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Paul Stuart Pottinger
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DETECTION OF THE FACTOR V LEIDEN MUTATION IN A NONSELECTED BLACK POPULATION.

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The purpose of this study was to determine the prevalence of the factor V Leiden mutation among black and non-black inpatients and outpatients at the Yale-New Haven Hospital. We had found no previous information on the prevalence of this mutation within the black population, although it had been predicted by some that the abnormality might be found predominantly in individuals of European extraction. Randomly selected blood samples were obtained from the Yale-New Haven Hospital hematology laboratory and ethnic background was determined from hospital records. In addition, we studied stored DNA samples from black individuals already available in the laboratory from previous population studies. Using previously described methods, genomic DNA was isolated and analyzed by PCR and restriction enzyme digestion to identify the factor V cleavage site gene associated with activated Protein C (APC) resistance. Results were obtained on 214 black individuals, of whom 3 (1.4%) were heterozygous for the Factor V Leiden mutation. The incidence of the mutation in a similarly selected group of 126 non-black patients was 1.6%, yielding a relative risk of 0.88 (Fisher Exact two-tailed test, P=1.0000, 95% confidence interval 0.12 to 7.64). These results do not support the hypothesis of a difference between the prevalence of the Factor V Leiden mutation in the black and non-black populations studied.

Acknowledgments

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Society itself, in order to present these data as an abstract and poster. Dr. Barry Wu was kind enough to invite me to present these findings before the Connecticut chapter of the American College of Physicians at their 1996 annual conference. And finally, I thank my wife, Julie, for her unwavering support regardless of my state of frustration, exhaustion, or elation.

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Introduction

Overview

Today we have a solid understanding of the physiology of blood coagulation in normal, healthy subjects. The clotting cascade – that dreaded maze of arrows and roman numerals which students diligently memorize in anticipation of exams – appears in textbooks as a gospel of established fact, and there is little mystery to the process in which blood turns from liquid to solid when vessels are damaged.

We know considerably less about the reasons why blood clots in apparently *healthy* tissue. Venous thromboembolism has presented clinicians with diagnostic and therapeutic challenges for generations. It is a common source of morbidity and mortality, with an annual incidence of approximately 1 in 1,000[2]. However, its molecular cause is rarely identifiable. Inborn deficiencies of the natural anticoagulant factors – Protein C, Protein S, Antithrombin III – and dysfibrinogenemia can be found in 5-10% of patients with venous thrombosis.[3-6] The majority remain idiopathic.

This changed somewhat in 1993 and 1994, with the publication of a series of elegant papers that demonstrated the presence of a previously unknown mutation in the gene for factor V.[7-14] This mutation quickly became known as "factor V Leiden," in honor of the city in the Netherlands where it was elucidated. Initial studies indicated that factor V Leiden was far more prevalent among the general population than dysfibrinogenemia or deficient Protein C, Protein S, or Antithrombin III combined. Factor V Leiden

was also found in many more patients with venous thrombosis than the previous disorders, so the wide gulf of "idiopathic hypercoagulation" was narrowed considerably by the identification of this mutation.

However, these initial studies were conducted in northern Europe, and at that time it was not clear whether the mutation was present to the same extent in other ethnic groups – for instance, American blacks. Our study was designed to address this question.

In order to place this experiment in its proper context, we should first consider the mutation's physiology and clinical significance.

What is Factor V?

Factor V was first described independently by Dr. Armand J. Quick in 1943, and by Dr. Paul A. Owren in 1947.[15] Quick noticed that the prothrombin time (PT) of plasma would increase after the sample was stored on a shelf for more than eight days.[16] He found that the PT would then return to normal if he added plasma from experimental animals to the stored sample. Prothrombin had been described since the early 1930s,[17] and Quick recognized that this chemical in the donor animal's plasma might be responsible for the normalization of prothrombin time. To eliminate this possibility, he fed bishydroxycoumarin to the donor animals, which would inactivate their own prothrombin activity. Even then, the normalization of stored plasma's PT persisted. He therefore postulated the existence of a "labile factor" in the

plasma distinct from prothrombin, which was lost during storage, but which would foster clot formation in life.

For Owren, the inspiration to consider the possibility of a new coagulation factor came in the form of a single patient, an unfortunate young woman with a lifelong tendency to bleed heavily.[18] Her prolonged PT could be normalized with donated, prothrombin-depleted plasma. This led him to conclude that she had a deficiency of some compound which he called "proaccelerin," since it sped up the PT. Because four proteins involved in the clotting process had already been described, he referred to proaccelerin as the "fifth coagulation factor," synonymous with today's "factor V."

The structure and function of Factor V have subsequently been well-described.[19-23] Indeed, the gene for factor V has been mapped to chromosome one (1q21-25),[24] and its DNA sequence was published more than a decade ago.[25] A brief review of Factor V physiology and regulation will be helpful before describing the Leiden mutation.

Factor V serves a crucial role in the clotting cascade. A linchpin at the intersection of the "intrinsic" and "extrinsic" pathways of coagulation, it functions as a potent pro-coagulant (see Figure 1).

When vessels are injured, tissue factor becomes exposed, and interacts with factor VII, leading to the conversion of prothrombin into thrombin.[16,17,26] Thrombin then activates platelets, converts fibrinogen to fibrin, and activates factors V and VIII. Under normal conditions, factor V circulates in the bloodstream as a biologically inactive single chain

