
Supplementary information

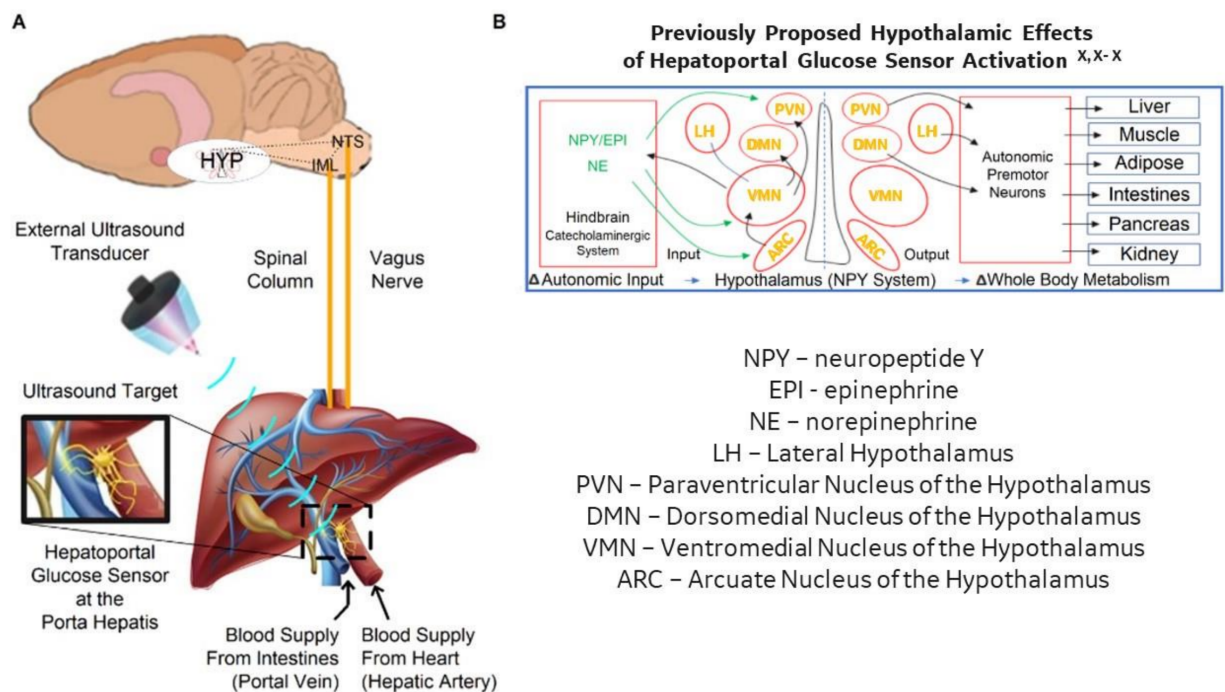
**Stimulation of the hepatportal nerve
plexus with focused ultrasound restores
glucose homoeostasis in diabetic mice, rats
and swine**

