


# Secretory antibodies to citrullinated peptides in plasma and saliva from rheumatoid arthritis patients and their unaffected first-degree relatives

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## Summary

The aim of this study was to evaluate secretory antibodies to citrullinated proteins (ACPA) in plasma and immunoglobulin (Ig)A ACPA in saliva from patients with rheumatoid arthritis (RA) and their unaffected first-degree relatives (FDRs). Patients with RA ( $n = 194$ ) and first-degree relatives unaffected by RA ( $n = 191$ ) were recruited for analysis of secretory antibodies to second-generation cyclic citrullinated peptides (anti-CCP) in plasma. From a subpopulation (25 RA patients, 21 first-degree relatives and 11 controls), saliva samples were obtained for IgA anti-CCP analysis. The presence of secretory ACPA was compared between subject categories, and related to genetic and environmental risk factors. Secretory ACPA occurred in 37 (19%) plasma samples from patients with RA, but only in two (1%) of FDRs. IgA ACPA in saliva was found in three of 25 (12%) patients with RA, but not in any of the 21 FDRs ( $< 5\%$ ). No significant associations were seen between the presence of secretory ACPA and SE or smoking, either among RA patients or among FDRs. Despite occurring in 19% of RA plasma, secretory ACPA was rare in both saliva and plasma among FDRs, even among those positive for conventional ACPA of non-mucosal origin. Longitudinal studies are warranted to determine whether circulating secretory ACPA occurs before or in parallel with the development of clinical arthritis.

**Keywords:** ACPA, mucosa, relatives, rheumatoid arthritis, secretory immunoglobulin

## Introduction

Mucosal involvement in the pathogenesis of RA has emerged as a highly attractive hypothesis in recent years [1]. Mucosal surfaces comprise interfaces between the environment and the host, and play a crucial role in digestion and uptake of nutrients as well as maintaining immune homeostasis [2]. According to the mucosal hypothesis, a diversity of triggering factors encountered at mucosal surfaces may lead to activation of the local, mucosal immune system with formation of antibodies to citrullinated proteins (ACPA). This is followed by a systemic immune response with circulating ACPA, which subsequently trigger joint symptoms and/or manifest arthritis [1].

Elevated circulating ACPA [3], as well as cytokine levels suggestive of immunopathology, can be found during a long presymptomatic period, which precedes manifest

arthritis and a clinical diagnosis of RA, indicating that other locations than joints are involved in the early phase of RA pathogenesis.

Several mucosal compartments have been implicated in the development of RA and data regarding the role of oral [4], lung [5] and gut inflammation indicate a mucosal site of origin of the disease. There is evidence especially for airway mucosal involvement, as smoking is associated with increased amounts of citrullinated proteins in bronchoalveolar lavage (BAL) fluid [6], and citrullination of proteins in BAL fluid is associated with lung abnormalities on high-resolution computed tomography (HRCT) [5]. ACPA has been found in sputum of both RA patients and healthy relatives, positive and negative for ACPA in serum [7], indicating that mucosal antibodies may precede circulating antibodies.

Secretory antibodies are produced at mucosal surfaces and transported through epithelial cells to the lumen. During this process, a protein chain called secretory component (SC) becomes attached to the antibody, making it more resistant to the enzymes present in secretions. Secretory antibodies can, however, also be detected in the circulation, and it has been demonstrated that these antibodies are of both immunoglobulin (Ig)A and IgM isotype [8]. The precise mechanism through which they reach the circulation is unknown. Recently we were able to demonstrate the occurrence of a secretory form of ACPA in saliva [9] as well as in the circulation [10] from patients with RA. To our knowledge, secretory ACPA has not been evaluated in serum or saliva of FDRs.

Previous studies have found a higher prevalence of conventional ACPA among FDRs than among healthy controls [11]. Interestingly, IgA ACPA has been found more commonly than IgG ACPA in FDRs compared to patients with RA, where IgG ACPA is far more common than IgA ACPA [12]. As IgA is strongly associated with mucosal surfaces, these findings support the view that mucosal immune reactions is part of the early phase of RA pathophysiology.

