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Determinants of Elevated Levels of Natural Anticoagulants in Healthy Subjects*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. The levels of protein C (PC), free protein S (PS) and antithrombin (AT) are evaluated during thrombophilia screening to exclude their deficiencies.
Objectives. The aim of this study was to investigate factors which determine the elevated levels of natural plasma anticoagulants in healthy individuals.
Material and Methods. The PC activity and antigen, free PS antigen and AT activity together with hematological, biochemical, genetic and immunological laboratory tests were assessed in 130 healthy adults (63 males) aged 20–60 (median 41) years. Individuals with personal or family history of cardiovascular diseases and venous thromboembolism were ineligible.
Results. The functionally active PC measured by chromogenic assay (values above 140%) was observed in 14 (11%) of subjects, while the PC antigen determined using the enzyme-linked immunosorbent assay (ELISA) was elevated in 5 (4%) of these patients. Free PS measured by immunoturbidimetry and ELISA was increased in 9 (7%) subjects (values above 139% in men and 114% for women) and in 6 (5%) patients (values above 130% in men and 111% for women), respectively. The AT activity above 118% was found in 5 (4%) subjects measured using chromogenic assay. None of the individuals had any deficiency of natural anticoagulants. Increased C-reactive protein (CRP) > 3.0 mg/L was associated with elevated PC activity (odd ratio [OR]: 11.14, 95% confidence interval [CI] 1.67–74.23). Increased free PS assessed by immunoturbidimetric assay and PC activity were associated with hypercholesterolemia (OR: 3.57, 95% CI: 1.09–10.06 and OR: 8.61, 95% CI: 1.07–69.04, respectively). Body mass index ≥ 25 kg/m^2 was independently associated with elevated PC activity (OR: 3.42, 95% CI: 1.01–11.52). No risk factors for elevated AT activity were identified.
Conclusions. Apart from hypercholesterolemia and overweight, increased serum CRP is associated with elevated PC activity in healthy adults. We confirmed that there are differences in the proportions of subjects with elevated PC and PS depending on the assay used (**Adv Clin Exp Med 2015, 24, 5, 791–800**).

Key words: protein C, protein S, antithrombin.

Protein C (PC), protein S (PS) and antithrombin (AT), which are natural anticoagulants, are of key importance in the regulation of haemostasis and thrombosis. PC is a vitamin K-dependent plasma serine protease mainly produced in the liver [1]. The circulating plasma PC exists in an inactive form as a single chain zymogen [2]. PC is converted to an active form when thrombin binds to thrombomodulin on endothelial cells. Active PC enhanced by its cofactors including PS, phospholipids and calcium ions, inactivates activated coagulation factors (F)Va and VIIIa. PS primarily produced in the liver circulates in 60% complexed with the complement C4b-binding protein (C4bBP) and 40% PS is a free fraction. Both these forms of PS serve as a cofactor for active PC, but free PS is more potent [3]. The major physiological inhibitor of thrombin and active factor X (Xa) is AT, a single-chain glycoprotein also mainly produced in the liver [4]. AT can also inactivate factors VIIa, IXa, XIa and XIIa in the presence of heparin [4, 5].

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All the 3 natural anticoagulants are commonly measured in patients suspected of inherited thrombophilia [6]. Deficiency of AT, PC and/or PS has been consistently linked with venous thromboembolism (VTE) [7]. The large cohort studies have demonstrated increased risk of VTE in asymptomatic individuals with the deficiency of AT, PC and/or PS and positive family history for VTE episodes [7, 8].

The plasma levels of natural anticoagulants exceeding the upper limit of reference ranges have been associated with age and gender [9–12]. Boerger et al. was the first who reported that free PS levels are higher in men than in women at all ages which has been confirmed by other studies [9, 13]. It has been also observed that the free PS levels increase with age in women while decrease in men older than 50 years [9, 14]. Similarly, PC activity increases with age in women but not in men [14, 15]. AT activity decreases in elderly men and rises in women after menopause [15]. It has been suggested that variations in PC activity, free PS levels and AT activity with regard to age and sex should be reflected in the reference ranges [15]. Moreover, serum lipids have been also associated with the levels of natural anticoagulants. Positive correlations of PC antigen with triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) have been reported in the population-based ARIC study, while the third Glasgow MONICA Survey has shown positive correlations of PS with TC and TG in healthy subjects [16, 17]. Some investigators have suggested that elevated concentrations of TC and TG were predictors of increased PC activity and free PS level in a healthy population [14, 17]. In contrast, Assmann et al. have failed to show any associations between serum lipids and PC or AT levels in healthy adults [18]. It has also been reported that in healthy volunteers elevated levels of natural anticoagulants could be associated with obesity [16, 17].

