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Humoral- and T-CellSpecific Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients With MS Using Different Disease-Modifying Therapies

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ABSTRACT

Objective: To evaluate the immune-specific response after the full SARS-CoV-2 vaccination of multiple sclerosis (MS) patients treated with different Disease Modifying drugs by the detection of both serological- and T-cell responses.

Methods: Health care workers (HCWs) and MS patients, having completed the two-dose schedule of an mRNA-based vaccine against SARS-CoV-2 in the last 2-4 weeks, were enrolled from two parallel prospective studies conducted in Rome, Italy, at the National Institute for Infectious diseases Spallanzani–IRCSS and San Camillo Forlanini Hospital. Serological response was evaluated by quantifying the Region-Binding-Domain (RBD) and neutralizing-antibodies. Cell-mediated response was analyzed by a whole-blood test quantifying interferon (IFN)-γ response to spike peptides. Cells responding to spike stimulation were identified by FACS analysis.

Results: We prospectively enrolled 186 vaccinated individuals: 78 HCWs and 108 MS patients. Twenty-eight MS patients were treated with IFN-β, 35 with fingolimod, 20 with cladribine, and 25 with ocrelizumab. A lower anti-RBD-antibody response rate was found in patients treated with ocrelizumab (40%, $p<0.0001$) and fingolimod (85.7%, $p=0.0023$) compared to HCWs and patients treated with cladribine or IFN-β. Anti-RBD-antibody median titer was lower in patients treated with ocrelizumab ($p<0.0001$), fingolimod ($p<0.0001$) and cladribine ($p=0.010$) compared to HCWs and IFN-β-treated patients. Importantly, serum neutralizing activity was present in all the HCWs tested and only in a minority of the fingolimod-treated patients (16.6%). T-cell-specific response was detected in the majority of MS patients (62%), albeit with significantly lower IFN-γ levels compared to HCWs. The lowest frequency of T-cell response was found in fingolimod-treated patients (14.3%). T-cell-specific response correlated with lymphocyte count and anti-RBD antibody

titer (rho=0.554, p<0.0001 and rho=0.255, p=0.0078 respectively). Finally, IFN-γ T-cell response was mediated by both $CD4^+$ and $CD8^+$ T cells.

Conclusion: mRNA vaccines induce both humoral and cell-mediated specific immune responses against spike peptides in all HCWs and in the majority of MS patients. These results carry relevant implications for managing vaccinations suggesting to promote vaccination in all treated MS patients.

Classification of Evidence: This study provides Class III data that COVID mRNA vaccination induces both humoral and cell-mediated specific immune responses against viral spike proteins in a majority of MS patients.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system and is a leading cause of disability in young adults [1] in western countries. Most people with MS are treated with immunomodulatory or immunosuppressive medications, which might increase the risk of opportunistic infections, infection-related hospitalization, and infection-related mortality rates [2-4].

The COronaVIrus Disease-2019 (COVID-19) pandemic caused by the 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 [5] and with still difficult clinical management [6,7].

Large-scale vaccination is the single most effective public health measure for controlling the COVID-19 pandemic and a global effort to develop and distribute an effective vaccine produced several effective options. Several data are now available about the efficacy of the mRNA platform vaccines, namely BNT162b2 and mRNA-1273 vaccines, in inducing strong antibody and cell-mediated immune responses in naïve healthy individuals [8-12]. The ability of vaccines to induce a coordinated induction of both humoral- and cell-mediated arms is fundamental for a more effective fighting of SARS-CoV-2 infection [13, 14]; this is

particularly crucial in people with MS treated with immunotherapy targeting pathogenetic inflammatory processes [15, 16].

Disease modifying treatments (DMTs) used in MS act at different levels of the immune system. Based on their mechanism of action they can be divided into: 1) immunomodulators: interferon-(IFN)β, glatiramer acetate, dimethyl fumarate and teriflunomide; 2) cell trafficking alteration molecules like S1P receptor modulators (i.e. fingolimod) and α4-integrin antibody (natalizumab); 3) depletive drugs (ocrelizumab, an anti-CD20 antibody, cladribine, a purine analog that interferes with DNA synthesis inducing a prolonged lymphocyte depletion, and alemtuzumab, an anti-CD52 antibody).

The overall effects of these DMTs in affecting the humoral and cell-mediated immune responses to SARS-CoV-2 vaccine is quite unknown. Preliminary data have been recently published suggesting that the antibody response to BNT162b2 vaccine is impaired in people with MS treated with fingolimod and ocrelizumab, whereas it is preserved in those treated with cladribine [17-19]. More recently, Guerrieri et al [20] in a real-word study on 32 people with MS have shown a higher frequency of the humoral response (62.5%) in patients treated with fingolimod. These data are essential for health decision and need to be confirmed and supplemented by the evaluation of the T-cell-specific response.

Aim of the present study was to evaluate the anti-Region-Binding-Domain (RBD) neutralizing antibodies and Spike (S)-specific T-cell response after the full SARS-CoV-2 vaccination of MS patients treated with different DMTs.

MATERIALS AND METHODS

Standard Protocol Approvals, Registrations, and Patient Consents

Human study protocols were approved by the Lazzaro Spallanzani National Institute for Infectious Diseases (INMI) Ethical Committee (approval numbers 297/2021 and 319/2021). The study protocols followed the ethics principles for human experimentation in agreement with the Declaration of Helsinki. Written informed consent was obtained from all participants in the study.

Study population

Subjects were enrolled from two parallel prospective studies conducted at the INMI Lazzaro Spallanzani. In detail, the studies evaluated the immune response to SARS-CoV-2 vaccination in both health care workers (HCWs) enrolled at INMI and in patients with MS enrolled at the MS Centre of the Department of Neurosciences of San Camillo Forlanini Hospital (Rome, Italy).

