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## Spike is the most recognized antigen in the whole-blood platform in both acute and convalescent COVID-19 patients



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### ABSTRACT

**Objectives:** To identify the best experimental approach to detect a SARS-CoV-2-specific T cell response using a whole-blood platform.

**Methods:** Whole-blood from 56 COVID-19 and 23 “NO-COVID-19” individuals were stimulated overnight with different concentrations (0.1 or 1  $\mu\text{g}/\text{mL}$ ) of SARS-CoV-2 PepTivator<sup>®</sup> Peptide Pools, including spike (pool S), nucleocapsid (pool N), membrane (pool M), and a MegaPool (MP) of these three peptide pools. ELISA was used to analyse interferon (IFN)- $\gamma$  levels.

**Results:** The IFN- $\gamma$ -response to every SARS-CoV-2 peptide pool was significantly increased in COVID-19 patients compared with NO-COVID-19 individuals. Pool S and MegaPool were the most potent immunogenic stimuli (median: 0.51, IQR: 0.14–2.17; and median: 1.18, IQR: 0.27–4.72, respectively) compared with pools N and M (median: 0.22, IQR: 0.032–1.26; and median: 0.22, IQR: 0.01–0.71, respectively). The whole-blood test based on pool S and MegaPool showed a good sensitivity of 77% and a high specificity of 96%. The IFN- $\gamma$ -response was mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and independently detected of clinical parameters in both hospitalized and recovered patients.

**Conclusions:** This easy-to-use assay for detecting SARS-CoV-2-specific T cell responses may be implemented in clinical laboratories as a powerful diagnostic tool.

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### Introduction

The COVID-19 pandemic caused by Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) has recently emerged as a new human-to-human transmissible disease with a serious global health impact (Braun et al., 2020). SARS-CoV-2 is an enveloped virus with a positive stranded RNA genome and four structural proteins, including spike glycoprotein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N) (Koblichke et al., 2020; Le Bert et al., 2020).

Most infected patients present mild-to-moderate symptoms and approximately 15–20% develop severe disease (Wu and McGoogan, 2020). The majority of patients infected with COVID-19 have normal or reduced white cell counts and lymphocytopenia, and those with severe disease show significantly elevated levels of neutrophils, with a continuing decrease in lymphocytes (Costela-Ruiz et al., 2020). SARS-CoV-2 infection activates innate and adaptive immune responses (Shah et al., 2020). Recent studies have highlighted the role of the adaptive immune response in viral control and immunopathogenesis during acute SARS-CoV-2 infection, and particularly the role of T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) in establishing durable protective immunity against reinfection (Vabret et al., 2020; Shrotri et al., 2021).

Currently, the evaluation of population immunity is based on seroprevalence studies; however, in the context of evidence for cellular responses in seronegative exposed individuals (Gallai

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et al., 2021; Freeman et al., 2004; Heller et al., 2013; Mizukoshi et al., 2008) and the potential waning of antibody responses over time (Shrotri et al., 2021; Ojeda et al., 2021), current surveillance methods are likely to be underestimating both exposure and immunity. On the other hand, recent studies have demonstrated the presence of SARS-CoV-2-reactive T cells in a large number of patients with COVID-19 and also in unexposed individuals (Mateus et al., 2020; Ni et al., 2020; Grifoni et al., 2020; Echeverría et al., 2021). Moreover, CD4<sup>+</sup> and CD8<sup>+</sup> T cells targeting structural viral proteins appear to confer broad and long-lasting protection against SARS-CoV (Liu et al., 2017). Thus, a better understanding of the role of T cells in the long-term protection from COVID-19 is crucial in estimating population-level immunity, vaccine development, and long-term surveillance of vaccine efficacy (Dan et al., 2021).

Cytokine-release-based tests in whole-blood have been employed for several infectious diseases (Kim, 2020; Goletti et al., 2018a,b; Mahmoudi et al., 2017; Petrone et al., 2017, 2021a; Dammermann et al., 2015). Recently, a whole-blood approach was scouted by the current (Petrone et al., 2021a,b,c) and other groups (Murugesan et al., 2020; Echeverría et al., 2021) to evaluate the specific immune response in COVID-19 patients. So far, different experimental approaches and clinical settings have been adopted, leading to different results. In particular, regarding the experimental procedures, peptides corresponding to different viral genome regions (spike, membrane, nuclear proteins or others), concentration of peptides, and read-out have been employed. Moreover, different clinical settings involving acute (Petrone et al., 2021a) or convalescent (Murugesan et al., 2020; Echeverría et al., 2021) COVID-19 subjects have been involved. This study used a whole-blood interferon (IFN)- $\gamma$  release assay (IGRA) to characterize the IFN- $\gamma$  response to different SARS-CoV-2 peptides in acute hospitalized and post-acute non-hospitalized COVID-19 patients, and to identify the best experimental approach to detect a SARS-CoV-2-specific T cell response.

## Materials and methods

### Study design

The prospective study was approved by the Ethical Committee of Lazzaro Spallanzani National Institute of Infectious Diseases (INMI) (approval number 59/2020) and was conducted between 10 December 2020 and 05 February 2021. Informed, written consent was required to consecutively enroll patients and controls by physicians. Demographic and clinical information were collected at enrollment. Inclusion criteria for COVID-19 patients were a diagnosis based on a positive nasopharyngeal swab for SARS-CoV-2 and a disease with the clinical characteristics already described [Lazzaro Spallanzani National Institute of Infectious Diseases (INMI) Recommendations for COVID-19 management] (Nicastri et al., 2020). Exclusion criteria were: HIV infection, inability to sign an informed consent and age <18 years.

The hospitalized COVID-19-patients were classified as mild, moderate, severe, and critical, according to WHO (WHO, 2020). This study reported the highest severity score of the disease occurring during hospitalization. Briefly, mild COVID-19 patients had symptoms but did not have viral pneumonia or hypoxia; moderate COVID-19 patients had pneumonia and SpO<sub>2</sub>  $\geq$  90% on room air; severe COVID-19 patients had pneumonia and a respiratory rate >30 breaths/min or severe respiratory distress or SpO<sub>2</sub> < 90% on room air; critical COVID-19 patients had acute respiratory distress syndrome. The “NO-COVID-19” group with 23 participants included 16 healthy blood donor (HD) volunteers from UOC Transfusion Medicine and Stem Cell Unit, San Camillo Forlanini Hospital (Rome, Italy), and seven patients with active

tuberculosis. Inclusion criteria for the NO COVID-19 group were negative SARS-CoV-2 serology and no symptoms of COVID-19. A portion of them also had a negative swab for the molecular identification of SARS-CoV-2.

