Analysis of the Gadolinium retention in the Experimental Autoimmune Encephalomyelitis (EAE) murine model of Multiple Sclerosis

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Abstract

Objectives: The aim of this study is to quantitatively investigate, at the preclinical level, the extent of Gd retention in the CNS, and peripheral organs, of immune-mediated murine models (Experimental Autoimmune Encephalomyelitis –EAE) of Multiple Sclerosis, compared to control animals, upon the injection of gadodiamide. The influence of the Gadolinium Based Contrast Agent administration timing during the course of EAE development is also monitored.

Methods: EAE mice were injected with three doses (1.2 mmol/kg each) of gadodiamide at three different time points during the EAE development and sacrificed after 21 or 39 days. Organs were collected and the amount of Gd was quantified through Inductively Coupled Plasma-Mass Spectrometry. Transmission electron microscopy (TEM) and MRI techniques were applied to add spatial and qualitative information to the obtained results.

Results: In the spinal cord of EAE group, 21 days after gadodiamide administration, a significantly higher accumulation of Gd occurred. Conversely, in the encephalon, a lower amount of Gd retention was reached, even if differences emerged between EAE and controls mice. After 39 days, the amounts of retained Gd markedly decreased. TEM validated the presence of Gd in CNS. MRI of the encephalon at 7.1 T did not highlight any hyper intense region.

Conclusion: In the spinal cord of EAE mice, which is the mostly damaged region in this specific animal model, a preferential but transient accumulation of Gd is observed. In the encephalon, the Gd

retention could be mostly related to inflammation occurring upon immunization rather than to demyelination.

Keywords

gadolinium retention, Multiple Sclerosis, Experimental Autoimmune Encephalomyelitis, Gadodiamide, Gadolinium based contrast agents.

Key points

- A damaged BBB leads to the detection of amounts of Gd significantly higher than in healthy mice.
- In the spinal cord of EAE mice, a preferential but transient accumulation of Gd is observed
- Ongoing inflammation in EAE mice may facilitate the retention of Gd in the CNS

List of abbreviations

MS: Multiple Sclerosis

GBCAs: Gadolinium Based Contrast Agents

EAE: Experimental Autoimmune Encephalomyelitis

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

TEM: Transmission Electron Microscopy

BBB: Blood Brain Barrier

CE-MRI: Contrast Enhanced Magnetic Resonance Imaging

EES: Extravascular Extracellular Space

PRAC: Pharmacovigilance Risk Assessment Committee

EMA: European Medicin Agency

FDA: Food and Drugs Administration

DN: Dentate Nucleus

IFA: Incomplete Freund's Adjuvant

MOG: Myelin Oligodendrocyte Glycoprotein

LOQ: Limit of Quantification

RARE: Rapid Acquisition with Refocussed Echoes

FOV: Field of View

MSME: Multi Slice Multi Echo

BCCAo: Bilateral Common Carotid Artery occlusion

Introduction

Multiple sclerosis (MS) is the most prevalent chronic inflammatory disease of the central nervous system (CNS) and currently incurable. MS is a demyelinating disease and a major cause of neurological disability because self-reactive immune cells, due to enhanced permeability of the blood-brain barrier (BBB), enter the CNS leading to myelin destruction and neuronal damage and the subsequent formation of lesions, also called plaques.

MRI is an important tool in the diagnosis of MS [1,2] as well as in the monitoring of disease and in the assessment of treatment efficacy. Clinical protocols for conventional MRI typically include T_1 contrast-enhanced MRI (CE-MRI) that is used to visualize blood–brain barrier breakdown, representing active lesions. Disruption of the BBB enables the extravasation of low-molecular weight contrast agents leading to locally increased signal intensity in T_1-weighted images. Enhancement persists for an average of 3 weeks and then resolves.

