mRNA COVID-19 vaccination does not exacerbate the disease or trigger an anti-neural antibody response in multiple sclerosis

eMethods:

1. **T-cellular response (IFN-ɣ ELISpot)**
2. **Immunohistochemistry on frozen sections of paraformaldehyde-perfused or post-fixed rat brain**
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1.T-cellular response (IFN-ɣ ELISpot)

Stimulation was conducted with $2x10^5$ peripheral blood mononuclear cells in X-VIVO 15 medium (Lonza) supplemented with 10% heat inactivated AB serum and PepTivator SARS-CoV-2 Prot_S (1 µg/mL, Miltenyi Biotec) that cover the immunodominant sequence domains of the spike ("S") glycoprotein of SARS-CoV-2 (GenBank MN908947.3, Protein QHD43423.2) and N peptide pools. The latter peptide pool N-ELISpot represents a control for exposure to the SARS-CoV-2 nucleocapsid phosphoprotein, as the mRNA-1273 vaccine is based on the S protein. Negative control wells lacked peptides, while positive control ones included anti-CD3-2 mAb. Cells were incubated overnight at 37°C 5% CO2 in pre-coated anti-IFNγ MSIP white plates (mAb 1-D1K, Mabtech). After incubation, plates were washed with PBS (Sigma-Aldrich) and incubated 2h at room temperature with horseradish peroxidase (HRP)-conjugated anti-IFN-γ detection antibody (1 µg/mL; clone mAb-7B6-1; Mabtech). After further washes with PBS, tetramethylbenzidine (TMB) substrate was added and spots were counted using an automated ELISpot Reader System (Autoimmun Diagnostika GmbH). To quantify positive peptide-specific responses, spots of the unstimulated wells were subtracted from the peptide-stimulated wells, and the results expressed as Spot forming units SFU/2×10^5 PBMCs. We determined SARS-CoV-2–specific spots by spot increment, defined as stimulated spot numbers >6 SFU/2×105 PBMCs against the N and the S protein. This cutoff was defined calculating the mean ± 2 standard deviations in a group of healthy donors obtained before the start of the pandemic of SARSCoV-2. Spot counting was done automatically and re-evaluated manually in all cases.[1]

2. Immunohistochemistry on frozen sections of paraformaldehyde rat brain

The presence of onconeuronal (Hu,Yo, Ri, CRMP5, amphiphysin, Ma2, Tr), GAD, and AK5 antibodies were examined using immunohistochemistry on sections of paraformaldehyde rat brain. Adult male Spraguey Dawley rats were anesthetized, sacrificed and perfused with 2% paraformaldehyde. Rat brains were removed, sagittally sectioned and fixed for 4 hours in 2% paraformaldehyde at 4°C. Subsequently, brains were cryoprotected with 20% sucrose for 48h, embedded in freezing medium, and snap frozen in isopentane chilled with liquid nitrogen. Seven-micron thick cryostat-cut sections from the cerebellum were defrosted for 20 minutes, washed once in PBS and then incubated with 10% goat serum diluted in 0.03% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 30 minutes. For IHC-c, sections were incubated with patients´sera (1:500, diluted in 10% goat serum in 0.03% Triton-X-100) for 3 hours at 37°C, then they were washed 2x in PBS, labeled with biotinylated goat anti-human IgG (Vector lab) (1:8000, diluted in PBS) for 30 minutes, washed 2x in PBS, and incubated with avidin-biotin peroxidase

for 30 minutes at room temperature. The reactivity was developed with diaminobenzidine for 1 to 1.5 minutes (Vector lab).[2] Samples were analyzed at T3 (1 month after the second dose), and at T6 (12 months after the first dose).