### Peptide pools

SARS-CoV-2 PepTivator<sup>®</sup> Peptide Pools (Miltenyi Biotec, Germany) were used, including: the spike protein (PepTivator<sup>®</sup> SARS-CoV-2 Prot\_S1, Prot\_S, and Prot\_S+) (pool S), the nucleocapsid phosphoprotein (PepTivator<sup>®</sup> SARS-CoV-2 Prot\_N) (pool N), and the membrane glycoprotein (PepTivator<sup>®</sup> SARS-CoV-2 Prot\_M) (pool M). The PepTivator<sup>®</sup> Peptide Pools comprise peptides of 15 amino acid length with 11 amino acid overlap. The peptides were grouped into different pools, including: pool S (equal amounts of Prot\_S1, Prot\_S, and Prot\_S+), pool N, pool M, and a MegaPool (MP) of equal amounts of all of these peptides.

### IFN- $\gamma$ whole-blood assay

Whole-blood (600  $\mu$ L) was stimulated or not with two different concentrations (0.1  $\mu$ g/mL and 1  $\mu$ g/mL) of different peptide pools. Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, Milan, Italy) (200 ng/mL) was used as a positive control. Plasma was harvested after overnight (20–24 h) stimulation in a 48-well flat-bottom plate at 37 °C (5% CO<sub>2</sub>) and stored at –80 °C. IFN- $\gamma$  levels were evaluated by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions ([www.quantIFERON.com](http://www.quantIFERON.com)). IFN- $\gamma$  values were subtracted from the unstimulated control. The lower and upper detection limits of the test were 0.065 and 10 IU/mL, respectively.

### PBMCs culture conditions and stimulations

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (Pancoll human, PAN Biotech, Germany) and resuspended in complete RPMI-1640 medium, with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (Euroclone S.p.A, Italy). To characterize the specific T cell response by flow cytometry, fresh  $1 \times 10^6$  PBMCs were resuspended in 1 mL of complete medium and stimulated with pools S, N, M at 0.1  $\mu$ g/mL, and SEB (200 ng/mL) as a positive control. Anti-CD28 and anti-CD49d monoclonal antibodies (mAbs) at 2  $\mu$ g/mL each were added to co-stimulate cells. After 1 h, 1  $\mu$ L/ $1 \times 10^6$  of Golgi plug (BD Biosciences San Jose, USA) was added to each sample to inhibit cytokine secretion, according to manufacturer's instructions. Following an incubation period of 16–24 h, cells were stained as described below.

### T cell phenotyping and intracellular staining

PBMCs were stained with fluorochrome conjugated antibodies according to the standard operating procedure. T cells were stained with the following antibodies: CD3 phycoerythrin-cyanine 7 (PE-Cy7), CD4 Brilliant Violet (BV)711 and CD8 allophycocyanin-H7 (APC-H7) (all from BD Biosciences). The Cytofix/Cytoperm kit (BD Biosciences) was used for sub-sequential intracellular staining of IFN- $\gamma$  allophycocyanin (APC). Dead cells were first excluded from analysis by side/forward scatter gating and then by Fixable Viability stain 700 (BD Biosciences). At least 100,000 gated events on living cells were analysed for each sample, whenever possible. Samples were acquired on a BD Lyric (BD Biosciences) cytometer. Data were analysed with FlowJo software, version 10 (Tree Star).

SARS-CoV-2 serology

SARS-CoV-2-specific IgM and IgG levels were measured by ELISA, according to manufacturer’s instructions (DIESSE Diagnostica Senese S.p.a., Monteriggioni, Italy). The ratio between the optical density (OD) of the sample and that of the cut-off reagent (index) was calculated. The samples were scored positive (index >1.1), doubtful (index between 1.1 and 0.9), and negative (index <0.9).

Statistical analysis

Data were analyzed using Graph Pad (GraphPad Prism 8 XML ProjecT) and Stata (Stata 15, StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). Medians and interquartile ranges (IQRs) were calculated. The following tests were used: Kruskal–Wallis test for comparisons among groups; Wilcoxon test for paired groups; Mann–Whitney U test with Bonferroni correction for pairwise comparisons; Friedman test to compare multiple paired groups; Chi-squared test for categorical variables; receiver operator characteristic (ROC) analysis for evaluating diagnostic performance; roccomp command in STATA for testing the equality of ROC areas; Spearman’s rank correlation for correlations:  $r_s >0.7$  high correlation,  $0.7 < r_s < 0.5$  moderate correlation and  $r_s < 0.5$  low correlation.

