Vasoconstrictor antagonism improves functional and structural vascular alterations and liver damage in rats with early NAFLD

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Supplementary materials and methods

Drugs

Animals that were sacrificed for liver samples were anaesthetised with isoflurane (IsoFlo®, Zoetis Belgium SA, Louvain-la-Neuve, Belgium), induction at 5% and maintenance at 3-3.5% at 0.2 L/min via vaporisation. For vascular corrosion casting, animals were sacrificed with 80 vol% ketamin (Ketalar®, Pfizer, Puurs Belgium) and 20 vol% xylazine (Rompun®, Bayer, Leverkusen, Germany), 2 mL/kg body weight intraperitoneally. All other animals were anaesthetised with natrii Pentobarbitalum (Nembutal®, 60mg/1mL Ceva Sante Animale, Brussels, Belgium), 30 mg/kg body weight intraperitoneally.

The isotonic Krebs-Ringer (Krebs) solution had the following composition: NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, CaEDTA 0.025 mM, and glucose 11.1 mM.

BQ-123 and BQ-788 were purchased from Merck Millipore, Darmstadt, Germany. Valsartan, methoxamine (Mx), endothelin-1 (ET-1), U46619, SC-560 and SC-236 were purchased from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. Angiotensin-II (ATII) was purchased from Enzo life sciences BVBA, Brussels, Belgium. All drugs were first dissolved in ultrapure distilled water before diluting them in the Krebs-Ringer solution to obtain the given concentrations. All solutions were freshly prepared on the day of the experiments.

Bosentan (Tracleer, Janssen-Cilag), valsartan (Diovane, Novartis) and celecoxib (Eurogenerics) for oral treatment were purchased at the pharmacy of the Antwerp University Hospital, Edegem, Belgium. Tablets were dissolved in tap water before oral gavage and the solutions were freshly prepared daily. All detailed information about drugs and laboratory animals can be found in the CTAT table.

