Original article

Persistently reduced humoral and sustained cellular immune response from first to third SARS-CoV-2 mRNA vaccination in anti-CD20-treated multiple sclerosis patients

Hamza Mahmood Bajwa [a,b,#,$], Frederik Novak [a,b,#,$], Anna Christine Nilsson [c,d], Christian Nielsen [c], Dorte K. Holm [c], Kamilla Østergaard [e], Agnes Hauschultz Witt [f], Keld-Erik Byg [d,g], Isik S. Johansen [d,8], Kristen Mittl [9], William Rowles [9], Scott S. Zamvil [9], Riley Bove [9], Joseph J. Sabatino [9], Tobias Sejbaek [a,b,$,*]

[a] Department of Neurology, Hospital Southwest Jutland, University Hospital of Southern Denmark, Esbjerg, Denmark
[b] Department of Regional Health Research, University of Southern Denmark, Odense, Denmark
[c] Department of Clinical Immunology, Odense University Hospital, Odense, Denmark
[d] Department of Clinical Research, University of Southern Denmark, Odense, Denmark
[e] Department of Neurology, Nordsjællands Hospital, Hillerød, Denmark
[f] Department of Neurology, Hospitalsenhed Midt, Viborg, Denmark
[g] Department of Rheumatology, Odense University Hospital, Odense, Denmark
[8] Department of Infectious Diseases, Odense University Hospital, Odense, Denmark
[9] Weill Institute for Neurosciences, Department of Neurology, University California San Francisco, San Francisco, USA

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ABSTRACT

Objective: To examine humoral and cellular response in multiple sclerosis patients on anti-CD20 therapy after third BNT162b2 mRNA SARS-CoV-2 vaccination.
Methods: A prospective longitudinal study design from first throughout third vaccination in Danish and American MS centers. All participants were treated with ocrelizumab. Antibody (Ab) levels were assessed before and after third vaccination using SARS-CoV-2 IgG II Quant assay (Abbott Laboratories). B- and T-lymphocytes enumeration was done with BD Multitest™6-color TBNK reagent. Spike-specific T-cell responses were measured through PBMC stimulation with spike peptide pools (JPT Peptide Technologies).
Results: We found that 14.0%, 37.7%, and 33.3% were seropositive after first, second and third vaccination. The median Ab-levels were 74.2 BAU/mL (range: 8.5–2427) after second vaccination, as well as 43.7 BAU/ml (range: 7.8–366.1) and 31.3 BAU/mL (range: 7.9–507.0) before and after third vaccination, respectively. No difference was found in levels after second and third vaccination ($p = 0.1475$). Seropositivity dropped to 25.0% of participants before the third vaccination, a relative reduction of 33.3% ($p = 0.0020$). No difference was found between frequencies of spike reactive CD4$^+$and CD8$^+$ T-cells after second (0.65 ± 0.08% and 0.95 ± 0.20%, respectively) and third vaccination (0.99 ± 0.22% and 1.3 ± 0.34%, respectively).
Conclusion: In this longitudinal cohort we found no significant increased humoral or cellular response with administration of a third SARS-CoV-2 mRNA vaccination. These findings suggest the need for clinical strategies to include allowance of B cell reconstitution before repeat vaccination and/or provision of pre-exposure pro-phylactic monoclonal antibodies.

1. Introduction

The ongoing Covid-19 pandemic raises concerns about its effects on the most vulnerable patients. Anti-CD20 medications such as ocrelizumab, rituximab, and ofatumumab are widely used to treat multiple sclerosis (MS), blood cancers, and autoimmune diseases. Anti-CD20 targets B-lymphocytes, thus leading to cell lysis, and thereby reduction of disease activity in both relapsing and progressive MS (Krumbholz et al., 2012). Furthermore, compared to other disease modifying therapies, anti-CD20 treatment is associated with more severe complications to SARS-CoV-2 infection (MP Sormani et al., 2021) e.g., higher rates of hospitalizations and severe disease course (Salter et al., 2021). A COVID-19-specific strategy for patients at specific risk in Denmark has therefore been to offer early re-vaccination to anti-CD20 treated patients.