Immunohistochemistry on frozen sections of post-fixed rat brain

The presence of neuronal cell-surface and glial antibodies (NMDAR, AMPAR, $GABA_BR$, $GABA_AR$, neurexin 3 α, IgLON5, CASPR2, LGI1, DPPX, mGluR1, mGluR5, and AQP4) were examined using immunohistochemistry on sections of post-fixed rat btrain. Adult female Wistar rats were sacrificed in a CO_2 chamber. Non-perfused rat brains were removed, sagittally sectioned and fixed for 1h in 4% paraformaldehyde at 4°C. Subsequently, brains were cryoprotected with 40% sucrose for 48h, embedded in freezing medium, and snap frozen in isopentane chilled with liquid nitrogen. Seven-micron thick cryostat-cut sections were defrosted for 20 minutes, washed once with PBS and then incubated with 0.3% hydrogen peroxide for 15 minutes. After washing 3x with PBS, slides were incubated with 5% goat serum in PBS for 1h, and then labeled with patients´or control sera (1:200, diluted in 5% goat serum) or CSF (1:2) at 4°C overnight. The next day, sections were washed 3x in PBS, labeled with biotinylated goat anti-human IgG (Vector lab) (1:2000, diluted in 5% goat serum) for 2h, washed 3x in PBS, and incubated with avidin-biotin peroxidase for 1h at room temperature. The reactivity was developed with diaminobenzidine for 7 minutes (Vector lab).[3,4] Samples were analyzed at T3 (1 month after the second dose), and at T6 (12 months after the first dose).

3.Cell based assay (CBA) for MOG-IgG detection

All samples were examined for MOG-IgG using an in-house CBA with HEK293 cells transfected with the full-length MOG C-terminally fused to EGFP. Live cells were incubated at 37ºC with serum (1:160 diluted with DMEM) for 35 minutes. After removing the media and washing with PBS, HEK cells were fixed with 4% PFA for 10 minutes and incubated with 0.3% Triton X-100 for 5 minutes. Then cells were immunolabeled with Alexa Fluor 594 secondary antibody against IgG- Fcγ fragment-specific (1:1000). A cut-off $\geq$1:160 was considered as MOG-IgG positive sample. For some experiments plasmids containing MOG cDNA from rat (rMOG) or mouse (mMOG) were used to detect the reactivity against rodent MOG.[5] Samples were analyzed at T3 (1 month after the second dose), and at T6 (12 months after the first dose).

4. Detection of anti-ganglioside antibodies

Patients' sera were screened for the presence of anti-ganglioside antibodies using a previously validated ELISA protocol.[6] Immulon 96-well ELISA plates (Thermo Fisher Scientific) were coated with the different gangliosides to be tested (in this case, GM1, GD1b and GQ1b) diluted in methanol, and incubated at room temperature for 4 hours. Then wells were blocked with 1% bovine serum albumin (BSA) in PBS for 2 hours at 4°C and incubated with the sera diluted in 0.1% BSA in PBS overnight at 4°C. The next day, the wells were incubated with peroxidase-conjugated anti-IgG or anti-IgM secondary antibodies for 2 hours at 4°C. The ELISA was developed with OPD solution (Sigma), and the reaction was stopped with 25% sulphuric acid. Optical density was measured at 490 nm with a Multiscan ELISA reader. Results were represented as the difference in optical density (ΔDO) between the sample well and the blank well (without any ganglioside). Samples were considered positive when they had a ΔOD greater than 0.1. Antibody titres were obtained by testing different dilutions of sera: 1/100, 1/500, 1/2500 and 1/12500. Anti-ganglioside antibodies were considered positive at a minimum titre of 1/1000. Samples were analyzed at T3 (1 month after the second dose).

