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Supplemental Information

Safety, Tumor Reduction, and Clinical Impact of Zika Virus Injection in Dogs with Advanced-Stage Brain Tumors

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Supplementary Methods

Inclusion Criteria for canine clinical trial protocol

Dogs were considered eligible for the oncolytic therapy if they were diagnosed by a veterinary neurologist with advanced CNS primary tumor with neural origin, excluding meningioma and other non-neural tumors (based on neurological symptoms and brain magnetic resonance images), had adequate organs function (hematocrit >25% and renal/hepatic function), with no neural infection diseases and whose owners were not pursuing chemotherapy or immediate euthanasia and consented to include the dogs in the clinical trial. Pre-treatment primary tumor size and location were obtained by brain magnetic resonance images (MRI) (Hitachi - Airis 0.3T Elite model) to establish a baseline for assessing tumor response.

Canine cell culture

D-GBM canine glioblastoma cell line were kindly provided by Dr. Michael Empl from University of Veterinary Medicine Hannover, Germany. Mesenchymal stem cell derived from normal canine adipose tissue (ADSC) were isolated and characterized as previously described[12]. All cells were cultivated in Dulbecco's Modified Eagle Media (DMEM) low glucose supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific), 100 U/mL Penicillin, 100 µg/mL Streptomycin and 250 ng/mL Fungizone® (Thermo Fisher Scientific) at 37° C at 5% CO^2 atmosphere.

Immunofluorescence

Tissue sections were blocked with 10% FBS, 5% bovine serum albumin (BSA), and 0.1% Triton X-100 in PBS for 1 h at room temperature and incubated at 4°C overnight with primary antibodies anti-Zika virus NS2B (GTX133308, Genetex, 1:500), anti-Alpha-tubulin (NB100-690, Novus Biologicals, 1:200), anti-IBA1 (ab107159, Abcam, 1:200) and anti-CD14 (MCA1042, Serotec/BioRad, 1:500). Fixed mesenquimal and glioblastoma cells (3,7% formaldehyde for 30 minutes) were permeabilized (0.1% Triton X-100 in 1 PBS for 2-hour) and blockaded (5% bovine serum albumin in PBS) before primary antibody 4°C overnight incubation. Tissue and cell culture were incubated with secondary antibodies goat anti-rabbit IgG (A11037, Thermo Fisher Scientific) Alexa Fluor 594 and Goat anti-mouse IgG Alexa Fluor 488 (A11001, Thermo Fisher Scientific) at a 1:1000 dilution for 1 h at room temperature. Tissues and cell culture were counterstained with 1 µg/mL DAPI for 2 minutes and microscope slides mounted in Vectashield medium (Vector Laboratories). All images were taken in confocal microscope (Zeinss LSM 800).

In vitro ZIKV infection and detection by flow cytometry

D-GBM ($8x10^4$ cells/well) or VERO cells ($5x10^4$ cells/well) were seeded in 6-well plate and 96-well plate, respectively at the day before of infection in DMEM or MEM culture media supplemented with 10% FBS. At the day of infection, the media was replaced with plain culture media containing the virus at MOI of 0.1, 1, 2, 5 and 10. After 1 hour

at 37 °C/5% CO2, the virus was removed and the cells washed once with 100ul of plain media and incubated in media supplemented with 2% FBS for 24, 48 or 72 hours. The cells were harvested using 50ul of trypsin/EDTA 2.5g/L solution (Vitrocell/Embriolife) and moved to a V-bottom 96-well plate containing 50ul of 1X PBS/2% FBS. The cells were pelleted at 700xg/5min/4 °C and washed with 100ul of 1X PBS/2% FBS. After, the cells were fixed and permeabilized using the BD Cytofix/Cytoperm method (BD Biosciences). Briefly, the cells were incubated on ice/15 minutes with 50 ul of Cytofix solution and washed twice with 100ul of 1X Cytoperm solution. The cells were then stained with the primary antibody 4G2 (0.5ug/well) followed by the secondary antibody goat anti-mouse-Alexa Fluor® 488 at 1:800 (Thermo Fisher Scientific) for 30 minutes each and washed as described above. Finally, the cells were resuspended in 200 ul of 1X PBS/2% FBS. The cells were acquired by LSR FortessaTM Analyser (BD Biosciences) and the data were using the FlowJo Software to determine the presence of E ZIKV protein intracellularly.

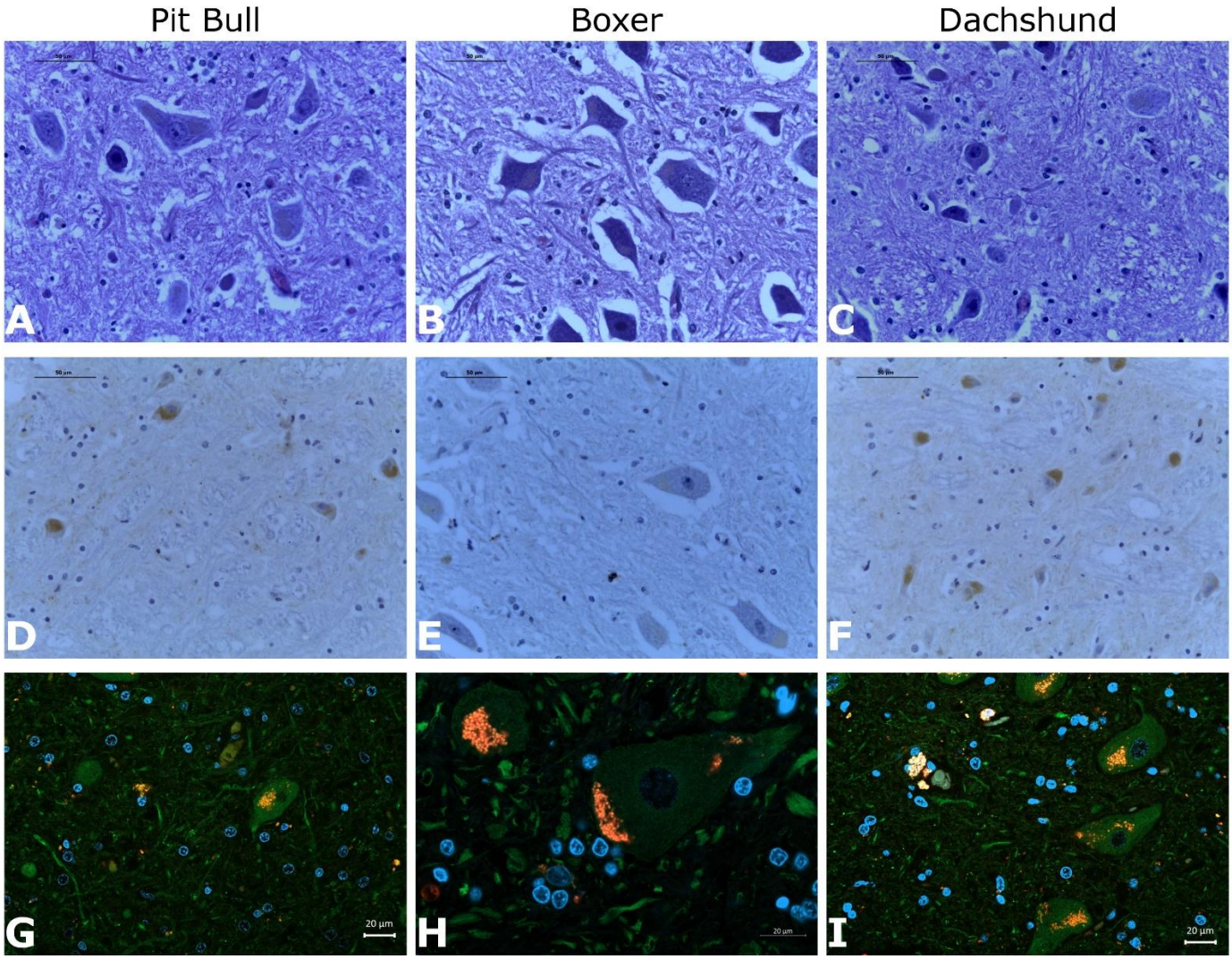
Cell proliferation Assay (Cell Growth Curve)

To access $ZIKV^{BR}$ influence on the D-GBM cells growing, $5x10^3$ cells per well were seeded in 96-well plate at a day before of infection. The cells were infected with $ZIKV^{BR}$ at MOIs of 1, 10 and 100 and their growth were evaluated at different time points (24h, 42h, 72h, 96h, and 120 hours). The $ZIKV^{BR}$ permissive VERO cells were infected at the same conditions serving as control to the assay. The cells were maintained in DMEM (D-GBM) or MEM (VERO) media supplemented with 10% FBS at 37 °C/5% CO_2. The culture media was removed, and the cells washed once with 200ul of 1X PBS and fixed with 100ul of 70% ethanol for 10 minutes. After, the cells were incubated with 40ul of 0.5% gentian violet (Laborclin) for 30 minutes. The cells were then washed 5 times with 200ul of Milli-Q water and lysed with 100 ul of 10% acetic acid. After 30 minutes, the absorbance was measured at 540 nm using the Epoch microplate spectrophotometer[37] (BioTek Instruments Inc).

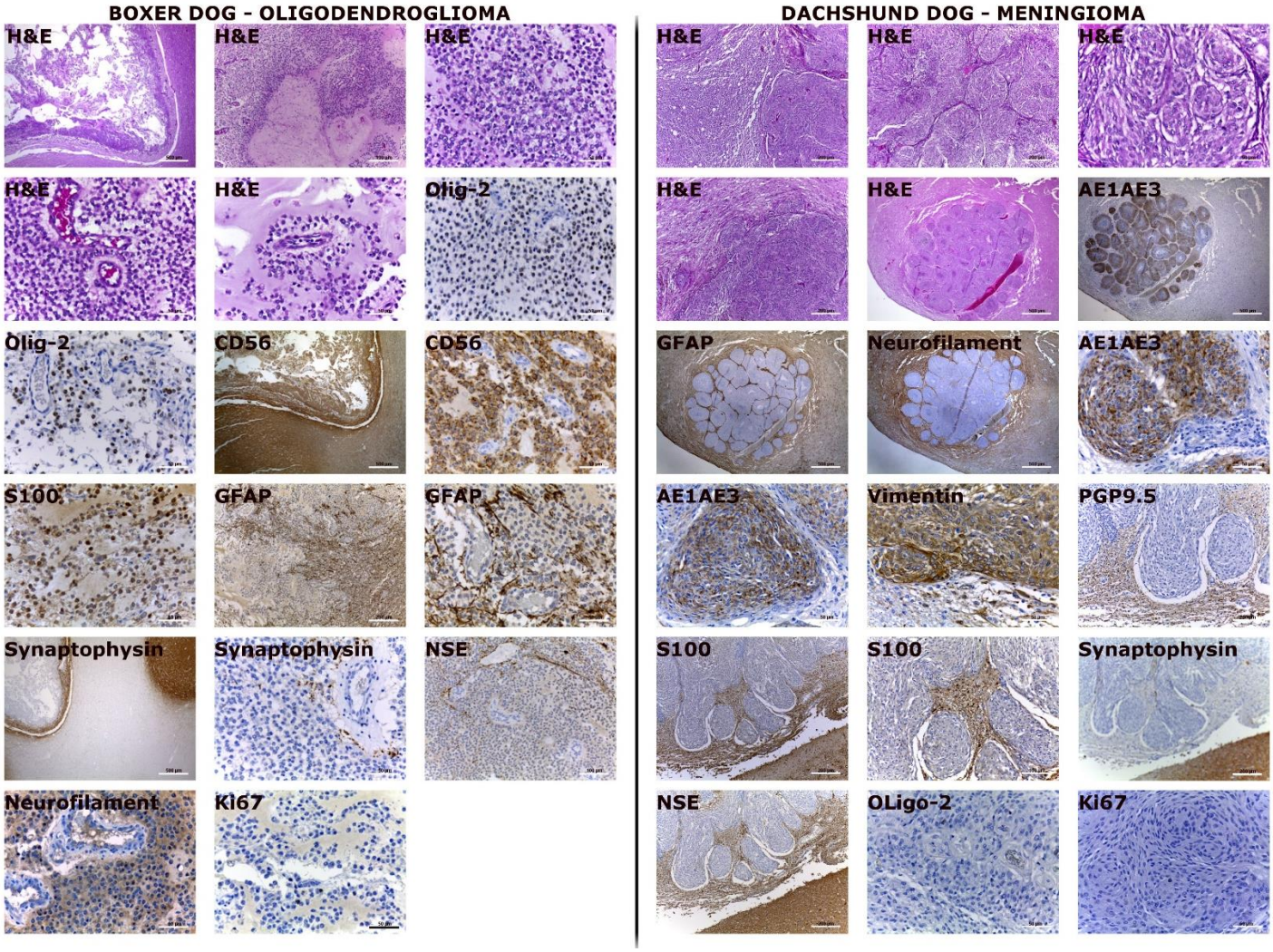
LIVE/DEAD D-GBM viability assay after in vitro ZIKV infection

D-GBM were seeded in 6-well plate (1×10^5 cells/well) at the day before of $ZIKV^{BR}$ infection in DMEM media supplemented with 10% FBS. After overnight cells incubation, the media was replaced with the culture media containing the virus at MOI of 0.1, 1, and 2. Cells were incubated for 72 hours for further cell viability analysis. For the positive control, cells were treated with a 20% DMSO culture media solution for 5 min prior to the cells labeling. Then, the total cells (adherent and floating cells) were harvested, washed, moved to a U-bottom 96-well plate and stained with the LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (#L34964), according to manufacturer's instructions. Finally, the cells were resuspended in 200 ul of 1X PBS/2% FBS and acquired by LSR FortessaTM (BD Biosciences) flow cytometer. Data were analyzed using the FlowJo software.

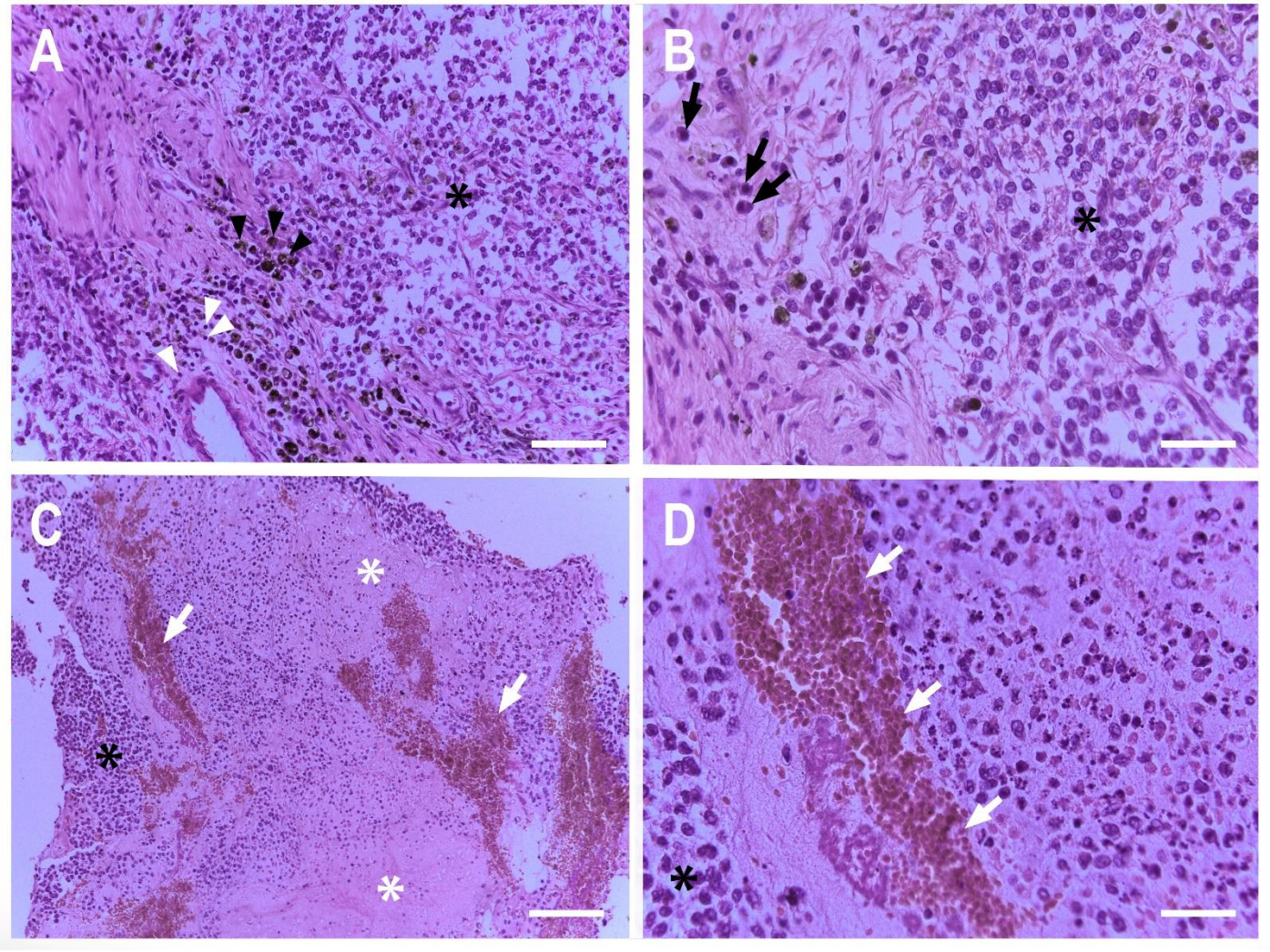
Supplementary Figures



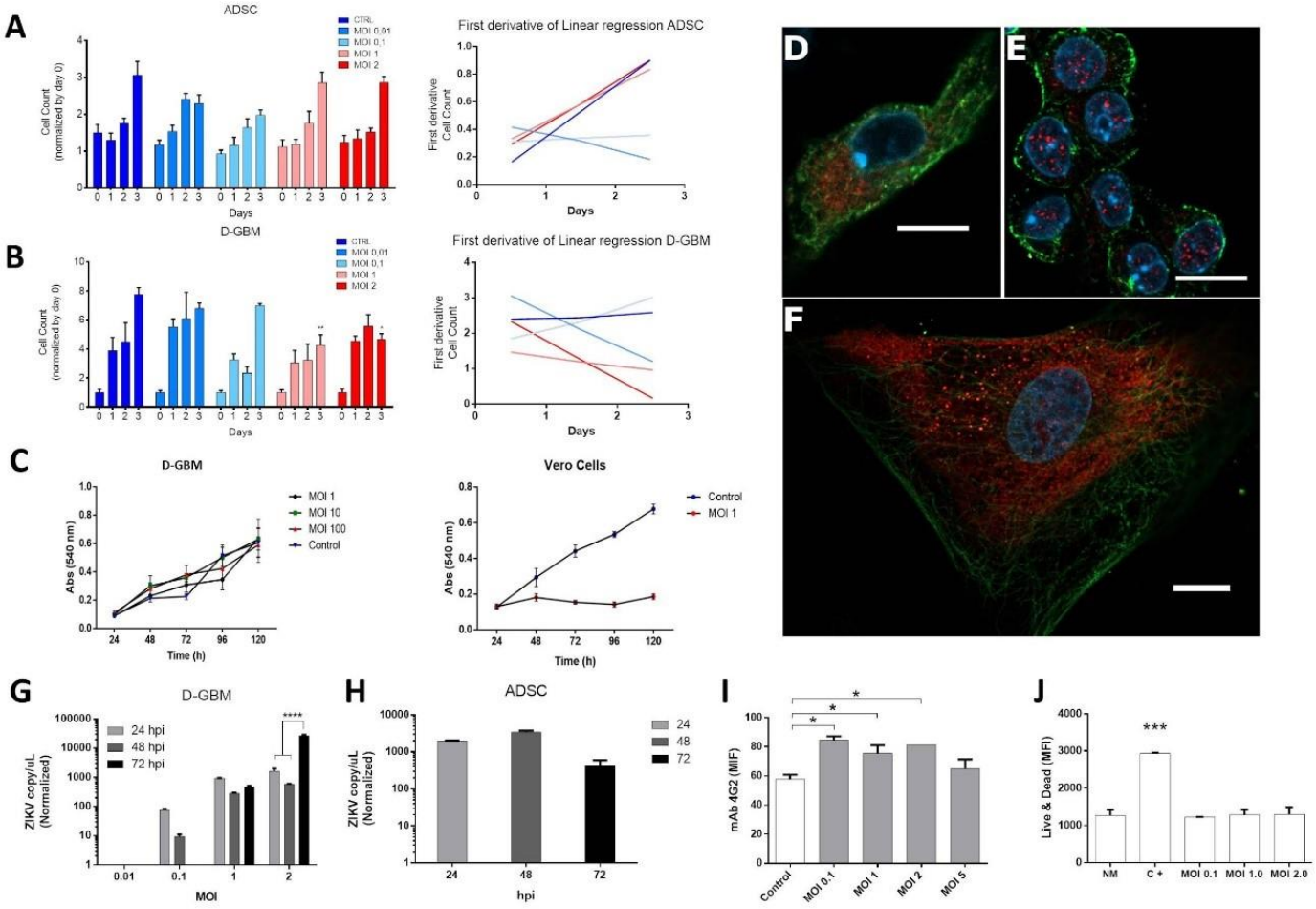
Supplementary Figure S1: Absence of viral infection in canine neurons after ZIKV^{BR} CNS infection. Representative images of neurons from CNS tissues H&E (A-C), immunohistochemistry immunolabeling for ZIKV^{BR} (D-F) and immunofluorescent immunolabeling (G-I) for ZIKV^{BR} (red), β3-TUBULIN cytoplasmatic (green) protein and nuclei DAPI (blue). Lipofuscin accumulation in neurons can be seen in post-mortem Pit Bull (A, D, G), Boxer (B, E, H) and Dachshund (C, F, I) in dogs CNS tissues samples after H&E and immunohistochemistry analysis, in brown, and in immunofluorescent analysis, in yellow, by unspecific-positive result for ZIKV^{BR} (red), β3-TUBULIN cytoplasmatic. Scale bar, 50 µm and 20 µm.



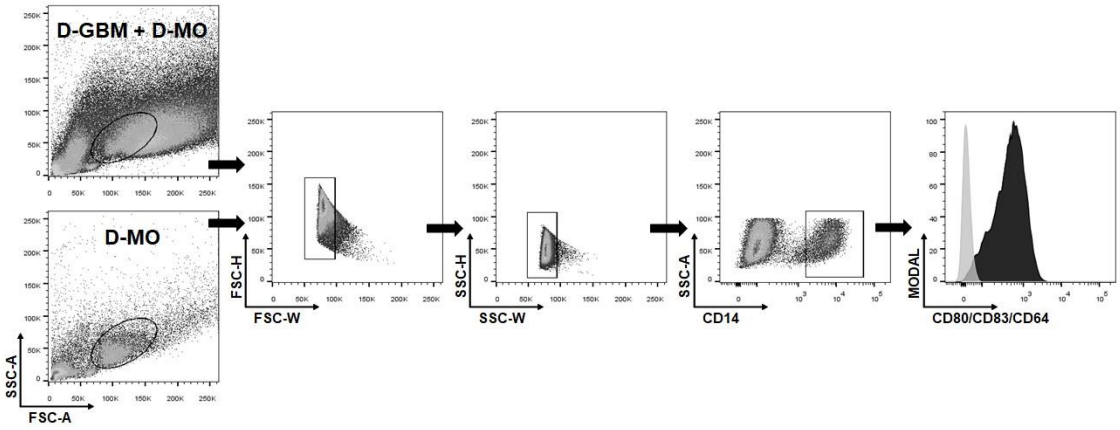
Supplementary Figure S2: Immunohistochemistry assay for histopathologic tumor diagnosis. Boxer dog: confirmed oligodendroglioma by positive immunolabeling in tumor cells for Olig2, CD56, S100, Ki67 and negative immunolabeling for GFAP, Synaptophysin, NSE and Neurofilament markers. **Dachshund dog:** confirmed meningioma by positive immunolabeling in tumor cells for CK Pan (AE1AE3), Vimentin and negative immunolabeling for GFAP, Synaptophysin, Neurofilament, NSE, S100, Olig2, PGP 9.5 and Ki67 markers.



Supplementary Figure S3: H&E representative images of Boxer dog tumor. A and B, Macrophages (black arrowhead), Lymphocytes (white arrowhead) and plasma cell (black arrow) presence in the tumor (black asterisk). Scale bar, 100 µm (A) and 50 µm (B). C and D, Large necrosis area (white asterisk) with haemorrhage (white arrow) alongside remaining tumor cells (black asterisk). Scale bar, 200 µm (C) and 50 µm (D).



Supplementary Figure S4: ZIKV^{BR} in vitro infection in canine cell lines. A-B, Total cell number of canine mesenchymal stem cell (ADSC)(A) and dog glioblastoma cell line (D-GBM) (B) infected by ZIKV^{BR} at different MOI conditions at 24, 48 and 72 hpi. First derivative of linear regression graphic analysis is followed at right. (*p<0.05, **p<0.01). **C,** Evaluation of cell death induced by ZIKV infection (MOI 1, 10 or 100) in D-GBM and VERO cells for up to 120 hours. **D-F,** Immunolabeling positive of ZIKV^{BR} (red), β3-TUBULIN cytoplasmatic (green) protein and nuclei DAPI (blue) in MOI 2 at 72 hpi of D-GBM (D-E) and ADSC (F) cells. Scale bar, 10 µm. **G-H,** Viral genomic copies on culture supernatants at 24, 48 and 72 hpi in MOI 0.01, 0.1, 1 and 2 for D-GBM(G) and MOI 2 for ADSC(H). (****p<0.0001). **I,** Intracellular staining of E ZIKV protein using the mAb 4G2 by flow cytometry (*p<0.05). **J,** Cell viability of ZIKV-infected D-GBM cell culture evaluated 48h after infection by flow cytometry. (***p<0.001)



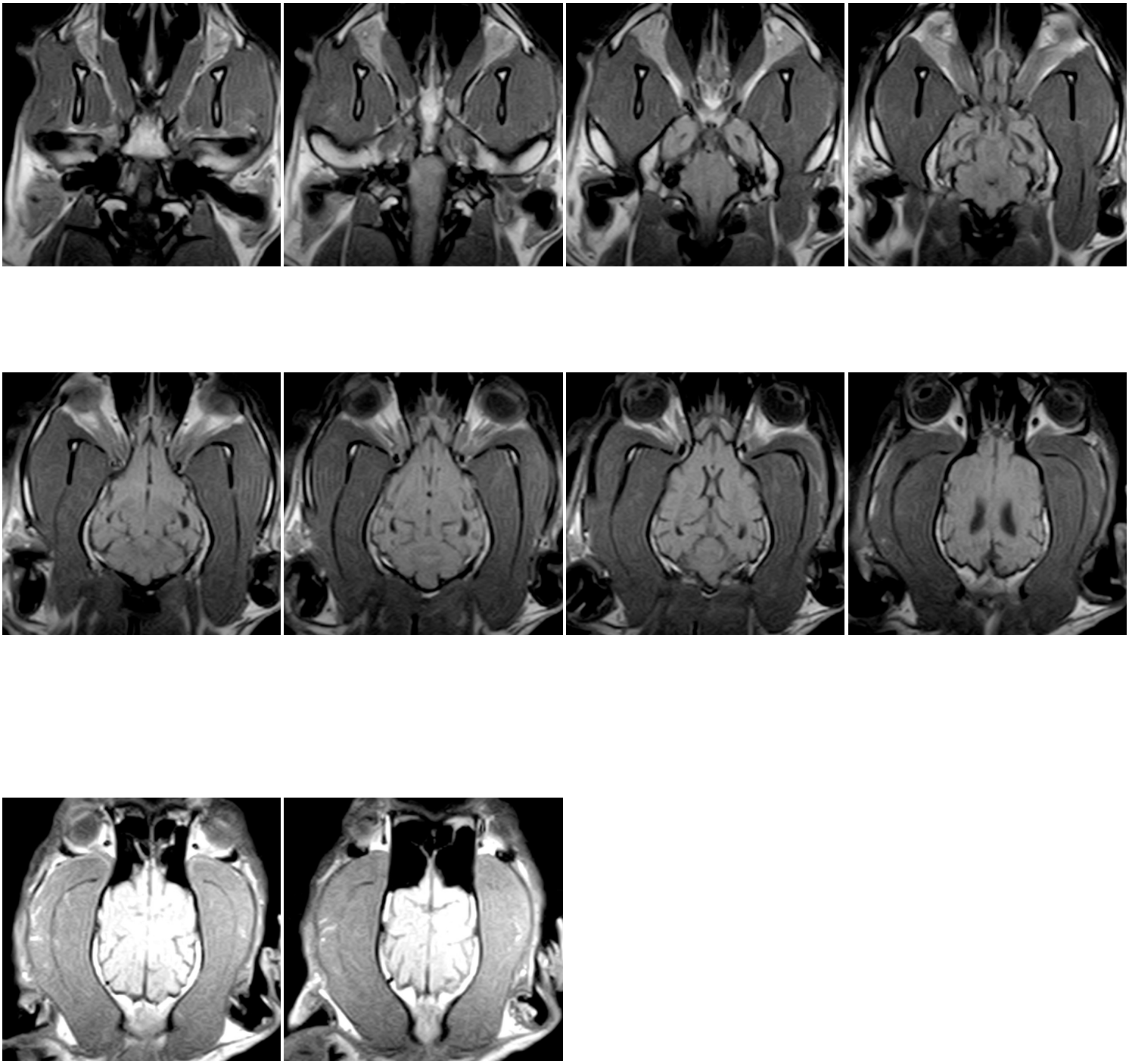
Supplementary Figure S5: Gating strategy for the evaluation of canine monocytes in the co-culture assay. Doublets were initially excluded from analysis by FSC and SSC parameters. D-GDM and monocytes were distinguished by $CD14^+$ expression. Cells were gated by the expression of $CD14^+$ and subsequently separated according to the $CD80^+$, $CD83^+$ and $CD64^+$ specific surface molecules expression and analyzed by mean of fluorescence intensity (MFI).