It has previously been shown by our and other investigators that patients on B-cell depleting treatments have significantly reduced humoral immunity after COVID-19 vaccines compared to healthy controls (Novak et al., 2021; Sabatino et al., 2022). Several studies confirm that vaccination, in general, generates a decreased humoral response in anti-CD20 treated patients (Achiron et al., 2021; Bar-Or et al., 2020; Hua et al., 2014; Ammitzbøll et al., 2021). Data indicate that higher levels of B-cells at the time of vaccination and longer intervals between anti-CD20 treatments improve the response to vaccination (Disanto et al., 2021). However, extending dosage interval is currently considered an off-label treatment, and the effect on relapse risk, while appearing to date to not be substantial, is still unknown (Ammitzbøll et al., 2021; Killestein et al., 2020; Nguyen et al., 2017; MP Sormani et al., 2021). One study has advocated that vaccination should occur one month before the next treatment infusion (Day et al., 2020). Still, the timing of treatment with anti-CD20 infusion and subject vaccination is still being debated (Novak et al., 2021; Rico et al., 2021).

The initial two vaccinations can lead to successful seroconversion in a subset of anti-CD20 patients (Novak et al., 2021). Accordingly, the Danish National Board of Health, European Medicines Agency (EMA), and the U.S. Food and Drug Administration (FDA) have all recommended or authorized an additional third vaccine for these patients.

The primary goal of this study is to determine whether additional mRNA SARS-CoV-2 vaccination can increase levels of specific SARS-CoV-2 spike receptor binding domain (RBD) antibodies (Abs) generated in MS patients treated with anti-CD20 therapy (ocrelizumab). We also assessed whether a third vaccine dose can increase T cell responses and the proportion of seropositive individuals among these participants. To address this question, we examined frequencies of spike-reactive T cells and levels of SARS-CoV-2 Abs before and after a third SARS-CoV-2 vaccination in a large cohort of anti-CD20-treated MS-patients from two international MS centers.

2. Method

2.1. Study population and design

In this observational study, we included prospectively adult participants (18 years or older) with confirmed MS (2017 McDonald Criteria) on ocrelizumab (anti-CD20) therapy. All participants had received two doses of mRNA SARS-CoV-2 vaccination and were enrolled prior to a third booster vaccine of the mRNA SARS-CoV-2 vaccine. Results from first and second vaccination were already published in a recent paper (Novak et al., 2021).

No other immunosuppressive treatment beyond infusion-related methylprednisolone was given to the participants during this study. Patients were included from two Danish MS clinics (Esbjerg, Viborg) and the University of California, centre for MS and Neuroinflammation, in San Francisco (USA). All participants followed standard clinical practice by their treating neurologist and interval of time between second and third vaccination was not standardised (Baden et al., 2021; Polack et al., 2020).

2.2. Sample collection

Blood samples were collected at two time points: 0–7 days before the third booster (V4) vaccination and two to four weeks after the third booster vaccination (V5).

Abs were compared with levels 0–7 days before the first vaccination (V1), 0–7 days before the second booster vaccination (V2), and 2–4 weeks after the second booster vaccination (V3) (Novak et al., 2021). Danish participants provided samples at all timepoints (V1-V5); North American participants at V1, V3, V4, and V5 (Fig. 1).

2.3. Data collection

Blood samples were collected following international guidelines for biobanking. Venous blood was procured from a cubital vein into evacuated K2-EDTA or heparinized tubes. Plasma was aliquoted in 500 µL Sarstedt polypropylene tubes and stored at −80 °C until batch analysis (Teunissen et al., 2009).

2.4. Human peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Lymphoprep™) and cryopreserved using DMSO-containing freezing medium and stored at –196 °C until flow cytometric analyses.

