RESEARCH ARTICLE

CLINICAL APPLICATION OF PLASMA PROTEIN C DETERMINATION

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Abstract

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Objective: Protein C, a vitamin K-dependent coagulation factor, is involved in blood coagulation. Activated protein C inactivates Va and VIIIa and stimulates fibrinolysis. In this process, protein S serves as an important factor for activated protein C. Furthermore, excess protein S drives cancer cell proliferation and cell survival through oncogenic receptor Axl (Anexelekto). We determined ranges of protein C both in healthy individuals and distinct hospitalized patients.
Methods: A total of 100 patients with different diagnostic diseases and 50 healthy individuals were included in their plasma protein C determination. A rabbit antibody against human protein C was used for the quantitative estimation of plasma protein C antigen by using rocket immunoassay.
Results: In healthy individuals protein C antigen (PC:Ag) ranges 0.6439-1.4752 μ/ml. The mean coefficient of variation (CV) of length of rocket was calculated to be 12.45%. PC:Ag within laboratory variation was 11.47%. Plasma protein C antigen was destroyed at 56℃ for 30 minutes, whereas no significant decrease of protein C was found at 4℃ refrigerator for one week.
Conclusion: The results showed that plasma protein C antigen was considerably high in 22 diabetes mellitus. On the other hand, the PC:Ag was significantly decreased in 19 liver cirrhosis ($p<0.001$) and was positively correlated with serum albumin levels ($p<0.05$). In 20 acute leukemias, on the average, there was slightly lower values in PC:Ag, and accompanied with significant decrease of PC:Ag in 5 FAB M5 subtype and in 9 hyper- leukocytes acute leukemias. However, the 3 acute promyelocytic leukemia (APL) with overt laboratory picture of DIC (disseminated intravascular coagulation) had protein C concentration no lower than the remaining 2 patients with infectious DIC, which suggested the coagulopathy in APL might be due to mechanisms different from other forms of DIC.
Keywords: Antigen, clinical determination, plasma protein C, rocket immunoassay.

INTRODUCTION

Protein C is a vitamin K-dependent protein which was discovered by Stenflo in 1976[1]. It was composed of a light and heavy chain linked by interchain sulfide bridges (Figure 1)[2]. In its activated form protein C inactivates the activated factor V (Va) and VIIIa and accelerates fibrinolysis[3]. Activated protein C (APC) requires the presence of protein S[4]. Protein S is a plasma protein that serve as an important cofactor for activated protein C in the blood anticoagulant system[4,5]. Protein S also act as a mitogen on distinct cell types and is a ligand for Tyro3, a member of the Axl family of oncogenic receptor tyrosine kinases[6,7]. The TAM family of proto-oncogenic receptor kinases (Tyro3, Axl and Mer) is implicated in many human cancers[8-11]. The full length Tyro-3, Axl, and Mer

protein contain 890, 894, and 999 amino acids, respectively[8]. Protein S is expressed in lung carcinoma tissue, predominantly in tumors of squamous cell origin, and in head and neck and oral squamous cell carcinoma[12]. Most of the protein S is synthesized in the liver by hepatocytes together with the closely related protein Gas6, as an activating ligand for TAM family. Gas6 and protein S share 43% amino acid sequence identity and have the same domain structure with the exception of thrombin cleavage sites which present in protein S but not Gas6[8]. The role of the TAM cognate ligands Gas6 and protein S was demonstrated in homeostatic regulation of the immune, vascular and nervous systems. Excess protein S drives oral squamous cell carcinoma proliferation and cell survival through high expression and the activation of oncogenic receptor Axl[7,11,13-16].
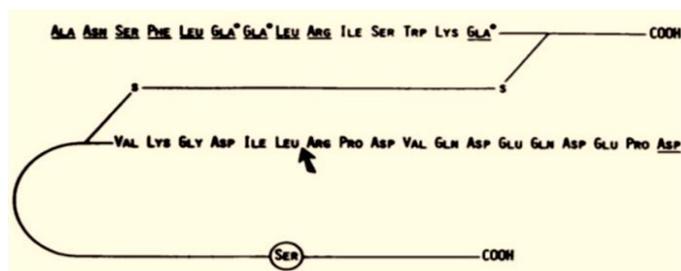

Figure 1: Partial structure of human protein C.
Residues that are underlined are identical to those found in bovine protein C. Gla (Gamma-carboxyglutamic acid) shown in positions 6,7, and 14 of the light chain is tentative. The arrow indicates the probable site of cleavage in the heavy chain of protein C by thrombin.