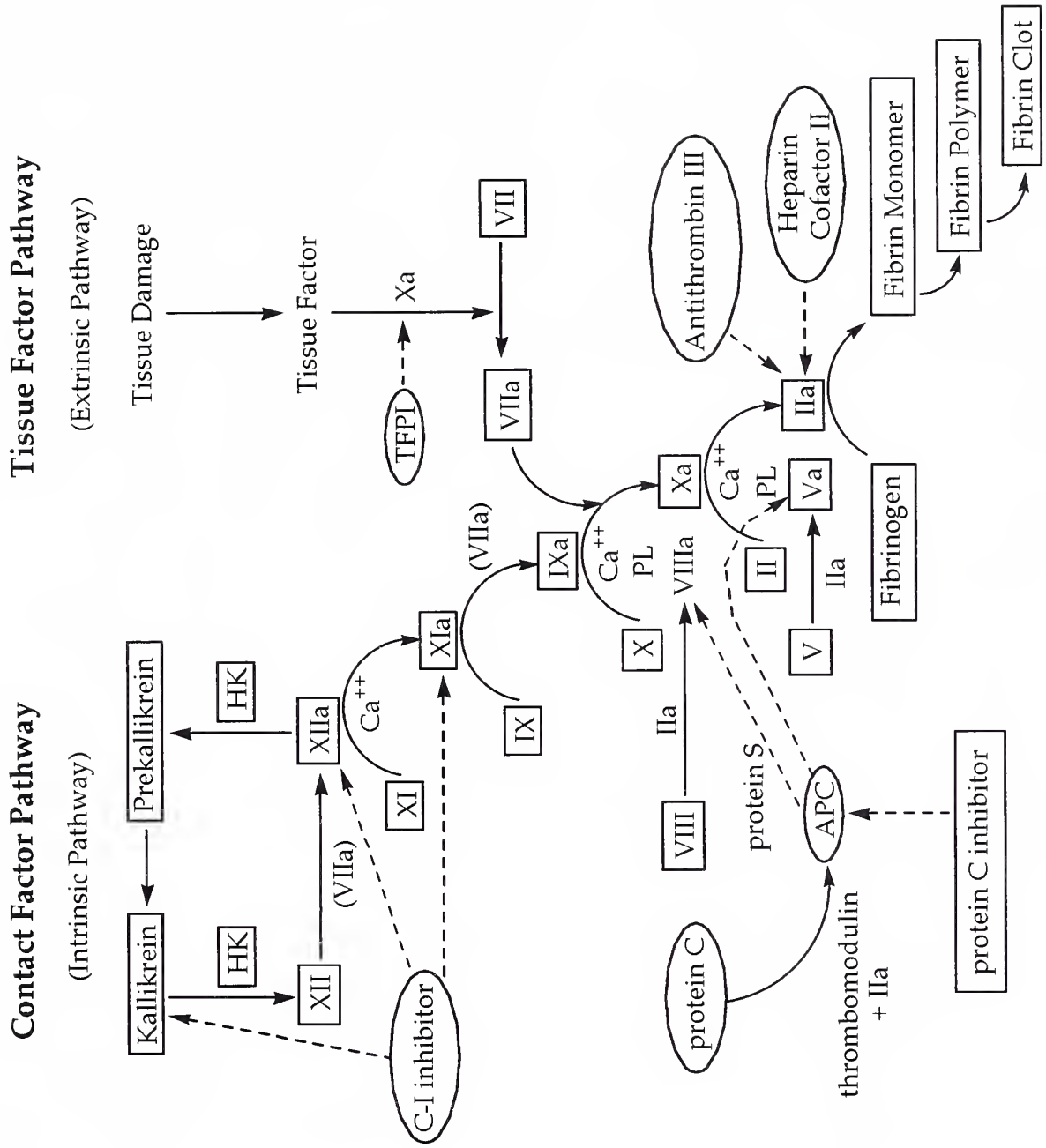


Figure 1. Schematic representation of the coagulation cascade. Inhibition reactions are indicated by dashed arrows. Notice that factors Va and Xa participate together in a reaction that is common to clotting that originates in either the intrinsic or extrinsic pathways, and thus are part of the "final common pathway." Adapted from Stieve-Martin EA, Lotspeich-Steininger CA, Koepke JA (eds): Clinical Hematology. Philadelphia, 1998, p.614.

glycoprotein with a molecular weight of 330,000.[23] It remains in this "dormant" state until it comes in contact with either thrombin or activated factor X (factor Xa). Although thrombin activates factor V at a rate approximately 100 times faster than factor Xa, the latter probably serves a more important role in the early stages of coagulation, when little thrombin has yet been generated.[27] Both of these enzymes cleave factor V into factor Va, a heterodimer whose heavy chain (M_r = 94,000) and light chain (M_r = 74,000) are bound non-covalently by Ca^{2+} ions.[23] Once activated, factor Va binds to activated platelets and becomes a receptor and cofactor for factor Xa. When factor Xa binds to factor Va, they together form a cleavage enzyme called "prothrombinase." Prothrombinase breaks prothrombin into thrombin, which will in turn activate more factor V, a cycle leading to the rapid amplification of the clotting cascade.[22]

Therefore, very small amounts of factor Va* lead to tremendous acceleration of the formation of platelet-fibrin clots. Without factor Va, clotting happens at only a small fraction of its potential: complete prothrombinase breaks prothrombin into thrombin 10^5 times faster than factor Xa alone.[28]

How is Factor V Regulated?

Factor Va's action is kept under strict control by an elegant series of enzymatic interactions.[26,29,30] Although thrombin serves to activate factor V in

*The plasma concentration of Factor V has been measured at approximately 10 mg/liter.[26]

damaged vessels, it has the opposite function in healthy ones, due to its interaction with a protein called thrombomodulin. Thrombomodulin is a multi-modular protein of 557 amino acids with a trans-membrane domain that anchors it to the luminal surface of arterial, capillary, venous, and lymphatic endothelial cells. It is found in highest concentration in the microcirculation, where the ratio of endothelial cell surface area to blood volume is 1,000 times that in the great vessels.[26]

Thrombomodulin protrudes from the vessel wall into the bloodstream, and functions as a receptor for thrombin. As thrombin circulates in the blood and enters capillaries, it binds to thrombomodulin. Once bound, thrombin no longer participates in clotting reactions; rather, it serves as an activator of another soluble protein called protein C.

The first attempts to purify protein C took place in 1960,[17] and today its structure and function are understood on a molecular level. It is a vitamin K-dependent zymogen comprised of a heavy chain and a light chain, with a molecular weight of 62,000 kDa. When protein C contacts the thrombin that is bound to thrombomodulin, its heavy chain is cleaved at a specific site near the amino terminus. This reaction exposes a Serine protease domain on the protein C molecule, converting it into a powerful cleavage enzyme referred to as "activated protein C" (APC) (see Figure 2). The protease action of APC is specific for three sites on the heavy chain of the factor Va heterodimer: when APC encounters factor Va, it cleaves the peptide bonds of the heavy chain at those sites, rendering it inactive as a cofactor for factor Xa. This process is

enhanced by the presence of protein S, a co-factor for APC whose precise mechanism of action remains unclear, and by factor V itself (see Figure 3).

In contrast, when protein C encounters thrombin not bound to thrombomodulin, its activation is 20,000 times less efficient. This serves to prevent the activation of protein C at sites of vascular injury, where there is an appropriately higher proportion of free, unbound thrombin, therefore allowing the pro-coagulant action of factor Va to predominate where it its needed.

In summary, factor V serves as a potent pro-coagulant when activated by cleavage at a specific site by thrombin or factor Xa. Factor Va activity, in turn, is regulated by the specific proteolytic action of APC at three different sites. The activity of APC is in turn downregulated at sites of vascular endothelial damage, because activation of protein C is much slower in the presence of thrombin which is not bound to thrombomodulin.