In the format provided by the
authors and unedited

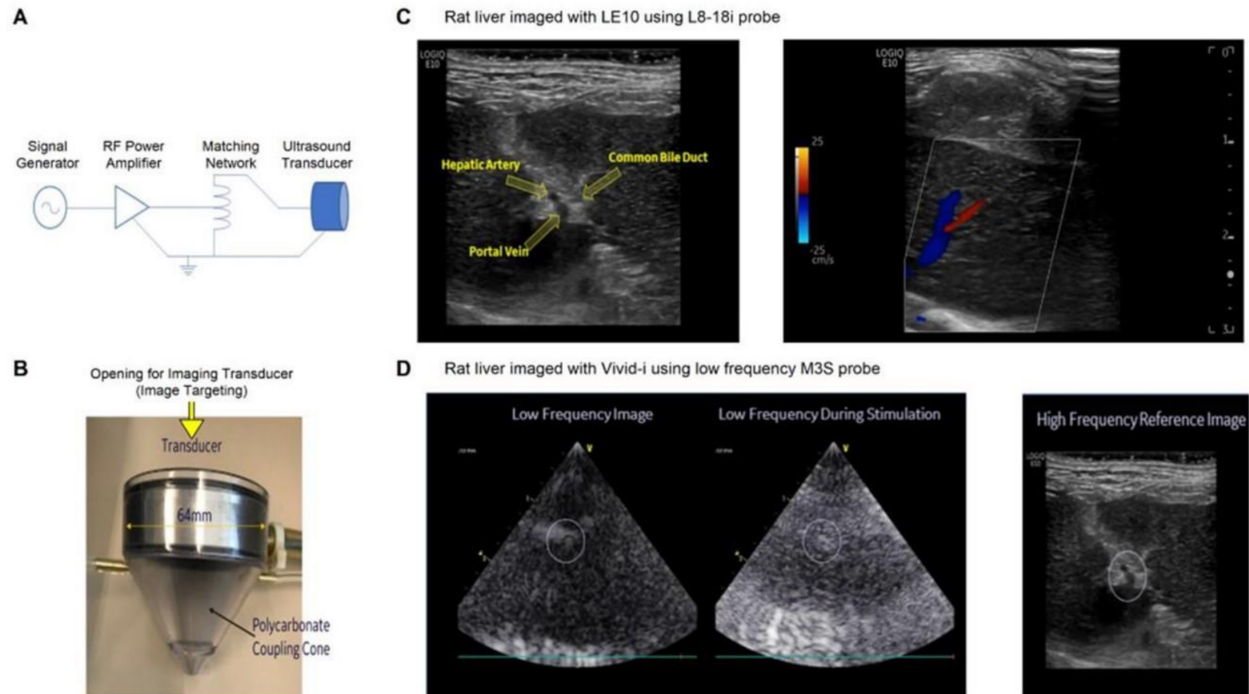
Contents

Supplementary Figs. 1–20

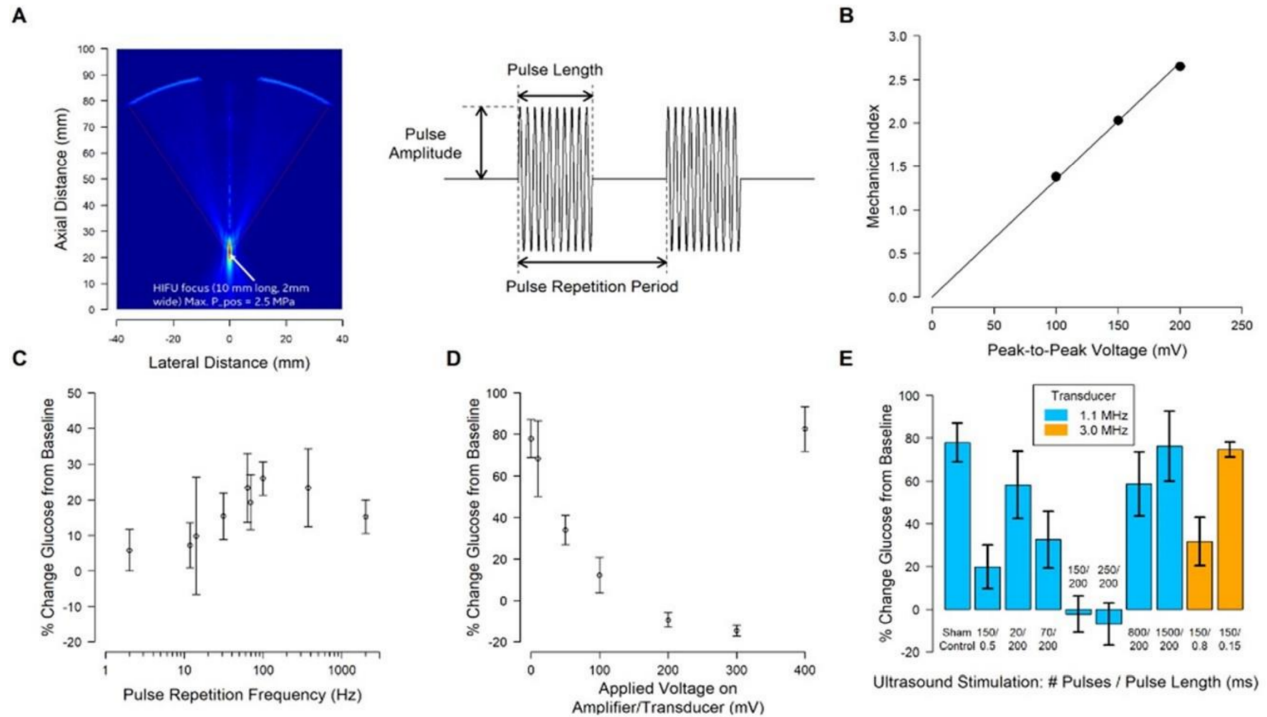
Supplementary Table 1



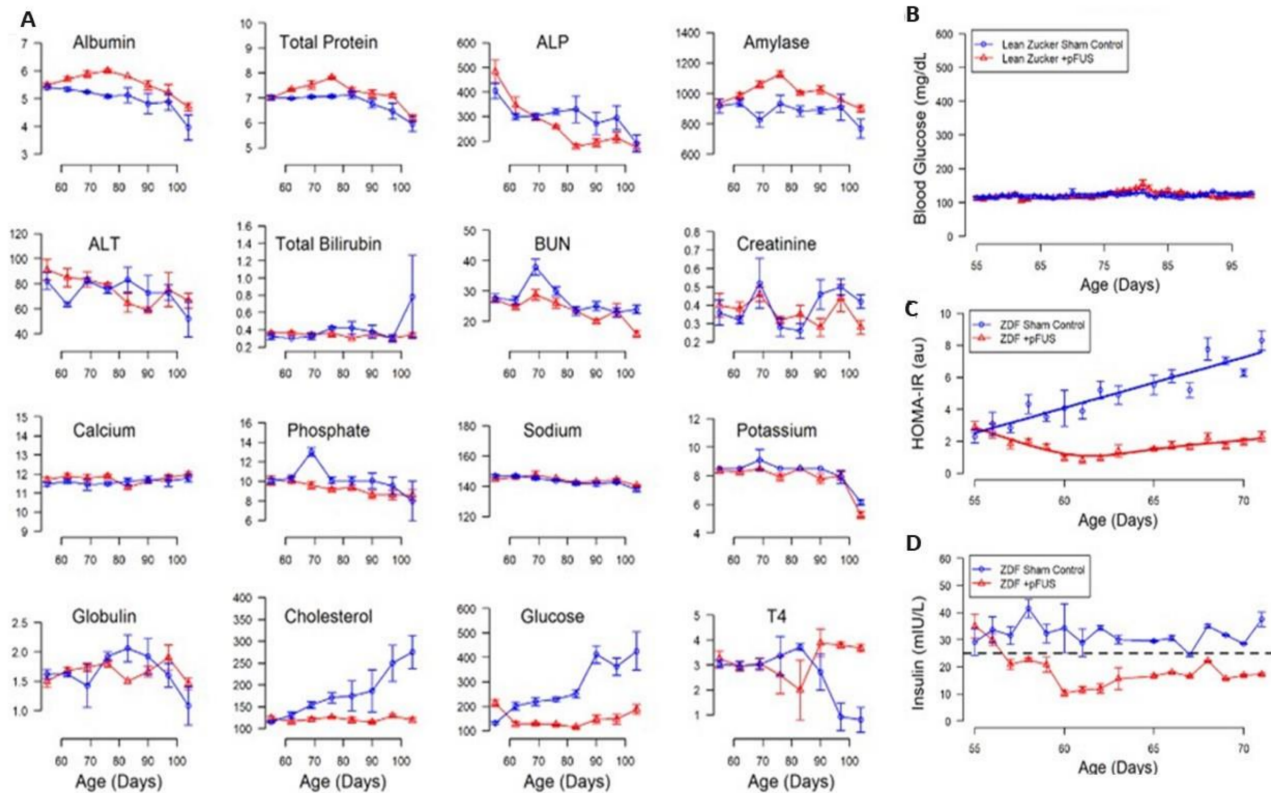
Supplementary Fig. 1 | Ultrasound stimulation of the liver-brain neural pathway and effects on glucose metabolism. A. A schematic of the ultrasound stimulus target at the porta hepatis, known to contain afferent sensory neurons associated with the hepatportal glucose sensing system.^{15,25–38,41} These neurons communicate through IML (intermediolateral nucleus) and NTS (nucleus tractus solitarius), modulate specific hypothalamic (HYP) metabolic nuclei, and alter out-going autonomic signaling important in maintaining energy homeostasis. **B.** Previous studies utilizing invasive hepatportal glucose clamps have provided evidence that communication between neurons in the porta hepatis and the hypothalamus is mediated by the hindbrain catecholaminergic system^{35–39,46,58} and affects the output of hypothalamic nuclei via the NPY system.^{46,57,60–62,141}



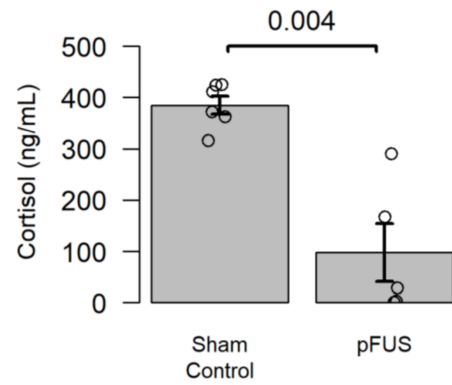
Supplementary Fig. 2 | A. Schematic of the 1.1 MHz focused ultrasound setup consisting of a signal generator (Model 33120A, Agilent Technologies Inc., Santa Clara, CA), a RF power amplifier (Model 350L, Electronics & Innovation Ltd., Rochester, NY) and a focused single-element ultrasound transducer (Model H102, Sonic Concepts Inc., Bothell, WA). The transducer is connected to the output of the power amplifier using an autotransformer matching network. **B.** The 64 mm diameter ultrasound transducer has a 20 mm diameter hole in the center for insertion of an imaging probe. The transducer is acoustically coupled to the animal through a 6-cm tall plastic standoff cone filled with degassed water. **C.** Ultrasound images of rat liver using a high-frequency L8-18i linear probe (GE Healthcare, Milwaukee WI) used for identifying the targeted location. Left: B-mode image showing the outline of the liver and the dark region corresponding to the portal vein. Right: Color Doppler image showing the blood flow in the portal vein. **D.** Ultrasound images of rat liver using a low frequency M3S phased-array probe (GE Healthcare, Milwaukee WI) as inserted into the opening of the focused ultrasound transducer.