**Table 1**  
Demographical and clinical characteristics of the enrolled subjects.

| Characteristics                                      | COVID-19  |                  | NO-COVID-19 | P value             |                       |
|--|---|------------------|-------------|---------------------|-----------------------|
|  | Hospitalized                                      | Non-Hospitalized |             |                     |                       |
| N (%)  | 47 (59.5)   | 9 (11.4)         | 23 (29.1)   |                     |                       |
| Age median (IQR)                                     | 61 (55–75)  | 57 (31–61)       | 45 (38–54)  | <0.0001*            |                       |
| Male N (%)   | 31 (65.9)   | 2 (22.2)         | 18 (78.2)   | 0.0112 <sup>§</sup> |                       |
| Origin N (%)   | West Europe                                       | 46 (97.9)        | 7 (77.8)    | 18 (78.3)           | 0.0658 <sup>§</sup>   |
|  | East Europe                                       | 0 (0)            | 1 (11.1)    | 2 (8.7)             |                       |
|  | Asia  | 1 (2.1)          | 1 (11.1)    | 1 (4.3)             |                       |
|  | Africa  | 0 (0)            | 0 (0)       | 2 (8.7)             |                       |
| Swab positive results at the time of enrolment N (%) | 37 (72.5)   | 3 (33.3)         | 0 (0)       |                     |                       |
| Days from symptom onset N (%)                        | Available   | 41 (87.2)        | 5 (55.6)    | –                   |                       |
|  | 1–7   | 13 (31.7)        | –           | –                   |                       |
|  | 8–14  | 12 (29.3)        | –           | –                   |                       |
|  | 15–30   | 11 (26.8)        | –           | –                   |                       |
|  | >30   | 5 (12.2)         | 5 (100)     | –                   |                       |
| Lymphocyte count N (%)                               | Available   | 47 (100)         | –           | –                   |                       |
| Lymphocyte count N (%) median (IQR)                  | <1 × 10 <sup>3</sup> /μL                          | 15 (31.9)        | –           | –                   |                       |
|  | 0.75 (0.58–0.92)                                  | 23 (49)          | –           | –                   |                       |
|  | ≤1 × 10 <sup>3</sup> /μL <2 × 10 <sup>3</sup> /μL | 1.53 (1.19–1.70) | –           | –                   |                       |
|  | ≥2 × 10 <sup>3</sup> /μL                          | 9 (19.1)         | –           | –                   |                       |
| Serology results IgM N (%)                           | 2.80 (2.51–4.51)                                  | 24 (51)          | 1 (11.1)    | 0 (0)               | 0.0871 <sup>§§</sup>  |
|  | IgM <sup>+</sup>                                  | 20 (42.6)        | 7 (77.8)    | 23 (100)            |                       |
|  | IgM <sup>–</sup>                                  | 3 (6.4)          | 1 (11.1)    | 0 (0)               |                       |
|  | IgM doubtful                                      | 37 (78.7)        | 7 (77.8)    | 0 (0)               | 0.892 <sup>§§</sup>   |
| Serology results IgG N (%)                           | IgG <sup>+</sup>                                  | 9 (19.2)         | 2 (22.2)    | 23 (100)            |                       |
|  | IgG <sup>–</sup>                                  | 1 (2.1)          | 0 (0)       | 0 (0)               |                       |
|  | IgG doubtful                                      | 46 (97.9)        | 7 (77.8)    | –                   | <0.0001 <sup>§§</sup> |
| Severity N (%) <sup>#</sup>                          | Available   | 1 (2.2)          | 6 (85.7)    | –                   |                       |
|  | Mild  | 8 (17.4)         | 0 (0)       | –                   |                       |
|  | Moderate  | 23 (50)          | 1 (14.3)    | –                   |                       |
|  | Severe  | 14 (30.4)        | 0 (0)       | –                   |                       |
|  | Critical  | 33 (70.2)        | 1 (11.1)    | –                   |                       |
|  | Available   | 0 (0)            | 1 (100)     | –                   |                       |
| Cortisone therapy N (%)                              | Mild  | 4 (12.1)         | –           | –                   |                       |
|  | Moderate  | 19 (57.6)        | –           | –                   |                       |
|  | Severe  | 10 (30.3)        | –           | –                   |                       |
|  | Critical  | –                | –           | –                   |                       |

COVID-19: coronavirus disease 19; N: number.

\* Kruskal–Wallis statistic test.

§ Chi-square test.

# WHO criteria (ref WHO).

§§ Chi-square test performed only on COVID-19 cohorts.

Results

Description of the studied population

Fifty-six (47 acute hospitalized and nine recovered non-hospitalized) COVID-19 patients and 23 NO-COVID-19 subjects were prospectively enrolled. COVID-19 patients were classified based on days from symptom onset and disease severity, as reported in Table 1. A higher proportion of acute-hospitalized patients had a severe or critical illness compared with recovered patients ( $p < 0.0001$ ). Demographic and clinical information are detailed in Table 1.

The IFN-γ response to SARS-CoV-2 peptides was increased in COVID-19 compared with NO-COVID-19 individuals

First, the study aimed to evaluate the optimum concentration of viral peptides to use in the whole-blood platform. It performed a dose concentration–response analysis of 0.1 μg/mL and 1 μg/mL concentrations of pools S, N, M, and MegaPool (MP) on a cohort of 23 COVID-19 and 22 NO-COVID-19 subjects (Figure S1 A–D). A significant difference was found in response to all SARS CoV-2 peptide pools between the concentrations tested both in COVID-19 (pool N  $p = 0.0005$ ; pool M  $p < 0.0001$ ; pool S  $p = 0.0003$ ; and MP  $p = 0.0007$ ) and NO-COVID-19 individuals (pool N  $p = 0.0047$ ; pool

M  $p = 0.0013$ ; pool S  $p = 0.0003$ ; and MP  $p < 0.0001$ ). After stimulation with SEB, employed as a non-specific stimulation to evaluate the immune ability to respond, no significant differences were found within the COVID-19 and NO-COVID-19 groups (median: 11.31, IQR 0.82–16.69 vs median: 12.77, IQR 12.45–12.93;  $p = 0.051$ , respectively) (Figure S2 A).

A ROC analysis was then performed to define the best concentration of each peptide pool for discriminating COVID-19 patients from NO-COVID-19 individuals. Similar and significant area under the curves (AUC) were obtained based on both concentrations tested for pools N (AUC = 0.73,  $p = 0.006$  vs AUC = 0.76,  $p = 0.003$ , respectively), M (AUC = 0.68,  $p = 0.036$  vs AUC = 0.72,  $p = 0.011$ , respectively), S (AUC = 0.85,  $p < 0.0001$  vs AUC = 0.85,  $p < 0.0001$ , respectively), and MP (AUC = 0.87,  $p < 0.0001$  vs AUC = 0.86,  $p < 0.0001$ , respectively) (Figure S1 F–I). Comparison of the ROC curves showed no significant differences in terms of accuracy between the whole-blood test based on peptide pools used at 0.1  $\mu\text{g/mL}$  and at 1  $\mu\text{g/mL}$  (Table 2). Therefore, the following experiments were performed using the 0.1  $\mu\text{g/mL}$  concentration for pools S, M, and MP. For pool N, a concentration of 1  $\mu\text{g/mL}$  was chosen due to a higher accuracy found at 1  $\mu\text{g/mL}$  compared with the 0.1  $\mu\text{g/mL}$  concentration.

Once the best concentration for each peptide pool had been identified, the initial cohort of subjects reaching the number of 56 COVID-19 and 23 NO-COVID-19 individuals was formed. As expected, it was found that the IFN- $\gamma$  levels in response to pools N, M, S, and MP stimulations were significantly higher in COVID-19 compared with NO-COVID-19 individuals ( $p < 0.0001$ ,  $p = 0.0005$ ,  $p < 0.0001$ , and  $p < 0.0001$ , respectively) (Figure 1A, C, E, G). In particular, pool S and MP were the most potent immunogenic stimuli (IFN- $\gamma$  response to pool S, median: 0.51, IQR: 0.14–2.17; to MP, median: 1.18, IQR 0.27–4.72) compared with pools N and M that showed a similar lower response (pool N, median: 0.22, IQR: 0.032–1.26; pool M, median: 0.22, IQR 0.01–0.71, respectively). The ROC analyses performed on the larger cohort confirmed the accuracy of the test for the diagnosis of COVID-19, showing significant and even high AUC for pools S (AUC = 0.90,  $p < 0.0001$ ) and MP (AUC = 0.89,  $p < 0.0001$ ), followed by pools N (AUC = 0.81,  $p < 0.0001$ ) and M (AUC = 0.74,  $p = 0.0008$ ) (Figure 1B, D, F, H). Based on the likelihood ratio, the cut-off for scoring purposes was defined (0.13 IU/mL for the IFN- $\gamma$  response to pool S and 0.24 IU/mL for MP) identifying a good sensitivity of 77% and a high specificity of 96% for both pool S and MP. For pools N and M, a cut-off of 0.13 IU/mL and 0.19 IU/mL was defined, respectively, which showed a sensitivity of 61% for pool N and 52% for pool M, and a specificity of 96% for pool N and 91% for pool M, respectively.

