

# Normal ranges and genetic variants of antithrombin, protein C and protein S in the general Chinese population. Results of the Chinese Hemostasis Investigation on Natural Anticoagulants Study I Group

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A complete list of the members of the Chinese Hemostasis Investigation on Natural Anticoagulants Study I Group is given in the Appendix.

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The online version of this article has a Supplementary Appendix.

## ABSTRACT

### Background

Inherited deficiency of antithrombin, protein C and protein S, three important, naturally occurring coagulation inhibitors, might play a major role in the occurrence of venous thromboembolism in Chinese. The establishment of age- and gender-related normal ranges of these inhibitors is crucial for an accurate diagnosis of these deficiencies.

### Design and Methods

We designed a prospective cross-sectional study recruiting healthy adults from four university-affiliated hospitals in China. Antithrombin, protein C and protein S were studied by measuring their activity. Gene analysis was performed when natural anticoagulant deficiency was suspected. Polymorphisms of the factor V gene were searched for among subjects who were positive for activated protein C resistance.

### Results

In 3493 healthy Chinese adults (1734 men, 1759 women; age 17-83 years), we found higher age-adjusted activities for protein C and protein S in men than in women but no sex difference for antithrombin. In women, mean protein C and protein S activities increased with age. In men, mean protein C levels increased with age up to the age of 49 but decreased after 50 years old; mean protein S levels decreased after 50 years of age. Antithrombin levels remained stable over time in women but decreased significantly after 50 years of age in men. Reference values according to age and sex allowed the identification of 15 genetic variants (protein C: 10, antithrombin: 3, protein S: 2) in subjects with protein activity below the 1<sup>st</sup> percentile.

### Conclusions

This is the largest survey ever conducted in the healthy general Chinese population. These normal ranges provide the essential basis for the diagnosis and treatment of thrombosis in Chinese.

**Key words:** antithrombin, protein C, protein S, normal ranges, genetics, Chinese.

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## Introduction

Venous thromboembolism is one of the leading causes of mortality and morbidity in the world. In the past, venous thromboembolism was thought to be rare in Asia as compared to in Western countries,<sup>1</sup> although according to recent studies, this is not true.<sup>2-6</sup> It is, however, becoming increasingly clear that there are differences in risk factors for thrombosis between Eastern and Western countries.<sup>7</sup> Factor V (FV) Leiden (FV R506Q) associated with activated protein C resistance (APC-R)<sup>8,9</sup> and factor II G20210A polymorphism,<sup>10</sup> the two most common genetic risk factors for venous thromboembolism in Western populations, are absent or very rare in Eastern populations.<sup>11-13</sup> By contrast, genetic deficiencies of the three main natural anticoagulants, antithrombin (AT), protein C (PC) and protein S (PS) are rare in Western countries but considered important risk factors for venous thromboembolism in Asian countries.<sup>14-20</sup> The diagnosis of AT, PC and PS deficiencies in Asians is, therefore, of substantial clinical interest.

The identification of people with low levels of these anticoagulants is based on the knowledge of reference ranges of AT, PC and PS plasma activities in the corresponding general population. Some publications have reported age- and gender-related normal ranges of AT, PC and PS activities in large series of healthy Western individuals.<sup>21-29</sup> However, such reference ranges have only been reported in small series of Asians, except in Japanese.<sup>20,30-32</sup> As variations can be expected between populations of different ethnic backgrounds, the results published for Caucasians should not be extrapolated to the Chinese and, likewise, results from Japanese series may differ from those for Chinese people.

In this prospective multicenter cross-sectional study, we explored the distribution of AT, PC and PS levels in a large series of healthy Chinese individuals in order to determine the normal activity ranges according to gender and age and to identify the underlying genetic variants.