In the present study we investigated demographic and biochemical factors which may affect the levels of natural plasma anticoagulants in healthy volunteers.

Material and Methods

Study Population

One hundred forty nine consecutive healthy volunteers, aged below 60 years, were recruited. The exclusion criteria were: personal and/or family history of cardiovascular diseases including VTE, myocardial infarction, angina, heart failure, sudden death, stroke, and any of chronic diseases. All subjects denied taking any medication on a long-term basis and within the previous month. Nineteen subjects were excluded from the final analysis due to elevated (> 40 U/L) alanine transaminase (ALT) levels (n = 8), elevated (> 10 mg/L) C-reactive protein (CRP) levels (n = 5) and hypothyroidism defined as high thyroid-stimulating hormone (TSH) above the upper limit of the reference range, i.e. 4.2 μIU/mL (n = 6).

Body Mass Index (BMI) was defined as the body mass divided by the square of the height. Overweight or obese subjects had BMI ≥ 25 kg/m^2. Hypercholesterolemia was defined as TC > 5.0 mmol/L or LDL-C > 3 mmol/L, hypertriglyceridemia as TG concentration above 1.7 mmol/L and hyperglycemia as serum glucose concentration above 5.5 mmol/L. Smoking was defined as the use of 1 or more cigarettes per day. The University Ethical Committee approved the study, and all the participants provided their written informed consent.

Laboratory Investigations

Fasting venous blood was drawn from patients between 7 and 9 a.m. Citrated blood samples (9:1 of 0.106 M sodium citrate) were centrifuged at 2,500 g at 4ºC for 20 min and stored in aliquots at –80ºC until analysis. Serum TC, LDL-C, high-density lipoprotein cholesterol (HDL-C), TG, aspartate transaminase (AST), ALT, glucose, TSH were assayed using a biochemical analyser Cobas 6000™ (Roche Diagnostics GmbH, Mannheim, Germany). Complete blood count was determined using the hematological analyser Sysmex XT2000i (Sysmex Corporation, Kobe, Japan). Fibrinogen was determined using the Clauss method. Hs-CRP was determined using immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany). Factor V Leiden (FVL) mutation was determined by the Real-Time PCR with the use of TaqMan Genotyping Assays in 7900 Fast Real-Time PCT System, Applied Biosystems (Foster City, California, USA). Prothrombin G20210A (PT G20210A) polymorphism was determined by the restriction fragment length polymorphism analysis with the use of HindIII restrictase, Fermentas (USA). Antinuclear antibodies (ANA) were tested using indirect fluorescent assay (IFA) in sera diluted at 1:160 (Euroimmun, Lübeck, Germany).

Measurements of PC

The quantitative determination of functionally active PC in plasma *via* chromogenic assay (Berichrom®Protein C, Siemens Healthcare Diagnostic, Marburg, Germany) on an automatic analyser BCS XP (Siemens Healthcare Diagnostics, Marburg, Germany) in citrated plasma was performed.

In that test the examined blood sample was activated by a specific snake venom activator and then the reaction with a chromogenic substrate (pyroglutamic acid-proline-arginine-methoxy-nitroanilide) was conducted. The activity was assayed in a kinetic test by measuring the increase of the absorbance value at a wavelength of 405 nm. The reference range for PC activity in accordance to the manufacturer's recommendation was 70–140%. PC antigen was determined in citrate plasma using an antigenic assay for the quantitative determination of PC by the enzyme-linked immunosorbent assay (ELISA, Asserachrom® Protein C, Diagnostica Stago, Asnieres-Sur-Seine, France). In that test the PC was captured by specific rabbit anti-human PC antibodies which were coupled with peroxidase bound to the remaining free antigenic determinants of PC. All unbound material was washed away and a peroxidase enzyme substrate (tetramethylbenzidine) was added. The intensity of the color was directly proportional to the level of PC. The reference range for PC according to the manufacturer's recommendation was 70–140%.