Gadolinium-Based Contrast Agents (GBCAs) are the most widely used contrast agents for positive MR imaging. All clinically available GBCAs are complexes containing the Gd(III) ion chelated by octacoordinating ligands. The "free" Gd ion is highly toxic because it acts as a Ca(II) antagonist.[3]

GBCAs are considered very safe products on the basis of more than 300 million administrations, with a very low frequency of acute adverse events. However, in 2006, a relationship between GBCAs and a new nosological entity, the Nephrogenic Systemic Fibrosis (NSF), was reported. Subsequent reports highlighted that the incomplete excretion and the prolonged presence in the body of Gd in patients with reduced renal function, could be associated to the insurgence of the pathology.[4]

More recently, it has been reported that tiny amounts of Gd(III) are retained in tissues also in absence of renal dysfunction.[5–7] Several publications followed these seminal observations and the Gd retention issue attracted more and more importance in the field of CE-MRI.[8,9] In November 2017, in fact, the European Medicines Agency (EMA) recommended suspending or restricting marketing authorizations for GBCAs based on linear chelators and warnings have been issued by the United States Food and Drug Administration (FDA).

Patients with MS undergo frequent CE-MRI thus receiving numerous GBCA administrations over their lifetime. Even if, in general, no clinical impact associated to Gd retention in brain has yet been reported, and, in the specific field of MS patients, there are studies suggesting that that Gd retention in the brain does not affect their clinical worsening,[10] several reports showed long-term increased T_1-signal in the dentate nucleus (DN) after administration of linear[11–13] and, although to a less extent, macrocyclic GBCAs.[14–16] More work appears necessary to shed light on this matter that remains the source of great concern among the MS patients.

Recently, a study[17] has been carried out at the pre-clinical level, on an immune-mediated murine model of MS, represented by the Experimental Autoimmune Encephalomyelitis (EAE), showing that, after repeated administrations of Gd-DTPA, EAE mouse brains retained higher levels of Gd with respect to healthy controls suggesting that ongoing inflammation may facilitate the retention of Gd

in the brain tissue. Nevertheless, this study can be considered only a pilot investigation as the small number of animals included in the experiments did not allow a quantitative analysis of the results. Notably, the well-characterized EAE model represents an invaluable tool to systematically study Gd retention in different organs and tissues.

Herein, our aim is to quantitatively assess, at the preclinical level in the EAE mice models of MS, the amounts of Gd retained with particular attention to the regions of CNS. The results obtained in the EAE mice are compared to two groups of control mice[18,19], namely, (I) the sham-immunized control group (SHAM), to verify that the effects observed in induced EAE mice are attributable to an immune response generated against myelin antigens able to induce demyelination, and (II) the healthy control group (h-CTRL), to compare the results with a physiological healthy condition.

Methods

1. *Animals*

All experimental procedures were carried out at Neuroscience Institute Cavalieri Ottolenghi (NICO), approved by the Ethical Committee of the University of Torino and authorized by the Italian Ministry of Health (authorization number: 808/2017-PR in 19/10/2017 and in 18/03/2020). The experiments have been carried out in accordance with the European Community Parliament and Council Directives of 24 November 1986 (86/609/EEC and 2010/63/EU). Female C57BL/6J mice used for all the experimental procedures were purchased from Envigo RMS srl (Udine, Italy).

2. *EAE induction and clinical evaluation*

To induce EAE, 6–8 week-old-female C57BL/6 mice were immunized according to the procedure reported in [20,21] and described in supporting material. Body weight and clinical score (0=healthy; 1=limp tail; 2=ataxia and/or paresis of hind limbs; 3=paralysis of hind limbs and/or paresis of forelimbs; 4=tetraplegia; 5=moribund or dead) were recorded daily by an investigator blind to group identity.

3. *Gadolinium Based Contrast Agent (GBCA) administration protocols*

In this study, a total of 3 intravenous injections of gadodiamide (Omniscan®, GE Healthcare, Gd(DTPA-BMA)), a linear neutral GBCA, were administered at a dose of 1.2 mmol/kg every 2 days to three different groups of mice: i) healthy (h-CTRL), ii) SHAM and iii) EAE mice. In mice the dose of 1.2 mmol/Kg is considered equivalent to the usual human dose of 0.1 mmol/kg of GBCA upon adjusting for body surface area as recommended by the FDA (Food and Drug Administration) guidelines.[22] The GBCA administration and the sacrifice were performed following four different protocols as detailed in supporting material.

4. Inductively Coupled Plasma-Mass spectrometry (ICP-MS) quantification of Gd content

The Gd content of the collected organs and tissues was measured by ICP-MS analysis (Element-2; Thermo-Finnigan, Rodano (MI), Italy) and the results expressed as nmol/g of wet tissue weight. The preparation of the samples and ICP-MS analysis were carried out according to the procedure reported in [23] and detailed in supporting material. The LOQ of the method in the different tissues/organs are reported in supporting material (Table S1).