Fig. S1

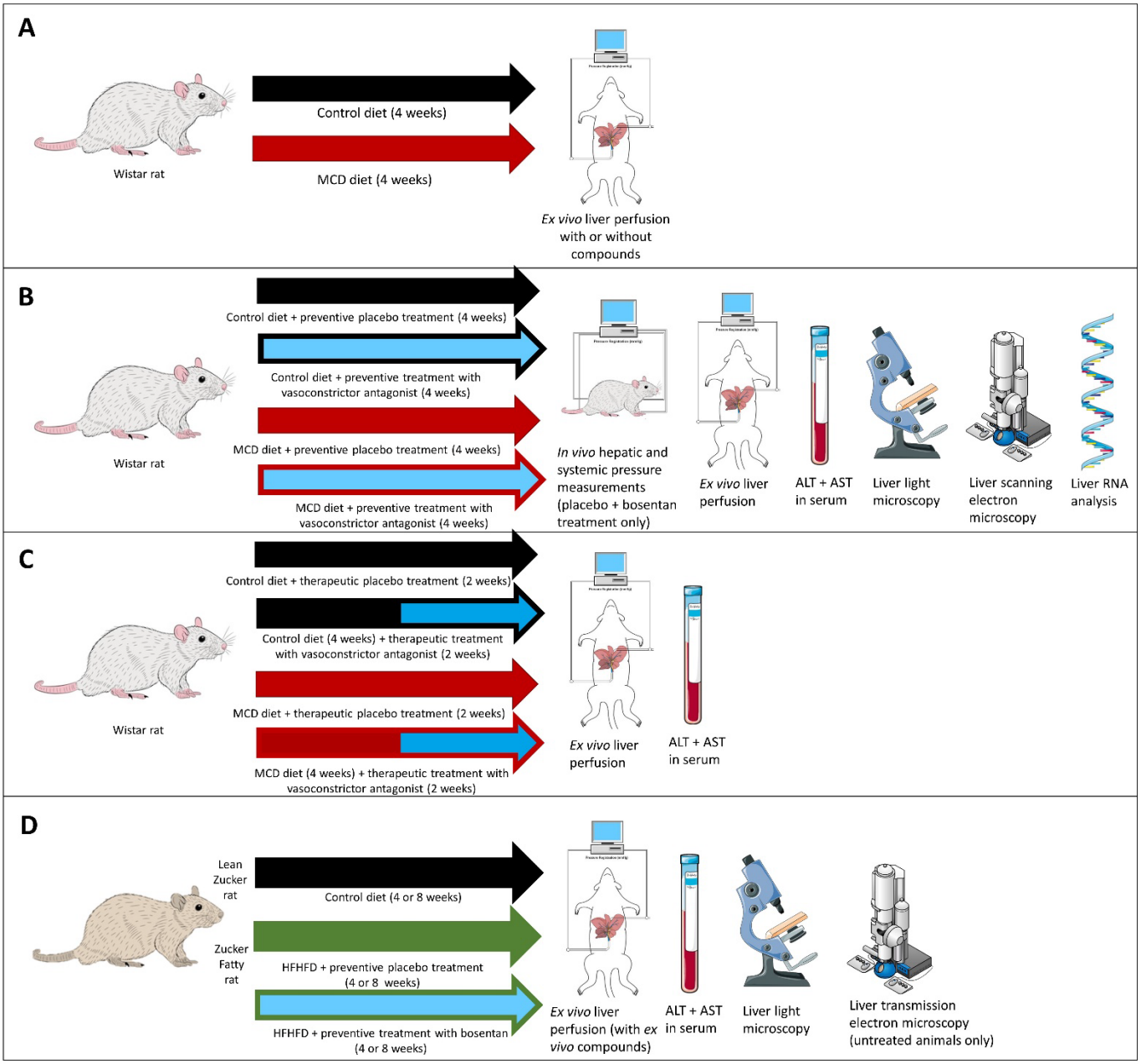


Fig. S1: Overview of the different experimental settings in time.

(A) In *ex vivo* vasoconstrictor experiments, Wistar rats were fed either a control or a methionine choline deficient diet (MCD). After 4 weeks of diet, *ex vivo* liver perfusion experiments were performed with the addition of vasoconstrictive agonists, antagonists or placebo to the Krebs fluid.

(B) In *in vivo* preventive treatment experiments, Wistar rats were treated with a vasoconstrictor antagonist or placebo during the complete 4 weeks of the MCD or control diet and subsequently the animals were submitted to *in* vivo hepatic and systemic pressure measurements, ex *vivo* liver perfusion experiments, blood analysis, liver histology, scanning electron microscopy of vascular corrosion casts and mRNA analysis.

(C) In *in vivo* therapeutic treatment experiments, Wistar rats were treated with a vasoconstrictor antagonist or placebo during the last 2 weeks of the 4 weeks during MCD or control diet respectively and subsequently the animals were sacrificed for *ex vivo* liver perfusion experiments, blood analysis and liver histology.

(D) Zucker fatty rats on high fat high fructose diet (HFHFD) or lean Zucker rats on control diet during 4 or 8 weeks were submitted to *ex vivo* liver perfusion experiments and a separate group of ZFR was treated with either bosentan or placebo during 4 or 8 weeks in a preventive setting before *ex vivo* liver perfusion experiments, blood analysis, liver histology and transmission electron microscopy.

Fig. S2

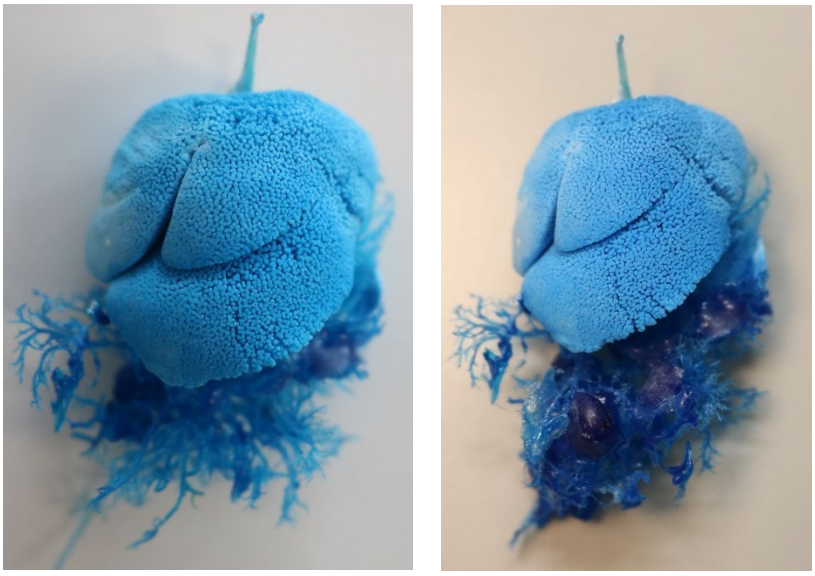


Fig. S2: examples of complete vascular corrosion casts

Rat livers before the preparation for subsequent scanning electron microscopy (SEM) imaging.

