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Association of factor V deficiency with factor V HR2

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A B S T R A C T

Background and Objectives. Factor V HR2 possesses decreased co-factor activity to activated protein C and an increased ratio of factor V1 to factor V2. Factor V HR2 is associated with a mild increase in the risk of venous thromboembolism although not all studies concur on this point.

Design and Methods. Inconsistencies in results of the epidemiological studies may stem from a failure to identify other variables in factor V which might contribute to an increased risk of thrombosis in selected HR2 carriers. The aim of this study was to establish whether factor V deficiency increases the risk of venous thromboembolism when associated with HR2.

Results. Four hundred and ninety-seven patients with venous thromboembolism and 498 controls were studied. HR2 was present in 12.5% of patients and 10.4% of controls. Factor V deficiency was associated with HR2 in 4.6% of patients and 1.0% of controls. The OR for venous thromboembolism in individual with HR2 alone was 1.2 (95% CI 0.8-1.8), while it was 4.7 (95% CI 1.8-12.5) for those with HR2 plus factor V deficiency.

Interpretation and Conclusions. Patients with HR2 and factor V deficiency developed a thrombotic event earlier (median age 35 years) than patients with HR2 alone (median age 43 years, $p = 0.018$). Double heterozygosity for HR2 and a factor V defect, including factor V deficiency, increased the thrombotic risk afforded by HR2.

Key words: factor V HR2, factor V deficiency, factor V Leiden, venous thromboembolism.

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A complex haplotype of human factor V, which includes 13 different polymorphisms throughout the gene, is termed HR2.^{1,2} Seven of the 13 base changes predict an amino acid change in factor V, and presumably lead to functional modifications of the protein.^{1,2} The product of the HR2 factor V gene has been characterized extensively: it possesses decreased co-factor activity for activated protein C in the degradation of factor VIIIa, and an increased ratio of factor V1 to factor V2, the former being the more procoagulant isoform.¹⁻³ Increased resistance to activated protein C (APC) and reduced factor V antigen and/or coagulant activity have been associated with HR2 factor V, although not consistently.⁴⁻⁷ The HR2 haplotype is very ancient and rather frequent, its prevalence throughout Asia, Europe and in native African populations ranging from 8 to 12%.⁴⁻⁹ A selective advantage for carrier-ship has been postulated for this gene, similarly to that for carrier-ship of factor V Leiden.

Based on the results of its biochemical characterization, it is reasonable to expect that carrier-ship of factor V HR2 would be associated with an increased risk of venous thromboembolism (VTE). Several studies have however, provided contrasting results.^{4-7, 9, 10} Most of them provide evidence of an increased, though mild, risk of VTE, and, more clearly, that factor VHR2 is a risk enhancer, i.e. it increases the risk of VTE afforded by factor V Leiden. This was observed in two case-control studies and in one prospective study.^{7, 11, 12} The association of factor V Leiden and HR2 is, however, rare, being expected in approximately 3 in 1000 individuals in the general population and 3 in 100 unselected patients with thrombosis. The mechanism by which HR2 increases the risk conferred by factor V Leiden is probably a synergy of actions. On the one side it is the sum of the products from two pathologic alleles that are both pro-thrombotic and both lack APC co-factor activity. On the other hand, part of the

effect is possibly due to the double heterozygote state, since factor V Leiden and factor V HR2 never reside on the same allele. Individuals with both defects, therefore, do not have any *normal* factor V.

The matter of the mechanism by which HR2 functions as a risk enhancer is interesting in that it allows this concept to be extended to all situations in which factor VHR2 is associated with a defective factor V and, by further extension, to factor V deficiency. In fact, one might suppose that the random association of factor V deficiency and factor V HR2 could confer an increased risk of VTE since the product of the HR2 gene would not be counterbalanced by *normal* factor V. Here we report the results of our study aimed at verifying this hypothesis.