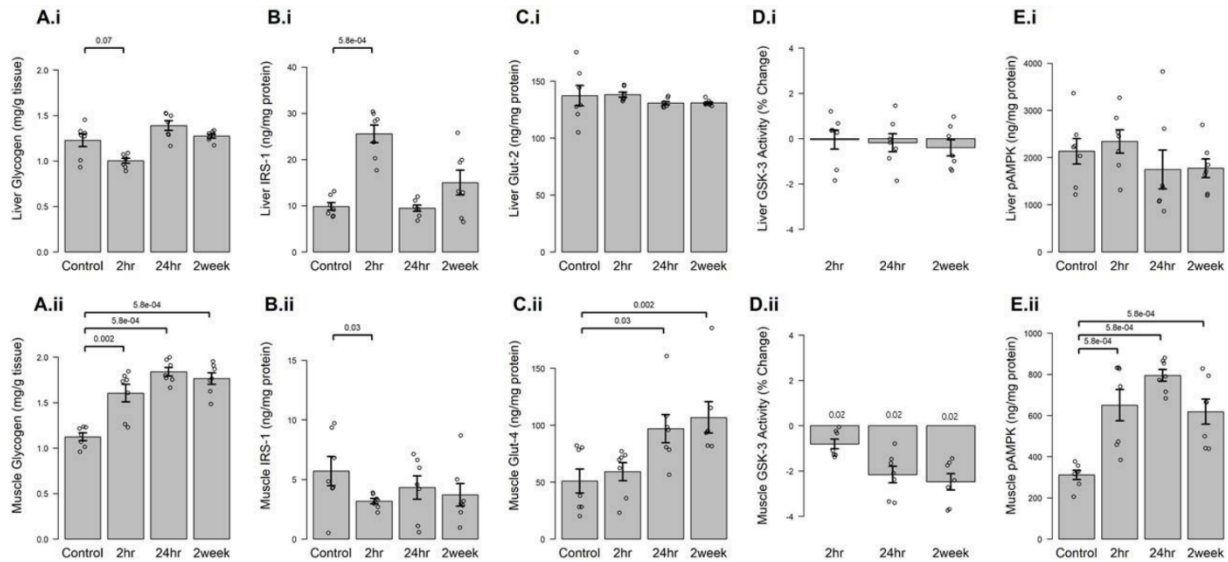
Supplementary Fig. 3 | A. The focal pressure profile of the focused ultrasound transducer (used in the rodent studies) has a full-width half-maximum amplitude of 1.8 mm laterally and depth of 12 mm as simulated using Field II.¹³⁸ **B.** The focused ultrasound is delivered in repeating bursts of the carrier. The waveform is governed by the selection of the Pulse Amplitude, Pulse Length and Pulse Repetition Period. The waveform is nominally delivered with a 135 mV pk-to-pk signal generator Pulse Amplitude, 150 μ sec Pulse Length and a 200 msec Pulse Repetition Period at the 1.1 MHz carrier frequency unless otherwise stated. The focused ultrasound transducer exhibits linearity of Mechanical Index (MI) over a range of input amplitudes up to 200 mV pk-to-pk as measured by an accredited laboratory (Acertara Acoustic Labs, Longmont, CO). **C.** Percent change from baseline in blood glucose in the LPS model of acute hyperglycemia after pFUS stimulation at various pulse repetition frequencies (Hz); at the standard pulse length, pulse amplitude, and carrier frequency used throughout the study (n=4; data shown as mean $\pm$ s.e.). **D.** Percent change from baseline in blood glucose in the LPS model of acute hyperglycemia after pFUS stimulation at various applied pulse amplitudes, at the standard pulse repetition frequency, pulse length, and carrier frequency used throughout the study (n=5; data shown as mean $\pm$ s.e.). **E.** Percent change from baseline in blood glucose in the LPS model of acute hyperglycemia after pFUS stimulation using various number of pulses (#) and pulse lengths (at both the standard carrier frequency (1.1. MHz) and 3.0 MHz; n=5; data shown as mean $\pm$ s.e.).



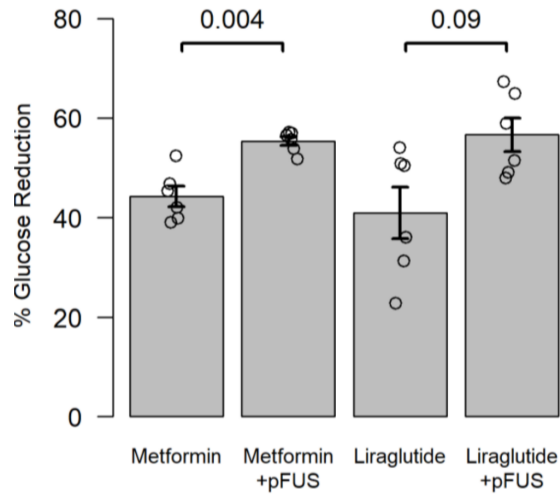
Supplementary Fig. 4 | A. Comprehensive metabolic analysis of circulating blood markers in the ZDF rat model collected on a weekly basis for 7 weeks not only showed no significant change in markers associated with liver or renal disease but also failed to show a change in circulating electrolytes, demonstrating the lack of tissue damage following chronic pFUS. Changes in cholesterol, glucose and the inactive form of thyroid hormone support an improvement in obesity and T2DM related co-morbidities, including metabolic syndrome and hypercholesterolemia (n=5; data shown as mean $\pm$ s.e.). **B.** pFUS treatment did not change circulating glucose levels as compared to Sham-CTRL in a naïve, non-diabetic animal model (SD rat; n=4; data shown as mean $\pm$ s.e.). **C.** Chronic pFUS treatment applied beginning in the prediabetic stage (age 55 days) and continued for 2 weeks (through age 75) significantly improved insulin sensitivity as determined by HOMA-IR scoring in a ZDF rat model of T2DM (n=5; data shown as mean $\pm$ s.e.). **D.** Chronic pFUS was associated with a rapid decrease in circulating insulin concentrations indicating improved insulin sensitivity following chronic pFUS treatment in the ZDF rat (n=5; data shown as mean $\pm$ s.e.). In panel A, we computed AUC for each animal (pFUS N=5 and Sham N=5) and performed a two sample Wilcoxon rank sum test. After correcting the p-values using the Bonferroni method, we observed no statistically significant difference between the pFUS and sham groups for Albumin, Total Protein, ALP, ALT, Total Bilirubin, Creatinine, Calcium, Phosphate, Potassium, Globulin, and T4. The corrected p-values for multiple testing for Amylase (p-value 0.13), BUN (p-value 0.13), Sodium (p-value 0.13), Cholesterol (p-value 0.13), and Glucose (p-value 0.13).



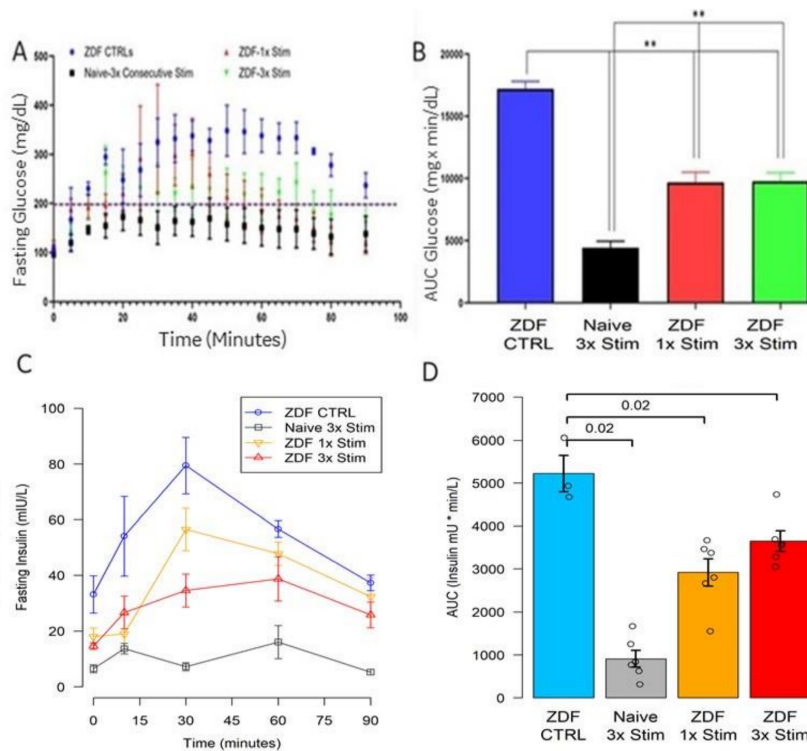
Supplementary Fig. 5 I Concentration of circulating cortisol levels (ng/mL) after 4-weeks of daily pFUS treatment in ZDF cohort (from Fig. 1). Circulating cortisol levels were compared between stimulated (pFUS) and unstimulated (Sham-Control) treatment groups. N=6 and data shown as mean $\pm$ s.e. All p-values shown are derived using nonparametric Wilcoxon rank-sum test (two-sided).