What is Factor V Leiden?

In 1993 Dahlbäck *et al* described the phenomenon of poor responsiveness to APC in some patients who experienced venous thrombosis.[7] In this study, the activity of protein C was tested *in vitro* by adding APC to the reagents used to determine the partial thromboplastin time (PTT), a test of the intrinsic and the final common pathway of coagulation. Because APC serves as an anti-coagulant, its addition to this reaction would be expected to increase the PTT. By comparing the PTT with

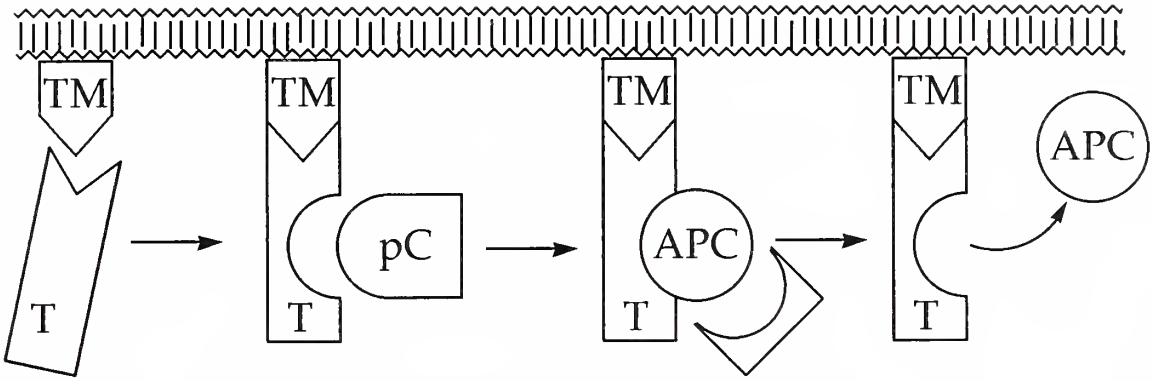


Figure 2. Schematic representation of the activation of protein C. Thrombin (T) circulates in the blood until it binds with thrombomodulin (TM), protruding from the vascular endothelium. Once bound, thrombin is able to bind protein C (pC) and enzymatically expose its serine protease moiety, thus converting it into activated protein C (APC), which can participate in the activation of factor V. Adapted from Dahlbäck B: Inhertited thrombophilia: Resistance to activated protein C as a pathogenic factor of venous thromboembolism (review). Blood 85:607-14, 1995.

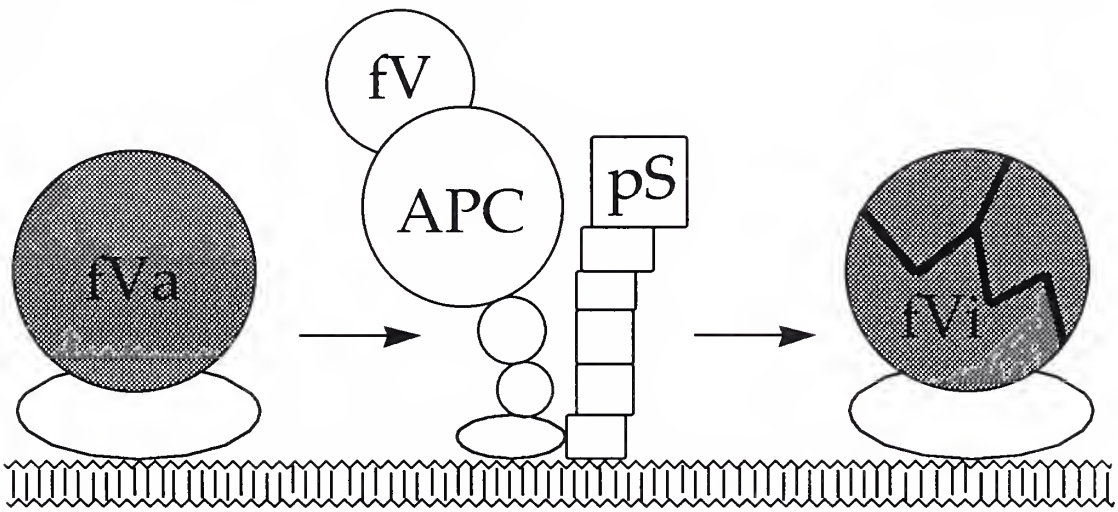


Figure 3. Model of the process by which the heavy chain of activated factor V (fVa) is broken into its inactivated form (fVi) by the proteolytic action of activated protein C (APC), with its cofactors protein S (pS) and factor V (fV). Events are believed to take place on the surface of phospholipid membranes. Adapted from Dahlbäck B: Inhertited thrombophilia: Resistance to activated protein C as a pathogenic factor of venous thromboembolism (review). Blood 85:607-14, 1995.

and without the addition of APC, a patient's response to APC can be quantified as the ratio between the two assays. Dahlbäck *et al* identified an individual with a strong personal and family history of multiple venous thromboses, and tested him and his family members for APC resistance in this manner. Among nineteen subjects tested, fourteen were found to have only a minimal increase in the PTT, placing them below the fifth percentile of control values. In other words, their blood was *resistant* to the anti-coagulant effects of APC. This observation was repeated in other studies; APC resistance was demonstrated again in 64 of 301 patients with thrombosis,[8] in 33% of 104 consecutive patients with a personal history of thrombosis.[14] The authors who first observed this phenomenon made the reasonable speculation that APC resistance might be explained by an inherited deficiency of some yet-undescribed cofactor for protein C.

Alternatively, it was postulated that the defect might lie in the targets of APC: factor V or factor VIII. In a study conducted by a team in the Netherlands,[8,9] patients with deep vein thrombosis and APC resistance were identified. Linkage analysis between the APC resistance trait and polymorphisms of the genes for various clotting factors revealed that APC resistance segregated with factor V but not factor VIII or von Willebrand factor, and therefore factor V became the focus of investigation. These patients' DNA encoding for factor V was analyzed. Careful sequencing and RFLP revealed the presence of a previously-unknown missense mutation which converted guanine to adenine at nucleotide 1,691 in the factor V

sequence. This in turn leads to a change in the codon sequence such that an arginine residue is replaced by a glutamine residue (CGA for arginine is replaced by CAA for glutamine). Because this residue is found at one of the three critical sites for proteolysis by APC, and because glutamine's acidic side chain renders it resistant to cleavage by the APC serine protease, the activated form of factor V Leiden is inactivated by APC at a much decreased rate. Therefore, it remains able to potentiate the clotting cascade even under circumstances in which it would normally be inactivated.

