Potential Use of Pregnancy-associated Plasma Protein-A and IMA as Biomarkers for the Early Stage of COVID-19

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Research

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Abstract

Background

This study has been undertaken with the urgent need for exploring reliable biomarkers for early infection of SARS-CoV-2. We performed a retrospective study analyzing the serum levels of the cardiovascular biomarkers N-terminal pro-B natriuretic peptide (NT-proBNP), cardiac troponin T (cTnT), Ischemia Modified Albumin (IMA) and pregnancy associated plasma protein A (PAPP-A), in 84 patients with COVID-19.

Methods

Patients were divided in three groups according to their RT-qPCR and IgG values in acute infection (n=35), early infection (n=25) or control subjects (n=24). Levels of biomarkers were analyzed in patient's serum samples by commercially available ELISA kits.

Results

Multivariate analysis and Receiver Operating Characteristic (ROC) curve showed that IMA and PAPP-A, had an excellent discrimination value for the early stage of COVID-19. Serum levels of IMA in early SARS-CoV-2 infected patients were significantly higher than in the control group with an area under the ROC curve (AUC) value of 0.94 (95% confidence interval (CI): 0.881- 0.999). Likewise, the serum level of PAPP-A was significantly higher in patients with early infection than in controls [AUC = 0.801 (95% CI: 0.673–0.929)]. The combined use of IMA and PAPP-A enhanced the sensitivity for total SARS-CoV-2 infected patients to 93%.

Conclusions

These results suggest that the levels of PAPP-A and IMA might be used as efficient biomarkers for the early stage of COVID-19 with high sensitivity and specificity. Importantly, when monitoring pregnancy and cardiovascular diseases by PAPP-A or IMA levels, an infection by SARS-CoV-2 should be discarded for proper interpretation of the results.

Background

It has been more than a year since the world health organization declared COVID-19 a pandemic (1). Since then, COVID-19 has quickly progressed to a global health emergency impacting the lives of billions of individuals. While 42 million vaccines are being administered daily (2), Covid-19 pandemic is a long way from over.

SARS-CoV-2 pathogenesis is triggered by a viral infection and amplified by a dysfunctional immune system, with a wide range of disease severity from asymptomatic to fatal outcome. Although respiratory illness is the dominant clinical manifestation of COVID-19, cardiovascular disease has been observed in

approximately 8–12% of all patients (3). Meanwhile, COVID-19 patients with preexisting cardiovascular diseases are often at a much higher risk of increased morbidity and mortality.

Despite RT-PCR remains the gold standard for SARS-CoV-2 infection, critical gaps still remain in screening asymptomatic people who are in the incubation phase of the virus. We wonder whether inflammatory or cardiovascular biomarkers involved in myocardial injury might help to detect early SARS-CoV-2 infected patients.

The exact mechanisms of how SARS-CoV-2 can cause myocardial injury are not clearly understood but the systemic inflammation and the exaggerated cytokine response (“cytokine storm") observed in many patients are important factors in the development of myocardial damage and arrhythmia (4, 5). In addition, coronary plaque destabilization, and hypoxia contribute to the damage of cardiomyocytes (5). Accordingly, multiple studies have shown increased proinflammatory cytokines as well as several cardiac biomarkers in the infected patients especially those with severe disease. In addition, the cardiovascular disease in the setting of COVID-19 can be associated with increased levels of biomarkers associated with myocardial stress and injury (6).

Currently the two best established markers in cardiovascular disease are the B type cardiac natriuretic peptides and cardiac troponins I (cTnI) and T (cTnT). There are several studies that have shown that TnT and NT-proBNP increase significantly in the period before death in COVID-19 patients that ultimately died but not in patients that survived (7). Even so, the rapid suspicion of cardiovascular complications remains a major challenge for patient management and therapeutic intervention. Hence, further studies are needed for finding prognostic and diagnostic molecules to monitor cardiac and cardiovascular dysfunctions in early stages of COVID-19.

We sought to determine whether other cardiac biomarkers increase in the early stage of SARS-CoV-2 infection. In the recent years, an increasing number of promising biomarkers have been identified to predict cardiovascular events. A recently developed biomarker for transient myocardial ischemia is the determination of Ischemia Modified Albumin (IMA), a form of human serum albumin in which the N-terminal amino acids are unable to bind transition metals (8). IMA plays an important role in the early diagnosis of cardiogenic ischemic diseases (9) although recent studies have shown that the serum level of IMA can also be significantly increased in non-cardiogenic ischemic diseases like community-acquired pneumonia (10)or obstructive sleep apnea (11).

