

# Closing the serological gap in the diagnostic testing for COVID-19: The value of anti-SARS-CoV-2 IgA antibodies

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## Abstract

During coronavirus disease 2019 (COVID-19) pandemic, the early diagnosis of patients is a priority. Serological assays, in particular immunoglobulin (Ig)M and IgG anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have today several applications but the interpretation of their results remains an open challenge. Given the emerging role of the IgA isotype in the COVID-19 diagnostics, we aimed to identify the SARS-CoV-2 IgA antibodies in a COVID-19 population seronegative for IgM. A total of 30 patients hospitalized in San Giovanni di Dio Hospital (Florence, Italy) for COVID-19, seronegative for IgM antibodies, have been studied for anti-SARS-CoV-2 antibodies. They all had a positive oro/nasopharyngeal swab reverse transcription-polymerase chain reaction result. Assays used were a chemiluminescent assay measuring SARS-CoV-2 specific IgM and IgG (S + N) and an ELISA, measuring specific IgG (S1) and IgA antibodies against SARS-CoV-2. Among the 30 patients, eight were positive for IgA, seven were positive for IgG (N + S), and two for IgG (S1), at the first point (5-7 days from the onset of symptoms). The IgA antibodies mean values at the second (9-13 days) and third (21-25 days) time points were even more than twice as high as IgG assays. The agreement between the two IgG assays was moderate (Cohen's K = 0.59; SE = 0.13). The inclusion of the IgA antibodies determination among serological tests of the COVID-19 diagnostic is recommended. IgA antibodies may help to close the serological gap of the COVID-19. Variations among anti-SARS-CoV-2 IgG assays should be considered in the interpretation of results.

## KEYWORDS

antiviral agents, immune globulin, immune responses, immunoglobulin, SARS coronavirus, virus classification

## 1 | INTRODUCTION

At the end of December 2019, 27 cases of pneumonia of unknown etiology were identified in Wuhan, China.<sup>1</sup> The causative agent was identified by the Chinese Center for Disease Control and Prevention in January 2020 directly from bronchoalveolar-lavage fluid samples and was then named severe acute respiratory syndrome coronavirus

2 (SARS-CoV-2). The disease-associated to it was referred to as coronavirus disease 2019 (COVID-19). Recently the World Health Organization (WHO) provided interim guidance to laboratories, showing the strategic use of diagnostic tests in different transmission scenarios of the COVID-19 outbreak, including how to justify their use when prioritizing patients due to the lack of proper facilities. The WHO document specifies the conditions necessary to consider a case

laboratory-confirmed by nucleic acid amplification tests (NAAT) for areas with not known or established SARS-CoV-2 circulation.<sup>2</sup>

It has been shown that the viral RNA can be detected from nasal and pharyngeal swabs, bronchoalveolar lavage, and blood plasma using real-time reverse-transcription polymerase chain reaction (RT-PCR).<sup>3-5</sup> The molecular diagnostic testing is particularly useful for the diagnosis and triage of patients, monitoring the spread of the disease, identifying strains and mutations, with the next-generation sequencing, and assessing the current infection status. These tests provide the message of whether the infection is active or not and can often be of great utility early in the course of infection as they are able to confirm the viral presence up to 2 days before the onset of symptoms.<sup>6</sup> Given that antibodies may not be detectable until 6 to 7 days after the symptom onset, molecular tests can accelerate the diagnostic window by up to 9 days. In cases where the NAAT is negative, the serological evaluation, both of the acute phase and the convalescence phase, may support and complete a diagnosis.<sup>7</sup> Serological assays are capable of verifying the immune response to SARS-CoV-2, the identification of seroconversion, and finally the characterization of the virus course.<sup>8</sup> The production of specific antibodies, in particular immunoglobulin (Ig)M, IgA, and IgG anti-SARS-CoV-2 should be used as an additional and noninvasive method, together with NAAT, in the COVID-19 diagnosis. It's increasingly evident the role of the serological testing also for disease surveillance, therapeutics, return-to-work screening tests, and vaccine applications. For all the above reasons, antibody tests commercially available are continuously increasing, with a wide variability of kits and test protocols.<sup>7</sup>

