Immunofluorometric Point-of-Care Assays for the Detection of Acute Coronary Syndrome-Related Noncomplexed Pregnancy-Associated Plasma Protein A

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Background: We recently reported that the pregnancy-associated plasma protein A (PAPP-A) form specifically related to acute coronary syndromes (ACS) is not complexed with the proform of eosinophil major basic protein (proMBP). The aim of this study was to develop rapid point-of-care immunoassays for the measurement of the noncomplexed PAPP-A.

Methods: We developed immunofluorometric noncompetitive dry-reagent assays for total PAPP-A with 2 PAPP-A subunit-specific monoclonal antibodies and for PAPP-A/proMBP complex with 1 PAPP-A subunit-specific antibody and 1 proMBP subunit-specific antibody. The concentration of noncomplexed PAPP-A was determined as the difference of the results obtained with the 2 assays.

Results: The assays were linear from 0.5 to 300 mIU/L. The analytical detection limit and functional detection limit (CV <20%) were 0.18 mIU/L and 0.27 mIU/L for total PAPP-A assay and 0.23 mIU/L and 0.70 mIU/L for PAPP-A/proMBP assay, respectively. The total assay imprecisions were <10%, and recoveries were 88%–107% for both assays. The mean difference (95% limits of agreement) between the new total PAPP-A assay and a previously reported total PAPP-A assay was −3.2% (−45.7% to 39.3%; n = 546; P = 0.0019). In serum samples from 159 non-ACS individuals, median concentrations (interquartile range) were 2.42 (1.14) mIU/L for total PAPP-A, 2.20 (1.18) mIU/L for PAPP-A/proMBP, and 0.18 (0.63) mIU/L for noncomplexed PAPP-A. Total PAPP-A and PAPP-A/proMBP, but not noncomplexed PAPP-A, correlated with age (r = 0.290, P = 0.0002; r = 0.230, P = 0.0035; r = 0.075, P = 0.3483, respectively).

Conclusions: The new assays described revealed that noncomplexed PAPP-A is found only in negligible amounts in non-ACS samples.

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Pregnancy-associated plasma protein A (PAPP-A), a zinc-binding metzincin metalloproteinase, has recently been associated with instability of atherosclerotic plaques (1, 2). Initially, PAPP-A was identified as a high-molecular-weight protein found in the circulation during pregnancy (3). However, increased concentrations of circulating PAPP-A were also detected in patients presenting with acute coronary syndromes (ACS) (2). Other studies confirmed the PAPP-A increase in ACS (4). Later, PAPP-A was found to be a powerful predictor of adverse cardiac events, especially in troponin-negative ACS patients with no biochemical evidence of myocardial necrosis (5), and also in troponin-positive patients (6). In patients with stable angina, PAPP-A predicts the extent of the coronary artery disease and plaque complexity (7, 8).

During pregnancy, PAPP-A is found in circulation as a 2:2 heterotetramer covalent complex of PAPP-A and the proform of the eosinophil major basic protein (proMBP) (9). We recently demonstrated that, in ACS patients, the PAPP-A form that causes the ACS-related PAPP-A in-
creases is not complexed with proMBP (10). However, to our knowledge, the PAPP-A assays that have been used thus far in all published clinical studies detect total PAPP-A (2, 4–8, 11), which includes all molecules containing the PAPP-A subunit, whether or not complexed with proMBP. The aim of our study was to develop rapid point-of-care assays for total PAPP-A and PAPP-A/proMBP complex that can be used together to determine the concentration of ACS-related noncomplexed PAPP-A.

**Materials and Methods**

**PAPP-A CALIBRATORS**

The PAPP-A calibrators were prepared from a filtered (0.22 μm) pool of 10 third-trimester pregnancy serum samples that were diluted in a buffer containing 50 mmol/L Tris-HCl, pH 7.75, 150 mmol/L NaCl, 0.5 g/L NaN₃, and 60 g/L bovine serum albumin (TSA-BSA buffer) and calibrated against the pooled third-trimester pregnancy serum-derived WHO IRP 78/610 standard for pregnancy-associated proteins (World Health Organization International Laboratory for Biological Standards, State Serum Institute, Copenhagen, Denmark). The calibrators were stored at −20 °C until use.