Another promising biomarker is the pregnancy-associated plasma protein A (PAPP-A) which increases with plaque instability and predicts the risk of acute coronary syndrome (12). PAPP-A is a zinc-binding matrix metalloproteinase that was originally identified in pregnant women. Recently, its ability to enhance local IGF bioavailability by cleaving IGF binding proteins (IGFBPs), specifically IGFBP-2, − 4, and − 5, has been revealed, conferring PAPP-A a relevant role in growth regulation. In addition, PAPP-A expression in human dermal fibroblasts has been shown to increase after injury and during tissue remodeling (13). Moreover, PAPP-A is expressed in vascular smooth muscle cells where may play a role in the development

of atherosclerotic lesions. Its circulating levels represent a marker of atheromatous plaque instability and extent of cardiovascular disease.

To our knowledge, the plaque destabilization marker PAPP-A, has not yet been reported in SARS-CoV-2 patients. In this article, we summarize reports on the plasma levels of cytokines IL-6, TNF-α and IL-28B and the biomarkers of cardiovascular disease, cTnT, NT-proBNP, PAPP-A and IMA in a retrospective study of 84 patients with COVID-19. We found that plasma levels of IMA and PAPP-A in the early phase of SARS-CoV-2 infection were significantly higher than in the control subjects and so we propose that measuring the level of IMA and PAPP-A from the incipience can be useful in detecting early stages of the disease and managing of COVID19 disease.

Methods

Serum samples

The study was approved by the Ethics Committees of the Principe de Asturias Hospital and Alcalá University (LIB21-2020 and CEI/HU/202/37) and conforms to the principles outlined in the Declaration of Helsinki.

Demographic data, and clinical features were available and collected according to the patient record system. Data collection of laboratory results were defined using the first-time examination at admission (within 24 h after admission).

The patients were diagnosed according to the World Health Organization interim guidance for COVID-19. The fluorescent reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was used to confirm each diagnosis made. Blood samples of 84 patients admitted to the Principe de Asturias Hospital were obtained at the time of hospital admission. Blood samples were collected and centrifuged for 10 min at 1000g; the resulting supernatant was transferred to barcode-labeled cryovials and immediately frozen at -80ºC.

Biomarkers determination in serum

Human IgG anti-S1 protein (RBD region) of Sars-CoV-2 were determined in patient serum samples by ELISA (GenScript USA, Inc., Piscataway, NY, USA).

IL-6 and TNF-α cytokines were analyzed in patient serum samples by commercially available ELISA kits (Affymetrix, Santa Clara, CA, USA). The IL-28B, cTnT, NT-proBNP, and PAPP-A biomarkers were determined using ultrasensitive ELISA kits provided by Cloud-Clone Corporation, (Katy, TX, USA). Analyses were performed according to the manufacture's protocol for each ELISA kit, assayed in triplicate, and read on a BioRad iMark™ Microplate Absorbance Reader at 450 nm (Hercules, CA, USA). Standard curves and individual well concentrations were determined using the Microplate Manager® 6, Version 6.3 software (BioRad, Hercules, CA, USA).

Ischemic Modified Albumin (IMA) was determined by competitive inhibition enzyme immunoassay technique (Cloud-Clone Corporation, Katy, TX, USA). Competition between sample's IMA and biotinylated IMA for a specific monoclonal antibody pre-coated onto the microplate, determined IMA concentration in the sample.

Statistical Analysis

Graph Pad Prism 9 (San Diego, CA) and IBM SPSS statistics version 27 (IBM Corp., Armonk, NY, USA) were used to analyze the experimental data. All experiments were performed at least three times for reproducibility. The results are expressed as mean ± standard deviation or standard error as indicated. ANOVA one way and Dunett multiple comparation test was used for comparisons between multiple groups. Obtained p value which was less than 0.05 was considered as statistically significant. ROC curve analysis was used to determine the diagnostic value of serum biomarker expression in patients with COVID-19. Other diagnostic parameters were also evaluated, including sensitivity, specificity, cut-off value, positive predictive value, negative predictive value, and area under the ROC curve (AUC) with 95% confidence interval (CI), to assess the discrimination power of biomarkers.

Results

Demographic and epidemiological characteristics of participants

Participants were aged 35–90 years, with a median age of 65 years of which 60% were men and 40% women. The median (interquartile range: IQR) age was 76 (63–86) years, being higher in women than in men, and 72.77% of patients were 65 years or older (Table 1).

Infection with SARS-CoV-2 was determined by RT-qPCR analysis of nasopharyngeal samples from patients of the study. From the same patients, we determined serum levels of IgG antibodies against the receptor binding domain (RBD) of the SARS-CoV-2 S1 spike protein, which are highly specific target of antibodies in SARS-CoV-2 patients (14, 15). As described, neutralizing anti-SARS-CoV-2 IgG antibodies are usually observed by day 9 after the onset of symptoms (16).