5. Detection of antibodies against nodo-paranodal antigens

Autoantibodies against NF140, NF186, NF155, CNTN1 and CASPR1 were tested by ELISA. Maxisorb 96-well ELISA plates (Thermo Fisher Scientific, NUNC, Denmark) were coated with 1µg/ml human recombinant CNTN1 protein (Sino Biological Inc., Georgia, USA), 1µg/ml NF155 protein (Origene, Maryland, USA), 1µg/ml NF140 protein (Sino Biological), 1 µg/ml NF186 protein (Origene) or 5µg/ml CASPR1 protein (R&Dsystems, MI, USA) overnight at 4 °C. Wells were blocked with 5% non-fat milk in PBS 0.1% Tween20 for 1 hour, incubated with sera diluted 1/100 in blocking buffer for 1 hour, and then incubated with peroxidase conjugated rabbit anti-human IgG secondary antibody (Invitrogen, CA, USA) for 1 hour at room temperature. ELISA was developed with tetramethylbenzidine solution (Biolegend, California, USA), and the reaction was stopped with 25% sulfuric acid. Optical density (OD) was measured at 450 nm in a Multiscan ELISA reader. Samples were considered positive by ELISA when they had a ΔOD higher than mean healthy control ΔOD plus two standard deviations. Cell based assays were used as a second confirmatory technique for questionable cases. Briefly, mammalian expression vectors encoding human NF140, NF186, NF155, CNTN1 or CASPR1 were transfected into HEK293

cells using Lipofectamine 2000 (Invitrogen). Cells were then fixed with 4% paraformaldehyde and blocked. ICC experiments were performed using patient's sera and appropriate primary and secondary antibodies.[7] Samples were analyzed at T3 (1 month after the second dose).

eTable 1. Summary of clinical features of the 3 patients with multiple sclerosis and MOG-IgG

	Patient #1	Patient #2	Patient #3
Sex	Female	Female	Female
Age of onset, y	26	26	46
Onset attack type	Right optic neuritis	Left optic neuritis	
Clinical course	Relapsing-remitting	Relapsing-remitting	Relapsing-remitting
No. of relapses	3	4	1
Brain MRI	>30 T2 lesions	20 T2 lesions	19 T2 lesions
Dawson´s fingers	Yes	Yes	Yes
Periventricular	Yes	Yes	Yes
Yuxtacortical	Yes	Yes	Yes
U-fibre lesions	Yes	No	No
Corpus callosum	Yes	Yes	Yes
Infratentorial	Yes	Yes	Yes
Spinal cord MRI	C1, C3, C5, C6	C6, C7	C4, C5
Oligoclonal bands	N.D	Yes	Yes
Treatment	First: glatiramer acetate Last: Dimethylfumarate	First: glatiramer acetate Last: Teriflunomide	Teriflunomide
Last EDSS score	2.0	1.5	2.0
Follow-up, y	7	17	9
MOG-IgG titer	1: 320	1:320	1:160
Confirmed in stored sample at onset	Yes	N.A.	Yes

N.D: not done; N.A: not available

eTable 2. Frequency of SARS-CoV-2 infections according to the therapy

Drugs	Overall infections No. (%)	Infections first period No. (%)	Infections second period No. (%)
Untreated n= 60	21 (35)	1 (1.6)	20 (33.3)
Anti-CD20[a] n= 100	39 (39)	3 (3)	36 (36)
Fingolimod[b] n= 47	25 (53)	5 (10.6)	21 (44.7)
Interferon Beta n= 38	14 (36.8)	0	14 (36.8)
Glatiramer acetate N= 35	14 (40)	1 (2.9)	13 (37)
Dimethylfumarate n=70	30 (42.8)	2 (2.8)	28 (40)
Teriflunomide n= 35	16 (45.7)	1 (2.8)	15 (42.8)
Cladribine n= 6	2 (28.6)	0	2 (28.6)
Natalizumab n= 21	8 (38)	0	8
Alemtuzumab n=2	0	0	0
Corticosteroids n= 12	0	0	0
Double IS[c] n= 9	3 (33.3)	0	3 (33.3)
Other IS[d] n= 14	5 (35.7)	1	4 (28.6)
Clinical trial n= 2	1 (50)	o	1 (50)

First period from April 17 to December 15, 2021; second period in which the Omicron variant became predominant from December 16 to July 5, 2022.