In 2004, Woo et al already described the longitudinal profiles of IgG, IgM, and IgA antibodies against the SARS-CoV nucleocapsid protein in patients with pneumonia due to SARS-CoV, with serum samples collected up to day 240 after the onset of the disease. The seroconversion times detected by the recombinant enzyme-linked immunosorbent assay (ELISA), intercepting the SARS-CoV nucleocapsid protein, and those detected through an indirect immunofluorescence assay (IIFA), were very similar. The median seroconversion times for IgG, IgM, and IgA detected by the IIFA were 17 days (17 days for ELISA), 16.5 days (20.5 days for ELISA), and 17.5 days (17 days for ELISA) after the disease onset, respectively. The levels of all three antibodies (IgG, IgM, and IgA) increased to detectable levels at the third week of illness.<sup>9</sup> On the contrary, an earlier study, in 2003, showed that the three isotypes began to be measurable at the second week.<sup>10</sup>

Since the outbreak of the pandemic COVID-19 in 2020, numerous research groups have become stubborn about the importance of kinetic of antibodies and about the immunological memory. However, the time required by serological tests is not negligible and the interpretation of the results is not always clear.<sup>11-13</sup> The group of Okba et al demonstrated that most PCR-confirmed SARS-CoV-2 infected patients were seroconverted by 2 weeks after the onset of the infection. Using a commercial ELISA method, they found that IgA antibodies showed higher sensitivity than IgG, but lower specificity.<sup>14</sup> IgA and IgG assays could both be used for serologic diagnosis, but IgG was longer lived, as previously

shown, after infection with SARS-associated coronavirus and was thus preferred for studies regarding the sero-surveillance.<sup>15</sup>

The group of Padoan et al<sup>16</sup> described the kinetics of IgM and IgG to SARS-CoV-2 using a chemiluminescent (CLIA) assay, showing a rapid increase of both IgM and IgG after 6 to 7 days from the onset of first symptoms. IgG presented 100% and IgM 88% sensitivity on day 12. In a subsequent study, coming from the same group, the characteristics of the kinetics of IgA antibodies in comparison to IgM were deepened from the COVID-19 onset. IgA response appeared and grew earlier, peaked at the third week, and maintained a response stronger and more persistent compared to the IgM one.<sup>17</sup>

Lippi et al studied the antibodies profile comparing anti-SARS-CoV-2 ELISA tests (IgA and IgG) to CLIA tests (IgG and IgM). In patients with symptoms onset  $\leq 5$  days the rate of positive antibodies was very low, whilst in those with symptoms onset between 5 to 10 days the rate of positive antibodies ranged between 15.4% and 53.8%. Notably, in patients with symptoms onset between 11 and 21 days, the rate of positive antibodies was 100% except for IgM antibodies, which was positive only in 60% of patients.<sup>18</sup>

With the viewpoint upcoming from Sethuraman et al<sup>19</sup> the two SARS-CoV-2 diagnostic screening tests most represented, such as RT-PCR and IgM and IgG ELISA, were analyzed in their variation over the time. They found that the total number of antibodies levels started to increase from the second week of symptoms onset. Although IgM and IgG antibodies were positive even as early as the fourth day after the first symptom onset, higher levels occurred in the second and third weeks. IgM and IgG seroconversion appeared in all patients between the third and the fourth week of clinical illness onset. IgM antibodies began to decline and reached lower levels by week 5 and almost disappeared by week 7, whereas IgG persisted beyond 7 weeks.<sup>20</sup> ELISA-based IgM and IgG antibody tests had greater than 95% specificity for the diagnosis of COVID-19, presenting a high sensitivity when used at the same time.<sup>21</sup>

The widespread of serological tests as quantitative determinations (ELISA, CLIA) can easily provide information about the kinetics of different immunoglobulin isotypes during the disease. Even if the majority of the literature studies just consider IgM and IgG antibodies as serological markers, there is evidence of the emerging role of the IgA isotype in the COVID-19 diagnostics. The main benefits of the anti-SARS-CoV-2 IgA antibodies are due to their early detection and to their high sensitivity.<sup>17,22</sup> For these reasons, this study aims to assess the temporal profile of specific anti-SARS-CoV-2 antibodies, searching for the IgA isotypes in a COVID-19 population seronegative for IgM, to investigate how it could close the serological gap.