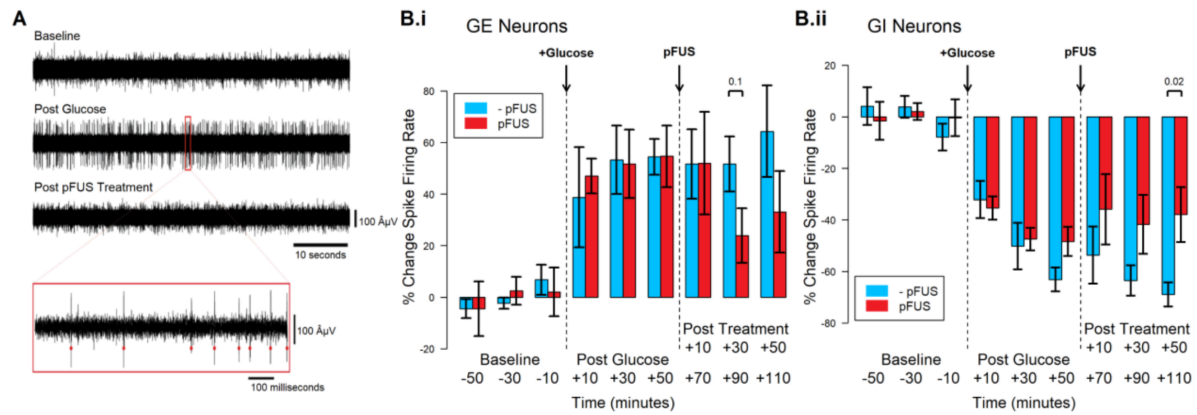
Supplementary Fig. 6 | A. Glycogen concentrations (mg/gram of tissue) in the liver (top) and muscle (bottom) in ZDF cohorts. Time points were as follows: (i) 2hr time points, representing a single stimulus with tissues collected 2-hours following pFUS (ii) 2-applications of pFUS (shown as 24-hr) where a second stimulus was applied 24-hours and a 2-hour incubation time allowed after the second treatment and (iii) 2-weeks of consecutive pFUS with samples collected 2-hours after the final pFUS treatment. Concentrations of glycogen were compared between pFUS stimulated animals across time points (2 hr., 24 hr. and 2-weeks) and unstimulated sham control animals (2-weeks of consecutive sham stimulations; n=7). **B-E.** % Change in IRS-1 (B, i. liver; ii. muscle; n=7), GLUT2 and GLUT4 (C, i. liver; ii. muscle; n=7), GSK-3 (D, i. liver, ii. muscle; n=7), and AMPK activity (E, i. liver, ii. muscle; n=7) following 2, 24 hours and 2 weeks timepoints during daily pFUS treatment. Animals were all age-matched to be 67 days old at study completion. Data shown as mean $\pm$ s.e.; All p-values shown are derived using nonparametric Wilcoxon rank-sum test (two-sided).



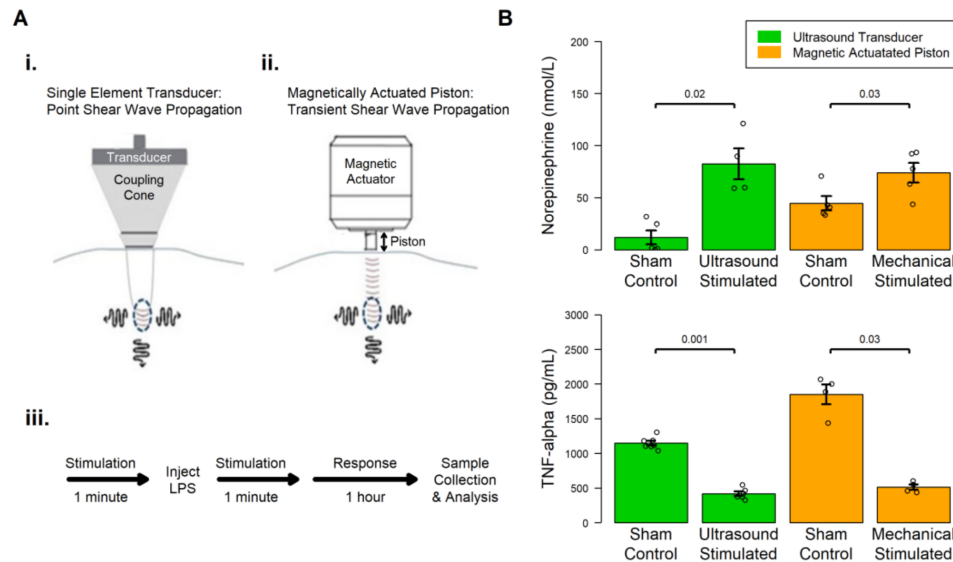
Supplementary Fig. 7 I Daily pFUS treatment was performed on age-matched cohorts of ZDF animals for 10 days (starting at age 60 days) in combination with daily administration of a diabetes drug (metformin or liraglutide; see materials and methods for drug concentrations and administration) and compared to cohorts receiving the drug treatment alone. Fig. 7 shows the percent change in blood glucose concentration (as compared to pre-stimulus glucose averages) for these leading pharmaceutical therapies⁵⁰ (metformin and liraglutide) with (+pFUS) and without pFUS. Addition of hepatopoortal pFUS to either metformin or liraglutide therapy was associated with a significant decrease in circulating blood glucose levels as compared to drug treatment alone. Data shown as mean $\pm$ s.e. (n=7); All p-values shown are derived using nonparametric Wilcoxon rank-sum test (two-sided).



