Increased levels of serum histone H4 and activated protein C in patients with active rheumatoid arthritis

W. Peng[1], X. Liang[1], Y. Zhong[2], Y. Zhou[3], L. Hu[1], W. Wang[1]

[1]Department of Clinical Laboratory, [2]Department of Nephrology, and [3]Department of Rheumatology, Xiangya Hospital, Central South University, Changsha, Hunan, China.

Abstract

Objective

We aimed to examine the levels of serum H4 and activated protein C (APC) in rheumatoid arthritis (RA) and other autoimmune conditions, and investigate the associations between H4 or APC levels and disease activity indicators in RA.

Methods

Serum H4 and APC distribution was examined in samples from patients with RA, systemic lupus erythematosus (SLE), polymyositis (PM), and ankylosing spondylitis (AS), as well as in samples from healthy controls, using commercial ELISA kits. Associations of serum H4 or APC levels with disease variables in patients with RA were evaluated. Receiver operating characteristic (ROC) curve analysis was performed to assess the discriminant capacity of APC against RA and non-RA.

Results

The patients with RA, PM, and AS showed higher serum levels of H4 and APC than those from the healthy control individuals, while the SLE patients showed higher serum levels of APC only. Moderate positive correlations between H4 levels and absolute neutrophil count (ANC), platelet count (PLT), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen (FIB), D-dimer (DD), and complement fraction 3 (C3) were observed. Positive correlations between APC levels and PLT, RF, DD, or DAS28 were additionally found. ROC curve analysis revealed that APC discriminated well between RA and non-RA.

Conclusion

H4 and APC concentrations are elevated in patients with chronic inflammatory autoimmune diseases. The observed associations between H4 and APC and disease variables in patients with RA support a role for H4 and APC in the inflammatory process of the disease.

Key words

histone H4, serum, activated protein C, rheumatoid arthritis, inflammation

*Wanchan Peng, MM**
*Xianghui Liang, MM**
Yong Zhong, MD
Yaou Zhou, MD
Litao Hu, MM
Wei Wang, MD

**These authors contributed equally to this study.*

Please address correspondence to:
Dr Wei Wang,
Department of Clinical Laboratory,
Xiangya Hospital,
Central South University,
87 Xiangya Road, Changsha,
Hunan 410008, China.
E-mail: weiweiwang1983@163.com

Received on June 20, 2017; accepted in revised form on October 6, 2017.

Funding: the study was supported by the National Natural Science Foundation of China (grant no. 81401731).

Competing interests: none declared

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with persistent inflammation of the synovium, which results in the destruction of cartilage and erosion of bone, and even leads to loss of function and deformity (1). The etiology and pathogenesis of RA is poorly understood at present; however, genetic predisposition and environmental factors might be involved (2-5).

Recently, it has been suggested that extracellular histones contribute to the pathogenesis of both infectious and non-infectious inflammatory diseases (6, 7). The histone family consists of 5 cationic proteins, namely, H1, H2A, H2B, H3, and H4, within the nuclei of eukaryotic cells: H2A, H2B, H3, and H4 are core histones, which assemble into an octamer comprising 2 copies of each. High circulating levels of histones are present in patients with autoimmune disorders (8), sepsis (9, 10), and trauma (11). Activated protein C (APC), a vitamin K-dependent serine protease, is a natural anticoagulant that plays an important role in coagulation homeostasis, as well as in the regulation of inflammation, by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory mediators (12, 13).

Elucidation of the function of histones and APC in chronic inflammatory conditions, as well as in the pathogenesis of infectious or non-infectious disease, has been the focus of much recent interest. Xu *et al*. (9) described extracellular histones as mediators of cell damage and organ dysfunction during sepsis; this was evidenced by the efficacy of a neutralising antibody against histone H4 in reducing mortality in several experimental models of murine sepsis. Extracellular histones might promote thrombus formation (14), and histones, especially H4, directly induce the aggregation of washed human platelets. In addition to the experiments of Xu *et al*. (9) showing that the co-injection of histones with APC abrogated the lethal effects of histone injection in mice, it has been demonstrated that APC degrades extracellular histones in an isolated system in sepsis. A study by Ekaney *et al*. (15) suggested an inverse correlation between plasma histone levels and endogenous APC levels in patients with sepsis (r=-0.58, $p<0.05$). Buisson-Legendre *et al*. reported that APC was elevated in RA synovial fluid and synovial joints (16). However, it is not known whether elevated levels of histone H4 or APC are present in the serum of patients with autoimmune disease. Neither is it clear whether histones and APC are linked with RA pathogenesis.

In the present study, we aimed to evaluate the serum histone and APC levels in patients with RA and other autoimmune diseases, with an emphasis on investigating the relationship between the levels of serum histones and/or APC and inflammatory processes in patients with RA to lay the foundation for further study of the pathogenesis of RA.