MS patients. One-hundred and eight subjects were enrolled. Inclusion criteria for the enrollment of MS patients were: 1) diagnosis of MS according to McDonald 2017 Criteria [21], 2) ongoing DMTs treatment with IFN-β, fingolimod, ocrelizumab or cladribine for at least six months before the study entry, 3) completed vaccination cycle (both doses) of an mRNA vaccine within the previous 2-4 weeks. In patients undergoing pulsed therapy (ocrelizumab and cladribine), the timing of vaccination after the last DMT administration was scheduled following the recommendation of both the Italian and European Academy of Neurology for COVID-19 vaccination. In particular, in patients with MS the drugs were provided with a delay of 3 months for ocrelizumab and of at least 4 weeks for cladribine. The therapies with IFN-β and fingolimod were not interrupted when vaccination was scheduled [22]. Blood tests and lymphocyte count were performed within one week from the time when the samples were taken for the immune-based assays. Percentage and absolute count of $CD19^+$ B cells and

serum IgG levels were collected in patients treated with ocrelizumab within one month from the study enrollment.

HCWs. A convenient sample of 78 HCWs from the cohort of vaccinated HCWs at INMI L. Spallanzani was included as healthy control group [12, 23]. Blood sampling and handling were performed following a standardized written protocol. Blood samples from all MS patients were collected at the MS Center of S. Camillo Forlanini Hospital, transported to INMI, and processed within 2 hours from collection. The same researchers' group at INMI processed all HCWs samples.

Peptide pools for the T cell-based tests

SARS-CoV-2 PepTivator® Peptide Pools (Miltenyi Biotec, Germany) covering the sequence of SARS-CoV-2 spike protein (PepTivator® SARS-CoV-2 Prot_S1, Prot_S, and Prot_S+) were used [24, 25, 26]. The PepTivator® Peptide Pools are constituted by peptides of 15 amino acid length with 11 amino acid overlap.

IFN-γ whole-blood assay

Whole-blood (600 μL) was stimulated with SARS-CoV-2 spike peptide pool in a 48-well flat-bottom plate according to the concentrations reported [24] and incubated at 37°C (5% CO2). Plasma was harvested after 20-24 h of stimulation and stored at -80°C until use. IFN-γ levels were quantified in the plasma samples using an automatic ELISA (ELLA, Protein Simple). IFN-γ values of the stimulated samples were subtracted from the unstimulated-control. The detection limit of this assay is 0.17 pg/mL.

Peripheral blood mononuclear cells (PBMCs) and in vitro stimulation

PBMCs from a small subset of the vaccinated individuals (8 MS patients and 7 HCWs) were isolated on density gradient centrifugation (SepMate-50 cat#85460 or SepMate-15

cat#85420, StemCell Technologies) according to manufacturer's procedure. The 7 HCWs, used as control group, were employed as controls in another publication [27]. All samples were frozen in heat-inactivated fetal bovine serum (FBS, Euroclone S.p.A. Italy) with 10% DMSO and stored in liquid nitrogen. PBMCs were thawed, counted, assessed for viability and rested for 2-4 hours at 37°C in RPMI+10% FBS prior to further use. Complete medium was freshly prepared as follows: RPMI-1640, 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (Euroclone S.p.A, Italy). Cells were seeded at a concentration of 2.5×10^6 cells/mL in a 96-multiwell flat-bottom plate (COSTAR, Sigma Aldrich) and stimulated with spike peptide pool at 1 μg/mL or Staphylococcal Enterotoxin B (SEB) at 200 ng/mL, as a positive control. Anti-CD28 and anti-CD49d monoclonal antibodies (BD Biosciences San Jose, USA) were added at 2 μg/mL to co-stimulate cells. After 1h of incubation at 37°C (5% CO_2), 1 μl/mL of Golgi Plug (BD Biosciences) was added to cell cultures to inhibit cytokine secretion. Following an incubation of 16-24 h, cells were stained as described below.

T-cell subpopulations and intracellular IFN-γ detection

PBMCs were stained with an appropriate combination of fluorochrome-conjugated antibodies prepared in Brilliant Stain Buffer (BD Biosciences). The Cytofix/Cytoperm solution kit (BD Biosciences) was used for the intracellular staining of IFN-γ, according to manufacturer's instructions (see eTable 1,http://links.lww.com/WNL/B668 for a complete list of antibodies and reagents). Dead cells were excluded from the 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 analyzed for each sample, whenever possible. Samples were acquired on a BD Lyric (BD Biosciences) cytometer. Data were analyzed with FlowJo software, version 10 (Tree Star). Cytokine background was subtracted to the stimulated conditions. The T-cell response was considered positive when SARS-CoV-2 spike stimulated PBMCs contained at least twofold higher frequencies of $CD4^+$ or $CD8^+$ T cells compared to the unstimulated control and at least 10 events were present in the IFN-γ gate [28].

Anti-SARS-CoV-2-specific IgG evaluation

Humoral response to vaccination was assessed by quantifying the anti-Nucleoprotein IgG and the anti-RBD IgG (Architect® i2000sr Abbott Diagnostics, Chicago, IL, USA). Anti-N-IgG were expressed as Arbitrary Units (AU)/mL and values ≥ 1.4 were considered positive. Anti-RBD-IgG were expressed as Binding Arbitrary Units (BAU)/mL and values ≥ 7.1 were considered positive.

Micro-neutralization assay (MNA)

Neutralizing antibodies to SARS-CoV-2 were assessed by a micro-neutralization assay with live SARS-CoV-2 virus (strain 2019-nCoV/Italy-INMI1; GISAID accession ID: EPI_ISL_412974). The assay is described in detail in [29], and is based on inhibition of Vero E6 cells infection by serum dilution curves, with cytopathic effect (CPE) determination at 48h post infection. Briefly, heat-inactivated and titrated sera (duplicate two-fold serial dilutions, starting dilution 1:10) were mixed with equal volumes of 100 $TCID_{50}$ SARS-CoV-2 and incubated at 37°C, 5% CO_2 for 30 min. Subsequently, 96-well tissue culture plates with sub-confluent Vero E6 cell monolayers were infected with 100 µL/well of virus-serum mixtures and incubated at 37°C and 5% CO_2. To standardize the inter-assay procedures, positive control samples showing high (1:160) and low (1:40) neutralizing activity were included in each MNA session. After 48 hours, microplates were observed by light microscope for the presence of CPE and then stained with Crystal Violet solution containing 2% Formaldehyde. Cell viability was measured by photometer at 595 nm (Synergy™ HTX Multi-Mode Microplate Reader, Biotek). The highest serum dilution inhibiting at least 90% of the CPE was indicated as the neutralization titer and expressed as the reciprocal of serum dilution (MNA_{90}).

