Establishing a Saudi Arabian Reference Range for Physiological anticoagulants Antithrombin III Protein C, Protein S and Resistance to Activated Protein C

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Abstract:

Objectives:

(1) To estimate the normal plasma levels of Antithrombin III, Protein C and Protein S in Saudi nationals.

(2) This study was carried out to assess the prevalence of Activated Protein C resistance in healthy Saudi blood donors in a University hospital and to compare the values with an unselected group of patients with various thromboembolic disorders.

Methods: In this prospective study Antithrombin III, Protein C, Protein S and activated PC resistance was estimated in 200 healthy potential blood donors and in 32 unselected patients with various thromboembolic disorders.

Results: Plasma from 200 blood donors, mean age 30.56 ± 8.55 years, minimum age 18 and maximum age 58 were tested. Mean value for activated protein C resistance was found to be 0.94 ± 0.293. The mean value from the 32 unselected patients with thrombotic disorders was found to be 0.65 ± 0.201. Mean level for PC was 104.0035 ± 22.0, antithrombin III 101.85 ± 17.6, and protein S 91.72 ± 26.4.

Conclusion: Activated Protein C resistance values show a normal distribution in normal healthy Saudis. We propose a reference range for APCR of 0.94 ± 0.293 for this population. Comparisons with results from other studies elsewhere are also discussed.
Introduction:

The blood coagulation system is composed of factors that foster the conversion of prothrombin to thrombin i.e. procoagulants, and factors that regulate this process i.e. natural anticoagulants. There is a unique balance between these two mechanisms and any major imbalance between procoagulant and anticoagulant activities can be associated with hemorrhage or thromboembolism\(^{(1)}\). Venous thromboembolism is a common disorder associated with significant morbidity and mortality, but a dramatic progress has been made in understanding the risk factors.

Rudolf Virchow proposed that the pathophysiology of thrombosis involved three interrelated factors: changes in the vessel wall, changes in blood flow and changes in the coagulability of blood. The first two of Virchow’s trial were based on his necroscopy findings. The third factor, reflects Virchow’s remarkable insight, that after 150 years later has been substantiated on molecular levels\(^{(2)}\). The term “hypercoaguable state” is a poorly defined condition that encompasses inherited or acquired (secondary) disorders associated with an increased risk of thrombosis\(^{(2,3)}\).

The secondary hypercoaguable states are a diverse group of conditions in which the mechanism of thrombosis is poorly understood and probably involves complex and multifactorial processes. Examples include prolonged immobilization, obesity, congestive heart failure, use of oestrogens, nephrotic syndrome, lupus anticoagulant and cancer etc.\(^{(2)}\). However, the inherited hypercoaguable states also known as inherited thrombophilia (or hereditary thrombophilia) are due to specific genetic abnormalities of the physiological anticoagulant proteins, which include qualitative or quantitative deficiencies of antithrombin III, Protein C and Protein S and disorders of the fibrinolytic system\(^{(4,5,6)}\). The inherited type should be suspected when a patient has recurrent thromboembolism, a family history of thrombosis, thromboembolic
phenomena at a young age, and thrombosis in unusual sites without any apparent acquired risk factors\(^{(2,15)}\). It should also be suspected if the patient is a woman who has a history of multiple abortions, stillbirths or both\(^{(5)}\). Acquired and genetic defects may interact making the diagnosis and management difficult\(^{(5)}\). Initially, investigations for inherited thrombophilias among patients with idiopathic venous thrombosis were disappointing since they constituted 5-20% of the later\(^{(1,3)}\). Therefore the etiology of 80 – 95% of thrombophilia cases remained undetermined\(^{(6,7)}\) until 1993 when Dalbőck et al described a novel anomaly associated with inherited thrombophilia known as activated Protein C resistance (APCR)\(^{(1,3-11)}\). This abnormality consists of a weak plasma response to the anticoagulant activity of activated protein C (APC)\(^{(1,3-11)}\). The field of thrombophilia had changed dramatically following this observation. This phenomenon of APCR was later shown in more than 90% of cases to be due to the replacement of Arginine in residue 506 with glutamine (an Arg → Gln mutation) in the factor V gene, called factor V Leiden (FVR506Q)\(^{(11,12,13)}\). This mutation abolishes the APC cleavage site at Arginine 506 of activated Factor V\(^{(11,12,13)}\). The heterozygous state for this mutation confers a 2.7 to 7 fold increase in the relative risk toward venous thromboembolism, and in homozygotes the risk is much higher\(^{(11,12,13)}\). Other changes in the factor V gene that lead to APCR include a rare mutation, G1091C, in the second APC cleavage site at Arginine 306 (Arg306 Thr substitution) and an array of polymorphisms designated HR2 haplotype\(^{(12)}\). The Factor V Leiden mutation has been found to be the most common abnormality in patients investigated for thrombophilia and a major factor in the development of venous thromboembolism\(^{(1,11)}\). The reports of APCR prevalence have varied from different parts of the world\(^{(14)}\). It showed a high prevalence in Europeans\(^{(14)}\). It was not found in any of the 1600 chromosomes from Africa, South
East Asia, Australia, the Americas and Saudi Arabia\textsuperscript{(14)}. In thrombophilic populations it varies between 8% to 64\%\textsuperscript{(5)}. The rates were found in the normal population to be between 5\% and 7\%\textsuperscript{(6,11)}.