Measurements of PS

The level of free PS antigen was assessed in citrated plasma on automated coagulation analyzer BCS XP (Siemens Healthcare Diagnostics, Germany) using an immunoturbidimetric assay (INNOVANCE® Free PS Ag, Siemens Healthcare Diagnostic, Marburg, Germany). To measure free PS antigen the polystyrene particles with two specific to free PS antigen monoclonal antibodies were used. When the examined sample contained free PS antigen aggregates were created and the degree of aggregation was measured turbidimetrically. The levels of free PS were directly proportional to the turbidity. The reference ranges for the levels of free PS antigen were 67–139% for male and 60–114% for female. In order to verify the elevated levels of free PS, the measurement were repeated in citrate plasma using an antigenic assay for the quantitate determination of free PS by the ELISA (Asserachrom Protein C, Diagnostica Stago, Asnieres-Sur-Seine, France). In that test the free PS was simultaneously captured by the monoclonal antibody immobilized in the microwells and by the monoclonal antibody coupled with peroxidase. The bound enzyme peroxidase was detected *via* its reaction with the substrate (tetramethylbenzidine). The intensity of the color was directly proportional to the level of free PS. The reference ranges were 60–130% for male and 58–111% for female.

Measurements of AT

The chromogenic assay (INNOVANCE™ ATIII, Siemens Healthcare Diagnostic, Marburg, Germany) for detection AT activity on an automatic analyzer BCS XP (Siemens Healthcare Diagnostics, Marburg, Germany) was performed. In that method an excess of active factor X (FXa) was added into citrate plasma. Then, in the presence of heparin, a portion of the enzyme was complexed and inactivated by AT which was presented in the sample. The uninhibited FXa cleaved a specific chromogenic substrate and caused the release of a dye. The rate of substrate cleavage was directly proportional to the absorbance value at a wavelength of 405 nm but inversely proportional to the inhibiting activity of the AT. The reference range for AT activity according to the manufacturer's recommendation was 83–118%.

Statistical Analysis

Continuous variables were presented as a median (interquartile range) and categorical variables were presented as numbers (percentages). Quantitative variables were checked for the normality of their distribution using the Kolmogorov-Smirnov test. The quantitative parameters were then compared *via* the unpaired *U*-Mann Whitney test. Comparisons of qualitative parameters were conducted using the two-tailed Fisher exact test, or χ^2 test, as appropriate. The correlations were calculated using the Spearman correlation coefficient. Significant results were subjected to an adjustment analysis using multiple logistic regression to determine the risk factors for the elevated levels of natural anticoagulants. Associations between the variables were expressed as odds ratios (ORs) with corresponding 95% confidence interval (CI). P values less than 0.05 were considered statistically significant.

Results

The study group included 130 subjects of both genders (63 men, 48%), between 20–60 years of age (median: 41). Thirty-six of them (28%) were overweight or obese. The study group included 18% smokers. The median level of PC activity obtained by chromogenic assay was 111.5% (102–128.0%) while the median level of PC antigen detected by ELISA was 99% (90–109%). The median level of free PS measured by immunoturbidimetric assay was 101.5% (95–108%) and by ELISA was 89% (76–96%). In the case of AT the median level was 99.0% (89–111.0%). The characteristics of the study group are presented in Table 1.

Table 1. Baseline characteristics of the study group (n = 130)

Variable	
Age [yr]	36 (29–43)
Female, n [%]	67 (52)
BMI [kg/m^2]	23.7 (21.15–25.6)
Overweight or obesity, n [%]	36 (28)
Smokers, n [%]	23 (18)
WBC [10^3/μL]	5.6 (4.9–9.3)
RBC [10^6/ μL]	4.8 (4.5–5.1)
Hemoglobin [g/dL]	14.1 (12.7–14.9)
Hematocrit [%]	41.6 (38.8–43.5)
PLT [10^3/μL]	240 (213–271)
Fibrinogen [g/L]	2.46 (2.17–2.91)
TC [mmol/L]	4.87 (4.23–5.40)
LDL-C [mmol/L]	2.90 (2.55–3.54)
HDL-C [mmol/L]	1.60 (1.43–1.91)
TG [mmol/L]	0.97 (1.36–1.92)
Glucose [mmol/L]	4.9 (4.5–5.2)
ALT [U/L]	19 (14–27)
AST [U/L]	19 (16–23)
CRP [mg/L]	0.80 (0.48–1.67)
TSH [mU/L]	1.73 (1.34–2.26)
Bilirubin [μmol/L]	10.3 (6.9–14.5)
Creatinine [μmol/L]	76 (67–86)
PC [%]	111.5 (102–128.0)
PS [%]	101.5 (95–108.0)
AT [%]	99.0 (89–111.0)
FVL, n [%]	9 (7)
PT G20210A, n [%]	10 (8)
Positive ANA, n [%]	25 (20)