2.5. Antibody assay and flow cytometry

We measured IgG antibodies against the SARS-CoV-2 spike RBD in plasma samples as described by Novak et al. (Novak et al., 2021) using the SARS-CoV-2 IgG II Quant assay (Abbott Laboratories), which is a quantitative chemiluminescent microparticle immunoassay (Abbott, 2021). The assay was performed using the Abbott Alinity I platform in accordance with the manufacturer's instructions. This assay has shown excellent correlation with the first WHO (World Health Organization) International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code 20/136) (Kumar et al., 2021), enabling the issuing of immunogenicity results in standardized units; binding antibody units (BAU)/mL for a binding assay format as the SARS-CoV-2 IgG II Quant assay. The mathematical relationship of the Abbott AU/mL unit to the WHO BAU/mL unit follows the equation BAU/mL = 0.142xAU/mL, corresponding to a cut-off at 7.1 BAU/mL. This assay has the documented ability to detect spike RBD IgG vaccine response in longitudinal samples from individuals with and without prior SARS-CoV-2 infection (Abbott, 2021; Kristiansen et al., 2021). Ab-levels above 506 BAU/ml were defined as sufficient levels (>80% protection). Values between <506 BAU/ml and >17 BAU/ml were considered intermediate (>50% protection) and below <17 BAU/ml as low (Feng et al., 2021).

Enumeration of B- and T-lymphocytes was performed using fresh EDTA blood stained from the Denmark cohort with the BD Multitest™ 6-color TBNK reagent in BD TruCount tubes. Samples were analyzed on a BD FACSCanto™ II flow cytometer with BD FACSDiva software. Flow cytometry was performed in participants included from Hospital Southwest Jutland, University Hospital of Southern Denmark within 24 h from collection.

2.6. T cell assay

We measured spike-specific $CD4^+$ and $CD8^+$ T cell responses by activation-induced marker (AIM) expression as previously described (Sabatino et al., 2022). Briefly, PBMCs were thawed, washed, and rested for 2 h at 37 °C prior to start of assay. PBMCs were stimulated for 24 h with 1 µg/ml spike peptide pools (JPT Peptide Technologies) or vehicle control (0.2% DMSO). Folllowing stimulation, cells were washed and

Fig. 1. Sample collection at visits (Vx) and vaccinations. V1= Visit 1 (0–7 days before the 1st vaccine), V2= Visit 2 (0–7 days before the 2nd vaccine), V3 = Visit 3 (2–4 weeks after the 2nd vaccine dose), V4 = Visit 4 (0–7 days before the 3rd booster) and V5 = Visit 5 (2–4 weeks after the 3rd booster). 1st, 2nd, and 3rd depict the order of vaccination. The time interval between 2nd and 3rd vaccination is non-standardized and is based on standard clinical practice when patients were offered the 3rd booster vaccine.

stained with the following antibody panel: CD4 Alexa 488 (OKT4), CD8 Alexa 700 (SK1), OX-40 PE-Dazzle 594, (ACT35), CD69 PE (FN-50), CD137 BV421 (4B4–1), CD14 PerCP-Cy5.5 (HCD14), CD16 PerCP-Cy5.5 (B73.1), CD19 PerCP-Cy5.5 (HIB19) (all BioLegend) and live/dead dye eFluor506 (Invitrogen). Cells were washed with FACS wash buffer, fixed with 2% paraformaldehyde (BD), and stored in FACS wash buffer in the dark at 4 °C until ready for flow cytometry analysis. The same gating strategy was employed as before (Sabatino et al., 2022).

AIM-positive T cells were defined by the co-expression of OX-40 and CD137 by CD4$^+$ T cells and co-expression of CD69 and CD137 by CD8$^+$ T cells. AIM expression following spike peptide pool stimulation was subtracted from AIM expression following stimulation with no peptide negative control to yield the frequencies of spike-specific T cells.