## 2 | MATERIALS AND METHODS

### 2.1 | Methods

To study the kinetics of SARS-CoV-2 specific antibodies, two different assays were used: CLIA for IgM and IgG detection, and ELISA for IgG and IgA detection.

SARS CoV-2 antibodies IgM and IgG CLIA kits were derived from YHLO Biotech Co Ltd (Shenzhen, China), with two antigens of SARS CoV-2, coated on the magnetic beads of the CLIA assay: Nucleocapsid (N) and Spike (S) proteins. All antibody tests were performed by iFlash1800 fully automatic chemiluminescence immunoassay analyzer coming from YHLO Biotech Co Ltd. The amount of anti-SARS CoV-2 antibodies IgG (N + S) and IgM can be evaluated quantitatively and is positively correlated with the relative light units (RLU) measured by the chemiluminescence analyzer. iFlash1800 CLIA analyzer automatically calculates the concentration (AU/mL) based on the calibration curve. The reference value proposed by the manufacturer was 10 AU/mL both for IgM and IgG antibodies: hence samples with IgM and IgG concentration  $\geq 10$  AU/mL are considered positive (reactive).

SARS CoV-2 antibodies IgG and IgA ELISA kits were by Euroimmun Medizinische Labordiagnostika AG (Luebeck, Germany). Both test kits use a recombinant S1 domain of the spike protein of SARS-CoV-2, as ELISA microplate coating. All antibody tests were performed by Analyzer I, a fully automated ELISA system from Euroimmun AG (Luebeck, Germany). The amount of anti-SARS CoV-2 antibodies IgG and IgA can be evaluated semiquantitatively by calculating a ratio of the extinction of the control or patient sample over the extinction of the calibrator. Analyzer I instrument automatically calculates the ratio value in according to the specific formula. For both IgG (S1) and IgA tests, the cut-off recommended from manufacturers is  $\geq 1.1$ : hence sample with IgG and IgA value  $\geq 1.1$  ratio are considered positive (reactive).

## 2.2 | Patients

This study enrolled a total of 30 patients (mean age  $64 \pm 19$  years; 20 men and 10 women) hospitalized in San Giovanni di Dio Hospital (Florence, Italy) for COVID-19.

All COVID-19 patients were confirmed to be infected with SARS-CoV-2 detected in oropharyngeal and nasopharyngeal swabs through the RT-PCR. Blood samples were selected for their IgM seronegativity at 5 to 7 days from the onset of symptoms (time point 1).

Serological profiles were also evaluated longitudinally testing anti-SARS-CoV-2 antibodies at 9 to 13 days (time point 2) and at 21 to 25 days (time point 3).

## 2.3 | Statistical analysis

Statistical analysis was performed with SPSS 25.0 software (SPSS, Chicago, IL).

The normality of distribution for antibodies values was assessed by Shapiro-Wilks normality test ( $P \leq .05$ ). All data met the normality requirements for parametric statistics and were thus summarized as mean  $\pm$  SD.

The agreement among methods was calculated by Cohen's Kappa agreement. Kappa was considered moderate from 0.41 to

0.60, good from 0.61 to 0.80, and excellent from 0.81 to 1.  $P$  value  $< .05$  was considered as significant.

## 3 | RESULTS

Among the 30 patients seronegative for IgM antibodies at time point 1 (5-7 days from the onset of symptoms), eight were positive for IgA, seven were positive for IgG (N + S), and two for IgG (S1). The distribution of positive sera by each isotype is shown through the Venn diagram reported in Figure 1. Three of the eight IgA positive patients and IgM negative were IgG negative.