Fig. S3

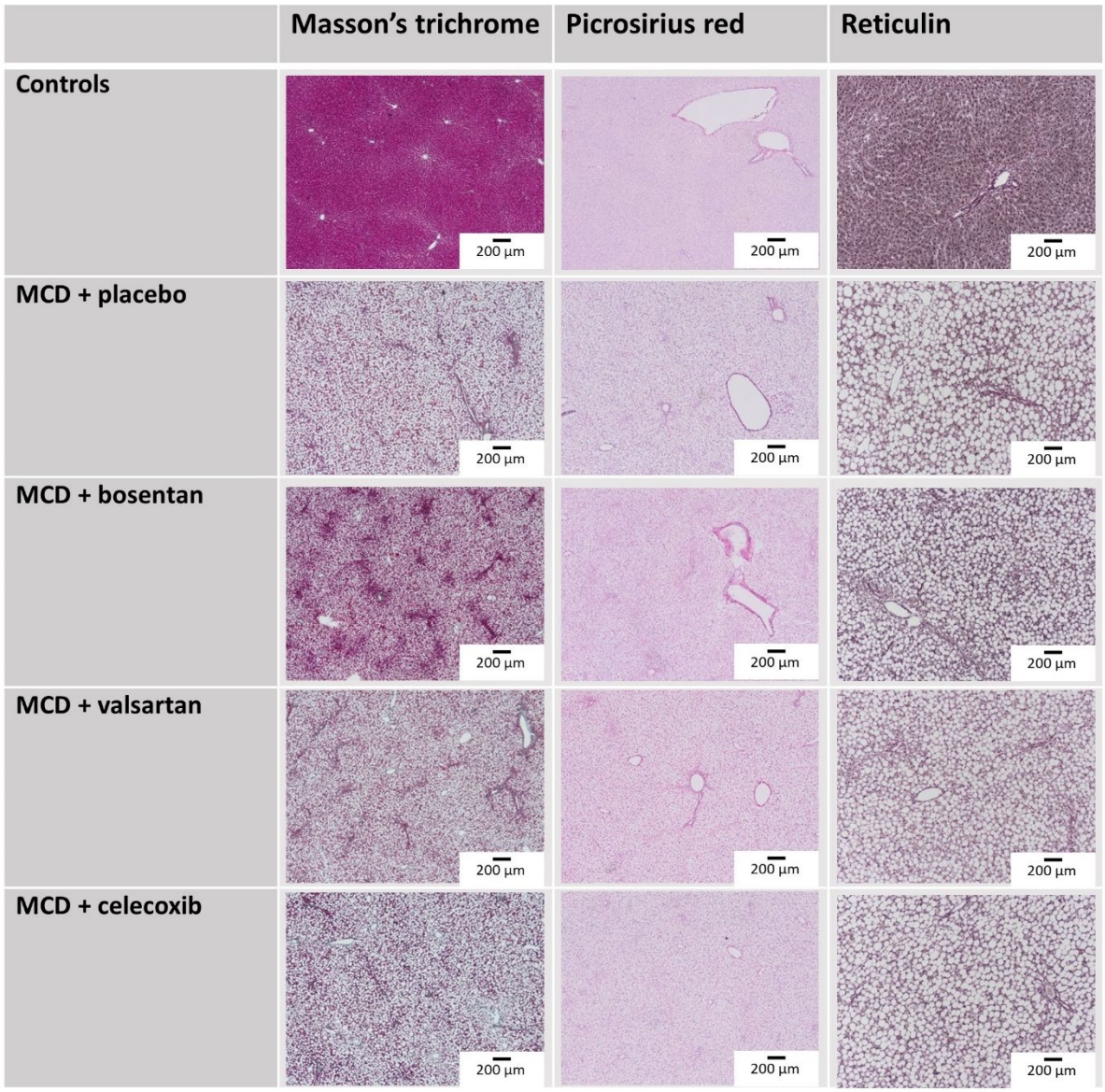


Fig. S3: Histological liver sections for fibrosis

Masson's trichrome stain, picrosirius red stain and reticulin stain (original magnification x4) of rat livers after control or methionine choline deficient diet (MCD) and preventive treatment with respective placebo, bosentan, valsartan or celecoxib.