Materials and methods

Human subjects

Patients with active RA (n=102: 76 women and 26 men; mean age, 58.6 years; range, 31–83 years; mean disease duration, 8.6 years; range, 3 months to 40 years) diagnosed according to the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria (17), were enrolled in this study. Control subjects (n=87) comprised healthy control individuals (HC, n=40) and patients with systemic lupus erythematosus (SLE, n=21), polymyositis (PM, n=14), and ankylosing spondylitis (AS, n=12). Diagnosis of SLE (18) was made based on the ACR criteria. The definition of PM was based on the Bohan and Peter criteria (19). AS was diagnosed according to the revised New York criteria (20). Serum samples were stored at -20°C until analysis. No significant differences with respect to sex or age were present between the test and control groups.

The following data for patients with RA were additionally collected: disease duration, rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) antibodies, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), complement fractions (C3 and C4), D-dimer (DD), fibrinogen (FIB), DAS28, absolute neutrophil count (ANC), and platelet count (PLT) in peripheral blood. In this study, subjects with RA for less than 3 years were considered

to have recent-onset disease (RO), and the remaining were considered patients with long-standing disease (LS). RF and CRP were detected using the immunonephelometric method. Values of >8 mg/L for CRP and >30 IU/mL for RF were considered positive. Anti-CCP antibodies were assessed using a commercial ELISA kit (Immunoscan CCPlus, Euro-Diagnostica, Malmo, Sweden) according to the manufacturers' recommendations. The cut-off value for a positive reaction was set at 25 U/mL, as suggested by the manufacturer. ESR was measured by the Westergren method; normal values for men and women were considered to be ≤15 mm/h and ≤20 mm/h, respectively. Complement fractions were detected by nephelometry. DD and FIB were detected by a STAGO STA-R automatic laboratory coagulometer. Normal values were 0–0.5 mg/L for DD and 2.0–4.0 g/L for FIB. The DAS28 was used to assess disease activity. Changes in disease activity were graded according to the following classification criteria: clinical remission (<2.6), low disease activity (≥2.6 to <3.2), moderate disease activity (≥3.2 to <5.2), and high disease activity (>5.2) (21). Only patients with RA with a DAS28 score of ≥3.2 were included in our study; accordingly, we compared patients with high-level disease activity with those with moderate-level activity. The demographic data for the disease groups are described in Table I.

Written informed consent was not obtained because of the nature of the study design, in which serum samples taken after routine tests were utilised. All subjects recruited in this study were informed of the nature of the project, and verbal informed consent was obtained from each patient. This was recorded by the physician who explained the study procedure. The study protocol and verbal consent documents were approved by the Ethics Committee of Xiangya Hospital, Central South University, where the study was performed.

Measurement of histone H4 and APC serum levels

Serum levels of human histone H4 and APC were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (USCN Life Science, Inc., Wuhan, China). Standards and patient samples were run in duplicates according to the manufacturer's instructions.

Table I. Demographic data and disease indicators of disease groups in the study.

	RA	SLE	PM	AS
Subjects	102	21	14	12
Females/males	76/26	20/1	11/3	8/4
Age, years[a]	58.6 ± 11.6	50.2 ± 11.2	52.5 ± 15.2	45.2 ± 7.6
Disease duration, years	8.6 ± 7.5	10.5 ± 6.3	7.9 ± 4.8	4.9 ± 3.2
Recent onset	31.40 %	-	-	-
RF, IU/mL	240.5 (28.9–857.7)	-	-	-
Anti-CCP	223.6 (102.2–356.2)	-	-	-
ANC, $\times 10^9$/L	(5.1 ± 3.0) × 10^9/L	(4.8 ± 2.3) × 10^9/L	(5.4 ± 3.8) × 10^9/L	(4.2 ± 2.7) × 10^9/L
ESR, mm/h	73.0 ± 32.3	25.3(2.1–120.2)	63.5 (11.5–110.0)	42.0 (14.5–96.7)
Elevated	78.40%	52.4%	71.4%	58.30%
CRP, mg/L	20.9 (6.9–50.9)	11.9 (1.0–71.7)	17.9 (2.6–52.8)	24.5 (5.7–40.6)
Elevated	69.60%	47.6%	64.2%	52.8%
C3, mg/L	1000.7 ± 206.1	743.4 ± 234.4	857.2 ± 164.1	-
C4, mg/L	200.8 ± 71.3	165.4 ± 81.5	172.7 ± 34.2	-
DD, mg/L	1.1 (0.5–1.8)	0.8 (0.1–1.9)	1.3 (0.1–2.1)	-
FIB, g/L	4.7 ± 1.6	3.2 ± 1.2	3.9 ± 1.7	-
DAS28	5.3 ± 1.2	n.a.	n.a.	n.a.
Highly active	48.00%	n.a.	n.a.	n.a.

RF: rheumatoid factor; SLE: systemic lupus erythematosus; PM: polymyositis; AS: ankylosing spondylitis; anti-CCP: anti-cyclic citrullinated peptide; ANC: absolute neutrophil count; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; C3: complement fraction 3; C4: complement fraction 4; DD: D-dimer; FIB: fibrinogen; DAS28: disease activity score in 28 swollen and 28 tender joints; n.a., not applicable.[a] Categorical variables are given as the %; normally distributed data are shown as the mean ± standard deviation (SD); other continuous variables are shown as the median (range).