## Design and Methods

### Population

The *Chinese Haemostasis Investigation on Natural Anticoagulants (C.H.I.N.A)* Study I recruited consecutive Han Chinese individuals over 17 years old among adult blood donors and healthy individuals evaluated for routine check-up in four university-affiliated hospitals in China in a period of 4 consecutive months. Of the potentially eligible subjects, 95% agreed to participate in the study and gave written informed consent. Subjects with abnormal renal function (serum creatinine levels >133  $\mu\text{mol/L}$ ) or liver function (serum transaminases >2 times the upper limit of normal) or previous thrombosis, individuals receiving anticoagulant therapy, oral contraceptives or hormonal replacement therapy, and pregnant women were excluded. The study was coordinated by Peking Union Medical College Hospital and University, Beijing, China, and approved by the Ethics Committee of this Center.

### Collection of blood samples

Venous blood (5.4 mL) was drawn directly by venipuncture into vacutainer tubes (Becton-Dickinson) containing 0.105 mol/L trisodium citrate (9:1, v/v) from each fasting participant in the morning. Each sample was immediately centrifuged at 2500g for

15 min; the supernatant platelet-poor plasma was separated into aliquots which were frozen quickly and stored at  $-70^{\circ}\text{C}$  until activity and antigen assays. Leukocytes were also isolated after centrifugation and stored at  $-70^{\circ}\text{C}$  for DNA extraction. Hemolysed or clotted samples were discarded.

### Plasma activity of antithrombin, protein C, and protein S, and activated protein C resistance assays

AT, PC, PS activities and APC-R were measured on a STA-R Instrument (Diagnostica Stago, Asnières, France) using the same batches of reagents (Diagnostica Stago). The activities of AT and PC were assayed using a chromogenic substrate method (STA-Stachrom ATIII and STA-Stachrom Protein C, respectively). PS and APC-R were assayed by a clotting method (STA-Staclot Protein S and Staclot APC-R). The STA-Staclot APC-R test is based on the activation of factor X by snake venom in the presence of activated protein C. The venom triggers the coagulation cascade downstream of factor X, thus eliminating the influence of all coagulation factors acting upstream. The results are given as clotting time in seconds. Activity levels below the 2.5<sup>th</sup> percentile were considered to be lower than normal. However, only subjects with activity levels below the 1<sup>st</sup> centile were strongly suspected of having an inherited deficiency and underwent further gene analysis.

### Genetic analysis of antithrombin, protein C, and protein S and factor V polymorphisms

Genomic DNA was isolated from leukocytes by phenol extraction. All the primers for PS, PC and AT genetic analyses were selected as previously published.<sup>33-35</sup> All the exons and 5'- and 3'-untranslated regions of the genes encoding for the three proteins were amplified. For each polymerase chain reaction (PCR), a total of 100 ng DNA template was amplified at  $95^{\circ}\text{C}$  (5 min), then  $95^{\circ}\text{C}$  (30 sec),  $50^{\circ}\text{C}$ – $68^{\circ}\text{C}$  (30 sec), and  $72^{\circ}\text{C}$  (30–60 sec) for 30 cycles, using 100 nmol primers, 1 $\times$ PCR buffer (containing  $\text{MgCl}_2$ ), 0.25 mmol/L dNTP 2.5  $\mu\text{L}$ , and 0.6 U Taq DNA polymerase in a 25  $\mu\text{L}$  final volume. A final extension step was performed at  $72^{\circ}\text{C}$  for 10 min. The PCR products of AT, PC and PS were then digested by shrimp alkaline phosphatase (SAP, USB Corporation, Cleveland, OH, USA) and exonuclease I (EPI-CENTRE Biotechnologies, Madison, WI, USA) at  $37^{\circ}\text{C}$  for 60 min and then at  $80^{\circ}\text{C}$  for 15 min. Finally, the amplified products were directly sequenced (ABI Prism<sup>®</sup> 377 DNA Sequencer, Applied Biosystems, Warrington, UK). Mutations were numbered according to the HGVS recommendations for mutation nomenclature (<http://www.hgvs.org/mutnomen/>). The reference sequences were: NM\_000312.2 for *PROC*, NM\_000488.2 for *SERPINC1* and NM\_000313.3 for *PROS1*.