Once RT-qPCR and anti-Spike S1 IgG antibodies were determined, patients were divided into three groups: no infection (PCR negative, IgG negative) (n = 24), early infection (PCR positive, IgG negative) (n = 25) and acute/active infection (PCR positive, IgG positive) (n = 35) groups (Table 1).

Cytokine levels in participants

We then evaluated the serum concentration for the cytokines IL-6, TNF-α and IL-28B. Levels of IL-6 and TNF-α increased significantly in the acute phase of SARS-CoV-2 infection (Fig. 1), in good agreement with previous observations showing elevated IL-6 in the setting of severe Covid-19 disease (17, 18). In line with this, a positive association between those cytokines and the severity of the viral infection and mortality rate has been described (18). By contrast, we found that IL-28B notably decreased in most patients with COVID-19 (PCR positive) compared to controls (Fig. 1).

Cardiovascular biomarkers in participants

We then investigated the levels of the classical cardiac damage biomarkers cTnT and NT-proBNP in patient's sera. Only 5 patients with acute infection had NT-proBNP levels higher than the cut-off value to predict the adverse outcome of severe COVID-19 (Supplemental Fig. 1), which was previously determined to be 88.64 pg/mL (19). No baseline cTnT elevations were detected in acute infection patients and only one patient in the early infection phase showed significant high levels of cTnT (Supplemental Fig. 1). In consequence, neither cTNT or NT-proBNP cardiac biomarkers significantly vary between control and PCR positive samples, which indicates that they were not useful to detect the early stages of infection.

We next sought to determine the levels of the novel biomarkers for cardiovascular events IMA and PAPP-A. IMA is a highly sensitive marker of hypoxia and detectable in the reversible early phase of myocardial ischemia, so we wonder whether it would be increased in SARS-CoV-2 infection, as ischemic stroke has been reported in patients with COVID-19 (20). Measurement of IMA levels in patient's sera revealed a notably increase in early infected patients (PCR + IgG-) (Mean 94.92 ± 4.80 mg/ml) compared to controls (PCR - IgG -) (Mean 44.03 ± 4.35 mg/ml) (Fig. 2A). In the acute infection (PCR + IgG +), IMA levels (Mean 77.73 ± 3.25 mg/ml) were higher than controls, although not as high as in the early infection (Fig. 2A).

Receiver operator characteristic (ROC) curve analysis showed that the area under the curve (AUC) was 0.867 (95% CI: 0.775–0.959) for IMA determination in the total of patients, and AUC = 0.940 (95% CI: 0.881–0.999) for IMA determination in the early phase of SARS-CoV-2 (Fig. 2B), which indicates an excellent discrimination. The optimum diagnostic cut off point which maximized the sensitivity, and the specificity was determined to be 59.26 mg/mL, with a sensitivity of 90 % and a specificity of 75%. These results indicate that IMA determination may facilitate diagnosis of COVID-19 with relatively high sensitivity and specificity.

We further investigated the levels of the Pregnancy-associated Plasma Protein, PAPP-A, in the sera of COVID-19 patients. As shown in Fig. 3A, PAPP-A concentration of early infection group (Mean 5.618 ± 2.281 ng/ml) was above the levels of the control (Mean 0.258 ± 0.043 ng/ml) and the acute infection (Mean 0.231 ± 0.034 ng/ml) groups. It is worthy to note that 35% of COVID-19 patients in the early phase, had remarkably high levels (an average of 65-fold increase over controls) of PAPP-A (Fig. 3A). This result suggested that PPAP-A could be used as a biomarker for the early phase of COVID-19. This was confirmed by plotting the ROC curve and calculating the AUC for PAPP-A values of early infected patients. As shown in Fig. 3B, AUC = 0.801 (95% CI: 0.673–0.929) for early infected patients, indicating a very good discrimination. The best cutoff value for PAPP-A was 0.40 ng/mL, with a sensitivity and specificity of 61% and 86%, respectively. However, levels of PAPP-A of patients in the acute infection phase, were similar as controls and not useful for diagnosis as AUC of ROC curve was 0.526 (Fig. 3B).

The sensitivity, specificity, positive predictive value and negative predictive value of IMA, PAPP-A and combined IMA and PAPP-A for the different patient groups were calculated (Table 2).

The combined use of IMA and PAPP-A significantly improved the sensitivity, specificity, positive predictive value and the negative predictive value of Total COVID-19 patients to 93%, 75%, 39% and 98% respectively. AUC for the combination of IMA and PAPP-A for total COVID-19 patients was 0.90 (Fig. 4), which is higher than PAPP-A value and similar to IMA.

These results indicate that serum levels of IMA and PAPP-A could be used as new biomarkers for COVID-19. The notably increase of IMA and PAPP-A observed in the first stages of SARS-COV-2 infection should be considered when determining these biomarkers to diagnose other conditions.