Statistical analysis

Statistical analysis was performed using Prism software v. 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Kruskal-Wallis or Mann-Whitney nonparametric tests were used to perform group comparisons. Correlations were calculated using Spearman's rank correlation. Data are expressed as mean ± standard deviation or median with the interquartile range (IQR), as appropriate. Finally, the ROC curve was used to evaluate the significance of APC levels in the diagnosis of RA. Youden index was calculated as sensitivity + specificity – 1. The best critical point was selected as the largest point of tangency of the Youden index. In all comparisons and correlations, $p<0.05$ was used to indicate a statistically significant result.

Results

Serum levels of H4 and APC

The distribution of histone H4 concentrations in patients with RA, SLE, PM, and AS, and in the healthy controls, is shown in Fig. 1a. The mean H4 concentration was significantly higher in patients with RA (147.70 (69.02–281.58) pg/mL, median (IQR); $p<0.001$), PM (347.10 (251.86–461.23) pg/mL, $p<0.001$), and AS (130.20 (92.55–266.90) pg/mL, $p=0.009$) than in the healthy controls (24.15 (0.00–106.53) pg/mL). However, no significant differences were observed between patients with SLE (63.20 (10.00–227.35) pg/mL, $p=0.114$) and healthy controls. Patients with RA had significantly lower H4 levels compared with those with PM ($p=0.037$). Among the patient groups with diseases other than RA, statistical differences in H4 concentrations were found between patients with SLE and those with PM ($p<0.001$). In addition, subjects with LS RA (117.73 (65.63–210.15) pg/mL) showed lower circulating H4 levels than those in the RO RA group (265.09 (106.62–402.10) pg/mL) ($p=0.033$); however, circulating H4 levels in both groups were higher than in the controls ($p<0.001$) (Fig. 1b).

The distribution of APC concentrations in patients with RA, SLE, PM, and AS and in the healthy controls is shown in Fig. 1c. The mean APC concentration was significantly higher in patients with RA (888.70 (334.90–1729.28) pg/

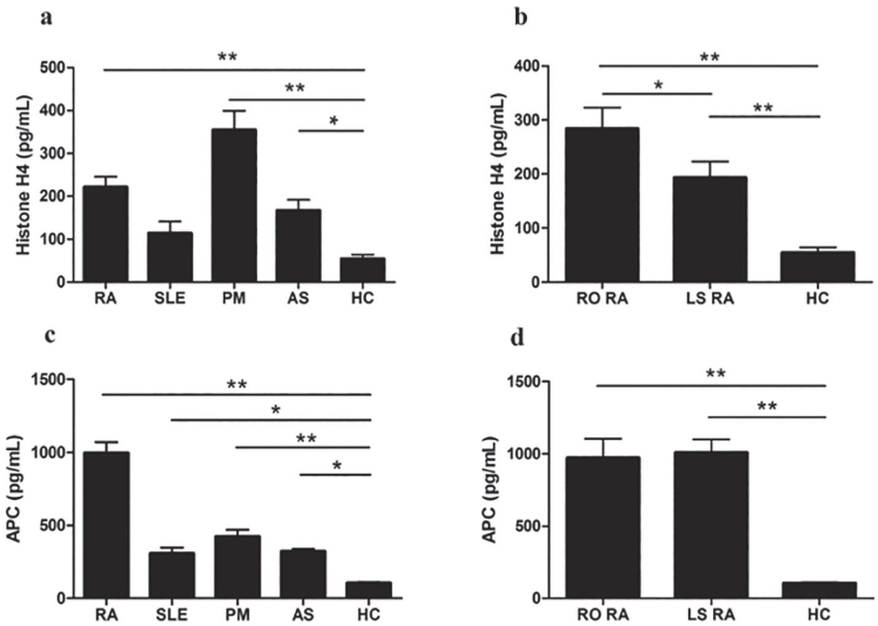

Fig. 1. Histone H4 (H4) and activated protein C (APC) levels are elevated in subjects with rheumatoid arthritis (RA). Levels of H4 and APC in all subjects with RA *versus* patients with other autoimmune diseases (SLE, PM, and AS) and matched healthy controls (HC) (a, c), or in RA subgroups separated by disease duration (b, d), are shown. Subjects were considered to have recent-onset disease (RO) if diagnosed within 3 years or long-standing disease (LS) if diagnosed more than 3 years prior to the study. Statistical significance is indicated by asterisks (*p<0.05; **p<0.001, Kruskal-Wallis test).

Table II. Associations of histone H4 levels with disease indicators in rheumatoid arthritis.