Statistical analysis

Data were analysed using Graph Pad (GraphPad Prism 8 XML ProjecT). Categorical variables were reported as count and proportion, whereas continuous variables, including IFN-γ levels and anti-RBD, anti-N and MNA_{90} titers, were reported as median and interquartile range (IQR). All data were investigated by non-parametric statistical inference tests. The Kruskal-Wallis test was used for between-group comparisons, Mann-Whitney U-test with Bonferroni correction for pairwise comparisons, and Chi-squared test for categorical variables. Correlations of demographic, clinical and laboratory variables with serological and S-specific T-response in mRNA-vaccinated individuals, as well as between-assay correlations, were assessed by non-parametric Spearman's Rank test before and after multivariable adjustment. Spearman's $r_{ho} > 0.7$ was considered high correlation, $0.7 < r_{ho} > 0.5$ moderate correlation and $r_{ho} < 0.5$ low correlation.

Two-tailed p-values <0.05 were considered significant, with except for subgroup analyses by type of MS-specific treatment, where a correction for multiplicity was applied according to Bonferroni method, yielding a significant two-tailed p-value threshold of 0.0125 (α/4).

RESULTS

Demographic and clinical characteristics of the enrolled subjects

We prospectively enrolled 186 vaccinated subjects: 108 MS patients and 78 HCWs. Demographic and clinical data were collected at enrollment (Table 1). No significant differences were found regarding age, sex and country of origin between the two groups.

Twenty-eight MS patients were treated with IFN-β, 35 were treated with fingolimod, 20 with cladribine and 25 with ocrelizumab. The median treatment duration at the first vaccine dose was 8.9 years (IQR: 6.9-13.5) for IFN-β, 6.5 years (IQR: 3.6-8.1) for fingolimod and 1.7 years (IQR: 1.1-2.3) for ocrelizumab. The median time elapsed from the first administration of cladribine to the first vaccine dose was 1.7 years (IQR: 1.2-2.0); 16 out of 20 patients (80%) completed the second year treatment cycle. The median time elapsed since the last drug assumption was 8.9 months (IQR: 7.7-12.7) for cladribine and 3.8 months (IQR: 2.8-4.3) for ocrelizumab.

HCWs received the BNT162b2 vaccine as well as 103 MS patients, whereas 5 MS patients received the mRNA-1273 vaccine. The median time elapsed from the second vaccine dose and the blood sample collection was 23 days (IQR: 21-26), without any difference across treatment subgroups.

As expected, lymphocyte count at the time of immune-based assays sampling was significantly decreased in patients treated with fingolimod compared to those treated with other DMTs ($p<0.0001$). Patients treated with ocrelizumab showed a very low percentage of $CD19^+$ B cells (median: 0.04 %; IQR: 0.03-0.09 %; normal range: 6-20%) and $CD19^+$ absolute count (median: 0.89 cells/μL; IQR: 0.38-1,67 cells/μL; normal range: 90-520 cells/μL, respectively). In these patients, the median IgG level, obtained within one month from the study enrollment, was 900 mg/dl (IQR: 829-1100 mg/dl), except for two patients with IgG levels below the lowest limit of the normal range (700-1600 mg/dl). No correlation was found between IgG levels and anti-RBD titer ($r_s= 0.26$, $p=0.19$).

Most of the enrolled HCWs were healthy (n=64, 82%); 93.5 % (n=73) were untreated, 4% (n=3) were treated with corticosteroids for a history of allergic diseases, whereas no clinical data were available for 2.5% (n=2) of the HCWs (see eTable 2,http://links.lww.com/WNL/B668).

Serological-specific response in vaccinated individuals

Anti-N antibodies were undetectable in both MS patients and HCWs confirming the absence of SARS-CoV-2 natural infection in the study population (eFigure 1,http://links.lww.com/WNL/B668).

A detectable anti-RBD antibody response was observed in all HCWs (100%). The majority of patients with MS (n=87, 80.5%) showed anti-RBD antibody response, although the percentage of seropositive patients and the quantitative specific response varied according to the ongoing DMTs. A detectable anti-RBD response was found in 10/25 (40%) patients treated with ocrelizumab, in 30/35 (85.7%) patients treated with fingolimod, in 27/28 (96.4%) patients treated with IFN-β and in all patients (100%) treated with cladribine (Table 2). Ocrelizumab and fingolimod-treated patients showed lower response rates compared to HCWs ($p<0.0001$ and $p=0.0023$, respectively).

The anti-RBD antibody median titer was significantly lower in MS patients treated with ocrelizumab ($p<0.0001$), fingolimod ($p<0.0001$) and cladribine ($p=0.01$) compared to HCWs. No differences in the serological median titer in comparison to HCWs were found in patients treated with IFN-β ($p=0.359$) (Figure 1A). In ocrelizumab-treated patients, a longer treatment duration was significantly associated with reduced anti-RBD antibody titers (rho=-0.529, $p=0.007$), whereas age, BMI and disease duration did not show any impact. Furthermore, none of these variables was associated with reduced anti-RBD antibody titers in patients treated with fingolimod or cladribine (Table 3).

In patients treated with cladribine and ocrelizumab, no correlation was found between the anti-RBD antibody titer and the time elapsed since the last treatment cycle (rho=0.111,

p=0.640 and rho=-0.014, p=0.946 respectively). Moreover, in those treated with ocrelizumab the anti-RBD titer did not correlate with serum IgG levels (p=0.19).

IFN-γ-T-cell-specific response in vaccinated individuals

All HCWs showed an IFN-γ-S-specific T-cell response (78/78, 100%) as compared with 67 (62%) in the MS cohort. Different proportions of T-cell-specific responses were found among MS patients: 92% (23/25 patients) in ocrelizumab-treated group, 89.3% (25/28 patients) in IFN-β-treated group, 70% (14/20 patients) in cladribine-treated group and 14.3% (5/35 patients) in fingolimod-treated group (p<0.0001). Cladribine- and fingolimod-treated patients response rates were significantly lower compared to HCWs (p<0.0001) (Table 2).

The IFN-γ-T-cell-specific response levels were significantly lower in MS-vaccinated individuals undergoing any DMTs than in HCWs (p<0.0001) (Table 2 and Figure 1B). In MS patients, sex, age, BMI, disease duration, DMTs treatment duration at the time of vaccination did not impact the IFN-γ-T-cell-specific response. No association was found between the IFN-γ-T-cell-specific response and the above-mentioned variables in the single MS-treated group (Table 3).