Supplementary Table 1: Primers sequence for the quantitative PCR for canine immune mediators

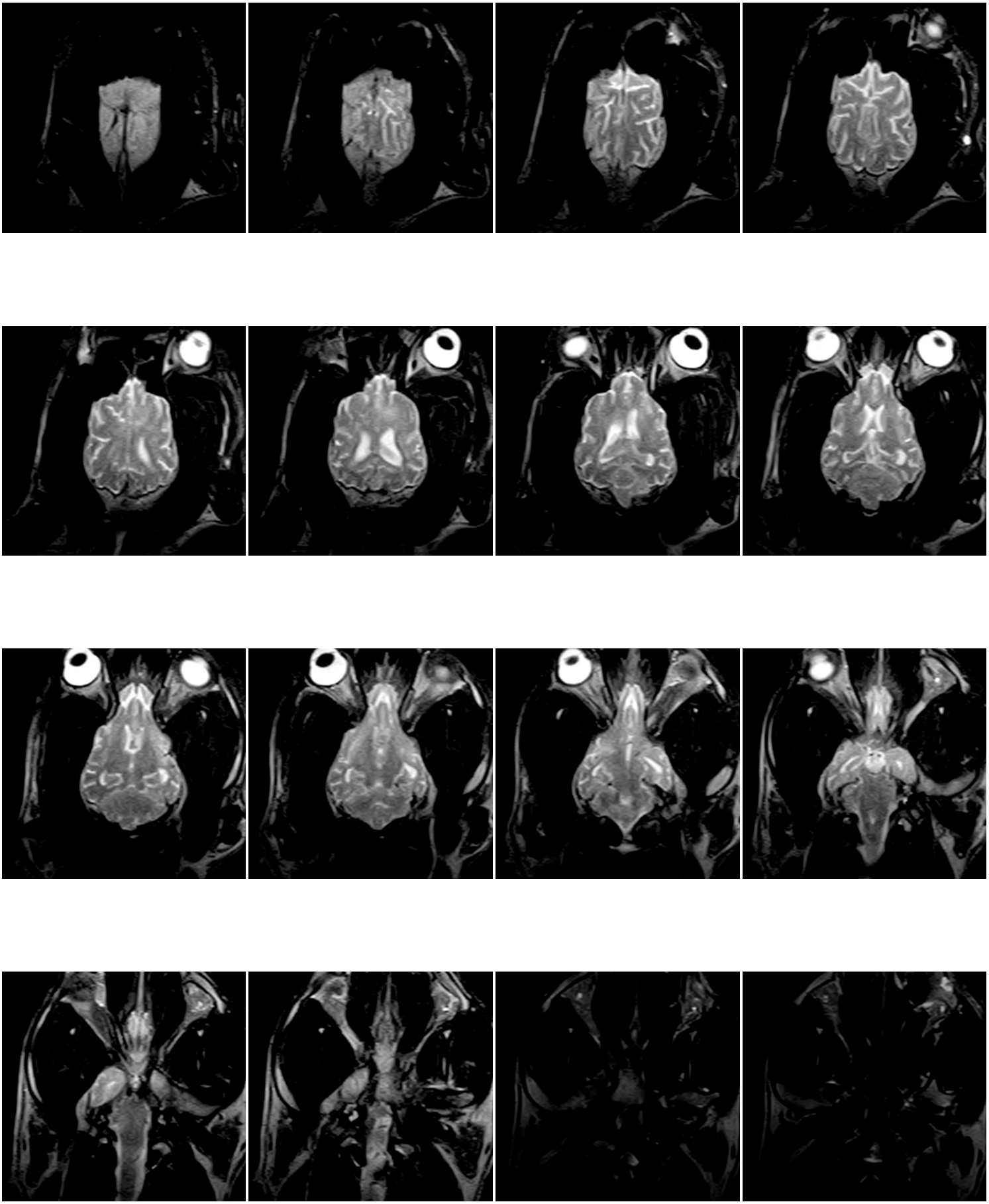
Canine Primers	Sequence
ZIKV	ZIKV 1086 forward: 5′-CCGCTGCCCAACACAAG-3 ZIKV 1162 reverse: 5′-CCACTAACGTTCTTTTGCAGACAT-3
MHC-1	forward 5′-CACCAACCTGTCCAAAGTTCC-3 reverse 5′-CCGGGCAGATCAAGAGAAGATA -3
GRANZYME B	forward 5′-GGAGAGATCATCGGGGGACATGA -3 reverse 5′-CTCCTGTTCCTTGATGTTGTGG-3
PD-1	forward 5′-ATGAGAATGTTTAGTGTCTT-3′ reverse 5′-TTATGTCTCTTCAAATTGTATATC-3′
PDL-1	forward 5′-AGGATGGCTCCTAGACTCCC-3′ reverse 5′-AGACGATGGTGGCATACTCG-3′
CCR2	forward 5′- ACATGCTGTCCACATCGCA -3′ reverse 5′- GGCGCGCTGTAATCATAGTC -3
STAT1	forward 5′- CTTACCCAGAAAGCCCTGATTA -3′ reverse 5′- CTGTATTCCTCTCGCTCACATC -3
NOS2	forward 5′- GGAAGCAGTAACAAAGGAGATAGA -3′ reverse 5′- CCTCCACCTGGTAGTAGTAGAA -3
IDO1	forward 5′- GTCTGCCTCCTATTCTGGTTTAT -3′ reverse 5′- GCAGTTTGGAGTTGCCTTTC -3
ARG1	forward 5′- GGTGGCAGAAGTCAAGAAGA -3′ reverse 5′- GGTGGGTTAAGGTAGTCAATAGG -3
CX3CR1	forward 5′- GACACATCAGACGTTCCCTTCCCAG -3′ reverse 5′- TGTCCCACAAATCACAGGCTTCA -3
CXCL10	forward 5′- TGGTACTCAAGGAATACCTCTCT -3′ reverse 5′- ATTGCTTTCACTAAACTCTTGATGG -3
CCR7	forward 5′- CCCTGACCTTTAGCAACATACA -3′ reverse 5′- AGCAAGGAGCCGAGATAGA -3
STAT1	forward 5′- CTTACCCAGAAAGCCCTGATTA -3′ reverse 5′- CTGTATTCCTCTCGCTCACATC -3
GATA3	forward 5′- TCTCCTTCCTCTTCTCCTCTTT -3′ reverse 5′- GGTACTTGATGCACTCCTTCTC -3
SOCS3	forward 5′- CAAGACCTTCAGCTCCAAGAG -3′ reverse 5′- GTAGTGATGCACCAGCTTGA -3
NK2D	forward 5′- GTTATTGTGGTCCGTGTCCTAA -3′ reverse 5′- ACTGCCAGGATCCATTTGTTGG -3
NKP30	forward 5′- CTTCTTGCCGTGTTCCTTCAA -3′ reverse 5′- CCAGAACCTCCACTCTGCACA -3
β-ACTIN	forward 5′- ACCAACTGGGACGACATGGAGA -3′ reverse 5′- AGGCATACAGGGACAGGACAG -3

Supplementary Data 1: MRI Images. Representative images of all MRI from the Pitbull, Boxer and Dachshund dogs post contrast T1 and T2 weighted dorsal, transversal and sagittal planes.

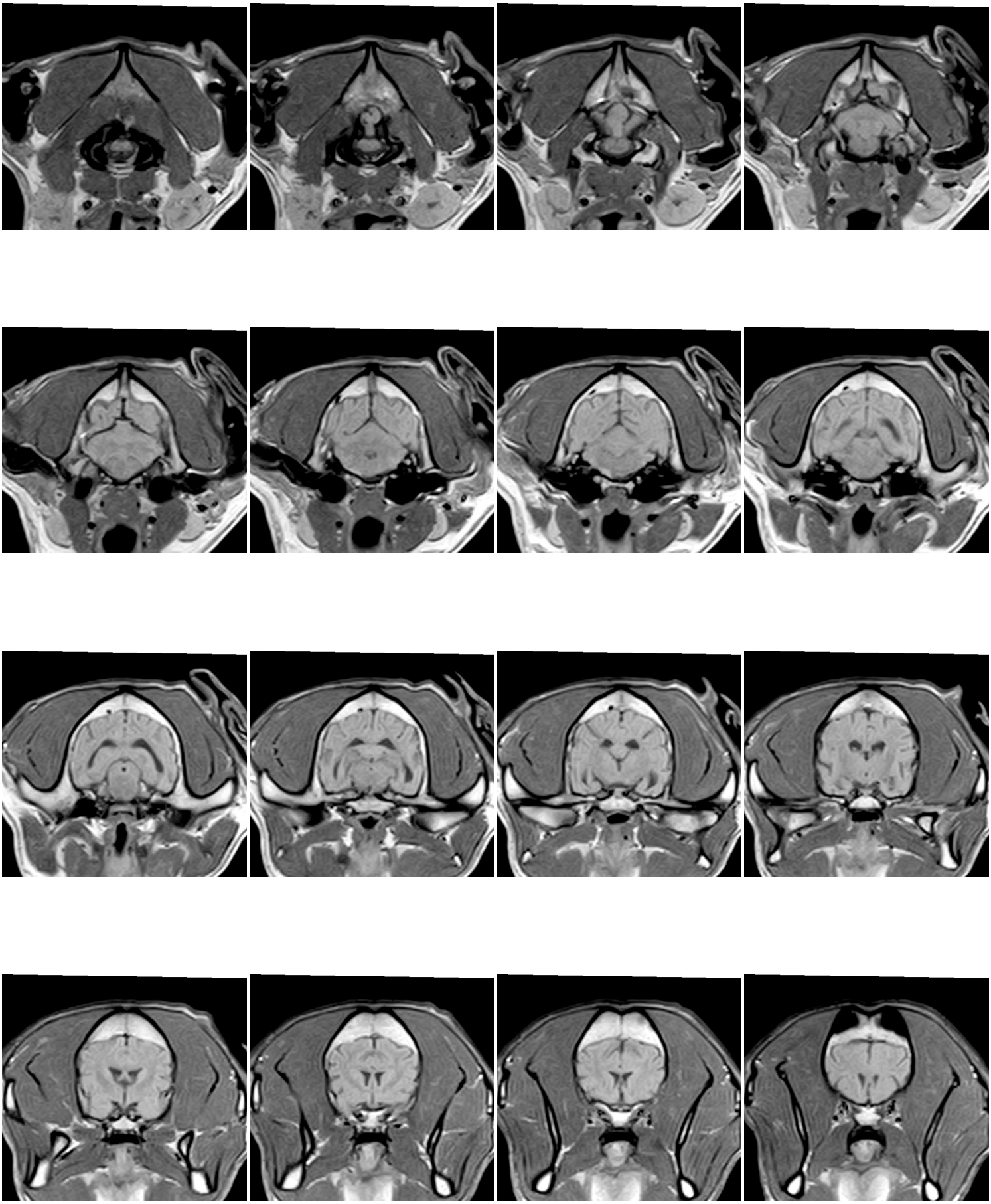
Pirata MRI 1 - **DAY 0** - Post contrast T1 weighted dorsal plane
Patient: Pirata (Pitbull dog)



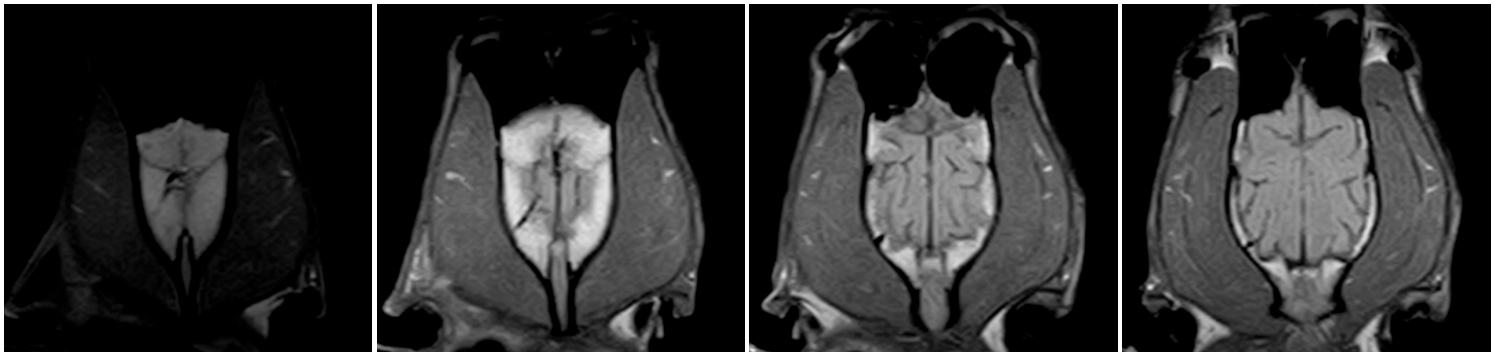
Pirata MRI 1 - **DAY 0** - Post contrast T2 weighted dorsal plane
Patient: Pirata (Pitbull dog)

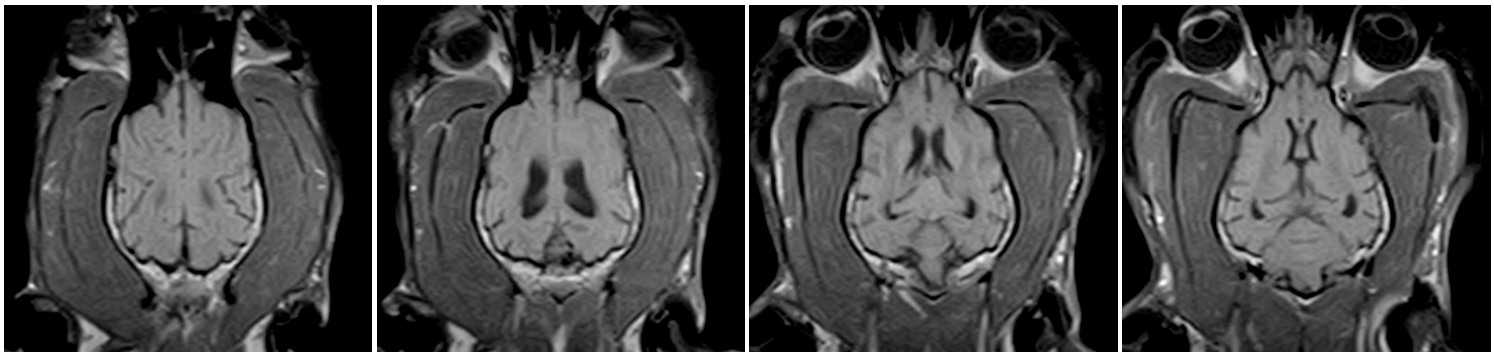


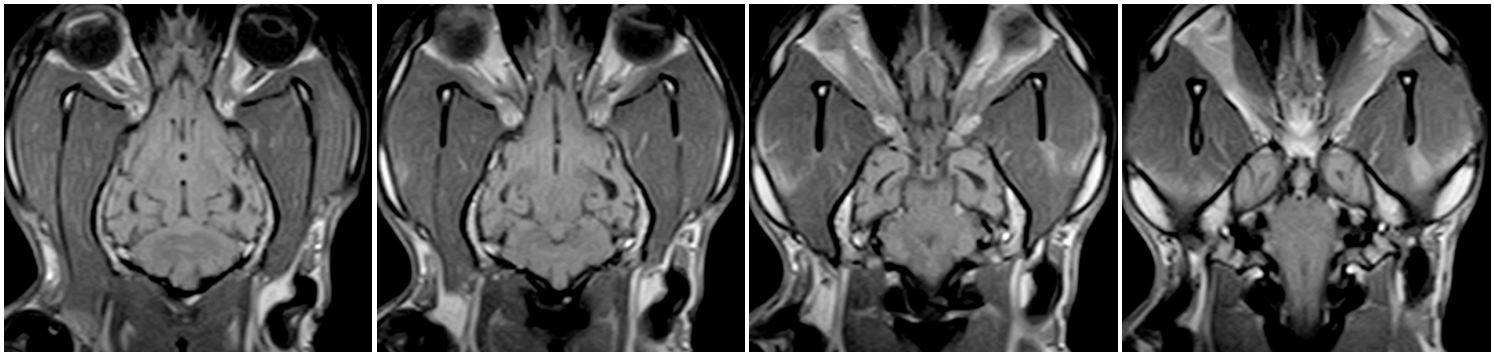
Pirata MRI 1 - **DAY 0** - Post contrast T2 weighted transversal plane
Patient: Pirata (Pitbull dog)

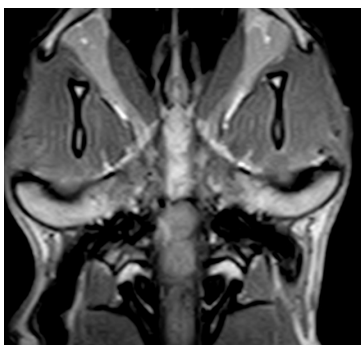


Pirata MRI 2 - **DAY 7** - Post contrast T1 weighted dorsal plane
Patient: Pirata (Pitbull dog)

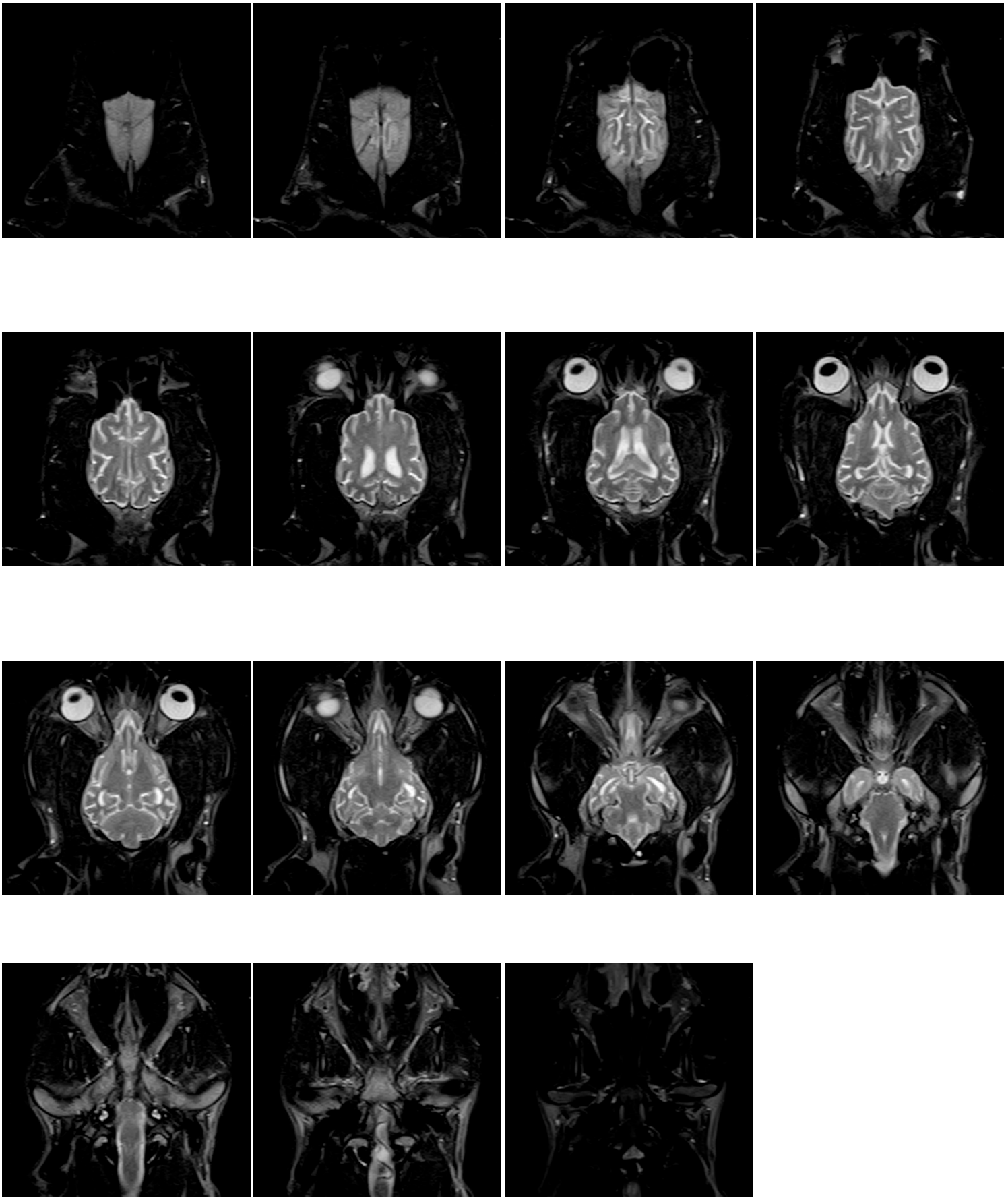




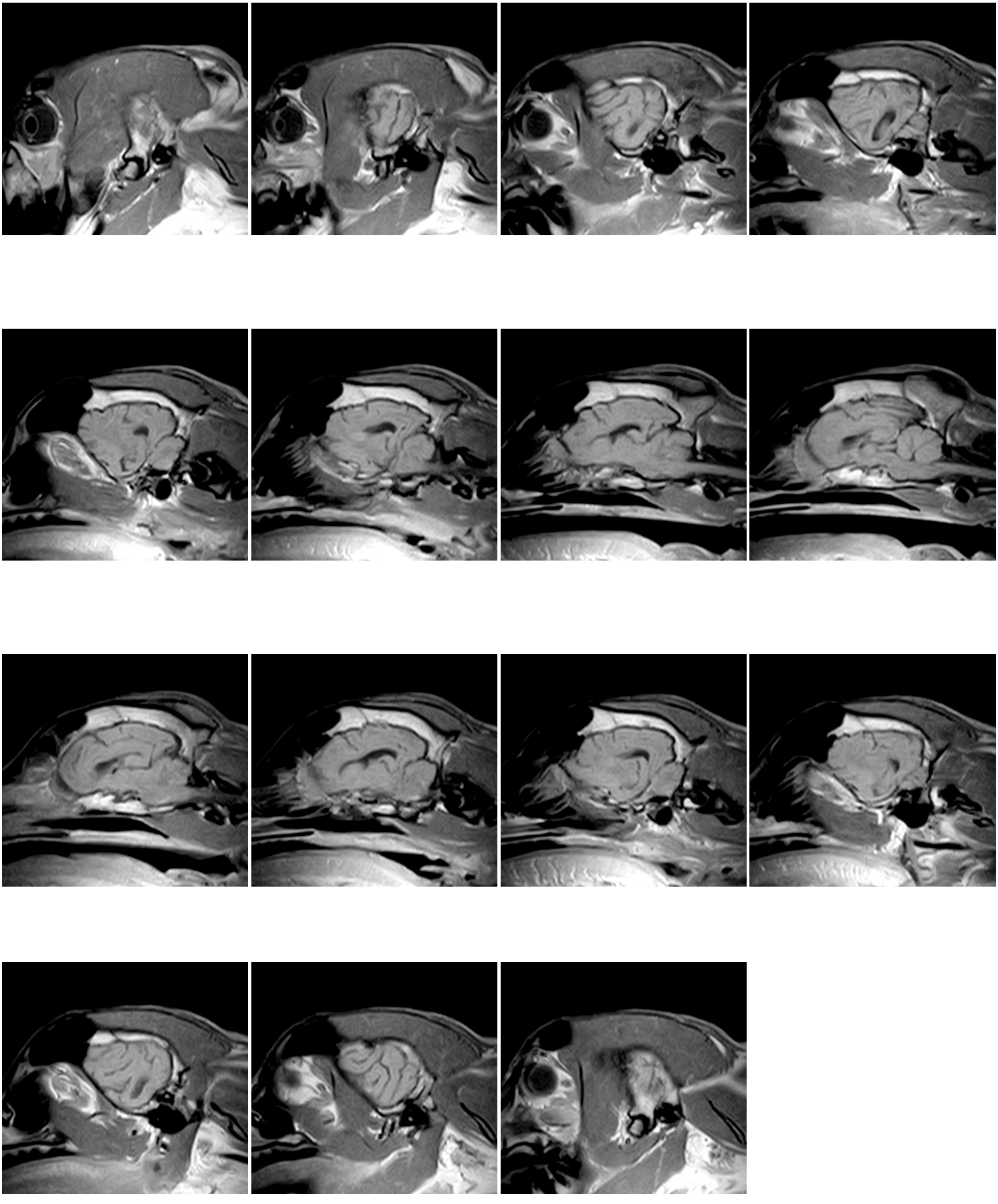




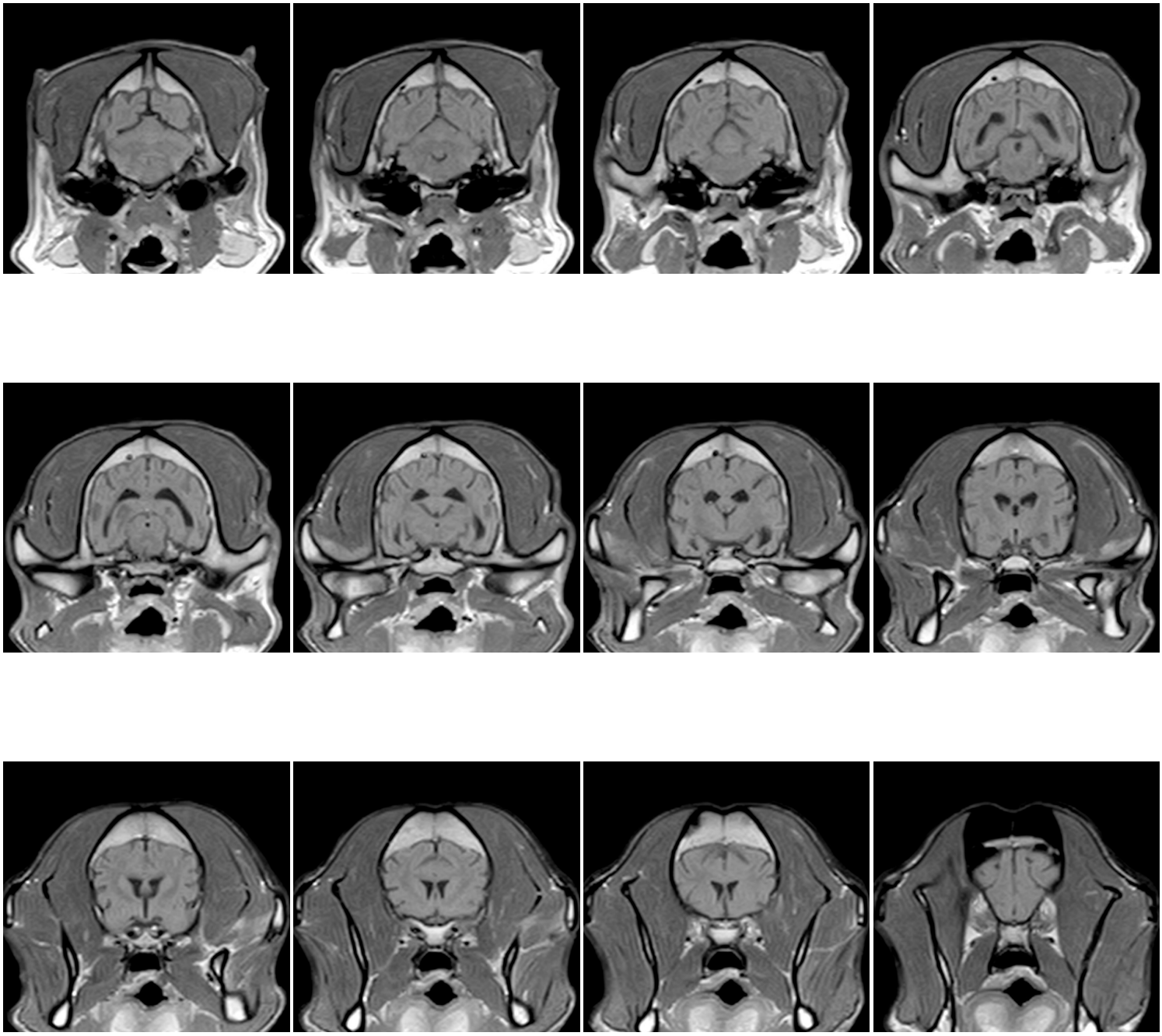
Pirata MRI 2 - **DAY 7** - Post contrast T2 weighted dorsal plane
Patient: Pirata (Pitbull dog)