	Histone H4 levels		p-value
Sex	Female: 150.42 (77.40–369.65)	Male: 124.54 (62.23–253.85)	0.207
Disease duration	RO: 265.09 (106.62–402.10) %	LS: 117.73 (65.63–210.15) %	0.004*
Anti-CCP	Positive: 149.97 (79.43–306.57) %	Negative: 65.63(41.65–223.74) %	0.061
RF	Abnormal: 149.96 (69.25–283.30) %	Normal: 97.33 (64.72–265.68) %	0.549
C3	Abnormal: 107.76 (67.89–310.63) %	Normal: 186.34 (101.86–371.30) %	0.129
ESR	Elevated: 153.60 (83.51–299.59) %	Non: 78.30 (51.83–179.46) %	0.024*
CRP	Elevated: 153.60 (89.05–342.64) %	Non: 80.11 (47.52–233.05) %	0.007*
D-D	Abnormal: 186.80 (102.99–376.18) %	Normal: 84.64 (54.77–156.67) %	0.002*
FIB	Abnormal: 166.40 (106.85–369.10) %	Normal: 85.55 (54.77–227.75) %	0.006*
DAS28	High-level: 157.23 (79.66–369.10) %	Moderate-level: 132.71 (54.77–264.50)%	0.178

Anti-CCP: anti-cyclic citrullinated peptide; RF: rheumatoid factor; C3: complement fraction 3; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DD: D-dimer; FIB: fibrinogen; DAS28: disease activity score in 28 swollen and 28 tender joints.
*p<0.05 was considered statistically significant.

mL, p<0.001), SLE (237.80 (213.60–484.85) pg/mL, p=0.011), PM (327.00 (311.63–531.80) pg/mL, p<0.001), and AS (307.00(260.30–372.00) pg/mL, p=0.004) patients than in healthy controls (109.42 (68.59–129.82) pg/mL). Patients with RA had significantly higher APC levels compared with those with SLE (p=0.030). Among the patient groups with diseases other than RA, no statistical differences in APC concentrations were found (p>0.05). Furthermore, the subjects with LS RA (989.47 (349.73–1715.05) pg/mL) and the RO RA group (625.92 (235.02–1806.22) pg/mL) showed higher circulating APC levels than those of the controls (p<0.001). However, no significant differences were observed among the RO, RA, and LS RA groups (Fig. 1d).

Association of H4 markers with other indicators of RA

The serum H4 levels based on various indicators of RA are listed in Table II. H4 levels were higher in patients with RO than in those with LS (p=0.004). Patients with elevated CRP and ESR levels showed higher serum H4 levels than those with normal indices (p=0.007 (CRP), p=0.024 (ESR)). In terms of coagulation function, H4 concentrations were significantly higher in subjects with abnormal levels of DD and FIB (p=0.002 (DD), p=0.006 (FIB)).

Correlation analysis revealed that the serum H4 level was positively associated with ANC (r=0.202, p=0.041), PLT (r=0.345, p<0.001), ESR (r=0.314, p=0.001), CRP (r=0.365, p<0.001), FIB (r=0.295, p=0.004), DD (r=0.274, p=0.007), and C3 (r=0.265, p=0.023) (Fig. 2). No correlations were found between H4 levels and C4, PLG, APTT, TT, RF, and anti-CCP.

Association of APC markers with other indicators of RA

The associations of serum APC levels with various indicators of RA are listed in Table III. APC levels were higher in male patients than in female patients (p=0.022). Furthermore, patients with abnormal RF and anti-CCP levels showed higher serum APC (p<0.001 (RF), p=0.012 (anti-CCP)). In addition, higher APC concentration was observed in patients with RA who had abnormal DD levels (p=0.001).

Correlation analysis revealed that the serum APC level was positively associated with PLT (r=0.205, p=0.045), RF (r=0.617, p<0.001), DD (r=0.468, p<0.001), and DAS28 (r=0.309, p=0.002) (Fig. 3). No correlations were found for APC with ANC, ESR, CRP, C4, C3, Fib, APTT, TT, or anti-CCP.

In addition, the predictive value of APC in RA *versus* non-RA or healthy controls was studied using univariate ROC analysis. The univariate area under the curve (AUC) for APC was 0.82 (95% CI 0.76–0.88) for discriminating between RA and non-RA. The best critical points of sensitivity and specificity of the marker APC were 71.58% and 85.00%, respectively (p<0.05; Fig. 4).

Discussion

We examined the serum histone H4 and APC levels in a broad spectrum of inflammatory autoimmune diseases, namely RA, SLE, PM, and AS; further-