5. Transmission Electron Microscopy (TEM)

TEM was carried out on cerebellum and spinal cord of h-CTRL (n=3) and EAE (n=3) mice injected during the chronic phase of the disease after the onset (i.e. at 19, 21 and 23 dpi) and sacrificed 21 days after the last administration. Conventional TEM was carried out as in [24]. Details are reported in supporting material.

6. Magnetic Resonance Imaging

MR images of the brain were acquired for healthy control mice (h-CTRL, n=5) not injected with gadodiamide and for four groups of mice 21 days after the last gadodiamide injection. Specifically, SHAM and EAE mice (n=3 per group), injected during the pre-symptomatic phase (i.e. at 4, 6 and 8 dpi) or in the time range between the onset and the peak of the disease (i.e. at 11, 13 and 15 dpi). Animals were deeply anesthetized and MR images were acquired at 7.1 T on a Bruker Avance 300 spectrometer equipped with a Micro 2.5 microimaging probe at room temperature (*ca.* 21 °C). Details on images acquisition are reported in supporting material.

7. Statistical Analysis

All data are expressed as the mean values of at least three independent experiments ± standard deviation (SD). Between group differences with respect to the mean Gd concentration were assessed using unpaired t test or two-way ANOVA followed by the Bonferroni's multiple comparison post-hoc test. Graph-Pad Prism 7.00 software was used for data analysis. Overall, statistical significance was defined as follows: *p<0.05, **p<0.01, ***p< 0.001; unless differently specified.

Results

1. ICP-MS quantification of retained Gd in healthy control, SHAM and EAE mice upon injection of gadodiamide in different disease phases

Figure 1 shows the clinical course (A, C) and the body weight loss (B, D) during time in h-CTRL, SHAM and EAE mice. All EAE mice had an onset of disability and weight loss. The h-CTRL and SHAM mice rightfully showed no disability and weight loss.

Figure 2 and table 1 report the amounts of retained Gd in the CNS organs of h-CTRL, SHAM and EAE mice injected with 3 doses (1.2 mmol/kg each) of gadodiamide.

In the spinal cord (Figure 2A), a low Gd retention was observed in the h-CTRL, in the whole SHAM group and in the EAE mice administered with GBCA in the pre-symptomatic phase. Conversely, upon the GBCA injection in the onset and chronic phase of the model, in EAE group, a significantly higher and progressive accumulation of Gd is observed (six times higher than in h-CTRL, in the chronic group). In the SHAM group the Gd retention was not significantly different from that of h-CTRL mice.

Conversely, in cerebrum (Figure 2B) and cerebellum (Figure 2C), a similar behaviour was observed for EAE and SHAM mice. For both groups of mice, a modest but significantly higher retention of Gd was observed, with respect to h-CTRL mice, when animals were injected with the GBCA in the pre-symptomatic phase and on the onset of the disease course but not in the chronic phase.

Figure 3 reports the amounts of retained Gd in the spinal cord of each EAE mouse as a function of its mean clinical score (calculated as the ratio between the respective cumulative score and the days of life between immunization and sacrifice). A positive correlation was observed (r = 0.55). The same analysis was carried out in cerebrum and cerebellum without finding any significant correlation (data not shown).

The assessment of the amounts of retained Gd was extended to other organs beside those of the CNS, namely to muscle, spleen, kidneys, liver and bone, (Figure S1 of Supporting Material). In muscle (Figure S1 A) and spleen (Figure S1 B), no differences emerged in the amounts of retained Gd either comparing the three groups (h-CTRL, SHAM and EAE) or the disease phases when mice were injected. In the kidneys (Figure S1 C), for SHAM mice injected in the pre-symptomatic and chronic phases and for EAE mice injected in the chronic phases, a significantly higher concentration of Gd was observed with respect to control mice. In the liver (Figure S1 D) and bone (Figure S1 E), a significantly lower concentration of Gd was observed for EAE mice injected in the chronic phase with respect to h-CTRL mice. In addition, a negative correlation was observed (r= -0.412) between the amounts of retained Gd in the bone of each EAE mouse as a function of its mean clinical score (Figure S2). The same analysis was carried out in all the other investigated organs without finding any significant correlation (data not shown).