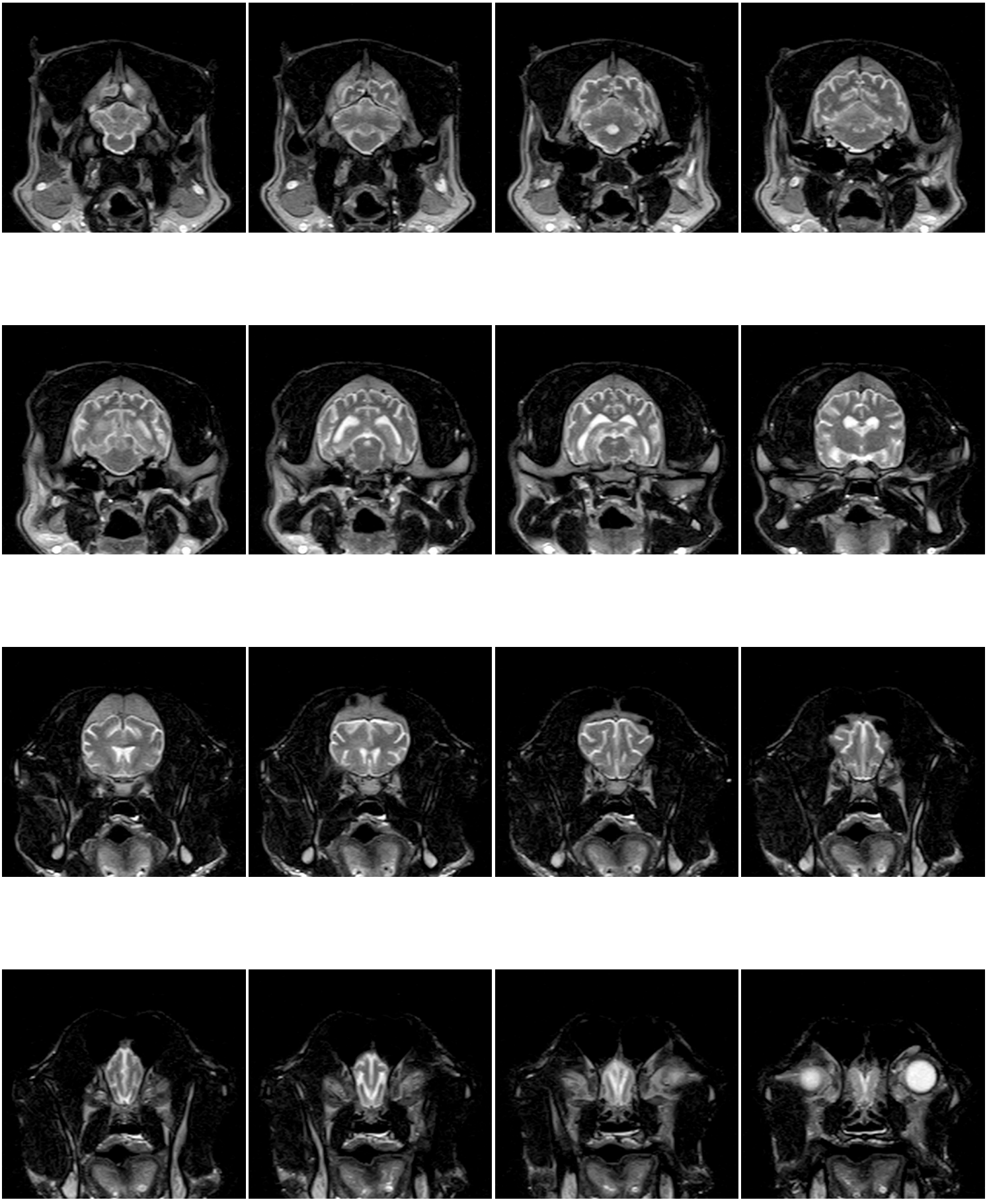
Pirata MRI 2 - **DAY 7** - Post contrast T2 weighted sagittal plane
Patient: Pirata (Pitbull dog)



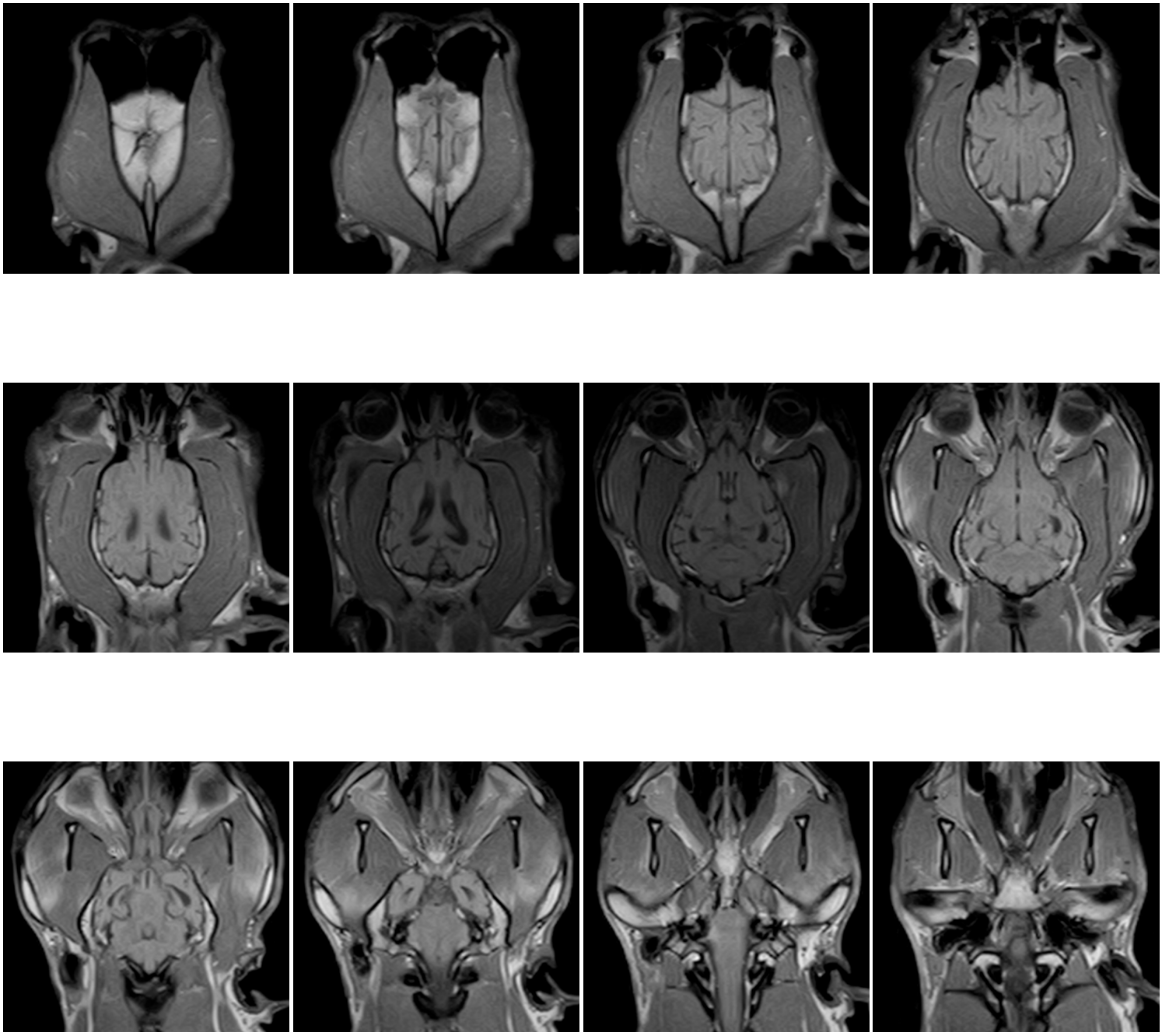
Pirata MRI 2 - **DAY 7** - Post contrast T1 weighted transversal plane
Patient: Pirata (Pitbull dog)



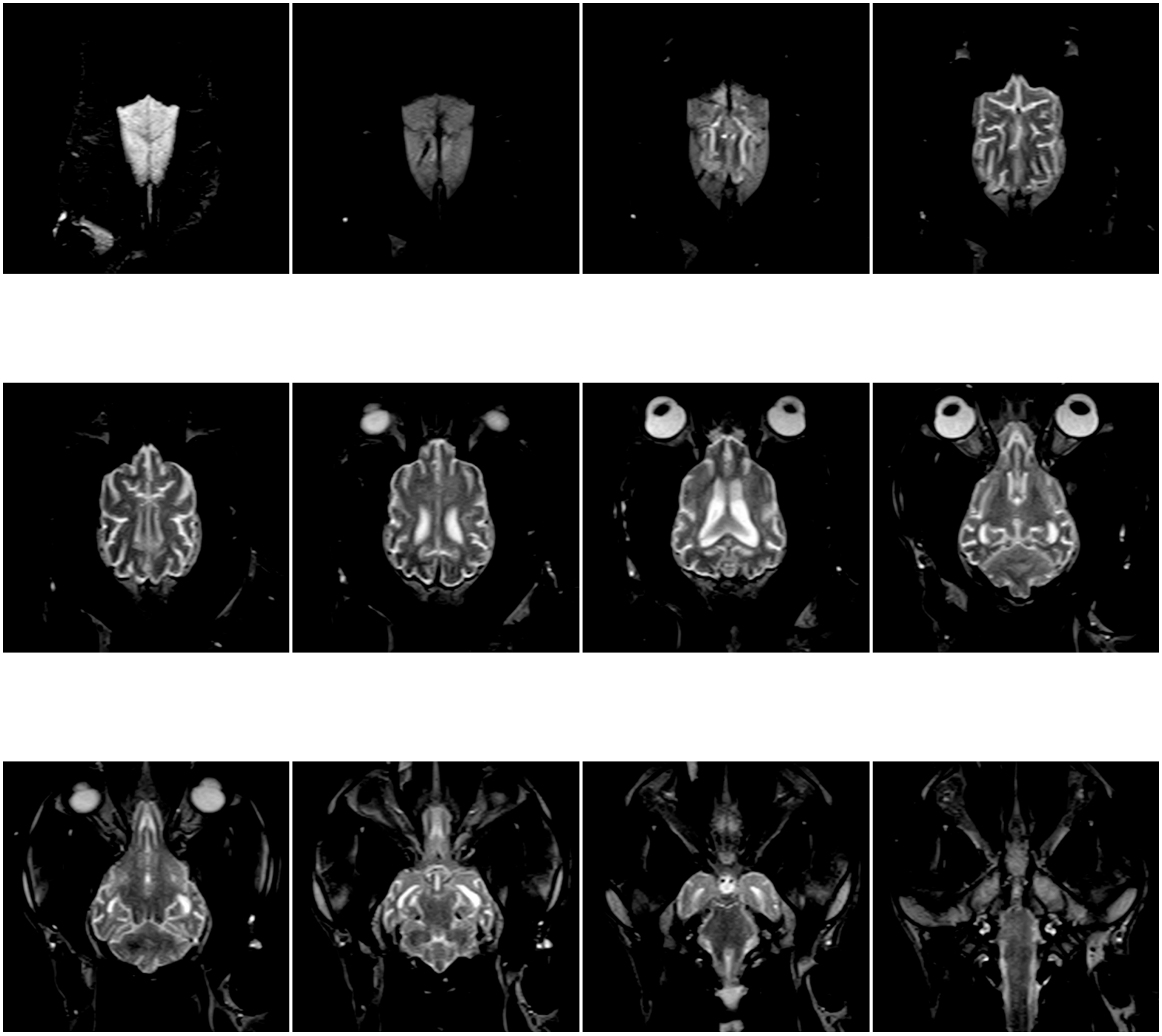
Pirata MRI 2 - **DAY 7** - Post contrast T2 weighted transversal plane
Patient: Pirata (Pitbull dog)



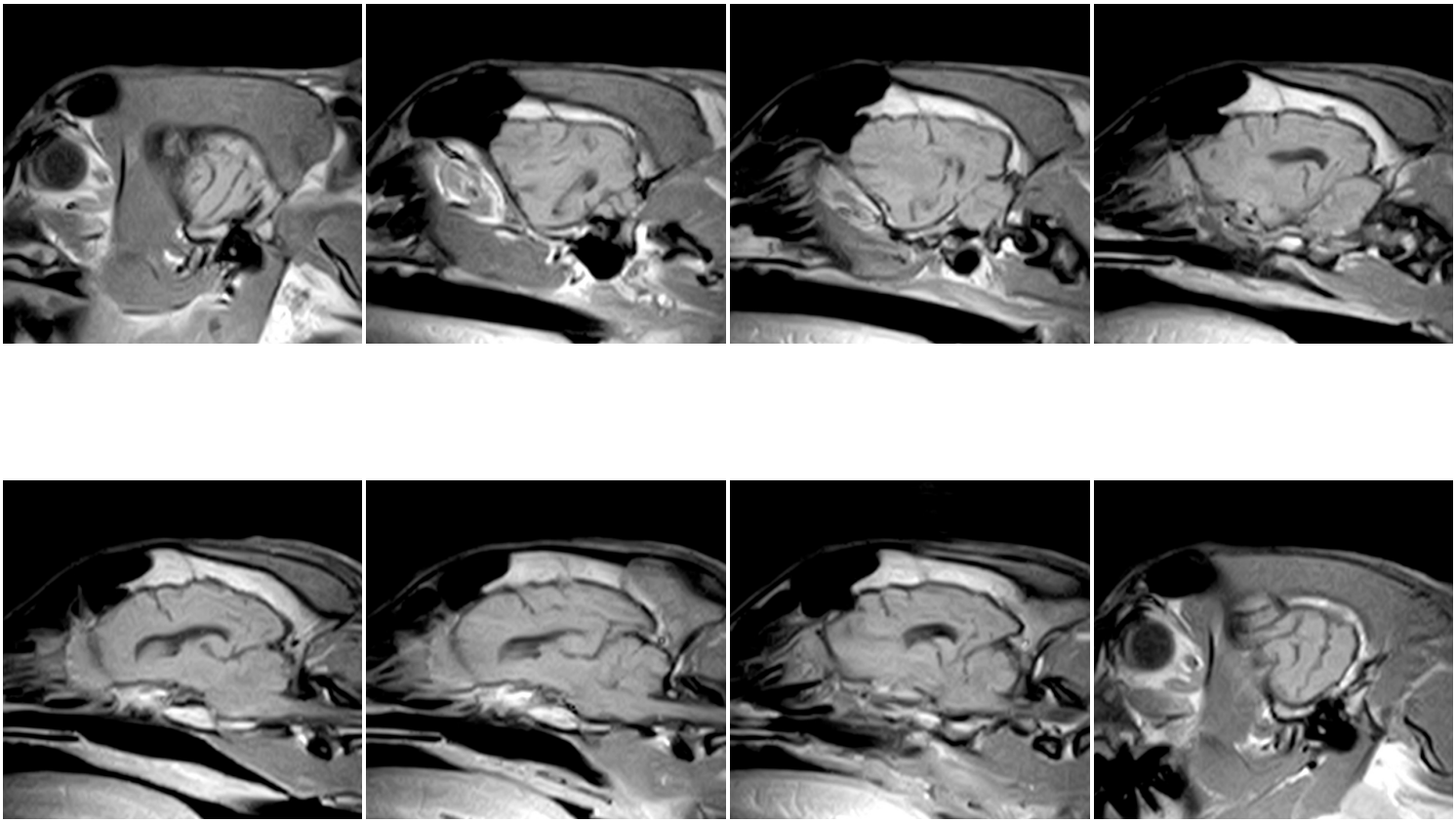
Pirata MRI 3 – **DAY 14** - Post contrast T1 weighted dorsal plane
Patient: Pirata (Pitbull dog)



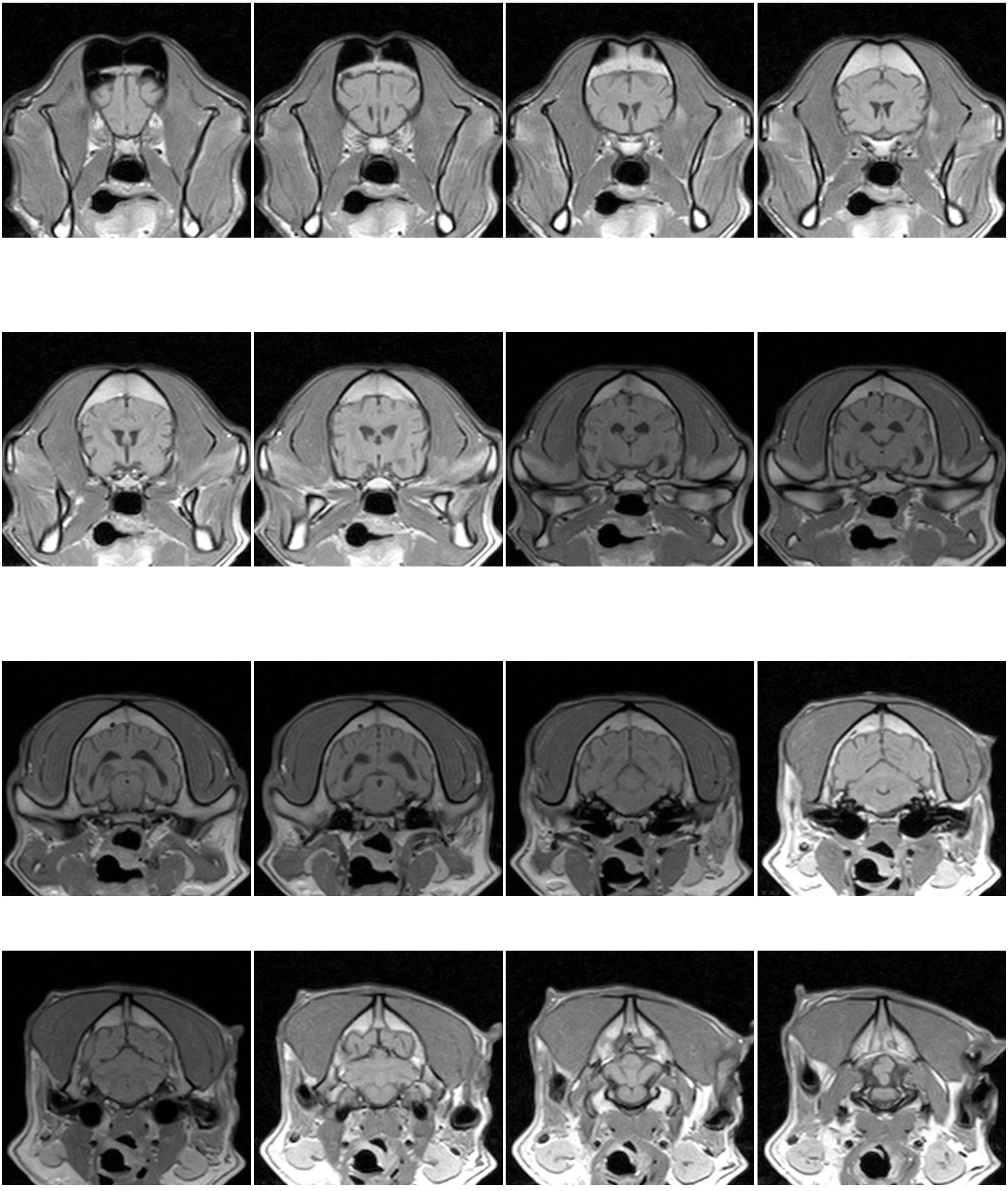
Pirata MRI 3 – **DAY 14** - Post contrast T2 weighted dorsal plane
Patient: Pirata (Pitbull dog)



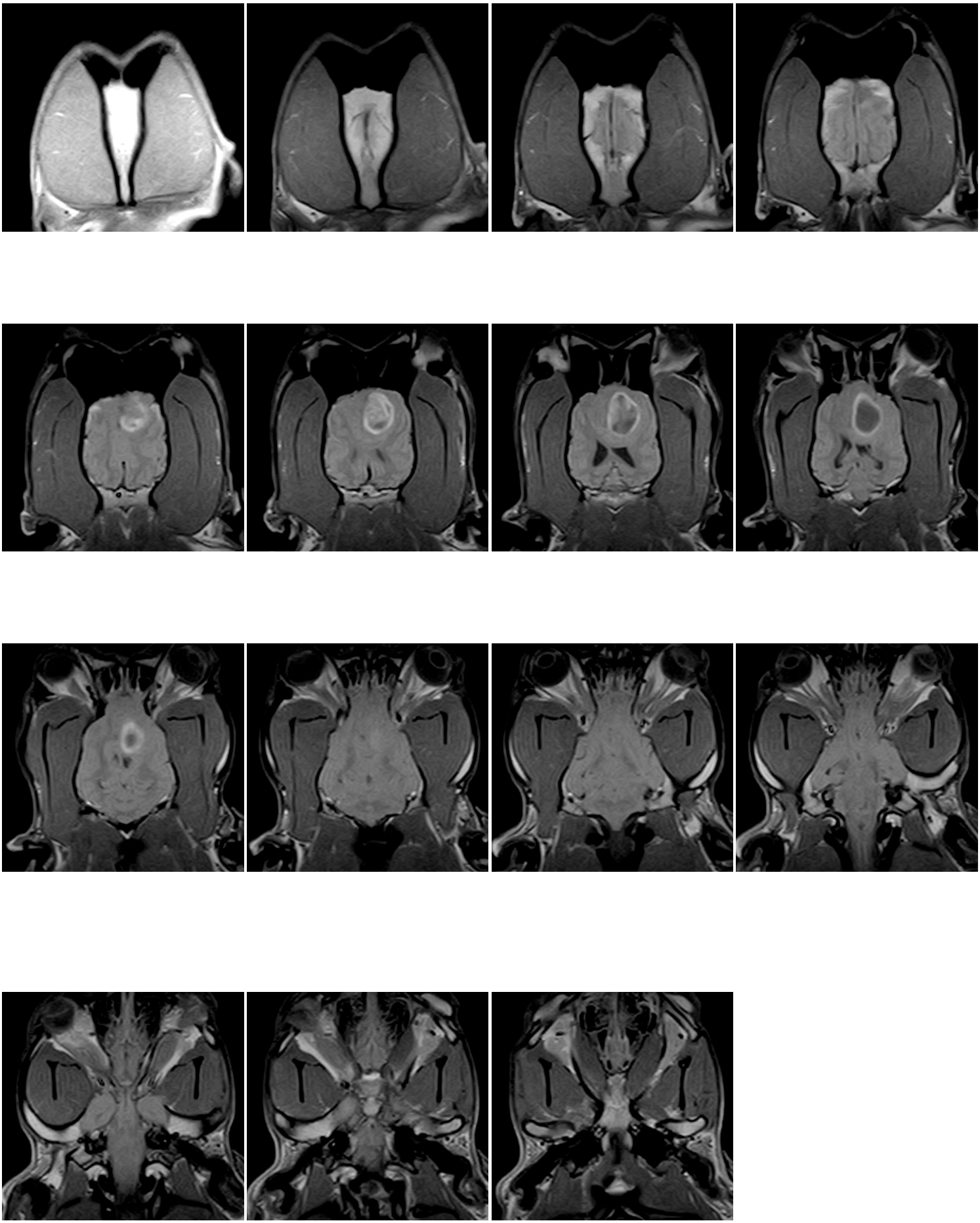
Pirata MRI 3 – **DAY 14** - Post contrast T1 weighted sagittal plane
Patient: Pirata (Pitbull dog)



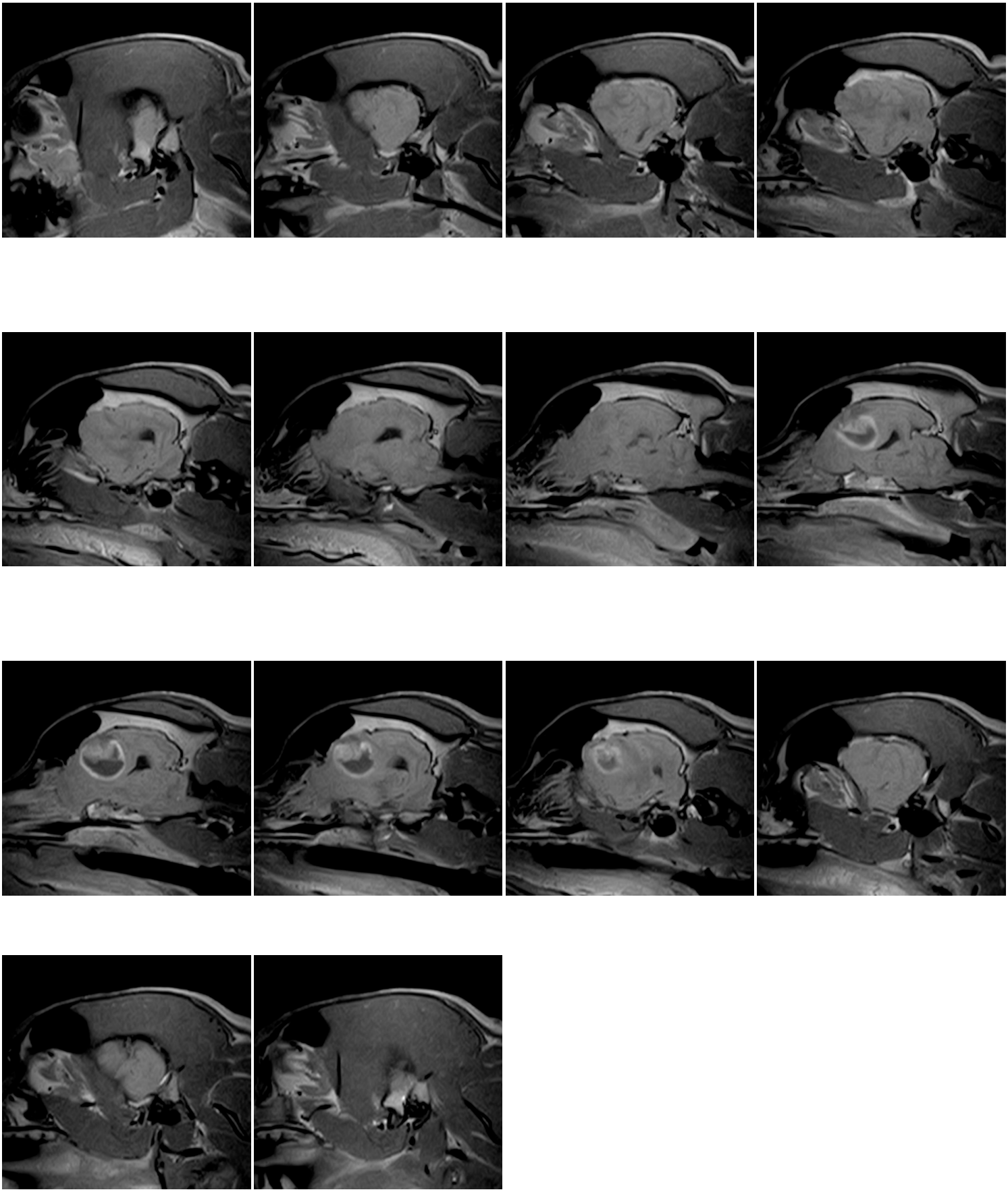
Pirata MRI 3 – **DAY 14** - Post contrast T1 weighted transversal plane
Patient: Pirata (Pitbull dog)



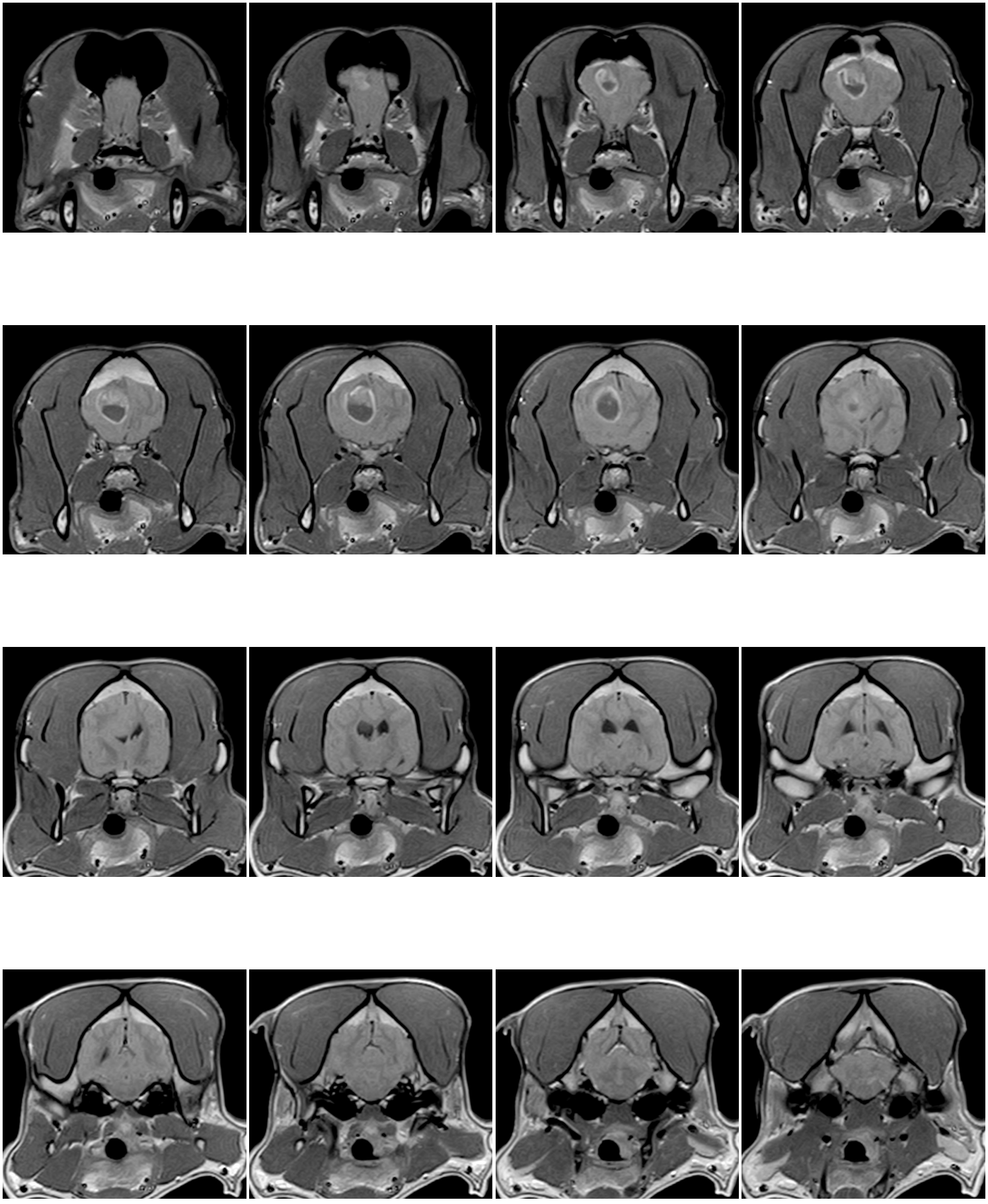
Matheus MRI 1 - **DAY 0** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