**CLINICAL SAMPLES**

We obtained a panel of 173 serum samples and corresponding heparin plasma samples by collection of samples from 39 patients with ST-elevation myocardial infarction (MI) at different time points after the onset of chest pain. From 200 consecutively hospitalized ACS patients presenting with ACS symptoms, we were able to collect 651 serum samples. A panel of 159 randomly chosen serum samples from apparently healthy non-ACS Finnish individuals was obtained from Labquality, Finland. Three first-trimester serum samples (gestational ages 5, 8, and 9 weeks) and a pool of third-trimester pregnancy sera were also included in the study. The local ethics committees approved the collection of the samples, and all blood samples were taken after patients gave informed consent. The pregnancy serum samples were stored at −20 °C and all other clinical samples at −70 °C before use.

**OTHER REAGENTS**

Monoclonal antibodies A1, A5, and A11 were from HyTest Ltd. Intrinsically fluorescent [2,2′,2″-terpyridine]-[2-(4-isothiocyanato-phenyl)ethyl]iminobis(methylene)bis[4-[[4-(α-glucopyranosyl)phenyl]ethyl]methylene-6,2-diyl]bis[methylene-nitrito] tetrakis(acetato)] europium (III) chelate and biotinylaton reagent, biotin isothiocyanate, were synthesized at the Department of Biotechnology, University of Turku, Finland. Streptavidin-coated single wells and all-in-one (AiO) buffer were from Innotrac Diagnostics. All other reagents used were of analytical grade.

**ASSAY DESIGN**

In a total PAPP-A assay, both monoclonal capture antibody A1 and monoclonal detection antibody A5 bind to epitopes on the PAPP-A subunit. Thus, the total PAPP-A assay detects PAPP-A whether or not it is complexed with proMBP. The PAPP-A/proMBP assay is constructed with the same capture antibody A1 and monoclonal detection antibody A11 that binds to the proMBP subunit of the PAPP-A/proMBP complex. Consequently, the PAPP-A/proMBP assay detects only PAPP-A in complex with proMBP. The concentration of ACS-related noncomplexed PAPP-A is calculated as the difference of the results given by the total PAPP-A assay and the PAPP-A/proMBP assay.

**LABELING OF ANTIBODIES WITH EUROPium CHelATE AND BIOTIN**

We labeled the detection antibodies A5 and A11 with 15-fold and 20-fold molar excess of the Eu³⁺ chelate in 50 mmol/L Na₂CO₃/NaHCO₃ buffer (pH 9.6), respectively. We also included ethylene glycol (100 ml/L) in the labeling reaction of A11. The reactions were incubated overnight, at ambient temperature, in the dark. We separated the labeled antibodies from the free chelate by gel filtration on a Superdex 200 26/60 column (GE Healthcare Bio-sciences), equilibrated and eluted with a buffer containing 50 mmol/L Tris-HCl (pH 7.75), 150 mmol/L NaCl, and 0.5 g/L NaN₃. The fractions containing the antibody were pooled, and europium concentration was determined against a europium calibrator. The labeling degrees of the labeled antibodies were 6–6.5 Eu³⁺ molecules per IgG molecule. The capture antibody A1 was biotinylated in 50 mmol/L Na₂CO₃/NaHCO₃ buffer, pH 9.6, with 15-fold molar excess of biotin isothiocyanate. After incubation, for 3 h at ambient temperature in the dark, the biotinylated antibody was purified with Superdex 200 26/60 gel filtration column using 50 mmol/L Tris-HCl buffer (pH 7.75) containing 150 mmol/L NaCl and 0.5 g/L NaN₃ as the eluent. The labeled antibodies were stabilized with 1 g/L bovine serum albumin, filtered (0.22 μm), and stored at 4 °C.

