno establemente ligado a la superficie leucocitaria, mayor contenido y expresión extracelular de factor tisular.

Estas características protrombóticas de los neutrófilos en los pacientes con sídromes mieloproliferativos parecería involucrar el *cross-talk* que se establece a partir de la P-selectina expresada sobre las plaquetas activadas de los pacientes estudiados.

Con el objetivo de confirmar esta teoría, se han estudiado los mismos pacientes luego del tratamiento con hidroxiurea (HU), ya que esta droga bloquea la interacción de la P-selectina con PSGL-1[14] y porque se observó una menor incidencia de eventos tromboembólicos en pacientes con PV y ET tratados con HU[15].

El efecto inibidor de la P selectina ejercido por la HU fue verificado ulteriormente, observando que los neutrófilos de individuos sanos incubados con HU pierden la capacidad de agregarse a las plaquetas activadas (independientemente del número de plaquetas) y de expresar factor tisular luego de ser estimulados con P-selectina purificada.

De manera paralela, se estudiaron nuevamente los pacientes con PV y TE después de 3 semanas de tratamiento con HU, observando que todos los marcadores de activación neutrofílica, la proporción de agregados de plaquetas y leucocitos, así como la expresión de factor tisular en la superficie leucocitaria, se normalizaron a pesar de la no variación en la expresión de P-selectina en las plaquetas circulantes en los pacientes estudiados[13].

Todas estas observaciones nos permitirían considerar la función neutrofílica como un nuevo ítem para abordar los estudios de trombofilia.

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