The research in the area of thrombophilia has witnessed an explosion in the past 7 years, with discovery and description of other recently identified causes of thrombosis namely a G to A mutation at position 20210 in the 3’ untranslated region of the prothrombin gene\textsuperscript{(15,16)}. Another disorder; hyperhomocysteinemia also predisposes to venous thrombosis, possibly through a complex interaction of multiple genetic or acquired traits, or both\textsuperscript{(17)} and is considered a mixed thrombophilic defect. The laboratory tests for evaluation of patients with thrombosis also exploited, especially the tests for detecting APCR including molecular testing for the Factor V Leiden Mutation. In a recent review, Bertina reported that a combination of tests for Protein C, protein S, antithrombin III, FV Leiden, and prothrombin 20210 identified a genetic risk factor for thrombosis in up to 25\% of unselected patients and up to 63\% of patients with a family history of thrombophilia\textsuperscript{(18)}. A recent study discussing primary thrombophilia in Saudi Arabia revealed that Protein S deficiency was the most common cause, followed by PC deficiency, APCR was present in only 2.2\% of their patients and two patients tested positive for the prothrombin mutation\textsuperscript{(19)}. The aim of this study was to assess the prevalence of APCR in a population of Saudi nationals from the Eastern Province and to compare the results with those from unselected patients with different thrombotic disorders as well as with other reports discussing this interesting area in Hemostasis and coagulation.

**Key words:** Thrombosis, Antithrombin III, Protein C, Protein S, APCR-R.
Materials and Methods:

Blood Sampling: Blood specimens from patients and donors were collected in vacutainer tubes containing 0.5 ml of 0.12 mol./L sodium citrate. Upon arrival of specimens to the Hematology coagulation laboratory, they are centrifuged at 2000 x g for 10 minutes.

Specimens were frozen, and then tested in batches. Samples at testing were thawed at 37°C and tested within one hour.

Any hemolysed or lipidemic samples as well as samples from patients on anticoagulants were rejected.

Investigations:

Antithrombin III:

Principle of the Method

The antithrombin III in the sample is converted into an immediate inhibitor by heparin and inactivates any thrombin present. The residual thrombin content is determined in a kinetic test by measuring the increase in absorbance at 405 nm. The absorbance decreases linearly with the amount of Antithrombin III present in the patient sample.

Protein C (PC):

Principle of the test:

PC in the patients sample is activated by a specific snake venom activator. The resulting PCa is assayed in a kinetic test by measuring the increase in absorbance at 405 nm.
Protein S (PS):

**Principle of the Method:**

Protein Ca proteolytically cleaves Fva which is generated during the activation of the coagulation cascade by RVVC (Venom of Vipera russelli). In this reaction protein S acts as a cofactor which powerfully accelerates the reaction. As a result, the coagulation time increases proportionally to the activity of PS in the sample.

**Activated PC resistance:**

Activated PC resistance was performed on the Dade-Behring Coagulation timer (BCT) instrument (Dade-Behring Marburg Germany) using a heparin insensitive silica based APTT that is prolonged by addition of a snake venom from AgKistrodon contortrix due to the activation of Protein C of the sample. The Protein C activation time (PCAT) in presence of the Protein C activator is set in relation to a parallel determination of a similar APTT --PCAT / O where buffer is added instead of the protein C activator reagent. For improved comparability and reliability of the results the ratio of clotting times of the samples are normalized (normalized ratio; NR) by testing a precalibrated normal plasma pool. The Pro C-Global – Reference range was found to be 0.69 – 1.56, with a median NR of 0.94.

Factor V/APC resistance (FV/APCR) was indicated by an APC resistance assay with factor-V-deficient plasma (chromogenix) using the KC10 (Amelung) and confirmed by a second APC resistance assay with factor-V-deficient plasma (ProC APC) using the BCT.

Prothrombin time (PT) and Activated Partial thromboplastin (APTT) time was also assayed in all blood donors as well as the patients.
A questionnaire was distributed to all blood donors with questions on demographic aspects e.g. relating to age, occupation, as well as questions relating to any history of thrombosis or a family history of any thrombotic disorders.
Results

During a 2 year period a total of 200 healthy potential male donors were accrued in this prospective study. The median age of the donors was $30.56 \pm 8.55$ years. The mean value for activated protein C resistance was found to be $0.94 \pm 0.293$. The mean level for Protein C (PC) was $104.035\% \pm 22$ antithrombin III (AT) $101.85\% \pm 17.6$ and Protein S (PS) $91.72\% \pm 26.4$.

Prothrombin time (PT) showed a mean value of $12.65 \pm 1.27$ seconds, partial thromboplastin time (PTT) showed a mean value of $24.47 \pm 13.5$ seconds.
Discussion

Establishment of reference values for Saudi nationals is very needed. We have been depending on values from many sources in the western literature. The aim of this study was to establish reference ranges for apparently healthy nationals to be a reference for laboratory as well as research use. A similar study \(^{20}\) showed functional levels of these proteins to be relatively similar to this study; the mean AT activity was 103.3 ± 11.8 (this study 101.85 ± 17.6), PC 94.8 ± 21.6 (this study 104 ± 22), while PS was 99.6 ± 20.3 (this study 91.72 ± 26.4). Our study showed a quite similar result in ATIII levels while PC was higher, while protein S was lower.
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References