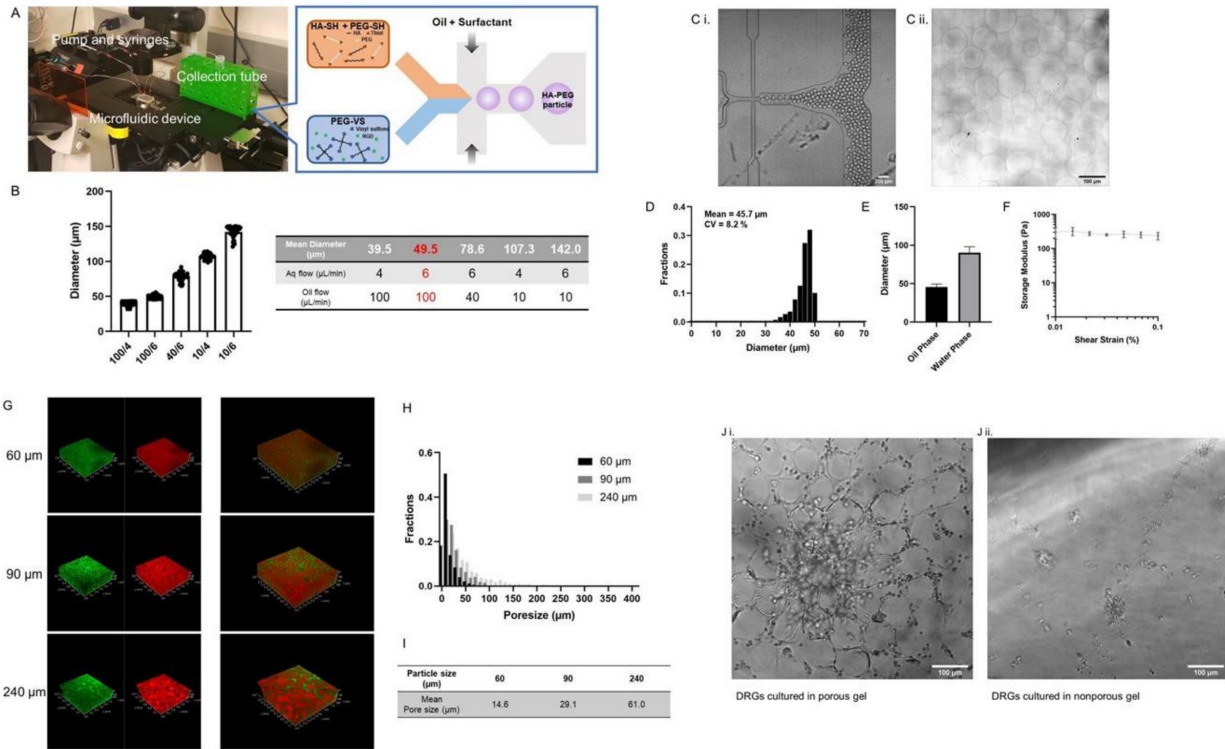
Supplementary Fig. 8 | A. pFUS treatment (labelled Stim) in the ZDF model resulted in the improvement of glucose tolerance (as measured by oral glucose tolerance tests (OGTTs) following both a single application (ZDF-1x) and three consecutive days (ZDF-3x) of pFUS. Both ZDF-1x and ZDF-3x treatment groups were within naïve (non-diabetic, SD rat) levels at 60-90 minutes following glucose challenge. **B.** Calculation of incremental area under the curve (AUC) for glucose showed a significant reduction in AUC following both a single (ZDF-1x) and three consecutive days of pFUS (ZDF-3x), and the naïve (SD rat) cohorts. p-values on panel B are derived from ANOVA using Tukey's multiple comparison test (** denotes p value less than 0.005; 95% confidence intervals: ZDF CTRLs (16041-18368), Naïve 3X Stim (3433 – 5428), ZDF 1X Stim (8084 – 11269), ZDF 3x Stim (8423 – 11105)). **C.** pFUS treatment resulted in a significant attenuation of hyperinsulinemia observed in ZDF rats. Both ZDF-1x and ZDF-3x showed significant reductions in circulating hyperinsulinemia within 30 minutes of treatment. **D.** Calculation of incremental areas under the curve (AUC) for insulin showed a significant reduction in AUC in pFUS treated-diabetic cohorts as compared to sham stimulated controls, suggesting an increase in whole body insulin sensitivity as demonstrated by reduced hyperinsulinemia. p-values on panel D are derived using nonparametric Wilcoxon rank-sum test (two-sided). n=6 on all panels (except for ZDF sham which was n=4); data shown as mean $\pm$ s.e.



Supplementary Fig. 9 | A. Sample electrode recordings in the PVN for data shown in Fig. 3B ii. Sample traces show 60s of recording in Baseline, Glucose+ and pFUS+ periods. Zoomed trace shows 1s of recording in the Glucose+ period with detected spikes indicated by red dots. **B.** Firing rates for GE (i.; $n=5$) and GI (ii.; $n=16$) for all time points before (-50 – 0) glucose infusion, and before and after pFUS. Data in Fig. 3C represents firing rates for GE and GI neurons following with and without pFUS at the +30-minute post-pFUS timepoint. All p-values shown are derived using nonparametric Wilcoxon rank-sum test (two-sided). Data shown as mean $\pm$ s.e.



Supplementary Fig. 10 | A. Activation and neuromodulation of the splenic cholinergic anti-inflammatory pathway (CAP) using both an ultrasound and a mechanical piston-based transducer.¹⁵ We have previously demonstrated use of pFUS to activate the splenic cholinergic anti-inflammatory pathway. In this figure, we demonstrate that pulsed mechanical ultrasound and direct mechanical pulsed stimuli (at similar pulse repetition frequencies) result in nerve activation and neuromodulation of CAP. This data enabled focused investigation of ion channel blockers that have previously been shown to inhibit channels affected by mechanical stimuli (such as TRPA1). i. A schematic of the ultrasound transducer described previously (and its acoustic coupling to the animal using the water-filled coupling cone). As described above, the transducer provides an ultrasound-based stimulus with a mechanical component based on point shear wave propagation at the focal point of the ultrasound stimuli.^{15,130,131} ii. A schematic of the non-ultrasonic, magnetically actuated piston described above to provide a mechanical stimulus when directly coupled to the animal (based on 1D transient shear wave propagation from the piston-tissue interface).¹³¹ iii. The timeline of ultrasonic or mechanical neuromodulation experiments performed in the LPS-induced inflammation model.¹⁵ **B.** (left) Concentration of splenic norepinephrine (NE, top) and TNF alpha (bottom) with and without ultrasound stimulation in LPS-treated animals versus sham controls. (right) Concentration of splenic norepinephrine (NE, top) and TNF alpha (bottom) with and without mechanical-piston based stimulation in LPS-treated animals versus sham controls. In evaluating CAP response to the mechanical-piston stimulus to ultrasound-based stimulation (as measured by CAP-related neurotransmitter (NE) and cytokine (TNF alpha) concentrations),¹⁵ the mechanical piston-based stimulation resulted in nearly identical elevation of NE and reduction of splenic TNF in response to the piston versus ultrasound transducer-based stimulus. All p-values shown are derived using nonparametric Wilcoxon rank-sum test (two-sided). Norepinephrine measurements are n=4 using the ultrasound transducer and n=5 using the magnetic piston. TNF measurements are n=6 for ultrasound transducer and n=4 for mechanical transducer. Data shown as mean $\pm$ s.e.



Supplementary Fig. 11 | A. Photograph of the setup for microparticle fabrication and scheme illustrating microgel formation that is utilized to provide an optimal growth environment for three-dimensional culture⁸⁴ of neurons (see Fig. 2) using a microfluidic water-in-oil emulsion system. A pre-gel and crosslinker solution are segmented into monodisperse droplets followed by in-droplet mixing and crosslinking via Michael addition. **B.** The operational regime for microfluidic microgel generation has a large dynamic range, spanning almost an order of magnitude in size while maintaining tight control at each condition, with Coefficient of Variation (CVs) < 7% in all cases ($n = \text{more than } 33$ ($n < 100/4 > = 105$, $n < 100/6 > = 171$, $n < 40/6 > = 68$, $n < 10/4 > = 45$; data shown as mean $\pm$ SD). We use 50-100 μm microparticles for experiments as this provided the best pore size for neuron culture. **C.i.** Representative images of microgel droplets in flow after generation, which are utilized to fabricate the three-dimensional neuron culture⁸⁴ (see Fig. 2). **C.ii.** Representative images of cross-linked microgels after phase transfer. **D.** Generation of HA microgel particles with highly defined sizes. **E.** HA microgel particles made with 50 μm diameter, when surrounded by oil swell in buffer after aqueous extraction from the oil phase ($n=11$; data shown as mean $\pm$ SD). **F.** Storage moduli of hydrogels. ($n=3$; data shown as mean $\pm$ SD). HA microgel particles were manufactured with an ideal storage modulus tuned for neuron culture (< 500 Pa). **G.** Confocal reconstructions of HA microgel-based neuron culture⁸⁴ scaffolds after incubation with high molecular weight ($M_w = 500$ kDa) FITC-dextran indicates interconnectivity of the void spaces through the scaffold. In all cases, FITC dextran is represented in green and HA microparticles are represented in red. Different size of particles yield different void spaces. **H. and I.** Different building-block sizes allow deterministic control over resultant microporous network characteristics. 30 μm , 50 μm and 100 μm diameter microgel particles yield 15 μm , 25 μm , and 60 μm mean pore size respectively. Since dissociated neurons are around 10 ~ 30 μm , we chose 50-100 μm microgel particles for further cell culture experiments. **J. i.** Bright field image of 3D culture of neuron cells within an HA-based microporous gel⁸⁴ (see Fig. 2). **J. ii.** Bright field image of 3D culture of neuron cells within a HA- based nanoporous gel (i.e., cell directly embedded in the gel).