Clinical Significance of Factor V Leiden

The robust pro-coagulant function of factor V Leiden leads to an increased risk of venous thrombosis for patients who carry it, particularly those with other identifiable risks for hypercoagulability. In the few years that have followed the elucidation of factor V Leiden, its implications for patients with a number of clinical conditions have been investigated, such as protein C deficiency. In order to determine whether factor V Leiden plays an additional role in thrombotic complications among patients known to have deficient protein C, Koeleman *et al* studied the segregation of the mutation and APC deficiency.[31] Out of 48 symptomatic patients with protein C deficiency, they detected 9 (19%) with factor V Leiden. Furthermore, in six families studied, 31% of subjects with protein C deficiency developed thrombosis, and 13% of subjects with factor V Leiden developed thrombosis; however, 73% of subjects with *both* conditions developed thrombosis, a

statistically-significant increase in risk. Although heterozygous protein C deficiency is itself found in only 0.1-0.5% of healthy blood donors,[32] these data suggest that it can have significant clinical implications when present in combination with factor V Leiden.

Factor V Leiden also fosters thrombosis in patients with other, far more common risk factors. For example, this mutation has an important impact on patients who use oral contraceptive pills (OCPs). OCPs have been associated with as increased incidence of venous thrombosis since their first use in the 1960s: it has been calculated that the incidence of thromboembolism among fertile, healthy women who did not use OCPs was approximately one in 20,000, whereas this value increased to one in 3600 among comparable women using the early generation of OCPs.[33] An increased risk persists even among users of later-generation OCPs, and the presence of factor V Leiden appears to greatly compound this risk, as demonstrated by Vandenbroucke *et al.*[34] Their retrospective analysis focused on 155 consecutive premenopausal women, aged 15 to 49, who had developed deep venous thrombosis in the absence of other underlying diseases. When compared with 169 population controls, subjects who used OCPs were four times more likely to develop a DVT, and subjects who later tested positive for factor V Leiden were eight times more likely. Those who *both* took OCPs and had factor V Leiden had a relative risk of 34.7 for experiencing thrombosis (CI 7.8-154). These data, and other series that corroborated them,[35-37] spurred a debate as to whether asymptomatic women should be screened for factor V

Leiden before beginning OCPs.[38,39] Considering the fact that approximately 9.9 million American women rely on oral contraception,[40] this is no small question. To date, a consensus seems to have been reached that general screening is not indicated. Cost-benefit analysis suggests that testing all patients for factor V Leiden before prescribing OCPs would be prohibitively expensive,[41] and should be reserved for those with a clear medical or family history of thrombosis.

Cigarette smoking may be another risk factor for thrombosis that is exacerbated by the presence of factor V Leiden. Among 84 women age 18 to 44 who experienced a first myocardial infarction (MI), 10% were heterozygous for factor V Leiden, as compared with 4% of 388 age-matched controls, yielding an overall odds ratio for the development of MI among factor V Leiden carriers versus non-carriers of 2.4 (95% CI 1.0 to 5.9).[42] Closer inspection revealed an interesting trend: the mutation had little effect among non-smokers, with an odds ratio for the development of MI among non-smoking factor V Leiden carriers versus non-smoking non-carriers equal to 1.1 (95% CI 0.1 to 8.5). However, among smokers the presence of factor V Leiden was a significant additional risk factor, with an odds ratio of 3.6 (95% CI 0.9 to 14.4), and a 32-fold increase in the risk of MI among smoking carriers versus non-smoking non-carriers.

Links have also been established between factor V Leiden and the Budd-Chiari syndrome,[43,44] childhood ischemic stroke,[45,46] fetal loss and preeclampsia,[47] retinal vein occlusion,[48] and venous thromboembolism in

concert with hyperhomocysteinemia.[49] Even when we ignore the mutation's synergistic effects with other identified risk factors, such as OCPs and protein C deficiency, the overall risks it poses are impressive. In the original Leiden thrombophilia study,[8] 301 patients with deep vein thrombosis were tested for APC resistance by modified PTT assay. 21% of these patients demonstrated APC resistance, as compared with 3% of control subjects. When the factor V Leiden mutation was identified the following year, those same APC resistant patients were tested for the presence of factor V Leiden: 47 were heterozygous and 6 were homozygous, as compared to three heterozygous controls.[9] It was therefore predicted that heterozygotes are at 5- to 10-fold the risk of venous thrombosis of non-carriers, and that homozygotes are at a 50- to 100-fold risk.[26]

The Question of Prevalence

From the earliest investigations, factor V Leiden seemed to be surprisingly common, not only among patients suffering from venous thrombosis, but also among the general population. In the Leiden thrombophilia study,[8] 64 of 301 consecutive Dutch patients with venous thrombosis tested positive for factor V Leiden, as did 14 of 301 healthy controls, suggesting a prevalence of about 5%. Subsequent studies of the general population in various countries demonstrated a peak carrier frequency of 13.4% in Greece[50] and 10.9% in Sweden,[51] with prevalence in other European nations ranging from 2.7% in Italy[50] to 6.8% in the United

Kingdom,[52] for a cumulative European average prevalence of 5.4%,[53] making factor V Leiden by far the most common identified hereditary risk for venous thrombosis.[26]

But as of the summer of 1995, we found no information on the prevalence of this mutation in the American black population. One of the earliest studies of factor V Leiden reported its presence in one African American individual,[10] but this observation was not quantified in a population-based study. It had been proposed in 1994 that a founder effect might be responsible for the mutation's high prevalence, because a disproportionate number of factor V Leiden carriers were also carriers for a marker allele: when digested with *Hin*f1, the DNA from 96% of 53 factor V Leiden carriers also had a cytosine at nucleotide 2,298, whereas only 73% of 69 non-carriers had this base pair (x^2_{diff} = 30.4, d.f. =1; P<0.001).[9] However, this analysis is not conclusive proof of a founder effect, nor does it serve as a guarantee of the mutation's absence among American blacks. Although the mutation's molecular nature was understood, and its clinical implications had begun to be quantified, its prevalence among American blacks remained unknown.