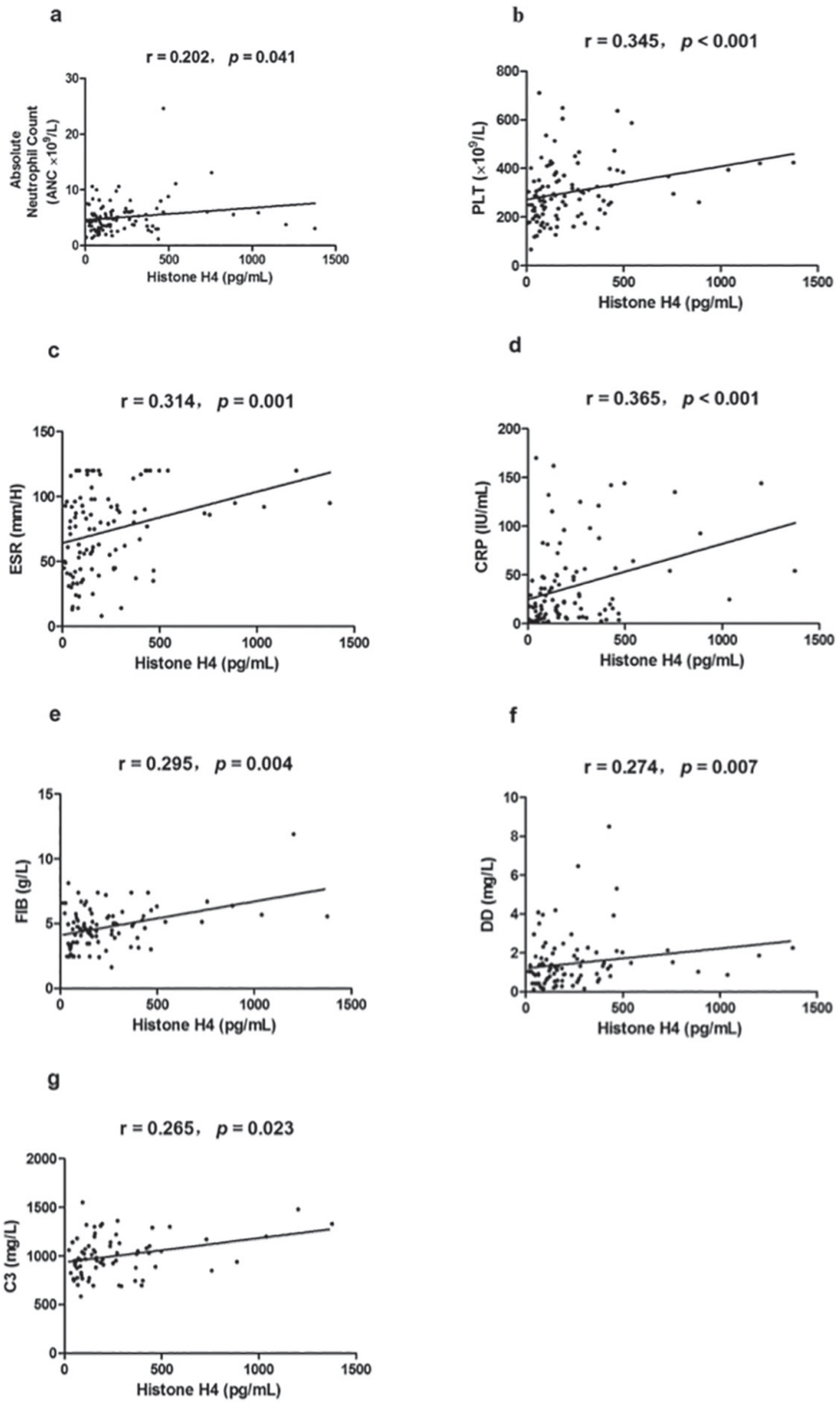

Fig. 2. Correlations of histone H4 levels with absolute neutrophil count (ANC), platelet count (PLT), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen (FIB), D-dimer (DD), and complement fraction 3 (C3) in patients with RA: the correlation between H4 levels and these indices in 102 patients with RA was assessed by Spearman's rank correlation coefficients. Results suggested moderate positive associations between H4 and ANC levels (r=0.202, p=0.041) (a), PLT (r=0.345, p<0.001) (b), ESR (r=0.314, p=0.001) (c), CRP (r=0.365, p<0.001) (d), FIB (r=0.295, p=0.004) (e), DD (r=0.274, p=0.007) (f), and C3 (r=0.265, p=0.023) (g).

more, we investigated the associations of serum H4 or APC levels with clinical indicators of disease activity in patients with RA. We found that the mean H4 concentration was significantly higher in patients with RA, PM, and AS than in the healthy controls. Meanwhile, patients with RA had higher serum levels of APC than patients with SLE, PM, and AS, as well as healthy control individuals. Furthermore, close associations between serum H4 or APC levels and the disease activity and coagulation function variables in the RA cohort were detected. Thus, H4 and APC may participate in inflammatory and coagulation-related processes in RA.

Histones are DNA-chained proteins buried deep within chromatin. These proteins may detach from DNA and be released extracellularly upon cell death, or from activated neutrophils as part of neutrophil extracellular traps (NETs) (22). Extracellular histones are increasingly being recognised as having profound cytotoxic and procoagulant effects (9, 23). Recently, an important role of extracellular histones has been implicated in inflammatory diseases; high serum levels of histones in systemic inflammation and RA (24, 25) are considered to be linked with disease severity. In the present study cohorts, patients with RA, PM, and AS showed higher H4 concentrations than the healthy controls, suggesting that high serum H4 levels might be associated with chronic inflammation. In addition, we observed that serum H4 levels were associated with coagulant indicators (DD and FIB) and elevated levels of inflammatory indicators (CRP and ESR) in patients with RA. Although these clinical observations do not establish cause–effect relationships, they do suggest that high levels of circulating histones play a role in activating or releasing anti-coagulant and fibrinolytic molecules from endothelial cells, as well as in the pathogenetic processes underlying inflammation (7). Notably, we observed a weak positive correlation between H4 levels and PLT. This result is not unexpected, because recent reports have shown that histone H1, H2A, H2B, H3, and H4 promote blood coagulation and thrombin formation mainly by inducing platelet activation and subsequent platelet-dependent thrombin formation (26, 27). Of these, H4 has the strongest impact on platelet activity (14). Recently published studies showed that anti-citrullinated protein/peptide antibodies (ACPA) from patients with RA target citrullinated