2.7. Standard protocol approvals, registrations, patient consents, and monitoring

Both written and oral consent was taken from all participants prior to inclusion. The Danish study followed national laws adhering to good clinical practice and was approved by the Danish National Committee on Health Research Ethics (Protocol no. S-20200068C) and Danish Data Protection Agency (journal no. 20/19,878). The study conducted by UCSF was made with the approval of the institutional review board (University of California, San Francisco, Committee on Human Research, protocol # 21–33,240).

2.8. Data availability

Anonymized data will be shared on request from any qualified investigator under approval from the Danish Data Protection Agency.

2.9. Statistical analysis

All data were analyzed for normal distribution with Shapiro-Wilk test and with a visual inspection. Continuous data were presented as the median with minimum and maximum values. Pairwise analysis of Abs levels between different visits were done with Wilcoxon matched pairs signed ranks test. In groups with non-paired data, Mann-Whitney test was used to compare age, time of vaccination after last ocrelizumab infusion and length of time between second and third vaccination. Simple linear regression analysis was used to determine correlation between levels of Abs and variables such as time between ocrelizumab infusion and the third vaccine; and time between second and third vaccination. Level of significance was defined as $p < 0.05$.

3. Results

We analysed IgG antibodies against the SARS-CoV-2 spike RBD in a total of 64 participants from 1st to 3rd vaccination. However, we did not achieve complete sample colletion at all timepoints and analysed material from 50 participants before and 54 participants after the third BNT162b2 vaccination. Samples before and after the third booster vaccination were collected between September 13 and November 25, 2021. All participants were vaccinated with BNT162b2. The median patient age was 47 years (range 24 - 67 years). Participant baseline demographic and clinical characteristics are shown in Table 1.

Table 1
Baseline clinical and demographic characteristics.

|	Denmark	USA	Total
n	41	13	54
Female, n (%)	31 (75.6)	11 (84.6)	42 (79.2)
Median age, years (min-max)	47 (24–67)	47 (26–54)	47 (24–67)
Time interval between ocrelizumab treatment and 3rd vaccine, median weeks (min-max)	15.5 (0.9–44.3)	20.1 (9.3–32.6)	15,8 (0.9–44.3)
Time interval between 2nd and 3rd vaccine, median weeks (min-max)	26 (12–38.3)	19.7 (9.1–27.3)	25.3 (9.1–38.3)

3.1. Antibody levels before third vaccination

We found detectable Abs in 14.0% of participants at V2 ($n = 43$). At V3 ($n = 53$) 37.7% had measureable Abs, the median Ab-level being 74.2 BAU/mL (range: 8.5–2427) (Fig. 2C) (Novak et al., 2021). At V4 ($n = 50$), the frequency of seropositive patients decreased to 24.0% with a median level of 43.7 BAU/ml (range: 7.8–366.1 BAU/ml). 33.3% of patients with detectable Abs at V3 no longer had detectable Abs at V4. The participants that converted to seronegative at V4 had lower levels of Abs at V3 with a median level of 13.8 BAU/mL (range: 8.5–70.1). The patients with detectable Abs at both visits had a median Abs level of 276.5 BAU/mL (range: 22.8 −2405 BAU/mL). The median time from V3 to V4 was 21.8 weeks (range: 4.1–25.1 weeks) (Fig. 1) and the median time from V3 to V4 in participants who converted to negative was 20.3 weeks (range: 7.7–24 weeks). Overall, the Ab levels were significantly lower at V4 compared to V3 ($p = 0.0020$) (Fig. 2C).

