To the Editor,

The reverse transcriptase polymerase chain reaction (RT-PCR) performed on nasopharyngeal (NP) swabs still remains the gold standard method for the diagnosis of SARS-CoV-2 infection [1]. The interest of measuring the nucleocapsid (N) SARS-CoV-2 antigen in blood has been investigated in some studies [2–5] but determination of the spike (S) antigen in blood received little attention. The aim of this study was to evaluate the clinical performance of two antigen assays for N and S performed in the same analytical platform and technology in blood of SARS-CoV-2 infected patients. The relationship between antigens and disease severity was also investigated.

A total of 81 unvaccinated patients with a documented molecular diagnosis of SARS-CoV-2 infection were included between April and July 2021. For each patient, a blood sample was obtained within 12 h of the NP sampling (median delta time = 1 min, IQR = −15 to 19 min). Thirty-six (44.4%) were women (median age = 77 years, min–max = 20–97) and 45 (55.6%) were men (median age = 76.5 years, min–max = 33–94). Eighteen patients were categorized as asymptomatic (WHO score of 1), 6 as symptomatic ambulatory mild disease (WHO score of 2 and 3), 43 as symptomatic hospitalized moderate disease (WHO scores of 4 and 5) and 14 as symptomatic hospitalized severe disease (WHO score of 6–10). Eleven patients died (WHO score of 10). A cohort of 71 pre-pandemic serum samples collected before February 2020 was also included to evaluate the specificity of antigen assays. The study protocol is available upon request and was in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee of Saint-Luc Bouge (Bouge, Belgium, approval number B0392020000005).

NP samples were collected using eSwab liquid preservation medium tubes (Copan Italia, Brescia, Italy) and analyzed by RT-PCR without any delay. Blood samples were collected in serum-gel tubes (BD SST II Advance®, Becton Dickinson, NJ, USA) and centrifuged for 10 min at 1740×g. Sera were stored in the laboratory serum biobank at −20 °C from the collection date. RT-PCR for SARS-CoV-2 determination in NP swab samples was performed on a LightCycler 480 Instrument II (Roche Diagnostics, Rotkreuz, Switzerland) using the LightMix Modular SARS-CoV E-gene set (for few samples originating from Clinique Saint-Luc Bouge) and on the GeneXpert instrument (Cepheid, CA, USA) using the Xpress SARS-CoV-2 assay targeting N2 and E genes (for samples originating from Clinique Saint-Luc Bouge and Clinique Saint-Pierre Ottignies). Cycle threshold (Ct) values obtained by RT-PCR were used as a proxy for the viral load. The SARS-CoV-2 N and S antigens were detected in patient sera by an electrochemiluminescent assay using an MSD QuickPlex SQ120MM instrument (Meso Scale Discovery, Maryland, USA). Samples were analyzed using the commercial S-PLEX SARS-CoV-2 N and S-PLEX SARS-CoV-2 S antigen assay kits. The results are quantitative and expressed as pg/mL. The LOD of the assay is 0.094 and 0.141 pg/mL for
the S-PLEX N and S-PLEX S, respectively. The within-run CV is less than 10% (manufacturer’s data). SARS-CoV-2 S IgG antibodies were quantified in patient sera by a Simoa immunoassay using the Simoa HD-X analyzer (Quanterix, Massachusetts, USA) with the commercial SARS-CoV-2 S IgG Advantage kit (item 103769). The positivity cut-off of 924 ng/mL corresponding to the maximal value obtained in pre-pandemic serum samples was used (manufacturer’s data). SARS-CoV-2 antibodies were available for 77 patients out of the 81 (95.1%) due to insufficient residual serum samples.

Based on the Youden Index recommended values, the following positivity cut-offs were found for the S-PLEX N and S-PLEX S assays: >0.76 and >0.77 pg/mL. The application of these cut-offs on the pre-pandemic cohort resulted in specificities of 100% (95% CI: 94.9–100). The mean Ct results were significantly lower in antigen positive samples (Figure 1A). The S-PLEX N assay had a clinical sensitivity of 100% for patients with a Ct <33 (Figure 1A). The S-PLEX S assay missed 10 patients with a Ct >33 leading to a clinical sensitivity of 85.3% (Figure 1A). A significant negative correlation was observed using both antigen assays compared to RT-PCR (Figure 1B, C) (r = −0.61 for the S-PLEX N and r = −0.55 for the S-PLEX S). S IgG were also significantly lower in case of antigen positivity (Figure 1D). Interestingly, the 10 patients with Ct >33 who were negative for the N antigen were also positive for IgG directed against the S protein (Figure 1A, D). Additionally, a significant negative correlation was observed using both antigen assays compared to S IgG (r = −0.55 for the S-PLEX N and r = −0.43 for the S-PLEX S) (Figure 1E, F). The inter-assay correlation was high (r = 0.80, p<0.0001) with an agreement of 86.4% and a Cohen’s kappa of 0.57. After having excluded patients with positive S IgG, both N and S antigens were significantly higher in severe patients compared to mild-moderate (p=0.008 for N; p=0.01 for S)