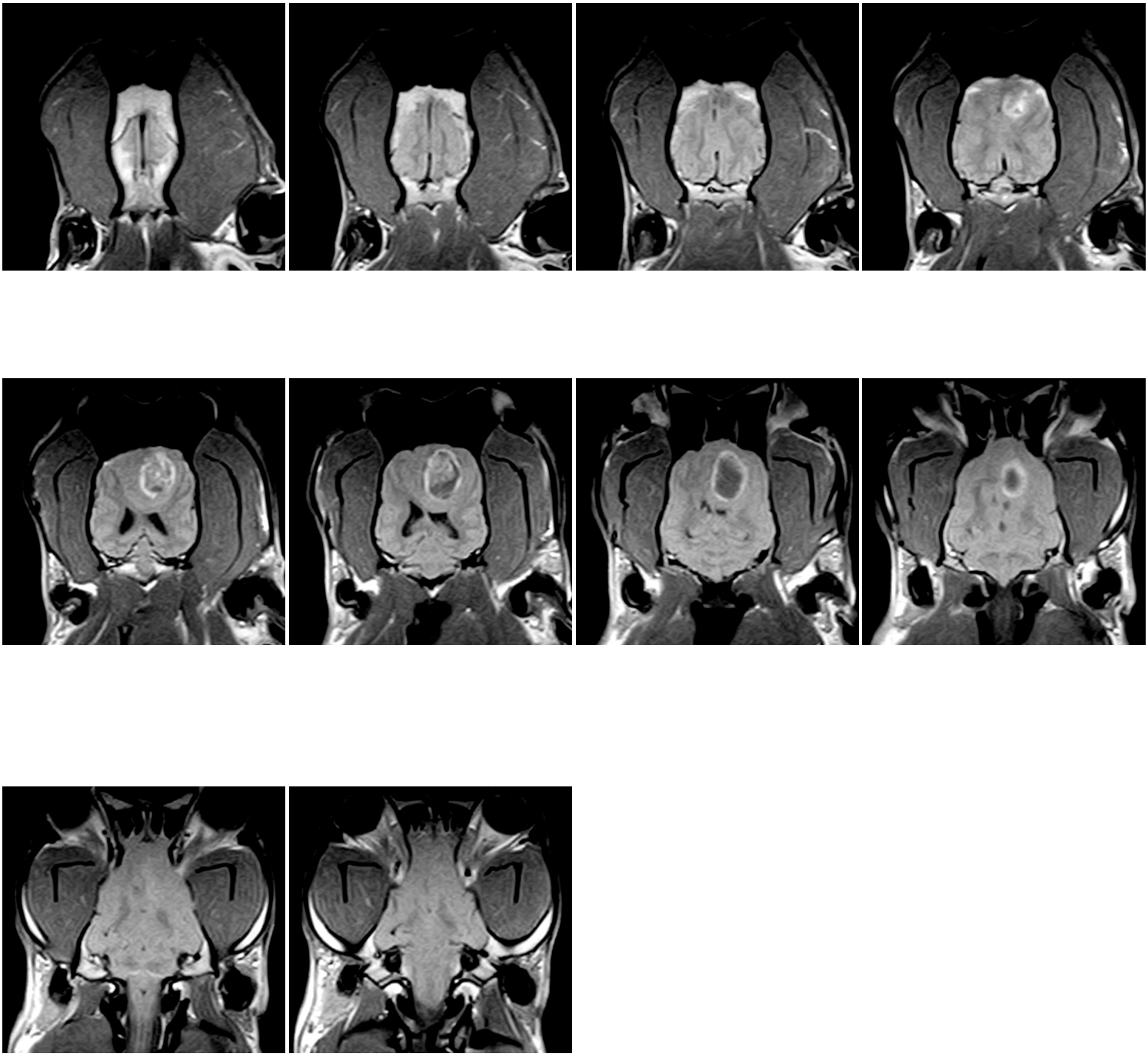
Matheus MRI 1 - **DAY 0** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



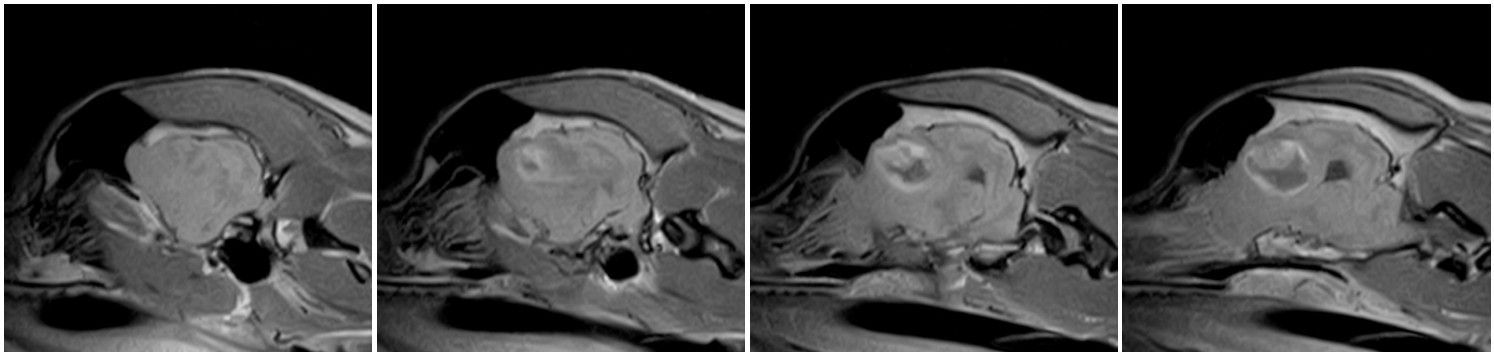
Matheus MRI 1 - **DAY 0** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)

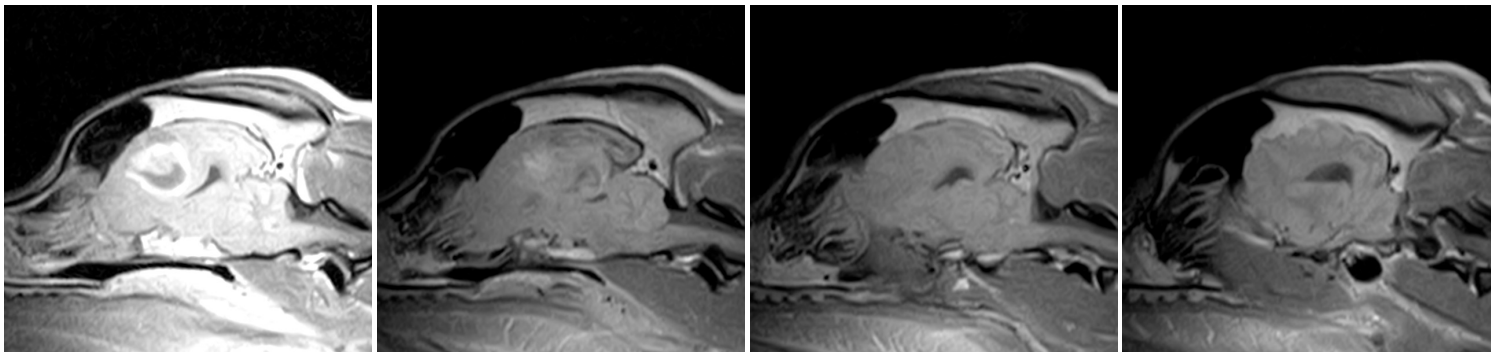


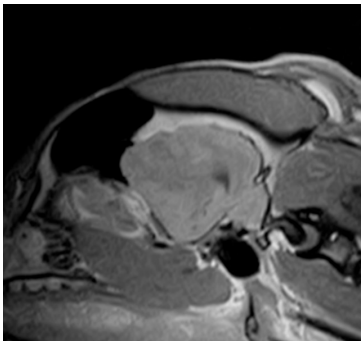
Matheus MRI 2 - **DAY 14** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



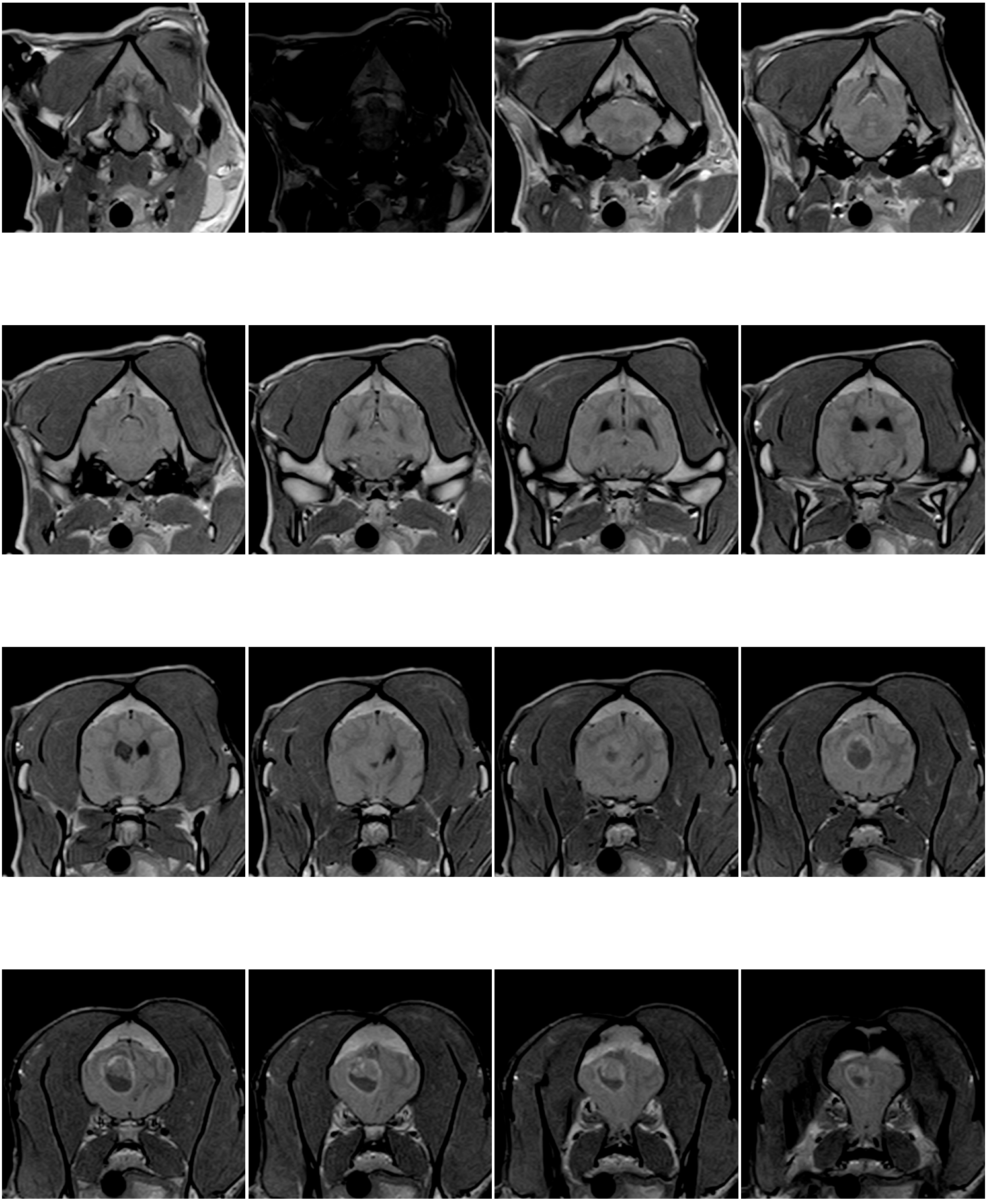
Matheus MRI 2 - **DAY 14** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



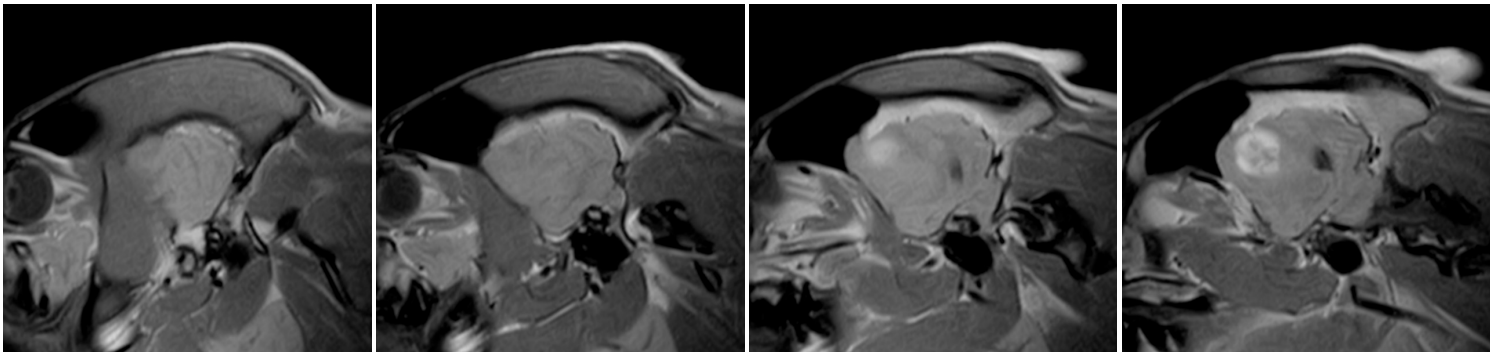


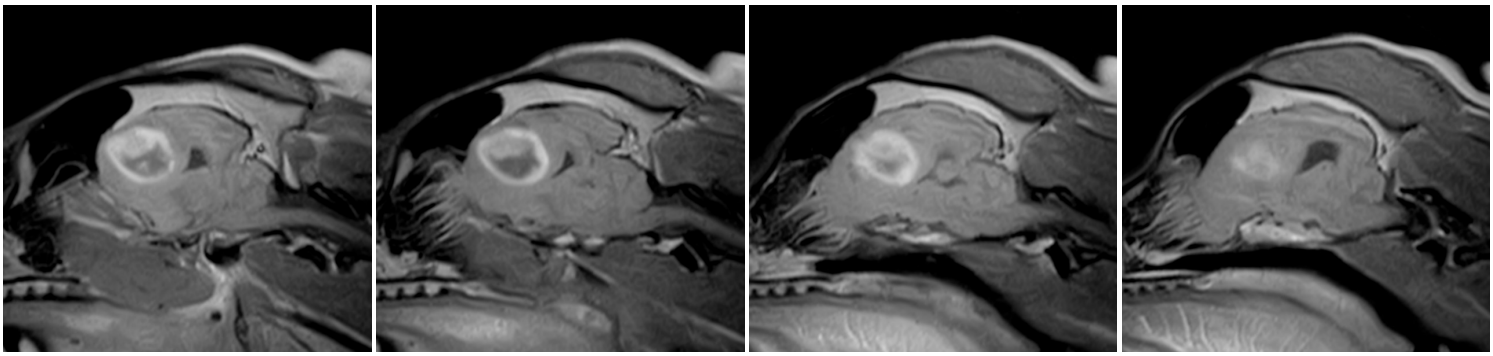


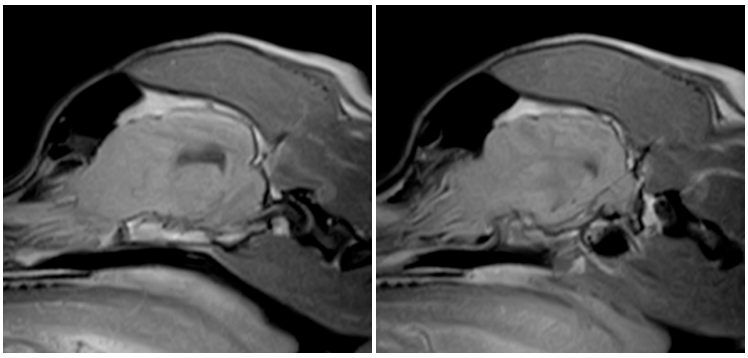
Matheus MRI 2 - **DAY 14** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



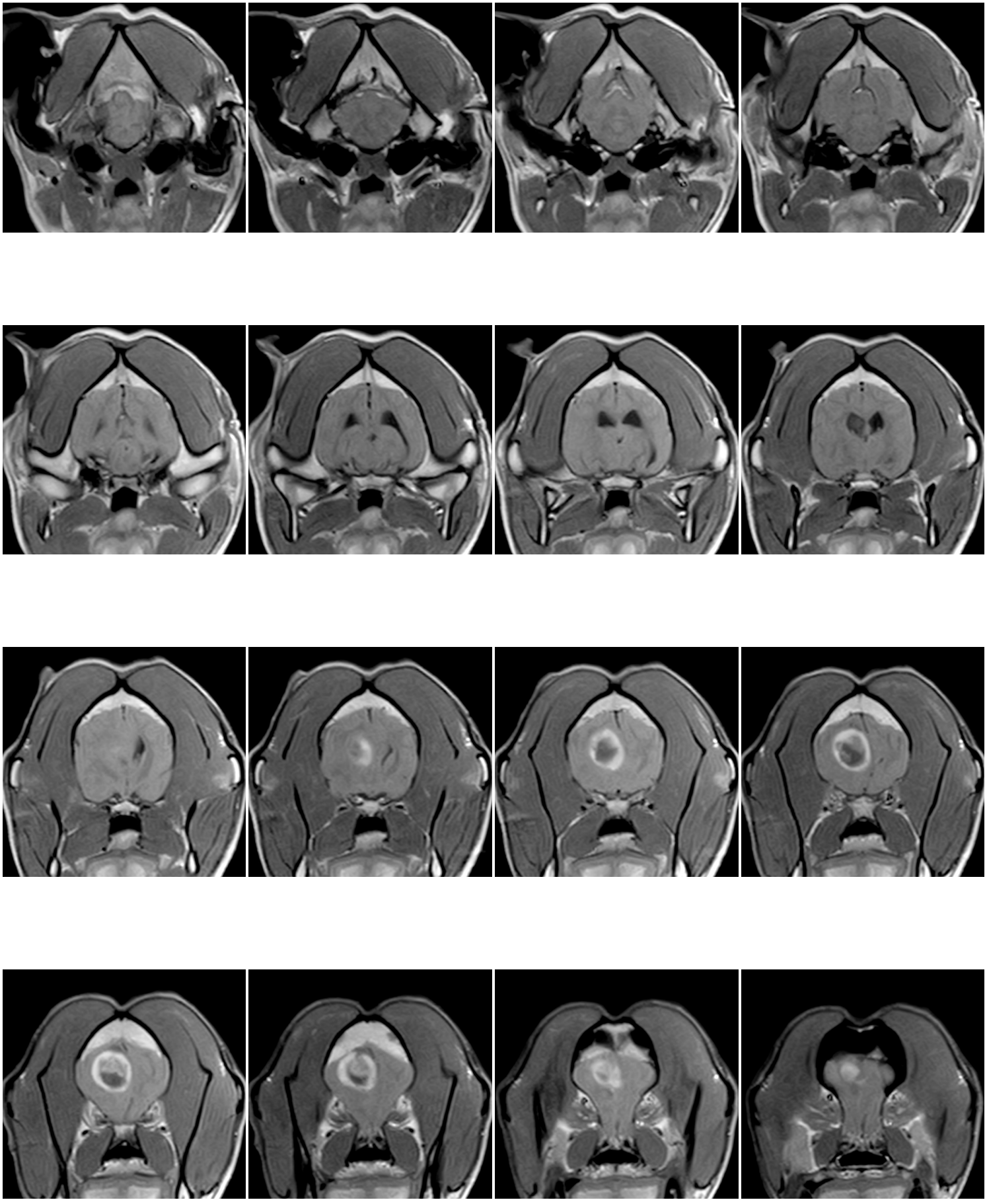
Matheus MRI 3 - **DAY 21** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



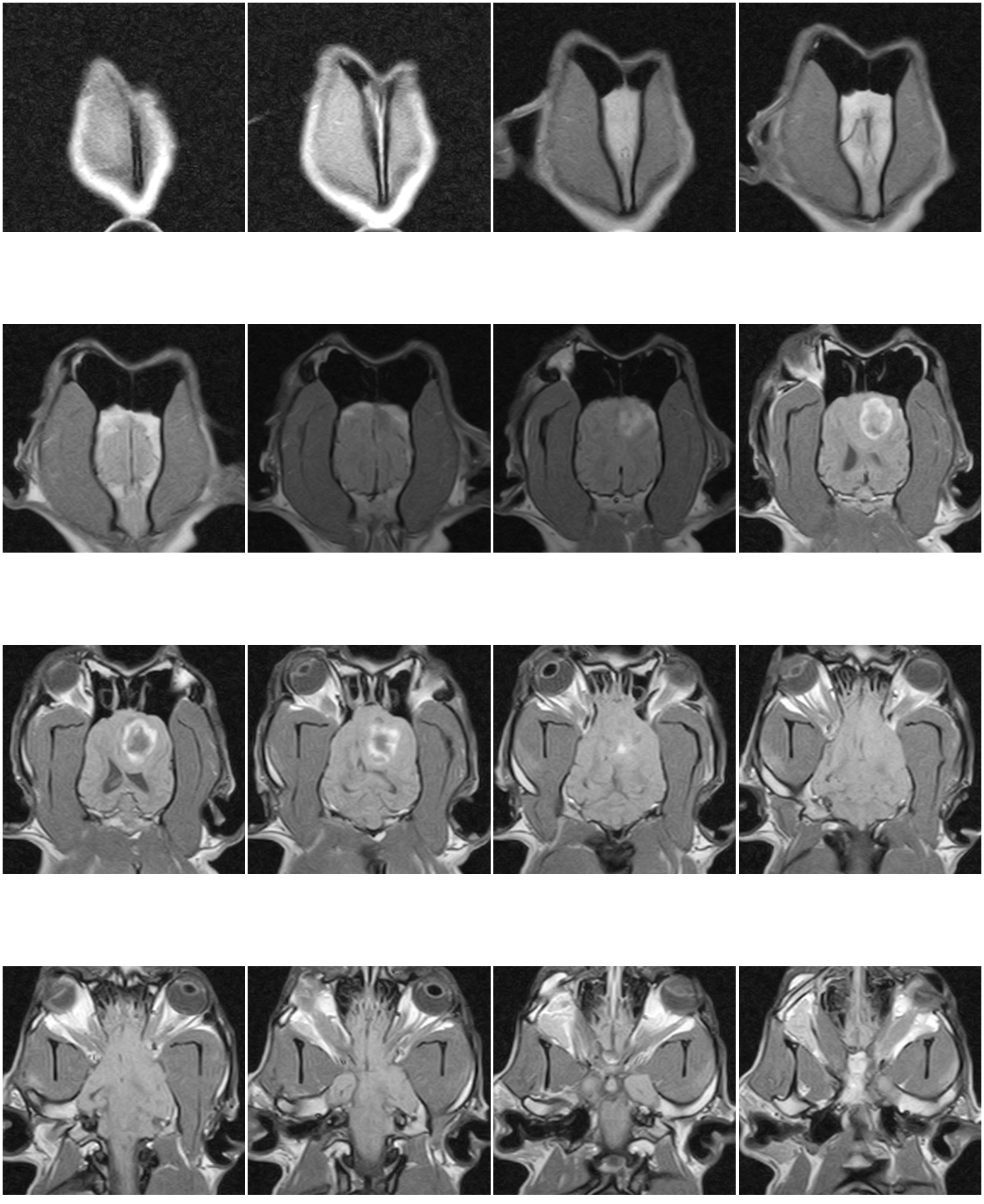




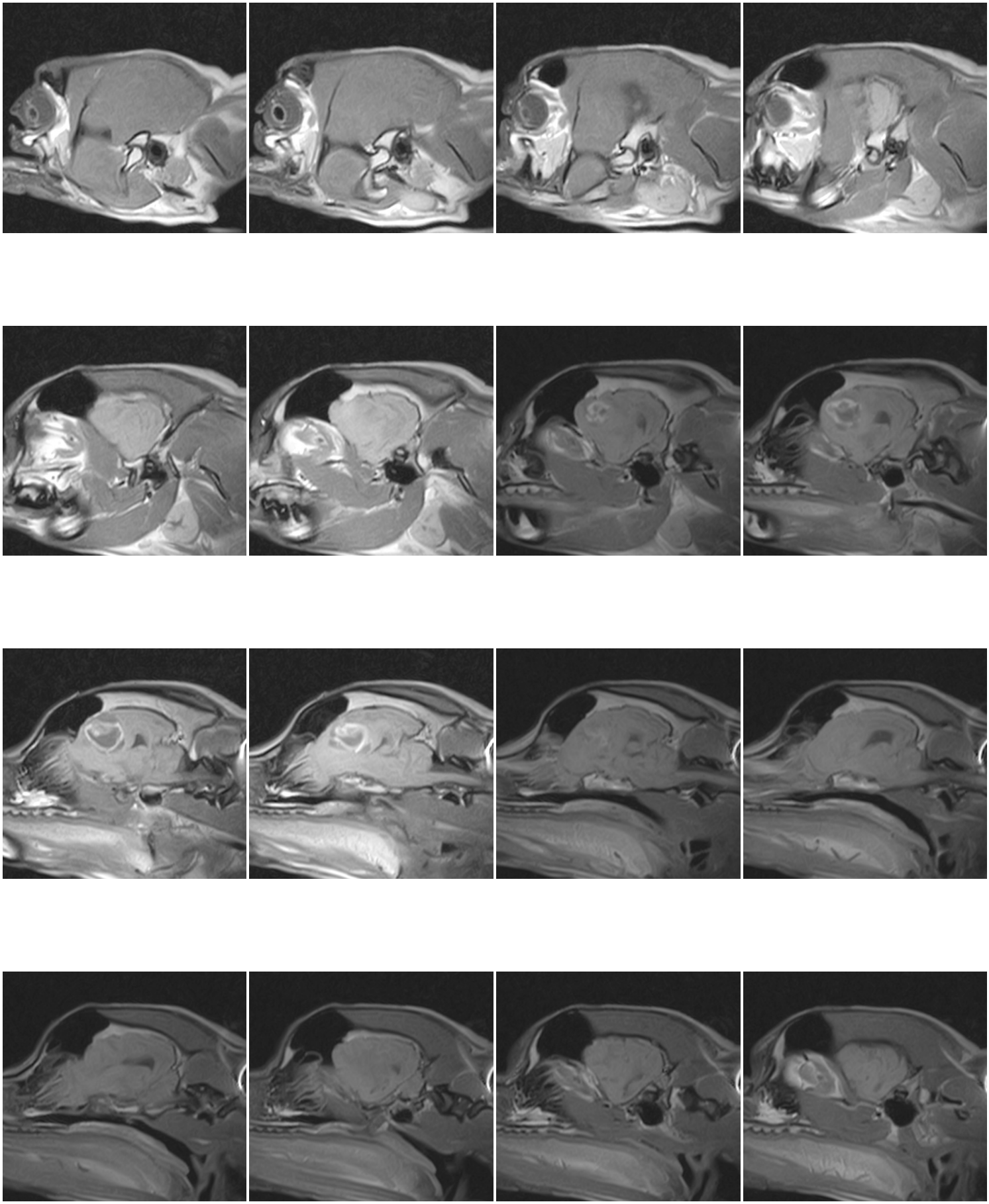
Matheus MRI 3 - **DAY 21** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