In patients treated with cladribine and ocrelizumab, the IFN-γ-T-cell-specific response was not related to time elapsed since the last treatment cycle (rho=-0.353, p=0.127; and rho=-0.271; p=0.189 respectively).

IFN-γ response is mediated by $CD4^+$ T cells and $CD8^+$ T cells

To evaluate whether the IFN-γ-T-cell-specific response was due to the $CD4^+$ or $CD8^+$ T-cell subset, we evaluated the IFN-γ-specific T-cell frequency in stimulated PBMCs of 8 MS patients (4 treated with IFN-β and 4 with cladribine) and 7 HCWs. We selected IFN-β- and cladribine-treated patients since they showed, as reported in Figure 1, good specific antibody and T-cell responses. T cells were gated as described in eFigure 2,http://links.lww.com/WNL/B668. In HCWs, IFN-γ-T-cell-specific response was mediated

by $CD4^+$ (Figure 2A) and $CD8^+$ T cells (Figure 2B) with a different magnitude of response (median CD4: 0.279%, IQR: 0.193-0.427 vs median CD8: 0.058%, IQR: 0.00-0.140, respectively) (Figure 2C). In MS patients, the IFN-γ response was mediated only by $CD4^+$ T cells (IFN-β: median 0.16%, IQR: 0.109-0.192 and cladribine: median 0.13%, IQR: 0.117-0.163) (Figure 2C). The frequency of antigen-specific $CD4^+$ or $CD8^+$ T cells was lower in MS patients compared to HCWs, although this difference was not significant (Figure 2C). A positive T-cell response to SEB, used as a positive control, was found in all subjects, and the percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells were comparable between HCWs and MS subjects (data not shown).

Correlation between anti-RBD antibody titer, IFN-γ-T-cell-specific response and lymphocyte count

A significant slight correlation was observed in MS patients between anti-RBD antibody titer and IFN-γ-Spike(S)-specific T-cell response (rho=0.255, p=0.0078) (Figure 3A), persisting after adjusting for sex, age, BMI and disease duration (rho=0.234, p=0.017). No significant correlations were found within the differently-treated MS groups (data not shown). There was no correlation between the lymphocyte count and the anti-RBD antibody titer (rho=0.132, p=0.211) (Table 3), whereas quantitative IFN-γ-T-cell-specific response correlated with lymphocyte count in the whole MS group (rho=0.569, p<0.001), but not in the single DMTs-treated subgroup (Figure 3B and Table 3).

Correlation between anti-RBD antibody titer and neutralization activity

We evaluated the neutralization activity in the sera of 69 HCWs (88.5%). All the enrolled HCWs showed detectable neutralizing antibodies, whose titer significantly correlated with anti-RBD titers (rho=0.754, p<0.001) (Figure 4A). Among MS patients, the neutralization test was performed only in 24 (68.6%) patients treated with fingolimod due to the low antibody titers, to characterize the neutralizing capacity of the specific antibodies

elicited by vaccination. Only 4/24 (16.6%) patients showed a neutralizing activity, although at low titer (Figure 4B), with a significant correlation between the neutralizing antibody and anti-RBD antibody titers (rho=0.591, p=0.0024).

In conclusion, this study provides Class III data that COVID mRNA vaccination induces both humoral and cell-mediated specific immune responses against viral spike proteins in a majority of MS patients.

DISCUSSION

To our knowledge, this is the first combined analysis of humoral- and cell-mediated immunity responses to SARS-CoV-2 vaccination in people with MS treated with different DMTs.

Mass vaccination against the SARS-CoV-2 is crucial for the control of the pandemic and is currently ongoing in large populations all over the world. A coordinated humoral- and cell-mediated response induced by specific vaccination is the only tool available for a more effective prevention of SARS-CoV-2 infection, symptom onset and severe disease outcome [13, 14]. In particular, the humoral response blocks viral replication itself, whereas the viral-specific T-cell response kills viral-infected cells [30].

Recently, Achiron et al [17] demonstrated in a cohort of 125 MS patients, the development of COVID-19 humoral response to the mRNA-based vaccine BNT162b2 in all untreated MS patients and in all patients treated with cladribine. In the same study, only 22.7% of patients treated with ocrelizumab and 3.8% of patients treated with fingolimod developed SARS-CoV-2 antibodies. These results were confirmed in a very small French cohort [31], and, only partially, in an Italian series of 32 MS patients showing the humoral response in 32.5% (6/16) patients treated with ocrelizumab, but also in 62.5% (10/16) patients treated with fingolimod [20].

In the present study, we demonstrate a normal qualitative and quantitative humoral response to COVID-19 vaccination in patients treated with IFN-β. We confirm that 100% of patients treated with cladribine developed a humoral response to mRNA-based vaccines, although the antibody titer with slightly lower than HCWs. Differently, we found a detectable humoral response in 85.7% of the patients treated with fingolimod and in 40% of those treated with ocrelizumab, both with an anti-RBD-antibody titer significantly reduced compared to HCWs. These findings are in agreement with previous results on vaccines other than COVID-19, showing a humoral response similar to healthy subjects in IFN-β-treated

patients [32], and a reduced antibody titer in patients treated with ocrelizumab [33] and fingolimod [34]. The higher proportion of positive specific anti-S serological response in MS patients treated with fingolimod and ocrelizumab compared to that reported by Achiron et al [17] might be due to the more accurate serological tests used here to detect the specific response.

Few data are available regarding the T-cell-specific response induced by COVID-19 vaccination both in treated or untreated MS patients. Recently, Apostolidis et al [35] showed that anti-CD20 agents significantly reduced spike- and RBD-specific antibody and memory B cell responses in most MS patients. This effect was dependent on the time from the last anti-CD20 treatment and from the extent of the B cell reconstitution [35]. Compared to this work, here, we report the evidence of quantitative and qualitative SARS-CoV-2-S-specific T-cell response in a larger cohort of MS patients (n=108 vs n=20) treated not only with anti-CD20 drugs, but also with other different DMTs. In the present study, the T-cell-specific response was observed in 92% of patients treated with ocrelizumab, 89.3% of the patients treated with IFN-β and in 70% of the patients treated with cladribine, but only in 14% of the fingolimod-treated patients. IFN-γ-T-cell-specific response was lower in all treated MS patients compared to HCWs, in agreement with Apostolidis et al describing the results from only one cohort under anti-CD20 treatment. Moreover, we analyzed the T-cell response by an easy-to perform assay on whole blood, quantifying the IFN-γ produced by T cells after specific stimulation [24,36]. Accordingly, these results correlated with the number of lymphocytes.