Samples positive for APC-R were screened for five single nucleotide polymorphisms in the FV gene (*F5*) predicting the following five substitutions in the FV protein: R306T or FV Hong Kong,<sup>36</sup> R306G or FV Cambridge,<sup>37</sup> R506Q, I359T, and H1299R. The single nucleotide polymorphisms were detected by restriction fragment length polymorphism analysis using five different restriction enzymes. The digestion products were then examined by agarose gel electrophoresis.

### Antigen assays of antithrombin, protein C, and protein S

Antigen levels were assayed only for the individuals who underwent genetic analysis. All the reagents were from Diagnostica Stago. AT antigen was measured by latex immunoassay (Liatest ATIII) and PC antigen by enzyme-linked immunosorbent assay (ELISA, Asserachrom Protein C). Antigen levels of total PS and free PS were also measured with ELISA kits (Asserachrom Total Protein S and Asserachrom Free Protein S).

### Statistical analysis

Statistical analyses were performed with SAS 9.1 (SAS Inc., Cary, North Carolina, USA) and STATA 10.0 (StataCorp, College Station, Texas, USA) for PC. Results are expressed as medians

(ranges) for quantitative variables and frequencies (percentages) for qualitative variables. Age-specific reference intervals were estimated using the parametric method described by Royston and Wright.<sup>38</sup> Details of the statistical analysis and equations used for estimating reference values are given in the *Online Supplementary Appendix* and *Online Table S1*, respectively.

**Table 1.** Demographic data of the study population.

	Men*	Women*
Total	1734 (49.6)	1759 (50.4)
Age: median (range)	37 (17-79)	37 (17-83)
<b>Age group (years)</b>		
<20	27 (1.6)	104 (5.9)
20-29	449 (25.9)	447 (25.4)
30-39	480 (27.7)	433 (24.6)
40-49	367 (21.1)	394 (22.4)
50-59	252 (14.5)	239 (13.6)
60-69	118 (6.8)	112 (6.4)
≥70	41 (2.4)	30 (1.7)
<b>Cigarette smoking</b>		
No	1294 (74.6)	1729 (98.3)
Yes	440 (25.4)	30 (1.7)