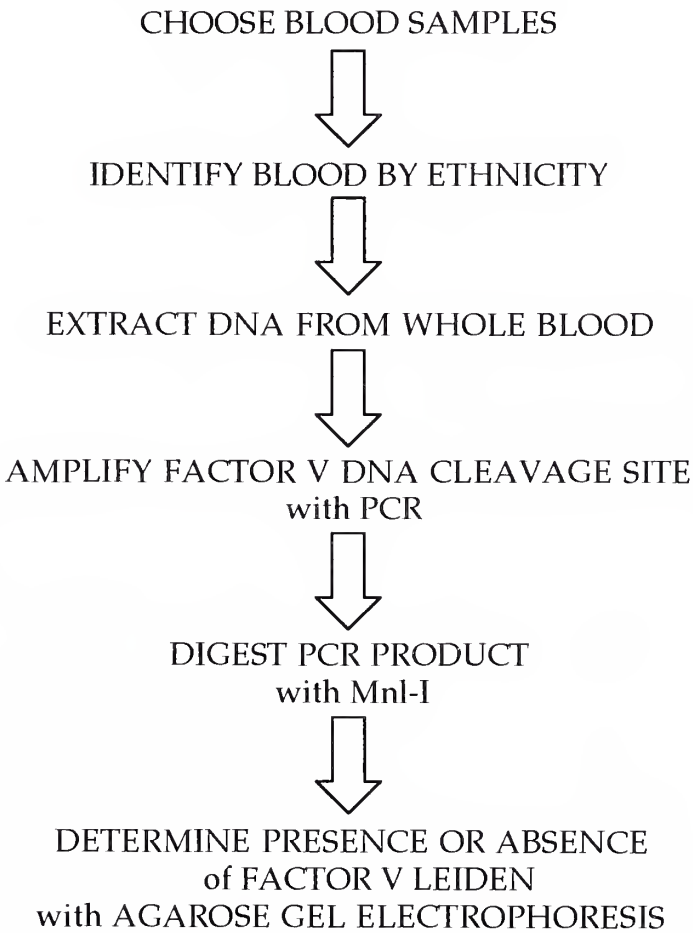
Statement of Purpose

At the time our study was conducted, Factor V Leiden had recently been described as the most common identifiable genetic risk for venous thrombosis in Caucasian patients. However, we found no data on the mutation's prevalence among American blacks. Therefore, the purpose of this study was to determine the prevalence of the Factor V Leiden mutation among non-selected black and non-black inpatients and outpatients at the Yale-New Haven Hospital. Our null hypothesis was that the mutation would not be detected among black patients to the same degree that it was detected among non-black patients.

Methods

Overview

In this study we chose samples of blood from the YNHH clinical hematology laboratory, identified the samples by ethnicity, extracted DNA from the blood, amplified a pre-determined portion of the gene for Factor V using the polymerase chain reaction (PCR), digested that DNA with a restriction enzyme, and used the restriction fragment length polymorphism (RFLP) to determine the presence or absence of the Factor V Leiden mutation. The techniques used were adapted from those described by Bertina *et al*[9] and Koeleman *et al*,[31] and can be summarized as follows:



Preparation

A proposal to conduct this study was submitted to the Yale University School of Medicine's Human Investigations Committee. Permission was granted to proceed, with the understanding that the identities of participants would remain secret. No study subjects would be aware of their participation, and none would be contacted at any time, regardless of individual results.

The YNHH admissions staff agreed to provide access to computerized hospital records. The hematology laboratory staff agreed to provide access to stored blood samples.

Dr. Berliner, Dr. Sigurdsson, and I agreed that this research would be conducted by me under their close supervision; unless otherwise indicated, I performed all of the hands-on work herein.

Patient Selection

Participants in this study were selected in one of two manners:

A. The hospital's clinical hematology laboratory routinely refrigerates tubes of blood for one week after they are drawn and analyzed, and these "remnant" blood samples were made available to us. In order to identify these anonymous blood samples by ethnic background, I first used the hematology laboratory computer to generate a list in chronological order of inpatients and outpatients whose blood had been drawn for unspecified assays after twelve noon on the previous day. The ethnicities of these

individuals was then verified using the hospital admissions laboratory computer, because all patients at Yale are required upon admission to describe their own ethnicity by choosing from the following categories: white, black, Spanish-American, Asian, Pacific Islander, Native American, or other. With knowledge of the patients' ethnicities, I then returned to the hematology laboratory to select appropriate blood samples for our study. Appropriate samples were at least 2cc of liquid whole blood in intact EDTA-anticoagulated Vacutainers® ("lavender-top tubes"). Over the course of several weeks, 180 blood samples were obtained from patients who had described themselves as "black," and 188 from control patients who did not describe themselves as "black"(166 "white," 15 "Spanish-American," 3 "Asian," 4 "other"). I recorded the donors' ethnicities, and numbered these samples in the order in which they were processed. All subsequent products of DNA analysis were labeled and referred to by these unique study numbers.

B. The research laboratory of Dr. Bernard Forget contains a bank of DNA from patients who have participated in unrelated population studies. Some of these are black patients who have been diagnosed with either hereditary spherocytosis or hereditary eliptocytosis. Their DNA was meticulously extracted by Dr. Forget's staff, using the "by hand" technique described below. Forty-five of these DNA concentrates were sampled under sterile technique, consecutively labeled, and included in the study group.

Extraction of DNA

DNA was extracted from whole blood by one of two methods:

QIAamp Protocol

In order to process high numbers of samples in a shorter time, I used the "QIAamp Blood Kit," manufactured by Qiagen.® Blood was processed according to the instructions which came with the kit. Each of the following steps was performed in a sterile fashion.

1) Blood in a lavender top (EDTA) tube was obtained from the YNHH clinical hematology laboratory, as described above. Blood was stored at 4°C until processed.

2) To increase the yield of DNA from whole blood, white blood cells were separated by centrifugation to obtain a buffy coat. This was done by gently agitating the sample on an automated tilt table for five minutes, then withdrawing 1.5ml into a 1.7ml eppendorf tube, and spinning at 4500g for 20 minutes.

3) 200λ of buffy coat was transferred into a new 1.7ml eppendorf tube.

4) To the buffy coat was mixed with 25λ proteinase (either QIA Protease or generic Proteinase K at 10μg/ml) and 200λ buffer AL, and vortexed immediately for ten seconds.

5) The sample was then incubated at 70°C for 10 minutes, either on a heating block or in a hot water bath.

6) The sample was then incubated at 95°C for an additional 15 minutes. This step was intended to deactivate the proteinase and kill any contaminating microbes.

7) The tube cooled for approximately five minutes, and then 210λ 100% isopropyl alcohol was added. The solution was vortexed.

8) The solution was transferred into a proprietary Qiagen® filter "spin column." This column was then placed in a 2ml collecting tube. The column and collecting tube were spun together at full speed for one minute, sending the liquid contents of the solution into the tube, and leaving the DNA behind.

9) The column and DNA were transferred to a clean collection tube. The used collection tube was discarded.