Design and Methods

Patients

Four hundred and ninety-seven patients (296 women and 201 men, median age 47 years, range 15–78) consecutively referred to the Thrombosis Centers of two large Italian cities in year 2001 for an episode of venous thromboembolism and thrombophilia screening were enrolled in the study. Any venous thrombotic event, as long as it was objectively confirmed, was considered, so that patients were unselected. Criteria for exclusion were liver or renal disease as ascertained by routine diagnostic tests. Four hundred and ninety-eight controls (278 women and 220 men, median age 46, range 16–84) were enrolled as well. In Milan, these control individuals were friends of patients asked to give blood if they had a personal negative history of thrombosis; in Vicenza, they were part of a larger population-based survey, the VITA study, described elsewhere.¹³ Oral anti-coagulants had been withdrawn at least one month before blood sampling and at least four months had passed since the thrombotic event. All the individuals underwent thrombophilia screening. In addition, the HR2 haplotype was searched for in all patients and controls. In patients positive for the HR2 haplotype, factor V coagulant levels were measured. Factor V was also measured in a group of patients who were not carriers of the HR2 haplotype but who were matched by sex and age to the HR2 carriers. All plasma samples from patients and controls were stored at -80°C . Informed consent to blood testing was obtained from all participants.

Screening for inherited thrombophilia

Factor V Leiden, prothrombin 20210A and HR2 were identified by standard methods. Due to linkage disequilibrium between the polymorphisms, detection of the A/G 4070 mutation (also termed R2), which pre-

dicts a His to Arg amino acid change in position 1299 within exon 13 (B domain) of factor V was considered sufficient to identify the HR2 haplotype. Resistance to APC was tested on an automated coagulometer (ACL 9000, Instrumentation Laboratory, Milan, Italy) using the original method reported by Svensson and Dahlbäck¹⁴ and modified as described elsewhere.⁴ This method is highly sensitive to factor V Leiden, but not totally specific, and picks up resistance to APC that is factor V Leiden-independent. Results are expressed as normalized ratios: lower ratios represent greater resistance to APC. The normal laboratory range for this variable is 0.76–1.18. The other tests of thrombophilia (including measurement of antithrombin, protein C and protein S) were carried out using established methods.

Factor V measurements

Due to the putative instability of factor V, the activity of this factor was measured over 6 months in subjects with normal and low factor V plasma levels. All plasma samples were kept at -80°C and rapidly thawed at 37°C prior to testing. Testing was performed at baseline, at weeks 1 and 2, and at 1, 2, 3 and 6 months. One-stage clotting tests (prothrombin-based) were carried out in an automated coagulometer (ACL 9000, Instrumentation Laboratory, Milan, Italy) using one dilution of test plasma. Factor V levels were extrapolated from a reference curve obtained from three dilutions of pooled reference plasma (see below). A factor V-deficient sample (from a known factor V-deficient patient) was run in each assay as a control. Totally factor V deficient plasma was purchased from Cabru (Peregallo di Lesmo, Italy) or from Instrumentation Laboratory (Milan, Italy). The normal laboratory range of factor V is 70–134% of pooled reference plasma and was obtained by determining factor V levels in 200 normal individuals (100 men and 100 women).

Statistical analysis

All variables were treated as dichotomous (presence/absence of a mutation, of a defect, of multiple defects, etc.) with the exception of factor V levels and APC normalized ratio, which in some cases (see below) were used as continuous variables. Odds ratios and their 95% CI were calculated by binary logistic regression, as an estimate of the risk of carriers of that defect developing a venous thromboembolic event compared with the risk of non-carriers doing so. Multiple logistic regression established which factors were independently associated with being a case rather than a control. Correlation analysis by non-parametric tests was also carried out for factor V levels and resistance to APC. Resistance to APC and factor V levels were compared in cases and controls and in carriers or non-

Table 1. Demographic and clinical characteristics of the 497 cases enrolled in the study.

Sex, women/men	296/201
Age, median (range) in years	47 (15-78)
Number of VTE at enrollment, absolute number (percentage of total)	
One	377 (76%)
More than one	120 (24%)
Type of first event, absolute number (percentage)	
Deep vein thrombosis of a limb	260 (52%)
Pulmonary embolism	70 (14%)
Pulmonary embolism and deep vein thrombosis	23 (5%)
Superficial thrombophlebitis	72 (15%)
Visceral vein thrombosis	11 (2%)
Cerebral vein thrombosis	12 (2%)
Retinal vein thrombosis	26 (5%)
Others [§]	23 (5%)
Patients with or without a circumstantial risk factor at first event, and type of event, absolute number (percentage)	
None	348 (70%)
At least one	149 (30%)
Oral contraceptives or hormonal replacement therapy	45 (30%*)
Surgery	27 (18%*)
Pregnancy or puerperium	36 (24%*)
Trauma or prolonged immobilization	41 (28%*)

[§]Axillary, mammary, jugular vein thrombosis; repeated abortion *of patients with a circumstantial risk factor.

carriers of HR2 by the Mann-Whitney U-test, since factor V levels were not distributed normally. Continuous variables are presented as median and range. The statistical package SPSS for Windows version 10.0 was used for the statistical analyses.