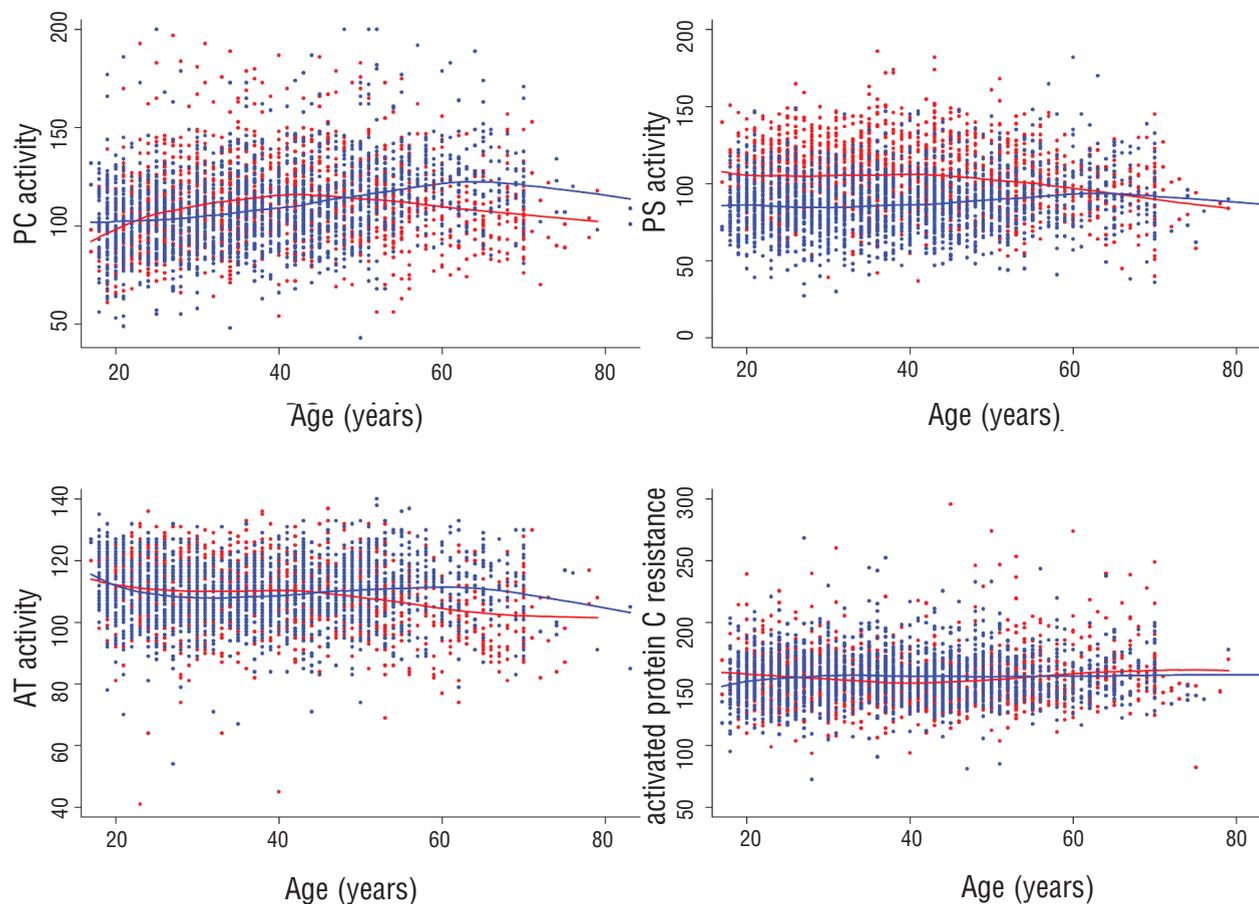
\* All results are indicated as numbers and percentages (between parentheses), except for the age of total men and women.