Discussion

In this study, we have measured the serum levels of several pro- and anti-inflammatory cytokines as well as markers of acute coronary syndromes in a cohort of 84 patients with laboratory-confirmed COVID-19. We reported here that the serum levels of TNFα, IL-6, IL-28B, IMA and PAPP-A change during SARS-CoV-2 infection whereas cTnT and NT-proBNP were not significantly different between the early phase and the active phase of infection. This result is not surprising since elevation of the cardiac biomarkers NT-proBNP and cTnT, predict poor clinical outcomes and elevated levels are rare in COVID-19 survivors with an uncomplicated course (21).

Our findings of raised TNF-α and IL-6 levels after SARS-CoV-2 infection are comparable to those obtained by other and are consistent with the cytokine storm described in COVID-19 (17, 22). Here we found that IL-28B levels decrease in COVID-19 patients. IL-28B (IFN-γ2) belongs to the type III interferon (IFN) subfamily. This cytokine is associated with the spontaneous clearance of HCV infection and has demonstrated antiviral activity against several viruses. Type III IFN are considered antiviral cytokines in innate immune responses, directly performing an antiviral immune response at epithelial surfaces limiting the replication of major human pathogenic viruses (23). It has been recently described that type III IFNs are able to inhibit SARS–CoV-2 replication (24). Although the studies that examine IL-28 are scarce, results from Vanderheiden et al. reported a lack of a type III interferon (IFN) response to SARS-CoV-2 infection (25). In line with this, research performed by Galani et al. in 32 COVID patients, demonstrated a diminished and delayed production of type III IFN (26), which is in good agreement with our results. The suppression of serum IL-28B found in SARS-CoV-2 infected patients could be a evasion strategy to impair the type III IFN induced antiviral action since it has been described that SARS-CoV-2 infected cells are sensitive to the antiviral effects of type III IFN (24, 25). Recent findings suggest that SARS-CoV-2 inhibits the production of IFN by a mechanism mediated by virus membrane M protein. SARS-CoV-2 M protein antagonizes type I and III IFN production by preventing the formation of the multiprotein complex and activation of IRF3 (27).

In our study, we have also found a notably increase of IMA in COVID-19 patients. The concentration of IMA was significantly higher in the early infected and acute groups compared to the control group and showed a good diagnostic value. Increased levels of serum IMA may be explained with hypoxia and tissue ischemia observed in SARS-CoV-2 infected patients (28). In a recent study by Yildiz et al. higher

levels of oxidative damage markers, including IMA, in COVID-19 patients were found in association with pulmonary involvement severity (29).

Pregnancy associated plasma protein A (PAPP-A) is a secreted metalloproteinase, originally discovered as a glycoprotein in pregnant women. It was introduced as a marker of Down Syndrome and later used in diagnosis of pre-eclampsia in early trimester. Several studies have indicated that PAPP-A is a novel biomarker for plaque instability and inflammation useful in early diagnosis, risk stratification, and prognostic prediction in patients with acute coronary syndrome (ACS). Herein, we have found that levels of PAPP-A increase in the early infection with SARS-CoV-2, and that it could be used for diagnosis accuracy of COVID-19 disease. Moreover, when using PAPP-A determination for pre-eclampsia and ACS monitoring, COVID-19 should be ruled out to avoid misdiagnosis.

Understanding the mechanistic level of PAPP-A increase in patients with COVID-19 may be helpful in disease management. Notably, PAPP-A expression can be potently induced in response to proinflammatory cytokines such as TNF-α and IL-1β in a time- and dose- dependent manner (13, 30). In fact, proinflammatory cytokines, interleukin- IL-1β, and TNF-α are potent stimulators of PAPP-A expression. By contrast, recent findings indicate that PAPP-A significantly stimulates the expression of TNF-α, and IL-6 in macrophages at both transcriptional and translational levels in a dose-dependent and time-dependent manner (31). This finding is in agreement with our study as we found that PAPP-A levels increase in the early infection whereas IL-6 and TNF-α increase in the acute infection phase, suggesting that PAPP-A may play a proinflammatory role in COVID-19 disease.

A common pathogenic phenomenon found in obstetric diseases, SARS-CoV-2 infected patients and COVID-19 pregnant women is the immune-mediated thrombosis (32). A systematic review on pregnancy outcomes in mothers infected with coronavirus SARS-CoV-2 revealed that pre-eclampsia was more common than in uninfected mothers (33). Altogether, these data suggest that the elevated PAPP-A found in COVID-19 patients could play a role in the thrombotic episodes and inflammation processes that worsen the COVI-19 outcome.