Ocrelizumab is an anti-CD20 monoclonal antibody that depletes B lymphocytes and interferes with the process of antibody production [37]. This mechanism leads to a reduced humoral response to vaccination, a higher risk of severe COVID-19 [2-4, 19] and the possibility of persistent SARS-CoV-2 infection in ocrelizumab-treated patients despite the induction of a SARS-CoV-2-specific T-cell response [38]. It is well known that serum IgG and IgM levels decrease with ocrelizumab treatment duration [39]; this is confirmed by our results showing a relationship between treatment duration and the entity of the humoral response to SARS-CoV-2-vaccine. Importantly, here, we show that ocrelizumab-treated patients mount an S-

specific T-cell response comparable to that developed in patients treated with IFN-β or cladribine. Whether the presence of a T-cell response, associated to an impaired humoral immunity, might be sufficient to control SARS-CoV-2 infection is still a matter of debate [38] and it is out of the purpose of this study. However, the preservation of the T-cell response might explain why the ongoing treatment with ocrelizumab is not always associated to an increased severity of SARS-CoV-2 infection [40], and why in a small cohort of anti-CD20-treated patients a low frequency of vaccine failure has been reported [31]. Lastly, the long-term potential beneficial effect of the SARS-CoV-2-specific T-cell response might potentially contribute to reduce COVID-19 disease severity.

Fingolimod is a sphingosine 1-phospate modulator that prevents T cells egress from lymph nodes reducing the number of circulating lymphocytes. This mechanism supports our results showing both reduced T-cell-specific and humoral responses in fingolimod-treated patients. Importantly, we demonstrate also that only a minority of patients treated with fingolimod have an anti-viral neutralizing capacity.

In vitro T-cell response to SARS-CoV-2 spike glycoprotein is mediated by both $CD4^+$ and $CD8^+$ T cells [11, 24] and was here confirmed in our vaccinated HCWs cohort [27]. Interestingly, $CD8^+$ T-cell response was not found in patients treated with cladribine or IFN-β. It is known that these treatments may have an impact on both $CD4^+$ and $CD8^+$ T-cell populations [41, 42] and that spike-specific response by the $CD8^+$ T cells is only one tenth of the $CD4^+$ T-cell response [24, 36] and therefore difficult to detect if present. These results need to be confirmed in larger cohorts of patients.

Despite the clinical and immunological interest for the results obtained, this study has some limitations. Firstly, the small size of the cohort restricts the power of the study, especially for the comparison of the effects of vaccination between different DMTs. Nevertheless, the enrolled subjects are representative of the MS patients and are well characterized, both clinically and immunologically. Secondly, the evaluation of immune responses was done at a single time point post-vaccination and the methodology used to

detect the T-cell response was based on the measurement of a single cytokine (IFN-γ), differently from published studies evaluating additional T-helper 1 cytokines [12, 23]. However, as we have already shown, the IFN-γ T-cell response correlates with RBD-antibody titers [12], therefore IFN-γ may be considered as a robust parameter to measure the T-cell-specific response induced after vaccination.

Importantly, one of the main strengths of this study, compared to prior works [17, 18, 20], is the evaluation of the humoral immune response using both the specific anti-RBD IgG and the SARS-CoV-2 neutralization tests, in addition to the characterization of the T-cell response in terms of both $CD4^+$ or $CD8^+$ T-cell involvement. The assays used in this study to detect SARS-CoV-2-specific response are easy and highly reproducible [24, 36], and therefore, compatible with the routine monitoring of vaccinated people. Indeed, the T-cell response was detected using a whole blood assay, whose platform is similar to current tests measuring the T-cell-specific responses against *Mycobacterium tuberculosis* [43].

In conclusion, this is the first study demonstrating the development of a T-cell-specific response to SARS-CoV-2 in the majority of DMTs-treated patients with the lowest rate in patients treated with fingolimod. Together with the observation concerning the humoral response, these data carry relevant implications for managing vaccinations in people with MS suggesting to promote vaccination in all treated MS patients. Future studies are needed to evaluate: 1) the longevity of the humoral and T-cell responses following COVID-19 vaccination in MS patients and 2) the impact of different time-window vaccination on immunity development in patients treated with ocrelizumab. These data will be key for defining the best vaccination strategy to balance the risk of MS disease progression and the protection against SARS-CoV2 infection.

Appendix 2: Coinvestigators

Name	Location	Role	Contribution

Daniele Lapa, MSc	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Massimo Francalancia, MLT	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Aurora Bettini, MLT	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Giulia Gramigna, MSc	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Federica Forbici, MSc	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Paola Gallì, MD	National Institute for Infectious Diseases (INMI)	Site investigator	Site of enrolment of HCWs set-up
Alessandra Marani, MD	National Institute for Infectious Diseases (INMI)	Site investigator	Site of enrolment of HCWs set-up
Adriano Possi, MSc	National Institute for Infectious Diseases (INMI)	Administration	Site of enrolment of HCWs set-up
Andrea Capri, MD	National Institute for Infectious Diseases (INMI)	Site investigator	Site of enrolment of HCWs set-up
Annapaola Santoro, MD	National Institute for Infectious Diseases (INMI)	Site investigator	Site of enrolment of HCWs set-up
Nicoletta Orchi, MD	National Institute for Infectious Diseases (INMI)	Site investigator	Site of enrolment of HCWs set-up
Ornella Butera, MLT	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Saeid Najafi Fard, PhD	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Linda Petrone, PhD	National Institute for Infectious Diseases (INMI)	Site investigator	Experimental set-up
Elisa Petruccioli, PhD	National Institute for Infectious Diseases (INMI)	Site investigator	Intellectual contribution

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Table 1. Demographical and clinical characteristics of the 186 enrolled subjects