### Results

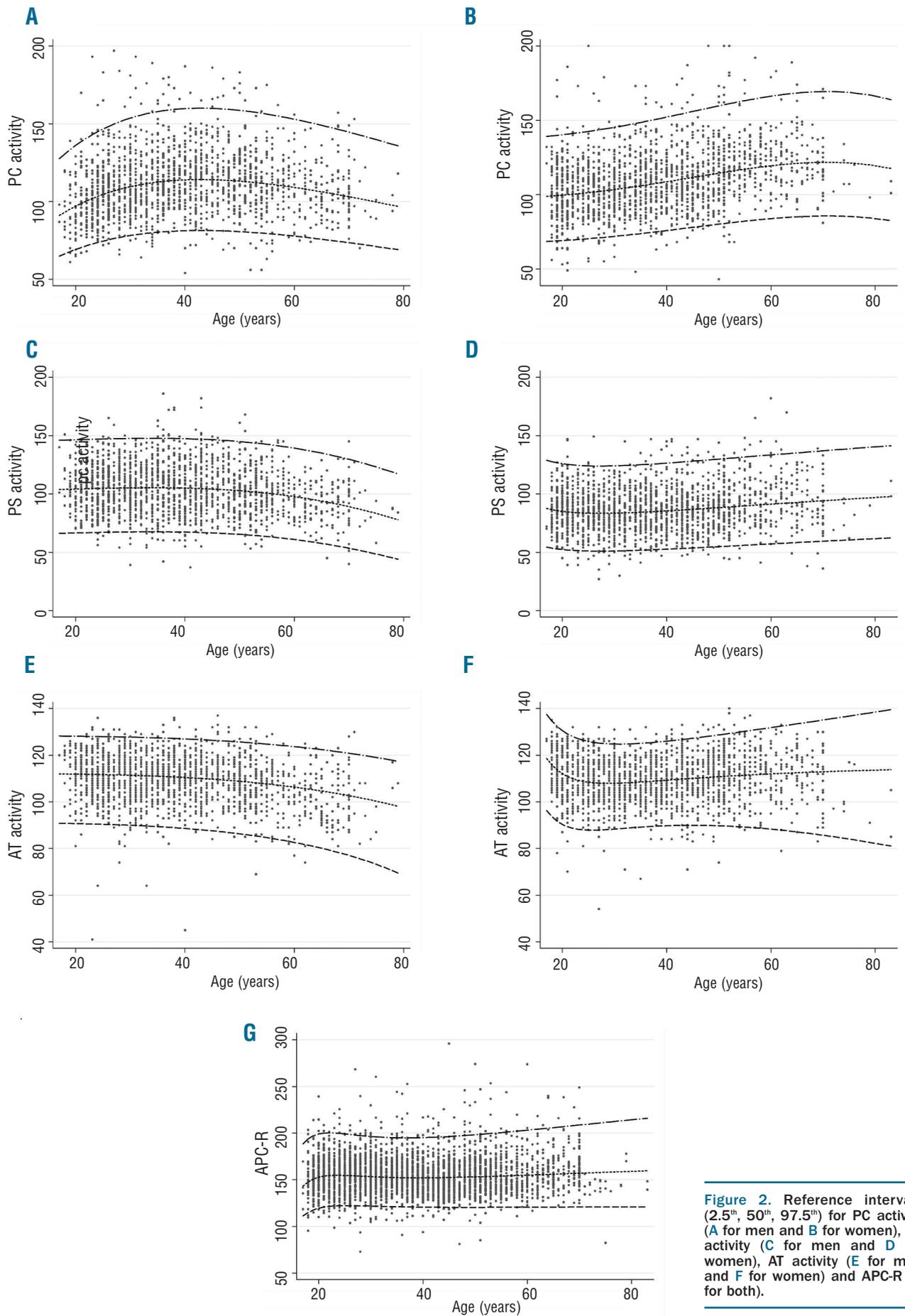
Among 3600 individuals who were asked to participate in the study and who signed the informed consent, 3493 were enrolled since 107 were excluded because of abnormal liver or renal function tests. The participants comprised 738 (21.1%) blood donors and 2755 (78.9%) adults who underwent voluntary health checkups. Detailed characteristics of the population are presented in Table 1. The median age of these participants was 37 years (range, 17-83 years).

### Plasma activity of antithrombin, protein C, and protein S, and activated protein C resistance assays

The distributions of AT, PC and PS activities and APC-R values according to age and sex are illustrated in Figure 1. The 2.5<sup>th</sup>-97.5<sup>th</sup> percentile reference values of AT, PC and PS activities according to sex and age are given in *Online*



**Figure 1.** Loess smoothing curves of PC, PS, AT activity and APC-R depending on age (in years) and sex. The X axis indicates age (years) and the Y axis indicates activity values (% for AT, PC, PS; sec for APC-R). Red points and lines: men; blue points and lines: women,



**Figure 2.** Reference intervals (2.5<sup>th</sup>, 50<sup>th</sup>, 97.5<sup>th</sup>) for PC activity (A for men and B for women), PS activity (C for men and D for women), AT activity (E for men and F for women) and APC-R (G for both).