Continuous variables are presented as a median (interquartile range) and categorical variables are presented as numbers (percentages).
PC – protein C activity, PS – free protein S, BMI – body mass index, TC – total cholesterol, LDL-C – low density lipoprotein cholesterol, HDL-C – high density lipoprotein cholesterol, TG – triglyceride, AST – aspartate transaminase, ALT – alanine transaminase, TSH – thyroid-stimulating hormone, CRP – C-reactive protein, WBC – white blood cells, RBC – red blood cells, PLT – platelets, FVL– factor V Leiden mutation, PT G20210A – prothrombin G20210A polymorphism, ANA – anti-nuclear antibody

Using chromogenic assay the elevated PC activities were found in 14 (11%) subjects while the elevated levels of PC antigen using ELISA test were confirmed in 5 (4%) subjects. A correlation between the chromogenic assay and ELISA for PC detection were observed (r = 0.44, p < 0.0001); however, all values obtained by ELISA were decreased when compared to those measured by chromogenic assay (Fig. 1). If the upper limit of the reference range is moved up by 10%, it contributes to reducing the occurrence of elevated results in chromogenic assay to 7 (5%) patients and eliminates all elevated values obtained by ELISA test.Free PS determined using immunoturbidimetric assay and ELISA was increased in 9 (7%) and in 6 (5%) subjects respectively. The correlation between immunoturbidimetric assay and ELISA was found (r = 0.69, p < 0.001); nonetheless, the values obtained by ELISA were lower than those determined by immunoturbidimetric assay (Fig. 2). Moreover, we observed an elevated AT levels in 5 (4%) patients using chromogenic assay. The increased levels of both PC and AT or PC and PS or PS and AT were noted in 3 patients (2%). None of the study subjects had all the 3 natural anticoagulants raised. PC levels increased with age in both sexes (r = 0.25, p = 0.004) and AT activity was raised (r = 0.24; p = 0.047) in women only. As expected, the levels of free PS were higher in men than women regardless of age (108.5% [99.0–121.3%] vs. 91.0% [79.0–101.0%], respectively; p < 0.001).

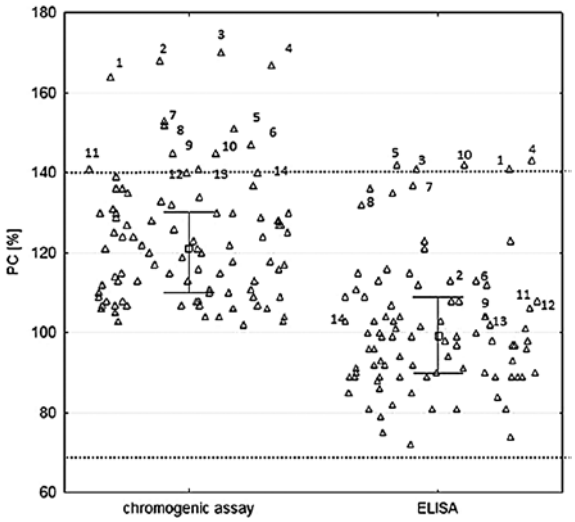

Fig. 1. Comparison between the levels of PC obtained by the chromogenic assay and ELISA test

PC – protein C, 1–14 patients with elevated levels of PC obtained by chromogenic assay with accompanied results obtained by ELISA test. The reference range is marked with the dotted lines.

