

## **Supplemental Methods**

### **Brief detailed methods in plasmid and MB preparation**

Large preparation of the plasmids was carried out by Aldevron (Fargo, North Dakota, USA) using standard industry techniques. Briefly, 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1, 2-distearoyl-sn-glycero-3-phosphate (DSPA), and N-(carboxymethoxypolyethyleneglycol 5000-DSPE) are mixed at a molar ratio of 82:10:8 (Avanti Polar Lipids, Alabaster, AL, USA). Lipids were then reconstituted, and gas exchange was performed to fill the headspace of each vial with octafluoropropane gas (American Gas Group, Toledo, OH, USA). MB size and concentration were measured using the qNano instrument with a NP2000 membrane (Izon Science, Christchurch, NZ). Immediately prior to use, MBs were activated by vigorous agitation for 45 seconds using a Vialmix™ (Lantheus Medical Imaging, N. Billerica, MA, USA).

### **Detailed method of catheter insertion in canine surgery**

Under general anesthesia, the access to the right jugular vein was established by puncturing the vein with a 21G needle (Cook Medical, Bloomington, IN, USA) under US imaging guidance (X-Porte; FUJIFILM Sonosite, Bothell, WA, USA). A 0.018 guidewire is passed through the needle and inserted into the jugular vein. After removing the needle, the guidewire was left in the vessel. The microcatheter dilator/introducer sheath of the micropuncture set (Cook Medical) was placed into the jugular vein over the 0.018 guidewire. The central stiffening dilator was unlocked, and removed simultaneously with the wire, leaving the 4-French introducer sheath in the vein, through which a 0.035-inch guidewire was inserted into the jugular vein. After removing the 4-French introducer

sheath, an 8-French sheath (Boston Scientific) was placed into the vein and sutured in place. Under the guidance of fluoroscopy (OEC 9900 Elite C-arm X; GE Healthcare, Little Chalfont, UK), through the 8-French sheath, a 5-French NIH angiographic catheter (Cook Medical) was placed into the left or middle hepatic vein. Through the 5-French catheter, a Back-Up Meier C-Tip guidewire (Boston Scientific, Marlborough, MA) was inserted for introducing a balloon catheter (20.0-mm length and 12.0-mm diameter). X-ray contrast agent (Visipaque; GE Healthcare) was injected to verify the position of the catheter. The balloon was inflated with x-ray contrast agent (Visipaque, GE Healthcare, Chicago, IL) to completely and temporarily occlude blood flow under fluoroscopy guidance (OEC 9900 Elite C-arm X, GE Healthcare, Little Chalfont, UK), which ensured the microbubble plasmid solution was pushed into the hepatic sinusoids under a constant pressure on the plunger of a 50-ml syringe.

### **Detailed method on FVIII expression analysis by ELISA**

Whole blood was collected from canines at multiple time points to isolate plasma. ELISA plates were coated with ESH-8 as the primary antibody (Biomedica Diagnostics, Windsor, NS Canada) diluted 1: 250 in 0.1M NaHCO<sub>3</sub> with NaOH (pH 9.5) and incubated overnight at 4 degrees Celsius. The plate was blocked 2 hours in 5% nonfat milk in Tris-Cl buffered saline (pH 7.5). The standard curve was made using a serial dilution of recombinant human FVIII in normal dog plasma (NDP). Plasma samples from each dog were run in triplicate or quadruplicate, and samples were incubated overnight at 4 degrees Celsius. The secondary antibody, biotinylated GMA- 8015 (Green Mountain Antibodies, Burlington, VT), diluted 1:500 in blocking buffer was added for 1.5 hours

incubation followed by HRP-Avidin diluted 1:250 in =blocking buffer for 2 hours and detected using K blue substrate. by Victor3™ plate reader (Perkin Elmer, Wellesley, MA).

### **Detailed method on the RNAscope® 2.5 High Definition (HD)—Red Assay**

Selected liver tissue sections were flash frozen in Optimal Cutting Medium. Seven-micron sections were produced by sectioning on the Leica CM3050S Research Cryostat and mounted on slides. These slides were subjected to the RNAscope® Red Assay according to protocols from ACD as follows. Sections were dehydrated in 50%, 70%, and 100% ethanol and stored in 100% ethanol overnight. Hydrogen peroxide (ACD, Newark, CA) was added for 10 minutes, then protease inhibitor IV (ACD) was added for 30 minutes at RT. Positive control probe (binds *Canis lupus familiaris* peptidylprolyl isomerase B (cyclophilin B) (PPIB) transcript variant 2 mRNA, a highly expressed protein in canine tissue), negative control probe (binds dihydrodipicolinate reductase (dapB) gene, a protein found in bacteria such as *E.coli*), and custom-designed experimental probe that specifically binds hF8-X10 mRNA, all made by ACD, were incubated on slides for 2 hours at 40° C. Slides were then exposed to the amplification series, AMP 1-6 (ACD) with alternating incubations of 15 and 30 minutes at 40 degrees Celsius until AMP 5, done at RT. Afterwards, slides were stained using fastRedA (ACD) for 10 minutes, rinsed and counterstained with 50% hematoxylin followed by 0.002% Ammonia, and imaged using a standard bright field microscope.