Characteristics		MS patients	Health care workers	P value
N (%)		108 (58.1)	78 (41.9)	
Age median (IQR)		47 (39-54)	44 (33-53)	0.098 *
Male N (%)		34 (31.5)	20 (25.6)	0.408 §
Origin N (%)	**West Europe**	105 (97.2)	76 (97.4)	
	East Europe	2 (1.9)	2 (2.6)	0.661 §
	Sud America	1 (0.9)	0 (0)	
BMI (kg/mq), median (IQR)		23.2 (20.9-26.5)	-	
MS duration, median (IQR)		13 (7-20)	-	
MS Course N (%)	**Relapsing-remitting**	98 (90.7)	-	
	Primary-progressive	10 (9.3)	-	
EDSS score, median (IQR)		2.0 (1.0-3.5)	-	
Multiple Sclerosis Treatment N (%)	**Ocrelizumab**	25 (23.2)	-	
	Fingolimod	35 (32.4)	-	
	Cladribine	20 (18.5)	-	
	IFN-β	28 (25.9)	-	
Lymphocytes count N (%)	**Available**	87 (80.5)	0 (0)	
Lymphocytes count N (%)	**Ocrelizumab**	25 (28.7)	-	
Median x$10^3/\mu$L (IQR)		1.46 (1.27-1.86)		
	Fingolimod	34 (39.1)	-	<0.0001 **
		0.66 (0.57-0.95)		
	Cladribine	20 (23)	-	
		1.11 (0.87-1.47)		
	IFN-β	8 (9.2)	-	
		1.60 (1.42-1.99)		

Footnotes: BMI: body mass index, EDSS: Expanded Disability Status Scale; IQR: Interquartile range; N: Number. * Mann-Whitney U-statistic test; § Chi-square test; ** Kruskal-Wallis test performed only on MS patients.

Table 2. Serological and T-cell-specific responses

Characteristics				MS patients	Health care workers	P value	
			N (%)	108 (58.1)	78 (41.9)		
Antibody response	**Qualitative response**	**anti-RBD abs responders N (%)**		87 (80.5)	78 (100)	<0.0001 §	
		anti-RBD abs responders within the subgroups N (%)	**Ocrelizumab**	10/25 (40)	-		**<0.0001** §
			Fingolimod	30/35 (85.7)	-		**0.0023** §
			Cladribine	20/20 (100)	-	<0.0001 §	>0.9999 §
			IFN-β	27/28 (96.4)	-		0.264 §
	Quantitative response	**anti-RBD abs BAU/mL Median (IQR)**		284.5 (18.8-1497)	2395 (1445-4089)	<0.0001 *	
			Ocrelizumab	3.40 (0.45-21.85)	-		**<0.0001 ***
			Fingolimod	48 (20.60-166.70)	-		**<0.0001 ***
			Cladribine	1360 (967.5-2177)	--	<0.0001 #	**0.010 ***
			IFN-β	2164 (1047-3504)			0.359 *
Spike-specific IFN-γ T cell response	**Qualitative response**	**anti-S responders N (%)**		67 (62)	78 (100)	<0.0001§	
		anti-S responders within the subgroups N (%)	**Ocrelizumab**	23/25 (92)	-		0.057 §
			Fingolimod	5/35 (14.3)	-	<0.0001 §	**<0.0001** §
			Cladribine	14/20 (70)	-		**<0.0001** §
			IFN-β	25/28 (89.3)	-		0.017 §
	Quantitative response	**anti-S IFN-γ pg/mL Median (IQR)**		53.09 (3.47-135.3)	343.8 (167-703)	<0.0001 *	
			Ocrelizumab	128.9 (49.5-268.7)	-		**<0.0001 ***
			Fingolimod	1.75 (0.18-5.3)	-	<0.0001 #	**<0.0001 ***
			Cladribine	60 (14.6-138.9)	-		**<0.0001 ***
			IFN-β	84 (51.2-385.6)	-		**0.0004 ***

Footnotes: N, Number; IQR, Interquartile range; §Chi-square test; *Mann-Whitney U-statistic test; #Kruskal-Wallis test; abs, antibodies; RBD, Receptor-Binding-Domain; S, spike.
In bold are only those values that were significant after multiplicity correction by the Bonferroni method (α/4=0.0125)

Table 3. Factors associated with antibody and T-cell-specific responses in MS patients

		All N=108	Ocrelizumab N=25	Fingolimod N=35	Cladribine N=20	IFN-β N=28
Age	**Anti-RBD abs, BAU/ml**	Rho=-0.145 P=0.134	Rho=-0.118 P=0.574	Rho=-0.059 P=0.734	Rho=-0.471 P=0.036	Rho=-0.002 p=0.990
	Anti-S IFN-γ, pg/ml	Rho=-0.103 P=0.287	Rho=0.025 P=0.906	-0.190 P=0.267	Rho=-0.168 P=0.478	Rho=-0.196 P=0.327
BMI	**Anti-RBD abs, BAU/ml**	Rho=0.060 P=0.535	Rho=0.361 P=0.076	Rho=-0.174 P=0.310	Rho=0.026 P=0.915	Rho=-0.249 P=0.211
	Anti-S IFN-γ, pg/ml	Rho=-0.162 P=0.095	Rho=-0.073 P=0.0727	Rho=-0.044 P=0.798	Rho=-0.217 P=0.359	Rho=-0.031 P=0.880
Disease duration	**Anti-RBD abs, BAU/ml**	Rho=0.097 P=0.319	Rho=0.159 P=0.447	Rho=0.083 P=0.631	Rho=-0.535 P=0.015	Rho=0.192 P=0.337
	Anti-S IFN-γ, pg/ml	Rho=-0.066 P=0.495	Rho=0.229 P=0.271	Rho=-0.170 P=0.323	Rho=-0.179 P=0.450	Rho=-0.120 P=0.550
Lymphocyte count	**Anti-RBD abs, BAU/ml**	Rho=0.132 P=0.211	Rho=-0.261 P=0.466	Rho=-0.012 P=0.944	Rho=0.185 P=0.435	-
	Anti-S IFN-γ, pg/ml	**Rho=0.569 P<0.001**	Rho=-0.316 P=0.374	Rho=0.099 P=0.564	Rho=-0.095 P=0.691	-
Treatment duration	**Anti-RBD abs, BAU/ml**	Rho=0.193 P=0.045	**Rho=-0.529 p=0.007**	Rho=-0.220 p=0.198	Rho=0.289 p=0.217	Rho=0.230 p=0.249
	Anti-S IFN-γ, pg/ml	Rho=-0.160 P=0.099	Rho=0.005 p=0.983	Rho=-0.313 p=0.063	Rho=-0.384 p=0.095	Rho=0.189 p=0.344

Footnotes: In bold are only those values that were significant after multiplicity correction by the Bonferroni method (α/4=0.0125).