*Supplementary Table S2.* Sex had a significant effect, when adjusted for age, on PS and PC activities, with men showing higher mean levels than women in the first 50 years of life (Figure 1). However, in men a trend to a change in this pattern could be observed with smoothing curves for AT, PC and PS activity levels after 50 years old. The effect of sex was also significant for AT activity as women had higher mean levels than men from around 50 years of age. Conversely, sex did not have an effect on mean APC-R after adjustment for age. The effect of age on APC-R levels was insignificant in both men and women ( $P>0.05$ ). Reference intervals (2.5<sup>th</sup>, 50<sup>th</sup>, 97.5<sup>th</sup>) for PC, PS, AT activities and APC-R values are shown in Figure 2, confirming the results of the Loess smoothing described above. No association between cigarette smoking and AT, PC and PS activities or APC-R values was identified. Interestingly, no subjects had combined low values of two or three anticoagulants.

### Genetic analysis of antithrombin, protein C, and protein S and factor V polymorphisms

All the subjects (total=95) with either AT, PC or PS activity below the first percentile (AT: women=15, men=16; PC women=16, men=16; PS women=17, men=15) were strongly suspected of bearing mutations and underwent genetic analysis. In addition, all the subjects with AT activity between the 1<sup>st</sup> and the 2.5<sup>th</sup> percentile (women=19, men=25) could also undergo a genetic analysis. A genetic variant was found in 15 subjects with an anticoagulant activity level below the 1<sup>st</sup> percentile: three mutations were found in the *SERPINC1* gene (2 missense substitutions and 1 small deletion), ten in the *PROC* gene (9 missense substitutions and 1 small deletion) and two missense substitutions in the *PROS1* gene. The characteristics of these 15 individuals are shown in Table 2. Nine of them with an activity/antigen ratio between 0.77 and 1.36 might be suspected of having a type I deficiency. Five of the subjects, BA36, SA31 (*PROC* gene substitutions), SA44, TA25 (*SERPINC1* gene substitutions) and BA24 (*PROS1* gene substitution) with an activity/ antigen ratio below 0.70 might be suspected of having a type II deficiency. Finally, JC2 had a high activity/antigen ratio, suggesting that the predicted mutated PC was not recognized fully by the antibody used for the ELISA. None of these subjects was symptomatic or had a strong family history of thrombosis. No recurrent genetic variants were found. Four substitutions in the *PROC* gene, c.247T>C, c.487G>A, c.865C>T, c.1069A>G, and two in the *PROS1* gene, c.906G>C, and c.1063C>T, were not found in previous reports and databases. One subject, BA24, was heterozygous for two genetic variants, one in PC and another in PS. According to these genetic results, the percentages of subjects with genetically confirmed low AT, PC and PS levels were at least 0.08%, 0.29% and 0.057%, respectively. No FV polymorphism was detected in the 41 APC-R positive samples.

### Discussion

To our knowledge this study is the largest cross-sectional survey ever performed with the aim of establishing normal ranges of the three main natural anticoagulants, AT, PC and PS in a large series of healthy Chinese Han people and of identifying their genetic variants. The

**Table 2.** Main characteristics of 15 subjects with PC or PS or AT mutation.

Gene	Subject ID	Age/gender	Protein activity (%)	Protein antigen (%)	Protein antigen (%)	Nucleotide mutation (cDNA)	Predicted amino-acid change
<i>PROC</i>	BA30	25/F	55	1.04	52.8	c.1069A>G	p.Thr357Ala
	BA36	55/M	63	0.69	91.3	c.970G>A	p.Gly324Ser
	BA24	40/M	54	0.83	64.5	c.541T>G	p.Phe181Val
	TA24	50/F	43	0.83	51.4	c.658C>T	p.Arg220Trp
	JC1	18/F	56	1.27	44.0	c.577_579del	p.Lys193del
	JC2	20/F	53	1.96	27.0	c.1019C>T	p.Thr340Met
	JC3	30/F	58	1.28	45.0	c.247T>C	p.Trp83Arg
	SA31	34/F	48	0.62	77.0	c.487G>A	p.Ala163Thr
	SA32	28/F	55	0.90	61.0	c.865C>T	p.Pro289Ser
	TA27	54/M	56	0.77	71.8	c.262G>A	p.Asp88Asn
<i>SERPINC1</i>	JD	40/M	45	1.02	44.0	c.1033_1035 del	p.Glu345del
	SA44	23/M	41	0.40	102.0	c.442T>C	p.Ser148Pro
	TA25	27/F	54	0.69	78.1	c.235C>T	p.Arg79Cys
<i>PROS1</i>					<b>TPS/FPS</b>		
	BA24	40/M	55	0.63	75.2/87.2	c.906G>C	p.Leu302Phe
	JB14	23/F	39	1.36	28.0/28.5	c.1063C>T	p.Arg355Cys