We hypothesize that mucosal immunization and formation of secretory ACPA is an early step in RA development, preceding the occurrence of conventional, non-secretory ACPA and that secretory ACPA would thus be prevalent in a large proportion of pre-RA individuals and therefore likely to be detected among FDRs. In this study we determined the presence of secretory ACPA in plasma and in saliva of both patients with RA and their unaffected FDRs.

## Patients and methods

### Study subjects

A total of 194 patients with a confirmed diagnosis of RA according to the 1987 ARA classification criteria and 191 unaffected FDRs were recruited from northern Sweden. All FDRs completed a questionnaire and those reporting symptoms or signs of joint disease were clinically assessed by a rheumatologist, as previously described [13]. Saliva samples were obtained by passive drooling from a subpopulation of 25 of the RA patients and 21 of the FDRs, as previously reported [9] for IgA anti-CCP analysis. Saliva samples were also obtained from 11 healthy controls. As disease controls, we recruited 70 cases of systemic lupus erythematosus (SLE), classified according to the 1982 American College of Rheumatology (ACR) and/or 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria, from the rheumatology unit in Linköping [14]. Demographic and laboratory characteristics for patients, FDRs and controls are presented in Table 1.

The regional ethics committee at the University Hospital, Umeå, Sweden approved the study and all participants gave their written informed consent.

### Antibody analyses

Samples from FDRs and the RA patients were collected at the same time and stored for the same length of time before analysis. Plasma samples were analysed for secretory antibodies to citrullinated peptides by using a modified second-generation cyclic citrullinated peptide (anti-CCP) immunoassay (EuroDiagnostica, Malmö, Sweden). We changed the secondary antibody to detect secretory component, as previously described [10]. This analysis does not differentiate between secretory IgA ACPA and secretory IgM ACPA. The citrulline-dependent specificity of plasma secretory anti-CCP antibodies was confirmed by testing reactivity to the corresponding, non-citrullinated peptide (cyclic arginine peptide, CAP). All samples were analysed in duplicate, and were re-analysed if the coefficient of variation was above 20%. The cut-off for a positive sample was set at 153 arbitrary units (AU)/ml, corresponding to the 99th percentile of 101 healthy blood donors [10].

Saliva samples were analysed for IgA antibodies using the same immunoassay as for plasma anti-CCP, but with a secondary antibody detecting human IgA (Dako-Cytomation, Glostrup, Denmark), as previously described [9]. To assess possible leakage of ACPA from the circulation to saliva, we have previously analysed saliva samples from patients positive for IgG ACPA in serum, with only one of 26 testing positive with a borderline value [9]. We have previously shown that saliva from RA patients contain both antibodies reacting with cyclic arginine peptides (CAP) and antibodies specific for CCP [9]. Further, we demonstrated that pre-incubation with soluble CCP attenuated CCP reactivity in patients with high CCP/CAP ratios, but not in patients with low CCP/CAP ratios, which clearly indicates specific reactivity to CCP. In order to report only citrulline-specific reactivity, the optical density (OD) value for IgA anti-CAP was measured and subtracted from the OD value for IgA anti-CCP. The difference between OD values for IgA anti-CCP and IgA anti-CAP ( $\Delta$  OD value) was calculated for RA patients, FDRs and healthy controls. IgA anti-CCP positivity in saliva was defined as  $> 2$  standard deviations (s.d.) above the mean  $\Delta$  OD value of the controls.

### Genetic analyses

Human leucocyte antigen (HLA)-DRB1 was genotyped as previously described [13]. Shared epitope (SE) was defined as HLA-DRB1\*01, \*0401, \*0404, \*0405 or \*0408.

### Statistical methods

For continuous variables, we used the Mann-Whitney U-test for two groups. Categorical variables were tested with Pearson's  $\chi^2$  test and Fisher's exact test as