2. Time dependence of Gd retention in EAE and SHAM mice

In Figure 4 the time dependence of Gd retention in spinal cord (A), cerebrum (B) and cerebellum (C) of EAE and SHAM mice injected with 3 doses (1.2 mmol/kg each) of gadodiamide during the chronic phase of the disease development were compared. In all the CNS analysed regions, a decrease of Gd retention is observed extending the time after the last GBCA injection, indicating an efficient washout mechanism. Specifically, the decrease of retained Gd is particularly evident in the spinal cord of EAE mice and, to a lesser extent, in SHAM mice.

3. *TEM analysis*

In order to qualitatively validate the presence of Gd in CNS, TEM was applied. Specifically, TEM analysis showed, Gd-spheroidal deposits with typical sea urchin–shaped in the basal lamina of blood vessels (Figure 5A and F) and intracellular inclusions (Figure 5A, B, D, F and G), in form of small electron-dense dots in dental cerebellar nuclei (Figure 5A-D) and in spinal cord (Figure 5E-F). Here, Gd-depositions have been shown in a not-damaged area of the cerebellum as indicated in the low magnification image (Figure 5A) and in a demyelinated region of the spinal cord (Figure 5F). Demyelination in the spinal cord is demonstrated by the marked reduction of myelin ensheathment (Figure 5F) in comparison to a not-demyelinated area (Figure 5E).

4. Magnetic Resonance Imaging

MR images of the brain were acquired before the sacrifice (*i.e.* 21 days after the last gadodiamide injection) at 7.1T on groups of mice which received GBCA injection in the pre-symptomatic phase and in the onset of disease progression compared to those of control CTRL mice not injected with gadodiamide (Figure S3). No significant difference in signal intensities was observed in all investigated groups of mice in cerebrum and cerebellum regions (Figure S4). We did not observe hyper-intense signals even in the dentate nuclei of all groups of mice.

Discussion

The main objective of this study was to investigate if the specific conditions related to MS pathology (i.e. BBB disruption, inflammation, formation of plaques) may have a role in the retention of Gd upon repeated injections of a GBCA in a murine MS model. The applied murine model induced chronic EAE and it is considered the most suitable choice to study MS pathology[25,26]. In our study, the model allows us to assess the role of GBCA injections during the pre-symptomatic, onset and chronic disease stage on the amount of retained Gd.

The BBB protects neurons from species present in the systemic circulation and maintains the highly regulated CNS internal milieu[27]. MS neuropathology is characterized by BBB breakdown which plays a crucial role on the pathogenesis of MS disease[28].

Even though several studies have dealt with Gd deposition in healthy animals, few data were available on animal models mimicking human neurodegenerative diseases or BBB damage [29].

Here we deal with a rigorous and reproducible animal study which allows for the comparison between healthy and diseased subjects leading to elucidate the extent of Gd-retention, to compare it to what observed in healthy animals.

With respect to the study published by Wang et al[17] on a closely related EAE model, several improvements were pursued, such as i) increasing the number of analysed mice in order to get a statistical significance of results, ii) increase the sacrifice time after the last administration in order to report on the amount of Gd effectively retained by the considered body districts, iii) explore the influence of GBCA administration timing during the disease progression on the amount of retained Gd and iv) using, beside healthy control mice, a sham immunized control group in order to discriminate between simple inflammation and BBB breakdown from the immune response mounted against antigens inducing demyelination.

In the present study, only one GBCA was examined as the attention was focussed on the information that can be gained upon comparing different injection protocols rather than on the comparison of different GBCAs. Gadodiamide was chosen because it is well established that, among the clinically used agents, it is the most prone to yield Gd deposition thus being able to highlight eventual differences among the applied protocols even upon only three GBCA administrations.

Though the correlation analysis between the amounts of retained Gd against the pathological signs in CNS have not been evaluated, some considerations can be made. It is known that, in C57BL/6 EAE model, spinal cord is the favourite target area for plaques formation and multifocal, confluent areas