Table III. Associations of APC levels with disease indicators in rheumatoid arthritis.

	APC levels		*p*-value
Sex	Female: 557.39 (265.02–1602.61)	Male: 1433.31 (765.62–1992.17)	0.022*
Disease duration	RO: 625.92 (235.02–1806.22) %	LS: 989.47 (349.73–1715.05) %	0.830
Anti-CCP	Positive: 1147.83 (356.22–1776.82) %	Negative: 287.50 (114.71–847.85) %	0.012*
RF	Abnormal: 1235.10 (412.60–1856.04) %	Normal: 342.50 (153.21–599.78) %	<0.001**
C3	Abnormal: 570.46 (214.83–1747.69) %	Normal: 570.39 (282.58–1461.99) %	0.919
ESR	Elevated: 939.66 (349.60–1806.22) %	Non: 636.50 (186.75–1369.89) %	0.217
CRP	Elevated: 1083.77 (345.93–1818.68) %	Non: 625.92 (218.55–1367.47) %	0.214
D-D	Abnormal: 1138.05 (379.13–1772.70) %	Normal: 289.18 (134.80–933.56) %	0.001**
FIB	Abnormal: 708.44 (304.33–1656.01) %	Normal: 782.79 (214.83–1640.28) %	0.891
DAS28	High-level: 1294.61 (294.15–1856.04) %	moderate-level: 590.66 (351.63–1369.89) %	0.077

Anti-CCP: anti-cyclic citrullinated peptide; RF: rheumatoid factor; C3: complement fraction 3; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DD: D-dimer; FIB: fibrinogen; DAS28: disease activity score in 28 swollen and 28 tender joints.
*p<0.05 and **p<0.001 were considered significant and highly significant, respectively.

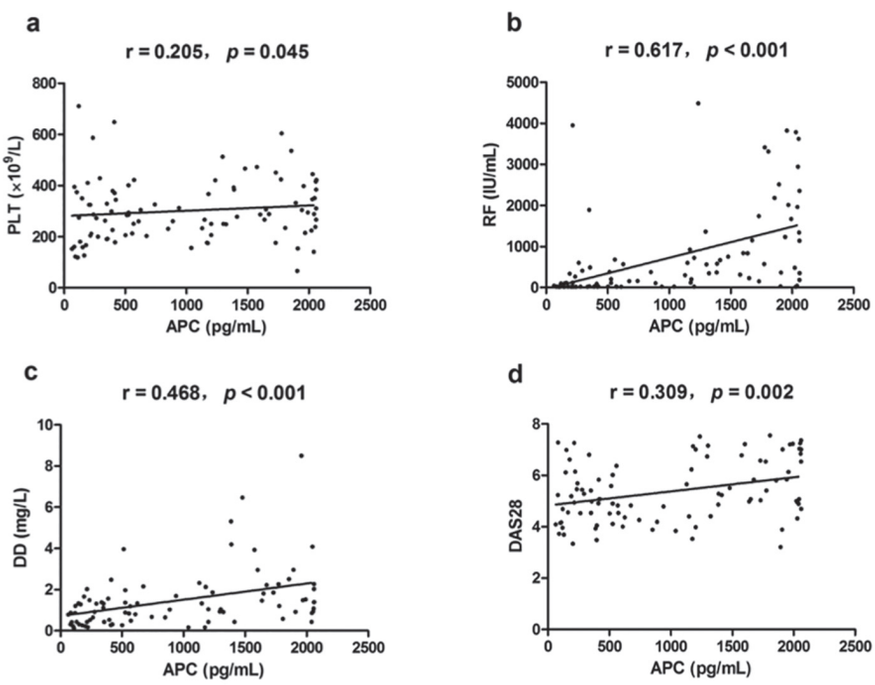

Fig. 3. Correlations of APC levels with platelet count (PLT), rheumatoid factor (RF), D-dimer (DD), and DAS28 in patients with RA: the correlation between APC levels and these indices in the 102 patients with RA was assessed by Spearman's rank correlation coefficients. Results suggested moderate positive associations between APC and PLT levels (r=0.205, p=0.045) (a), RF (r=0.617, p<0.001) (b), DD (r=0.468, p<0.001) (c), and DAS28 (r=0.309, p=0.002) (d).