The kinetics of IgA, IgM, IgG (S1), and IgG (S + N) antibodies were longitudinally studied at three different time points: after 5 to 7 (T1), 9 to 13 (T2), and 21 to 25 (T3) days from the onset of COVID-19 symptoms. The population was stratified according to the IgA positivity. In group 1 (IgM negative/IgA positive) eight patients serologically negative at time point 1 for IgM but positive for IgA antibodies were included and in group 2 (IgM negative/IgA negative) 22 patients serologically negative at time point 1 for both IgM and IgA antibodies. The different sample concentrations were compared using the ratio (value/cutoff) to allow the comparison. In the group 1 (IgM negative/IgA positive) the IgA antibodies mean values at the second and third-time points were more than double the mean values of both IgG (Figure 2 and Table 1); in the group 2, all patients were positive for IgA at time T3 (Figure 3 and Table 2), as well as for IgG antibodies.

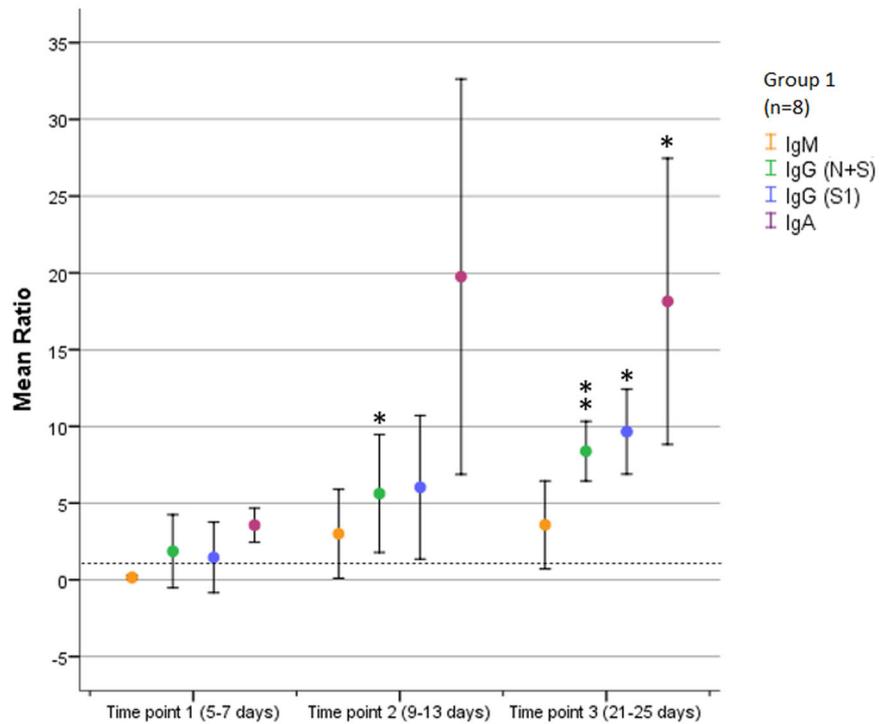
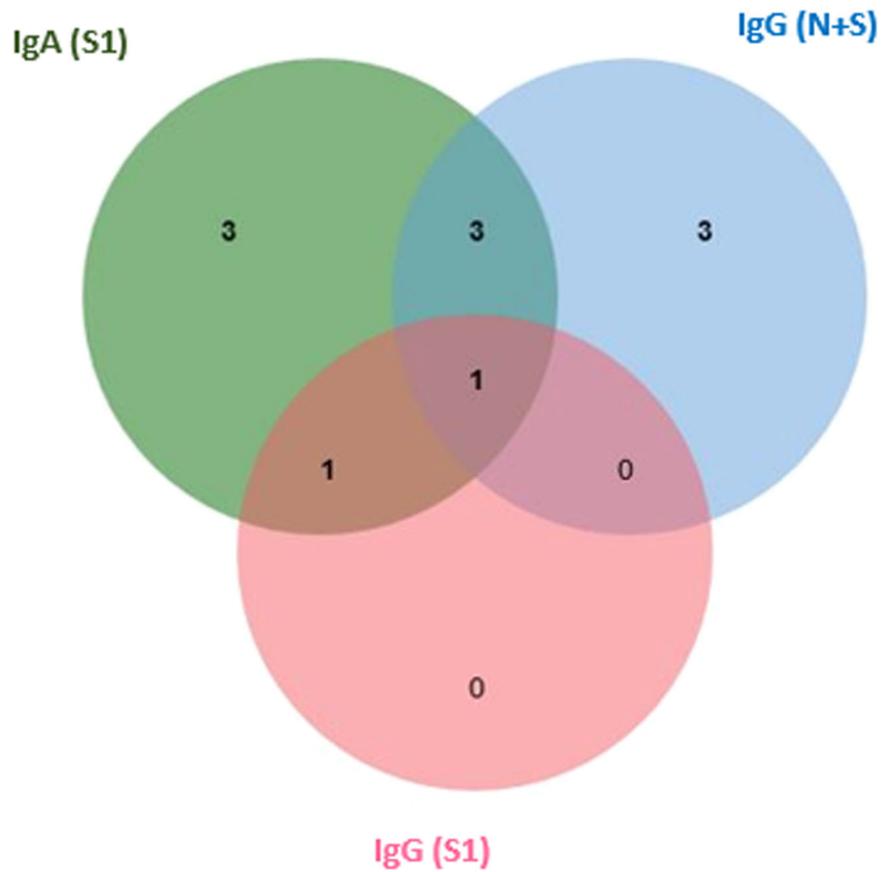
The agreement between the two assays, CLIA and ELISA, for measuring IgG antibodies, using N + S and S1 antigens respectively, was moderate (Cohen's  $K = 0.59$ ;  $SE = 0.13$ ).