3.2. Antibody levels after the third vaccination

After third SARS-CoV-2 vaccination (V5), 18 out of 54 (33.3%) participants demonstrated positive Abs with a median of 31.3 BAU/mL (range: 7.9–507.0 BAU/mL). Paired results between V3 and V5 were available in 43 participants, of whom 11 out of 43 (25.6%) had detectable Abs at both V3 and V5 with a median of 41.8 BAU/mL (range: 12.6–238.5 BAU/mL). However, the proportion of seropositive patients between V3 and V5 was not significantly different ($p = 0.1475$) (Fig. 2C). Furthermore, 53 participants had paired results between V3/ V4 and V5. In participants with previous undetectable AB-levels at V3/ V4, 7 out of 53 (13.2%) participants had detectable Ab at V5 with a median of 14.4 BAU/mL (range: 7.9–18.8 BAU/mL). Mean Ab levels were significantly higher at V5 compared to V4 ($p = 0.0313$) (Fig. 2C).

3.3. Antibodies levels of protection after third vaccine (at V5)

When we categorized Ab levels according to protection against infection, 7.4% ($n = 4$ out of 54) participants had low Ab levels between 7.1 and 17.0 BAU/ml, that would thereby be considered as providing less than 50% protection. In the intermediate group, 24.1% ($n = 13$) had levels between 17 and 506 BAU/ml and are considered to have above 50% protection. One (1.8%) patient had more than 506 BAU/ml and is considered to have protection >80%.
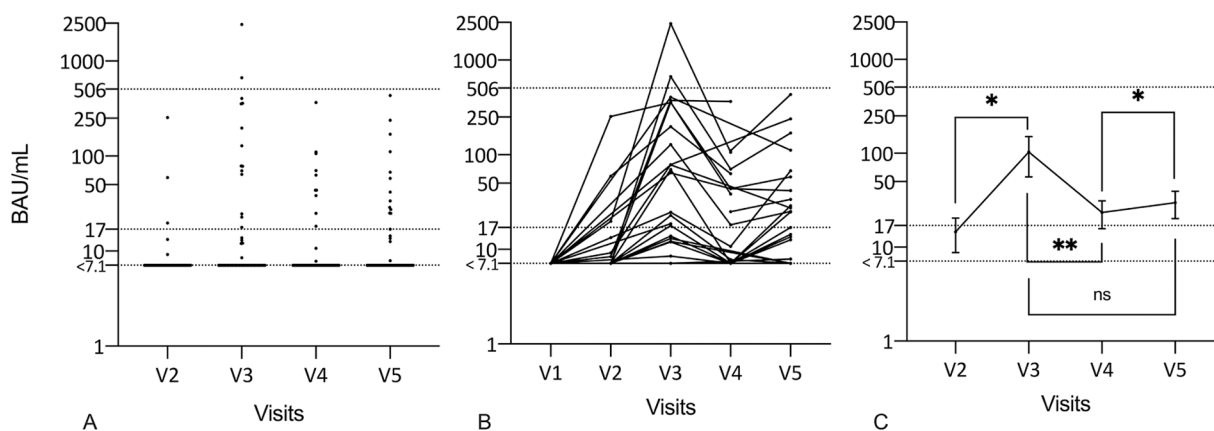
Fig. 2. Depicts levels of antibodies (Abs) given in BAU/mL- Y-axis with logarithmically scaled. BAU/ml = Binding antibody unit per ml. V1= Visit 1 (0–7 days before the 1st vaccine), V2= Visit 2 (0–7 days before the 2nd vaccine), V3 = Visit 3 (2–4 weeks after the 2nd vaccine dose), V4 = Visit 4 (0–7 days before the 3rd booster vaccine) and V5 = Visit 5 (2–4 weeks after the 3rd booster). Cut-offs are depicted with dotted lines. We defined seronegative (undetectable Abs) as 〈 7.1 BAU/mL, low levels of Abs as 7.1–17 BAU/mL, intermediate levels of Abs as 17–506 BAU/mL and high levels of Abs as 〉 506 BAU/mL.

Graph A: Ab-Levels after 1st vaccination and before 2nd vaccine at V2, after 2nd vaccine at V3, before 3rd booster at V4 and after the 3rd booster at V5. Each dot corresponds to the measured Ab-level.