*The IFN- $\gamma$  production in response to SARS-CoV-2 specific stimulations was mediated by CD4<sup>+</sup> and CD8<sup>+</sup> T cells*

To define the T cell subsets responsible for the SARS-CoV-2 immune response, the ability of T cells to produce IFN- $\gamma$  in response to stimulation with pools S, N, and M was evaluated by

flow cytometry. The analysis was performed on fresh PBMCs isolated from a cohort of three hospitalized COVID-19 patients. It was demonstrated that all COVID-19 patients responded to SEB stimulation (Figure 2A) and that SARS-CoV-2 response was mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 2B and C).

*The IFN- $\gamma$  response to SARS-CoV-2 peptides was detected in COVID-19 patients independently of disease severity, symptom onset and lymphocyte count*

To evaluate whether the IFN- $\gamma$  response was associated with the acute phase of the disease, the COVID-19 patients were stratified according to their hospitalization status. No significant differences were observed in the IFN- $\gamma$  levels in response to all stimuli between acute hospitalized and post-acute non-hospitalized (recovered) COVID-19 patients (Figure 3). Within acute hospitalized COVID-19 patients, the characteristics of patients were evaluated as potential factors impacting the IFN- $\gamma$  response to SARS-CoV-2 peptides (Table 3). Patients were stratified based on disease severity (mild/moderate, severe or critical), COVID-19 symptom onset within 1–7 days, 8–14 days, 15–30 days or >30 days in respect to whole-blood stimulation, serology or cortisone therapy. The IFN- $\gamma$  response to all SARS-CoV-2 peptides was independent of the characteristics of patients. A positive and significant low correlation between IFN- $\gamma$  levels and the number of lymphocytes ( $r_s = 0.30$ ,  $p = 0.048$ ;  $r_s = 0.31$ ,  $p = 0.037$ , respectively) or IgG index ( $r_s = 0.29$ ,  $p = 0.043$ ;  $r_s = 0.29$ ,  $p = 0.052$ , respectively) were found only for pool M and SEB.

*The IFN- $\gamma$  response to SARS-CoV-2 peptides was detected in COVID-19 patients with both positive and negative SARS-CoV-2 serology*

Acute hospitalized COVID-19 patients were stratified based on serology results. Patients who had doubtful serology only for IgG ( $n = 1$ ) or IgM ( $n = 3$ ) were included in the IgG<sup>+</sup>/IgM<sup>+</sup> group. No significant differences in the IFN- $\gamma$  levels were found in response to pools N ( $p = 0.43$ ), M ( $p = 0.39$ ), S ( $p = 0.63$ ), and MP ( $p = 0.34$ ) (Figure 4). Interestingly, the IFN- $\gamma$  response was detected in seven patients who scored IgG-negative and IgM-negative that showed a higher IFN- $\gamma$  response to pool S and MP (median: 1.22, IQR 0.20–5.41; median: 1.54, IQR 0.57–9.65, respectively) compared with pools N and M (median: 0.17, IQR 0.03–1.60; median: 0.26, IQR 0–0.51, respectively). Surprisingly, among the patients who scored negative to IgG/IgM there was one patient whose symptom onset dated back to 6 days before the time of blood stimulation and became IgG-positive after 5 days from the IFN- $\gamma$  blood test.

## Discussion

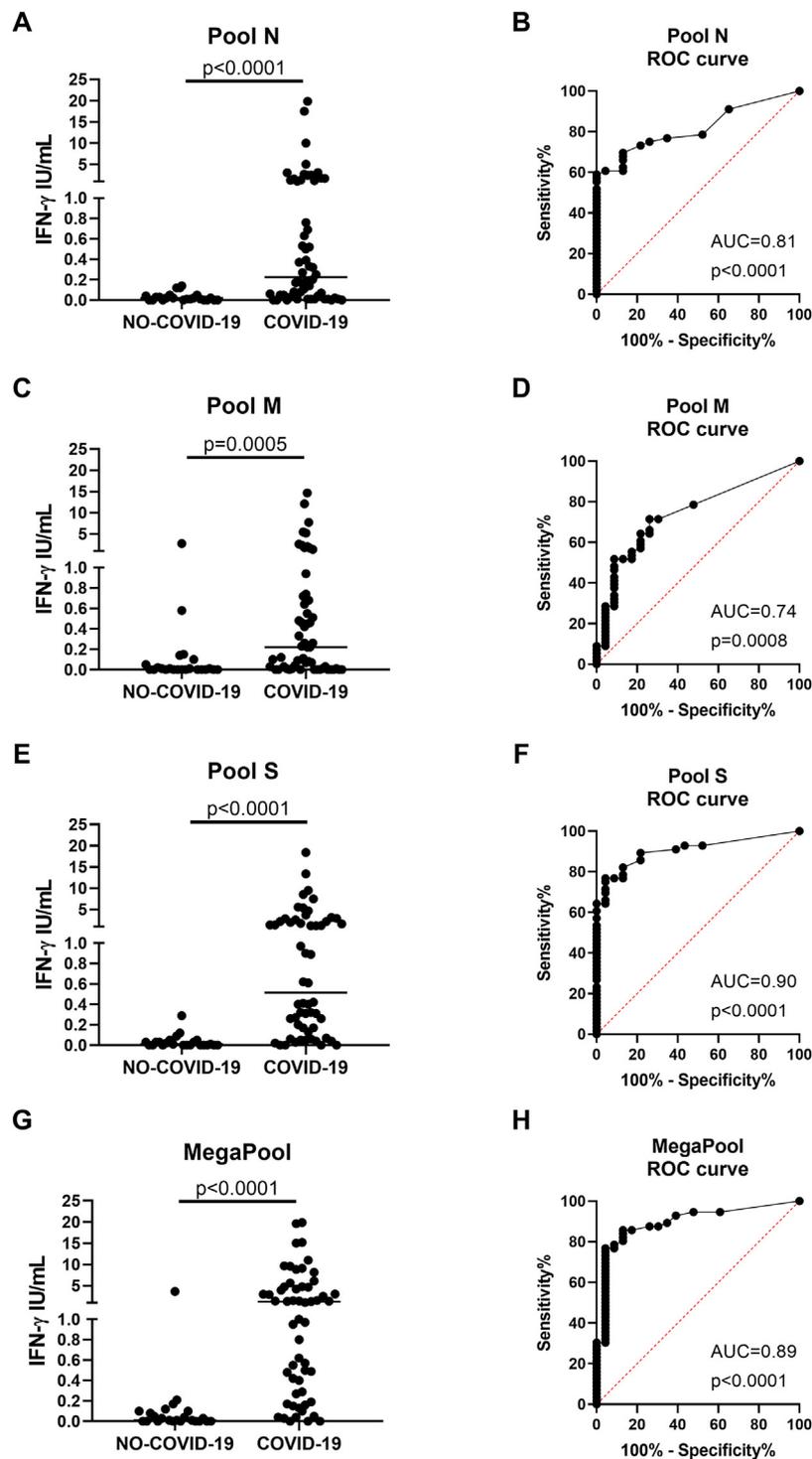
This study demonstrated in a cohort of hospitalized COVID-19 patients, COVID-19-recovered individuals and COVID-19-unexposed subjects that an IFN- $\gamma$  test based on whole-blood stimulated with SARS-CoV-2-specific peptide pools corresponding to spike,

