# To the editor:

## Increased resistance to activated protein C in women taking third-generation oral contraceptives?

In a recently published paper in *Blood*,[1] Kemmeren et al elegantly studied the effect of second- and third-generation oral contraceptives (OCs) on hemostatic variables that probe the activity of the protein C system. The authors showed that, because the progestagen poorly counteracted the effect of the estrogen component, desogestrel-containing OCs significantly increased activated protein C (APC) resistance, thus perhaps explaining the higher thrombotic risk associated with third-generation oral contraceptives.

The design of this double-blind study is perfect. However, the intensity of the plasma anticoagulant response to activated protein C was determined by quantifying the effect of activated protein C on thrombin generation and was given *only* as the APC sensitivity ratio (APC-sr; the higher the values are, the lower the plasma anticoagulant response is), as described by Rosing et al,[2] a choice that, for us, calls for some comments.

The APC-sr parameter is obtained by calculating the P/N ratio. P is obtained, for a given patient's plasma, by dividing the value of the endogenous thrombin potential (ETP) performed in the presence of a constant amount of purified activated protein C (ETP-aPC) by the basal value of the ETP. N corresponds to P performed on a normal reference plasma. For a given patient's plasma, the APC-sr parameter is finally the $[(\text{ETP-APC})/\text{ETP}]_{\text{patient}}$–$[(\text{ETP-APC})/\text{ETP}]_{\text{normal plasma}}$ ratio. The variations of the APC-sr parameter must be analyzed basically according to the variations of the various components of the formula and depend mainly on the variations of the $(\text{ETP-APC})_{\text{patient}}$ and the $\text{ETP}_{\text{patient}}$ components. No data in the paper by Kemmeren et al are given that would allow this cautious analytic interpretation to be performed.

We thus studied the single paper from this team that used the same technical methodology but that also contained enough crude data to allow the analysis of the variations of the APC-sr parameter.[2] Results are given in Table 1. As detailed, the values of the $[(\text{ETP-APC})/\text{ETP}]_{\text{patient}}$ ratios obtained in women taking third-generation oral contraceptives are obviously higher than those obtained in women taking second-generation oral contraceptives (nearly the same values as those found in factor $V_{\text{Leiden}}$ heterozygous carriers), quickly leading to the claim of a lower plasma response to activated protein C. However, the striking analytic facts are that the ETP-APC values are consistently higher in women taking third-generation OCs than in women taking second-generation OCs and that the values of the absolute differences calculated between ETP-APC and ETP values (Table 1, "ETP–ETP-APC") are *identical*—midway between the one found in women not taking OCs and the one found in heterozygous factor $V_{\text{Leiden}}$ female carriers.

**Table 1. Analysis of the data contained in the initial paper from Rosing et al[2]**

| Origins of pooled plasmas (no.) | ETP | ETP-APC | ETP-APC/ETP | ETP-ETP-APC |
|---|---|---|---|---|
| Normal plasmas (90) | 458 | 43 | 9.4 | 415 |
| Men (23) | 442 | 39 | 8.8 | 403 |
| **Women** | | | | |
| No OCs (27) | 473 | 45 | 9.5 | 428 |
| Triphasic OCs (28) | 533 | 105 | 19.7 | 428 |
| Monophasic OCs | | | | |
| Second generation (28) | 483 | 85 | 17.6 | 398 |
| Third generation (25) | 554 | 155 | 28 | 399 |
| Factor $V_{\text{Leiden}}$ +/− (23) | 486 | 141 | 29 | 345 |

ETP indicates endogenous thrombin potential; ETP-aPC, endogenous thrombin potential in presence of a constant amount of purified activated protein C; and OCs, oral contraceptives.

Thus, women taking third-generation OCs are probably not less responsive to activated protein C than are women taking second-generation OCs but probably have only significantly higher basal values of the endogenous thrombin potential. Studies of the variations of the APC-sr parameter induce a bias of interpretation if they are not analyzed in relation to the variations of the parameter's components. We imagine that the search for coagulation-related indicators, in hopes of understanding the higher prothrombotic risk in women taking third-generation OCs, is more likely to be informative if performed in order to understand what explains the effect of the progestagen desogestrel on the basal endogenous thrombin potential itself rather than in order to analyze the induced variations of sensitivity to activated protein C.