of mononuclear inflammatory infiltration and demyelination in the peripheral white matter of the spinal cord are frequently observed.[30,31] Interestingly, here, it was observed that the spinal cord shows a Gd retention up to six times higher than inflamed and healthy condition. However, cerebrum and cerebellum accumulate Gd at lower extent in comparison to spinal cord and they do not report differences between EAE and SHAM mice. These results could suggest that, in regions characterized by minor demyelination, such as the brain, the lower but significant increased Gd retention (almost doubled for both EAE and SHAM) observed with respect to h-CTRL mice could be associated to the general inflammation state, induced by IFA and Mycobacterium tuberculosis administration or by the intravenous injection of Pertussis toxin, which characterizes both EAE and SHAM mice. Conversely, where massive demyelination occurs, such as in the spinal cord, Gd retention increases dramatically. The hypothesis is also supported by the fact that we have not reported an increase in the amount of Gd retention in the spinal cord of the EAE mice injected with GBCA in the pre-symptomatic phase, when demyelination shouldn't have happened yet. Interestingly, in cerebrum and cerebellum, we determined an increased Gd retention only when EAE and SHAM mice were injected with gadodiamide in the pre-symptomatic phase and on the onset of the disease, i.e. at a relatively short time after BBB disruption. But rather, if the GBCA injections are moved to the chronic phase of disease the amount of retained Gd is almost the same as that retained by healthy mice. This datum supports the concept that the temporal proximity between BBB disruption and GBCA injection could influence the Gd retention in CNS.

This hypothesis is further supported by the positive correlation, in the spinal cord, between the amount of retained Gd and the clinical score of each EAE mice. This result seems to be highly relevant for translation to human MS patients, as it recalls the attention on possible enhanced Gd retention in the regions mostly damaged by MS pathology.

Importantly, this preferential accumulation of Gd in the spinal cord of EAE mice has demonstrated to be transient, as, moving the sacrifice time to 39 days after the last administration, a marked decrease in Gd retention is observed indicating an efficient washout mechanism.

An interesting result was obtained looking at the amount of retained Gd in the bones of EAE mice. Contrary to what observed in the other body districts, the extension of retained Gd is inversely proportional to the disease progression. Likely, this observation can be associated to the disability fostered by the pathological conditions of the model and to the lower motility of diseased mice.

The TEM analysis allowed us to add spatial and qualitative information to the obtained results. Accordingly to [32] we observed Gd-containing deposits with typical sea- urchin shape in the basal lamina of blood vessels and as intracellular inclusions. In addition, for the first time, we highlighted the characteristic shape of Gd-deposition not only in the cerebellum but also in the spinal cord of EAE mice. However, further TEM systemic analysis will be necessary to clarify the exact location of retained Gd in the damaged CNS of EAE mice.

The increased retention of Gd in the brain and spinal cord of EAE (and SHAM) mice, as determined by ICP-MS analysis, was not accompanied by an enhanced signal intensity in the corresponding MR T_{1w} images (Figure S3 and S4). This is not an unexpected result as, due to the limited number of injections, the administered dose (total dose 3.6 mmol/Kg) is likely not sufficient to give rise to a visible signal enhancement 21 days after the last administration.

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References

1. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: Guidelines from the International Panel on the Diagnosis of Multiple Sclerosis. Ann. Neurol. 2001;50(1):121–127.

2. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann. Neurol. 2011;69(2):292–302.

3. Idée JM, Port M, Raynal I, et al. Clinical and biological consequences of transmetallation induced by contrast agents for magnetic resonance imaging: A review. Fundam. Clin. Pharmacol. 2006;20(6):563–576.

4. Yang L, Krefting I, Gorovets A, et al. Nephrogenic systemic fibrosis and class labeling of gadolinium-based contrast agents by the Food and Drug Administration. Radiology. 2012;265(1):248–253.

5. Kanda T, Ishii K, Kawaguchi H, et al. High signal intensity in the dentate nucleus and globuspallidus on unenhanced T1-weighted MR images: Relationship with increasing cumulative dose of a gadoliniumbased contrast material. Radiology. 2014;270(3):834–841.

6. Errante Y, Cirimele V, Mallio CA, et al. Progressive increase of T1 signal intensity of the dentate nucleus on unenhanced magnetic resonance images is associated with cumulative doses of intravenously administered gadodiamide in patients with normal renal function, suggesting dechelation. Invest. Radiol. 2014;49(10):685–690.

7. Mcdonald R, Mcdonald JS, Kallmes DF, et al. Intracranial gadolinium deposition after contrast-enhanced MR imaging. Radiology. 2015;275:772–782.

8. McDonald RJ, Levine D, Weinreb J, et al. Gadolinium retention: A research roadmap from the 2018 NIH/ACR/RSNA workshop on gadolinium chelates. Radiology. 2018;289(2):517–534.