10) The column was washed with 500λ proprietary Buffer AW. The tubes were spun at full speed for one minute, sending the buffer wash into the collection tube and leaving the DNA behind.

11) The collection tube was discarded and replaced with a sterile one, again washed with 500λ proprietary Buffer AW. This time the tube was spun at full speed for two minutes.

12) The used collection tube was discarded, and the column was transferred to a sterile 1.7ml eppendorf tube.

13) 200λ T.E., preheated to 70°C, was added to the spin column. When spun at full speed for one minute, the T.E. carries the DNA residue with it into the collection tube.

14) To verify that this process succeeded in extracting DNA, 7λ of the DNA/T.E. solution was mixed with 3λ of loading buffer containing marking dyes. Samples were then analyzed on a standard 1% agarose gel with ethidium bromide at 100mv for approximately 30 minutes. If present, DNA would show up as a sharp, intense, high-molecular-weight band.

15) DNA was stored in the short run at 4°C, and at -20°C once PCR analysis was complete (see below).

Triton X Protocol

Early in our study DNA from 24 subjects was extracted with the following method.

1) Blood in a lavender top (EDTA) tube was obtained from the YNHH clinical hematology laboratory, as described above. Blood was stored at 4°C until processed.

2) Triton X solution was prepared as follows:

0.32 M Sucrose	109.5 g/L
10 mM Tris HCl, pH 7.5	10 cc/L of 1 M Stock
5 mM $MgCl_2$	5 cc/L of 1 M Stock
1% Triton X 100	10 cc/L

Solution was autoclaved and stored at 4°C.

3) 5 cc whole blood was transferred into a 50 ml conical tube. Tube was filled to 50 ml with Triton X solution precooled to 4°C, then mixed by gentle shaking.

4) Mixture was spun at 1000 g for 10 minutes at 4°C.

5) Supernatant was discarded. Nuclear pellet could then be frozen at -70°C and stored indefinitely.

6) 1 liter of RSB solution as follows:

1M Tris	10 ml
5M NaCl	20 ml
0.5M EDTA	20 ml
10% SDS	50 ml
H_2O	900 ml

7) Nuclear pellet was resuspended in 5 ml RSB.

8) Proteinase K was added at 200 μg/ml, and the solution was incubated overnight at 37°C.

9) 2.5 ml phenol and 2.5 ml chloroform were added to the tube. Tube was agitated gently. After the formation of an aqueous-organic fluid level, the top layer was discarded.

10) Step nine was repeated once.

11) To achieve better purification, 5 ml chloroform was added to the tube, Tube was agitated gently. After the formation of an aqueous-organic fluid level, the top layer was discarded.

12) Step eleven was repeated once.

13) 0.5 ml 3 M NaOAc was added.

14) DNA was precipitated with 13 ml 100% EtOH.

15) A p1000 pipetman was used to move precipitate into a fresh 1.7 ml eppendorf tube.

16) Precipitate was washed with 70% EtOH, and allowed it to air dry.

17) Precipitate was re-suspended in 1.5 ml T.E.

18) To verify that this process succeeded in extracting DNA, 7λ of the DNA/T.E. solution was mixed with 3λ loading buffer containing marking dyes. I then ran this mixture on a standard 1% agarose gel with ethidium bromide at 100mv for approximately 30 minutes. If present, DNA would show up as a sharp, intense, high-molecular-weight band.

19) DNA was stored in the short run at 4°C, and at -20°C once PCR analysis was complete (see below).

Amplification of Factor V APC cleavage site with PCR

In order to determine whether the Factor V Leiden mutation was present or absent in a given subject's DNA, it was necessary to analyze the RFLP pattern of the DNA that encoded for the APC cleavage site. The first step in this analysis involved the amplification of that site via PCR, as follows:

1) DNA primer oligonucleotide primers were synthesized. These primers were the same as those used by Bertina *et al*[9] in their analysis of the factor V

Leiden mutation. They served to amplify a segment of the gene for factor V that includes the APC cleavage site.

The primers had the following sequence:

3′: CTTGAAGGAAATGCCCCATTA

5′: TGCCCAGTGCTTAACAAGACCA

2) A master mix of ingredients for PCR was prepared according to the number of tubes to undergo the process. Each tube contained the following sterile ingredients:

0.5λ DNA (obtained from either the QIAamp protocol or the Triton X protocol outlined above).	
0.2 U/λ Taq Polymerase	0.25λ
0.1μg/λ 3′ oligo	1λ
0.1μg/λ 5′ oligo	1λ
10X buffer	5λ
2 mM dNTPs	5λ
25 mM $MgCl_2$	10λ (This is equivalent to a 5mM solution.)
H_2O	30 λ
	52.75λ Total Volume

In addition to the tubes containing study DNA, one tube contained no DNA ("negative control tube") and one tube contained DNA that had been amplified successfully on previous occasions ("positive control tube").

3) These tubes were subjected to PCR according to the following program:

Denature at 91°C x 40 seconds
Anneal at 60°C x 40 seconds
Extend at 71°C x 1 minute
Repeat x 30 cycles
Extend at 71°C x 5 minutes
Soak at 4°C until tubes are removed from machine

4) 6λ PCR product was mixed with 2λ loading buffer containing marking dyes and electrophoresed through a 1% agarose gel with ethidium bromide for approximately 20 minutes. A base-pair "ladder" of HindIII and ∅X174 was run in each gel to determine fragment length. If present, the APC cleavage site would appear as a band of 220 base pairs.

5) If the negative control lane displayed any bands, the experiment was considered contaminated, and all PCR products were discarded.

6) If no bands appeared in any lane, or if bands appeared with lengths other than 220 base pairs, the PCR products from that tube were considered contaminated, and were discarded.

Analysis of PCR Products with RFLP

RFLP analysis was performed in order to determine whether the patient's amplified DNA was wild-type or factor V Leiden.

1) A master mix was prepared, and the PCR product was digested as follows:

3λ 10X BSA
3λ 10X Buffer (New England Biolabs buffer #2)
0.5λ Mnl-I (New England Biolabs, Equivalent to 2.5 U)
20λ H_2O
4λ PCR product

30.5λ total volume.

2) Tubes were spun briefly to move all fluid into the bottom.

3) Tubes were then incubated at 37°C for two hours.

4) Digestion was visualized on 2% agarose gel as follows: 6λ loading buffer containing marking dyes was added to each tube, and the whole volume of digestion (approximately 36λ) was loaded into large wells in the gel. One lane was loaded with undigested PCR product (the "negative control"), and another was loaded with digested PCR product from a patient with known to have Factor V Leiden (the "positive control"). A base-pair "ladder" was made with HINDIII and ∅X174 Electrophoresis was performed in TBE at 150 mv for approximately 30 minutes.