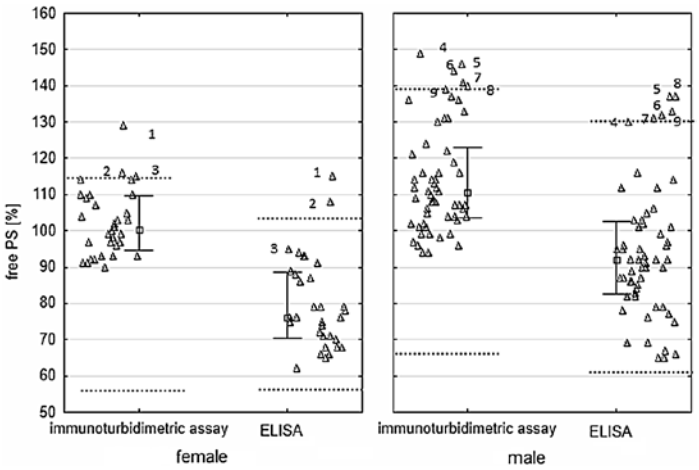

Fig. 2. Comparison between the free PS levels obtained by the immunoturbidimetric assay and ELISA test

Free PS – protein S, 1–9 patients with elevated levels of free PS obtained by immunoturbidimetric assay with accompanied results obtained by ELISA. The reference range are marked with the dotted lines

PC

Increased PC activity was observed commonly in older subjects who had a higher BMI and elevated levels of TC, LDL-C and TG (all $p < 0.05$; Table 2). After adjustment for sex and age the differences regarding LDL-C levels and BMI were significant ($p < 0.0001$ and $p = 0.0003$, respectively). Increased PC antigen was observed only in patients with higher BMI ($p = 0.048$) and after adjustment for sex and age this difference remained significant ($p = 0.046$). Multiple logistic regression showed that the elevated PC activity was independently associated with elevated concentration of CRP (> 3 mg/L), overweight or obesity and hypercholesterolemia (OR = 11.14, 95%CI: 1.67–74.23; OR= 3.42, 95% CI: 1.01–11.52; OR = 8.61, 95% CI: 1.07–69.04 respectively, Table 3)

Free PS

Elevated free PS levels measured *via* immunoturbidimetric assay were found in men and in subjects with increased levels of TC, LDL-C and TG (all $p < 0.05$; Table 2). After adjustment for age and sex, the differences regarding LDL-C concentration and BMI were observed ($p = 0.023$ and $p = 0.028$ respectively). The elevated levels of free PS obtained by ELISA test were only found in men ($p = 0.03$). Multiple logistic regression showed that the elevated free PS levels (immunoturbidimetric assay) were independently associated with male sex and hypercholesterolemia (OR = 4.13, 95% CI: 2.83–11.73; OR = 3.57, 95% CI: 1.09–10.06 respectively; Table 3).

AT

Increased AT activity was noted in subjects with elevated TG ($p = 0.04$, Table 2) and after adjustment for sex and age this difference remained significant ($p = 0.03$). Multiple logistic regression did not display any significant associations for elevated AT activity (data not shown).

Discussion

In the current study we showed for the first time that increased concentration of CRP is associated with elevated PC activity in healthy Polish adults. We also demonstrated that elevated concentration of serum lipids may cause the rise in PC activity obtained by chromogenic assay and free PS levels measured by immunoturbidimetric assay in healthy subjects. Moreover our study confirmed the influence of demographic parameters like age, gender, and overweight or obese on the levels of natural anticoagulants.

In this study the chance of the elevated activity of PC was 11.14 times higher among patients with the CRP concentration above 3 mg/L than in patients with CRP concentration within the reference range. CRP is one of the most sensitive indicators of an acute-phase reaction, which facilitates the interaction between monocytes and endothelial cells and encourages plasminogen activator inhibitor-1 (PAI-1) and tissue factor (TF) formation [19–21]. To prevent serious events caused by inflammation-coagulation interactions, the increase activity of PC, which has an important function in modulating inflammation, has been observed [22, 23]. This anticoagulant decreases the leukocyte chemotaxis, suppresses inflammatory cytokine elevation or maintains endothelial cell barrier [25]. The above associations may suggest that the rise of PC activity together with CRP levels might be the defense mechanism against coagulation activation.

