Metabolic profiling of RG2 glioma using in vivo 1H MRS and ex vivo HRMAS 1H MRS

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Introduction.
^{1}H MRS is a powerful analytical method to investigate the complex metabolic consequences of cancer disease. *In vivo* ^{1}H MRS can provide information regarding glioma growth and response to treatment. A wider range of metabolites can be obtained *ex vivo* in biopsies using ^{1}H High Resolution Magic Angle Spinning (HRMAS) ^{1}H MRS. Besides, the metabolic data can also be interpreted and classified using multivariate pattern recognition methods, such as Projection to Latent Structure-Discriminant Analysis (PLS-DA) [1]. Comparison of metabolic profiles between ^{1}H MRS and HRMAS ^{1}H MRS is essential [2] and the ability of both approaches to discriminate tumoral from normal tissue with statistical tools such as PLS-DA might be of great help for diagnosis. Here, we have used this approach to analyse *in vivo* and *ex vivo* spectra in the rat RG2 model of glioma.

Materials and methods. Experimental design: Fischer rats (n= 7, 8 weeks old) were orthotopically implanted with RG2 glioma (5.10^3 cells) at day 0 under 2.5% isoflurane anesthesia. *In vivo* ^{1}H MRS was performed on well established tumor at day 14. At day 15, animal were deeply anesthetized with 5% isoflurane and quickly decapitated. The tumor bulk and the contralateral striatum were rapidly isolated (in less than 3 minutes), subsequently frozen and kept in liquid nitrogen. For HRMAS ^{1}H MRS experiments, about 15 mg of the frozen biopsies were rapidly introduced in a 4 mm ZrO_2 rotor, and a cold 1 mM D_2O solution of 3-(trimetylsilyl) propionic-2,2,3,3-d4 acid (TSP) was added (δ_{TSP} = 0 ppm).

Data acquisition:
In-vivo: ^{1}H MRS experiments were performed on a 7 Tesla Bruker BioSpec AVANCE III (Ettlingen, Germany) MRI system. Tumor and contralateral spectra were acquired with short TE PRESS sequence in a 3x3x3 mm^3 voxel with TE/TR=20/2500 ms, 4006 Hz bandwidth, 2048 data points, 400 averages resulting in a total acquisition time of 16m40s. The PRESS localization was preceded by water suppression and outer volume suppression modules. The localization of the VOI was based on T2-weighted RARE images (TE/TR=33/4000 ms, 20 slices 1mm thick, 117x117 um^2 in plane resolution, **Fig. 1**). *Ex-vivo:* the HRMAS ^{1}H MRS experiments were performed at 9.4 Tesla on a Bruker Avance III spectrometer using a 4 mm ^{1}H-^{13}C-^{31}P probehead. Samples were spun at 4 KHz and the temperature maintained at 4°C. 1D spectra were all acquired with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to attenuate macromolecule and lipid resonances, synchronized with the spinning rate (interpulse delay 250 µs, total spin echo time 30 ms) [3]. The residual water signal was presaturated during the 2s relaxation delay. Total acquisition of one spectrum with 256 scans lasted 16 min. Resonance assignment was performed as previously described [4].

Metabolite quantification: the signals were processed using the "substract-QUEST" algorithm [5] of the jMRUI-sofware (http://www.mrui.uab.es/mrui/). The procedure involves a simulated metabolite data basis set. For HRMAS ^{1}H MRS spectra 20 metabolites were included in the basis set: acetate (Ace), alanine (Ala), aspartate (Asp), creatine and phosphocreatine (tCr), choline (Cho), ethanolamine (Eth), γ-amino-butyric acid (GABA), glutamate (Glu), glutamine (Gln), glutathione (Gsh), glycerophosphocholine (GPC), glycine (Gly), hypotaurine (Hyp), lactate (Lac), myo-inositol (m-Ins), N-acetylaspartate (NAA), phosphoethanolamine (PE), phosphorylcholine (PC), scyllo-inositol (Syll), taurine (Tau). For MRS spectra 10 metabolites were included in the basis set: Asp, tCr, tCho (Cho+PC), GABA, Glu, Gln, Lac, m-Ins, NAA, Tau. For both analysis the total spectrum intensity was used for normalization.