5) The gel was visualized and photographed.

6) The predicted sizes of the digested PCR products from patients with wild-type factor V DNA are 163, 67, and 37 base pairs.

7) Because the factor V Leiden mutation destroys a restriction enzyme site within this fragment, the predicted sizes of the digested PCR products from patients with factor V Leiden DNA are 153 and 67 base pairs. ***Therefore, the difference between 153 base pairs and 67 base pairs was diagnostic for Factor V Leiden.*** (See Figure 4.)

8) Any uncut DNA was found at 220 base pairs (matching the band found in the "negative control" lane). If 220 was the only band found in a lane, that digestion was considered a failure, and more PCR product was digested and visualized.

Time Course

This research was conducted in June, July, and August of 1995.

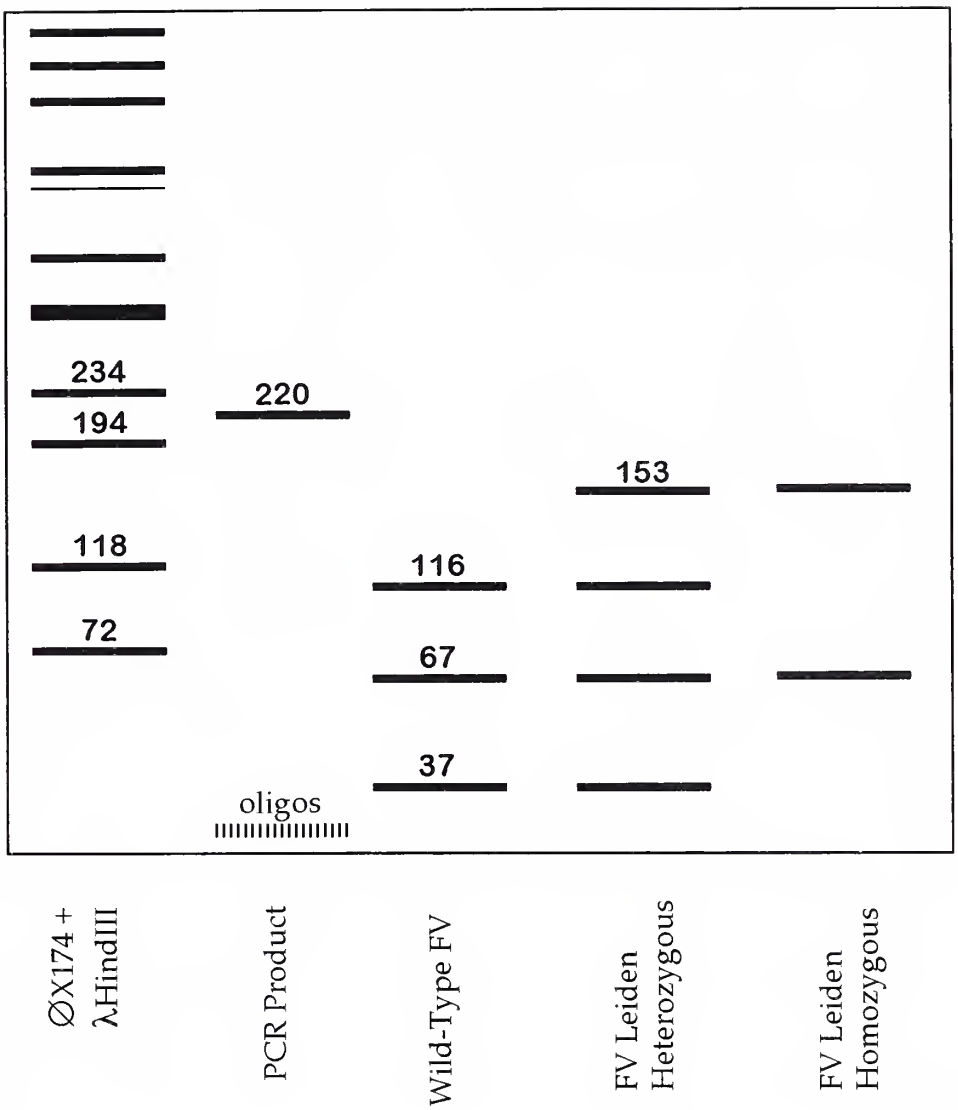


Figure 4. Appearance of an agarose gel slab after undergoing electrophoresis with the DNA products of our experiment. A base-pair "ladder"was used at left to gage the lengths of DNA fragments. Proper amplification of the factor V cleavage site region yielded a band of 220 base pairs, and a faint band at the bottom of the gel, representing oligonucleotides left over from the PCR process. After undergoing digestion with the Mnl-I restriction enzyme, three patterns were possible: DNA from patients with two wild-type copies of the factor V gene would appear as three bands at 116, 67, and 37 base pairs; if patients were heterozygous for factor V Leiden, a band of 153 base pairs would appear in addition to the three bands found in the wild-type DNA; in patients who were homozygous for factor V Leiden, only bands at 153 and 67 base pairs would be expected.

Results

The blood of 188 anonymous patients who did not describe themselves as "black" was obtained and analyzed as described above. Of these samples, four became contaminated, 45 were not amplified successfully via PCR, and 13 were not digested successfully with Mnl-I. Of the remaining 126 samples, 124 were negative for the factor V Leiden mutation, two were heterozygous positive, and none was homozygous positive.

The blood of 180 anonymous patients who described themselves as "black" was obtained and analyzed as described above. Of these samples, three became contaminated, five were not amplified successfully via PCR, and six were not digested successfully with Mnl-I. Of the remaining 166 samples, 165 were negative for the factor V Leiden mutation, one was heterozygous positive, and none was homozygous positive.

DNA from 49 patients who described themselves as "black" and had participated in unrelated studies was obtained from another laboratory and analyzed as described above. Of these samples, one was not digested successfully with Mnl-I. Of the remaining 48 samples, 46 were negative for the factor V Leiden mutation, two were heterozygous positive, and none was homozygous positive.

In total, then, of the 214 samples from black patients that were analyzed successfully, three (1.4%) tested positive for the factor V Leiden mutation. By comparison, the prevalence of the mutation in 126 samples from non-black patients that were analyzed successfully was two (1.6%).

Therefore, the relative risk of carrying a single copy of the factor V Leiden Mutation is 0.88 (Fisher Exact two-tailed test, P = 1.0000, 95% confidence interval 0.12 to 7.64). (See Table 1.)

