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Rosendaal, F.R.

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SHORT REPORT

The effect of plasma caeruloplasmin levels on the sensitivity for activated protein C

MARIEKE C H DE VISSER,[1] JOHN H M SOUVERIJN,[2] FRITS R ROSENDAAL[1,3] AND ROGIER M BERTINA[1]
[1]*Hemostasis and Thrombosis Research Center, Department of Hematology, and Departments of* [2]*Clinical Chemistry and* [3]*Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands*

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Summary. The effect of caeruloplasmin levels on the sensitivity for activated protein C (APC), measured by a clotting assay based on the activated partial thromboplastin time, was investigated in a large group of healthy individuals without factor V Leiden. A modest inverse association between caeruloplasmin and normalized APC sensitivity ratio was found (regression coefficient $\beta = -0.33 \times 10^{-2}$, 95% confidence interval, -0.42×10^{-2} to -0.24×10^{-2}). After adjustment for sex and oral contraceptive use, this association weakened ($\beta = -0.19 \times 10^{-2}$, 95% CI -0.34×10^{-2} to -0.05×10^{-2}). After additional adjustment for factor VIII levels, which are known to influence the assay, the effect of caeruloplasmin on APC sensitivity completely disappeared.

Keywords: caeruloplasmin, APC resistance, Factor V Leiden, Factor VIII

The protein C pathway is an important anticoagulant mechanism. Activated protein C (APC) acts as an anticoagulant by proteolytic cleavage of the procoagulant proteins factor Va and factor VIIIa. APC resistance is defined as a poor anticoagulant response of plasma to APC (Dahlback *et al*, 1993). Most cases of APC resistance are caused by a mutation in one of the APC cleavage sites (Arg506) of factor V (Bertina *et al*, 1994), resulting in an activated factor V variant (factor V Leiden), which is inactivated more slowly than activated wild-type factor V. The factor V Leiden mutation is a common risk factor for venous thrombosis. A reduced sensitivity for APC not due to factor V Leiden is also associated with an increased risk of venous thrombosis (Rodeghiero & Tosetto, 1999, de Visser *et al*, 1999). Causes of non-factor V Leiden-related APC resistance are lupus anticoagulants, pregnancy, oral contraceptive use and high factor VIII levels, as well as other currently unidentified factors.

Recently, Ripoll *et al* (1998) reported on a small study in which they observed an inverse correlation between APC sensitivity ratios and plasma caeruloplasmin levels, suggesting that high caeruloplasmin levels may also lead to a reduced sensitivity for APC. Caeruloplasmin is a monomeric blue α-2 glycoprotein that contains more than 95% of the total circulating copper in blood plasma. Its physiological role is not fully known. However, it has been proposed that it may have roles in copper transport, conversion of Fe(II) to Fe(III) (ferroxidase activity) for subsequent uptake by transferrin and that it exhibits pro-oxidant activity towards low density lipoproteins under some circumstances (for a review, see Floris *et al* 2000). Caeruloplasmin is an acute-phase protein. The plasma concentration increases during inflammation, oestrogen therapy and pregnancy, largely as a result of effects on hepatic caeruloplasmin gene expression. The protein is composed of three identical A domains that show sequence identity with the A domains of coagulation cofactors V and VIII (Church *et al*, 1984). The carboxy termini of the light chain A domains of these activated cofactors are involved in binding to APC (Walker *et al*, 1990). Caeruloplasmin contains significant sequence homology to these terminal A domains, and may therefore compete with factors Va and VIIIa for binding to APC, leading to reduced inactivation of factors Va and VIIIa (Walker & Fay, 1990).

We investigated the effect of caeruloplasmin levels on the normalized APC sensitivity ratio in a large group of individuals without a history of venous thrombosis (the control group of the Leiden Thrombophilia Study, LETS).

MATERIALS AND METHODS

Subjects. The investigated subjects were 474 healthy controls (202 men and 272 women) of a population-based case–control study on venous thrombosis (LETS) (Koster

Correspondence: M. C. H. de Visser, Hemostasis and Thrombosis Research Center, Department of Hematology, Leiden University Medical Center, D2-25, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: M.C.H.de_Visser@lumc.nl

et al, 1993) The median age was 47 years (range, 16–73 years)

To investigate the relationship between caeruloplasmin levels and oral contraceptive use, an additional selection was made, as described before (Vandenbroucke *et al*, 1994) Pre-menopausal women aged 15–49 ($n = 153$) were selected after exclusion of women who were pregnant ($n = 10$), within 30 d post partum ($n = 14$) with a recent miscarriage ($n = 2$) or who had used only depot contraceptives ($n = 3$) at the index date