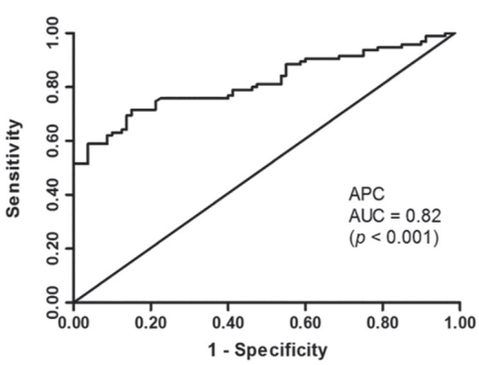

Fig. 4. Area under receiver operating characteristic (ROC) curve (AUC) for APC in predicting rheumatoid arthritis: ROC curve analysis was used to assess the ability of APC to distinguish between RA and other non-RA diseases or healthy controls. The reference curve is also shown; RA: rheumatoid arthritis. APC: activated protein C, AUC: area under the curve.

histone 4 (citH4) contained in NETs, indicating that citH4 might serve as a source of autoantigen to induce ACPA production (28). However, in the present study, we failed to find a significant correlation between serum levels of histone H4 and anti-CCP. Histone citrullination is believed to be initiated in the rheumatoid synovium, contributing to the perpetuation of chronic inflammation; thus, synovial fluid might be the representative sample for exploring the associations between histone H4 and anti-CCP (29). Nevertheless, we found a significant p-value (p=0.061) when comparing serum levels of histone H4 between anti-CCP-positive and anti-CCP-negative RA patients. A larger sample size is required to estimate the correlation, and cytological and molecular studies might help reveal the relationships among ACPA, histone H4, and NETs.

We found that APC levels in patients with RA, SLE, PM, and AS were significantly higher than in the healthy controls, suggesting that during systemic inflammatory states or in autoimmune diseases, serum APC levels could rise, corresponding to its anti-inflammatory character, as reported by Sarangi *et al*. (30). Interestingly, APC levels in patients with RA were higher than in patients with other autoimmune diseases in our study cohort. While the reasons for this discrepancy are not clear, it might be related to the differences in the pathogenetic mechanisms involved in the different diseases. In addition, there might be some undefined relationship between APC concentration and the disease process of RA. We also detected a statistically significant positive correlation between RF and APC levels (r=0.617, p<0.001) and a weak positive correlation between DAS28 scores and APC levels (r=0.309, p=0.002) in the patient group. DAS28 (17) was used to assess disease activity. Patients with RA have a relatively long disease history and suffer from inflammation for the longest period among the other disease groups. Accordingly, we expected that increased levels of APC in patients with RA are related to high disease activity and disease severity. However, there were not enough data to support

this expectation. The ability of APC to discriminate between RA and non-RA was calculated by ROC analysis (Fig. 4), and APC levels represented a good basis for distinguishing between RA and non-RA, with an AUC of 0.82. Thus, APC represents a potentially useful serological marker for the prediction of RA.

Unexpectedly, we found no significant correlation between serum histone H4 and APC levels in patients with RA ($p>0.05$). These results are in contrast with those of Xu (9) and Ekaney (15). These conflicting results might be attributed to the heterogeneity between the study cohorts, in terms of disease variety, proportion of patients with highly active disease, pattern of therapy, genetic background, and/or disease process. It is noteworthy that subjects with LS RA showed lower circulating H4 levels than the RO RA group, while the LS and RO RA groups had higher APC levels, but this was not statistically significant. The discrepancy between H4 and APC levels and disease duration suggests that APC blocks histones by cleaving histone proteins at later stages of the disease, as shown by Xu *et al*. (9). Our data indicate that serum histone H4 might be associated with inflammatory processes in RA, while serum APC was associated with some disease predictors of RA, such as anti-CCP ($p=0.012$) and RF ($r=0.617$, $p<0.001$). Similarly, both H4 and APC levels were significantly correlated with the coagulant indicator DD, suggesting that H4 and APC are involved in coagulation and anti-coagulation. On the basis of the findings of previous studies, we propose that H4 and APC have a complex relationship in the context of inflammation and coagulation during disease progression in RA; further studies are warranted to precisely elucidate these relationships.

In conclusion, the findings of our study demonstrate that serum concentrations of histone H4 and APC are elevated in chronic inflammatory autoimmune diseases, particularly in patients with active RA. Moreover, the associations between serum H4 or APC levels and various disease factors in patients with RA indicate that H4 is involved in the process of thrombus formation and inflammation, while APC is related to anti-coagulation and anti-inflammatory processes in this disease. However, whether serum H4 can serve as a supplementary indicator for disease activity, or how H4 and APC interact each other in RA and other chronic inflammatory diseases, must be validated in a larger study population via further experimental studies.