A question that arises is whether PAPP-A could contribute to the pathogenesis of SARS-CoV-2 since PAPP-A is a membrane-bound metalloproteinase and SARS-CoV-2 pathogenesis depends on proteolytic activity. SARS-CoV-2 S protein interaction process is mediated by proteases such as transmembrane protease serine 2 (TMPRSS2), cellular protease furin and cathepsin L. Interestingly, we have measured PAPP-A expression in nasopharyngeal samples of 90 COVID-19 patients, but we have not observed any change between controls and patients (data not shown) and so, this not seems to be the case.

Taken together our results indicate that PAPP-A and IMA emerged as novel biomarkers of early phase of SARS-CoV-2 infection. To the best of our knowledge this is the first study showing an increase of PAPP-A in COVID-19 patients.

Conclusions

In conclusion, our findings suggest that IMA and PAPP-A levels can be used to estimate the severity of COVID-19. If necessary, the levels of IMA and PAPP-A should be measured, as they can help diagnose the severity of adult COVID-19 patients. Moreover, by increasing our knowledge of biomarkers involved in SARS-CoV-2 infection, we can improve our understanding of the potential mechanisms underlying COVID-19, paving the way towards the development of preventative and therapeutic solutions.

Several limitations of our study should be considered to properly interpret its findings. First, the sample size (84 patients) was relatively small and then further investigations are highly recommended in a larger cohort study for validation of the present findings. Second, IMA assay in the current research has been based on an immunoassay technique using a monoclonal antibody specific to IMA. However, the IMA test extensively used for hypoxia research is the albumin cobalt binding assay that is based on IMA's inability to bind to cobalt when has been modified by hypoxia and this should be considered when comparing results from different laboratories.

Declarations

Ethical approval and consent to participate

The study was approved by the Ethics Committees of the Principe de Asturias Hospital and Alcalá University (LIB21-2020 and CEI/HU/202/37) and conforms to the principles outlined in the Declaration of Helsinki. Consent to participate is not applicable, since according to Spanish National Bioethics on the ethical-legal requirements in research with health data and biological samples in the framework of the COVID-19 pandemic, informed consent of patients is not required and retrospective investigation of stored samples is permitted.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing Interests

The authors declare no conflict of interests in relation to this article.

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Authors' contributions

AB and BGS performed the experimentation and the ELISA analysis. JMG and MS-C analyzed and interpreted the patient data. IDL and AB made substantial contributions to the conception of the study. IDL was a major contributor in writing the manuscript. All authors read and approved the final manuscript

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Tables

Due to technical limitations, table 1 and 2 pptx are only available as a download in the Supplemental Files section.