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2. Rosing J, Tans G, Nicolaes GAF, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol 1997;97:233-238.

# Response:

## APC fails to adequately down-regulate thrombin generation in oral contraceptive users

The letter by Gris and colleagues raises an interesting point about the interpretation of activated protein C (APC) resistance determined with the endogenous thrombin potential (ETP)–based APC resistance test. Following an earlier debate paper,[1] they argue that the normalized APC sensitivity ratio (nAPC-sr) determined with the ETP-based APC resistance test induces bias of interpretation unless analyzed in relation to the variation of its components, which are the endogenous thrombin potentials (ETP = time integral of thrombin generation) determined in the absence and presence of APC.

Before addressing the question of interpretation, we would like to point out that the nAPC-sr determined with the ETP-based APC resistance test and calculated according the equation used in the paper that we recently published in *Blood*[2] (1) correlates very well with the risk of venous thrombosis for a wide variety of risk factors and combinations of risk factors[3] and (2) predicts the occurrence of venous thrombosis in both men and women, also in the absence of the factor $V_{Leiden}$ mutation, a conclusion that is based on analysis of the Leiden thrombophilia study.[4]

The fact that, in the latter study, the predictive value of the ETP-based APC resistance test was the highest in women who were using oral contraceptives (OCs) supports our interpretation regarding the association between changes in APC resistance and the risk of venous thrombosis in women using second- and third-generation OC.

We agree with Gris et al that the variation of the individual components that comprise the nAPC-sr may provide additional information with respect to the mechanistic basis and the thrombotic risk of acquired APC resistance that occurs during OC use. Actually, their argument applies to virtually all functional APC resistance tests, since it is common use in these assays to normalize the hemostatic parameter (clotting time or ETP) determined in the presence of APC through division by the same parameter determined in the absence of APC. Some laboratories subsequently reduce the effect of day-to-day variation on the APC-sr by introducing a "second normalization step" by dividing the APC-sr of patient plasma by the value determined in normal pooled plasma, which yields the so-called normalized APC sensitivity ratio (nAPC-sr).

In retrospect, one may question whether the common practice of expressing APC resistance as an APC sensitivity ratio is a proper action, because this mathematic operation tends to diminish differences observed in the presence of APC (Table 1). In fact, since the protein C pathway is an essential component of the in vivo regulation of thrombin formation, a clotting time or ETP obtained in the presence of APC (ie, with the protein C pathway in action) is likely to be more representative of the physiologic situation and thus may have a higher predictive value for the risk of venous thrombosis than the clotting time (eg, the activated partial thromboplastin time) or ETP determined in the absence of APC. Thus, taking the ratio of clotting time or ETP obtained with and without APC may actually diminish the association with the thrombotic risk.

Therefore, a better measure to evaluate the capability of APC to control coagulation in a given plasma might be the parameter determined in the presence of APC divided by that determined in normal pooled plasma in order to evaluate its deviation from normal. In the case of the ETP-based assay this would result in the following equation: nAPC-sr = ETP + APC (patient plasma)/ ETP + APC (normal pooled plasma).

When the data from Table 1 in the letter of Gris et al are used to calculate nAPC-sr according to the "classical method" (nAPC-sr1) or the alternative method described in the previous paragraph (nAPC-sr2), the results presented in our Table 1 are obtained.

Table 1 shows that in both OC users and heterozygous carriers of the factor $V_{Leiden}$ mutation, nAPC-sr's calculated according the alternative method, in which only the ETPs determined in the presence of APC are taken into account, are actually higher than nAPC-sr's calculated via the classical method. In other words, acquired APC resistance in OC users and the difference between users of second- and third-generation OCs become even more pronounced when only the ETP components determined in the presence of APC are used for evaluation of the anticoagulant effect of APC in plasma.