The present work showed that PC activity measured by chromogenic assay and free PS levels obtained by immunoturbidimetric assay were higher in subjects with increased concentrations

Table 2. The relationship between levels of natural anticoagulants with demographic and biochemical parameters in the study group

Variable	Subjects with normal PC n = 116 (89%)	Subjects with elevated PC n = 14 (11%)	p value	Subjects with normal free PS n = 121 (93%)	Subjects with elevated free PS n = 9 (7%)	p value	Subjects with normal AT n = 125 (96%)	Subjects with elevated AT n = 5 (4%)	p value
Age [yr]	36 (27–42)	42 (32–56)	0.01	36 (29–43)	31 (27–42)	0.44	36 (29–43)	39 (28–40)	0.98
Female [%]	62 (52.5)	5 (42)	0.06	64 (53)	3 (33)	< 0.001	65 (52)	2 (40)	0.09
BMI [kg/m^2]	23.51 (21.02–25.36)	27.09 (24.15–31.42)	< 0.001	23.51 (21.10–25.3)	25.83 (23.60–27.93)	0.06	23.68 (21.17–25.62)	22.95 (21.32–26.43)	0.84
Smoking [%]	22 (19)	1 (7)	0.26	22 (18)	1 (10)	0.19	23 (18)	1 (20)	0.64
WBC [10^3/μL]	5.7 (4.9–6.8)	5.6 (5.1–6.5)	0.96	4.9 (4.5–6.6)	5.9 (5.1–7.2)	0.47	5.6 (4.98–6.77)	6.0 (4.22–7.22)	0.95
RBC [10^6/μL]	4.7 (4.5–5.1)	5.0 (4.6–5.2)	0.19	4.7 (4.5–5.1)	5.0 (4.8–5.3)	0.59	4.78 (4.49–5.12)	4.72 (3.96–4.82)	0.18
Hemoglobin [g/dL]	14.1 (12.7–14.4)	14.8 (13.1–15.2)	0.17	14.0 (12.6–14.8)	14.9 (14.3–15.4)	0.07	14.2 (12.7–14.9)	13.6 (11.25–14.6)	0.47
Hematocrit [%]	41.5 (38.7–43.4)	42.5 (40.3–44.1)	0.63	41.3 (38.2–43.4)	43.2 (42.1–46.5)	0.09	41.6 (38.8–43.5)	40.3 (34.4–43.1)	0.49
PLT [10^3/μL]	241 (214–269)	231 (180–289)	0.66	255 (230–290)	244 (223–277)	0.9	240 (213–271)	255 (204–327)	0.73
Fibrinogen [g/L]	2.46 (2.18–2.90)	2.56 (2.08–3.94)	0.81	2.42 (2.16–2.89)	2.64 (2.28–3.16)	0.13	2.45 (2.17–2.95)	2.21 (2.12–2.47)	0.19
TC [mmol/L]	4.84 (4.20–5.35)	5.12 (4.40–6.11)	0.04	4.68 (4.20–5.29)	5.72 (4.67–6.44)	0.03	4.96 (4.20–5.35)	5.04 (4.36–6.05)	0.22
LDL–C [mmol/L]	2.87 (2.45–3.49)	3.69 (2.82–4.21)	0.02	2.84 (2.45–3.4)	3.95 (2.88–4.54)	0.001	2.90 (2.47–3.49)	3.16 (2.52–4.14)	0.35
HDL-C [mmol/L]	1.67 (1.36–1.92)	1.47 (1.39–1.76)	0.14	1.69 (1.38–1.93)	1.45 (1.3–1.57)	0.08	1.67 (1.36–1.92)	1.65 (1.32–1.84)	0.27

Table 2. The relationship between levels of natural anticoagulants with demographic and biochemical parameters in the study group (cont.)