**Table 2**

Comparison of the ROC curves generated from COVID-19 and NO-COVID-19 groups stimulated with SARS-CoV-2 peptide pools used at 0.1  $\mu\text{g/mL}$  and 1  $\mu\text{g/mL}$ .

| Peptide pools |                      | AUC    | 95% CI        | P value | ROC comparison p value |
|---------------|----------------------|--------|---------------|---------|------------------------|
| Pool N        | 0.1 $\mu\text{g/mL}$ | 0.7372 | 0.5860–0.8883 | 0.0064  | 0.5487                 |
|               | 1 $\mu\text{g/mL}$   | 0.7579 | 0.6104–0.9054 | 0.0030  |                        |
| Pool M        | 0.1 $\mu\text{g/mL}$ | 0.6828 | 0.5251–0.8405 | 0.0357  | 0.4439                 |
|               | 1 $\mu\text{g/mL}$   | 0.7213 | 0.5692–0.8735 | 0.0110  |                        |
| Pool S        | 0.1 $\mu\text{g/mL}$ | 0.8518 | 0.7354–0.9681 | <0.0001 | 0.8177                 |
|               | 1 $\mu\text{g/mL}$   | 0.8587 | 0.7449–0.9725 | <0.0001 |                        |
| MegaPool      | 0.1 $\mu\text{g/mL}$ | 0.8775 | 0.7686–0.9863 | <0.0001 | 0.5221                 |
|               | 1 $\mu\text{g/mL}$   | 0.8597 | 0.7503–0.9691 | <0.0001 |                        |

ROC: receiver-operator characteristic; AUC: area under the curve; CI: confidence interval.

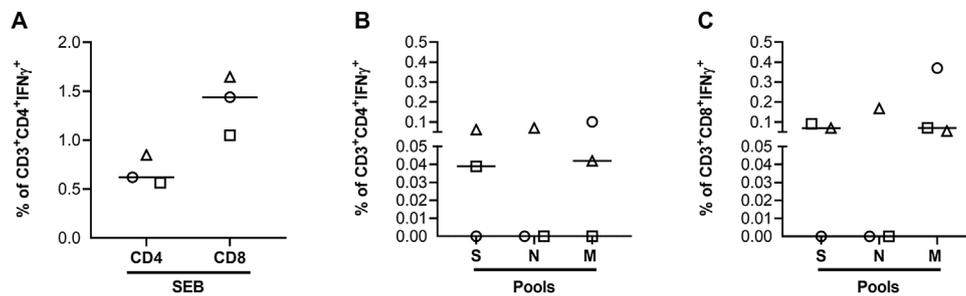


**Figure 1.** Spike and MegaPool peptides were the most immunogenic SARS-CoV-2 antigens in the whole-blood platform. The IFN- $\gamma$  level in response to SARS-CoV-2 peptides was significantly increased in COVID-19 patients compared with NO-COVID-19 individuals after stimulating whole-blood with 1  $\mu$ g/mL of pool N (A) and 0.1  $\mu$ g/mL of pool M (C), pool S (E) and MegaPool (G) antigens. The ROC analysis shows significant AUC results for pool N (AUC = 0.81,  $p < 0.0001$ , B), pool M (AUC = 0.74,  $p = 0.0008$ , D), but mainly for pool S (AUC = 0.90,  $p < 0.0001$ , F) and MegaPool (AUC = 0.89,  $p < 0.0001$ , H) antigens. For scoring purposes, the cut-off of 0.13 IU/mL and of 0.19 IU/mL were chosen for pools N and M antigens, respectively, that led to 61% sensitivity/96% specificity for pool N and 52% sensitivity/91% specificity for pool M. For scoring purposes, the cut-off of 0.13 IU/mL and of 0.24 IU/mL were chosen for pool S and MegaPool antigens, respectively, that led to 77% sensitivity/96% specificity for both antigens. IFN- $\gamma$  was measured by ELISA in stimulated plasma. The horizontal lines represent the median; statistical analysis was performed using the Mann-Whitney test and ROC analysis, and p-value was considered significant if  $\leq 0.05$ .

IFN, interferon; COVID-19: coronavirus disease 19; N: nucleocapsid; M: membrane; S: spike; ROC: receiver operating curve.

nucleoprotein, membrane or a mix of them has good accuracy to discriminate COVID-19-hospitalized or -recovered patients from healthy unexposed individuals. Among the stimuli that were used, the best was pool S, followed by MP, pool N, and pool M.

Interestingly, the T cell response was also found in individuals who scored negative to SARS-CoV-2 serology. Moreover, flow cytometry showed that the specific response to pools S, N and M was mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This T cell-based test may



**Figure 2.** The Sars-CoV-2-specific response was mediated by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Freshly isolated PBMCs were stimulated overnight with different stimuli to evaluate by flow cytometry the CD4<sup>+</sup> or CD8<sup>+</sup> T cell-specific IFN- $\gamma$  response.