**Table 1.** Demographic and laboratory characteristics of the study participants

	FDR secretary ACPA: <i>n</i> = 191		RA patients secretary ACPA: <i>n</i> = 194		<i>P</i> -value*	Healthy controls		SLE disease controls	
	Conventional ACPA and RF: <i>n</i> = 157	Conventional ACPA and RF: <i>n</i> = 163	Conventional ACPA and RF: <i>n</i> = 163	Conventional ACPA and RF: <i>n</i> = 163		<i>n</i> = 101	<i>n</i> = 70		
Age, median (IQR)	60 (26-0)	66 (18-3)	66 (18-3)	66 (18-3)	< 0.001	43.5 (12-5)	58 (15-0)	43.5 (12-5)	58 (15-0)
Female <i>n</i> (%)	111 (58.1)	136 (70.1)	136 (70.1)	136 (70.1)	0.014	51 (50.5)	61 (87.0)	51 (50.5)	61 (87.0)
Shared epitope, <i>n</i> (%)	83 (53.9)	116 (71.2)	116 (71.2)	116 (71.2)	0.001	n.a.	n.a.	n.a.	n.a.
Smoker ever, <i>n</i> (%)	81 (47.9)	109 (58.0)	109 (58.0)	109 (58.0)	0.057	n.a.	n.a.	n.a.	n.a.
Secretary ACPA AU/ml (s.d.)	7.9 (2.1)	45.7 (116.3)	45.7 (116.3)	45.7 (116.3)	< 0.001	24.5 (35.0)	26.3 (41.6)	24.5 (35.0)	26.3 (41.6)
Positive <i>n</i> (%)	2 (1.0)	37 (19.1)	37 (19.1)	37 (19.1)	< 0.001	1 (1.0)	2 (3.0)	1 (1.0)	2 (3.0)
IgG ACPA AU/ml (s.d.)	2.2 (1.7)	235 (542.0)	235 (542.0)	235 (542.0)	< 0.001	n.a.	68.1 (302.8)	n.a.	68.1 (302.8)
Positive <i>n</i> (%)	34 (21.7)	140 (85.9)	140 (85.9)	140 (85.9)	< 0.001	n.a.	7 (10.0)	n.a.	7 (10.0)
IgA ACPA AU/ml (s.d.)	1 (0.8)	2.8 (11.7)	2.8 (11.7)	2.8 (11.7)	< 0.001	0.67 (0.19)	n.a.	0.67 (0.19)	n.a.
Positive <i>n</i> (%)	42 (26.8)	118 (72.4)	118 (72.4)	118 (72.4)	< 0.001	1 (1.0)	n.a.	1 (1.0)	n.a.
IgM ACPA AU/ml (s.d.)	27.9 (39.6)	51.7 (117.5)	51.7 (117.5)	51.7 (117.5)	< 0.001	n.a.	n.a.	n.a.	n.a.
Positive <i>n</i> (%)	35 (22.3)	74 (45.4)	74 (45.4)	74 (45.4)	< 0.001	n.a.	n.a.	n.a.	n.a.
IgM RF AU/ml (s.d.)	5.2 (6.3)	114 (543)	114 (543)	114 (543)	< 0.001	n.a.	n.a.	n.a.	n.a.
Positive <i>n</i> (%)	22 (14.0)	121 (74.2)	121 (74.2)	121 (74.2)	< 0.001	n.a.	n.a.	n.a.	n.a.
IgA RF AU/ml (s.d.)	1.4 (1.4)	7.4 (15.3)	7.4 (15.3)	7.4 (15.3)	< 0.001	n.a.	n.a.	n.a.	n.a.
Positive <i>n</i> (%)	35 (22.3)	123 (75.5)	123 (75.5)	123 (75.5)	< 0.001	n.a.	n.a.	n.a.	n.a.

FDR = first-degree relatives of RA patients; RA = rheumatoid arthritis; IQR = interquartile range; ACPA = antibodies to cyclic citrullinated peptides and RF rheumatoid factor; s.d. = standard deviation; n.a. = not applicable.

\*Statistical difference between FDR and RA patients, as calculated with the Mann-Whitney *U*-test.

appropriate. Two-sided  $P$ -values  $< 0.05$  were considered statistically significant. Logistic regression analyses were performed to identify associations between antibodies and subject categories and are presented as odds ratios (ORs) and 95% confidence intervals (95% CIs). Statistical calculations were performed using SPSS for Windows, version 24. Sensitivity, specificity, positive and negative predictive values were calculated using the package EpiR version 0.9.99 in the R software version 3.5.2.