## 4 | DISCUSSION

Our results showed that anti-SARS-CoV-2 IgA rises rapidly and reach concentrations markedly higher (over 18-fold the cut off) than those observed for IgM and IgG, at all the analyzed time points. At a longitudinal evaluation, anti-SARS-CoV-2 IgA was positive in 26.7% of IgM-negative patients at time T1, and in 100% at time T3. This suggests that IgA antibodies may play a role in bridging the serological gap of the disease, even a few days after the onset of symptoms. The earliness of the appearance and the high concentrations of IgA, persisting up to over 25 days from the disease onset, could greatly help the identification of COVID-19 patients. This would prompt the inclusion of the IgA antibodies determination among serological tests in the diagnostic work-up of the disease to increase its diagnostic accuracy, above all in patients with atypical symptoms, in asymptomatic, and in acute cases with repeatedly negative RT-PCRs.

The question of the possible prognostic significance of IgA antibodies has been recently addressed.<sup>23</sup> However, further studies evaluating their correlation with the trend and the severity of clinical manifestations are needed. IgA is both present in peripheral blood

**FIGURE 1** Venn diagram of SARS-CoV-2 specific antibodies in a population seronegative for IgM antibodies. IgG was tested with two different tests: ELISA (S1 antigen) and CLIA (N + S antigens). CLIA, chemiluminescent assay; ELISA, enzyme linked immunosorbent assay; Ig, immunoglobulin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