Animals

34 (all males) Obese Zucker diabetic fatty (ZDF) rats (Lepr gene mutation)

10 (all males) Lean Zucker rats

11 (6 males, 5 females) High-fat diet-induced obesity (DIO) rats

Treatment protocols

- Obese ZDF Control 3d
- Obese ZDF US3dAMx1
- Obese ZDF US3dAMx10
- Obese ZDF US4wAMx10
- Obese ZDF US4wAMPx1
- Obese ZDF US4wAMPx10
- Obese ZDF Control 7w
- Obese ZDF US7w
- DIO-stz Control 7w
- DIO-stz US7w
- Lean Zuckers Control 7w
- Lean Zuckers US7w

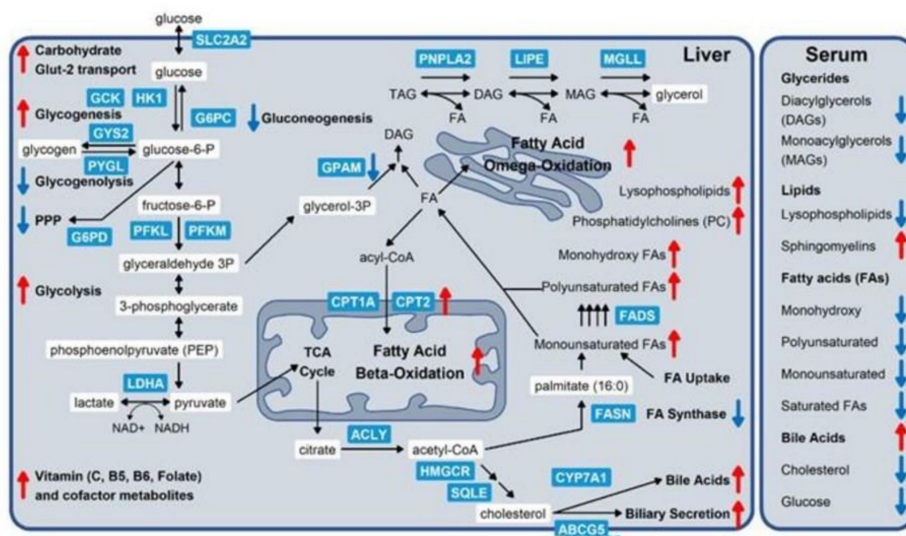
Standardized organ tissue sample collection methods

Tissues that underwent RNA-Seq (transcriptomics) and * for untargeted metabolomics

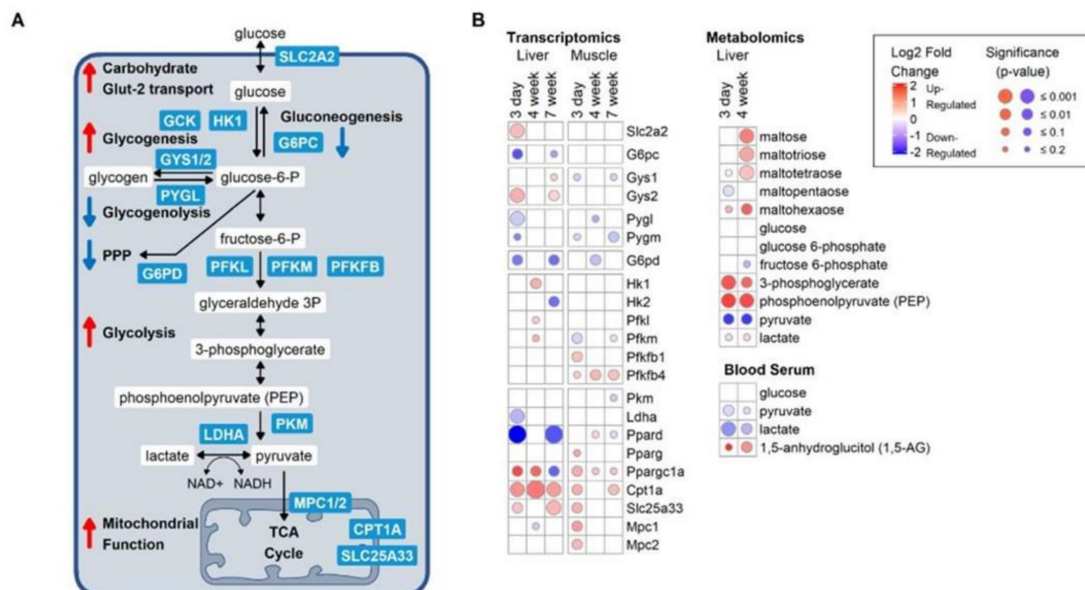
- Liver *
- Adipose
- Muscle
- Intestine
- Pancreas
- Kidney
- Brain (hypothalamus brain punch)
- Blood cells and blood serum *

All tissue samples were maintained at -80°C until processed.

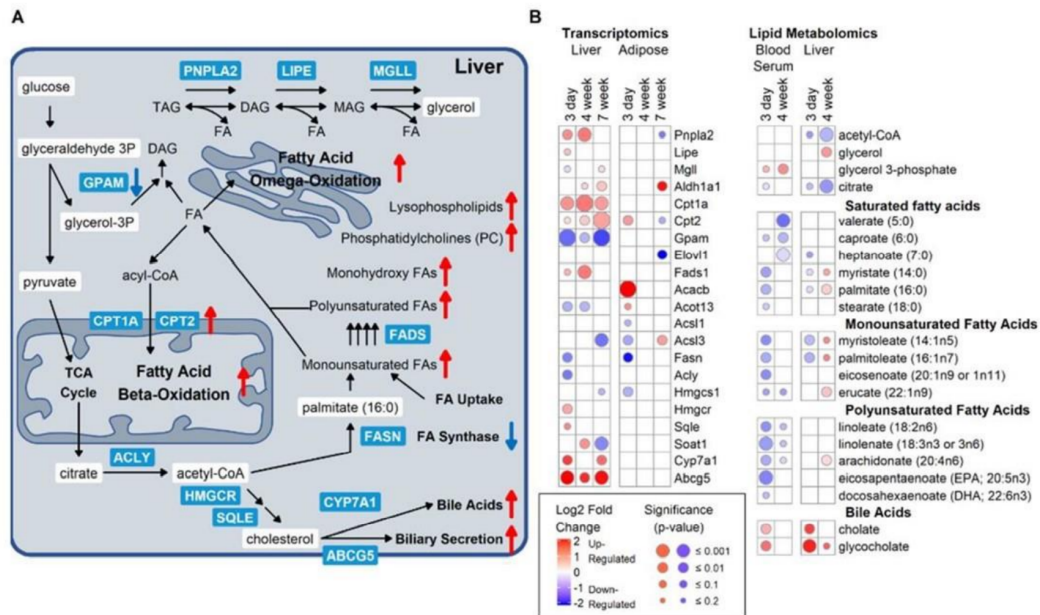
Supplementary Fig. 12 | Sample list and description of metabolomic and transcriptomic samples; including controls.