Graph B: Outlines the Ab-development from V1-V5. All patients at V1 were seronegative.

Graph C: Depicts the mean of levels of Abs at each visit with standard error of mean. Lines demonstrates levels of significance (p-value) between visits in paired samples V2 vs V3 ($p = 0.03$)*, V3 vs V4 ($p = 0.002$)**, V3 vs V5 (p= ns) and V4 vs V5 ($p = 0.03$)*.

3.4. Clinical and demographic predictors of AB detectability

3.4.1. B-cells and T-cells

We compared levels of B-cells and T-cells between participants with or without detectable Abs at V4. No correlation were found in the levels of B-cells, $CD4^+$ and $CD8^+$ T lymphocytes between the seronegative and seropositive individuals (See Table 2, Appendix 1).

3.4.2. Age, time between ocrelizumab infusion and third vaccination, and time from second to third vaccination

There was no difference in median age between seropositive (45 years, range: 26–58) and seronegative participants (47 years, range: 24–67) ($p = 0.2254$). In addition, there was no difference between seronegative and seropositive participants in median time interval since the last infusion with ocrelizumab and the timing of third vaccination (15.7 weeks, range: 0.9–26.9 vs. 15.9 weeks, range: 3–44.3, $n = 52$). Linear regression analysis revealed no correlation ($r^2 = 0.04008$, $p = 0.44$) between Abs level and the time between ocrelizumab infusion and third booster vaccine (Fig. 3).

Finally, we compared levels of Abs and the time interval between the second and the third vaccination. There was no significant difference in median time intervals between seropositive (24.5 weeks, range: 9.1-27.4) and seronegative (26.0 weeks, range: 12–38.3), participants. In addition, we found no correlation between Abs level and time from second to third vaccination (r^2=0.00097, $p = 0.90$) (Fig. 3).

3.4.3. Spike-specific CD4+ and CD8+ t cells

We also measured the frequencies of spike-reactive T cells at V1 ($n = 28$), V3 ($n = 31$) and V5 ($n = 23$). Consistent with prior reports, the mean frequencies spike-specific $CD4^+$ and $CD8^+$ T cells at V3 (0.65×10^9 cell/$l \pm 0.08\%$ and 0.95×10^9 cell/$l \pm 0.20\%$, respectively) were significantly increased compared to V1 (0.02×10^9 cell/$l \pm 0.01\%$ and 0.06×10^9 cell/$l \pm 0.02\%$, respectively). While there was a marginal increase in spike-specific CD4+ and CD8+ T cell frequencies at V5 (0.99×10^9 cell/$l \pm 0.22\%$ and 1.3×10^9 cell/$l \pm 0.34\%$, respectively), this was not significantly increased compared to V3 (Fig. 4).
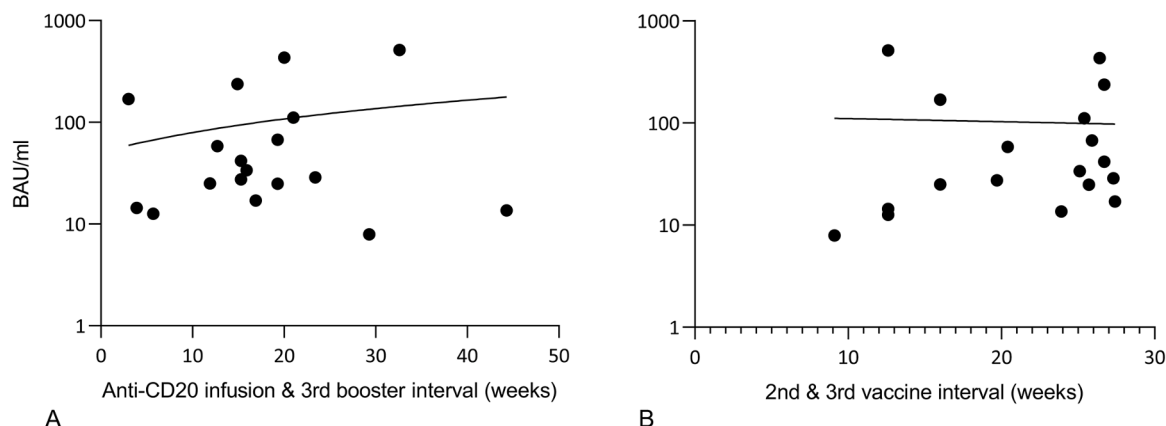
Fig. 3. Graph A: Linear regression analysis of antibody levels 2–4 weeks after third vaccination in seropositive patients with time interval from the last ocrelizumab infusion. $r^2= 0.03779$, $p = 0.4395$.