FIGURE LEGENDS

Figure 1. Antibody and T-cell responses after SARS-CoV-2 vaccination in patients with MS. Evaluation of antibody response (**A**) in 78 HCWs and 108 MS patients stratified according to drug treatment in four groups: ocrelizumab (n=25), fingolimod (n=35), cladribine (n=20) and IFN-β (n=28). SARS-CoV-2-specific anti-RBD Abs were quantified in plasma or sera samples. Anti-RBD-IgG were expressed as Binding Arbitrary Units (BAU)/mL and values ≥ 7.1 were considered positive. Evaluation of IFN-γ response to spike antigen (**B**). IFN-γ was measured by automatic ELISA in plasma harvested from stimulated whole-blood samples and shown as median after subtracting the background. Dashed lines identify the cut-off of each test (spike 16 pg/mL and anti-RBD 7.1 BAU/mL). Each black dot represents one sample. The red horizontal lines represent the median; statistical analysis was performed using the Mann-Whitney test, and p value was considered significant if ≤ 0.0125. Footnotes: Abs, antibodies; RBD, Receptor-Binding-Domain; HCWs, Health Care Workers; IFN, Interferon.

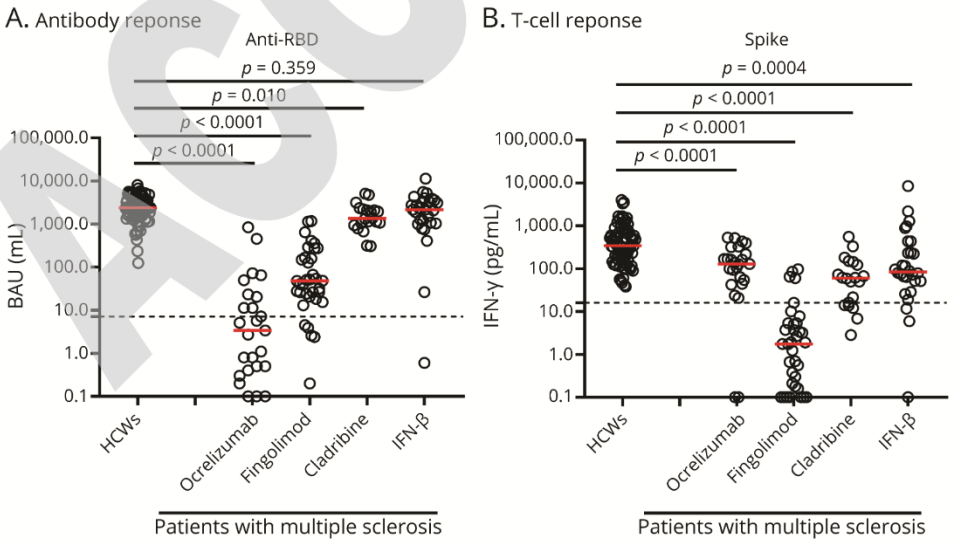


Figure 2. Evaluation of IFN-γ-S-specific T-cell response by flow cytometry. HCWs (n=7) and MS patients (IFN-β-treated n=4; cladribine-treated n=4) were stimulated for 24h with spike peptide pool and the frequency of IFN-γ-specific T cells was evaluated by flow cytometry. Plots show the frequency of IFN-γ-specific T cells in a representative HCW subject, MS patient treated with IFN-β and MS patient under cladribine within the $CD4^+$ subset (**A**) and $CD8^+$ T-subset (**B**). Frequency of the $CD4^+$ and $CD8^+$ T- cell responses (after subtraction of the unstimulated-condition value) is shown in HCWs and MS patients. Each dot represents a different HCW or MS individual and black lines represent medians. Statistical analysis was performed using the Mann-Whitney test and p value was considered significant if ≤ 0.05. Footnotes: IFN, Interferon; MS, multiple sclerosis; HCWs, Health Care Workers.