Supplementary Fig. 13 | Schematic of key protein enzymes (blue rectangles), metabolites (white rectangles) and metabolic pathways (e.g., gluconeogenesis, fatty acid and bile acid metabolism, oxidative phosphorylation, mitochondrial transport) that are either upregulated (red upward arrows) or downregulated (blue downward arrows).

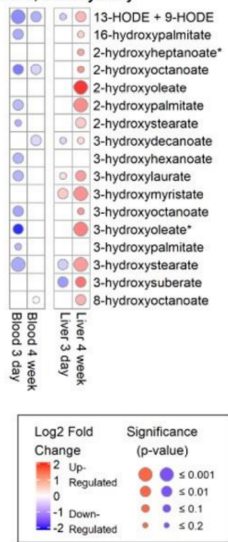


Supplementary Fig. 14 | A. Schematic of key protein enzymes (blue rectangles), metabolites (white rectangles) and metabolic pathways (e.g., gluconeogenesis, fatty acid and bile acid metabolism, oxidative phosphorylation, mitochondrial transport) that are either upregulated (red upward arrows) or downregulated (blue downward arrows) within the liver-muscle axis. **B.** (left) Differential RNA transcripts (i.e., genes) from liver and muscle tissue of ZDF animals undergoing 3 days, 4 weeks, or 7 weeks of daily pFUS stimulation vs sham controls. The protein encoded by these differentially expressed genes is presented in the schematic diagram. (right) The changes in metabolites in liver tissue at 3 days and 4 weeks between ultrasound treated vs controls. The size of each circle represents the p-value of differentiation between the pFUS stimulated group (n=5) vs sham controls (n = 5). The color of the circle represents the degree of being upregulated (red) or downregulated (blue). For differential transcript analysis, p-values shown are derived from the Wald test. For metabolomic data, p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

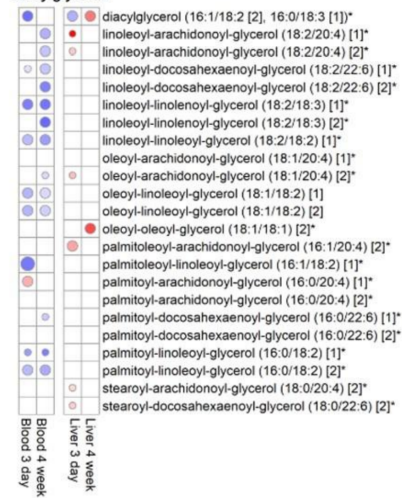


Supplementary Fig. 15 | A. Schematic of key protein enzymes (blue rectangles), metabolites (white rectangles) and metabolic pathways (e.g., gluconeogenesis, fatty acid and bile acid metabolism, oxidative phosphorylation, mitochondrial transport) that are either upregulated (red upward arrows) or downregulated (blue downward arrows) within the liver-adipose axis. **B.** (left) Differential RNA transcripts (i.e., genes) from liver and adipose tissue of ZDF animals undergoing 3 days, 4 weeks, or 7 weeks of daily pFUS stimulation vs sham controls. The protein encoded by these differentially expressed genes is presented in the schematic diagram. (right) The changes in metabolites in liver tissue at 3 days and 4 weeks between ultrasound treated vs controls. The size of each circle represents the p-value of differentiation between the pFUS stimulated group (n=5) vs sham controls (n = 5). The color of the circle represents the degree of being upregulated (red) or downregulated (blue). For differential transcript analysis, p-values shown are derived from the Wald test. For metabolomic data, p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