Griffin *et al*.,[17] reported the first family with a congenital protein C deficiency. Bertina and Broekmans *et al*.,[4,18,19] reported the first Dutch family with a congenital protein C deficiency. Using an immunologic assay for protein C[20], we carried out the measurement of protein C in patients with clinical various diseases especially bleeding diathesis.

MATERIALS AND METHODS

Blood collection:
Venous blood was collected in 1/10 volume of 0.11 M sodium citrate. Plasma was prepared by centrifugation of blood at 3000 rpm for 10 min. Platelet free plasma of 100 patients and 50 healthy volunteers were stored at -20°C for the assessment of protein C levels. Three tubes with rabbit anti-protein C serum were kindly supplied by Prof. RM Bertina, Leiden University Hospital, The Netherlands. The period of experimental procedure was completed in early 1986-89.
Procedures:
  i. **Preparation of antibody-containing agarose gels.**
  A 12.5 ml of 1% indubiose A37 or commercial agarose solution is obtained by boiling in gel buffer (PH8.8 Tris-BB or Tris-BB-EDTA) during continuous stirring. The solution is allowed to cool to 55°C, and a 60 μl amount of rabbit protein C antiserum and a 0.05ml of 0.11 mol/l sodium citrate solution was added during careful mixing. A 100 x 60 x 2 mm gel is prepared on small glass plate by pouring antibody- containing agarose (50～55°C) into a coated glass plate placed on a horizontal table. After 10 minutes the solidified gel is placed in a refrigerator (4°C) in a moist atmosphere. The gel should harden before wells can be punched.
  ii. **Punching out the wells.**
  Wells are punched out with a gel puncher, using a template.
  iii. **Application of the samples.**
  A row of wells (about 4 mm in diameter) with their centers at least 5 mm apart is punched in the gel. After appropriate dilution the samples (13 μl) are deposited in the wells. The ionic strength of the samples should preferably be approximately the same as that of the electrophoresis buffer. Subsequently, samples are applied after the glass plate has been placed on the electrophoresis apparatus and a low voltage has been applied (0.5

V/cm). Standards in suitable dilutions are deposited in the middle holes of the row.
  iv. **Electrophoresis, staining and drying.**
  Electrophoresis is performed at 120 V (4-6 mA/plate) at room temperature 12~30°C for 20 hours overnight.
  v. **Calculation of the test results.**
  The length of rocket immunoprecipitate was measured by using a ruler, which is linearly correlated to the amount of antigen. Results (including normal controls and patients) are plotted on log-log paper (length of rocket in mm versus percentage antigen), based on the construction of the standard curve.

RESULTS

Protein C antigen in healthy individuals
Protein C antigen was measured in the plasma of 50 healthy individuals who contributed to our pooled normal plasma. A mean protein C antigen of 1.0578 μ/ml was calculated with a SD of 0.1886 μ/ml; individual protein C values ranged from 0.6439 μ/ml to 1.4752 μ/ml. The distribution of age on the protein C antigen concentration was evaluated in 50 healthy individuals. The results are summarized in Table 1, which indicated that no statistical differences in protein C antigen were found for the different age groups ($p>0.5$). Also, no difference was observed between men (mean 1.0613 μ/ml) and women (mean 1.0550 μ/ml). Data from those in Table 1 are the representation of Mean±Standard deviation without significant difference of mean for the different age groups ($p>0.5$).

Table 1: Plasma protein C antigen value distribution among age groups

Age groups	No	Protein C antigen (μ/ml)	
		mean±SD	Range
18−25	9	1.0505±0.225	0.6439~1.3588
26−30	11	1.0623±0.2066	0.6870~1.4269
31−40	16	0.9767±0.1040	0.8047~1.1649
41−50	13	1.1268±0.1841	0.8785~1.4298
51	1	1.4752	
Total	50	1.0578±0.1886	0.6439~1.4752

Data are the representation of Mean±Standard deviation without significant difference of mean for the different age groups

Table 2: Experimental results of PC peak height following a consecutive 18 assays from pooled normal plasma.

