New tools for amyloid plaques detection by MRI: Gadolinium-VHH antibody conjugates

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Target Audience: Scientists interested in diagnosis of Alzheimer's disease by MRI as well as by molecular imaging.

Purpose: The early diagnosis of Alzheimer's disease (AD) is critical for the evaluation of new therapies and patient follow-up. Aggregation of $A\beta_{1-40}$, $A\beta_{1-42}$ peptides in amyloid plaques, one of the major hallmarks of the pathology, begins more than 15 years before neuropsychological symptoms [1]. Few methods have been developed to detect amyloid plaques by MRI but none of them are transposable to human notably due to invasive procedures [2, 3, 4]. Heavy chain only antibody fragments (VHH) are nanobodies that can cross the blood brain barrier [5]. Here, in order to detect amyloid plaques by MRI, we developed VHH with high specificity against $A\beta_{1-40}$, $A\beta_{1-42}$. Then, we conjugated VHH with Gadolinium (Gd) via a DOTA chelate and evaluated the resulting contrast agent (VHH-DOTA/Gd) for its specificity against Aβ peptides. Finally, we demonstrated that MR images recorded after *in vitro* incubation or *in vivo* intracerebroventricular (ICV) injection of VHH-DOTA/Gd reveal amyloid plaques.

Methods: Production and characterization of VHH were performed as previously described [5]. After immunization with Aβ fibrillar form, alpaca lymphocytes were isolated and VHH encoded genes were amplified by RT-PCR. Specific VHH for $A\beta_{1-40}$ and $A\beta_{1-42}$ were selected by phage display, before expression in *E.coli*. VHH-DOTA/Gd were synthesized by conjugation of DOTA on the VHH amino groups, followed by Gd chelation. HPLC/MS was used to monitor the process and to evaluate the number of Gd in the final conjugate. VHH-DOTA/Gd was evaluated by ELISA and IHC to confirm binding properties. PS2APP mice, known to develop amyloid plaques around 40 weeks, were used (n=2/experiment, 75-85 weeks-old, Roche, Switzerland). MRI experiments were performed on a 7T-Agilent spectrometer by using birdcage and mouse brain surface coils (RapidBiomed GmbH). Brain images were recorded after 3 different protocols. 1. *In vitro* procedure: performed by incubating one hemisphere with VHH-DOTA/Gd (0.1 mg/ml in PBS + Triton 0.2%) overnight, followed by 6x30min wash-out before MR images. 2. *Ex vivo* procedure: 6h after *in vivo* ICV injections of VHH-DOTA/Gd (1 µg/side), mice were perfused (PFA 4%) and their brains were extracted prior to e*x vivo* MR images. For each procedure, controls were performed by using an equivalent solution of Gd (i.e: 0.01mM). 3. Gd-staining procedure: after each experiment brains were soaked in a Gd-solution (2.5 mM) for 2days and imaged by MRI for detection of amyloid plaques [3]. Gd-stain images were used as the reference to register labeling observed with VHH-DOTA/Gd on MR images from procedure 1 and 2. All MR images were recorded using a high-resolution 3D-GE sequence (25*25*100µm^3, TR/TE=40/15ms, flip angle=20°, Nex=16, Acq time=11h39min). Finally, control of the diffusion of the agent and the labeling of amyloid plaques were performed by IHC.

Results: VHH-DOTA/Gd were obtained with an average of 3-4 DOTA/Gd per VHH, and were confirmed to bind to both Aβ by ELISA and IHC compared to the unlabeled VHH and to a reference antibody anti-Aβ (4G8). MR images obtained in the frontal area following the *in vitro* procedure with VHH-DOTA/Gd (Fig. 1A) and with Gd-staining (Fig. 1B) revealed numerous hypointense spots. Moreover several hypointense spots were colocalized on both images (red arrows). IHC confirmed the large diffusion of VHH-DOTA/Gd and the labeling of amyloid plaques in the same area (Fig. 1C, white arrows), even if the distortion induced by the paraffin procedure did not allow point-to-point registration between MRI and IHC. We then recorded *ex vivo* MR images following *in vivo* ICV injection of VHH-DOTA/Gd (Fig. 2). Hypointense spots could also be detected on these images, although they were less numerous compared to *in vitro* conditions (Fig. 2A). Several spots could be registered with the amyloid plaques detected on Gd-stained MRI (Fig. 2B, red arrows). IHC confirmed the labeling of amyloid plaques with VHH-DOTA/Gd and the diffusion from the frontal areas to the hippocampus after ICV injection (Fig. 2C, white arrows).