References

1. FIRESTEIN GS: Evolving concepts of rheumatoid arthritis. *Nature* 2003; 423: 356-61.
2. TRAYLOR M, CURTIS C, PATEL H *et al*.: Genetic and environmental risk factors for rheumatoid arthritis in a UK African ancestry population: the GENRA case-control study. *Rheumatology* (Oxford) 2017.
3. GLANT TT, MIKECZ K, RAUCH TA: Epigenetics in the pathogenesis of rheumatoid arthritis. *BMC Medicine* 2014; 12: 35-9.
4. ANGELOTTI F, PARMA A, CAFARO G, CAPECCHI R, ALUNNO A, PUXEDDU I: One year in review 2017: pathogenesis of rheumatoid arthritis. *Clin Exp Rheumatol* 2017; 35: 368-78.
5. ESSOUMA M, NOUBIAP JN: Is air pollution a risk factor for rheumatoid arthritis? *Journal of Inflammation* 2015; 12: 48-54.
6. KAMOSHIDA G, KIKUCHI-UEDA T, TANSHO-NAGAKAWA S *et al*.: Acinetobacter baumannii escape from neutrophil extracellular traps (NETs). *J Infect Chemother* 2015; 21: 43-9.
7. ALHAMDI Y, TOH CH: The role of extracellular histones in haematological disorders. *Br J Haematol* 2016; 173: 805-11.
8. HAKKIM A, FURNROHR BG, AMANN K *et al*.: Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA* 2010; 107: 9813-8.
9. XU J, ZHANG X, PELAYO R *et al*.: Extracellular histones are major mediators of death in sepsis. *Nat Med* 2009; 15: 1318-21.
10. ALHAMDI Y, ABRAMS ST, CHENG Z *et al*.: Circulating histones are major mediators of cardiac injury in patients with sepsis. *Critical Care Medicine* 2015; 43: 2094-103.
11. ABRAMS ST, ZHANG N, MANSON J *et al*.: Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med* 2013; 187: 160-9.
12. YUDA H, ADACHI Y, TAGUCHI O *et al*.: Activated protein C inhibits bronchial hyperresponsiveness and Th2 cytokine expression in mice. *Blood* 2004; 103: 2196-204.
13. TOLTL LJ, BEAUDIN S, LIAW PC *et al*.: Activated protein C up-regulates IL-10 and inhibits tissue factor in blood monocytes. *J Immunol* 2008; 181: 2165-73.
14. CARESTIA A, RIVADENEYRA L, ROMANIUK MA, FONDEVILA C, NEGROTTO S, SCHATTNER M: Functional responses and molecular mechanisms involved in histone-mediated platelet activation. *Thromb Haemost* 2013; 110:1035-45.
15. EKANEY ML, OTTO GP, SOSSDORF M *et al*.: Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. *Crit Care* 2014; 18: 543-52.
16. BUISSON-LEGENDRE N, SMITH S, MARCH L, JACKSON C: Elevation of activated protein C in synovial joints in rheumatoid arthritis and its correlation with matrix metalloproteinase 2. *Arthritis Rheum* 2004; 50: 2151-6.
17. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010; 62: 2569-81.
18. TAN EM, COHEN AS, FRIES JP *et al*.: The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-7.
19. BOHAN A, PETER JB: Polymyositis and dermatomyositis. *N Engl J Med* 1975; 292: 344-7.
20. GOIE THE HS, STEVEN MM, LINDEN SM, CATS A: Evaluation of diagnostic criteria for ankylosing spondylitis: a comparison of the Rome, New York and modified New York criteria in patients with a positive clinical history screening test for ankylosing spondylitis. *Br J Rheumatol* 1985; 24: 242-9.
21. VAN GESTEL AM, HAAGSMA CJ, RIEL PL: Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. *Arthritis Rheum* 1998; 41: 1845-50.
22. SAFFARZADEH M, JUENEMANN C, QUEISSER MA *et al*.: Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One* 2012; 7: e32366.
23. ALLAM R, KUMAR SV, DARISIPUDI MN, ANDERS HJ: Extracellular histones in tissue injury and inflammation. *J Mol Med* (Berl) 2014; 92: 465-72.
24. ZHANG Y, WEN Z, GUAN L *et al*.: Extracellular histones play an inflammatory role in acid aspiration-induced acute respiratory distress syndrome. *Anesthesiology* 2015; 122: 127-39.
25. MONACH PA, HUEBER W, KESSLER B *et al*.: A broad screen for targets of immune complexes decorating arthritic joints highlights deposition of nucleosomes in rheumatoid arthritis. *Proc Natl Acad Sci USA* 2009; 106: 15867-72.
26. SEMERARO F, AMMOLLO CT, MORRISSEY JH *et al*.: Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* 2011; 118: 1952-61.
27. LAM FW, CRUZ MA, LEUNG HC, PARIKH KS, SMITH CW, RUMBAUT RE: Histone induced platelet aggregation is inhibited by normal albumin. *Thromb Res* 2013; 132: 69-76.
28. PRATESI F, DIONI I, TOMMASI C *et al*.: Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis* 2014; 73: 1414-22.
29. KHANDPUR R, CARMONA-RIVERA C, VIVEKANANDAN-GIRI A *et al*.: NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013; 5: 178ra140.
30. SARANGI PP, LEE H, KIM M: Activated protein C action in inflammation. *Br J Haematol* 2009; 148: 817-33.