## Results

### Secretory ACPA in plasma

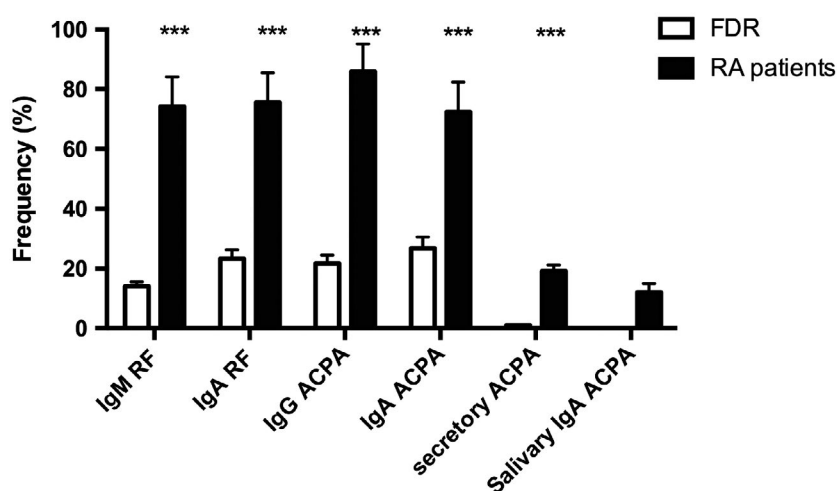
Demographic and laboratory characteristics of the study participants are shown in Table 1. Among the 194 RA patients, 37 (19%) were positive for secretory ACPA in plasma, whereas among the 191 FDRs, two (1%) tested positive (Fig. 1). Positive secretory ACPA test were also found in two of 70 SLE cases (3%) and one blood donor (1%). Levels of secretory ACPA and other RA-related autoantibodies are graphically shown in Supporting information, Fig. S1. In RA patients, occurrence of circulating secretory ACPA was clearly associated with higher levels (Supporting information, Fig. S2) and more isotypes of conventional ACPA and RF (Supporting information, Table S1). Among RA patients positive for conventional ACPA of one or more isotypes, 21% were also positive for secretory ACPA, while RA patients positive for secretory ACPA were all positive for conventional ACPA (Supporting information, Table S1).

This FDR population has been characterized previously [13] concerning RF and conventional ACPA, demonstrating that 34 (22%) were positive for IgG ACPA and 42 (27%) for IgA ACPA. Among RA patients, 140 (86%) were

positive for IgG ACPA and 118 (72%) for IgA ACPA (Table 1). When comparing the presence of conventional, non-secretory, ACPA (i.e. IgA, IgM and IgG) to the presence of secretory ACPA, it can be seen that among FDRs negative for conventional ACPA, none was positive for secretory ACPA. Among the 77 FDRs who tested positive for conventional ACPA of any isotype, only two individuals (3%) were positive for secretory ACPA (Supporting information, Table S1). One was a 33-year-old male, non-smoker, IgM RF-positive and IgM ACPA-positive, but negative for IgG and IgA ACPA and IgA RF. The other was a 69-year-old woman, smoker, positive for IgA, IgG and IgM ACPA as well as for IgA and IgM RF. After a 10-year follow-up, five FDRs had developed RA, although none of the two who tested positive for secretory ACPA.

When comparing different circulating ACPAs and RFs in logistic regression analyses with participant status as dependent variable (RA patient *versus* FDR), secretory ACPA in plasma showed the numerically strongest association with being an RA patient [unadjusted odds ratios (ORs): secretory ACPA = 22.2 (95% CI = 5.3–93.9), IgG ACPA = 22.0 (12.3–39.4), IgA ACPA = 7.2 (4.4–11.8), IgM ACPA = 2.9 (1.8–4.7), IgM RF = 17.7 (10.0–31.3) and IgA RF = 10.7 (6.4–18.0)]. Adjusted ORs are presented in Fig. 2. As shown in Table 2, the highest positive predictive value to identify patient status was seen for plasma secretory ACPA (95%), while the highest negative predictive value was seen for conventional IgG ACPA in plasma (84%).