TPS: total PS; FPS: free PS.

strength of this study lies not only in the large size of the population sample but also in the wide age range of the participants (17-83 years), the similar numbers of representatives of both genders, the calibrated assays for measuring the activity of the three major natural coagulation inhibitors with the same batches of reagents and controlled equipment, and the selection of unquestionable statistical methods. Using the method of Royston and Wright, one of the methods recommended by the World Health Organization,<sup>39</sup> effects of age and gender on plasma activities of anticoagulant proteins and APC-R were identified. The main findings were that PS and PC activities were lower in women than in men (in particular, for PS), while AT levels remained constant over time in women but decreased significantly in men after 50 years. PC and PS increased constantly with age until old age in women but decreased after 50 years in men. The remarkable combined decline of all three inhibitors in men over 50 years old might favor an increased risk of thrombosis with age in Chinese males, as indeed has already been reported.<sup>40</sup> A small effect of age on APC-R levels was found in both genders but the effect of sex on mean APC-R level was lost after adjustment for age. The cut-off value for the 2.5<sup>th</sup> percentile differed for each 5-year age group in both sexes. Interestingly, the variability of 2.5<sup>th</sup> percentiles for men (PC: 66 to 81%, PS: 46 to 66%, AT: 71 to 91%) and for women (PC: 69 to 86%, PS: 54 to 62%, AT: 81 to 95%) may reflect a difference in thrombotic risk between men and women.

Among the 15 genetic variants found in our series, nine had been reported previously and have been found to be associated with venous thromboembolism.<sup>41-44</sup> Six novel genetic variants were identified in subjects without previous thrombotic episodes, which might be explained by

the young age of these subjects and/or the absence of thrombogenic challenges. The ongoing follow-up and pedigree studies of these subjects will help to define the genotype-phenotype correlation and the significance of these genetic variants for thrombotic risk. No FV Leiden or any of the four other FV polymorphisms were detected in the 41 APC-R-positive subjects.

As the demographic composition of the population studied was very similar to that of the general Chinese Han population (about 92% of the total Chinese population), it is reasonable to believe that the results are accurately representative of the Han population.

The differences in protein activities between men and women, especially for PS, might indicate a role of sex hormones, most likely estrogens, in the regulation of anticoagulant factors levels under physiological conditions, as already reported,<sup>45,46</sup> although other mechanisms have been proposed. Falkon *et al.*<sup>47</sup> found no relationship between estradiol levels and total or free PS concentration and suggested that low PS levels in young women might result from a combined effect of diverse stimuli on PS synthesis and/or metabolism. Nevertheless, they measured PS antigen only and did not identify any precise molecular mechanism. Variations of other parameters such as triglycerides and cholesterol with sex and age might also play a role.<sup>27</sup> Most of the genetic variants were missense substitutions, as listed in the databases of these inhibitors. Mechanisms by which the genetic variants may be deleterious will be presented in detail in another report. The absence of recurrent genetic variants suggests that there was no founder effect, but larger prospective studies are necessary to confirm this.