Plasma dilution	Repeat No.	Rocket height (x±s)(cm)	CV*(%)	Mean CV*(%)
1:1	18	1.6217±0.1463	9.02	12.45
1:2	18	1.1722±0.1136	9.69	
1:4	18	0.8783±0.1082	12.30	
1:8	18	0.6256±0.1181	18.8	

*Mean CV=S/X x 100%, S: total CV% X: 4 different dilution.

The qualitative evaluation of protein C antigen determination

a) For investigation, to perform the consecutive numerous assays on mixed pooled normal plasma as standard curve, the mean coefficient of variation (CV) of length of rocket was calculated to be 12.45% (Table 2).

b) To estimate the within laboratory variation, 4 different plasma with PC:Ag level 0.9695, 0.5583,0.2894 and 0.0581 μ/ml were measured by EIA at different gel plate, each plasma sample was measured twice. The average within laboratory precision (CVp) was calculated using the following formula:

$$CVp = 1/x\sqrt{\sum(X1-X2)^2/2n} \times 100$$

Where, x is the calculated PC antigen level. (X1−X2) is the difference in protein C antigen level observed at different gel plate, and n refers to the numbers of assay. Using this formula the CVp for 4 different plasma could be calculated to be 6.69%,18.03%,4.2% and 15.7% respectively, the total mean CVp 11.16%. Meanwhile, another 3 plasma with low (0.1841 μ/ml), middle (0.9799 μ/ml) and high (1.3098 μ/ml) PC antigen level, and each plasma was measured 5 times by EIA at the same gel plate. The calculated mean CVs was 2.64%. The total coefficient of variation (CV_T) in the calculated mean PC antigen levels within laboratory consisted of PC antigen level by EIA detection at the same gel plate variation (CV within a batch, Batch CV) and between different gel plate variation (interassay CV). Based on the formula:

$$CV_T = \sqrt{(CVs)^2 + (CVp)^2}$$

CV_T in PC antigen levels within laboratory was calculated to be 11.47%.

c) Influence of PC antigen level in electrophoresis buffer with or without EDTA. To determine whether electrophoresis buffer with or without EDTA could influence the peak height of PC antigen assay, pooled normal plasma was a dilution of 1:1,1:2,1:4 and 1:8, no significant difference in results obtained by EIA in the presence of EDTA-laurell method or without EDTA from the electrophoresis buffer (*p*>0.5). When normal plasma was kept at 56℃ for 24,12,6, 2.5, 1 hour and 30 minutes respectively, no rocket-like immunoprecipitate was seen,

which indicated that plasma protein C antigen could be destroyed at 56℃ for 30 minutes. After plasma frozen at 4℃ for 1, 3, 6, 9, 12, and 17 days, by using EIA assay, protein C antigen level could be measured at 1.0405, 0.9779, 1.0560, 0.7008, 0.8774 and 0.8950 μ/ml. The results suggested no significant decrease of protein C antigen when stored at 4℃ refrigerator for one week.

d) Linear correlation and regression coefficient analysis. In this current study, protein C antigen concentration was measured according to Laurell's rocket immunoelectrophoresis, and further Prof. Bertina's modification. Electro-immunoassay (EIA) or rocket immunoelectro-phoresis is a rapid and producible method for identification and quantitation of even minor amounts of proteins. When electrophoresis of an antigen leaves the wells and enters the agarose gel containing the corresponding antibody, and eventually the antigen-antibody complex precipitates. In particular, a 'rocket-like' shape is seen. The majority of the antibody-antigen precipitate is indeed at the head of the rocket. More detail protocol, when 3 various concentration of PC:Ag antibodies (60 μl, 75 μl, 90 μl) were mixed with 12.5 ml of 1% indubiose A-37 agarose solution in a gel plate, consequently, pooled diluted normal plasma was applied for EIA. Three linear regression equation regarding protein C antigen could be drawn (Figure 2), and no statistical difference in results was obtained by comparative analysis of linear regression between each other, respectively (*p*>0.05). According to Prof. RM Bertina experience and our laboratory condition, an addition of 60 μl amounts of rabbit protein C antiserum was better available in preparation of antibody-containing agarose gels.
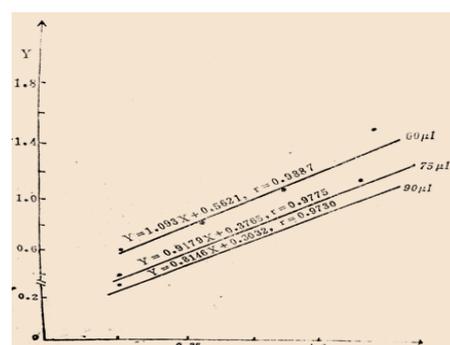

Figure 2: The linear regression equation between the peak height and protein C antigen concentration at various dilution of rabbit PC antiserum.