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**Table 1. nAPC-sr calculated according the classical and alternative method**

| Origins of pooled plasmas | No. | ETP − APC, nM/min | ETP + APC, nM/min | nAPC-sr1 | nAPCsr2 |
|---|---|---|---|---|---|
| Normal plasmas | 90 | 458 | 43 | 1.00 | 1.00 |
| Men | 23 | 442 | 39 | 0.94 | 0.91 |
| Women, no OCs | 27 | 473 | 45 | 1.01 | 1.05 |
| Triphasic OCs | 28 | 533 | 105 | 2.10 | 2.44 |
| **Monophasic OCs** | | | | | |
| Second generation | 28 | 483 | 85 | 1.87 | 1.98 |
| Third generation | 25 | 554 | 155 | 2.98 | 3.60 |
| Factor $V_{Leiden}$, no OCs | 23 | 486 | 141 | 3.09 | 3.28 |

Data were obtained from Rosing et al.[5]
nAPC-sr1 = [ETP + APC (patient plasma)/ETP + APC (normal pooled plasma)] × [ETP − APC (normal pooled plasma)/ETP − ATP (patient plasma)].
nAPC-sr2 = [ETP + APC (patient plasma)/ETP + APC (normal pooled plasma)].

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# To the editor:

## A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype

We read with special interest the article of Busiello and colleagues in *Blood*.[1] They described atypical features of familial hemophagocytic lymphohistiocytosis (FHLH) in a patient/family presenting 2 different perforin gene alterations: an already known homozygous A91V and a novel heterozygous R231H exchange. Since the identification of this gene, which is responsible for the disease in a subgroup of patients,[2] several groups have reported on the identification of novel mutations.[3-6] Small deletions and nonsense and missense mutations were described and scattered both coding exons of the gene. In this family, the homozygous A91V and the heterozygous R231H exchange was detectable in both twins, one of them presenting typical signs of hemophagocytic lymphohistiocytosis (HLH) at the age of 11 years with a rapidly fatal course of the disease. The second twin, with the same perforin mutation pattern, had yet no signs of HLH, including a normal natural killer (NK) cell activity. The 272C>T transition was further detectable at the heterozygous level in the remaining healthy family members (father, mother, and 2 sisters). The R231H exchange was found in the father and, as mentioned before, also in the healthy twin. The authors conclude that due to the identical genetic pattern in the second twin, a late onset of the disease may still be possible in this child.

From our studies, we present evidence that the A91V exchange represents a polymorphism in the perforin gene not causative of the HLH phenotype. We analyzed exon 2 in a series of 86 control DNA samples from healthy unrelated Caucasian individuals by denaturing high-performance liquid chromatography (DHPLC) and found a heterozygous 272C>T transition in 15 cases (17.5%). Additionally, Feldmann et al reported on a homozygous A91V in a nonaffected subject.[3] Finally, Molleran Lee et al confirmed the observation of a polymorphism at this nucleotide in the perforin gene by analyzing a large cohort of controls with a heterozygosity of 3% (7 out of 202 investigated cases).[7] In contrast to these data, Clementi et al described a family including 2 brothers with late onset of the disease and a compound heterozygous pattern of mutations in the perforin gene.[8] In parallel to a W374X mutation leading to a premature stop, the heterozygous A91V exchange was found in both twins. NK cell activity and perforin expression were markedly reduced in both patients. Taken together, the A91V transition has been described either as polymorphism (Feldmann et al,[3] Molleran Lee et al,[7] and our own observations) or as disease causing mutation in 2 families including 4 patients with late onset of the disease.[1,8] The frequency of this transition differed between the geographic or ethnic origin of the samples. With the assumption of a pathologic role of A91V and an allelic frequency of about 9% in our healthy population, the incidence of HLH should be much higher than observed in Germany. However, the real disease prevalence is not yet determined exactly because of a possible underestimation of the diagnosis due to atypical phenotypic presentations. A reduced perforin expression may also occur in heterozygous carriers or may be due to additional genetic defects in the regulatory region of the gene (eg, exon 1).

The presence of a noncausative A91V polymorphism described in the paper by Busiello et al is underlined by the fact that the healthy twin, who has genetically the same mutation pattern as his affected sister, has a completely healthy phenotype, including a normal NK cell activity. This supports our findings that the described genotype is not responsible for the onset of the disease. In conclusion, we show very strong evidence that A91V represents a polymorphism rather than a relevant mutation. This should be taken into account for further genetic counseling in affected families.

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