**PREPARATION OF AIO! DRY-REAGENT WELLS**

We immobilized 300 ng of biotinylated capture antibody A1 to streptavidin-coated single wells in 50 μL of 0.2 mol/L phosphate buffer (pH 7.8), with overnight incubation at ambient temperature. After washing, we added 40 μL of insulating solution containing 37.5 mmol/L Tris, 120 mmol/L NaCl, 0.375 g/L NaN₃, 0.6 g/L bovine γ-globulin, 25 g/L bovine serum albumin, 50 g/L D-trehalose, 0.1 g/L native mouse IgG, 0.05 g/L denatured mouse IgG, and 2 g/L casein (pH 7.75) to the wells, and dried them overnight at 35 °C and 5% relative humidity. The europium-labeled detection antibody (200 ng of A5 or 300 ng of A11 per well) was added in 1 μL on top of the insulating layer and dried immediately in a stream of hot air. The dry-reagent wells were packed in analyte pens and stored at 4 °C, protected from humidity until use. The calibrator wells were prepared similarly, but a known
concentration of PAPP-A calibrator was added to the insulating solution.

**ASSAY PROCEDURE**
We conducted the one-step sandwich-type immunoassays for total PAPP-A and PAPP-A/proMBP complex in dry-reagent wells in an Aio! Immunoanalyzer (Innotrac Diagnostics), which performed the automated assays with a total assay time of 18 min. In the assay procedure, 20 μL of sample and 10 μL of Aio! buffer were dispensed into a dry-reagent well. After incubation for 15 min at 36 °C with shaking, the well was washed 6 times and dried in a stream of hot air. Time-resolved fluorescence was measured from the surface of the well. The concentrations of unknown samples were obtained by calibrating their fluorescence signals against a calibration curve constructed with the calibrator wells.

**ASSAY EVALUATIONS**
The assay imprecisions were determined according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) Guideline EP5-A. The total PAPP-A assay imprecision was studied with 2 pools of serum from ACS patients (mean total PAPP-A concentrations, 4.9 mIU/L and 15.9 mIU/L). The PAPP-A/proMBP assay imprecision was studied with pooled pregnancy serum diluted with BSA-TSA buffer to achieve PAPP-A/proMBP concentrations relevant in ACS (mean PAPP-A/proMBP concentrations, 3.1 mIU/L and 10.0 mIU/L). The serum pools were analyzed with the assays twice a day for 20 days in duplicate. For recovery studies ACS serum or pregnancy serum containing high concentrations of PAPP-A (43.7 mIU/L and 162.3 mIU/L, respectively) was added to 3 serum samples from ACS patients. The added volume was 5% of the total sample volume. The initial total PAPP-A concentrations of the samples were 4.36, 2.52, and 8.37 mIU/L, while the initial PAPP-A/proMBP concentrations were 3.59, 1.23, and 1.34 mIU/L, respectively.

**STATISTICAL ANALYSIS**
All P values are 2-tailed, and P values <0.05 were considered significant. We assessed the normality of variable distributions with the Kolmogorov–Smirnov test and probability plots. We evaluated Spearman rank-correlation to determine the relation between 2 quantitative variables with nongaussian distribution. The nonparametric Mann–Whitney U-test was used to compare differences between continuous variables with nongaussian distribution. Statistical significance of the relative differences between the results obtained with different assays and sample matrices was assessed with a 1-sample Student t-test. We performed the statistical analyses with statistical software SAS 9.1 with SAS Enterprise guide 3.0 (SAS Institute).

**Results**
The calibration curves for the total PAPP-A and PAPP-A/proMBP assays are shown in Fig. 1. The curves were determined with pregnancy-serum-derived PAPP-A calibrators and were linear for both assays in the aimed range of 0.5–300 mIU/L. The analytical detection limits, calculated as the mean background signal + 3 SD, were 0.18 mIU/L and 0.23 mIU/L for total PAPP-A assay and PAPP-A/proMBP assay, respectively. Linearities and functional detection limits of the 2 assays were determined with serum samples diluted with TSA-BSA buffer (see Fig. 1 in the Data Supplement that accompanies the online version of this article available at http://www.clinchem.aacc.org/content/vol52/issue9). Three serum samples obtained from ACS patients were used with total PAPP-A assay. However, in PAPP-A/proMBP assay evaluations, 3 first-trimester pregnancy serum samples were applied because the PAPP-A/proMBP concentrations of ACS-patient serum samples are generally very low (<5 mIU/L). The assay responses of the successively diluted samples were linear (r = 0.998–1.000). The functional detection limit, defined as the lowest concentration measured with a CV <20%, was estimated to be 0.27 mIU/L for total PAPP-A and 0.70 mIU/L for PAPP-A/proMBP assay. Total assay imprecisions were 6.0%–7.2% for total PAPP-A assay and 6.7%–9.7% for PAPP-A/proMBP assay. Within-run imprecisions were 3.6%–5.4% and 4.1%–6.9% for total PAPP-A assay and PAPP-A/proMBP assay, respectively. The total PAPP-A assay recoveries were 102%–107%, and the PAPP-A/proMBP assay recoveries were 88%–96%.