The head of the rocket is clear, higher peak, save the amounts of antibodies and easy to be measured by a ruler. It has been found that a good correlation between the peak height and protein C antigen level. After a consecutive 18 assays, the linear regression equation:

$$Y = 1.093X + 0.5621, r = 0.9887 \text{ (Figure 2)}.$$

Protein C antigen in distinct diseases
Diabetes Mellitus (DM)
In a group of 22 patients with diabetes mellitus (DM) an average value of protein C antigen was 1.4238 µl/ml, which is considerably higher than the average value in healthy individuals (1.0578 µ/ml). Among 8 DM, the protein C antigen level was over 1.4752 µl/ml. The increased plasma protein C antigen was in turn diabetic ketoacidosis (n=4, 1.5361 µl/ml, range 1.3516 ~1.8434 µ/ml), diabetic angiopathy (n=5, 1.4551 µl/ml, range 1.1888~2.0894 µl/ml), DM without vascular complication (n=9, 1.4403 µl/ml, range 1.0834 ~ 1.8926 µl/ml) and diabetic ketosis (n=4, 1.2352 µl/ml, range 1.1955~1.2663 µl/ml). Plasma PC:Ag level was positively correlated with urine glucose level (r=0.9581, $p<0.001$).

Pregnancy-Induced Hypertension Syndrome (PIHS)
In 15 pregnancy-induced hypertension syndrome (PIHS), 10 severe preeclampsia were observed, and a significant increase of protein C antigen (PC:Ag) value (1.4673 µ/ml, range 0.7781 ~2.2525 µl/ml), while in 2 moderate PIHS the PC:Ag has normal limit range (0.7787, 0.9481 µl/ml respectively), another 3 mild patients with PIHS had decreased plasma PC:Ag level (0.5603 µl/ml). Intriguing, repeat PC:Ag level was recovered to normal value (1.1455 µl/ml) in one mild patient with PIHS after partum. Plasma protein C antigen was positively correlated with urine protein (r=0.9518, $p<0.001$).

Uremia
In a group of 10 uremia there were no significant changes of protein C antigen concentration when compared to normal controls (1.1387 vs 1.0578 µ/ml, $p>0.2$)

Thrombotic diathesis
To screening congenital protein C deficiency, we detected 6 patients with cerebral haemorrhagic infarction and 3 deep venous thrombosis, no PC deficiency was found in this study.

Table 3: Experimental data of plasma PC:Ag determination in patients and normal healthy subjects.

Groups	No	Mean±SD (µ/ml)	Range (µ/ml)	p
DM	22	1.4238±0.2556	1.0834~1.8434	<0.001
PIHS	15	1.2054±0.4930	0.5163~2.2525	<0.05
Uremia	10	1.1387±0.2512	0.7787~1.5507	>0.2
Thrombotic diathesis	9	1.1166±0.2699	0.7637~1.6893	>0.2
Acute leukemias	20	0.9349±0.3388	0.3971~1.9220	>0.05
DIC: APL	3	0.8707	1.0019	1.2445
Infectious diseases	2	0.2328	0.1646	
Liver cirrhosis	19	0.5501±0.2536	0.1436~1.0883	<0.001
Viral liver cirrhosis	14	0.5068±0.2514	0.1436~0.9291	
PC deficiency	1	<0.01		
Healthy individuals	50	1.0578±0.1886	0.6439~1.4752	

The results are Mean±Standard deviation with significant difference of mean at $p<0.05$, and show where there is significant ($p<0.05$) difference in the DM and liver cirrhosis groups when compared to healthy individuals. DM: diabetes mellitus PIHS: Pregnancy-induced hypertension syndrome. *including 1 nodular panniculitis and 1 septic shock.