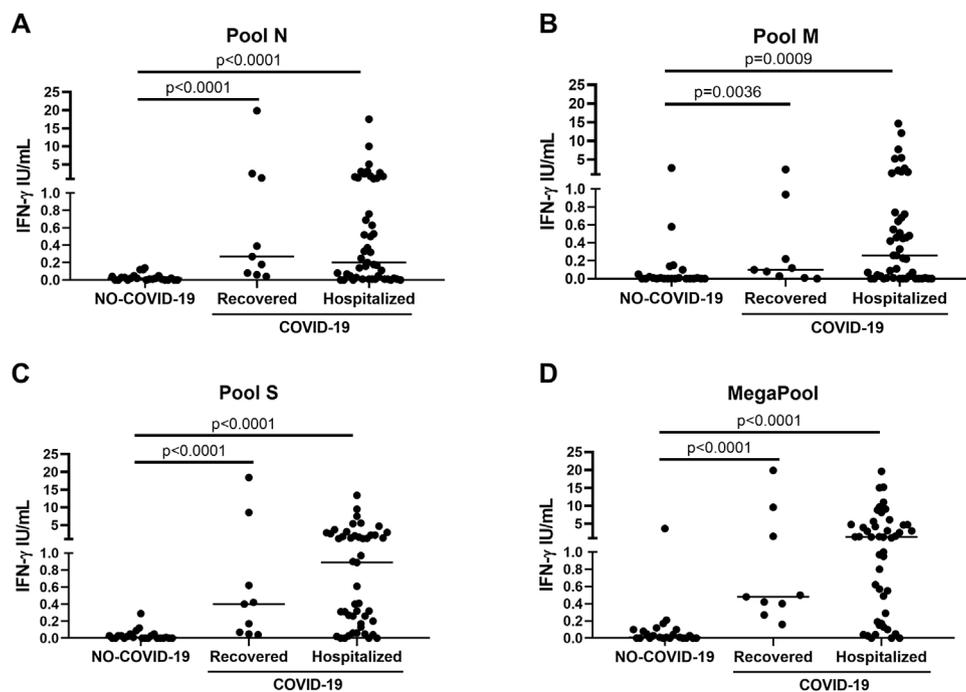
A) Percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells producing IFN- $\gamma$  in response to the positive control SEB.

B) Percentage of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in response to pools S, N and M.

C) Percentage of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells in response to pools S, N and M.

In all graphs the IFN- $\gamma$  values were subtracted from the unstimulated control. The horizontal lines represent the median and statistical analysis was performed using the Friedman test and  $p \leq 0.05$  was considered significant.

Footnotes: IFN, interferon; N, nucleocapsid; M, membrane; S, spike.



**Figure 3.** The IFN- $\gamma$  response to pools N, M, S and MegaPool was detectable in both recovered and hospitalized COVID-19 patients. Analysis of the IFN- $\gamma$  response to pools N (A), M (B), S (C) and MegaPool (D) antigens in COVID-19 patients either hospitalized (n = 47) or recovered (n = 9). No significant differences were observed in response to all stimuli between the two groups of COVID-19 patients. IFN- $\gamma$  was measured by ELISA in stimulated plasma. Statistical analysis was performed using the Mann-Whitney test and  $p \leq 0.05$  was considered significant.

IFN, interferon; COVID-19: coronavirus disease 19; N, nucleocapsid; M, membrane; S, spike.

be a good approach with which to study the specific response in COVID-19 patients during the acute phase, at recovery and likely in SARS-CoV-2-vaccinated individuals (Manisty et al., 2021).

The assay described in the present study had a higher accuracy compared to whole-blood tests that have been previously described (Petrone et al., 2021a,b,c; Murugesan et al., 2020; Echeverría et al., 2021). The specificity of this test to detect SARS-CoV-2 infection was high. Based on the peptide concentration used, SARS-CoV-2-specific T cells were rarely found in NO-COVID-19 individuals, as differently reported in previous reports (Grifoni et al., 2020; Weiskopf et al., 2020; Sette and Crotty, 2020; Echeverría et al., 2021).

Different from an earlier study (Petrone et al., 2021a), this study analysed different peptide pools covering the whole spike region; additionally, it included other peptides from other viral proteins, as

the nucleocapsid and membrane proteins. Based on this peptide selection, the IFN- $\gamma$ -based test in response to spike was shown to be more accurate for SARS-CoV-2 detection of infection, with 77% sensitivity and 96% specificity compared with 60% sensitivity and 86.2% specificity reported in previous work (Petrone et al., 2021a). Different from other studies using a whole-blood platform (Murugesan et al., 2020; Echeverría et al., 2021), beside the different peptide used (Echeverría et al., 2021) or the different protocol performed, the current study enrolled both hospitalized and recovered patients, and evaluated the immune response based on the clinical stage, time of symptom onset, and, being an immune-based test, on lymphocyte counts or cortisone therapy. It showed that the IFN- $\gamma$  response was independently detected of these clinical parameters. Therefore, the test seems robust and this is important for future clinical applications.

**Table 3**  
Impact of the characteristics of hospitalized COVID-19 patients on IFN- $\gamma$  response induced by pools N, M, S and MegaPool.