Control livers do not show any histological abnormalities. In MCD-fed rats, there is severe steatosis but no portal or lobular inflammation, no ballooning and no significant

fibrosis. Besides decreased steatosis with bosentan, treatment did not affect liver histology.

Fig. S4

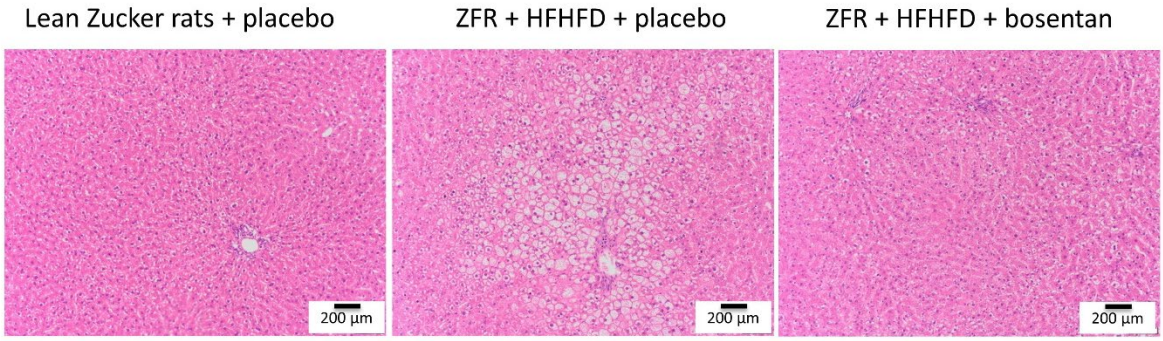


Fig. S4: Histological liver sections

Haematoxylin-eosin stain (original magnification x10) of lean Zucker rats fed a standard diet and Zucker fatty rats (ZFR) fed a high fat high fructose diet (HFHFD) after preventive treatment with placebo or bosentan.

Control livers do not show any histological abnormalities. In ZFR, moderate steatosis is demonstrated but without any histological signs of portal of lobular inflammation, ballooning or significant fibrosis. The degree of steatosis is decreased after bosentan treatment compared to placebo.