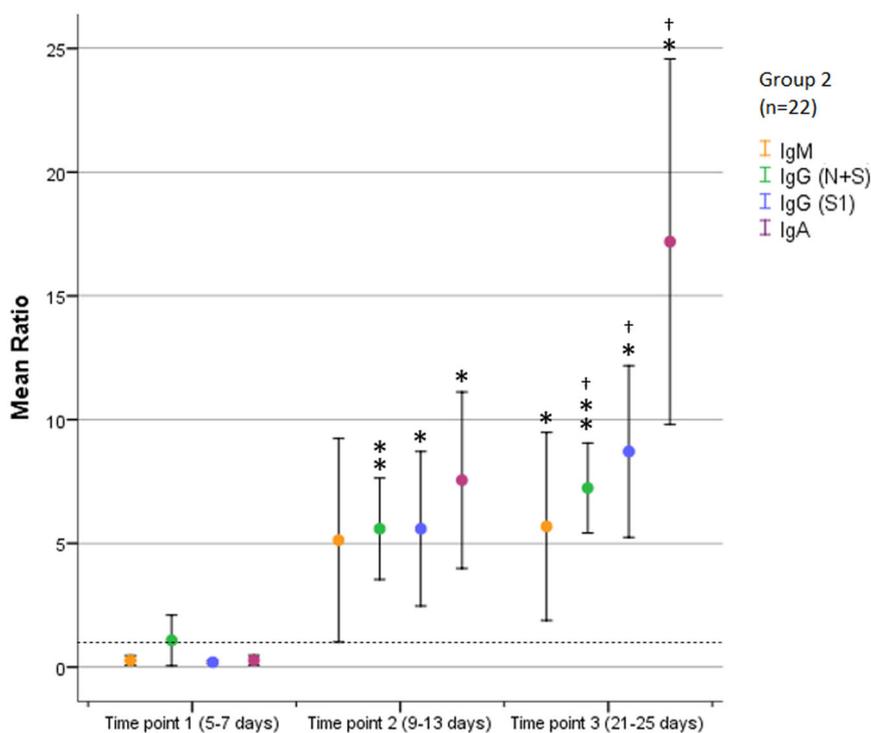


**FIGURE 2** Kinetics at three-time points of IgM, IgG (N + S), IgG (S1), and IgA expressed as mean (SD) ratio (value/cutoff) of group 1 (IgM neg/IgA pos). \*  $P < .05$  compared to Time point 1; \*\*  $P < .001$  compared to Time point 1. Ig, immunoglobulin

**TABLE 1** Mean values (SD) and frequencies of positive tests of the of group 1 (IgM neg/IgA pos) at three serial antibody determinations

	IgM Mean (SD)  Number of positive tests (%)	IgG (N + S) Mean (SD)  Number of positive tests (%)	IgG (S1) Mean (SD)  Number of positive tests (%)	IgA Mean (SD)  Number of positive tests (%)
<b>Time point 1</b>	0.15 (0.13)  0 (0%)	1.86 (2.38)  4 (50%)	1.46 (2.30)  2 (25%)	3.56 (1.10)  8 (100%)
<b>Time point 2</b>	3.00 (2.89)  4 (50%)	5.62 (3.84)  5 (62.5%)	6.03 (4.68)  4 (66.6%)	19.75 (12.88)  8 (100%)
<b>Time point 3</b>	3.58 (2.86)  5 (62.5%)	8.38 (1.94)  8 (100%)	9.65 (2.76)  8 (100%)	18.14 (9.32)  8 (100%)

Abbreviation: Ig, immunoglobulin.

**FIGURE 3** Kinetics at three-time points from the onset of symptoms of IgA, IgG (N + S), IgG (S1), and IgM expressed as mean (SD) ratio (value/cutoff) of group 2 (IgM neg/IgA neg). \*  $P < .05$  compared to Time point 1; \*\*  $P < 0.001$  compared to Time point 1; †  $P < .05$  compared to Time point 2. Ig, immunoglobulin**TABLE 2** Mean values (SD) and frequencies of positive tests for the of group 2 (IgM neg/IgA neg) at three serial antibody determinations

	IgM Mean (SD)  Number of positive tests (%)	IgG (N + S) Mean (SD)  Number of positive tests (%)	IgG (S1) Mean (SD)  Number of positive tests (%)	IgA Mean (SD)  Number of positive tests (%)
<b>Time point 1</b>	0.27 (0.29)  0 (0%)	1.1 (1.54)  3 (13.6%)	0.20 (0.11)  0 (0%)	0.28 (0.30)  0 (0%)
<b>Time point 2</b>	5.13 (6.13)  14 (64%)	5.59 (3.05)  20 (91%)	5.59 (4.65)  14 (64%)	7.55 (5.31)  20 (91%)
<b>Time point 3</b>	5.68 (5.65)  20 (91%)	7.24 (2.71)  22 (100%)	8.71 (5.16)  20 (91%)	17.19 (11.00)  22 (100%)