### **Detailed method in plasmid copy number analysis**

Total DNA was extracted from canine liver tissue using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and digested with 100 mg/mL RNase A (Macherey Nagel

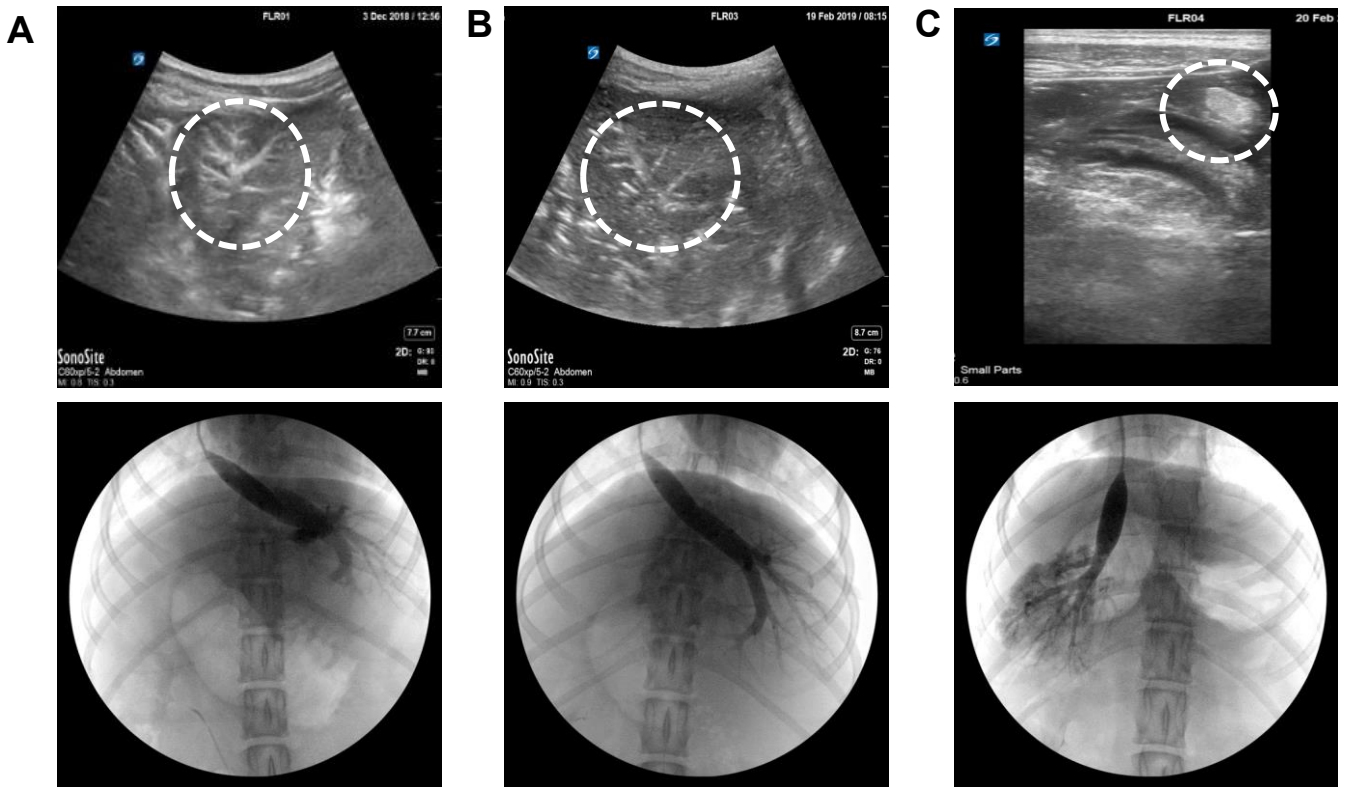
Inc., Duren, Germany). Extracted DNA concentration was measured using a Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA). qPCR was performed according to manufacturer specifications, except that the annealing/extension step was reduced to 30 seconds per cycle to reduce non-specific amplification. DNA from an un-transfected lobe was used as a negative control. Plasmid DNA diluted in control canine genomic DNA were used to establish a standard curve. Plasmid copy numbers were determined by qPCR using a Step One Plus Real-Time PCR System (Applied Biosystems, Waltham, MA) and Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA). FVIII primers were designed using the Primer-Blast (National Institutes of Health) including FVIII (forward) GACCCACCGTTACTGACTCG, FVIII (reverse) TCGGCTCGAGGATCAATTCG . GAPDH was chosen as a control housekeeping gene and its primer sequences were based on Song et al.<sup>23</sup> including GADPH (forward) TCCACCCACGGCAAATTC, and GADPH (reverse) GCATCACCCCATTTGATGTTG. Primer specificity to FVIII gene was confirmed using restriction enzyme (ApaLi) digest of DNA amplified by PCR with PTC-200 Peltier Thermal Cycler (MJ Research).