9. Kanal E, Tweedle MF. Residual or retained gadolinium: Practical implications for radiologists and our patients. Radiology. 2015;275(3):630–634.

10. Cocozza S, Pontillo G, Lanzillo R, et al. MRI features suggestive of gadolinium retention do not correlate with Expanded Disability Status Scale worsening in Multiple Sclerosis. Neuroradiology. 2019;61(2):155–162.

11. Tanaka M, Nakahara K, Kinoshita M. Increased signal intensity in the dentate nucleus of patients with multiple sclerosis in comparison with neuromyelitisoptica spectrum disorder after multiple doses of gadolinium contrast. Eur. Neurol. 2016;75(3–4):195–198.

12. Schlemm L, Chien C, Bellmann-Strobl J, et al. Gadopentetate but not gadobutrol accumulates in the dentate nucleus of multiple sclerosis patients. Mult. Scler. 2017;23(7):963–972.

13. Forslin Y, Shams S, Hashim F, et al. Retention of gadolinium-based contrast agents in multiple sclerosis: Retrospective analysis of an 18-year longitudinal study. Am. J. Neuroradiol. 2017;38(7):1311–1316.

14. Stojanov DA, Aracki-Trenkic A, Vojinovic S, et al. Increasing signal intensity within the dentate nucleus and globuspallidus on unenhanced T1W magnetic resonance images in patients with relapsing-remitting multiple sclerosis: correlation with cumulative dose of a macrocyclic gadolinium-based contrast age. Eur. Radiol. 2016;26(3):807–815.

15. Splendiani A, Perri M, Marsecano C, et al. Effects of serial macrocyclic-based contrast materials gadoteratemeglumine and gadobutrol administrations on gadolinium-related dentate nuclei signal increases in unenhanced T1-weighted brain: a retrospective study in 158 multiple sclerosis (MS) patients. Radiol. Medica. 2018;123(2):125–134.

16. Kelemen P, Alaoui J, Sieron D, et al. T1-weighted Grey Matter Signal Intensity Alterations After Multiple Administrations of Gadobutrol in Patients with Multiple Sclerosis, Referenced to White Matter. Sci. Rep. 2018;8(1):1–8. Available at: http://dx.doi.org/10.1038/s41598-018-35186-w.

17. Wang S, Hesse B, Roman M, et al. Increased Retention of Gadolinium in the Inflamed Brain after Repeated Administration of GadopentetateDimeglumine: A Proof-of-Concept Study in Mice Combining ICP-MS and Micro- A ndNano-SR-XRF. Invest. Radiol. 2019;54(10):617–626.

18. Bittner S, Afzali AM, Wiendl H, et al. Myelin Oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. J. Vis. Exp. 2014;(86):3–7.

19. Hasselmann JPC, Karim H, Khalaj AJ, et al. Consistent induction of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice for the longitudinal study of pathology and repair. J NeurosciMethods. 2018:71–84.

20. Montarolo F, Raffaele C, Perga S, et al. Effects of Isoxazolo-Pyridinone 7e, a Potent Activator of the Nurr1 Signaling Pathway, on Experimental Autoimmune Encephalomyelitis in Mice. PLoS One. 2014;9(9):e108791. Available at: https://doi.org/10.1371/journal.pone.0108791.

21. Montarolo F, Perga S, Martire S, et al. Nurr1 reduction influences the onset of chronic EAE in mice. Inflamm. Res. 2015;64(11):841–844.

22. Food and Drug Administration center for Drug Evaluation and Research (CDER). Guidance for

industry Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. Pharmacol. Toxicol. 2005;(July).

23. Di Gregorio E, Ferrauto G, Furlan C et al. The Issue of Gadolinium Retained in Tissues Insights on the Role of Metal Complex Stability by Comparing Metal Uptake in Murine Tissues Upon the Concomitant Administration of Lanthanum- and Gadolinium-Diethylentriamminopentaacetate, *Invest. Radiol.* 2018; (53): 167-172.

24. Lombardi M, Parolisi R, Scaroni F, et al. Detrimental and protective action of microglial extracellular vesicles on myelin lesions: astrocyte involvement in remyelination failure. ActaNeuropathol. 2019;138(6):987–1012. Available at: https://doi.org/10.1007/s00401-019-02049-1.