![Figure 1: Relations between N/S antigens, S IgG and Ct results.](image)

(A) Positive and negative antigen results in serum according to RT-PCR cycle threshold values in NP samples. The red dotted line corresponds to a cycle threshold of 33. (B and C) Linear regression of cycle threshold results vs. antigenemia measured on the S-PLEX N and S-PLEX S. The grey dotted lines correspond to the positivity cut-off of antigen assays and the red dotted line corresponds to a cycle threshold of 33. Samples in orange are the ones with a cycle threshold <33 and with a negative antigen result in serum. (D) Positive and negative antigen results in serum according to S IgG values in serum. The blue dotted line corresponds to the positivity cut-off for S IgG. (E and F) Linear regression of S IgG results vs. antigenemia measured on the S-PLEX N and S-PLEX S. The grey dotted lines correspond to the positivity cut-off of antigen assays and the blue dotted line corresponds to the positivity cut-off for S IgG. Samples in orange are the ones with negative S IgG and with a negative antigen result in serum.
and asymptomatic patients (p=0.02 for N; p=0.03 for S) (Figure 2A, B). Of importance, the eight patients with negative S antigen were only observed in non-severe patients (Figure 2B). The Ct results were however not significantly different between the three severity categories (p-values ranging from 0.42 to 0.98) (Figure 2B).

In the literature, clinical sensitivities for N antigen assays have been reported to range from 85.2 to 100%, depending on the timing of inclusion of patients since RT-PCR [2, 5–7]. The strength of our study was the inclusion of patients with paired NP and blood samples allowing the direct comparison of the performance between antigen assays and RT-PCR. We found that the clinical sensitivity for the N antigen assay was 100% if considering Ct <33, a threshold considered as a surrogate of contagiousness since patients with low viral loads (i.e. Ct >33) have a very low risk of being infective and capable of transmitting the virus [8, 9]. In our study, we identified 10 patients with negative N antigen along with Ct results >33 (mean = 39, min–max: 33.5–44.2). These patients were also positive for S IgG, probably meaning that the “positive” RT-PCR results were attributed to a residual low viral load from a past-infection. Importantly, the timing of antigen detection in blood is crucial since it significantly decreases after approximately 14 days since symptoms [2, 4, 5, 7, 10]. It is therefore important to measure antigen in blood of patients in the first days after the onset of symptoms. The association of SARS-CoV-2 antibodies might therefore be useful in such settings.

We also found that the antigen assay targeting the N protein performed better compared to the S antigen assay (Figure 1A).

The higher clinical sensitivity of the N assay is probably due to the larger copy number of N proteins per viral particle (around 1,000). Furthermore, the large number of mutations in the S protein might also explain why assays targeting the S protein may lost the recognition capacity [4].

As observed in previous studies, higher concentrations of N antigens were observed in more severe patients [3–7, 10]. We also showed that severe patients had more S antigen compared to mild-moderate or asymptomatic patients. A better discrimination compared to RT-PCR regarding the severity of the disease was also observed.

In conclusion, the measurement of SARS-CoV-2 antigens in blood represents a valuable alternative to RT-PCR for the diagnosis of COVID-19, especially considering the measurement of N antigens. Additionally, antigens were significantly higher in more severe patients. The possibility of using antigenemia to facilitate patient triage to optimize better intensive care utilization deserves more investigations.

Figure 2: Antigenemia and RT-PCR results according to the disease severity. Panels (A and B) refer to N- and S-antigens in serum, respectively. Panel (C) refers to Ct results obtained on NP samples. The grey dotted lines correspond to the positivity cut-off of antigen assays.
Research funding: None declared.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The study protocol is available upon request and was in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee of Saint-Luc Bouge (Bouge, Belgium, approval number B0392020000005).

References