Among patients with RA, no significant difference in disease duration could be seen between patients positive for secretory ACPA in plasma (21.5 years) and patients negative for secretory ACPA in plasma (14 years),  $P = 0.057$ . Patients positive for secretory ACPA in plasma were aged median 69 years and patients negative for



**Fig. 1.** Frequencies of rheumatoid factors [immunoglobulin (Ig)M and IgA] and anti-citrullinated protein antibodies (IgG and IgA) in first-degree relatives (FDR;  $n = 157$ ) and patients with rheumatoid arthritis (RA;  $n = 163$ ), secretory ACPA in plasma ( $n = 191$  and  $n = 194$ , respectively) and IgA-ACPA in salivary samples ( $n = 21$  and  $n = 25$ , respectively). Error bars represent 95% confidence intervals.

secretory ACPA were aged 65 years,  $P = 0.068$  (Supporting information, Table S2).

### Associations between secretory ACPA in plasma and smoking or HLA-SE

Among RA patients positive for secretory ACPA, 67.6% were ever smokers compared to 55.6% among RA patients negative for secretory ACPA. The difference was not significant ( $P = 0.187$ ). Regarding HLA-SE, 76.7% of RA patients positive for secretory ACPA were SE-positive, compared to 69.9% among RA patients negative for secretory ACPA, non-significant ( $P = 0.462$ ) (Supporting information, Table S2). As only two FDRs were positive for secretory ACPA, no calculations regarding association to smoking or SE were performed.

### Secretary ACPA in saliva

Salivary IgA ACPA was detected in three of 25 (12%) RA patients, but in none of the 21 FDRs ( $P = 0.239$ ) (Fig. 1). Among healthy controls, salivary IgA ACPA was detected in one of 11 individuals (9%). FDRs with saliva samples were younger than those without (53 versus 61 years,  $P = 0.031$ ). RA patients with saliva samples were less often positive for IgA RF than those without

(56 versus 79%,  $P = 0.014$ ). There were no further significant differences regarding age, gender, smoking, shared epitope, conventional ACPA, RF or secretory ACPA between individuals with and without saliva samples.

### Discussion

Secretary ACPA could be found in plasma from a substantial proportion (19%) of patients with RA, which confirms previous findings in two separate RA cohorts, where 15 and 19%, respectively, tested positive for circulating secretory ACPA [10].

This is the first study, to our knowledge, investigating secretory ACPA in FDRs of RA patients, and the main finding is that secretory ACPA could be detected in only 1% of the FDRs. Furthermore, even in the subgroup of FDRs positive for conventional IgA ACPAs in plasma, circulating secretory ACPA only occurred in 3%.

This raises interesting questions regarding the role of circulating, secretory ACPA with regard to RA pathophysiology. According to the mucosal hypothesis, the formation of secretory ACPA at mucosal sites would precede formation of conventional circulating ACPA. Our findings do not support this, as secretory ACPA was rare among the FDRs, and in no instance occurred in the absence of conventional ACPA. This study confirms that mucosal immune responses against citrullinated proteins occur in a proportion of patients with RA, but only in those where a systemic ACPA production is already established. In contrast to our hypothesis, the results do not support the idea of secretory ACPA to be common in a cohort of individuals with increased risk of developing RA.

However, alternative explanations to these findings are possible, e.g. that the presence of secretory ACPA in plasma does not fully reflect mucosal ACPA production. Secretary ACPA may be present at mucosal sites in these individuals, although failing to reach the circulation. However, as none of the FDRs tested positive for ACPA in saliva, the oral cavity is probably not such a compartment, with early

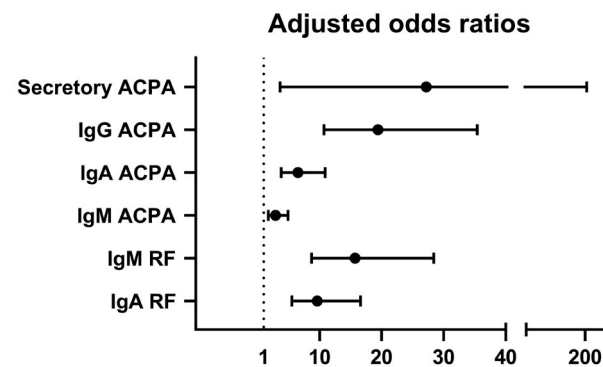


Fig. 2. Adjusted odds ratios for having rheumatoid arthritis, based on antibody positivity, with first-degree relatives as reference. Results from regression analyses, one separate analysis for each antibody.