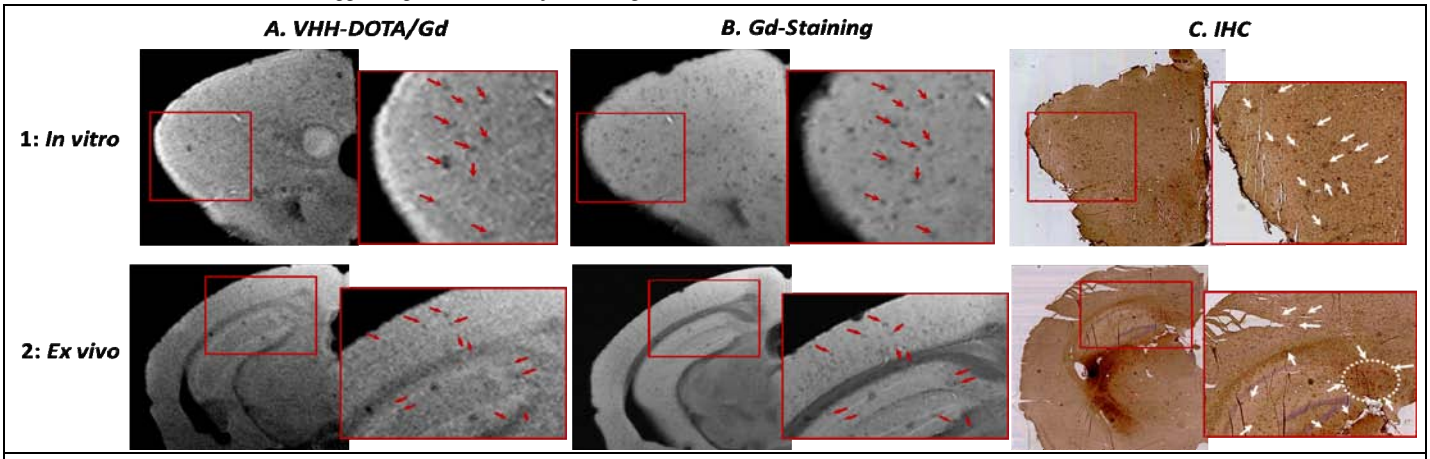

Figure: Hypointense spots are observed on MR images after *in vitro* incubation (1.A) or *in vivo* ICV injection (2.A) with VHH-DOTA/Gd. For each procedure, several spots were colocalized with amyloid plaques detected on Gd-stained MRI (1.B and 2.B, red arrows). IHC confirmed the diffusion of the VHH-DOTA/Gd and the labeling of amyloid plaques in the same areas (1.C and 2.C, white arrows). Red squares show the magnified areas.

Discussion/Conclusion: We report a new tool for the detection of amyloid. The small size and the specific properties of VHHs allow a large diffusion of the contrast agent in the whole brain following *in vitro* incubation or *in vivo* ICV injection. Hypointense spots revealed on high resolution MR images were registered with amyloid plaques detected by a reference method (Gd-stained MRI). VHH-DOTA/Gd conjugates thus appear as promising tools, with translational opportunities, for *in vivo* detection of amyloid deposits.

Reference: [1] Villemagne et al. *Lancet Neurol* 12(4):357-67 (2013); [2] Ramakrishnan et al. *Phar Res* 25(8):1861-72 (2008); [3] Petiet et al. *Neurobiol Aging 33(8):1533-44* (2012); [4] Wadghiri et al, *Plos One* 8(2):e57097 (2013); [5] Li et al. *FASEB* 26(10):3969-79 (2012)