25. Lassmann H. The changing concepts in the neuropathology of acquired demyelinating central nervous system disorders. Curr. Opin. Neurol. 2019;32(3):313–319.

26. Manu Rangachari and Vijay K. Kuchroo. Using EAE to better understand principles of immune function and autoimmune pathology. J Autoimmun. 2013;23(1):1–7.

27. Melanie D. Sweeney, Abhay P. Sagare and BVZ. Blood–brain barrier breakdown in Alzheimer's disease and other neurodegenerative disorders. Physiol. Behav. 2018;176(1):1570–1573.

28. Nishihara H, Engelhardt B. Brain Barriers and Multiple Sclerosis: Novel Treatment Approaches from a Brain Barriers Perspective. In: Handbook of Experimental Pharmacology.; 2020.

29. Arena F, Bardini P, Blasi F, et al. Gadolinium presence, MRI hyperintensities, and glucose uptake in the hypoperfused rat brain after repeated administrations of gadodiamide. Neuroradiology. 2019;61(2):163–173.

30. Ehud L, Constantinescu CS. Experimental models of multiple sclerosis. Exp. Model. Mult. Scler. 2005:1–901.

31. Constantinescu CS, Farooqi N, O'Brien K, et al. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br. J. Pharmacol. 2011;164(4):1079–1106.

32. Rasschaert M, Schroeder JA, Wu T Di, et al. Multimodal Imaging Study of Gadolinium Presence in Rat Cerebellum: Differences between Gd Chelates, Presence in the Virchow-Robin Space, Association with Lipofuscin, and Hypotheses about Distribution Pathway. Invest. Radiol. 2018;53(9):518–528.

Figure and table legends:

Table 1: Quantitative total gadolinium concentrations in the CNS organs of EAE, SHAM and h-CTRL mice determined by ICP-MS analysis. Data are reported as mean (in nmol/g of tissue) ± standard deviation.

Figure 1: The clinical score (A, C) and body weight (B, D) of healthy control (h-CTRL), SHAM and EAE mice were daily measured and reported. (A-D) Syringes indicate the days when mice were injected with doses corresponding with 1.2 mmol/Kg Gd. Specifically, Gadodiamidewas intravenously administered in the pre-symptomatic phase (4, 6 and 8 days post immunization), in the onset (11, 13 and 15 days post immunization) and in the chronic phase (19, 21 and 23 days post immunization). Crosses indicate the sacrifice time. (A-B) In the short experiment the sacrifice of h-CTRL (n=16), SHAM (n=27) and EAE (n=27) mice occurred 21 days after the last administration. In the long experiment only the Gd administration in the chronic phase (19, 21 and 23 days post

immunization) is performed and the sacrifice of SHAM (n=5) and EAE (n=7) mice occurred 39 days after the last administration (A-B).

Figure2: Amounts of retained Gd in spinal cord (A), cerebrum (B) and cerebellum (C) of h-CTRL, SHAM and EAE mice administered with three doses of gadodiamide at days 4, 6, 8 p.i. (pre-symptomatic), 11, 13, 15 p.i. (onset) and 19, 21, 23 p.i (chronic). Mice from all experimental groups were sacrificed 21 days after the last GBCA injection.

Figure 3: Correlation line of the amounts of retained Gd in the spinal cord of each EAE mouse as a function of its mean clinical score (calculated as the ratio between the respective cumulative score and the days of life between immunization and sacrifice). ($n= 16$, $p<0.05$)

Figure 4: Amounts of retained Gd in spinal cord (A) cerebrum (B) and cerebellum (C) of EAE and SHAM mice administered with three doses of gadodiamide at 19, 21, 23 p.i (chronic phase) and sacrificed 21 or 39 days after the last GBCA injection.

Figure 5. TEM tissue localization of Gd-containing deposits in the dentate cerebellar nuclei (A-D) and in the spinal cord (E-G) of EAE mice that received Gd. TEM evaluation showed Gd-containing deposits with typical sea urchin–shaped in the basal lamina of blood vessels (A and F) and intracellular inclusions of Gd (A, B, D, F and G), in form of small electron-dense dots. Representative low magnification images showed not-damaged areas of the cerebellum (A) and spinal cord (E) and a demyelinated regions of the spinal cord (F). Scale bars 10 um (A), 1um (B, E and F) and 500nm (C, D, G and H).