| Characteristics                  |   | SEB              |                |              | Pool S        |                |       | MegaPool      |                |       | Pool N        |                |       | Pool M        |                |              |
|----------------------------------|---|------------------|----------------|--------------|---------------|----------------|-------|---------------|----------------|-------|---------------|----------------|-------|---------------|----------------|--------------|
|                                  |   | median (IQR)     | r <sub>s</sub> | p            | median (IQR)  | r <sub>s</sub> | p     | median (IQR)  | r <sub>s</sub> | p     | median (IQR)  | r <sub>s</sub> | p     | median (IQR)  | r <sub>s</sub> | p            |
| Gender                           | Male  | 10.7 (1.1–12.0)  | na             | 0.849        | 1.8 (0.2–2.8) | na             | 0.329 | 1.4 (0.6–5.6) | na             | 0.369 | 0.3 (0.0–1.1) | na             | 0.551 | 0.3 (0.0–0.7) | na             | 0.457        |
|                                  | Female  | 10.8 (2.1–12.1)  |                |              | 0.4 (0.1–2.1) |                |       | 0.7 (0.2–4.4) |                |       | 0.1 (0.0–1.7) |                |       | 0.1 (0.0–0.9) |                |              |
| Age                              |   | na               | –0.14          | 0.349        | na            | 0.0            | 0.996 | na            | 0.01           | 0.947 | na            | –0.01          | 0.637 | na            | –0.04          | 0.790        |
| Cortisone                        | No  | 10.4 (2.7–12.0)  | na             | 0.710        | 1.0 (0.3–2.2) | na             | 0.515 | 1.0 (0.2–4.6) | na             | 0.954 | 0.2 (0.0–1.7) | na             | 0.953 | 1.1 (0.0–0.5) | na             | 0.086        |
|                                  | Yes   | 10.9 (1.1–12.1)  |                |              | 0.6 (0.2–2.2) |                |       | 1.4 (0.3–4.7) |                |       | 0.3 (0.0–0.8) |                |       | 0.3 (0.0–1.4) |                |              |
| Days from symptom onset*         |   | na               | 0.12           | 0.467        | na            | 0.07           | 0.670 | na            | 0.1            | 0.655 | na            | 0.01           | 0.958 | na            | 0.19           | 0.232        |
| Severity of disease <sup>a</sup> | Mild/Moderate                                   | 10.9 (10.8–13.4) | na             | 0.598        | 1.2 (0.3–2.9) | na             | 0.478 | 4.0 (1.3–5.6) | na             | 0.195 | 0.8 (0.1–1.6) | na             | 0.242 | 0.4 (0.2–0.5) | na             | 0.260        |
|                                  | Severe  | 10.6 (1.5–12.1)  |                |              | 0.4 (0.1–3.1) |                |       | 1.4 (0.2–6.1) |                |       | 0.2 (0.0–1.1) |                |       | 0.5 (0.0–1.8) |                |              |
|                                  | Critical  | 6.8 (0.8–11.9)   |                |              | 0.9 (0.2–2.0) |                |       | 0.9 (0.0–2.9) |                |       | 0.2 (0.0–0.3) |                |       | 0.0 (0.0–0.3) |                |              |
|                                  |   |                  |                |              |               |                |       |               |                |       |               |                |       |               |                |              |
| Lymphocytes (x10 <sup>3</sup> )  | Number  | na               | 0.31           | <b>0.037</b> | na            | 0.02           | 0.872 | na            | 0.16           | 0.286 | na            | 0.17           | 0.260 | na            | 0.30           | <b>0.048</b> |
| Serology                         | IgG index                                       | na               | 0.29           | <b>0.052</b> | na            | 0.10           | 0.503 | na            | 0.18           | 0.219 | na            | 0.21           | 0.150 | na            | 0.29           | <b>0.043</b> |
|                                  | IgG score: Negative                             | 10.1 (3.4–10.8)  | na             | 0.474        | 1.2 (0.2–3.7) | na             | 0.490 | 1.0 (0.6–4.8) | na             | 0.979 | 0.1 (0.0–0.5) | na             | 0.343 | 0.2 (0.0–0.5) | na             | 0.166        |
|                                  | Positive  | 10.9 (1.1–12.1)  |                |              | 0.5 (0.2–2.2) |                |       | 1.4 (0.2–4.6) |                |       | 0.3 (0.0–1.2) |                |       | 0.3 (0.0–1.4) |                |              |
|                                  | IgM index                                       | na               | 0.14           | 0.360        | na            | 0.05           | 0.738 | na            | 0.06           | 0.711 | na            | 0.15           | 0.306 | na            | 0.11           | 0.461        |
|                                  | IgM score: Negative                             | 10.3 (0.8–12.0)  | na             | 0.445        | 1.0 (0.2–3.3) | na             | 0.533 | 1.2 (0.2–7.5) | na             | 0.855 | 0.2 (0.0–1.1) | na             | 0.763 | 0.3 (0.0–0.6) | na             | 0.714        |
|                                  | Positive  | 10.9 (1.5–12.1)  |                |              | 0.4 (0.1–2.2) |                |       | 1.3 (0.3–4.0) |                |       | 0.2 (0.0–1.2) |                |       | 0.2 (0.0–1.4) |                |              |
|                                  | IgG <sup>+</sup> /IgM <sup>+</sup>              | 10.9 (1.5–12.1)  | na             | 0.781        | 0.5 (0.3–2.2) | na             | 0.339 | 1.4 (0.5–4.0) | na             | 0.626 | 0.3 (0.0–1.2) | na             | 0.435 | 0.2 (0.0–1.4) | na             | 0.395        |
|                                  | IgG <sup>+</sup> /IgM <sup>–</sup>              | 9.1 (0.8–12.0)   |                |              | 0.6 (0.1–2.1) |                |       | 1.3 (0.1–7.5) |                |       | 0.3 (0.0–1.7) |                |       | 0.4 (0.0–1.3) |                |              |
|                                  | IgG <sup>–</sup> /IgM <sup>+</sup> <sup>b</sup> | 3.8              |                |              | 0.0           |                |       | 0.0           |                |       | 0             |                |       | 0             |                |              |
|                                  | IgG <sup>–</sup> /IgM <sup>–</sup>              | 10.3 (1.7–11.5)  |                |              | 1.6 (0.7–4.6) |                |       | 1.2 (0.7–7.2) |                |       | 0.2 (0.0–1.1) |                |       | 0.2 (0.0–0.5) |                |              |

Mann–Whitney or Kruskal–Wallis test for categorical variables; Spearman's correlation for continuous variables; r<sub>s</sub>: Spearman's correlation coefficient; na: not applicable. + and – are related to the IgG and/or IgM scores.

<sup>a</sup> Missing values: days from symptom onset, 4; severity of disease, 1.

<sup>b</sup> IgG<sup>–</sup>/IgM<sup>+</sup> there was one patient.