Fig. S5

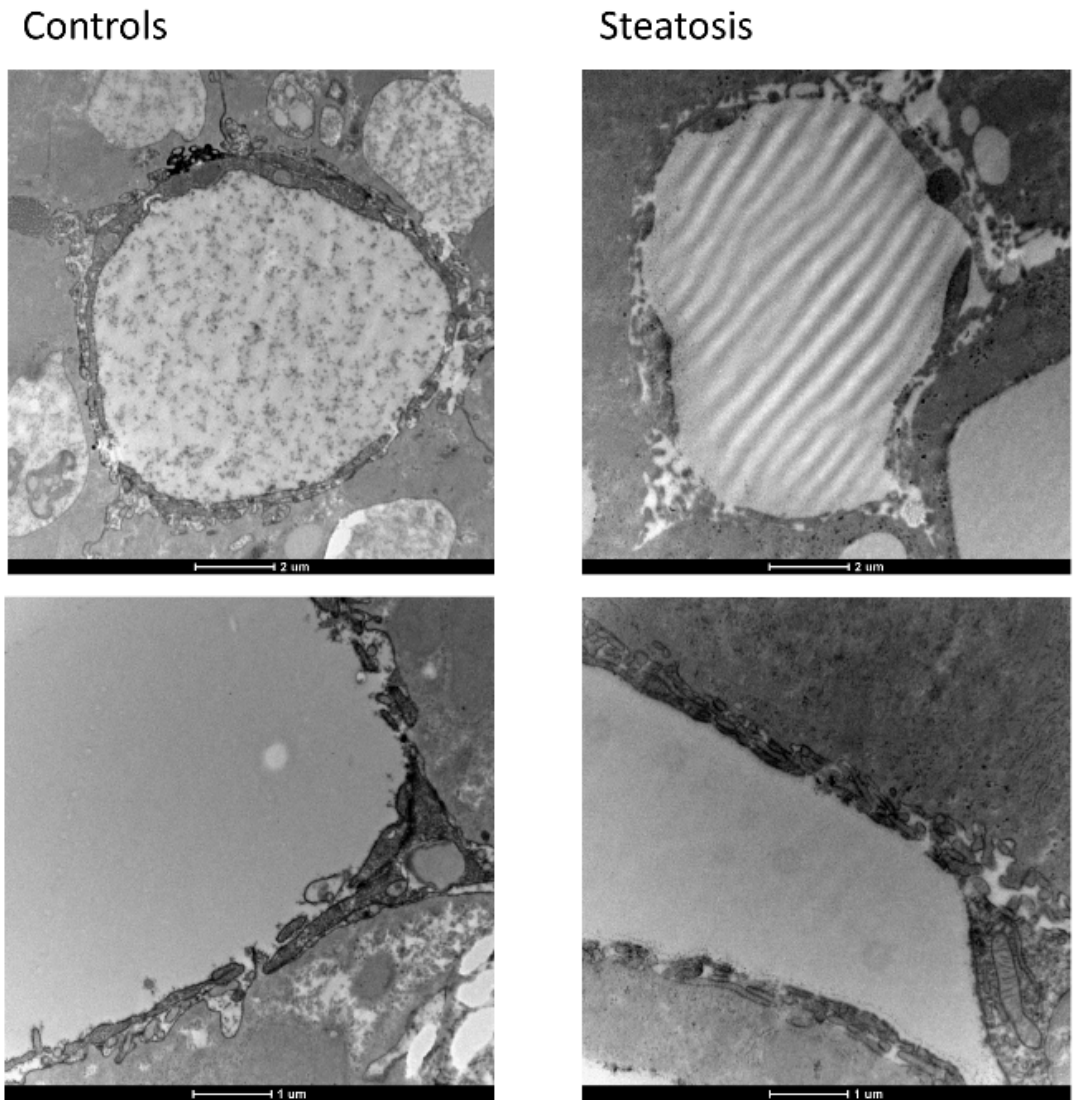


Fig. S5: Transmission electron microscopy images of Zucker fatty rats fed a high fat high fructose diet during 4 weeks (steatosis) compared to lean Zucker rats fed a control diet (controls). Original magnification 2000x for the upper row, 2500x for the bottom row.