Abbreviation: Ig, immunoglobulin.

and at the mucosal side, where are locally produced to inhibit bacterial and viral barrier adhesion and invasion. Moreover, IgA is the only immunoglobulin capable to penetrate epithelial cells to neutralize intracellular viruses.<sup>24</sup> Notably, SARS-CoV2 is able to damage the respiratory mucosal barrier, entering in an alveolar cells by ACE2 receptor, and respiratory symptoms are between the main complaints in infected patients. Also, the gastrointestinal mucosa can be affected, with diarrhea described as quite frequent during the first phase of the disease.<sup>25</sup> Thus, it can be hypotized that high circulant IgA levels represent a mirror of this mucosal infiltration and of the reactive immune activation. It has to be clarified how, in COVID-19, the dual capacity of IgA antibodies, anti-inflammatory on one side and pro-inflammatory on the other, is modulated and whether the pro-inflammatory capacity of IgA, eventually resulting not only in innate immunity but also in T-cell (notably, T helper-1 cell) activation, can affect IgG production and long-term immunity.

Our study focuses on patients seronegative for IgM anti-SARS-CoV-2 and highlights the early positivity for IgA and IgG antibodies. Other studies in the literature report data relating to COVID-19 patients who demonstrated a previous seroconversion for IgG compared to IgM<sup>26</sup> but, it is not clear, whether this evidence is attributable exclusively to a lower sensitivity of IgM tests compared to that for IgG.

As regards the IgG antibody response, two tests were used with different methods and different SARS-CoV-2 antigenic targets. In all the cases studied longitudinally, the IgG (S1) antibodies appeared later compared to the IgG (N + S), reaching 93.3% positivity at time T3 compared to 100% IgG positivity (N + S). In our total results, the agreement between the two assays, CLIA for IgG (N + S) and ELISA for IgG (S1), was moderate (Cohen's K = 0.59) at variance with the data published by Padoan et al<sup>17</sup> who reported a better agreement (Cohen's K = 0.83). Such difference may likely be due to a difference in timing: in our study, the first point was very early (5-7 days), whereas in Padoan's study the late-stage occurred much later (up to 16 weeks). However, these observations focus our attention, together with consolidated literature data, on the ability of a number of viruses to induce prompt antibody production by B lymphocytes, regardless of the activation of T lymphocytes. Such antibody production is due to repetitive viral epitopes capable of inducing cytokine production/release by innate immunity cells. These antibodies are usually short-lived IgG. However, the cooperation of T cells is considered necessary for the development of sustained, long-lasting antiviral antibody responses, thanks to the formation of plasma cells and memory B cells.<sup>27-29</sup> The responses of CD4 T cells have been shown to be mainly directed towards protein spikes, and it is known that the production of neutralizing antibodies by plasma cells requires interactions with CD4 T cells specific for the same B lymphocyte activation protein.<sup>30</sup> On the basis of these literature data, it could be hypothesized that the production of IgG (N + S) antibodies, earlier than the appearance of IgG (S1) antibodies, would be attributable to T-cell-independent antibody production. IgG (S1) antibodies could represent the neutralizing and important humoral component in the development of immunity.

Prospective data with a long-term follow-up of antibody response in COVID-19 patients and Neutralization Test (PRNT) could elucidate the possible relationship between IgA response and sustained IgG response, likely protective.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## AUTHOR CONTRIBUTIONS

MI, SF, MM, and GP participated in the design of the study and drafted the manuscript. SP supported data analysis and revised the manuscript. VG, BL, MB, AD, AF, EMM, and MP participated in the design and revised the manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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