Blood collection and laboratory analysis Venous blood was collected into tubes containing 0 1 volume 0 106 mol/l trisodium citrate Plasma was prepared by centrifugation for 10 min at 2000 *g* at room temperature and stored at −70°C

The sensitivity of the plasma activated partial thromboplastin time (APTT) to APC was measured as described before (Koster *et al*, 1993) Results were expressed as normalized APC sensitivity ratios (APC-SR) The APC sensitivity ratio was defined as the APTT in the presence of APC divided by the APTT in the absence of APC The normalized APC-SR was calculated by dividing the APC-SR of the sample by the APC-SR of pooled normal plasma that was measured in the same run

Caeruloplasmin levels were measured in plasma by immunological turbidimetric assay (Boehringer Mannheim Tina-quant®) by a two-point end-point measurement on a Hitachi 911 analyser (Hitachi, Tokyo, Japan) The normal range was 18–45 mg/dl

Factor VIII coagulant activity (expressed in U/ml) was measured by a one-stage clotting assay with factor VIII-deficient plasma and automated APTT (Organon Teknica, Durham, NC, USA) on an Electra 1000

Statistical analysis To assess the effect of caeruloplasmin levels on the normalized APC-SR, linear regression analysis was performed with normalized APC-SR as dependent variable and caeruloplasmin level as independent variable in 455 subjects without factor V Leiden To control for the effects of sex and oral contraceptive use, multiple linear regression analysis was performed (in 429 subjects for whom oral contraceptive use was known) with normalized APC-SR as dependent variable and caeruloplasmin level, age, sex and oral contraceptive use (yes or no) as independent variables Because it was demonstrated previously that the normalized APC-SR is affected by factor VIII level (de Visser *et al*, 1999) we also performed a regression analysis with factor VIII level as additional independent variable

RESULTS

Determinants of caeruloplasmin levels

Mean caeruloplasmin level was 36 mg/dl (range, 8–79), it was 30 mg/dl (range, 8–55) in men ($n = 202$) and 40 mg/dl (range, 15–79) in women ($n = 272$) Caeruloplasmin levels were higher in women than in men and oral contraceptive use further increased plasma caeruloplasmin level (Table I) No effect of age on caeruloplasmin levels was found

Relation between caeruloplasmin and normalized APC-SR

The effect of caeruloplasmin on the normalized APC-SR was investigated in subjects without factor V Leiden ($n = 455$) The scatterplot (Fig 1) with regression line (regression coefficient $\beta = -0\,33 \times 10^{-2}$, 95% CI $-0\,42 \times 10^{-2}$ to $-0\,24 \times 10^{-2}$) shows a modest inverse association between caeruloplasmin and normalized APC-SR As shown in Table I normalized APC-SRs and caeruloplasmin levels were both influenced by sex and oral contraceptive use Women tended to have higher caeruloplasmin levels and lower normalized APC-SRs than men, with the highest caeruloplasmin levels and the lowest APC ratios in women using oral contraceptives Multiple regression analysis (with sex, age and oral contraceptive use as additional variables) yielded a regression coefficient for caeruloplasmin of $-0\,19 \times 10^{-2}$ (95% CI $-0\,34 \times 10^{-2}$ to $-0\,05 \times 10^{-2}$) Because the normalized APC-SR is known to be influenced by factor VIII levels (de Visser *et al*, 1999), regression analysis was performed with factor VIII as independent variable in addition to caeruloplasmin level, age, sex and oral contraceptive use After this addition of factor VIII level to the model, the regression coefficient for caeruloplasmin level was no longer significant ($\beta = -0\,06 \times 10^{-2}$, 95% CI $-0\,34 \times 10^{-2}$ to $-0\,05 \times 10^{-2}$) The regression coefficient for factor VIII level in this model was $-0\,20 \times 10^{-2}$ (95% CI $-0\,22 \times 10^{-2}$ to $-0\,17 \times 10^{-2}$)

DISCUSSION

A reduced sensitivity for APC not caused by the factor V Leiden mutation is associated with an increased risk of

Table I Caeruloplasmin level and normalized APC sensitivity ratios (APC-SR) according to oral contraceptive (OC) use

	Men ($n = 202$)	Post-menopausal women −OC ($n = 90$)	Pre-menopausal women −OC ($n = 99$)	Pre-menopausal women +OC ($n = 54$)
Mean caeruloplasmin (mg/dl) (95% CI)	30 (29–31)	35 (34–36)	36 (34–37)	57 (54–60)
Mean *n*-APC-SR* (95% CI)	1 06 (1 04–1 07)	1 00 (0 98–1 02)	1 03 (1 01–1 06)	0 95 (0 93–0 97)