### **Statistical Methods for analyzing PCR data**

Data from dogs treated with the same transducer and ultrasound conditions was pooled. FVIII copy numbers from all sections in a lobe were averaged to obtain lobe averages. Treated lobes were defined as those that had a significantly higher copy number per ng than the normal dog genomic DNA used as a negative control. All other lobes were defined as untreated. The lobe average distribution was tested for fit to a Gaussian curve using the D'Agostino-Pearson omnibus normality test with a confidence interval of 95%. Not all data passed the normality test. Thus, a Kruskal-Wallis test for non-

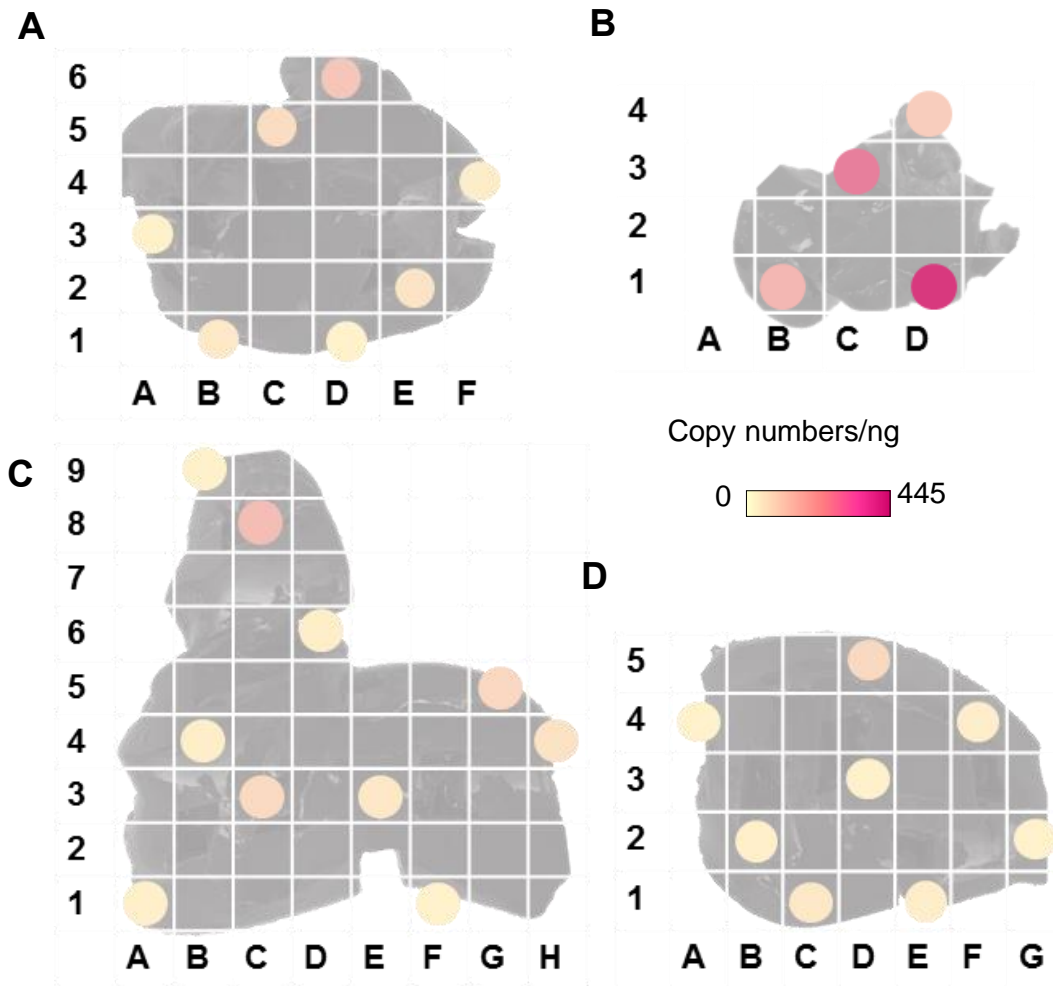
normally distributed data was applied to determine which lobes were treated and which were not.