Compared with antigen assays, functional assays to determine the levels of AT, PC and, especially, PS are subject to the influence of other coagulation factors.<sup>48</sup> For example, it is known that the presence of APC-R or FV Leiden can cause artifactually low functional PS levels. However, consistently with other studies in Chinese,<sup>12,15,18</sup> no subjects with FV Leiden were identified in our population. Furthermore, there was no correlation between low PS levels and APC-R-positive results either in the whole study population or in the selected APC-R-positive individuals (*data not shown*). Of more importance, functional assays can identify all types of defects (quantitative and qualitative). APC-R-positive tests cannot be explained by either FV gene mutations or by low PS or PC levels because no FV gene mutation and no interference of low PS or PC activity was observed in these individuals. Interestingly, no interference of low PS level has been found with the Staclot APC-R assay.<sup>49</sup> There might be other causes of APC-R, such as still unknown genetic polymorphisms (single nucleotide polymorphisms) especially in the Chinese population in which FV Hong Kong has been discovered.

A bibliographic search was made of PubMed for articles written in English and published between 1975 to 2009. The following key words were used: antithrombin, protein C protein S, Asia population, genetic populations, database, reference ranges. This search did yield some reports of normal ranges for small Asian or Caucasian groups but only a few reports concerned large series of healthy cohorts for Western<sup>21-29</sup> or Eastern<sup>20,31,32</sup> countries (*Online Supplementary Table S3*). The largest studies from Asia were conducted in Japan. Age- and sex-related tendencies were found to be similar in the Chinese population and three other populations from Scotland,<sup>28</sup> Italy<sup>27</sup>

and Japan.<sup>20</sup> These four studies and a number of other reports<sup>26,45-46</sup> were concordant in showing that PS levels were lower in women than in men, with the values being lowest before the age of 45 years,<sup>50</sup> as in our study. Interestingly, the findings of the Japanese studies were most closely similar to those in our study. However, no mutation found in the Chinese population was reported in the Japanese population. Our study was not designed to give information about the prevalence of AT, PC and PS deficiencies in the Chinese population. Nevertheless, if we consider people with a confirmed anticoagulant deficiency (low activity level and mutation), PC deficiency (n=10) was more frequent in Chinese subjects (0.29%) than in Japanese ones (0.13%), while AT (n=3) and PS (n=2) deficiencies were less common among the Chinese (0.08% and 0.056%, respectively) than among the Japanese (0.15% and 1.12%, respectively).

Our study has some limitations. First, the clinical relevance of the results should be interpreted cautiously because there is no consensus about the cut-off activity value of the three natural anticoagulants under which there is a true thrombotic risk. Although statistically significant differences were documented for PC and AT activities between the various sex and age groups, the absolute differences were relatively slight, as reported for the Scottish population.<sup>22</sup> By contrast, if the same cut-off value was to be applied to make a diagnosis of PS deficiency whatever the sex, this might lead to misdiagnosis of PS deficiency in women. Secondly, since DNA analysis was only performed in subjects with a level of PC or PS activity below the 1<sup>st</sup> percentile, some true PC and PS hereditary deficiencies might have been missed because some subjects with a level of PC or PS activity between the 1<sup>st</sup> and 2.5<sup>th</sup> percentiles might have a genetic defect. A possible overlap of AT, PC or PS activity levels between heterozygotes and individuals without mutations should also be mentioned. In addition, some genetic variants, such as large deletions, might have been missed by direct sequencing. Finally, due to financial, logistic and local reasons, only one sample was obtained for each participant: however, given the large number of samples, one could argue that supposed over- and under-estimated results for some samples would balance each other out.

In conclusion, for the first time we have established sex and age-related normal ranges for levels of AT, PC and PS activity in a large series of 3493 healthy Chinese Han subjects. Fifteen subjects (4.3 per 1000) with a genetic variant were detected by direct sequencing. These reference ranges are crucial in daily clinical practice for the detection of Chinese people with deficiency of the naturally occurring anticoagulant proteins and for better prevention and treatment of venous thromboembolism in the Chinese population.

## Appendix

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## Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with

the full text of this paper at [www.haematologica.org](http://www.haematologica.org).

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