Graph B: Linear regression of antibody levels 2–4 weeks after third vaccination in seropositive patients with time interval between second and third vaccine. r^2=0.00097, $p = 0.9$.

BAU/ml = Binding antibody unit per ml.
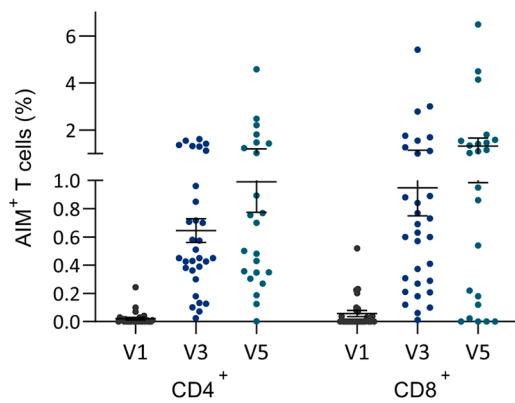
Fig. 4. Scatter plot graph depicting frequencies of spike-reactive CD4^{+} and CD8^{+} cells mean with SEM at visit 1 (V1), visit 3 (V3) and visit 5 (V5). Y-axis is scaled in two segments from 0 to 1 percent and 12–6 percent.

V1= Visit 1 (0–7 days before the first vaccine), V3 = Visit 3 (2–4 weeks after the second vaccination), and V5 = Visit 5 (2–4 weeks after the third vaccination).

AIM = Activation-induced markers of positive T-cells

SEM = standard error of the mean.

4. Discussion

In this cohort of patients treated with ocrelizumab before mRNA SARS CoV-2 vaccination, we found minimal change in seroreactivity following a third vaccination, both in terms of seroconversion and antibody titers compared to healthy controls (Meschi et al., 2021). In addition, the timing of anti-CD20 infusion and a third vaccination did not impact the antibody response.

We previously demonstrated that the majority of ocrelizumab treated patients converted from negative to positive Abs after the second vaccination. Thus, we expected a higher proportion of seropositive patients after the third vaccination (Novak et al., 2021) (Fig. 1). Here we compared proportions of SARS-CoV-2 Abs-positive participants after the first, second, and third BNT162b2 mRNA SARS-CoV-2 vaccine. We found that 14.0%, 37.7%, and 33.3% were seropositive, respectively. Seropositivity dropped to 25.0% of participants before the third vaccination, which is likely related to low antibody titers at V3 and to the median time of 25 weeks between the second and third vaccination (Table 1). Circulating Ab-levels are known to decrease from month two to six, also in healthy individuals (Levin et al., 2021). To prevent conversion from positive to negative, a shortened interval between repeat vaccinations may be indicated in B-cell depleted patients.

A total of 26.7% participants in the seropositive group at V5 had no detectable levels of Abs at the previous visits (V1-V4), suggesting a certain degree of humoral reactivity can be achieved even in previously seronegative patients. Conversely, 20% of participants showed fluctuating seropositivity, converting from seropositivity at V3, to seronegativity at V4 and again to seropositivity at V5. These participants had low levels of Abs at V3, likely accounting for the fluctuating seropositivity. The same tendency is not observed in healthy controls (Goel et al., 2021; Canaday et al., 2021; Falsey et al., 2021). This suggests that even in those patients who achieve low titer seroconversion, the protective effects may be transient at best. The remaining proportion of the group was seropositive throughout V3-V5.