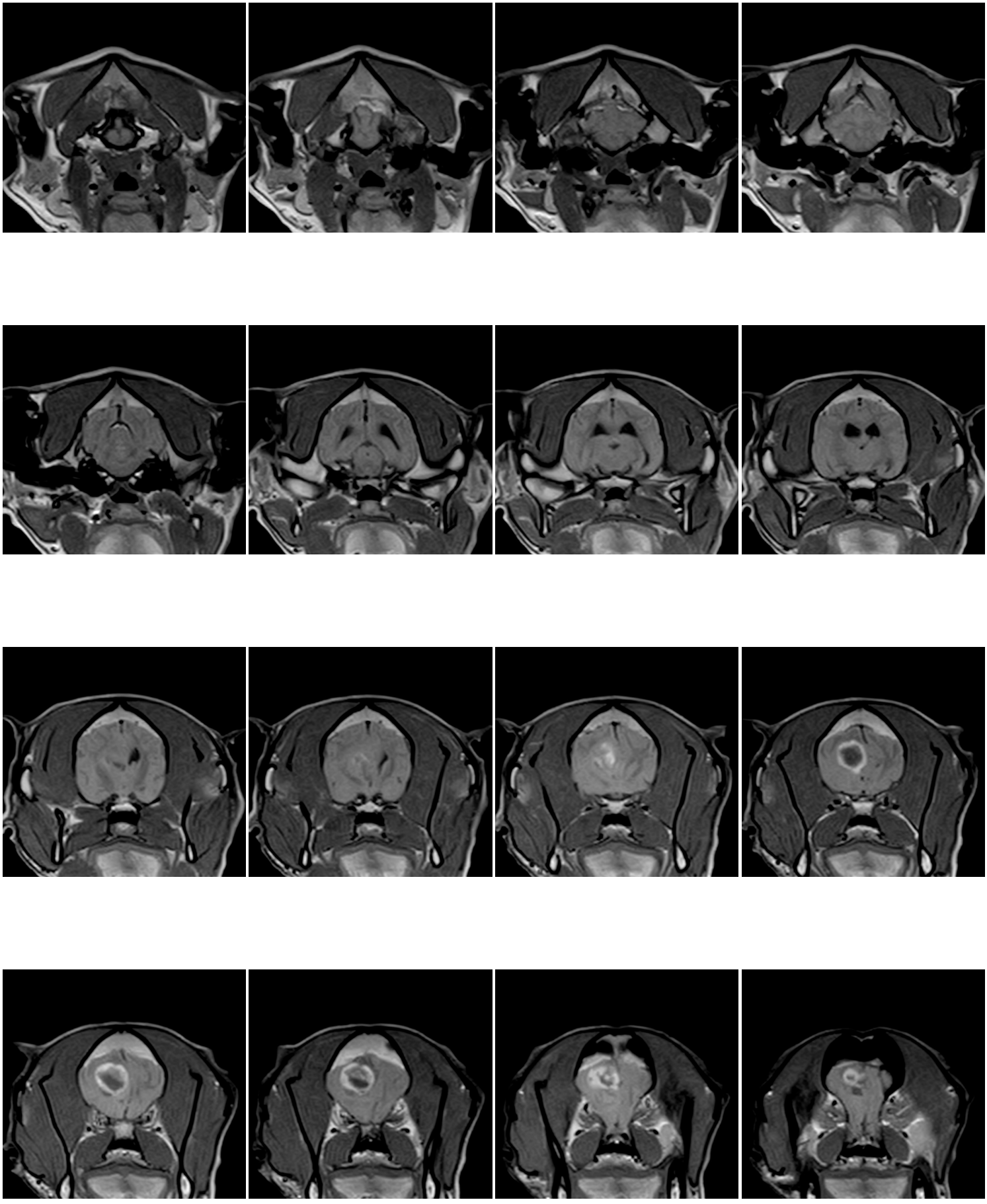
Matheus MRI 4 - **DAY 35** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



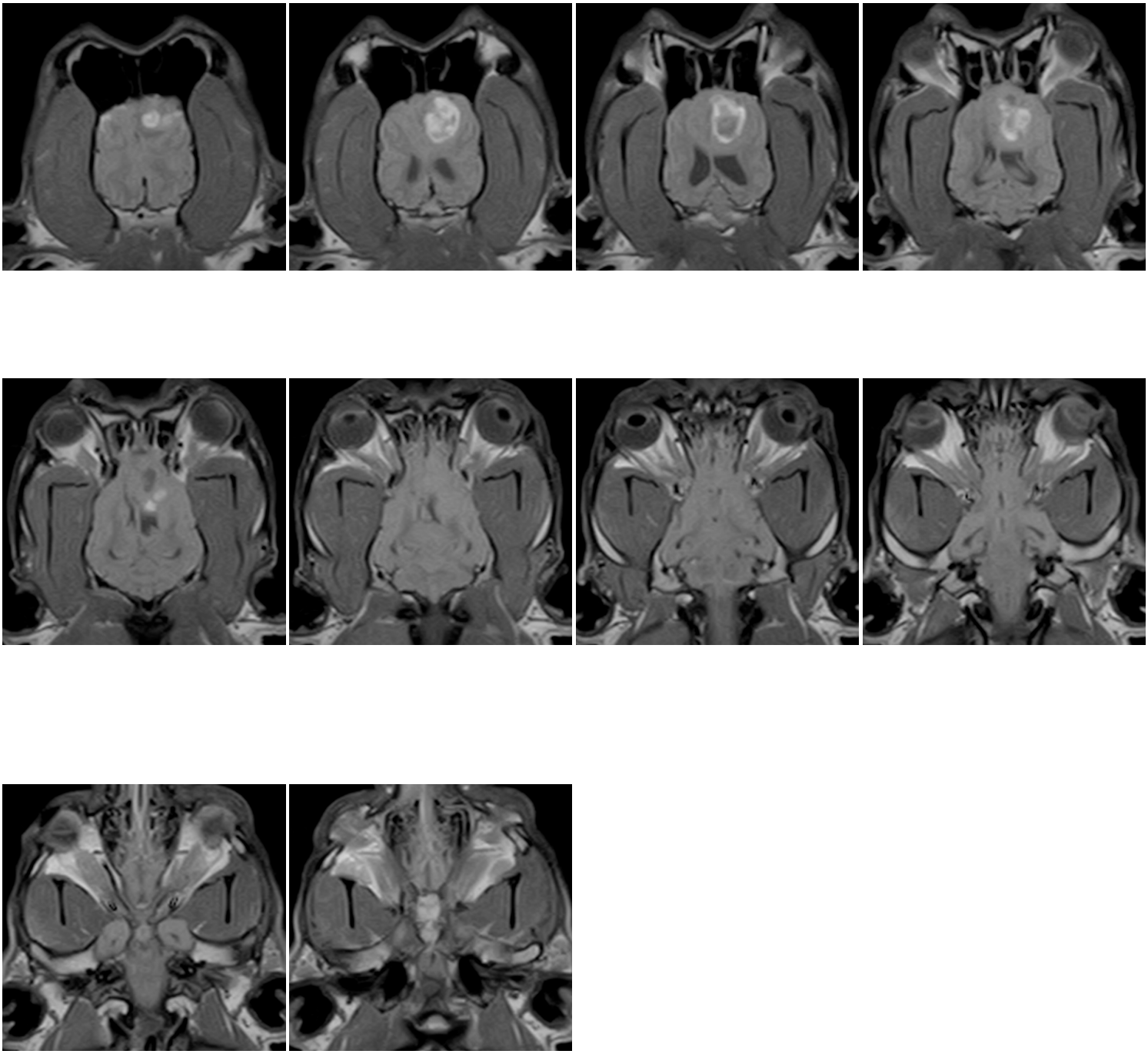
Matheus MRI 4 - **DAY 35** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



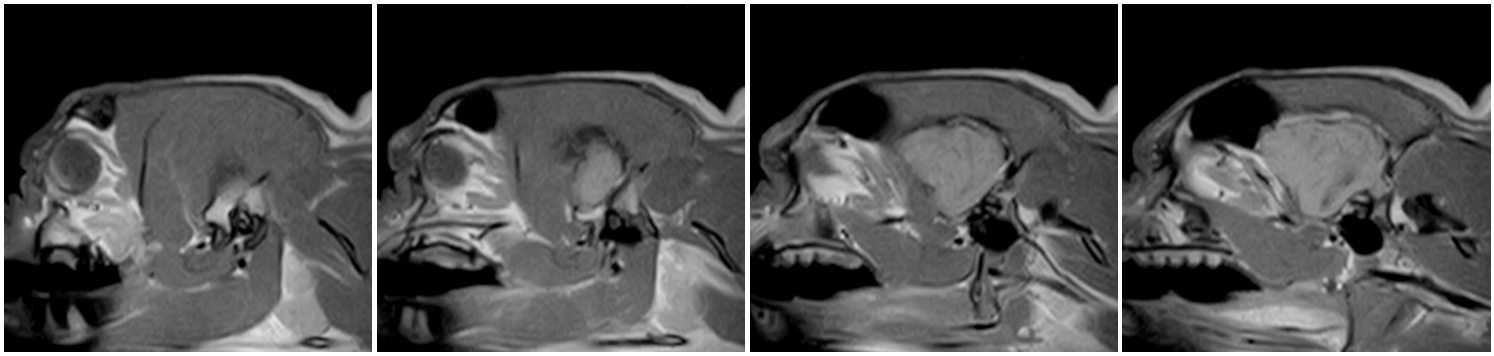
Matheus MRI 4 - **DAY 35** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)

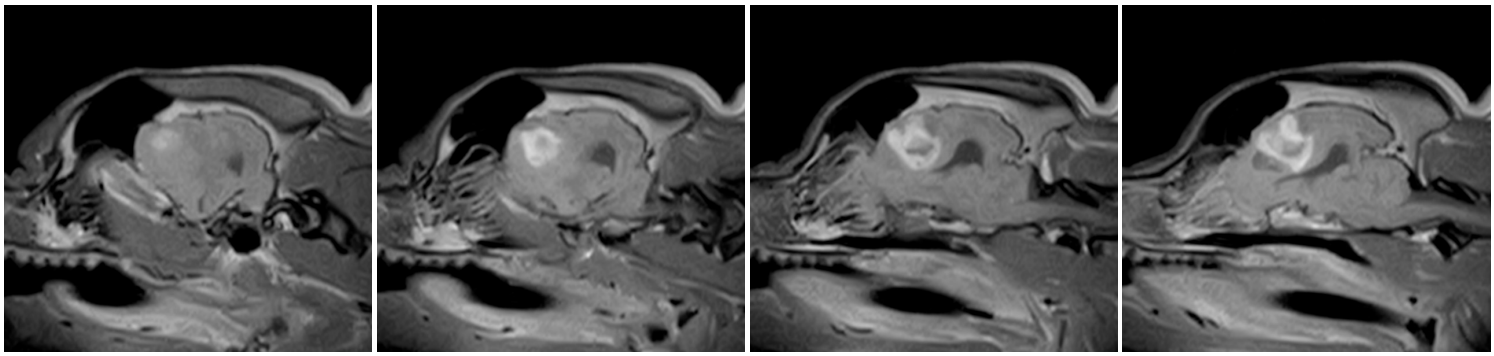


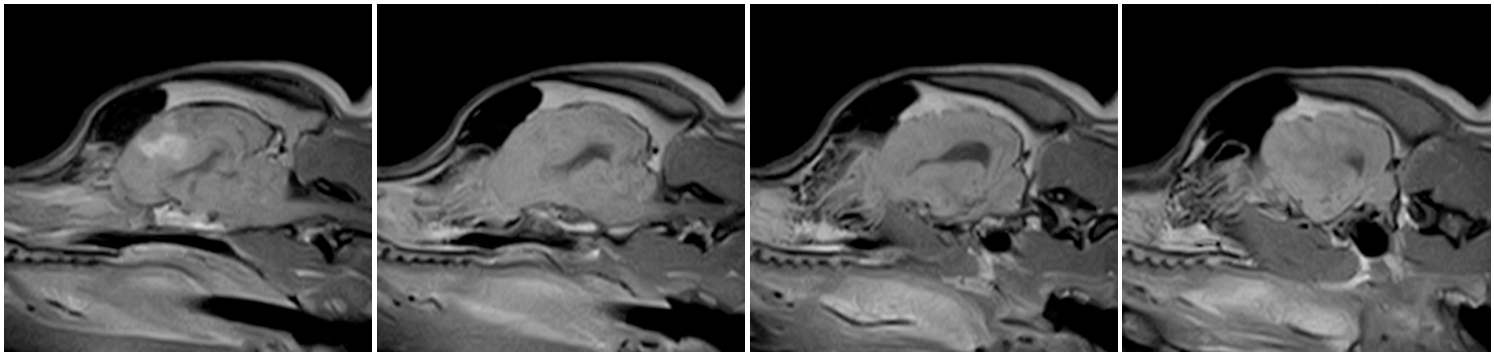
Matheus MRI 5 - **DAY 60** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)

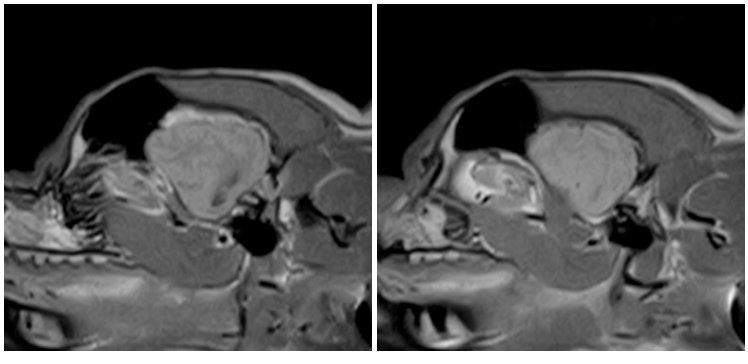


Matheus MRI 5 - **DAY 60** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)

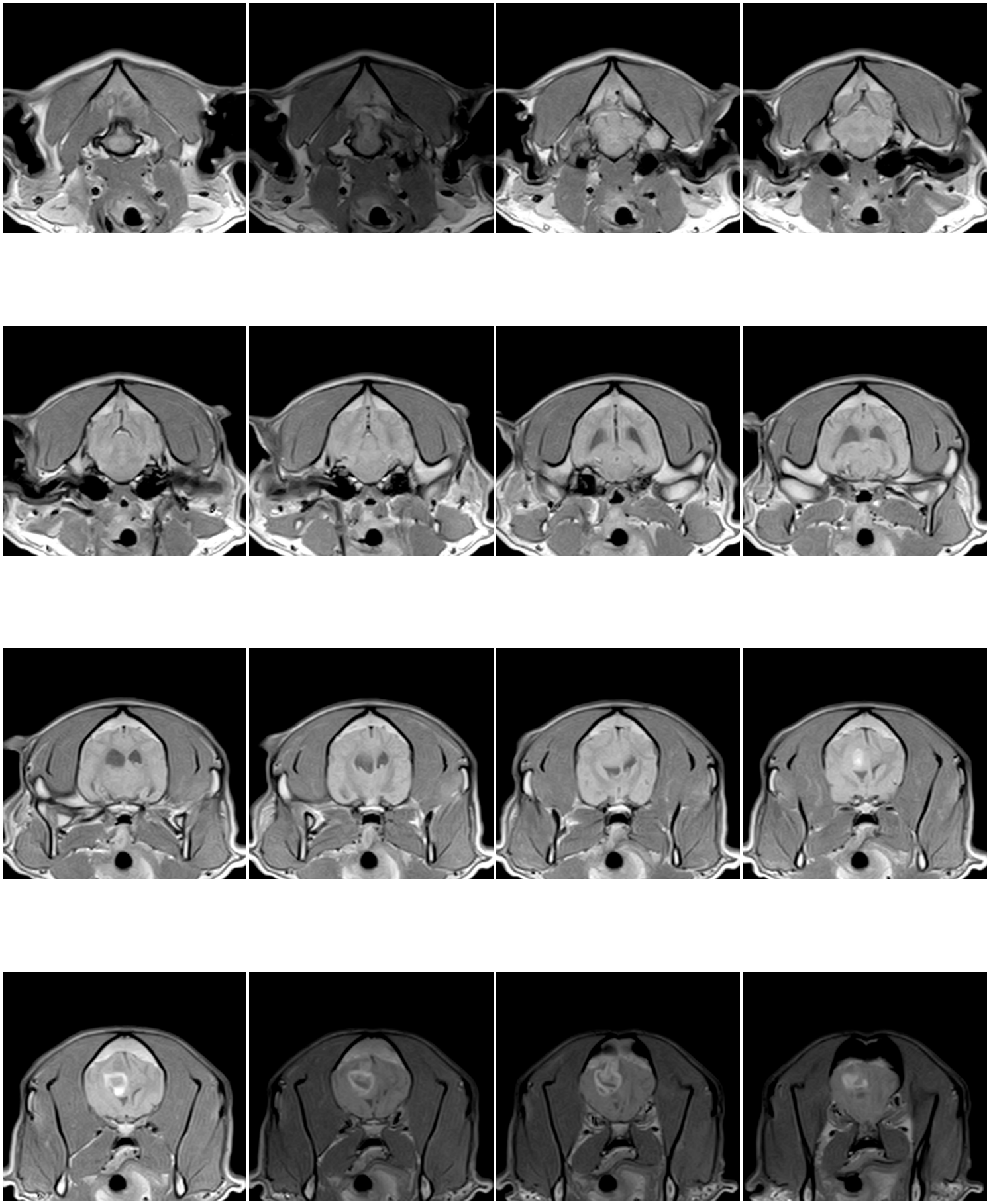




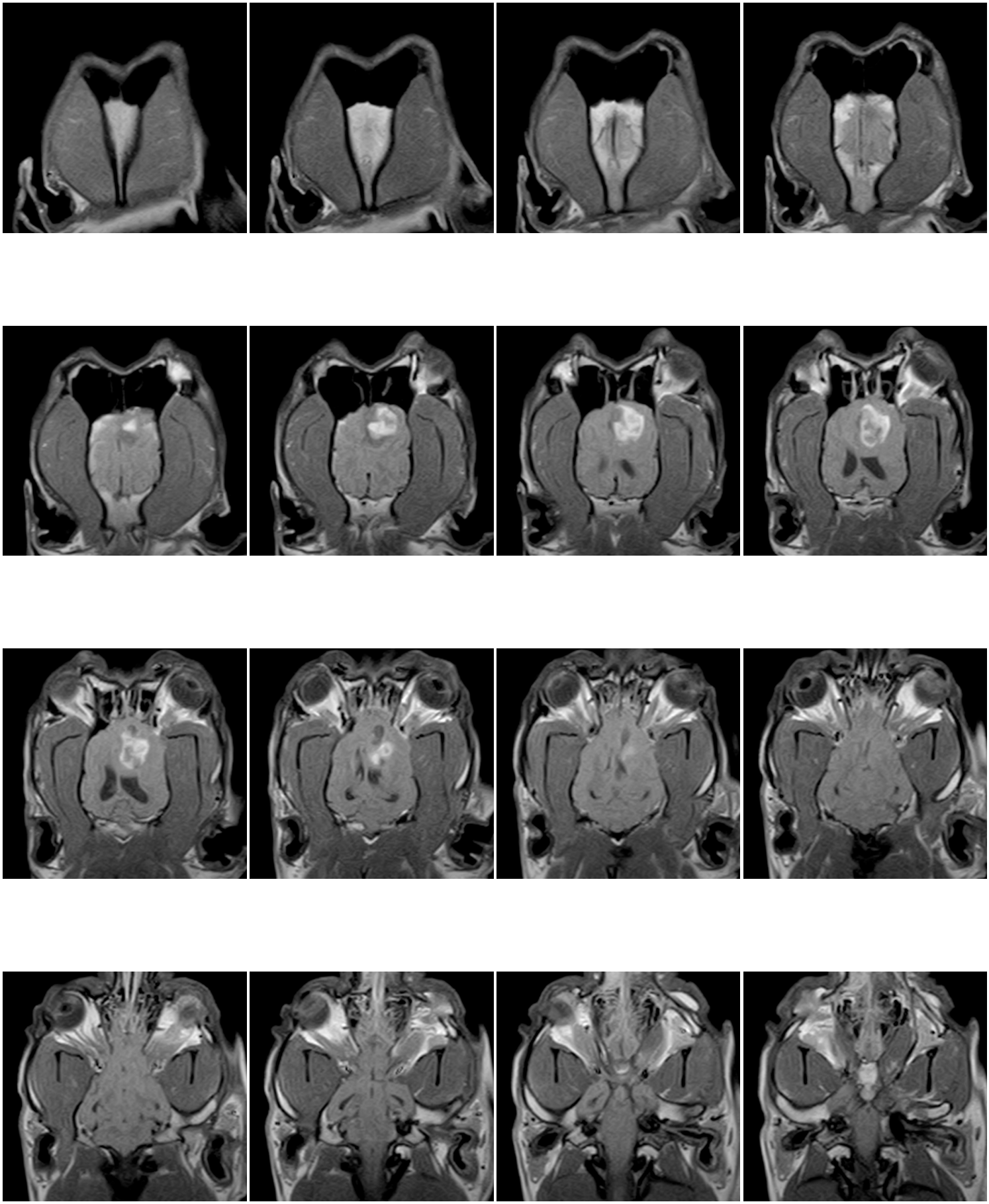




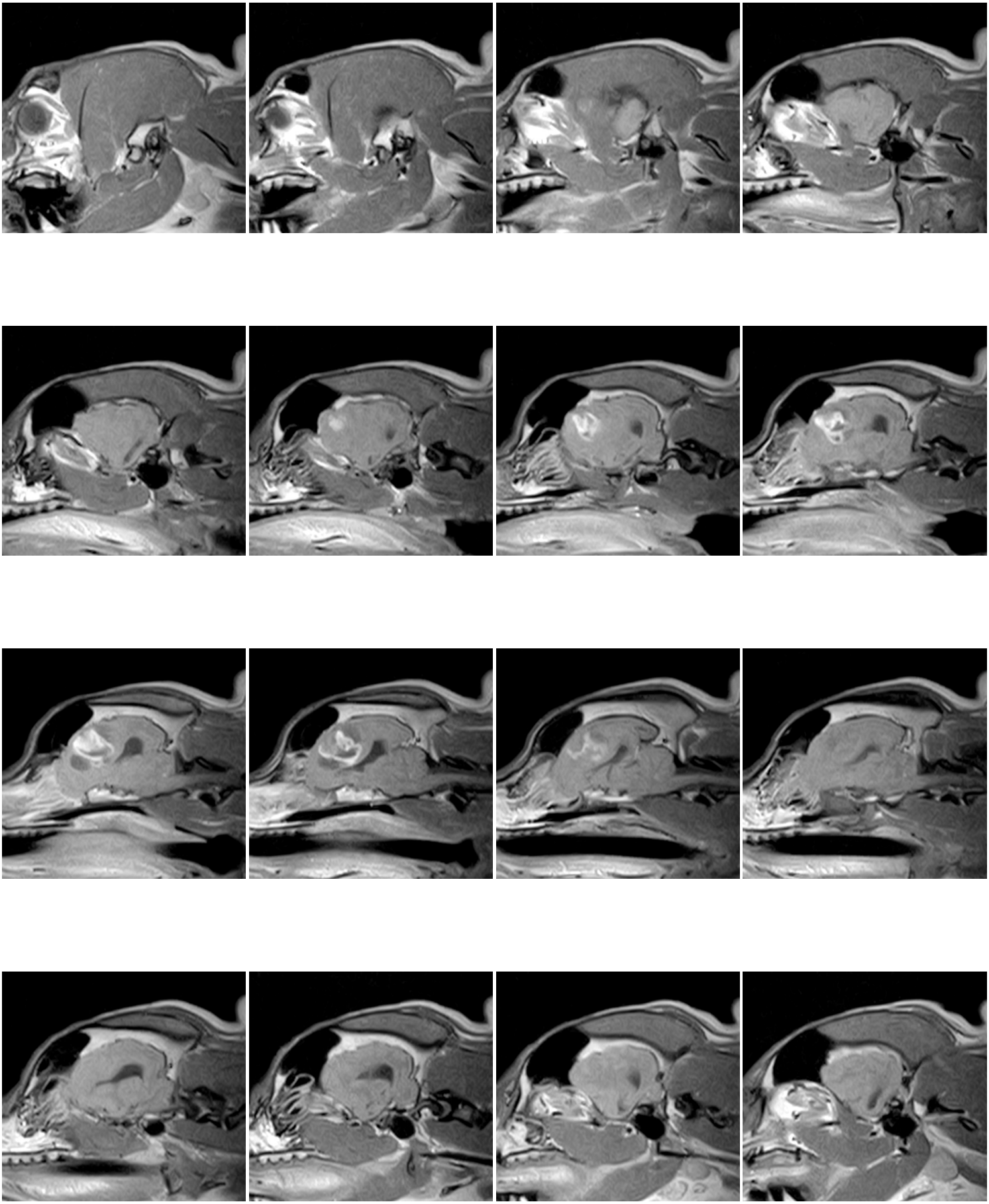
Matheus MRI 5 - **DAY 60** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



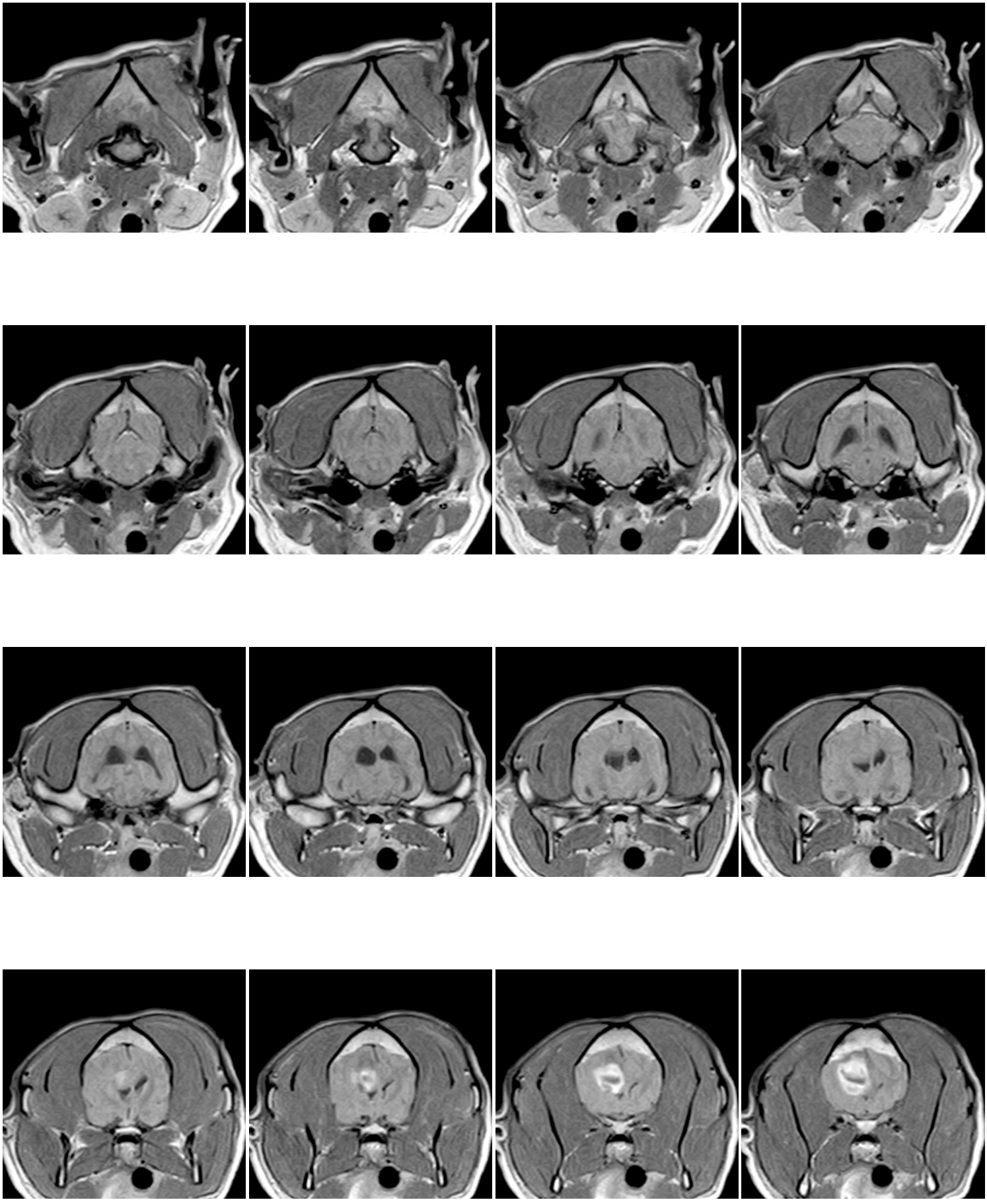
Matheus MRI 6 - **DAY 90** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