Fig. S6

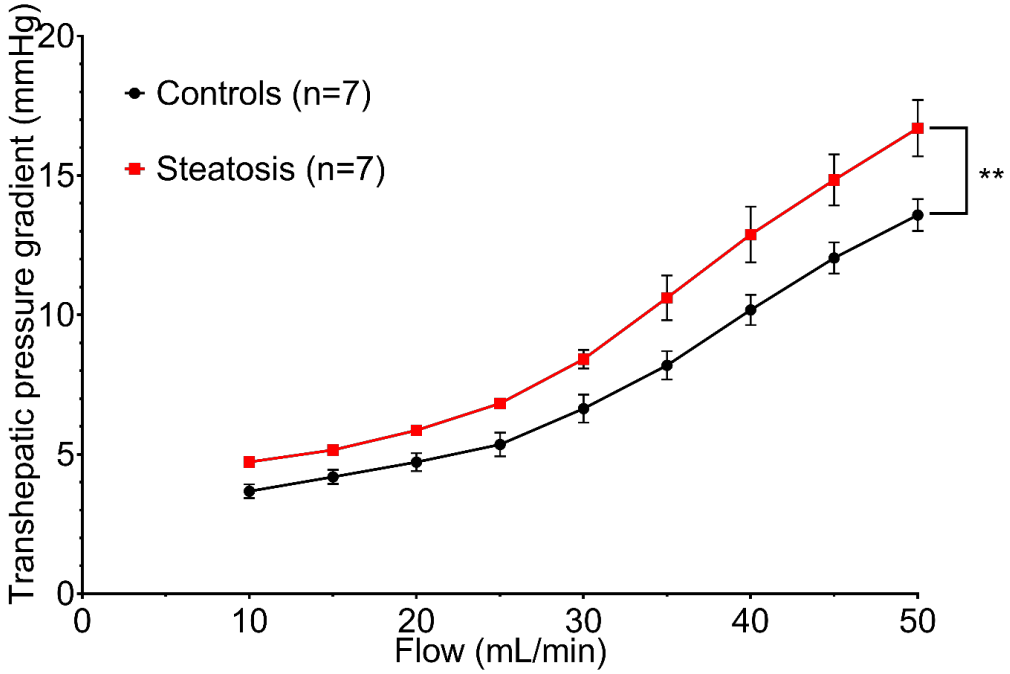


Fig. S6: Basal flow-pressure curves

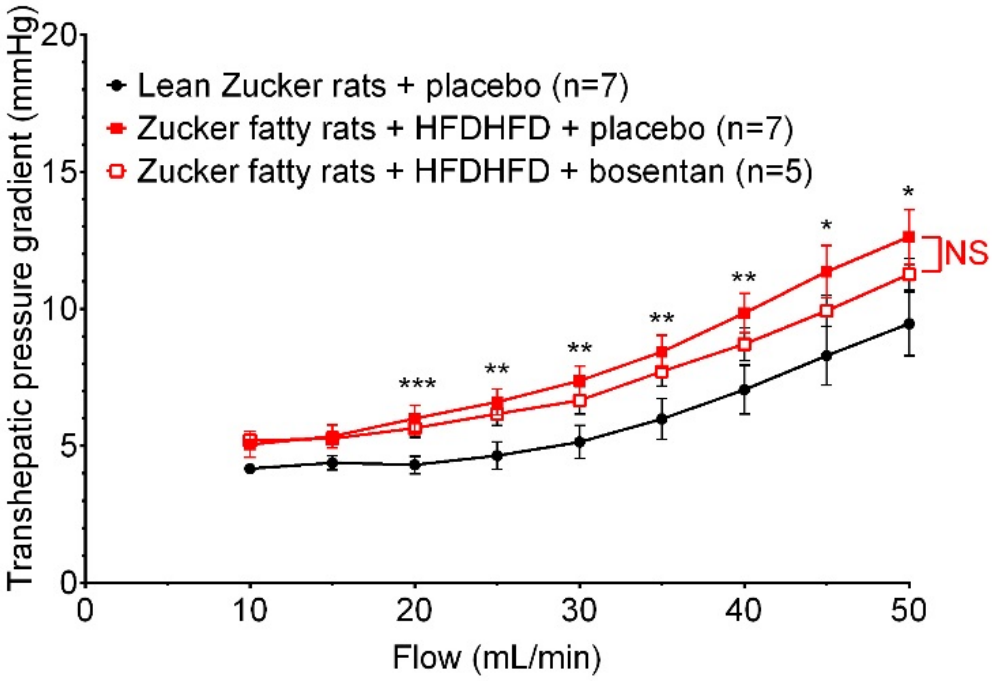
This graph shows the flow-pressure curve of the transhepatic pressure gradient (THPG) of male Wistar rats with control diet and Wistar rats with methionine choline deficient (MCD) diet.

All data were analysed using the generalised estimating equation model.