Variable	Subjects with normal PC n = 116 (89%)	Subjects with elevated PC n = 14 (11%)	p value	Subjects with normal free PS n = 121 (93%)	Subjects with elevated free PS n = 9 (7%)	p value	Subjects with normal AT n = 125 (96%)	Subjects with elevated AT n = 5 (4%)	p value
TG [mmol/L]	0.96 (0.72–1.21)	1.15 (0.90–1.57)	0.02	0.93 (0.70–1.20)	1.14 (0.93–1.47)	0.01	0.97 (0.72–1.22)	1.27 (0.9–1.82)	0.04
Glucose [mmol/L]	4.8 (4.5–5.2)	5.3 (5.0–5.5)	0.06	4.8 (4.5–5.2)	5.0 (4.6–5.4)	0.23	4.9 (4.5–5.2)	5.1 (4.7–5.2)	0.54
ALT [U/L]	18 (13–26)	26 (21–33)	0.09	19 (13–25)	27 (18–40)	0.06	19 (14–27)	19 (11–37)	0.77
AST [U/L]	19 (16–23)	20 (18–24)	0.23	19 (16–23)	21 (18–27)	0.26	19 (16–23)	21 (17–26.5)	0.38
CRP [mg/L]	0.79 (0.48–1.43)	1.54 (0.60–3.84)	0.06	0.80 (0.50–1.75)	0.90 (0.50–1.50)	0.09	0.82 (0.52–1.74)	0.38 (0.37–2.29)	0.11
TSH [mU/L]	1.77 (1.34–2.35)	1.61 (1.05–1.99)	0.21	1.71 (1.37–2.27)	1.81 (1.32–2.33)	0.74	1.76 (1.37–2.29)	1.33 (1.12–1.66)	0.19
Bilirubin [µmol/L]	10 (7–14)	14 (7–19)	0.28	10 (7–15)	12 (8–20)	0.32	10 (7–15)	12 (4–17)	0.67
Creatinine [µmol/L]	76 (67–84)	81 (64–87)	0.56	80 (76–84)	86 (83–90)	0.09	76 (67–86)	59 (65–87)	0.57
FVL, n [%]	8 (7)	1 (7)	0.59	8 (7)	1 (11)	0.69	5 (4)	0 (0)	–
PT G20210A, n [%]	9 (8)	1 (7)	0.93	9 (7.5)	1 (11)	0.77	5 (4)	0 (0)	–
ANA, n [%]	22 (19)	3 (21)	0.63	24 (20)	1 (11)	0.43	25 (20)	0 (0)	–

Continuous variables are presented as a median (interquartile range) and categorical variables are presented as numbers (percentages).
PC – protein C activity, PS – free protein S, BMI – body mass index, TC – total cholesterol, LDL-C – low density lipoprotein cholesterol, HDL-C – high density lipoprotein cholesterol, TG – triglyceride, AST – aspartate transaminase, ALT – alanine transaminase, TSH – thyroid-stimulating hormone, CRP – C-reactive protein, WBC – white blood cells, RBC – red blood cells, PLT – platelets, FVL – factor V Leiden mutation, PT G20210A – prothrombin G20210A polymorphism, ANA – anti-nuclear antibody.

Table 3. The OR with 95%CI for the elevated levels of PC% and free PS%

Variable	PC%			Free PS%		
	OR	95% CI	p value	OR	95% CI	p value
Overweight or obesity	3.42	1.01–11.52	0.008	2.78	0.98–7.84	0.06
Age > 50 years	2.18	0.93–17.61	0.15	2.45	0.59–10.15	0.41
Males	1.55	0.46–7.21	0.47	4.13	2.83–11.73	< 0.001
Hypercholesterolemia	8.61	1.07–69.04	0.043	3.57	1.09–10.06	0.027
Hypertriglyceridemia	1.85	0.27–6.88	0.46	1.5	0.38–5.88	0.84
Hyperglycemia	1.25	0.66–9.50	0.82	0.95	0.02–5.37	0.41
hsCRP > 3.0 mg/L	11.14	1.67–74.23	0.013	1.09	0.124–9.56	0.94
ALT > 40 U/L	–	–	–	2.5	0.27–23.87	0.41
Creatynine > 90 μmmol/L	–	–	–	1.45	0.12–5.94	0.32

PC – protein C activity, PS – free protein S, BMI – body mass index, TC – total cholesterol, LDL-C – low density lipoprotein cholesterol, HDL-C – high density lipoprotein cholesterol, TG – triglyceride, AST – aspartate transaminase, ALT – alanine transaminase, TSH – thyroid-stimulating hormone, CRP – C-reactive protein, WBC – white blood cells, RBC – red blood cells, PLT – platelets, FVL – factor V Leiden mutation, PT G20210A – prothrombin G20210A polymorphism, ANA – antinuclear antibody.