Figures

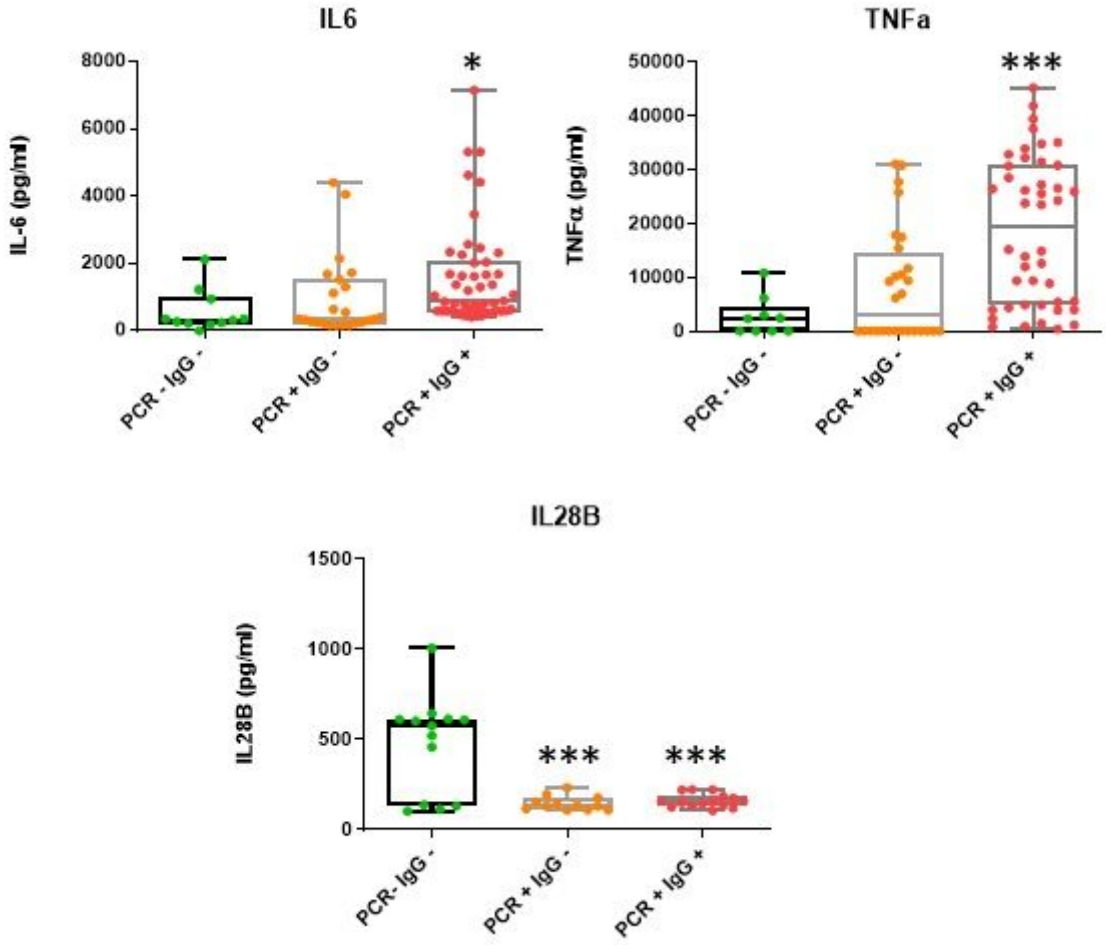
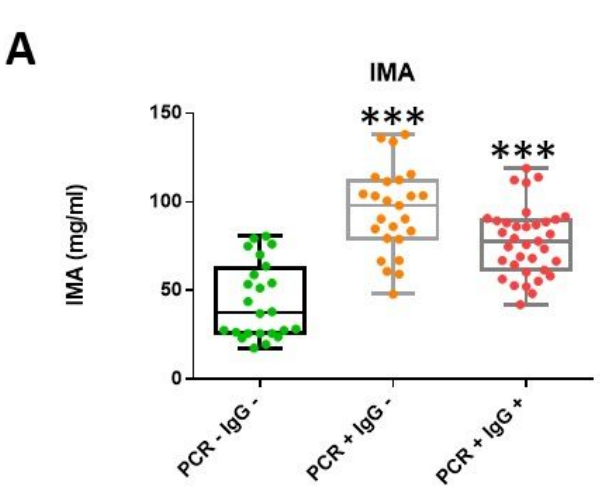


Figure 1

Determination of cytokine concentrations in patient samples. Box- and-whisker plots of IL-6, TNF-α and IL-28B levels in the serum of patients . PCR- IgG-, patients with a discharge diagnosis of COVID-19; PCR+ IgG-, patients with early SARS-CoV-2 infection, PCR+ IgG+, patients with active SARS-CoV-2 infection. Data represent the mean ± S.D. * $p \leq 0.05$; *** $p \leq 0.001$.



Ischemia Modified Albumin (IMA)						
	Mean mg/ml	Median	25% Percentile	75% Percentile	Lower 95% of CI	Upper 95% of CI
PCR- IgG-	44.03 ± 4.35	37.72	25.92	62,66	35.03	53.03
PCR+ IgG-	94.92 ± 4.80	98.03	79.34	112.05	85.00	104.83
PCR+ IgG+	77.73 ± 3.25	77.95	61.74	89.42	71.11	84.35