## Supplemental Figure 1



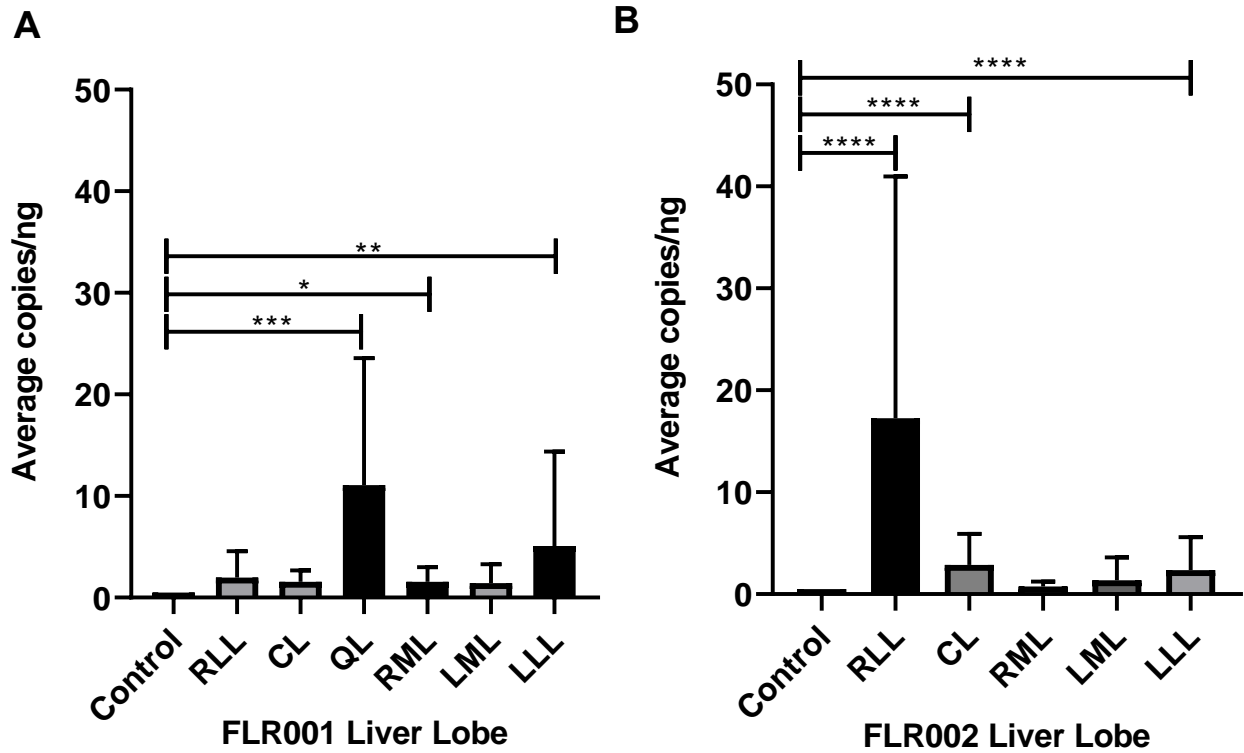
**Supplementary figure 1.** *Additional fluoroscopy and diagnostic ultrasound images.* For each canine, one representative diagnostic ultrasound image (top row) and one representative fluoroscopy image (bottom row) is shown. Microbubble dispersion is circled in white for diagnostic ultrasound images, with vascular structure visible in images from FLR001 (A) and FLR003 (B), both showing the left lateral lobe. Diagnostic US image and fluoroscopy image from treatment of the right medial lobe are shown for FLR004 (C).

## Supplemental Figure 2



**Supplementary figure 2. Plasmid Copy Number Distribution In FLR004 Liver Lobes.** FLR004 lobes: the right lateral lobe (A), the quadrate lobe(B), the right medial lobe (C), and the left medial lobe (D). Distribution maps show plasmid copy numbers per nanogram in transfected FLR004 liver lobes which were sectioned according to an alphanumeric grid upon necropsy. Lighter colors indicate lower plasmid copy number, and darker colors indicate higher plasmid copy number. The right lateral, quadrate, and right medial lobes are untreated lobes, and the quadrate lobe was a treated lobe.

### Supplemental Figure 3



**Supplemental Figure 3.** Average Plasmid Copy Number in FLR001 and FLR002. Average plasmid copy number per nanogram of genomic DNA is plotted on a bar graph, showing all lobes and untreated control canine genomic DNA (control). The right lateral lobe (RLL), caudate lobe (CL), quadrate lobe (QL), right medial lobe (RML), left medial lobe (LML), and left lateral lobe (LLL) are shown. Treated lobes are shown in black, while untreated lobes are shown in grey. Error bars indicate standard deviation.