Liver cirrhosis
19 patients with liver cirrhosis included viral liver cirrhosis 14 cases and other 1 primary biliary cirrhosis, 1 schistosomal cirrhosis, 1 alcoholic liver cirrhosis and 1 primary hepatocellular carcinoma, respectively. The average value of protein C antigen was 0.5501 µ/ml (range: 0.1436~0.9291 µ/ml), which was significantly lower than the average normal controls (1.0578 µ/ml). Moreover, 13(68.4%) liver cirrhosis the protein C antigen was below the lower limit of normal control (range: 0.1436~0.6188 µ/ml). 14 patients with viral liver cirrhosis the protein C antigen was 0.5068 µ/ml. Three severe liver cirrhosis had its the lowest value of protein C antigen 0.1436, 0.1846 and 0.1919 µ/ml respectively. The PC:Ag level was 1.0883 µ/ml in one liver cirrhosis complicated with diabetes mellitus. Plasma PC:Ag level was positively correlated with serum albumin value (r=0.9680, $p<0.05$).

Leukemias
In 20 acute leukemias, there was slightly decreased in protein C antigen level (0.9340 vs 1.0578 µ/ml), but no statistical difference was found between acute leukemia and normal controls. When further analysis of acute leukemia with different cytological subtypes morphology according to FAB classification, it was shown that there was remarkably decrease of PC:Ag (0.8879 µ/ml, $p<0.05$) in 5 FAB M5 subtype. The decreased PC:Ag level (0.7833 µ/ml) was also found in 9 hyper-leukocytes acute leukemias (WBC 23.6~280 x109/l). Intriguing, there were no significant decrease in terms of protein C antigen among 3 acute promyelocytic leukemia (APL) with DIC, whereas 2 patients with infectious DIC (1 nodular panniculitis and 1 septic shock) the average value of protein C antigen was 0.2328 and 0.1646 µ/ml respectively.

Protein C deficiency plasma
1 vial protein C deficient plasma which provided by RM. Bertina was used to measure the protein C antigen <0.01%.

DISCUSSION

This underlines the need for each laboratory to construct its own reference values for PC antigen in the laboratory determination of different methods[5,21]. We

set up successfully the electroimmunoassay (EIA) of the protein C antigen with the laurell method. Based on our laboratory condition, the mean PC:Ag level in 50 healthy individuals was 1.0578±0.1886 µ/ml, which was slightly higher PC antigen levels than that of Bertina's results. To evaluate the performance and specificity of PC:Ag assays[5,21], the ICTH subcommittee on protein C organized an international collaborative study in which 13 lymphilized plasma samples were distributed among 17 different laboratories. Three different types of methods has been used: ELISA's using polyclonal anti-protein C IgG (n=9), electro- immunoassays (n=10) and other methods (three laboratories used monoclonal antibodies against protein C, one laboratory performed a RIA with affinity purified polyclonal antibodies against protein C and one laboratory used an immunoradio- metric assay of protein C antigen). No statistically significant results were obtained with these three different methods. ELISA, RIA and IRMA methods were found to be more sensitive than the electro-immunoassay (EIA). The mean coefficient of variation (CV) for protein C antigen in various plasma was calculated to be 22%, with CV within laboratory variation 11.7% and between laboratory variation 17.8%. In this current study, the mean CV in the construction of standard curve for protein C antigen was 12.45%, with CV within laboratory 11.47%. The results indicated that the protein C antigen measurement was specific, practice and available in clinical useful. The measurement of plasma PC:Ag has become relevant in clinical medicine since the important demonstration that hereditary protein C deficiency is linked to a history of recurrent venous thrombosis[22]. PC is found to be low in a number of acquired condition such as during the postoperative and neonatal periods[23], DIC[21,23,24], liver disease[21,23-25] and oral anticoagulant treatment (e.g. warfarin)[4,17,26]. Elevated PC levels, on the other hand, have been detected in a number of unrelated condition, such as ischemic heart disease, diabetes, late pregnancy[27] and in women taking oral contraceptive with a high estrogen content[28].