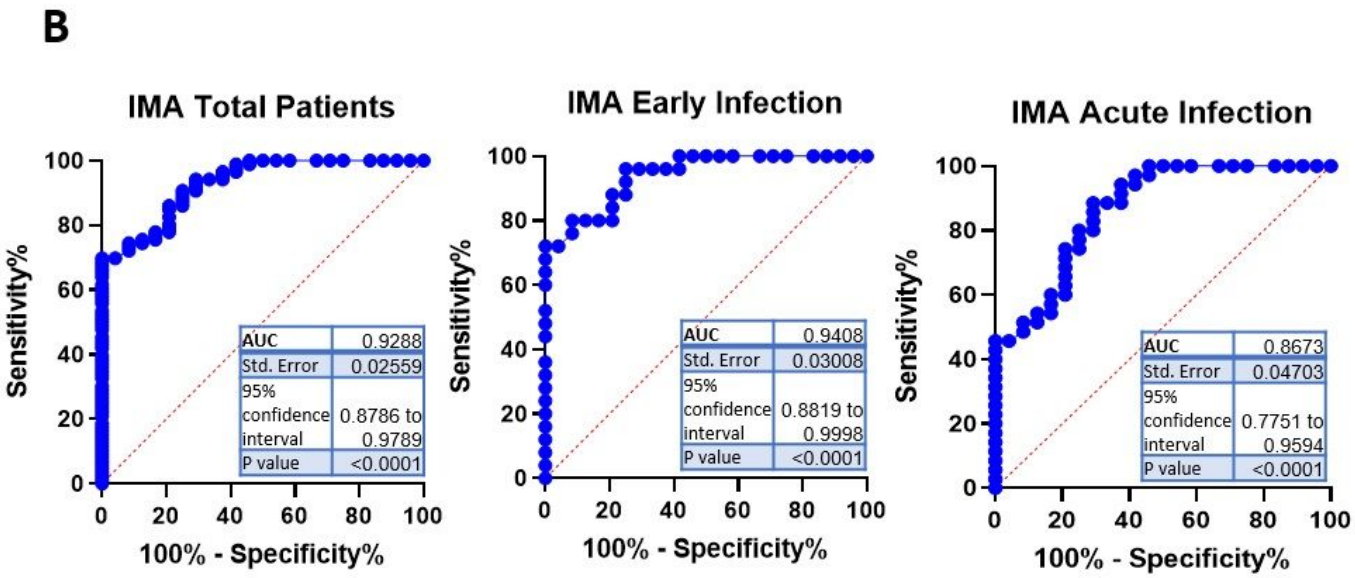
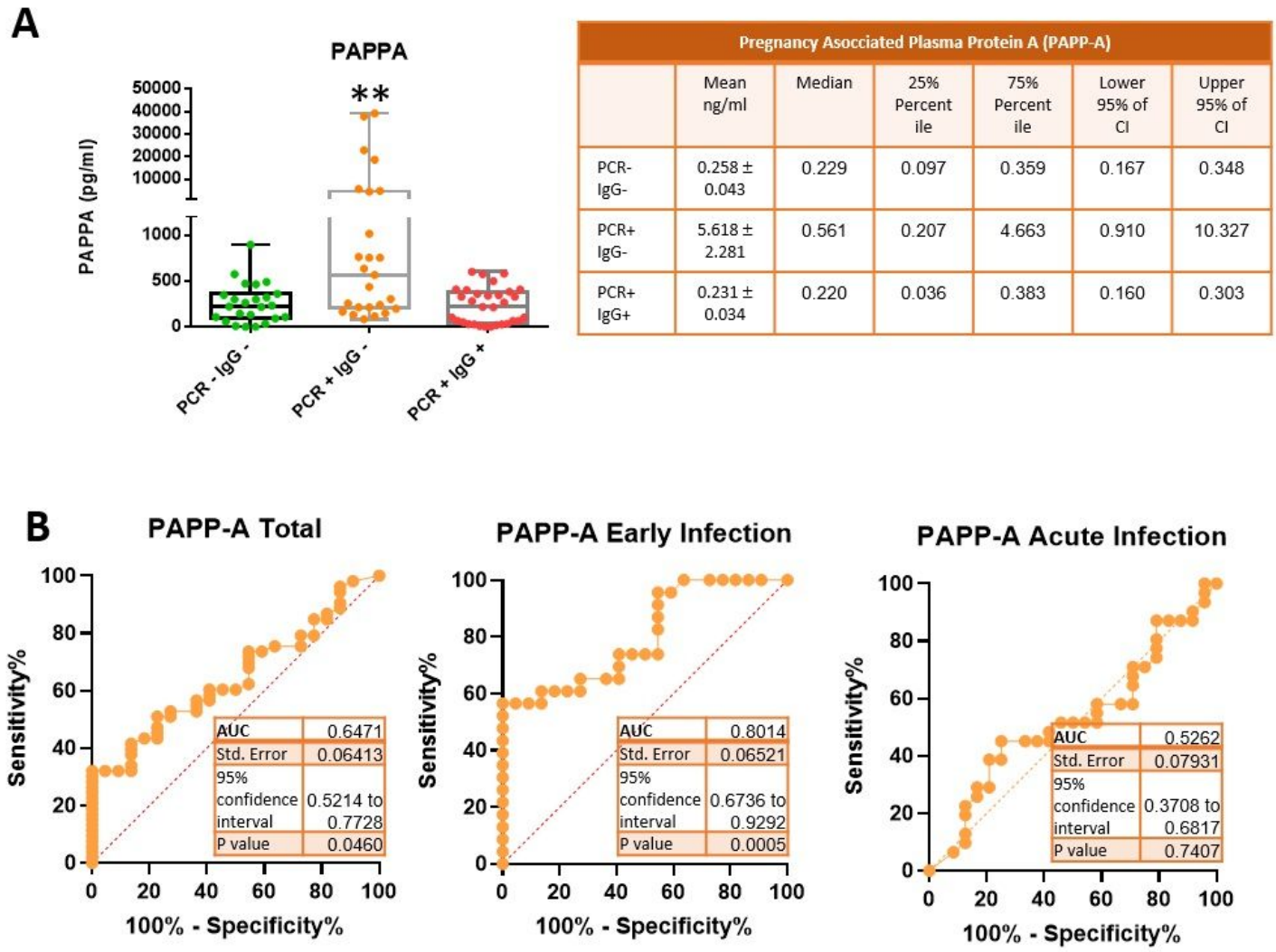