*Subjects carrying the factor V Leiden mutation or with undetermined normalized APC-SR were excluded (six men three post-menopausal women −OC six pre-menopausal women −OC and one pre-menopausal woman +OC)

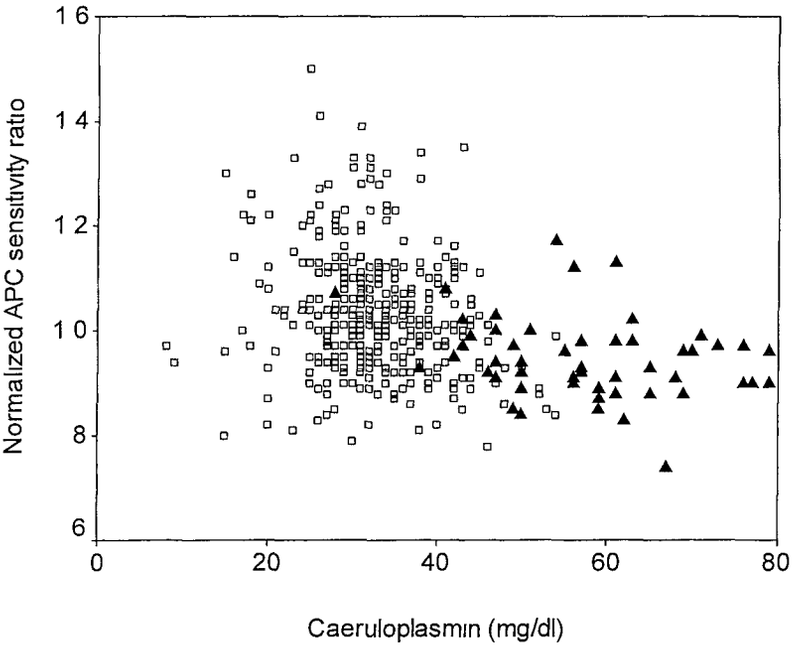

Fig 1 Caeruloplasmin level and normalized APC sensitivity ratio in healthy controls Pre menopausal women using oral contraceptives are depicted by a triangle (▲) Regression coefficient $\beta = -0.33 \times 10^{-2}$ (95% CI -0.42×10^{-2} to -0.24×10^{-2})

venous thrombosis (de Visser *et al* 1999) Recently Ripoll *et al* (1998) investigated the effect of caeruloplasmin levels on APC sensitivity in non-factor V Leiden carriers An inverse relationship between caeruloplasmin and APC sensitivity ratios was found but the results were based on a small study population ($n = 83$) In another study with 19 pregnant women without factor V Leiden no association between caeruloplasmin and APC sensitivity was found indicating that acquired APC resistance in pregnancy is not due to elevated caeruloplasmin levels (Hung *et al* 1999)

We examined the effect of caeruloplasmin levels on APC sensitivity in a large group of healthy subjects without a history of venous thrombosis and without factor V Leiden A modest inverse association (regression coefficient $\beta = -0.33 \times 10^{-2}$ 95% CI -0.42×10^{-2} to -0.24×10^{-2}) was found between caeruloplasmin level and the normalized APC-SR Women particularly oral contraceptive users had both an increased caeruloplasmin level and a decreased sensitivity for APC compared with men Because both caeruloplasmin level and APC ratio were affected by sex and oral contraceptive use we performed multiple regression analysis to control for these determinants This adjustment weakened the association between caeruloplasmin and the normalized APC-SR ($\beta = -0.19 \times 10^{-2}$ 95% CI -0.34×10^{-2} to -0.05×10^{-2}) So similar to Ripoll *et al* (1998) we observed an association between caeruloplasmin level and APC sensitivity However our results indicated that the overall association between caeruloplasmin and normalized APC-SR was to a large extent explained by an effect of sex and oral contraceptive use on both APC sensitivity and caeruloplasmin level After additional adjustment for factor VIII levels which are known to influence the assay (de Visser *et al* 1999) the effect of caeruloplasmin on the APC sensitivity completely disappeared So the overall effect of caeruloplasmin level on the normalized APC-SR was insignificant compared with the effect of factor VIII levels on the assay

In conclusion we saw a modest association between caeruloplasmin levels and the normalized APC-SR which largely depended on the effects of sex and oral contraceptive use on both caeruloplasmin level and APC sensitivity However the effect of caeruloplasmin levels on the normalized APC-SR was negligible compared with the effect of factor VIII levels on the APC sensitivity

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