of TC, LDL-C and TG when compared to those with normal lipids profiles while AT showed only association with TG concentration. We found that the odds for the elevated activity of PC and the elevated levels of free PS (obtained by immunoturbidimetric assay) were 8.61 and 3.57, respectively, times higher among patients with hypercholesterolemia. These relationships were not found in the case of PC antigen and free PS levels obtained by ELISA test, which may suggest that both chromogenic and immunoturbidimetric assays are more susceptible to hypercholesterolemia than ELISA test. The possible reason for the described phenomenon may be the interference of lipids with the immunotubidimetric and the chromogenic assays reagents. The chromogenic assays, used for assessment of PC and AT activities, are the kinetic tests based on the spectrometric measurement and may be exposed to interfere with lipids, which are capable to absorb the light, especially in a lower wavelength (the absorbance from lipids decrease from 700–700 nm, without specific absorption peaks) [24]. In the current study, the free PS was determined using immunoturbidimetry. During this measurement lipids might interfere with antigen-antibody binding and/or may elevate the turbidity [25]. In both immunoturbidimetric and chromogenic assays, the interference of lipemia can be minimized by using proper sample dilutions, the removal of the lipids by ultracentrifugation, and extraction with organic solvents or enzymatic cleavage by lipase [26]. The most frequent cause of lipemic samples is inadequate time of blood drawing after the meal [27].

The associations of PC activity and free PS level with TG and TC have been reported by MacCallum et al. [14]. They have shown that 1.0 mmol/L rise in serum TC using a chromogenic assay was accompanied by the increase in PC activity of 0.07 U/mL, while a rise in TG of 1.0 mmol/L was associated with increasing free PS levels, obtained by electroimmunoassay, of 0.1 U/mL. However, they have suggested that those observations were associated with chronic abnormalities rather than caused by the fact that patient did not fast before blood drawing or an artefact occurred during measurement [14]. Kim et al. have also found that PC activity measured by chromogenic assay and free PS levels obtained by immunoassay significantly correlated with TG concentrations, whereas activities of PC and AT, measured by chromogenic assay substantially correlated with TC concentration. They also noted the elevated levels of the procoagulant factors: II, V, VII, IX, X, and XI in patients with hypercholesterolemia and hypertriglyceridemia. Based on above observations, they have suggested that increased levels of the anticoagulant factors might be a natural defense mechanism against elevated levels of procoagulant factors [28].

In the current study, we observed that PC activity was independently associated with elevated BMI. We observed that overweight or obese subjects were 3.42 times more likely to have increased PC activity. A large epidemiological study performed on healthy subjects has shown the positive correlations between BMI and PC and PS in both sexes, and also between BMI and AT only in women [16]. Moreover, Solá et al. have reported that PC

activity has risen in obese patients and decreased after a 3-month diet [29]. It has also been suggested that this phenomenon is a protective mechanism intended to counteract the elevated activity of pro-thrombotic factors [30].

In the present study the distribution of free PS levels was similar to that reported by other authors [9, 12]. We noted higher levels of free PS in men than in women; men were 4.13 times more likely to be exposed to elevated levels of free PS than women. A number of researchers have demonstrated the differences in free PS levels between men (higher level) and women (lower level) in healthy populations [9, 12]. Moreover, the present study showed that the PC activity elevates with age in both sexes, while AT activity increases with age in women only. Franchi et al. have reported a relevant rise of PC activity, measured by chromogenic assay, with age but only in women. Similarly, they have observed increased AT activity measured by chromogenic assay, with age in women, which was associated with menopause and the decrease with age in men [15]. Pertaining to the observation described above, during the interpretation of laboratory results, clinicians should consider the influence of demographic variables. Moreover, laboratories should develop their own age- and sex-specific reference ranges for PC activity, free PS level and AT activity. The falsely increased values can lead to an incorrect interpretation of the laboratory tests. It may be important in VTE patients suspected of inherited thrombophilia in whom the elevated levels of PC activity, free PS and AT in 17 (8%), 12 (5.5%) and 13 (6%) have been found (Undas A., unpublished data). In these cases, when the levels of natural anticoagulants are monitored, even a slight difference in these parameters is a key factor in the therapeutic process.

Our study has several limitations. The size of the study groups was limited particularly in the subgroup analysis. Moreover, the study group included the small number of individuals above 50 years.

In summary, there is the association between elevated concentration of CRP and increased activity of PC in healthy adults. Additionally the activities of AT, PC, and the level of free PS are dependent on the demographic factors. The normal ranges which take into account variables such as gender, age and BMI can be more useful in order to better discriminate any abnormalities in natural anticoagulants levels. Moreover, the elevated levels of those factors connected with the increased levels of TG, TC and LDL-C may be caused by preanalitycal errors. It is important to take the concentrations of serum lipids into account during the interpretation of the obtained results. In order to minimize errors associated with postprandial lipemia it is recommended that the patient should be fasting before the determination of natural anticoagulants levels.

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