Results

Patients

Table 1 illustrates the characteristics of the patients enrolled in the study, the type of first thrombotic event and the associated circumstantial risk factors. The most frequent event was deep vein thrombosis of a lower limb, followed by pulmonary embolism. The most frequent circumstantial risk factors associated with the first thrombotic event were prolonged immobilization and, in women, oral contraceptive use and pregnancy.

Factor V levels over time

Factor V clotting levels over time in a sample of normal plasma and in plasma from a patient with factor V deficiency did not change significantly (in normal plasma: 82, 75, 75, 72, 77, 79, 79%; in factor V-deficient plasma: 58, 60, 58, 54, 57, 56, 57%, at baseline,

week 1 and 2, month 1, 2, 3 and 6, respectively). This time course allowed us to establish that factor V levels are stable up to at least six months if the plasma is stored at -80°C . As a consequence, factor V was tested in plasma samples before the six-month storage time had expired.

Distribution of risk factors between cases and controls and odds ratios

Table 2 shows the distribution of established inherited risk factors for venous thromboembolism in cases and controls and the prevalence of factor V HR2. As can be seen from the table, the prevalence of risk factors in cases was that expected of an unselected population with venous thrombosis. Table 2 also reports odds ratios for venous thromboembolism according to carriership of any one of the defects. Resistance to APC and low factor V levels were dichotomized: the defect was considered to be present for levels of factor V below 70% and APC ratios below 0.76. All defects were also taken together (without HR2) and included prothrombin 20210A, factor V Leiden, antithrombin deficiency (four cases), protein C deficiency (seven cases), and protein S deficiency (eight cases). Factor V HR2 by itself was not a risk factor for venous thromboembolism. It was not possible to estimate an odds

Table 2. Distribution of risk factors for venous thromboembolism in cases and controls and odds ratios for carriership of thrombophilic defects. APC resistance is defined as an APC ratio <0.76 and factor V deficiency as factor V activity levels <70%.

	Cases N=497	Controls N=498	Odds ratios (95% CI)
	N (%)		
Factor V Leiden			
heterozygotes	82 (16.5%)	18 (3.6)	5.4 (3.2-9.1)
homozygotes	9 (1.8%)	0	NC
Prothrombin 20210A			
heterozygotes	57 (11.5)	18 (3.6)	3.4 (2.0-6.0)
homozygotes	1 (0.2)	0	NC
Resistance to activated protein C (all) *	96 (19.3)	18 (3.6)	6.3 (3.8-10.7)
Resistance to activated protein C (no factor V Leiden)	9 (1.8)	0	NA
Any defect [§]	155 (31.2)	34 (6.8)	6.2 (4.2-9.2)
Factor V HR2**			
heterozygotes	61 (12.5)	52 (10.4)	1.2 (0.8-1.8)
homozygotes	3 (0.6)	1 (0.2)	NC
Factor V HR2 with factor V Leiden	12 (2.4)	0	NA
Factor V HR2 (heterozygotes) with low factor V clotting activity (below 70%)	23 (4.6)	5 (1.0)	4.7 (1.8-12.5)

*4 missing determinations in factor V Leiden carriers; **9 missing determinations; [§]HR2 not included; includes antithrombin, protein C and protein S deficiency (see text); NA: not applicable (none observed with defect in controls); NC: not calculated.

ratio for the association between factor V Leiden and factor V HR2 because none of the controls was doubly heterozygous. However, there was a statistically significant difference in the distribution of the double defect (HR2 and factor V deficiency) between cases and controls ($p < 0.001$). When factor V HR2 was associated with low factor V levels, a risk of approximately 5 was estimated (Table 2, bottom row). To ascertain that this risk was not confounded by other prothrombotic factors, multiple logistic regression was performed taking into account both the double defect (HR2 and factor V deficiency) and any other prothrombotic defect. Logistic regression showed that factor V HR2 plus factor V deficiency remained statistically significant, i.e. was independently associated with an increased risk of venous thromboembolism ($p = 0.0023$).

