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Appendix E1

Region of Interest Methodology

Sites for intraoperative sampling were identified and marked on thin-section T2-weighted images, which were then merged with postgadolinium BRAVO images on the surgical navigation system (StealthStation S7 and S8: Medtronic, Minneapolis, MN). Two sites within each participant's tumor were marked for sampling; one site was chosen in an area of T2/T2* hypointense signal that reflected strong ferumoxytol-induced signal change, and the other site was chosen in an area of less T2/T2* hypointense signal that reflected less ferumoxytol-induced signal change. The inclusion of two sites, each with a different signal intensity, within a given tumor allowed for a broader range of postferumoxytol signal intensity that could be correlated with histopathology. These regions of interests (ROIs) were visually recorded and saved as image captures, along with a record of the image slice number of each ROI on the T2 images. As DICOM imaging coordinates could not be saved by the surgical navigation system, a method was devised to confirm the location of the target ROI for image analysis: two perpendicular lines were drawn from the center of each target ROI to a point in the calvarium, one in an anterior-posterior and the other in a left-right direction, and then measurements of the line distances were saved as image captures (Fig 3).

Using OsiriX MD software (version 7.0.4, Pixmeo SARL, Bernex, Switzerland) operator-defined circular ROIs with 3.5 mm diameter (approximately 10 mm^2 area) were manually reproduced at the sampled sites on the thin-section T2-weighted images. ROI size was fixed to minimize variability in ROI analysis across all of the samples. Specifically, the image slice number of each ROI recorded during surgery was used to find the target image slice on the thin-section T2-weighted images. Next, an ROI was drawn at the location of the target site on the T2-weighted images based on visual comparison with a previously recorded image of the ROI. To ensure accuracy of ROI placement, two perpendicular lines were drawn from the center of the ROI to points in the calvarium, replicating the method described above. The ROI was visually adjusted as needed to ensure that the ROI and perpendicular lines matched the positioning on the previously saved image captures. The T2-weighted images were then automatically aligned with the postprocessed quantitative susceptibility mapping (QSM), R2*, and R2 images on Osirix MD. ROIs on the T2 images were subsequently transferred onto the output QSM, R2*, and R2 images. The values generated by an ROI on QSM, R2*, and R2 images represented the mean susceptibility, R2*, and R2 values, respectively.

Immunohistochemistry Technique

Dual staining is a well-established and uniquely informative technique that is used to advantage in this study. After conventional processing by formalin fixation, paraffin embedding, sectioning, deparaffinization, and antigen retrieval, slides were stained with antibodies to the macrophage markers CD68 (Dako, mouse anti-Human CD68 Clone KP1, catalog# M0814) (1:100) or CD163 (Novocastra, mouse anti CD163, catalog# NCL-L-CD163) (1:100), or the astrocyte marker glial fibrillary acidic protein (GFAP) (Dako, rabbit anti-GFAP, catalog# Z0334) (1:500). After incubation with a polymer-HRP secondary, 3,3′-diaminobenzidine chromogen was used to

visualize the staining. Slides were subsequently counterstained with Prussian Blue via standard histochemistry technique, thus representing the dual staining referenced above, which allowed us to determine the exact location of iron particles in the tumor in relation to macrophages containing phagocytosed iron particles or, when combined with GFAP immunohistochemistry, the relationship between iron particles and GFAP+ tumor cells or astrocytes.

Table E1: Quantitative MRI Measurements of Susceptibility, R2*, R2, and R2' from ROIs Placed at the Sites of Tissue Sampling

	Imaging variables			
	Susceptibility (ppm)	R2* (*sec*-1)	R2 (*sec*-1)	R2' (*sec*-1)
Sample Site 1				
Participant 1	0.01	21.69	3.00	18.69
Participant 2	−0.09	18.72	10.76	7.97
Participant 3	0.22	79.56	6.28	73.29
Participant 4	−0.13	48.13	7.81	40.32
Participant 5	0.31	118.08	13.56	104.52
Participant 6	−0.04	17.84	8.33	9.51
Participant 7	0.01	14.52	4.59	9.93
Participant 8	0.06	84.32	14.71	69.61
Participant 9	0.18	70.97	16.95	54.02
Participant 10	0.22	91.83	13.13	78.70
Sample Site 2				
Participant 1	0.01	9.25	2.62	6.63
Participant 2	−0.03	13.56	3.45	10.10
Participant 3	0.02	14.93	3.38	11.55
Participant 4	−0.08	40.79	7.76	33.03
Participant 5	0.06	63.37	11.15	52.22
Participant 6	0.47	116.74	11.05	105.69
Participant 7	N/A	N/A	N/A	N/A
Participant 8	0.37	116.83	19.04	97.79
Participant 9	0.29	135.20	17.87	117.33
Participant 10	0.42	121.80	14.82	106.98

Two sites were chosen within each study participant's tumor. See manuscript and supplemental text for methodology.

Note.—Numbers represent mean values of the ROIs. N/A = not available, ppm = parts per million, ROI = region of interest; sec = second

Table E2: Histopathologic scores of CD163+ and CD68+ iron-containing macrophages using the following semiquantitative scoring system: 0—no iron-macrophages on any 400× high power field (hpf) across the entire sample, 1—one hpf containing iron-macrophages, 2—two hpfs containing iron-macrophages, and 3—three or more hpfs containing iron-macrophages

	CD163-Prussian Blue				CD68-Prussian Blue			
	Sample 1	Sample 2	Sample 3	Majority Score	Sample 1	Sample 2	Sample 3	Majority Score
	Sample Site 1				Sample Site 1			
Participant 1	0	0	0	0	0	0	0	0
Participant 2	0	0	0	0	0	0	0	0
Participant 3	3	3	3	3	3	3	3	3
Participant 4	1	N/A	N/A	1	1	N/A	N/A	1
Participant 5	3	2	2	2	2	2	2	2
Participant 6	0	0	0	0	0	0	0	0
Participant 7	0	N/A	N/A	0	0	N/A	N/A	0
Participant 8	0	2	0	0	0	1	0	0
Participant 9	3	0	0	0	2	0	0	0
Participant 10	1	1	1	1	1	1	1	1
	Sample Site 2				Sample Site 2			
Participant 1	0	0	0	0	0	0	0	0
Participant 2	0	0	0	0	0	0	0	0
Participant 3	0	0	0	0	0	0	0	0
Participant 4	1	0	0	0	0	0	0	0
Participant 5	0	0	1	0	0	0	0	0
Participant 6	3	2	2	2	3	1	1	1
Participant 7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Participant 8	3	3	1	3	2	2	1	2
Participant 9	0	0	0	0	0	0	0	0
Participant 10	0	2	2	2	0	1	1	1

Abbreviations: hpf = high power field, N/A = not available.