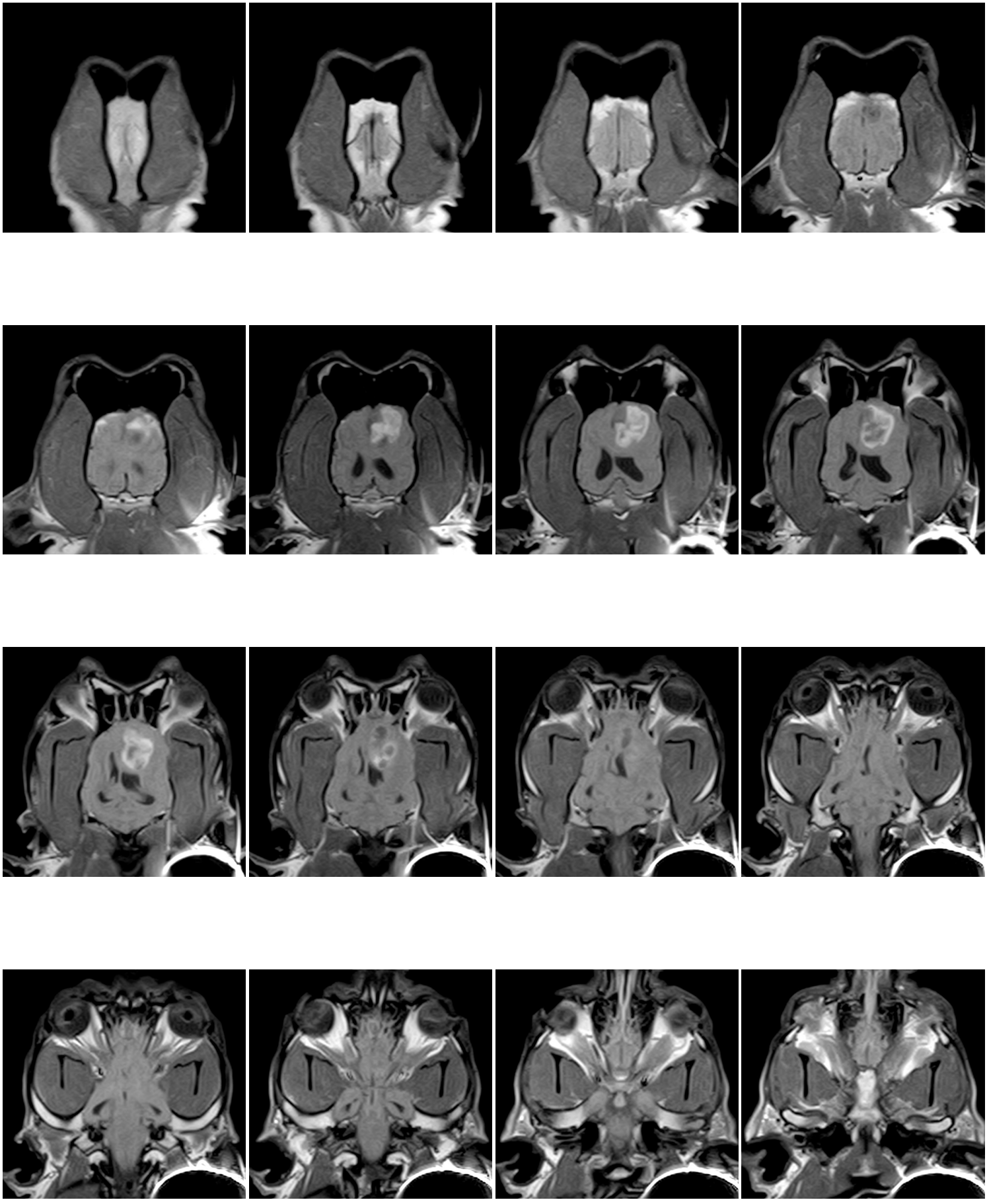
Matheus MRI 6 - **DAY 90** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



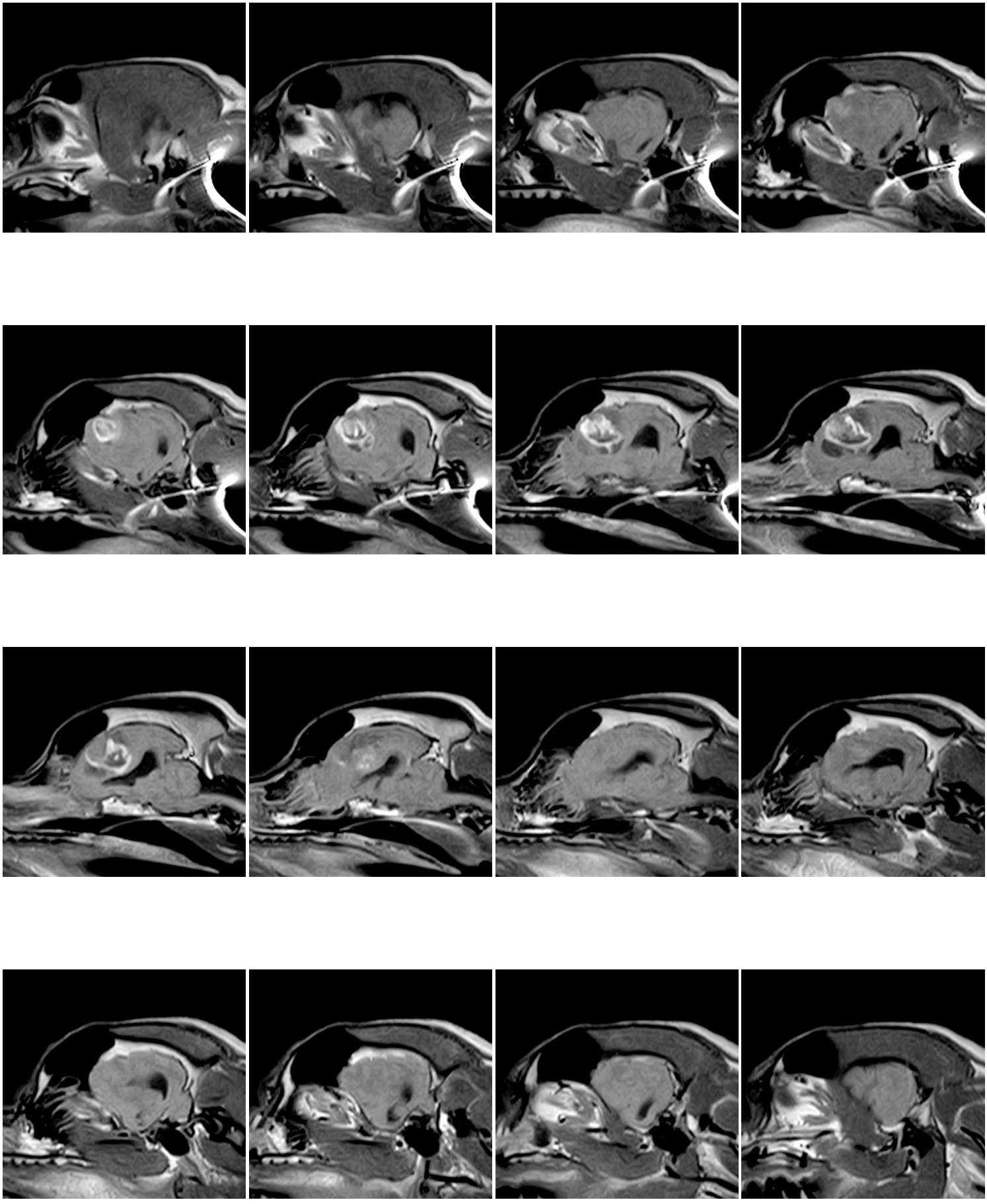
Matheus MRI 6 - **DAY 90** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



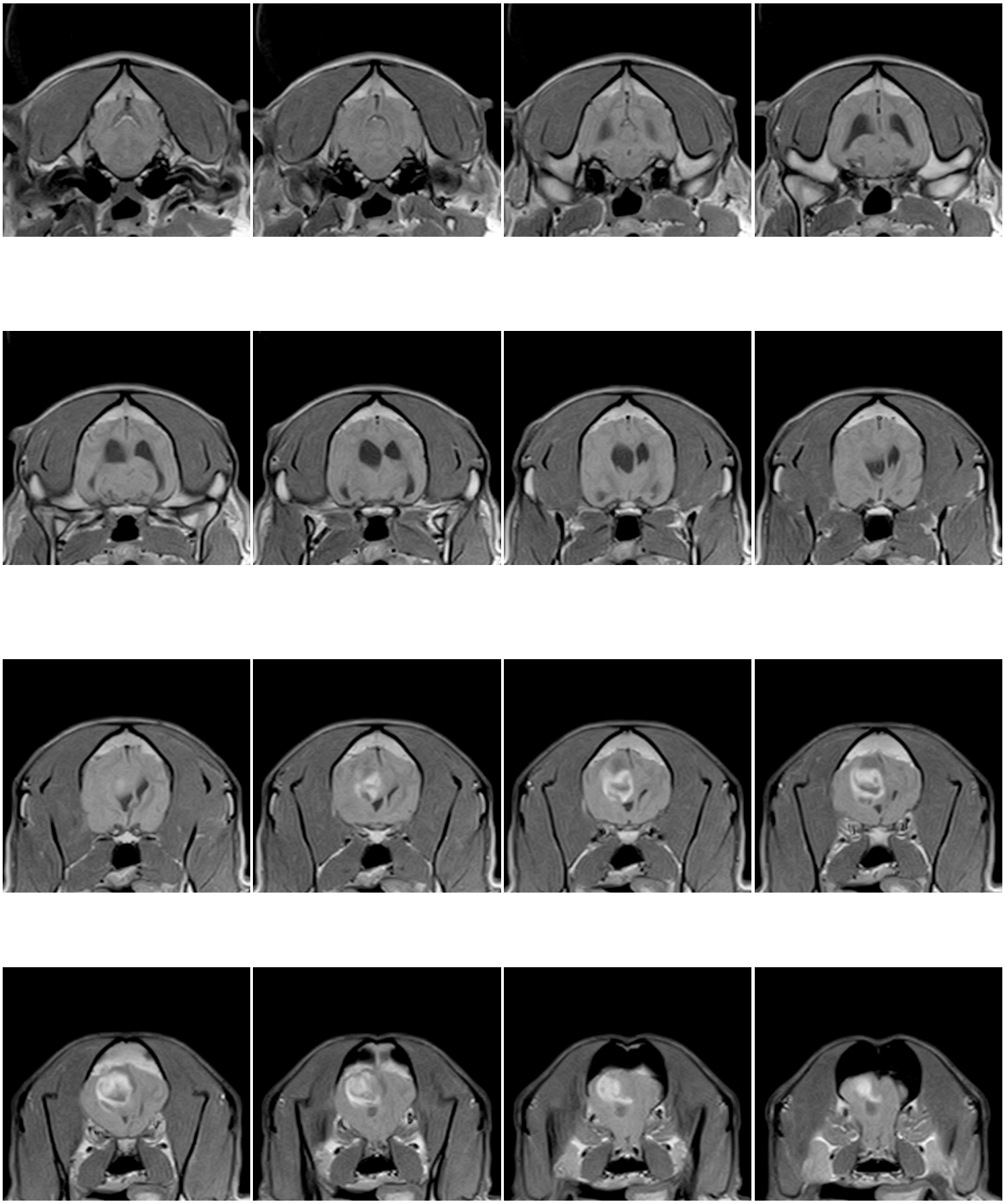
Matheus MRI 7 - **DAY 120** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



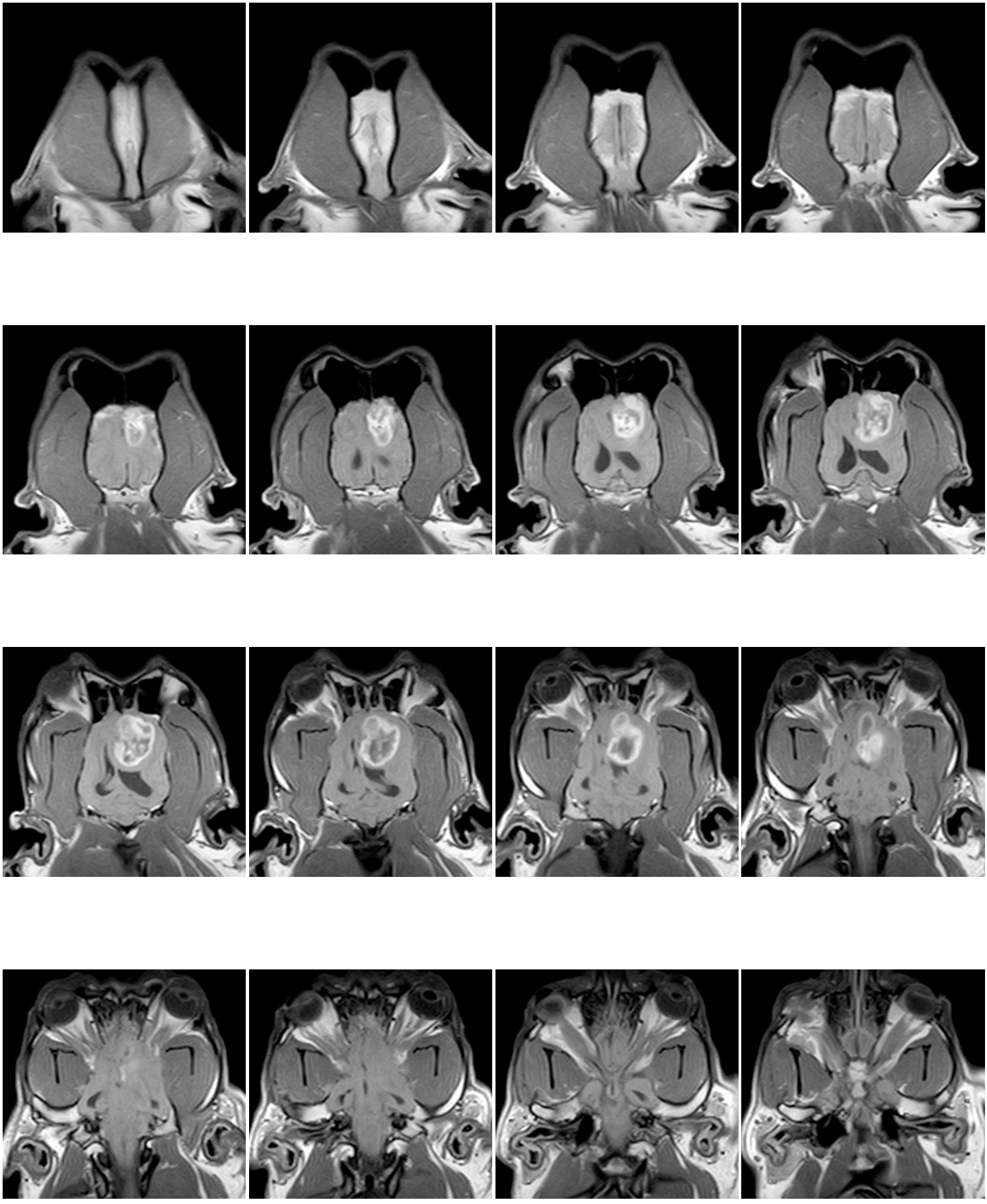
Matheus MRI 7 - **DAY 120** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



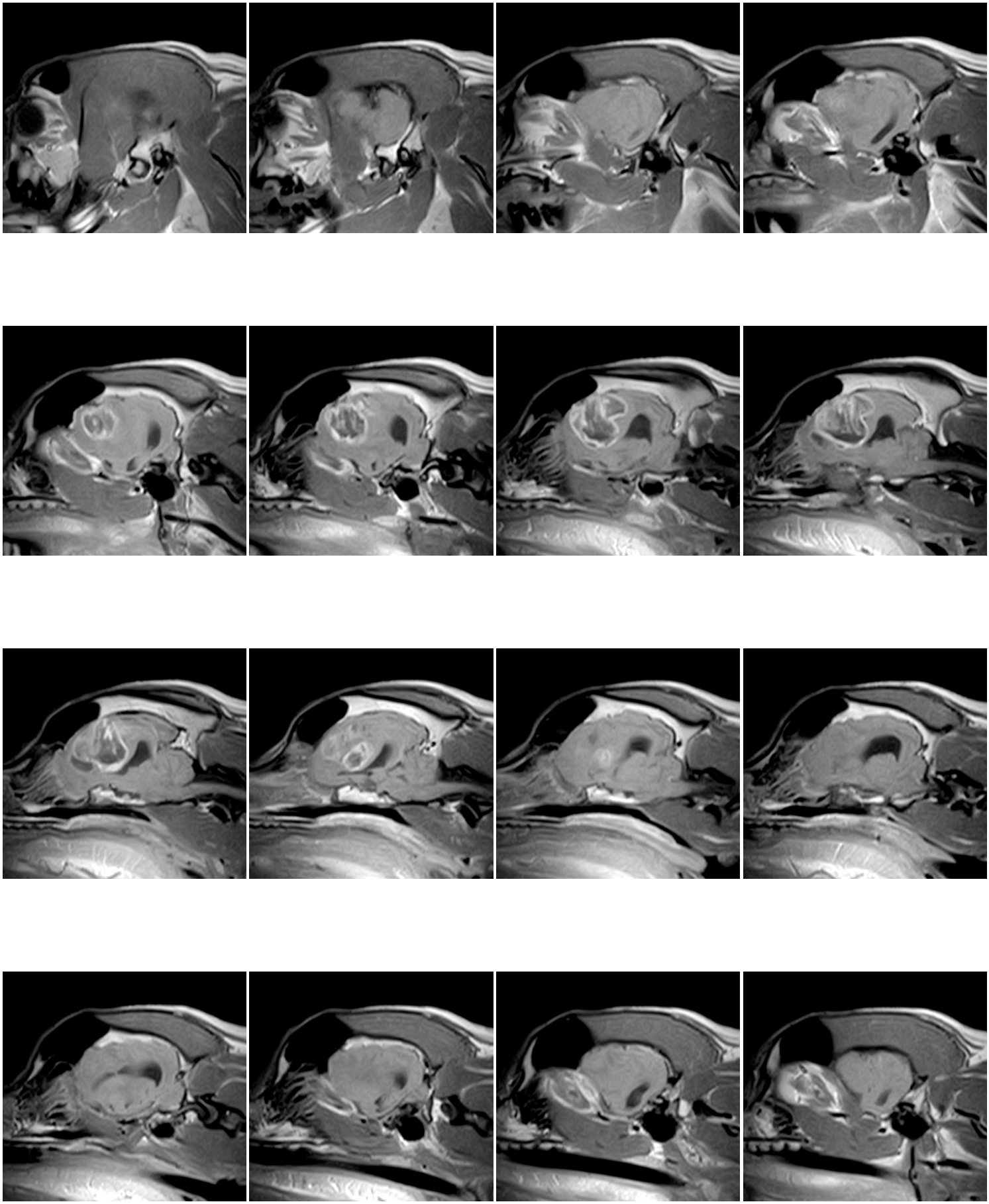
Matheus MRI 7 - **DAY 120** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



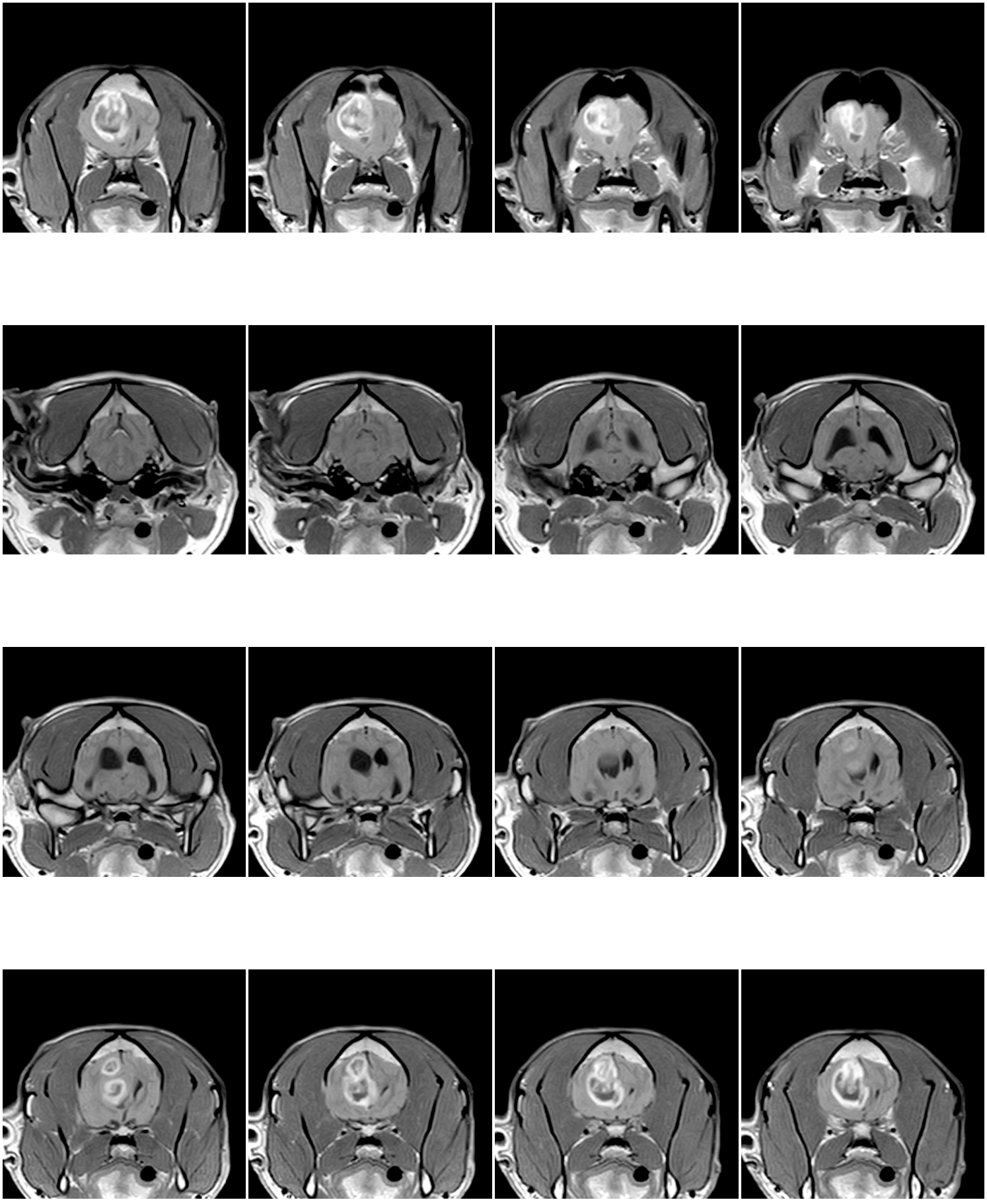
Matheus MRI 8 - **DAY 150** - Post contrast T1 weighted dorsal plane
Patient: Matheus (Boxer dog)



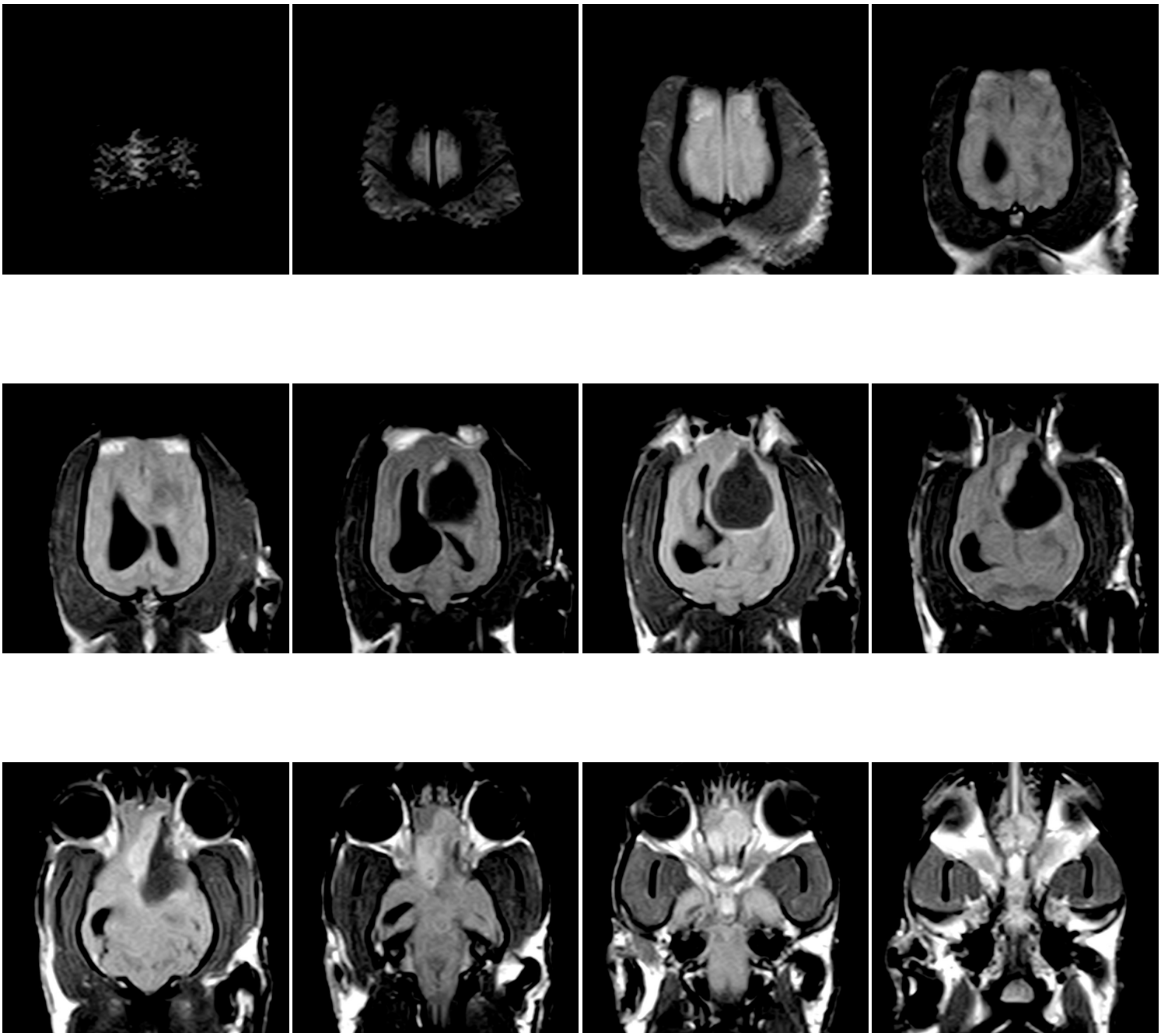
Matheus MRI 8 - **DAY 150** - Post contrast T1 weighted sagittal plane
Patient: Matheus (Boxer dog)



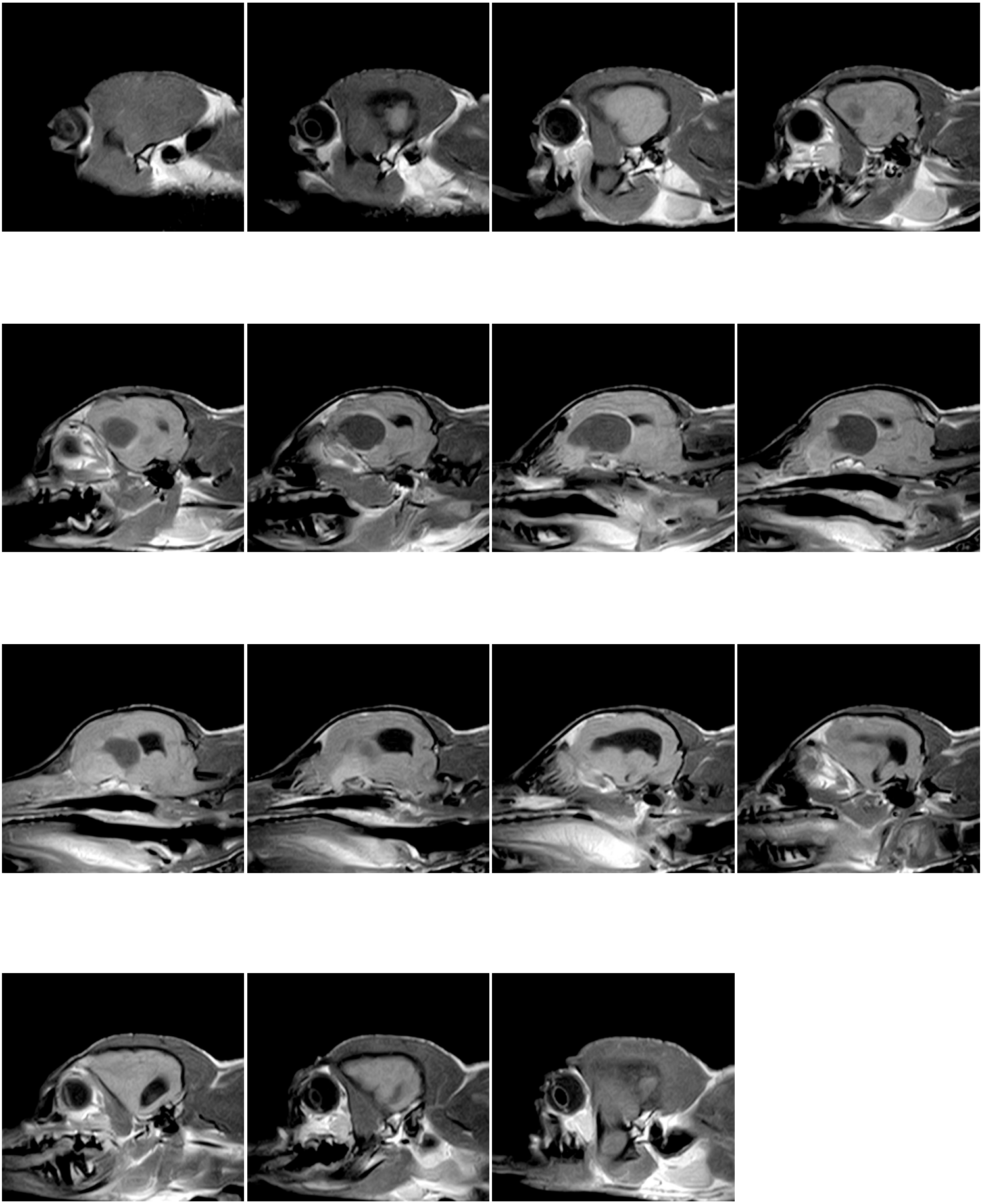
Matheus MRI 8 - **DAY 150** - Post contrast T1 weighted transversal plane
Patient: Matheus (Boxer dog)