Multivariate statistical analysis: Quantified data were loaded in the SIMCA-P software version 12 (Umetrics, Umea, Sweden) as variables and scaled to unit variance before PLS-DA analysis. All PLS-DA models were cross-validated, to allow evaluation of the statistical significance of the model. Cross validation is a procedure during which the model is iteratively rebuilt using only 6/7 of the data as training set. The model is then used to predict the class of the remaining 1/7 data which serves as a test set. Cross-validation lead to the calculation of the Q2 and R2Y factors. R2Y is a quality factor while Q2 is a predictive factor, i.e. a good Q2 (>0.5) allows the model to be used for prediction. The results were visualized by plotting the first two principal components of the analysis against each other in a scatter plot. Each point in a scatter plot represents one individual animal.

Results. *In vivo* and *ex vivo* spectra gave similar results in tumor with an increase of tCho and a decrease of NAA, tCr (**Fig. 2**). Interestingly the increase of Lac observed *ex vivo* confirmed the Lac increase observed *in vivo* at 1.3 ppm that is usually controversial. *Ex vivo* HRMAS ^{1}H MRS provided additional information in tumor metabolism: an increase of Ala and Gly, a decrease of GABA, the identification of choline components (Cho, PC, GPC) and the emergence of Hyp. Both analyses yielded robust statistical models with a clear separation between tumor and contralateral striatum (**Fig. 3**).

Figure1: T2W image with depict ROIs (3 x 3 x 3 mm^3 voxel). Dotted line show tumor bulk

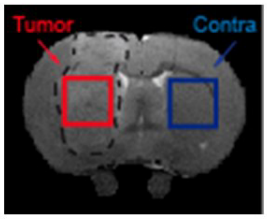


Figure2: Representative ^{1}H spectra obtained with (A) single voxel MRS and (B) HRMAS MRS. Tumor spectra are shown in red and contralateral spectra are shown in blue.

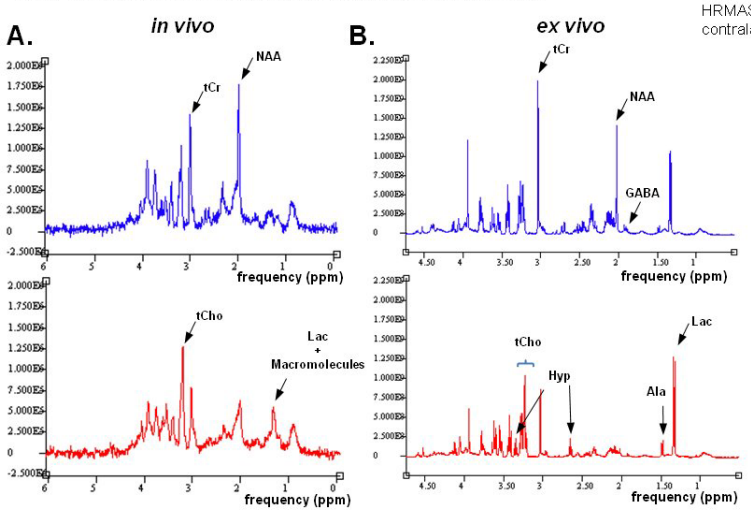
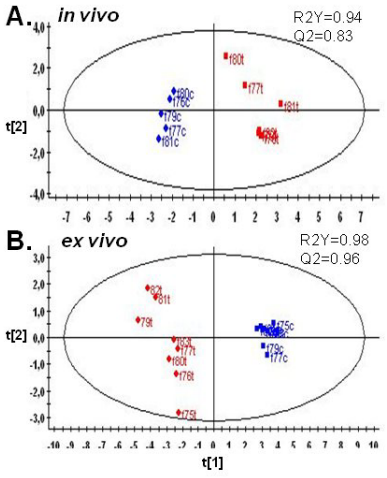


Figure3: Score plot of PLS-DA analysis based on (A) 10 metabolites for single voxel MRS or (B) 20 metabolites for HRMAS MRS. Tumor samples are represented in red and contralateral samples in blue.



Conclusion: Quantification with jMRUI is reliable since metabolic profiles from contralateral striatum are well grouped. The PLS-DA models are robust despite inter-individual variability. These models are highly predictive (high Q2 values) and could be a powerful tool for evaluation of treatment efficiency of glioma in preclinical research. Further studies are needed to validate this approach.

References: [1] Holmes et al., *NeuroRx*, 2006, [2] Opstad et al., *j. mag. reson. imaging,* 2010, [3] Wieruszeski et al., *j. mag. reson.,* 2001, [4] Rabeson et al., *mag. reson. med.,* 2008, [5] Ratiney et al., *NMR biomed.*, 2005.