The performance of the assays with serum and heparin plasma matrices was studied with 173 serum and heparin plasma samples from MI patients with ST elevation. We found that the PAPP-A concentrations measured with the total PAPP-A assay and PAPP-A/proMBP assay with
serum samples agreed well with the results obtained with the corresponding heparin plasma samples (Fig. 2). For the regression line of total PAPP-A assay, the slope (95% confidence interval) was 1.059 (1.043 to 1.075), and the intercept was $-0.114 (-0.491$ to 0.263) ($r = 0.995, S_{yx} = 2.088$); and for the regression line of PAPP-A/proMBP, the slope was 1.058 (1.026 to 1.091), and the intercept was $0.009 (-0.072$ to 0.091) ($r = 0.980, S_{yx} = 0.293$). The mean relative differences (95% limits of agreement) were 4.4% (-19.8% to 28.6%) for total assay and 5.7% (-20.8% to 32.3%) for PAPP-A/proMBP assay. The relative differences were statistically significant ($P < 0.0001$). One outlier relative difference result obtained with total PAPP-A assay was excluded from the $t$-test to achieve gaussian distribution. The new total PAPP-A assay was compared with a previously reported point-of-care total PAPP-A assay (12). From 200 ACS patients, we analyzed 651 samples with both assays. The functional detection limit (CV $< 20\%$) was 1.5 mIU/L for the older total PAPP-A assay (5), and therefore, only samples that had total PAPP-A concentration $> 1.5$ mIU/L ($n = 546$) measured with the older assay were included in the correlation analysis. The assay results for the 2 assays correlated well. Regression analysis of the assays yielded a slope (95% confidence intervals) of 1.063 (1.043 to 1.082) and an intercept (95% confidence intervals) of $-0.018 (-0.189$ to 0.154) ($r = 0.977, S_{yx} = 1.579$) (Fig. 3A). Difference analysis revealed a mean difference (95% confidence limits) of $-3.2\% (-45.7\%$ to 39.3%) (Fig. 3B). The difference was statistically significant ($P = 0.0019$). Two outlier relative difference results were excluded from the $t$-test to achieve gaussian distribution. The same samples were also analyzed with the new PAPP-A/proMBP assay, and there was no strong correlation between the results obtained with this assay and the results obtained with either the older total PAPP-A assay ($r = 0.545$) (Fig. 4) or the new total PAPP-A assay ($r = 0.532$).

A panel of 159 serum samples from apparently healthy non-ACS individuals (79 men, 80 women) were analyzed with the total PAPP-A and PAPP-A/proMBP assays, and the concentrations of ACS-related noncomplexed PAPP-A were calculated as the difference of the results of these assays. The samples had been grouped so that there were 41 samples from individuals 31–50 years of age, 79 samples from individuals 51–70 years of age, and 39 samples from individuals $>70$ years of age. The groups had equal sex distribution, and the mean (SD) age for the whole population was 57.5 (12.9) years. PAPP-A and PAPP-A/proMBP concentrations had non-gaussian distribution in the study population, whereas the difference variable noncomplexed PAPP-A had gaussian distribution. In the whole population, total PAPP-A varied between 1.0 and 5.9 (median 2.4 mIU/L), PAPP-A/proMBP between 1.1 and 5.9 (median 2.2 mIU/L), and noncomplexed PAPP-A between $-1.1$ and 2.0 (median 0.2 mIU/L) (Fig. 5). The concentrations of total PAPP-A, PAPP-A/proMBP, and noncomplexed PAPP-A (mIU/L) were significantly higher in men than in women [median (interquartile range), total PAPP-A: 2.8 (1.1) vs 2.1 (0.8), $P < 0.0001$; PAPP-A/proMBP: 2.6 (0.7) vs 2.0 (0.7), $P = 0.0001$; noncomplexed PAPP-A: 0.3 (0.6) vs 0.0 (0.6), $P = 0.0025$]. There was weak but statistically significant correlation between total PAPP-A concentrations and age (Spearman $r = 0.290, P = 0.0002$) and PAPP-A/proMBP concentrations and age (Spearman $r = 0.230, P = 0.0035$).
The concentrations of ACS-related noncomplexed PAPP-A did not correlate with age (Spearman $r = 0.075$, $P = 0.3483$). The 97.5th percentile in the whole group was 4.85 mIU/L for total PAPP-A and 1.29 mIU/L for noncomplexed PAPP-A.