Type 2 Diabetes mellitus is characterized by vascular complication with accelerated micro and macro vascular thrombotic disease. A hypercoagulable state is appreciated in this disease. In this study, PC:Ag levels of patients especially in ketoaccidosis and diabetic angiopathy were significantly higher than that of the control group (*p*<0.001). The results were consistent with the reports by Vigno[27], Takahashi[30], Saito[31], and Garcia[32]. Moreover, evaluation of protein C and protein S levels in patients with DM receiving well- managed T2DM (type 2 DM) had significantly higher antigen levels and activity of PC (5.78 vs 4.64 µl/ml), PS and ATIII compared to those that were poorly- managed[33]. The decreased PC antigen was observed in type I diabetes mellitus[34,35]. This may reflect the possibility that diabetic ketoacidosis (or angiopathy) and hypercoagulate may cause endothelial injury, which lead to elevated levels of thrombomodulin (TM) and compensatory increase the PC synthesis by hepatocytes. Patients with the nephrotic syndrome and uremia are at increased risk of developing renal venous and arterial thromboembolism. In the measurement of PC:Ag levels in 10 uremia, there was no significant difference compared to normal controls. Cosio[36] measured the plasma concentration of protein C in 17 patients with severe proteinuria, the results was not significantly difference between the healthy subjects and 14 patients with chronic renal insufficiency (CRI). But protein C was elevated in 71% of patients with proteinura, and was inversely correlated with serum albumin concentrations. Demaschell[37] found that PC functional activity and the antigen level were normal in 30 uremic patients before dialysis. Data of current study are consistent with the previous observation. In this current study, PC:Ag levels in 19 different types of liver cirrhosis were significantly lower than those in healthy individuals (0.5501 vs 1.0578 µl/ml, *p*<0.001). 68.4% of liver cirrhosis had PC:Ag level below the lower limit of normal controls. Moreover, plasma PC:Ag level was positively correlated with serum albumin values. In earlier study, Griffin[24] reported that eleven of 15 patients with clinically severe liver disease (9 with positive FDP, 6 with negative FDP) have decreased plasma protein C levels. Mannucci[21,23] also found that in 58 chronic liver diseases protein C levels were lower than 60 healthy subjects, the decreased degrees roughly proportional to the severity of the disease. These findings indicated that protein C deficiencies occur the dysregulation of hepatic PC synthesis, and might play a role as a predictor index in liver diseases[38].

Acute leukemias varied in PC antigen level. In this current study, mean PC antigens in 20 acute leukemias were slightly lower than that of the normal individuals. Among FAB subtypes, the decreased PC:Ag concentration were found in those with M5 subtype and hyperleukocytic acute leukemias. Moreover, the PC:Ag had no lower in a limited 3 patients with APL complicated by DIC, which suggested the coagulopathy in APL might be due to mechanisms different from other forms of DIC such as infectious disease (e.g. septic shock)[39,40]. The results was similar to previous studies by Griffin *et al*.,[24] and Rodeghiero *et al*.,[25]. In clinics, two types of hereditary protein C deficiency can be recognized[4, 22]. In patients with type I protein C deficiency, the plasma concentration of protein C activity and/or protein C antigen will be below the lower limit of the normal range. A type I protein C deficiency inherits as an autosomal dominant trait. In type II protein C deficiency, the patients protein C antigen will be within the normal range, while the protein C activity and the ratio between protein C activity and protein C antigen are below the lower limit of the normal range. The presence of 1 vial PC deficiency plasma which was provided by RM Bertina was the lower than 0.01 µ/ml, and which was consisted with the criteria of type I severe protein C deficiency[41-45]. In Dutch family, more than 80 patients from 30 unrelated families have been identified with a type I protein C deficiency. Individual protein C values may vary between 0.30 and 0.76 µl/ml[4,22]. Up to now, only five patients with a type II protein C deficiency have been reported[22]. It seems likely the main

prevalence of clinical type I protein C deficiency. Like protein C deficiency, familial protein S deficiency is also reported[46-49]. At present, some case reports and case series suggest that protein C concentrates may improve the outcome in patients with congenital or acquired protein C deficiency[45,50-52]. The use of protein C concentrates in adult septic shock[53-55] is also testable.

CONCLUSIONS

The results showed that plasma protein C antigen was considerably high in 22 diabetes mellitus. On the other hand, the PC:Ag was significantly decreased in 19 liver cirrhosis (p<0.001) and was positively correlated with serum albumin levels (p<0.05). In 20 acute leukemias, on the average, there was slightly lower values in PC:Ag, and accompanied with significant decrease of PC:Ag in 5 FAB M5 subtype and in 9 hyper-leukocytes acute leukemias..

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AUTHOR'S CONTRIBUTION

Zhu G: writing original draft, literature survey. **Broekmans AW:** methodology, conceptualization. **Bertina RM:** formal analysis, review. All authors revised the article and approved the final version.

DATA AVAILABILITY

The data and material are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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