Factor V levels

Factor V levels were measured in all individuals with HR2, both cases and controls, if plasma was available (three samples were missing among cases, six among controls). Twenty-three of 61 cases with HR2 and 5/47 controls with HR2 had factor V levels below the lower limit of the normal range. Sixteen of 23 cases and 1/5 controls had factor V levels below 50%, suggesting a genetically based factor V deficiency. When factor V levels were compared between cases and controls with HR2, a significant difference was detected (fac-

tor V levels, median and range, in cases with HR2: 80%, 26-111; in controls with HR2: 96, 49-135, $p < 0.001$). Since factor V levels could be lower in cases than controls independently of HR2 status, factor V levels were also measured in a group of patients without HR2, matched by age and sex to the patients with HR2. Factor V levels were within the normal range in this group (median factor V: 107%, range 77-130).

Resistance to APC

Normalized ratios were lower in carriers of HR2 than in non-carriers, independently of case/control status, after excluding factor V Leiden: the median levels were 0.97 vs 1.00, respectively ($p = 0.045$). To check whether this difference could be related to the lower factor V levels in HR2 carriers than in non-carriers, the correlation between factor V levels and resistance to APC was evaluated in HR2 carriers. No correlation was found either including ($\rho = 0.339$ by Spearman's test) or excluding factor V Leiden carriers ($\rho = 0.180$ by Spearman's test) from the analysis. Within HR2 carriers, APC normalized ratios were lower in cases than controls (median levels 0.94 vs 1.03, $p = 0.003$).

Clinical characteristics of carriers of HR2

Twenty-three patients who were carriers of HR2 had factor V levels below 70% of normal, whereas the remaining 38 had levels above of normal. No differ-

ences could be detected in the type of thrombotic events between the two groups: deep vein thrombosis ± pulmonary embolism 57% vs 52%, superficial thrombophlebitis 35% vs 30%, cerebral vein thrombosis 4% vs 6%, retinal vein thrombosis 4% vs 6% in the group with low factor levels compared to the group with normal factor V levels group (6% of the former group also had miscellaneous type thrombosis). The age at the time of the first event was 35 years (range 19–46) in the group with lower factor V levels compared to 43 years (range 18–74) in the group with high factor levels ($p = 0.018$ by the Mann-Whitney test). Circumstantial risk factors at the first thrombotic event were present in 42% and 43% of patients with low and normal factor V levels, respectively.

Discussion

HR2 factor V has rather unique features in the coagulation field, in that it is frequent, being found in approximately 10% or more of the studied populations, and it possesses the biochemical characteristics of a prothrombotic factor. Similarly to what has been postulated for factor V Leiden, which is rarer than HR2 in most populations, its evolutionary stability may be due precisely to its mildly prothrombotic features which constitute an advantage rather than a disadvantage for mankind. An exception to this statement is the Western society of today, in which inherited prothrombotic factors interact strongly with frequently occurring acquired prothrombotic factors (oral contraceptives, prolonged age, to mention some) to generate events. In contrast to factor V Leiden, factor V HR2 is perhaps not a risk factor by itself, but possibly interacts with other factor V defects. This has been shown for the association of factor V HR2 with factor V Leiden^{11,12} and was postulated for the association between factor V HR2 and factor V deficiency.¹⁵

In the present study we report that factor VHR2 and factor V deficiency together increase the risk of venous thromboembolism more than four-fold. Patients who carry both defects seem to develop events earlier than do carriers of HR2 alone. No difference in the type of thrombotic event was evidenced. Though these results are typically expected when two risk factors interact, they need to be confirmed prospectively.