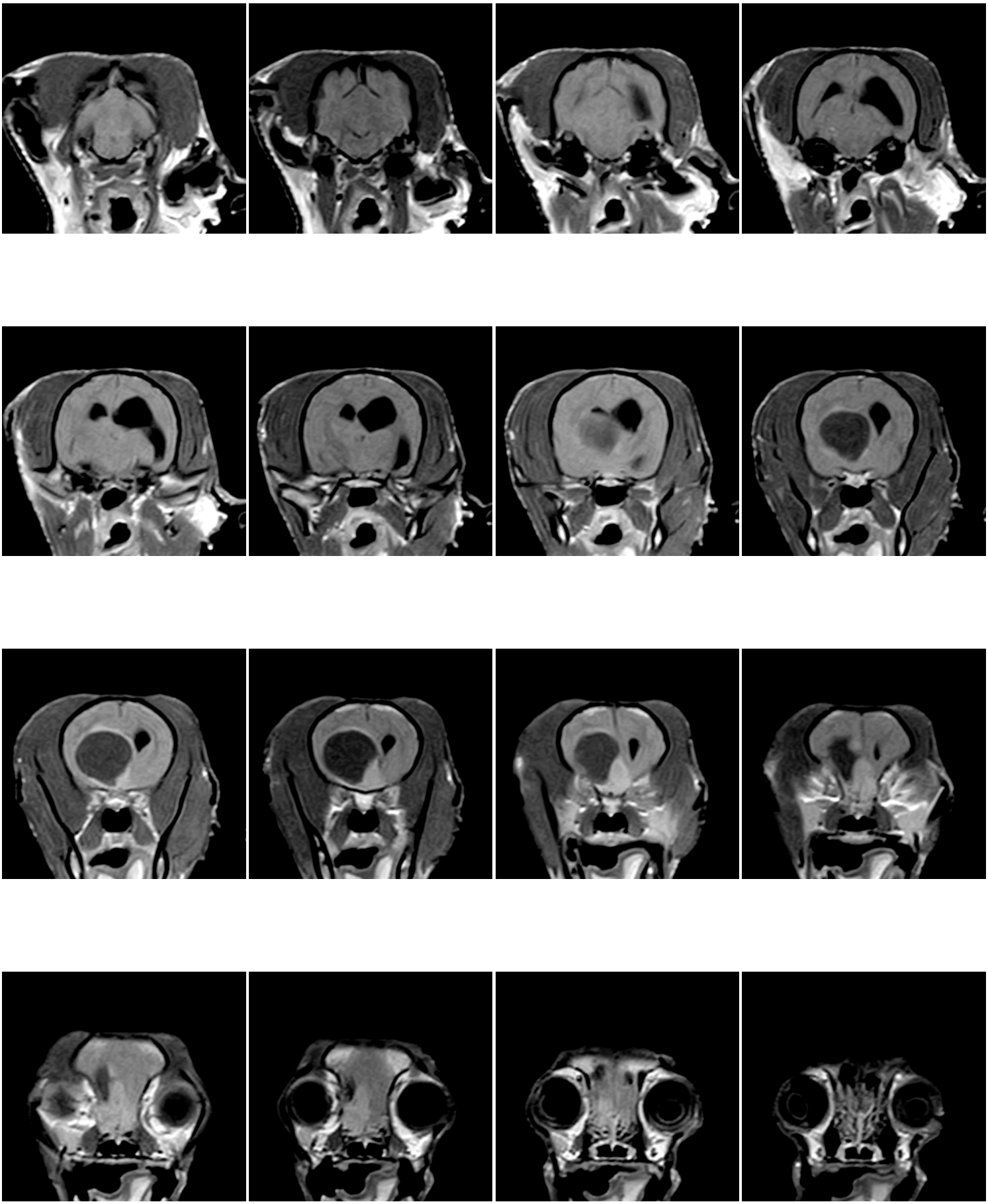
Nina MRI 1 - **DAY 0** - Post contrast T1 weighted dorsal plane
Patient: Nina (Dashchund dog)



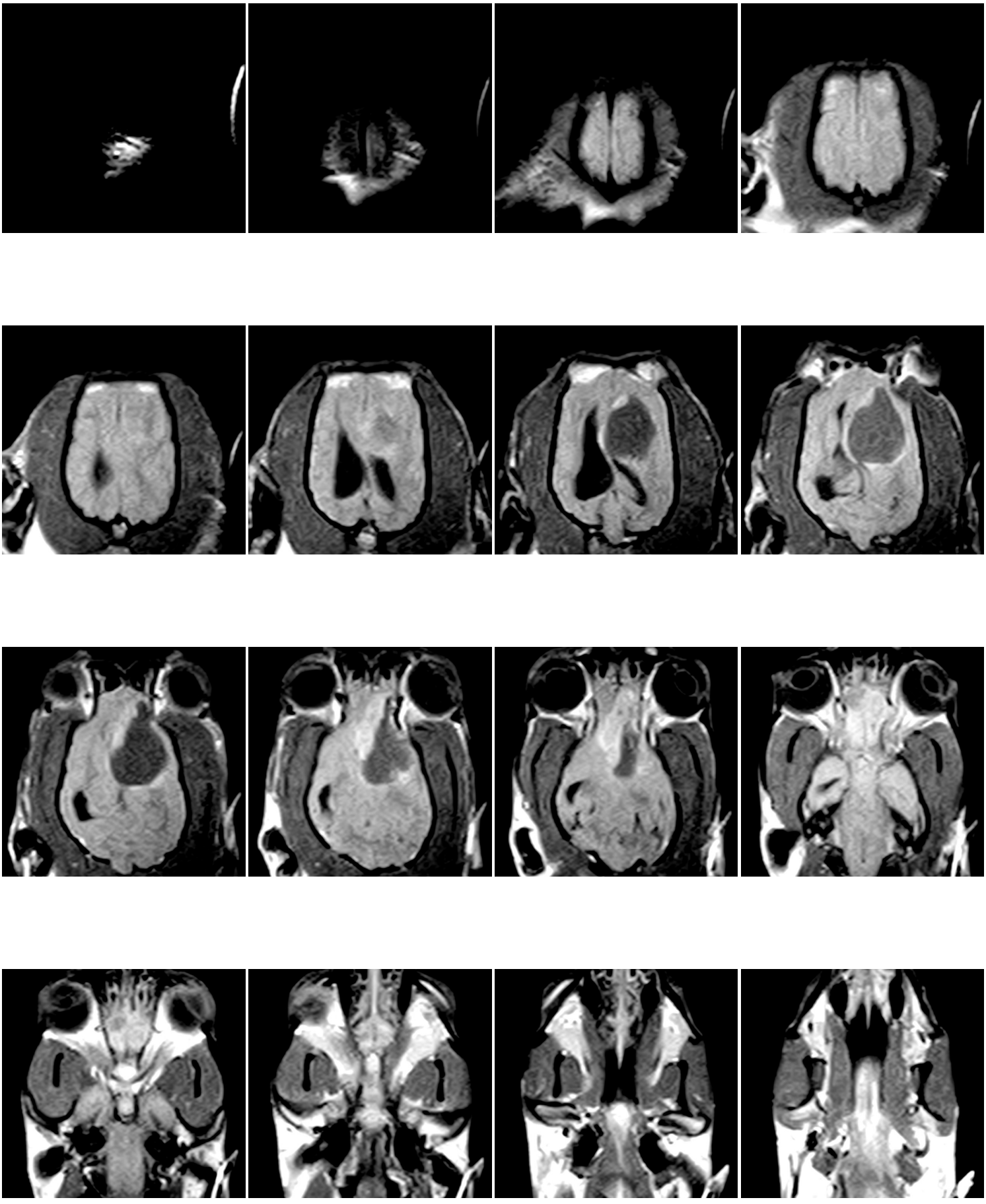
Nina MRI 1 - **DAY 0** - Post contrast T1 weighted sagittal plane
Patient: Nina (Dashchund dog)



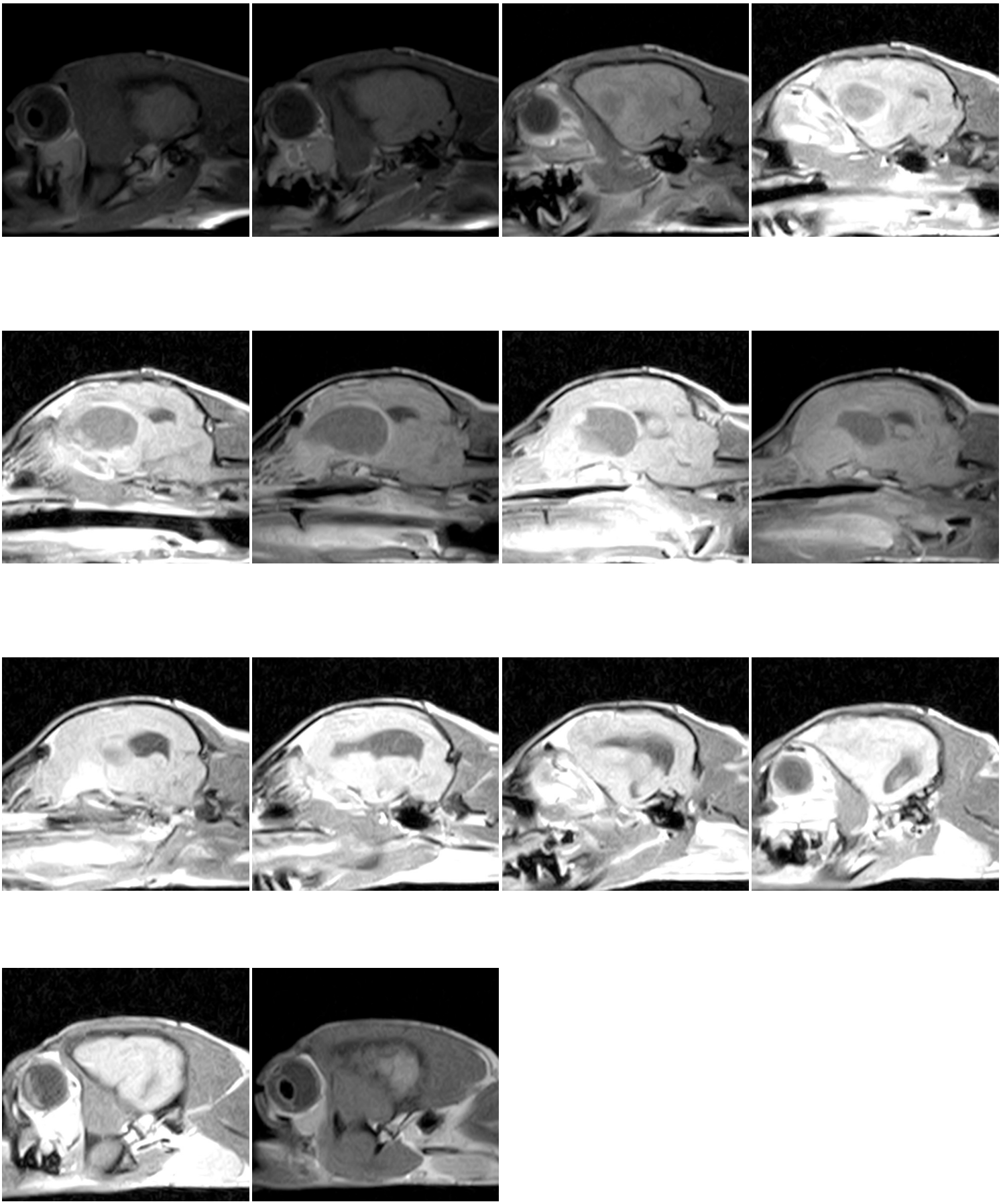
Nina MRI 1 - **DAY 0** - Post contrast T1 weighted transversal plane
Patient: Nina (Dashchund dog)



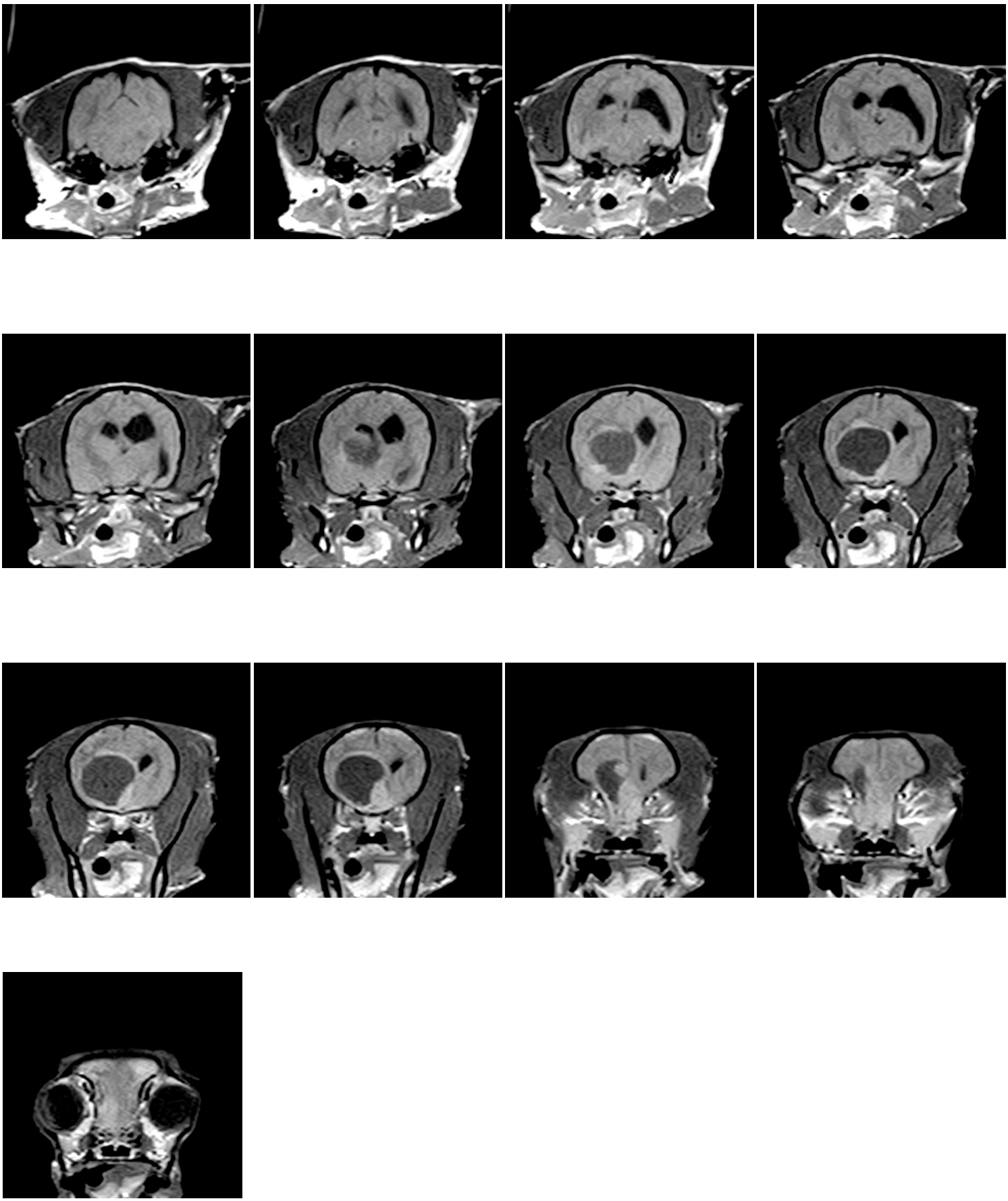
Nina MRI 2 - **DAY 14** - Post contrast T3 weighted dorsal plane
Patient: Nina (Dashchund dog)



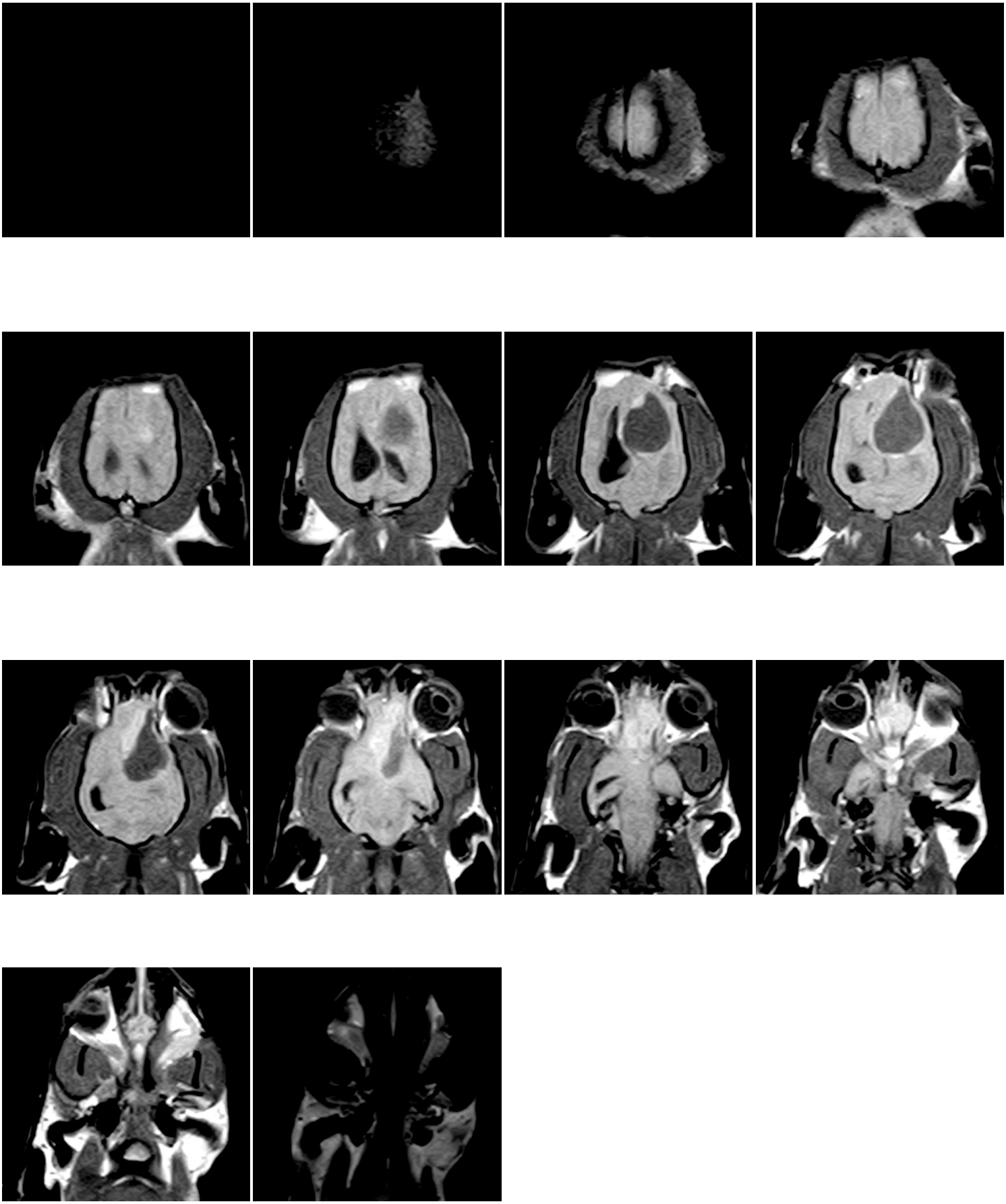
Nina MRI 2 - **DAY 14** - Post contrast T3 weighted sagittal plane
Patient: Nina (Dashchund dog)



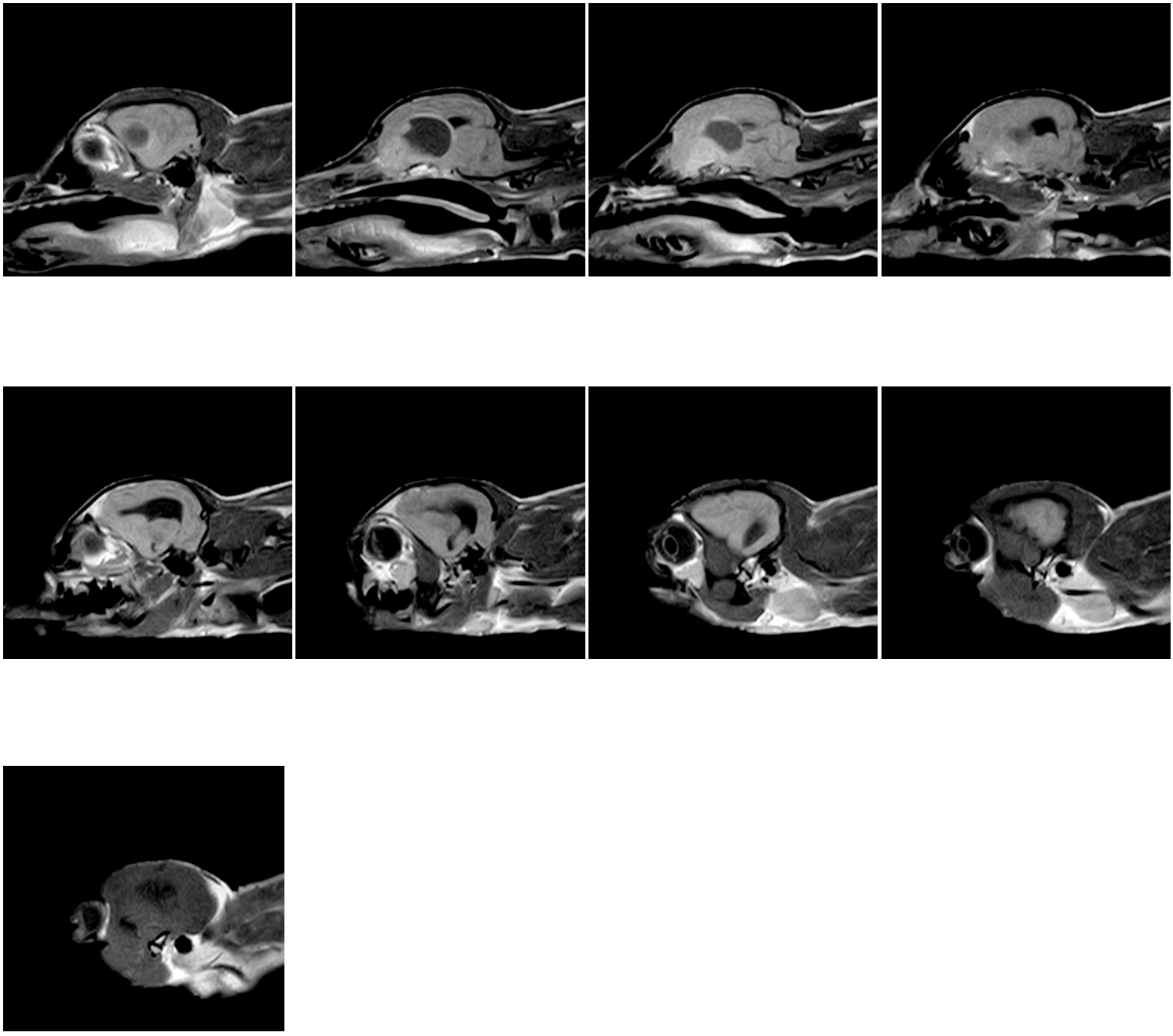
Nina MRI 2 - **DAY 14** - Post contrast T3 weighted transversal plane
Patient: Nina (Dashchund dog)



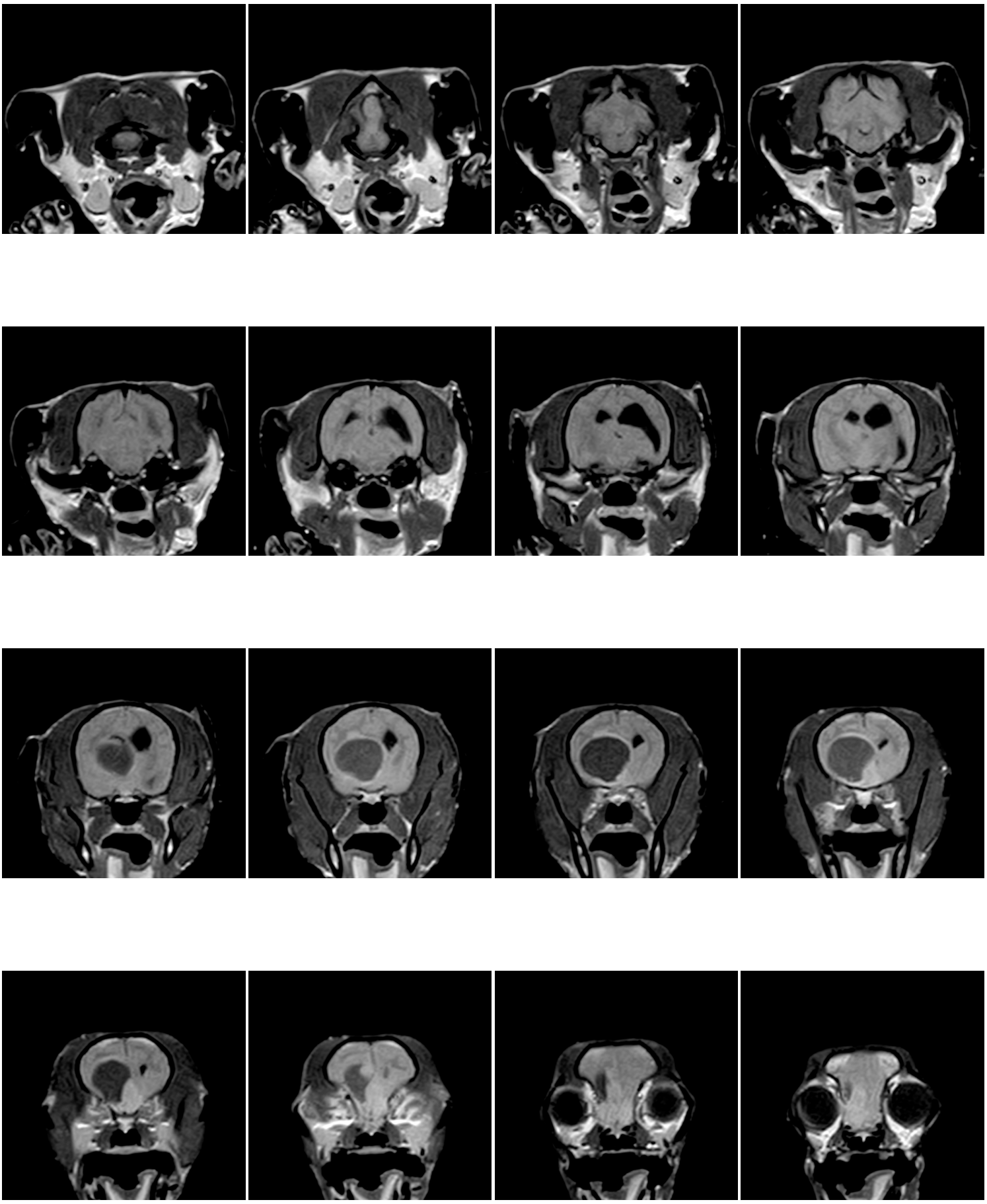
Nina MRI 3 - **DAY 21** - Post contrast T1 weighted dorsal plane
Patient: Nina (Dashchund dog)



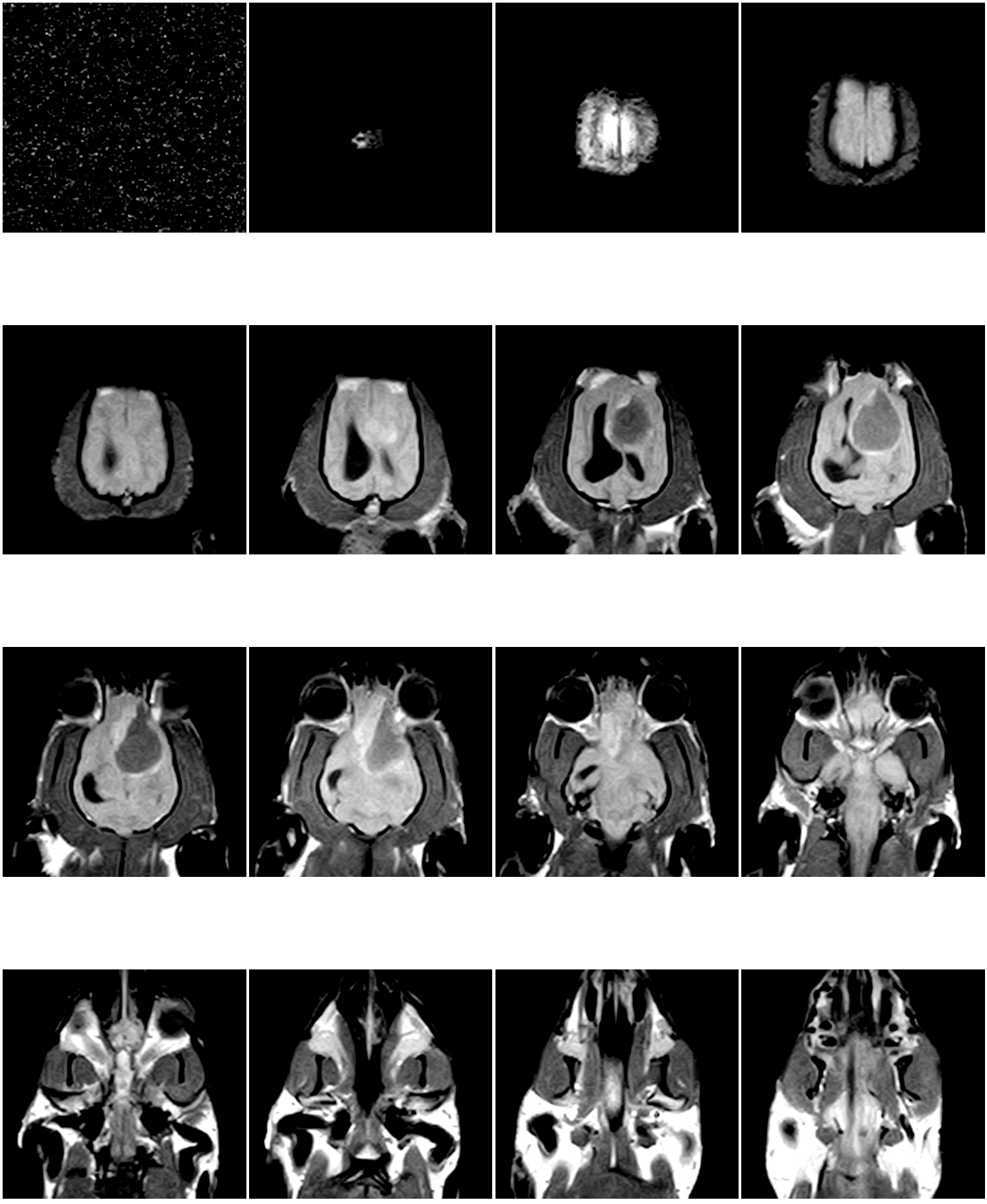
Nina MRI 3 - **DAY 21** - Post contrast T1 weighted sagittal plane
Patient: Nina (Dashchund dog)