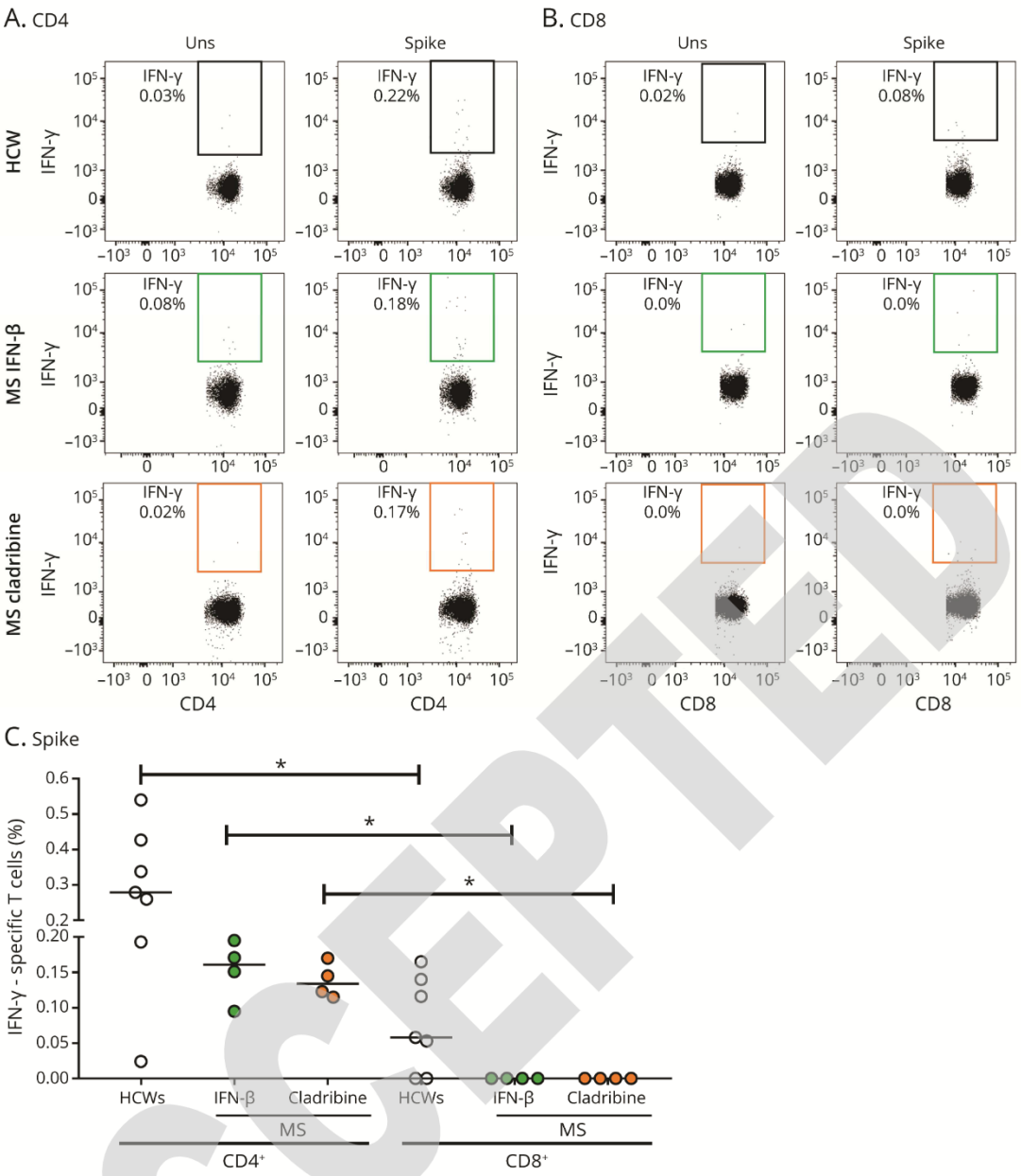


Figure 3. Correlations across humoral, cell-mediated immunity and lymphocyte count. Evaluation of correlation between IFN-γ levels in response to spike and anti-RBD antibodies in 108 MS patients (**A**). Anti-RBD-IgG were expressed as Binding Arbitrary Units (BAU)/mL and values ≥ 7.1 were considered positive. A slight significant correlation was found in MS patients (rho=0.255, p=0.0078). Evaluation of correlation between IFN-γ levels in response to spike and lymphocyte number in a subgroup of MS patients (n=87) (**B**). IFN-γ levels correlate with lymphocyte number in MS patients (rho=0.569, p<0.0001). Dashed lines identify the cut-off of each test (spike 16 pg/mL and anti-RBD 7.1 BAU/mL). Each black dot represents one sample. Correlations between assays were assessed by non-parametric Spearman's rank tests. A 2-sided p value <0.05 was considered statistically significant. Footnotes: Abs, antibodies; RBD, Receptor-Binding-Domain; HCWs, Health Care Workers; MS, multiple sclerosis.

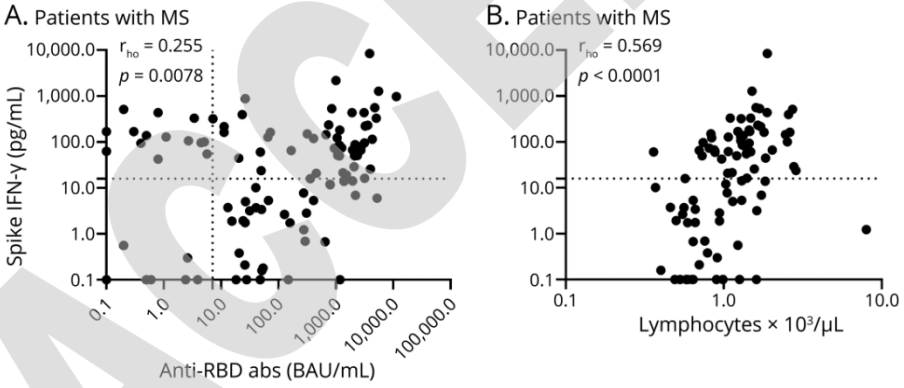
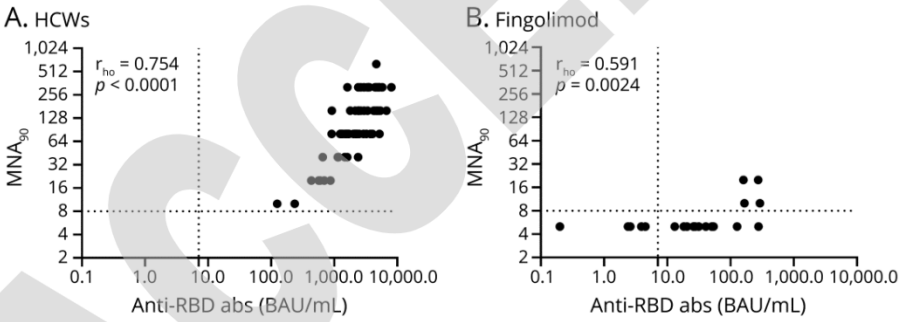


Figure 4. Correlations within humoral levels (anti-RBD IgG and MNA_{90}). The correlation between anti-RBD IgG levels and neutralizing antibodies was evaluated in a subgroup of the enrolled HCWs (n=69) (**A**) and of MS patients under fingolimod therapy (n= 24) (**B**). Anti-RBD-IgG were expressed as Binding Arbitrary Units (BAU)/mL and values ≥ 7.1 were considered positive; neutralizing antibodies were expressed as the reciprocal of dilution and values ≥ 10 were considered positive. A strong significant correlation was found in HCWs (rho=0.754, p<0.0001), whereas a moderate correlation was found in MS patients (rho=0.591, p=0.0024). Dashed lines identify the cut-off of each test (anti-RBD 7.1 BAU/mL and MNA_{90} 8). Each black dot represents one sample. Correlations between assays were assessed by non-parametric Spearman's rank tests. A 2-sided p value <0.05 was considered statistically significant. Footnotes: Abs, antibodies; RBD, Receptor-Binding-Domain; HCWs, Health Care Workers.



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