[a]Anti-CD20: ocrelizumab (35)+ riruximab (59)+ofatumumab (6); [b]Fingolimod: fingolimod (45)+ponesimod (2)

[c]Double IS: double immunosuppressor: rituximab+prednisone (4); rituximab+prednisone+mycophenolate mofetil (1); azathioprine+prednisone (1); IV immunoglobulins+prednisone (1); mycophenolate mofetil+prednisone (2)

[d]Other IS: other immunosuppressor: Intravenous (IV) immunoglobulins (5); azathioprine (4); mycophenolate mofetil (2); tocilizumab (1); eculizumab (1); receptor of autologous hematopoietic stem cell transplantation (1);

eTable 3. Overall frequency of local and systemic adverse events

	After first dose No. (%)	After second dose No. (%)	After third dose No. (%)	Survey 1 vs Survey 2 p-value*	Survey 1 vs Survey 3 p-value	Survey 2 vs Survey 3 p-value	All three groups p-value
Total sample	306	304	171				
Side effects							
Pain	266 (87)	254 (84)	144 (84)	<0,0001	0,028	<0,0001	<0,0001
Redness	34 (11)	61 (20)	33 (19)	<0,0001	0,017	0,017	<0,0001
Inflammation	45 (15)	74 (24)	37 (22)	<0,0001	0,008	0,001	0,001
Fever	17 (6)	118 (39)	43 (25)	0,049	0,441	<0,0001	<0,0001
Headache	61 (20)	114 (38)	56 (33)	<0,0001	<0,0001	<0,0001	<0,0001
Fatigue	110 (36)	178 (59)	89 (52)	<0,0001	0,007	0,007	<0,0001
Myalgias	45 (15)	109 (36)	62 (36)	<0,0001	0,198	<0,0001	<0,0001
Arthralgias	27 (9)	83 (27)	41 (24)	<0,0001	0,430	<0,0001	<0,0001
Nausea	13 (4)	30 (10)	11 (6)	0,820	0,999	<0,0001	0,002

eTable 4. Frequencies of local and systemic adverse events in adolescent patients with multiple sclerosis

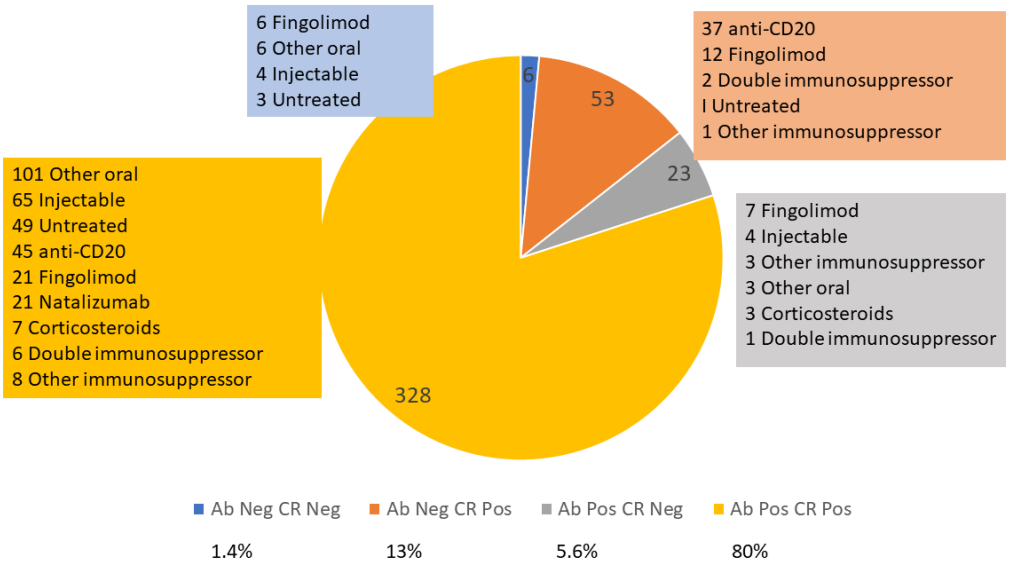
	After first dose No. (%)	After second dose No. (%)	After third dose No. (%)
Total sample	35	34	17
Side effects			
Pain	21 (60)	14 (41)	7 (41)
Redness	1 (3)	0 (0)	0 (0)
Inflammation	0 (0)	0 (0)	0 (0)
Fever	32 (91)	7 (21)	3 (18)
Headache	0 (0)	1 (3)	1 (6)
Fatigue	1 (3)	5 (15)	3 (18)
Myalgias	0 (0)	1 (3)	0 (0)
Arthralgias	0 (0)	0 (0)	0 (0)
Nausea	0 (0)	0 (0)	0 (0)

eTable 4. Frequencies of local and systemic adverse events in adult and adolescent patients with multiple sclerosis