**Discussion**

In an earlier study (10), we demonstrated that the PAPP-A form that increases in ACS is not complexed with proMBP. In the present study we have developed and evaluated new point-of-care immunoassays for total PAPP-A and PAPP-A/proMBP complex that can be used together to determine the concentration of the ACS-related noncomplexed PAPP-A. In this study, we also revealed that, in healthy populations, low concentrations of circulating PAPP-A are found that consist mostly, if not completely, of the PAPP-A/proMBP complex, and thus, the concentration of noncomplexed PAPP-A in non-ACS individuals is close to zero. Total PAPP-A concentrations $>2.9$ mIU/L predicted higher risk for adverse cardiac events in our previous study (5). In the present study, the total PAPP-A 97.5th percentile for the non-ACS population was 4.85 mIU/L. Therefore, the healthy baseline
concentrations of PAPP-A and the concentrations of PAPP-A important in the management of ACS patients seem to overlap considerably. With ACS-related noncomplexed PAPP-A, the overlap does not exist or is, at least, less pronounced (Fig. 5) because fluctuations in the concentration of PAPP-A/proMBP do not interfere with the results. Consequently, false-positive as well as false-negative results can be largely decreased with noncomplexed PAPP-A. Moreover, age does not affect noncomplexed PAPP-A concentrations in the same way it affects total PAPP-A concentrations.

In our study, we compared the newly developed total PAPP-A assay to another previously reported point-of-care total PAPP-A assay (12). The results obtained from both assays with serum samples from ACS patients correlated well. The older assay was designed to be used for Down syndrome screening in the first trimester of pregnancy, and the functional detection limit for the assay (CV% <20) was 1.5 mIU/L (5). In the first trimester of pregnancy, the concentrations of PAPP-A are rarely <100 mIU/L, and therefore, this limit is well suited for pregnancy-related applications. However, in the case of ACS and especially when the concentration of noncomplexed PAPP-A is calculated based on the total PAPP-A results, the functional sensitivity of total PAPP-A assay should be as low as possible. There was no strong correlation between the PAPP-A/proMBP and total PAPP-A concentrations measured in the ACS serum samples. This was an expected result because, as we have previously shown (10), the noncomplexed PAPP-A is responsible for the total PAPP-A increases associated with ACS while PAPP-A/proMBP concentrations remain stable.

PAPP-A (as total PAPP-A) has been detected in unstable atherosclerotic plaques but not in stable plaques (2), suggesting that PAPP-A may have a role in plaque development in a vulnerable state. Increased PAPP-A expression has also been associated with vascular repair and wound healing after induced injury, and a similar mechanism of tissue remodeling may also take place in unstable plaques (13, 14). The known substrates of the enzymatic activity of PAPP-A in humans are insulin-like growth factor binding protein-4 and -5 (15, 16), which regulate the activity of insulin-like growth factors. The formation of the covalent complex between proMBP and PAPP-A inhibits the activity of PAPP-A (17). To have a special function in the unstable plaques, PAPP-A should be enzymatically active, which means that it should exist in plaques in the active noncomplexed form. This reasoning is supported by the evidence that complexation of PAPP-A and proMBP is inhibited in an oxidizing environment (18), and that oxidative stress is pronounced within the atherosclerotic plaques (19). On the basis of the evidence presented above, we believe that the noncomplexed PAPP-A in ACS patients is released from disrupted plaque(s) and is thus a specific marker of plaque rupture.