Table 2. Sensitivity, specificity, positive predictive value and negative predictive value of the different ACPAs and RFs tested

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Secretary ACPA in plasma, positive $n$ (%)	19	99	95	55
IgG ACPA, positive $n$ (%)	86	78	80	84
IgA ACPA, positive $n$ (%)	72	73	74	72
IgM ACPA, positive $n$ (%)	45	78	68	58
IgM RF, positive $n$ (%)	74	86	85	76
IgA RF, positive $n$ (%)	75	78	78	75

PPV = positive predictive value; NPV = negative predictive value; ACPA = antibodies to cyclic citrullinated peptides and rheumatoid factor (RF). Data for secretory ACPA is based on all 194 rheumatoid arthritis (RA) patients and 191 first degree relatives of RA patients (FDRs), and data for conventional ACPA and RF on 163 RA patients and 157 FDRs.

secretory ACPA formation undetectable in the circulation. A possible protective role of (mucosal) IgA antibodies has also been hypothesized, as recently presented by Holers *et al.* [15]. One argument is that mucosal ACPA was found in a considerable number of individuals at risk, exceeding the number of individuals statistically likely to develop RA. The results of the current study do not support this hypothesis.

FDRs of RA patients are at increased risk of developing RA, and the risk has been estimated to be three- to nine-fold [16]. They are usually regarded as a population of potential pre-RA patients or at-risk individuals. However, unless they are followed until they develop RA, they might as well constitute a population of (genetically predisposed) individuals where protective factors against RA actually are enriched. Thus, one drawback with the current study is the cross-sectional design, which prevents us from separating FDRs that will develop RA from those that will not. Also, the limited number of participants with salivary samples available, and the low prevalence of IgA ACPA therein, hampered further analyses due to low statistical power. It was recently shown that secretory ACPA predominantly occurs as IgM, and to a lesser extent IgA [8]. Given the low proportion of samples positive for secretory ACPA, it appears unlikely that isotype-specific secretory ACPA analysis would yield further information. In further studies, analyses of antibodies to other post-translationally modified antigens as well as antibodies against *Porphyromonas gingivalis* or Leukotoxin A from *Aggregatibacter actinomycetemcomitans* would be interesting to explore among FDRs.

In conclusion, as secretory ACPA among FDRs was rare (in plasma) or absent (in saliva), we reject our hypothesis stating that secretory ACPA would be prevalent in a large proportion of FDRs. Instead, secretory ACPA in plasma was almost exclusively found among RA patients, and showed the highest OR and PPV for identifying RA patients *versus* relatives. Longitudinal studies are warranted to determine whether circulating secretory ACPA occurs before or in parallel with the development of clinical arthritis.

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## Disclosures

There are no conflicts of interest to declare.

## Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design: A. S., A. K. and S. R. D.; acquisition of data: S. R. D., M. B., C. S., K. M. K. M. and K. R. L.; analysis and interpretation of data: A. S., M. B., C. S., K. M. K. M. and A. K. A. S. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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## Supporting Information

Additional supporting information may be found in the on-line version of this article at the publisher's web site:

**Fig. S1.** Levels of different rheumatoid arthritis (RA)-related antibodies in first degree relatives (FDR) and RA patients. Panel A shows levels of IgG-ACPA and IgM-RF in 157 FDR and 163 RA patients. Panel B shows IgM-ACPA and IgA-ACPA and IgA-RF in 157 FDR and 163 RA patients, and circulating secretory ACPA in 191 FDR and 194 RA patients.

**Fig. S2.** Levels of conventional ACPAs and RF versus status of circulating secretory ACPA in RA patients. Panel (a) shows IgG ACPA, (b) IgA ACPA, (c) IgM ACPA, (d) IgM RF and (e) IgA RF.

**Table S1.** Secretory ACPA status versus number of isotypes of conventional ACPAs and RFs in patients with rheumatoid arthritis (RA) and their first degree relatives (FDR).

**Table S2.** Characteristics of RA patients positive and negative for secretory ACPA