Figure 2

A, Box- and-whisker plots of Ischemia Modified Albumin (IMA) concentrations in patient samples. PCR- IgG-, patients with a discharge diagnosis of COVID-19; PCR+ IgG-, patients with early SARS-CoV-2 infection, PCR+ IgG+, patients with active SARS-CoV-2 infection. Data in the graph represent the mean ± S.D. *** $p \leq 0.001$. The right table shows the mean ± standard error, the median, 25% Percentile, 75% Percentile, Lower 95% of CI, Upper 95% of CI of IMA values. B, Receiver Operator Characteristic (ROC) curves and area under the curve (AUC) for Ischemia Modified Albumin in SARS-CoV-2 infected patients (Total, early infection and acute infection).



Pregnancy Asocciated Plasma Protein A (PAPP-A)						
	Mean ng/ml	Median	25% Percentile	75% Percentile	Lower 95% of CI	Upper 95% of CI
PCR- IgG-	0.258 ± 0.043	0.229	0.097	0.359	0.167	0.348
PCR+ IgG-	5.618 ± 2.281	0.561	0.207	4.663	0.910	10.327
PCR+ IgG+	0.231 ± 0.034	0.220	0.036	0.383	0.160	0.303

Figure 3

A, Box- and-whisker plots of Pregnancy-associated plasma Protein A (PAPP-A) concentrations in patient samples. PCR- IgG-, patients with a discharge diagnosis of COVID-19; PCR+ IgG-, patients with early SARS-CoV-2 infection, PCR+ IgG+, patients with active SARS-CoV-2 infection. Data in the graph represent the mean ± S.D. ** $p \leq 0.01$. The right table shows the mean ± standard error, the median, 25% Percentile, 75% Percentile, Lower 95% of CI, Upper 95% of CI of PAPP-A values. B, Receiver Operator Characteristic (ROC) curves and area under the curve (AUC) for Pregnancy Associated Plasma Protein in SARS-CoV-2 infected patients (Total, early infection and acute infection).

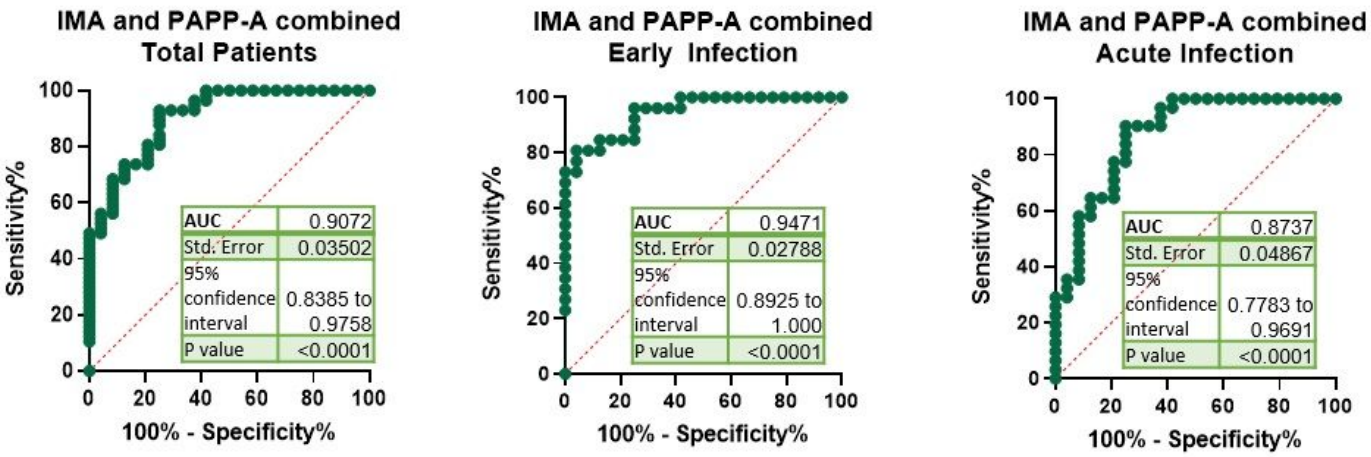


Figure 4

Receiver Operator Characteristic (ROC) curves and area under the curve (AUC) comparing the potential of combination of Ischemia Modified Albumin (IMA) and Pregnancy Associated Plasma Protein-A (PAPP-A) to diagnose COVID-19 (Total, early infection and acute infection).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure1.pptx
- Table1.pptx
- Table2.pptx