	Wild Type FV	Heterozygous FV Leiden	Homozygous FV Leiden
Black	211	3	0
Non-Black	124	2	0

Table 1. Results were obtained for 214 Black individuals, of whom 3 (1.4%) were heterozygous for the Factor V Leiden mutation. By comparison, the prevalence of the mutation in a group of 126 non-Black patients selected in the same manner was 2 (1.6%), yielding a relative risk of 0.88 (Fisher Exact two-tailed test, 95% CI 0.12 to 7.64, P = 1.0000). No patients were found to be homozygous for factor V Leiden.

Statistical analysis was computed with Epi Info.©

Discussion

These data do not support the hypothesis of a difference between the incidence of the factor V Leiden mutation in the black and non-black populations studied. Rather, the proportion of these populations that carries a single copy of the factor V Leiden allele is very close: 1.4% among blacks and 1.6% among non-blacks, a difference that is not statistically significant.

Other studies confirm approximately the same prevalence of factor V Leiden among American blacks. Dilley *et al*[54] tested for the prevalence of this mutation in 93 non-selected blacks, and found one heterozygous carrier, equivalent to a prevalence of 1.1%. Hooper *et al*[55] looked for the mutation among 76 blacks without a history of thrombosis, and found one heterozygote, for a prevalence of 1.4%. Austin *et al*[56] assayed 130 African-Americans, and found one who was heterozygous for factor V Leiden, for a prevalence of 0.8%. The largest study to date on prevalence of the mutation among African Americans was published by Ridker *et al* last year.[57] In this experiment, blood samples from 1,608 men participating in the Physicians' Health Study and 2439 women participating in the Women's Health Study were assayed using techniques of PCR and RFLP similar to those used in our study. Subjects were free of myocardial infarction, stroke, or venous thrombosis at time of enrollment. Six-hundred and fifty of them described themselves as African-American; 1.23% tested positive for factor V Leiden. (See Table 2.)

Factor V Leiden Mutation

Author (ref.)	Location	Yes	No	Carrier Prevalence	95%CI
present study (1)	New Haven	3	211	1.4%	0.12-7.64
Dilley (54)	Atlanta	1	92	1.1%	*
Hooper (55)	Atlanta	1	75	1.4%	0.02-3.60
Austin (56)	Atlanta	1	129	0.8%	0.01-2.1
Ridker (57)	USA	8	642	1.2%	0.53-2.41
Total		14	1,149	**1.2%**	

Table 2. Cumulative data from this and other studies of the prevalence of the factor V Leiden mutation in non-selected patients who described themselves as "black" or "African American." The total prevalence of 1.2% is close to the 1.4% found in our study.

However, our control data are not in agreement with the prevalence found in other studies. In the Ridker data, for instance, 2,468 participants described themselves as Caucasian; 128 tested heterozygous positive for factor V Leiden, and 2 tested homozygous positive, for a total carrier rate of 5.27% (95% CI 4.42 to 6.22). This is significantly different from our carrier rate; even when only "white" controls are included, the carrier rate is 1.8% (110 subjects, 2 heterozygous positive). We have no explanation for this discrepancy.

As more information has become available on the mutation's prevalence in different ethnic groups around the world, persuasive

* Preliminary data published as an abstract at the 1995 annual conference of the American Society of Hematology. No statistical analysis performed.

hypotheses have been made regarding its evolutionary genetics. Reviews such as that by Rees[53] compile evidence that points strongly to a single origin of the mutation, or "founder effect," with subsequent genetic mixing to explain the mutation's existence in the new and old world. For instance, studies of more than 550 individuals from Sub-Saharan Africa have revealed no carriers of factor V Leiden,[53,58] a marked contrast from the prevalence in American blacks, which would be consistent with genetic mixing rather than separate *de novo* mutations in the old and new worlds.

If we wanted to apply our data to the question of whether factor V Leiden is present in blacks because of the mixing of genetic information from Europe or because of a *de novo* mutation, we would require extensive genetic pedigrees from all participants. We have no pedigrees on any study participants, nor do we have precise information on their ethnic backgrounds; we might imagine that patients of African, African-American, Caribbean, or Latin heritage might describe themselves as "black." New Haven does have a considerable African-American population, so it is not unreasonable to postulate that the majority of patients who described themselves as "black" would also describe themselves as "African-American" if given that option. However, we cannot take that assumption for granted. So the question of where the factor V Leiden mutation came from is beyond the scope of this study.

Nor was that our intention. The origins and population drift of factor V Leiden pose fascinating questions, but those were not *our* questions.

Regardless of how or when Factor V Leiden entered the black population of New Haven, our data show that it is present to a significant degree.

How, then, do we use this knowledge to best serve our black patients? Does it make sense to seek the mutation in every black resident of New Haven? Apparently it does not. It has been estimated that the cost of preventing one thromboembolic death by screening a general population with a factor V Leiden prevalence of 2% would cost over $44 million.[59] Furthermore, anticoagulating all factor V Leiden carriers would probably do more harm than good, because the morbidity of lifelong warfarin therapy would surpass the inherent thrombotic risk of the mutation.[60,61] On the other hand, it does not seem prudent or ethical to ignore these data. Some middle ground must be sought, in which individual patient risk factors are considered. Patients with factor V Leiden should be counseled as to the risk factors that it entails, as should their families.

So far, the risk factors among black carriers seem to be the same as those in the non-black population. At the time we conducted this study, it was unknown whether the detection of factor V Leiden among African Americans would hold implications specific to that group. For instance, patients with sickle cell disease would seem to benefit from screening for factor V Leiden; because both conditions are associated with abnormal blood flow, a synergistic effect and propensity towards more crises might be predicted. Interestingly, this has not been demonstrated. Wright *et al* found only one carrier of factor V Leiden among 165 patients with steady-state sickle

cell disease.[62] Kahn *et al*[63] looked for factor V Leiden in 82 patients with either HgbSS, SC, or S-beta thalassemia, 19 of whom had a history of stroke. Only one of the patients *without* a history of stroke had factor V Leiden, for a carrier prevalence of 1.2%. Although such data do not implicate factor V Leiden in the genesis of sickle crises, they do not rule out such an interaction either.

In the few years since its description there has been a boom in information about the mutation: a Medline© search today for the subject "factor V Leiden" yields more than 250 results, and there is every indication that the research will continue. And with good reason. In a sense, the story of factor V Leiden is emblematic of much genetic research in the 1990s: we can identify the molecular lesion, we can quantify the clinical risks it entails, but we cannot repair the DNA. Until that becomes possible, we are obligated to pursue the mutation's presence in patients with thrombophilia, and make treatment decisions based on individual risk factors. Our data indicate that factor V Leiden is a risk factor that should be considered in both black and non-black patients.

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