The first report on HR2 stemmed from the search, in patients with factor V deficiency, of molecular defects in the factor V gene.¹⁶ Since then, studies have focused on the prothrombotic aspects of HR2, but reports have not provided consistent results regarding the association with low factor V levels. When confirmed, levels of factor V in HR2 carriers were lower than in non-carriers but nonetheless within the normal laboratory

range. In parallel, results from studies on the stability of factor V derived from the HR2 gene did not show any difference compared to normal factor V,⁴ and a search for mutations in the promoter region of HR2 failed to identify any.⁷ Recently, a study showed a decrease of steady-state expression levels or of secretion rates of factor V carrying any of three missense mutations which are part of the HR2 haplotype (Asp2194Gly, His1299Arg, Met385Thr).¹⁷ In our study factor V deficiency was strongly associated with factor V HR2, since patients with a previous thrombotic event who were not carriers of HR2 had normal factor V levels. However, patients with a previous thrombotic event and HR2 had lower levels of factor V than did controls carriers of HR2. It does, therefore, seem unlikely that the observed low factor V levels are due uniquely to the molecular alterations of HR2. Acquired causes of factor V deficiency were ruled out and measurements were reliable since plasma was stored correctly, as shown by the stability of factor V levels over time. Moreover, intra-individual variability of factor V is reported to be very low.¹⁸ An alternative explanation is that factor V deficiency is independently and randomly associated with factor VHR2, and that when this happens it concurs to the thrombotic risk. Patients who carry both defects can be considered as double heterozygotes for a defect in APC co-factor activity towards factor VIII and homozygotes for a prothrombotic defect, since the HR2 allele product is not counterbalanced by normal factor V.

The diagnostic and clinical relevance of these findings remains unclear. Folsom and co-workers, who recently confirmed in a prospective study that the association of HR2 and factor V Leiden confers an increased risk of venous thrombosis,¹² concluded their paper by underlining the rarity of this association (3% of patients with thrombosis) and thus wonder whether testing for HR2 is worthwhile. A similar issue can be raised for the association of HR2 and factor V deficiency. First of all, no clear data on the prevalence of factor V deficiency are available. One of the problems connected with establishing this estimate is the variability of hemorrhagic symptoms associated with the defect, so that low levels may go undetected in asymptomatic patients. Moreover, not all laboratory reagents used to measure prothrombin time or activated thromboplastin time are sensitive to 50–70% factor V levels so that during routine screening the deficiency state might be overlooked. Of the 23 patients with low factor V levels identified in our study, only 2 had prolonged prothrombin or activated thromboplastin times. Variability in the prevalence of factor V deficiency in different geographical areas (and thus variability in the prevalence of the association) might explain the differences in risk estimates of HR2 reported by sev-

eral groups. Conversely, one could speculate that the association of HR2 with factor V deficiency might partly account for the variability of clinical expression of hemorrhagic symptoms in factor V-deficient patients. Regarding the association of APC resistance with HR2, another debated topic, we confirm in this study that HR2 carriers had slightly lower levels of APC resistance than did non-carriers. This difference did not seem to be related to factor V levels and was more marked in patients than controls. It is hard to explain this finding, which is not consistent in the different studies. Possibly the method of detection of APC resistance plays a role. It has been elegantly shown that the resistance induced by factor V HR2 is related to the inability to inactivate factor VIII (decreased co-factor activity of HR2) rather than factor V.^{2,6,8} Thus, a test based on factor VIII inactivation would perhaps be better suited to pick up this defect. The classical factor V-based APC resistance test, on the other hand, is sensitive to factor V Leiden and, specifically, to vari-

ous prothrombotic factors: elevated factor VIII levels, elevated prothrombin levels, and lupus anticoagulant, which are all variably associated with thrombophilia.^{19,20,21} Thus, the slightly more elevated resistance to APC in HR2 carriers might be related to slower factor VIII inactivation and, in some cases, to a *thrombophilia* status.

In conclusion, we believe that factor HR2 is a mildly prothrombotic factor which can be clinically relevant when associated with other factor V defects. Whether it is worthwhile screening patients with thrombosis for HR2 depends on costs and health policy issues which can only be resolved locally.

EMF, GC and FR conceived and designed the manuscript, drafted the article, and gave approval of final version to be submitted; DA was responsible for enrolling patients, for processing of blood, for running laboratory tests, and gave approval of final version to be submitted. FL: conception and design, statistical analysis and interpretation of data, and gave approval of final version to be submitted. The authors reported no potential conflicts of interest.

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