The kinetic of SARS-CoV-2 serology is not fully defined in terms of appearance of IgG and IgM (Sun et al., 2020; Fu et al., 2021; Long et al., 2020). The median seroconversion time of specific IgM and IgG against SARS-CoV-2 varies, ranging from 5 to 13 days and 11–14 days, respectively, after symptom onset (Fu et al., 2021). It is well documented that both humoral and cellular immune responses are crucial for SARS-CoV-2 infection and containment (Ni et al., 2020) and it has been found that cellular responses can also be detected in seronegative COVID-19 patients (Gallais et al., 2021; Freeman et al., 2004; Heller et al., 2013; Mizukoshi et al., 2008). Interestingly, the present study found T cell responses in seven hospitalized COVID-19 patients who scored negative to both IgM and IgG SARS-CoV-2 serology. Within this group there were five patients whose symptom onset dated back to 1–8 days before blood stimulation. Particularly, one patient had IgG seroconversion 5 days after the test. These data suggest that the IFN- $\gamma$  T cell response might anticipate, in some cases, the B cell response as detected by antibody. This result is interesting and may offer clinical diagnostic applications. Considering that SARS-CoV-2-IgG or IgM levels are not constant over time (Sethuraman et al., 2020; Xiao et al., 2020), beside serology, the IFN- $\gamma$  release assay (IGRA) may be a potential additional immune tool for further diagnostic and more in-depth clinical evaluations. The test may be used as an

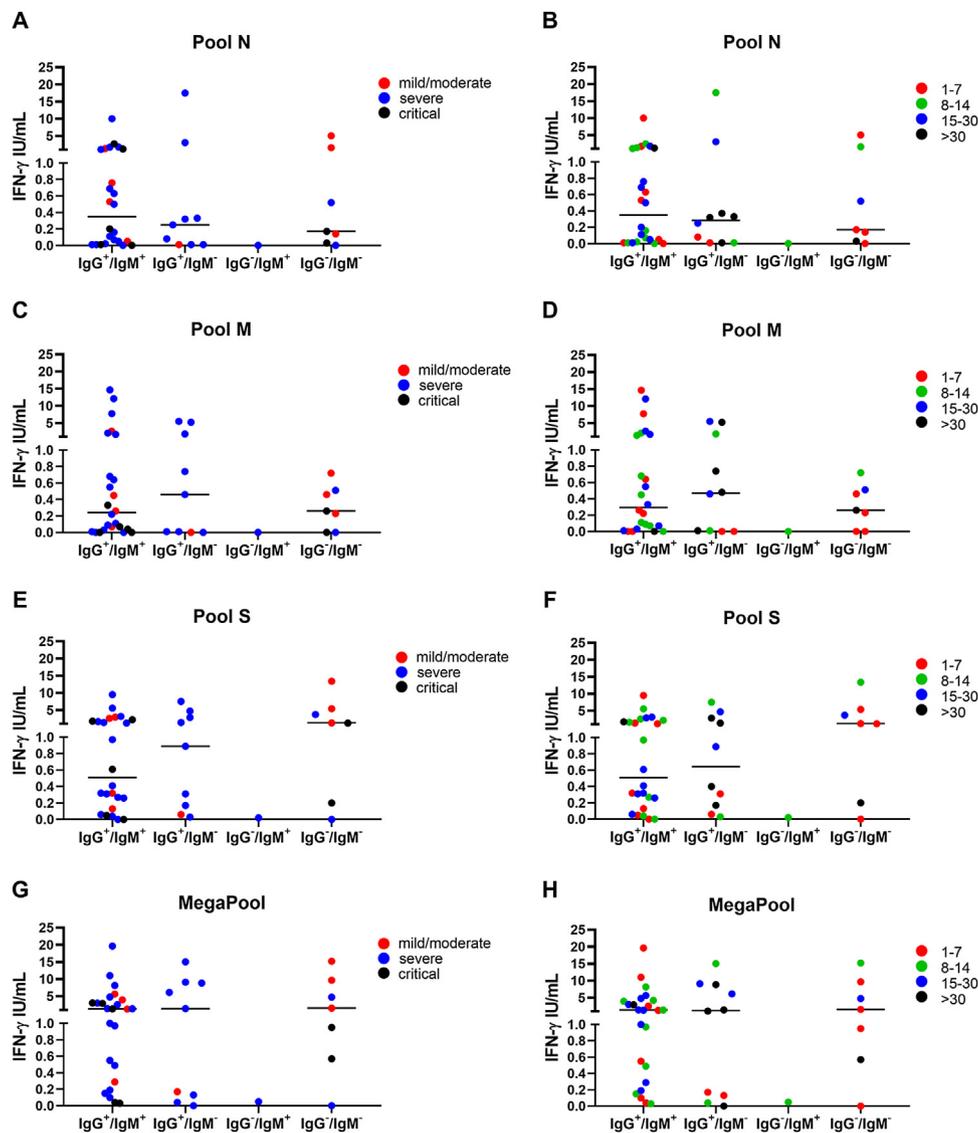
easy-to-use assay to better understand the prevalence of T cell-specific immunity in the population, access to pre-existing T cell immunity in seronegative individuals, vaccine-induced immunity, and also to pre-evaluate the SARS-CoV-2 vaccine candidates in clinical trials.

This study had some limitations. The sample size was relatively small (79 subjects) and not representative of the whole COVID-19 or NO-COVID-19 population. Moreover, it did not perform any longitudinal analyses of specific T-cell responses and antibodies to evaluate the levels of specific immunity over time. These issues may be assessed in future studies.

In conclusion, this study provided a simple IGRA platform for SARS-CoV-2-specific immune response detection, which can easily be scaled up for population-based studies. This test can be implemented in clinical laboratories as a powerful diagnostic tool and for understanding vaccine efficacy and potentially for surveillance strategies.

**Authors' contributions**

Study conception and design: DG; acquisition of data: AA, SNF, CF, VV, GC, GG, SM, EN, LP, DG; analysis and interpretation of data: AA, SNF, CF, AN, EP, LP, DG.



**Figure 4.** The IFN- $\gamma$  response to pool S and MegaPool peptides can be detected in COVID-19 patients with a negative IgG/IgM serology. Evaluation of the IFN- $\gamma$  response to pools N (A–B), M (C–D), S (E–F) and MegaPool (G–H) antigens in hospitalized COVID-19 patients (n = 41) stratified according to the IgG and IgM serology score and disease severity (A, C, E, G) or symptom onset (B, D, F, H). The IFN- $\gamma$  response to pool S and MegaPool antigens can be detected in COVID-19 patients with negative IgG and IgM serology. IFN- $\gamma$  was measured by ELISA in stimulated plasma. The horizontal lines represent the median; statistical analysis was performed using the Mann–Whitney test, and p-value was considered significant if  $\leq 0.008$ . IFN, interferon; N, nucleocapsid; M, membrane; S, spike.

Drafting the article: AA, SNF, CF, LP, DG; critically revising the article for important intellectual content: AA, SNF, EP, LP, VV, CF, GC, AN, GG, SM, LP, EN, DG; final approval of the version of the article to be published: AA, SNF, EP, LP, VV, CF, GC, AN, GG, SM, LP, EN, DG.

**Conflict of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Ethical approval**

The Ethical Committee of National Institute of Infectious Diseases (INMI) Lazzaro Spallanzani-IRCCS approved the study (approval number 59/2020).

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ijid.2021.04.034>.

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