	Adults No. (%)	Adolescents No. (%)	ǂp-value
Total sample	695	86	
Side effects			
Pain	622 (89.5)	86 (49)	<0.0001
Redness	127 (18.3)	1(1.2)	0.0001
Inflammation	156 (22.4)	0	<0.0001
Fever	165 (23.7)	42 (49)	<0.0001
Headache	229 (32.9)	2 (2.3)	<0.0001
Fatigue	368 (52.9)	6 (7.0)	<0.0001
Myalgias	215 (30.9)	1 (1.2)	<0.0001
Arthralgias	151 (21.7)	0	<0.0001
Nausea	54 (8)	0	0.0065

Data frequency after plotting the 3 surveys (after first, second, and third vaccine dose)

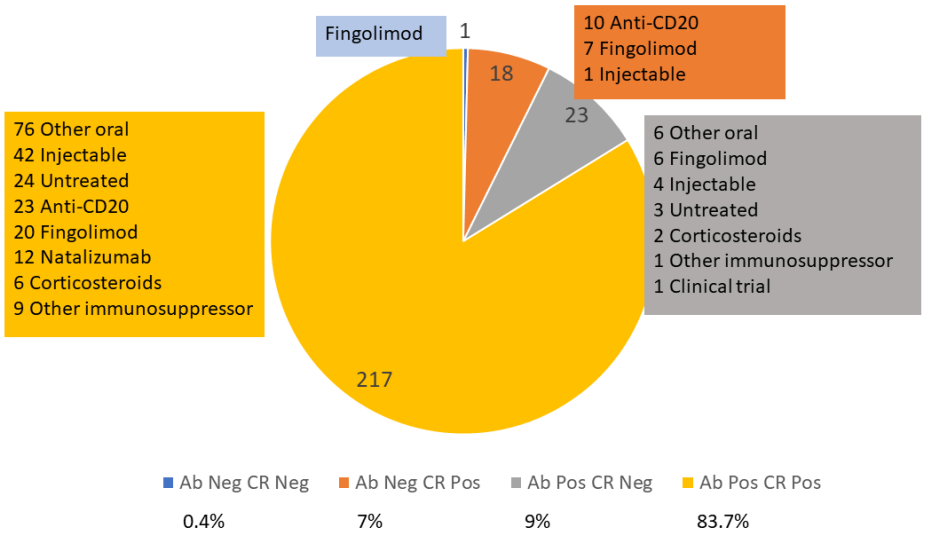
eFigure1. Humoral and cellular response 1 month after two doses



Abbreviations: Ab Neg CR Neg: humoral and cellular response negative; Ab Neg CR Pos: humoral response negative and cellular response positive; Ab Pos CR Neg: humoral response positive and cellular response negative; Ab Pos CR Pos: humoral and cellular response positive

Humoral and cellular response positive in 20/30 (66%) patients on ocrelizumab vs 15/44 (34%) on rituximab, *P*= 0.27, and in the 5 patients on ofatumumab analyzed.

eFigure2. Humoral and cellular response at 12 months



Abbreviations: Ab Neg CR Neg: humoral and cellular response negative; Ab Neg CR Pos: humoral response negative and cellular response positive; Ab Pos CR Neg: humoral response positive and cellular response negative; Ab Pos CR Pos: humoral and cellular response positive

Humoral and cellular response positive in 12/17 (70.5%) patients on ocrelizumab vs 7/12 (58%) patients on rituximab, *P*= 0.7; and in the 4 patients on ofatumumab analyzed

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