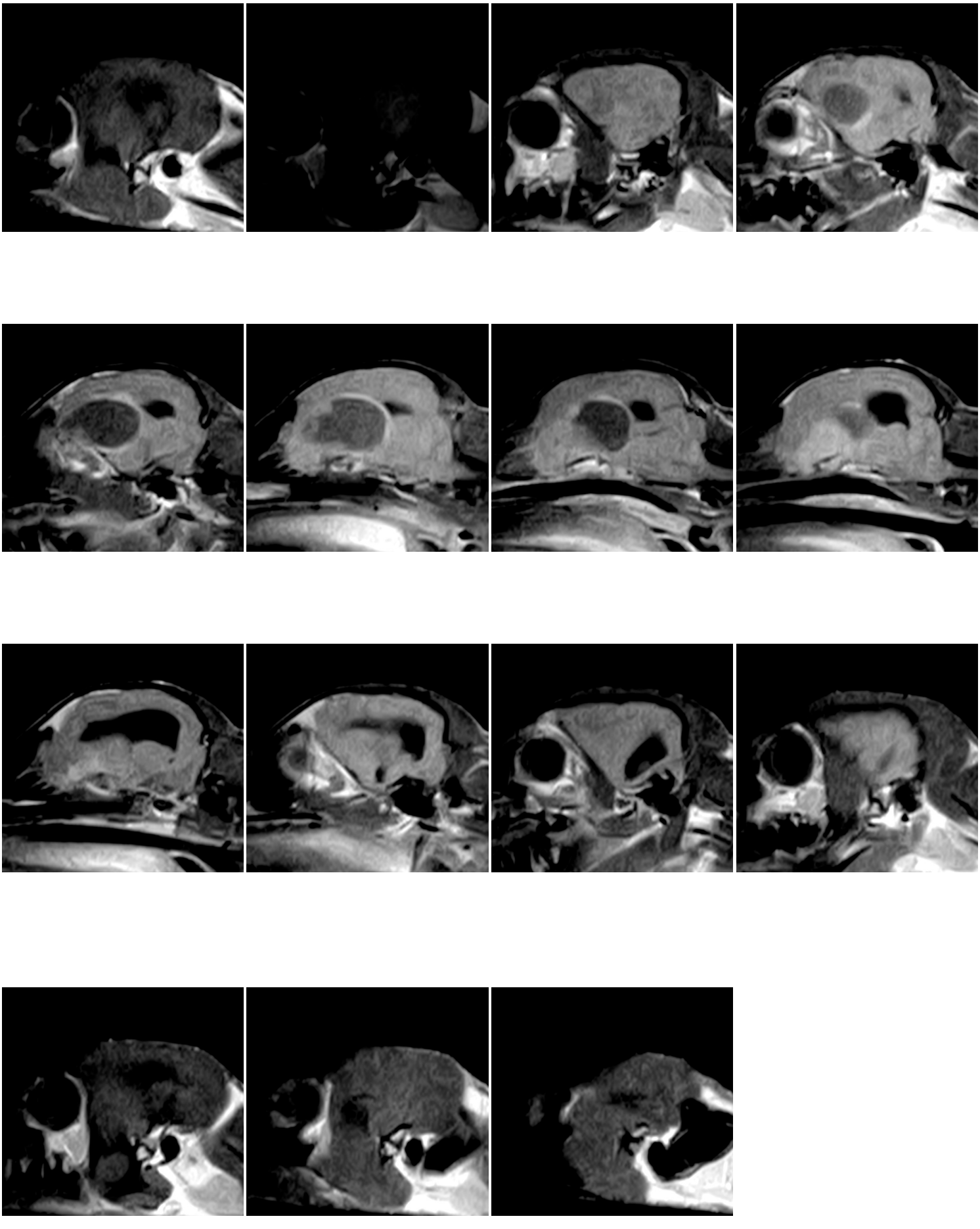
Nina MRI 3 - **DAY 21** - Post contrast T1 weighted transversal plane
Patient: Nina (Dashchund dog)



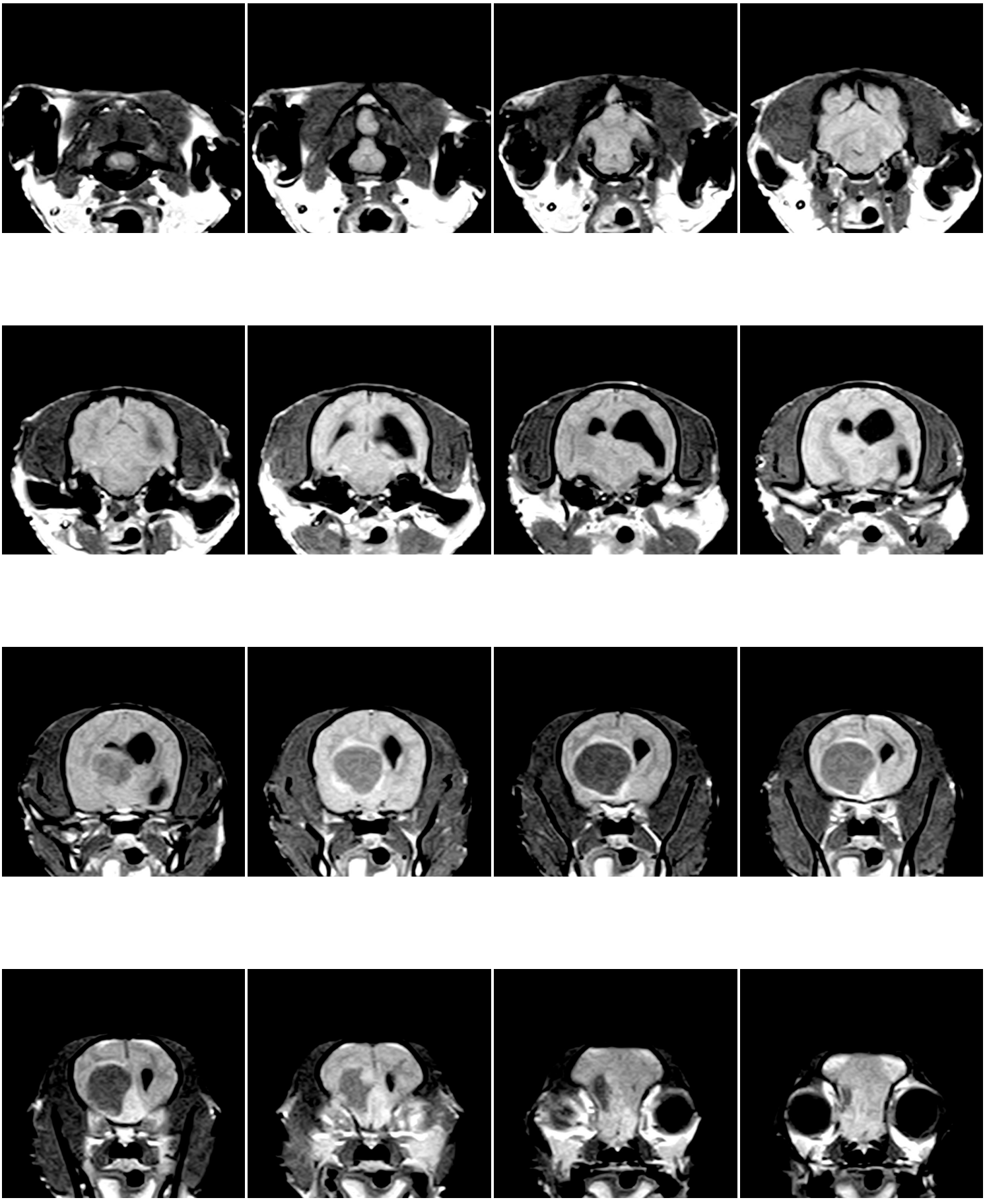
Nina MRI 4 - **DAY 42** - Post contrast T1 weighted dorsal plane
Patient: Nina (Dashchund dog)



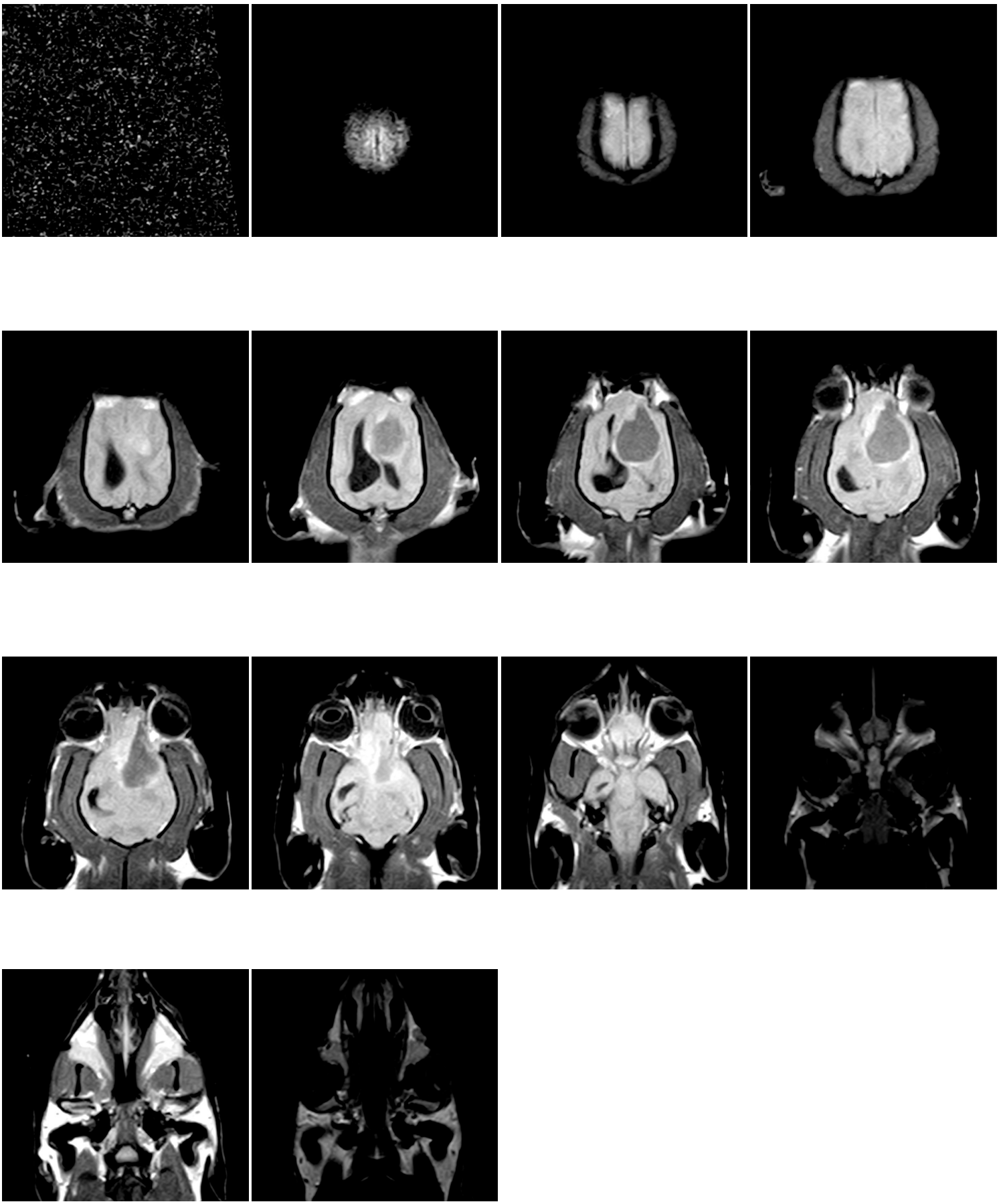
Nina MRI 4 - **DAY 42** - Post contrast T1 weighted sagittal plane
Patient: Nina (Dashchund dog)



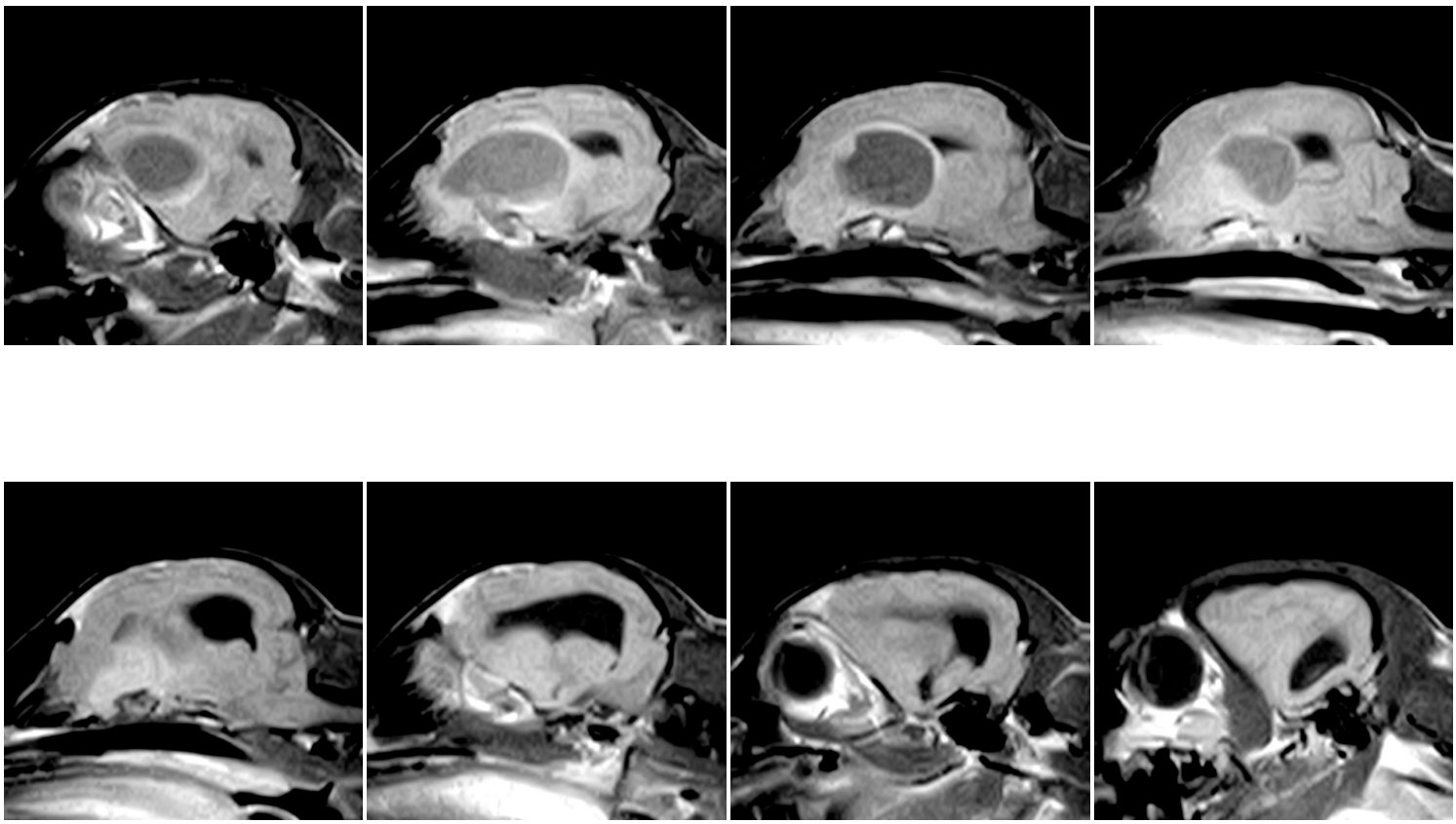
Nina MRI 4 - **DAY 42** - Post contrast T1 weighted transversal plane
Patient: Nina (Dashchund dog)



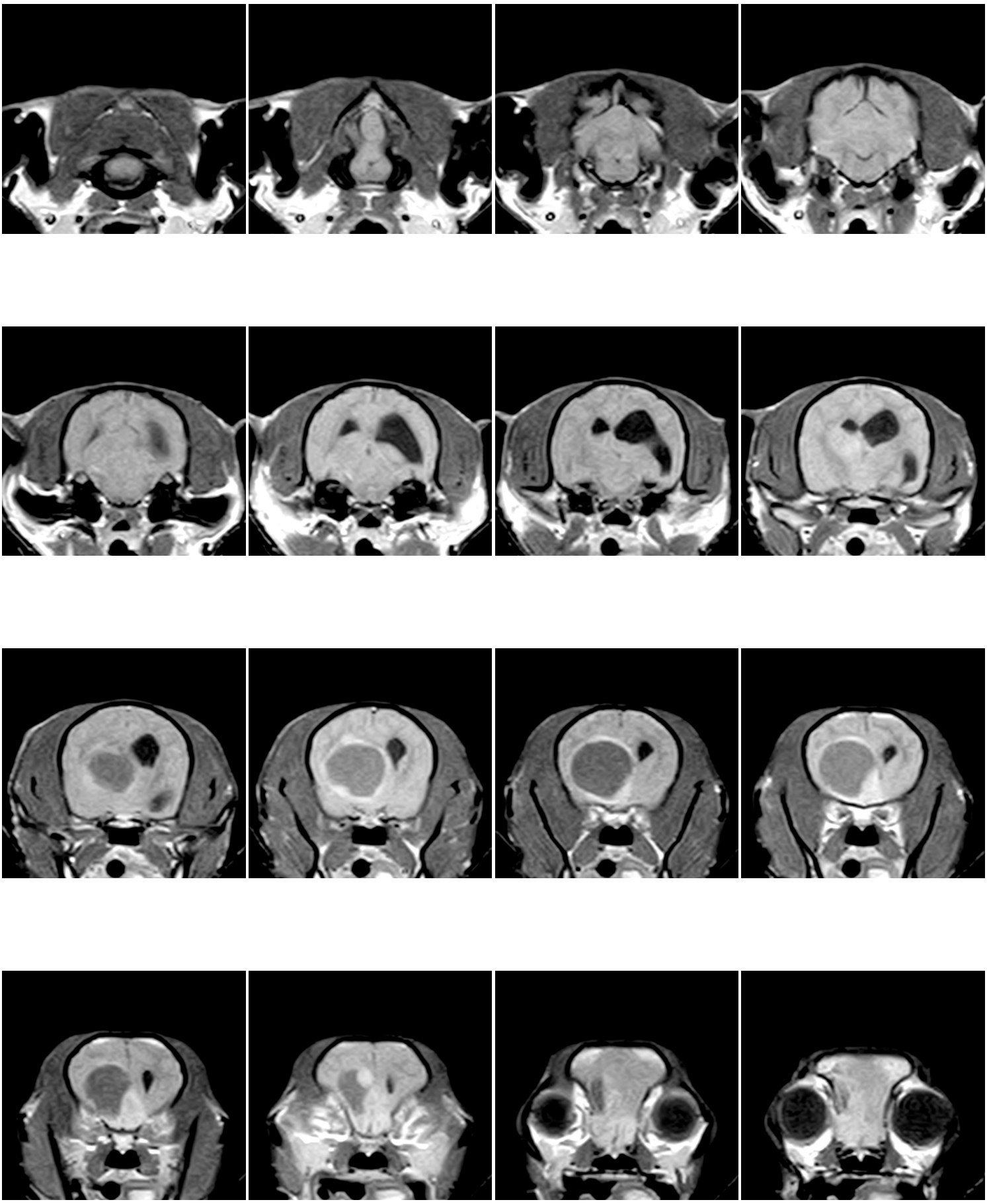
Nina MRI 5 - **DAY 52** - Post contrast T1 weighted dorsal plane
Patient: Nina (Dashchund dog)



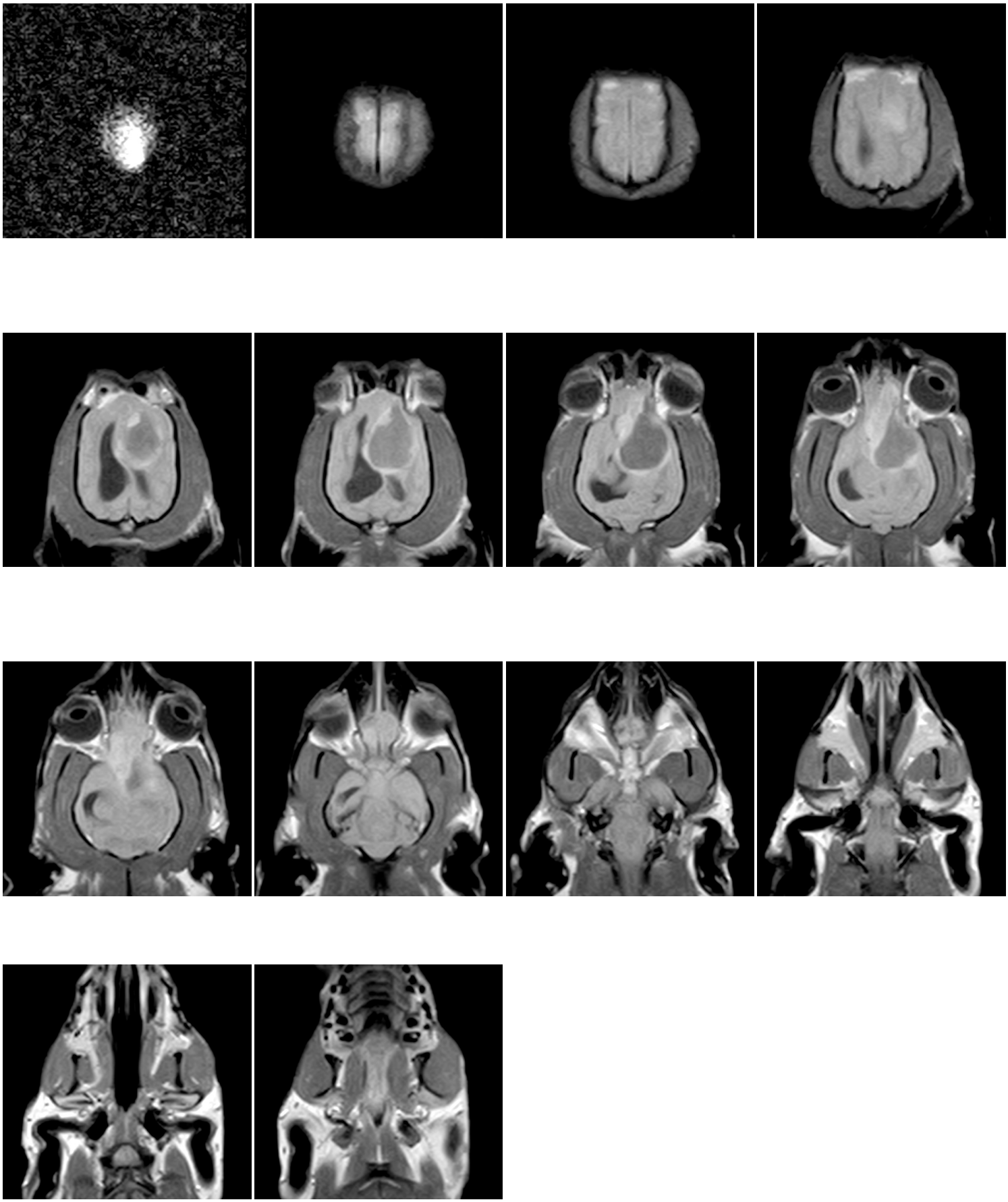
Nina MRI 5 - **DAY 52** - Post contrast T1 weighted sagittal plane
Patient: Nina (Dashchund dog)



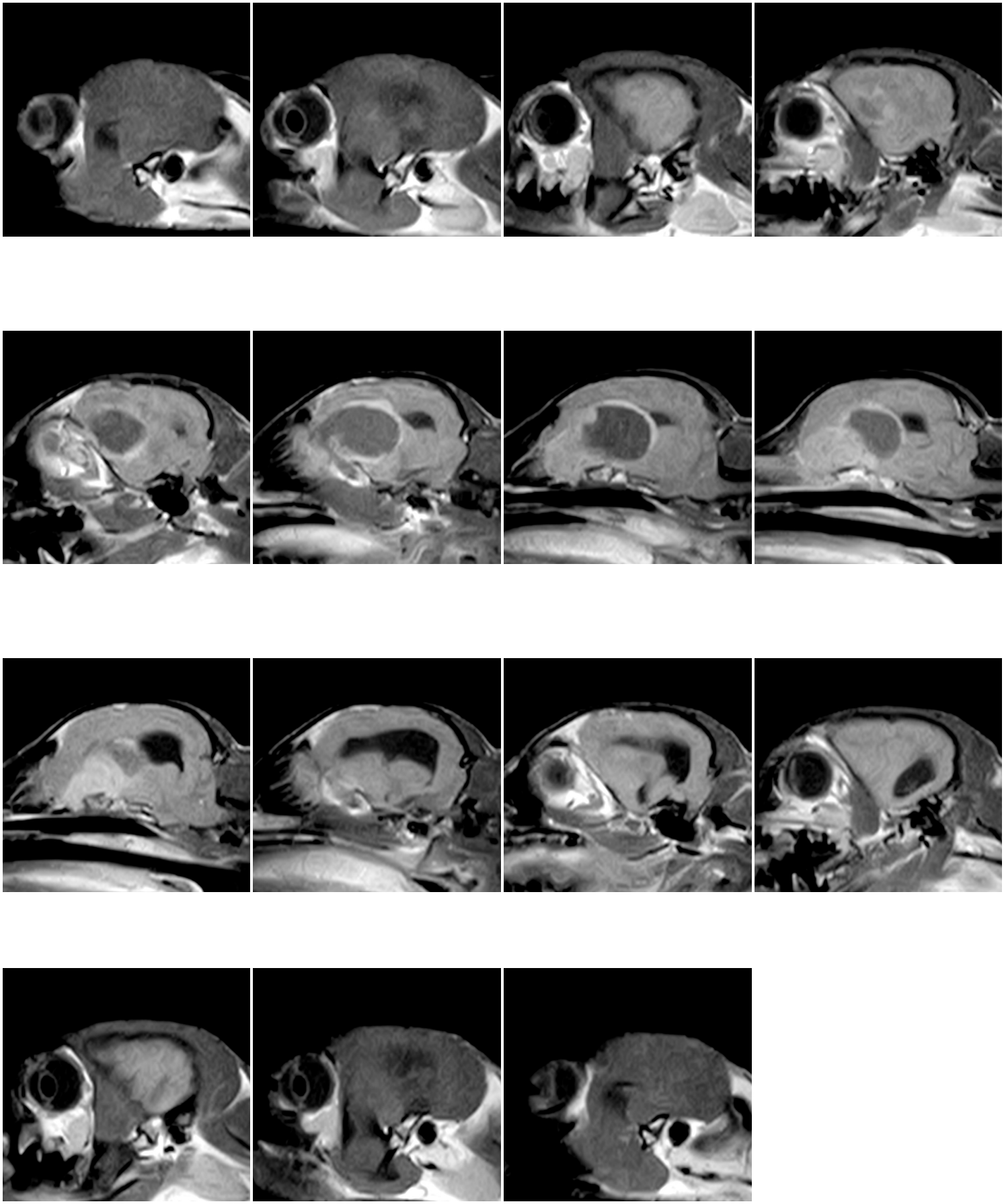
Nina MRI 5 - **DAY 52** - Post contrast T1 weighted transversal plane
Patient: Nina (Dashchund dog)



Nina MRI 6 - **DAY 78** - Post contrast T1 weighted dorsal plane
Patient: Nina (Dashchund dog)



Nina MRI 6 - **DAY 78** - Post contrast T1 weighted sagittal plane
Patient: Nina (Dashchund dog)



Nina MRI 6 - **DAY 78** - Post contrast T1 weighted transversal plane
Patient: Nina (Dashchund dog)