Our study did not find a correlation between anti-spike RBD Ab-levels and the time interval between vaccinations. More studies with different strategies, such as additional vaccination or permitting B cell reconstitution, are needed to optimize humoral responses to SARS-CoV-2 vaccination.

Several recent studies on third SARS-CoV-2 vaccine responses in anti-CD20 treated patients revealed seroconversion in a subset of individuals who had remained seronegative after two vaccinations (Sidler et al., 2021; König et al., 2021; Achtnichts et al., 2021). In contrast, our study

of a longitudinal cohort from two international sites demonstrates that both the proportion of patients with positive Abs and the Ab-levels are overall unchanged compared to after the initial two dose vaccinations and only a minority of patients that were previously negative have detectable antibodies after the third vaccination.

Other studies have suggested that an extended dosage interval of ocrelizumab could improve the humoral response (Disanto et al., 2021; Ali et al., 2021). Extended dosing of ocrelizumab has been suggested safe in selected patient population with low short-term disease activity (Rolfes et al., 2021; AbdelRazek et al., 2022; Van Lierop et al., 2021). It is however not standard practice in Denmark and USA since the risk of disease activity with extended treatment interval has only been examined in non-randomized trials.

We found no correlation between levels of B- and T-cells and Ab-levels. B-cells are related to humoral response, and other studies have proven relationship between levels of B-cells and SARS-CoV-2 Abs (Fig. 2) (Disanto et al., 2021). Currently, the Danish Health Authority recommends the third vaccination 4.5 months after the second vaccination and US Centers for Disease Control and Prevention (CDC) recommends additional vaccine shots to be given after 5 months from third vaccination. Such recommendation will result in re-vaccination more than twice a year and extended treatment intervals in this scenario is logistically not possible and could be harmful to the patient.

Our study also assessed T cell responses following third SARS-CoV-2 vaccination in anti-CD20-treated MS patients. While spike-specific CD4^{+} and CD8^{+} T responses remained high after third vaccination, they were not significantly increased compared to the responses after the second vaccination. These findings therefore suggest that additional vaccinations do not significantly augment the cellular response in anti-CD20-treated patients. Other studies compared MS patients on ocrelizumab with both MS patients on first line treatment and healthy controls and found no significant difference in T-cell responses after second vaccination. This is similar to our results, before and after second and third vaccine (Habek et al., 2022; Apostolidis et al., 2021).

There were limitations to our study. The short inclusion timeframe resulted in a few missing samples at V4 and V5. Furthermore, the cut-off levels presented in this study reflect reactivity against alpha variants of SARS-CoV-2, but not more recent variants of concern, including the delta or omicron variants.

In summary, in contrast to prior studies, in this longitudinal cohort we found no significant increased protective benefit from a humoral or cellular perspective with administration of a third SARS-CoV-2 mRNA vaccination. These findings have important clinical implications and suggest the need for clinical strategies to include allowance of B cell reconstitution before repeat vaccination and/or provision of pre-exposure prophylactic monoclonal antibodies.

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Author contributions

H Bajwa, F Novak, T Sejbaek, J Sabatino, I S Johansen, KE Byg, and R Bove all contributed to study design

H Bajwa, F Novak, T Sejbaek, J Sabatino, W Rowles, AH Witt, K Østergaard all contributed to patient recruitment and patient data

F Novak, T Sejbaek, J Sabatino, A C Nilsson, C Nielsen, K Mittl, and W Rowles all contributed to sample processing

H Bajwa, F Novak, T Sejbaek, J Sabatino, A C Nilsson, C Nielsen, I S Johansen, KE Byg, AH Witt, K Østergaard, S Zamvil, and R Bove all contributed to data analysis/interpretation

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2022.103729.

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