Prompt diagnosis of patients with or at high imminent risk of MI is important to limit the extent of cardiac tissue damage. Biochemical markers of myocardial tissue necrosis, primarily cardiac troponins, are routinely used for diagnosis of MI. However, as a marker of value in identifying patients at high risk for adverse cardiac events, PAPP-A is useful in triage and risk stratification of ACS patients, particularly when troponins are negative. The new point-of-care assays presented here allow rapid and reliable measurement of ACS-related noncomplexed PAPP-A, leading potentially to earlier diagnosis and risk stratification of the patients presenting with ACS symptoms.

Anticoagulation treatment with either unfractionated heparin or low–molecular-weight heparin is commonly used in the clinical management of ACS. PAPP-A contains glycosaminoglycan binding site that actively binds heparin and thus heparin may interfere some PAPP-A assays (12, 20). The new assays described here with heparin plasma samples gave results that were very similar to results obtained with serum samples, which indicates that these assays are not affected by heparin.

Several studies have shown a connection between circulating PAPP-A and coronary artery disease (5–7, 21). However, contradictory results have also been published by Dominguez-Rodriguez et al. (22). In their study, the concentrations of PAPP-A in admission serum samples of ST-segment elevation myocardial infarction (STEMI) patients and in healthy controls did not differ significantly, a finding that may be related to the assay (DRG PAPP-A ELISA) they used. According to the manufacturer’s specification, this assay is intended for use in Down syndrome screening, with analytical sensitivity of 0.19 mg/L, which equals 42 mIU/L (by manufacturer’s conversion factor: 1 mIU = 4.5 μg). Such an assay is obviously inadequate for PAPP-A measurement in ACS samples, because serum PAPP-A concentrations found in ACS are very low, usually <30 mIU/L. We tested the performance of the DRG ELISA with 4 ACS serum samples (from 4 AMI patients) that contained high concentrations of PAPP-A (19.2 mIU/L, 22.8 mIU/L, 49.4 mIU/L, and 145.0 mIU/L, respectively) measured with our total PAPP-A assay, and we found that only the sample that had a PAPP-A concentration of 145.0 mIU/L was actually measurable with the DRG ELISA. Thus, the negative study results obtained by Dominguez-Rodriguez et al. are quite understandable and highlight the importance of the selection of proper immunoassays for PAPP-A measurement in the ACS samples, as we previously pointed out (23).

A limitation in the current study is that whole blood samples were not tested with the assays because no whole blood samples were available from ACS patients. However, there are several reports demonstrating that the Aio! assays perform well regardless of sample matrix (12, 24–26). For the Aio! Immunoanalyzer, the use of whole blood samples does not require any additional processing step compared with the use of serum and plasma samples.
Furthermore, preliminary tests with samples from healthy individuals with added PAPP-A show that the concentrations of total PAPP-A and PAPP-A/proMBP measured in heparinized whole blood and in corresponding plasma correlate very well ($r \geq 0.99$), with only a minor increase (~10%) in the results obtained with whole blood after hematocrit correction (data not shown). We believe that the results with original patient samples will be quite similar, but further studies to confirm this are warranted. There are no published data about the effect of the time from sample collection to serum or plasma separation on the concentrations of PAPP-A in the samples. Because whole blood samples from ACS patients were not available for us, we could not study this with the assays described. However, such effects are unlikely because no significant expression of PAPP-A in circulating cells has been reported.

Determination of the ACS-related noncomplexed PAPP-A in our approach is based on the use of 2 assays. However, the 2 assays have their own variations, thereby increasing the total variation of the measured concentrations of noncomplexed PAPP-A. Therefore, a single assay that specifically detects the noncomplexed PAPP-A would be ideal, because it would improve the sensitivity of the noncomplexed PAPP-A measurement as well as decrease costs and time required for the assay. However, because there are no antibodies specific for noncomplexed PAPP-A, such an assay is currently not available.

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