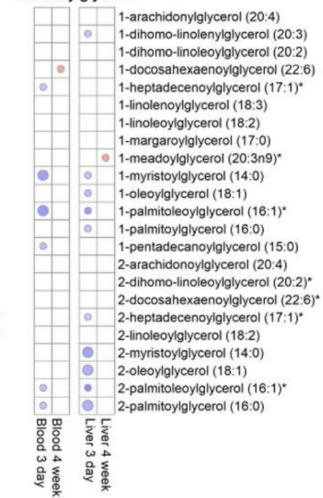
Fatty Acid, Monohydroxy



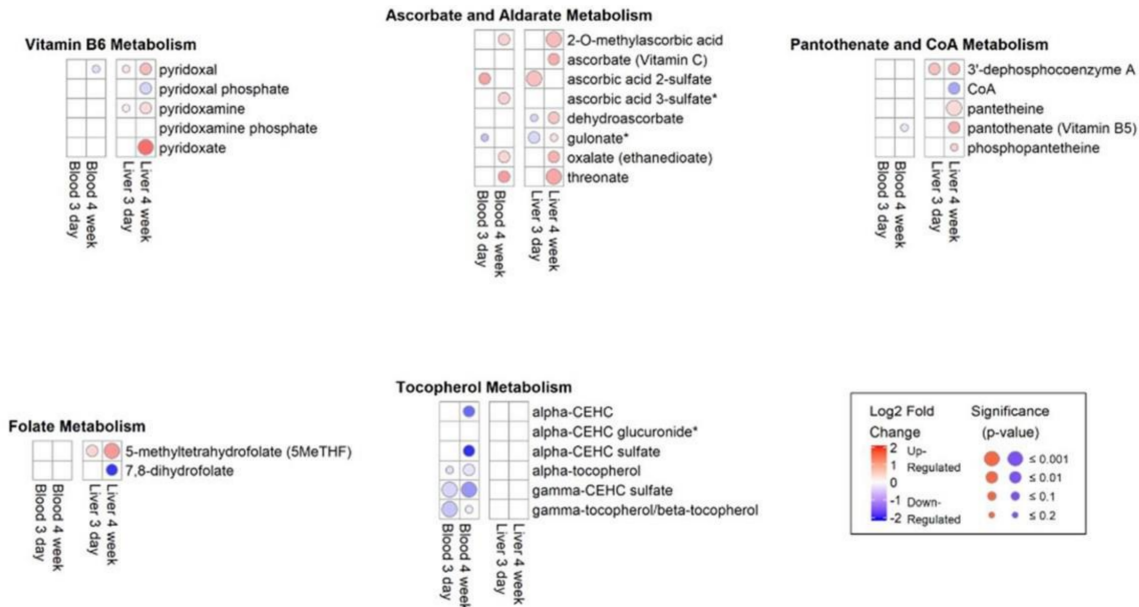
Diacylglycerol



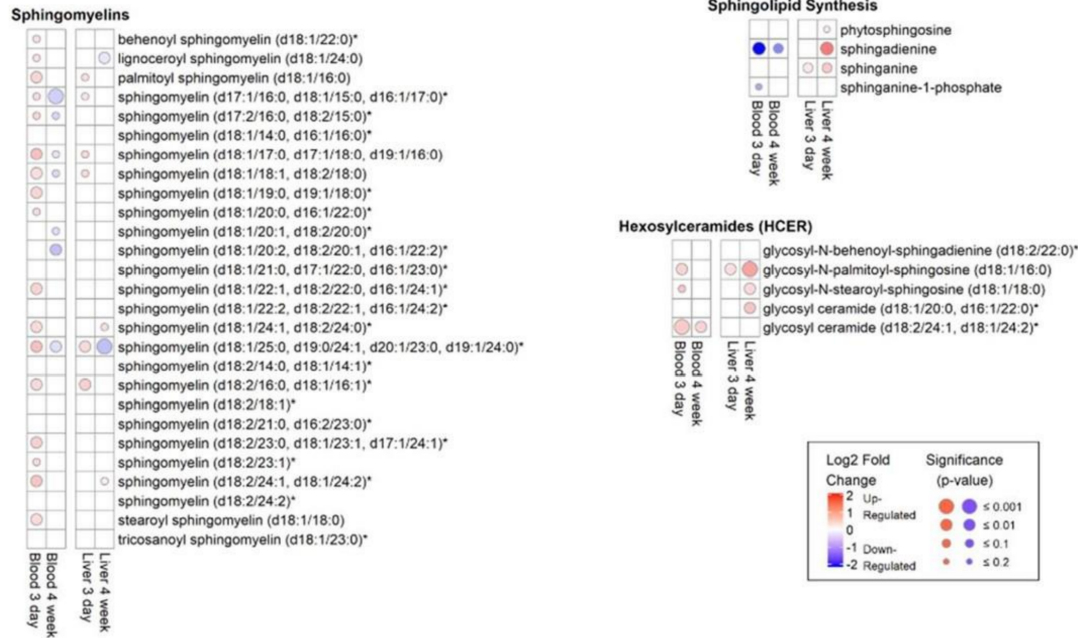
Monoacylglycerol



Supplementary Fig. 16 I Changes in monohydroxy fatty acids, Diacylglycerols (DAGs), Monoacylglycerols (MAGs) in blood serum and liver tissue at 3 days and 4 weeks between ultrasound treated vs control ZDF animals. p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

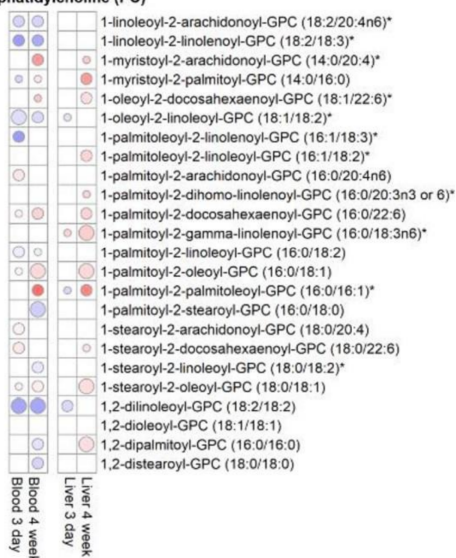


Supplementary Fig. 17 | Changes in vitamin and cofactor metabolites in blood serum and liver tissue at 3 days and 4 weeks between ultrasound treated vs control ZDF animals. p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

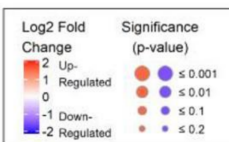
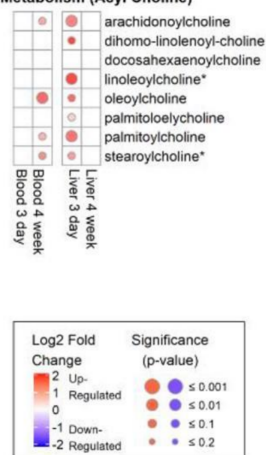


Supplementary Fig. 18 I Changes in Sphingomyelins, hexosylceramides (HCER), and metabolites involved in sphingolipid synthesis in blood serum and liver tissue at 3 days and 4 weeks between ultrasound treated vs control ZDF animals. p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

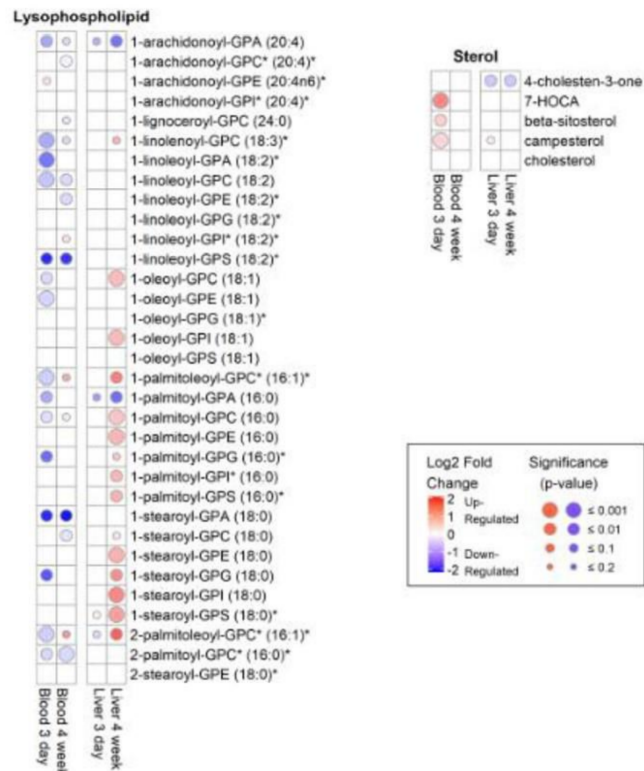
Phosphatidylcholine (PC)



Fatty Acid Metabolism (Acyl Choline)



Supplementary Fig. 19 | Changes in phosphatidylcholines (PC) and acyl cholines from fatty acid metabolism in blood serum and liver tissue at 3 days and 4 weeks between ultrasound treated vs control ZDF animals. p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).



Supplementary Fig. 20 | Changes in lysophospholipids and sterols in blood serum and liver tissue at 3 days and 4 weeks between ultrasound treated vs control ZDF animals. p-values are derived using nonparametric Wilcoxon rank-sum test (two-sided).

T2D Associated Defects		Drug Mode(s) of Action (MOA)						Portal-Sensor (MOA)
Organ Site	T2D Pathophysiology	GLPs	DPP4s	SGLT2s	TZDs	SUs	Metformin	Liver-Brain Pathway
Pancreas (Beta)	Reduced Insulin Secretion; Decreased Beta Cell Mass	X	X			X		X ^{34,142}
Intestines	Decreased Incretin Effect	X	X					X ^{34,143,144}
Pancreas (alpha)	Increased Glucagon Secretion	X	X					X ¹⁴⁵
Adipose	Increased Lipolysis				X		X	X ^{33,41,146}
Muscle	Reduced Glucose Uptake (Insulin Resistance)				X		X	X ^{26,41,42}
Liver	Increased Glucose Production; Liver-Adipose Pathway	X			X		X	X ^{32,35,72}
Brain	Insulin Resistance; Neurotransmitter Dysfunction	X					X	X ^{15,30,32,35,39,58,72}
Colon/ Biome	Abnormal Microbiota; Effect on Incretins	X	X				X	X ^{147,148}
Inflammation	Beta Cell Function; Beta Cell Mass, Fat-Liver Pathway	X	X					X ¹⁴⁸⁻¹⁵⁰
Stomach and Intestine	Increased Glucose Absorption or Gluconeogenesis	X					X	X ^{33,147,148}
Kidney	Increased Glucose Reabsorption or Kidney gluconeogenesis			X				X ^{135,151}

Supplementary Table 1 | The table contains descriptions and references^{34,142; 34,143,144; 145; 33,41,146; 26,41,42; 32,35,72; 15,30,32,35,39,58,72; 147,148; 148–150; 33,147,148; 145,151} on the known afferent and efferent nerve pathways that are associated with hepatportal glucose sensing and the hepatportal plexus (right column). These are organized by organ system (left most column) and type II diabetes pathophysiological (second column from left). The current T2D drug medications are also listed (middle column), where each X denotes that the drug mode of action is known to act on that particular T2D pathophysiology (row). The cited liver-brain pathway (right column) references each used portal glucose/hormone infusions to activate the hepatportal plexus and investigate metabolic effects. These studies have shown significant involvement with hepatportal glucose sensing across organ systems, and T2D pathophysiology.

References

(Numbered following the final reference number in the main text, which is 140.)

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