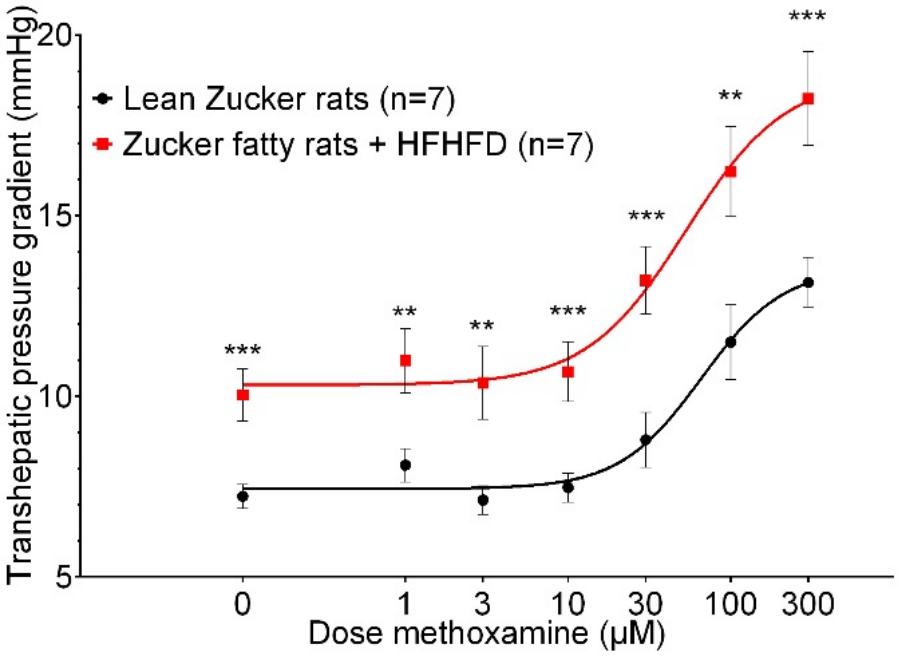
**=p<0.01

Fig. S7

A


Transhepatic pressure gradient (mmHg)
Flow (mL/min)
Lean Zucker rats + placebo (n=7)
Zucker fatty rats + HFDHFD + placebo (n=7)
Zucker fatty rats + HFDHFD + bosentan (n=5)
NS

B


Transhepatic pressure gradient (mmHg)
Dose methoxamine (μM)
Lean Zucker rats (n=7)
Zucker fatty rats + HFHFD (n=7)

C

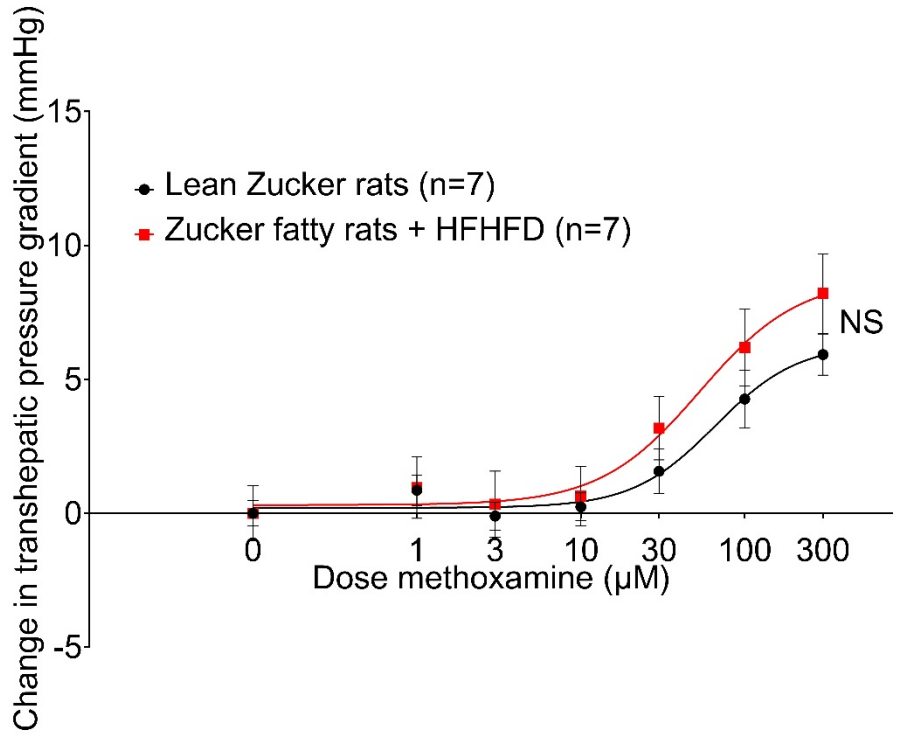


Fig. S7: Basal flow-pressure curve and methoxamine dose-response curve of the high fat high fructose-diet model of steatosis.

(A) Flow-pressure curve of the transhepatic pressure gradient (THPG) of Zucker fatty rats with high fat high fructose diet (HFHFD) with bosentan or placebo treatment and lean Zucker rats with control diet and placebo treatment in preventive set-up.

(B) Dose-response curve of the transhepatic pressure gradient (THPG) to alpha1-adrenergic agonist methoxamine or Krebs in Zucker fatty after HFHFD and lean Zucker rats after control diet.

(C) Baseline-corrected dose-response curve of the relative change in transhepatic pressure gradient (THPG) to alpha1-adrenergic agonist methoxamine or Krebs in Zucker fatty after HFHFD and lean Zucker rats after control diet.

All data were analysed using the generalised estimating equation model. Significances between Zucker fatty rats and lean Zucker rats are indicated in black. Significances between Zucker fatty rats with or without treatment are indicated in red.

*=p<0.05, **=p<0.01, ***=p<0.001, NS=not significant

Fig. S8

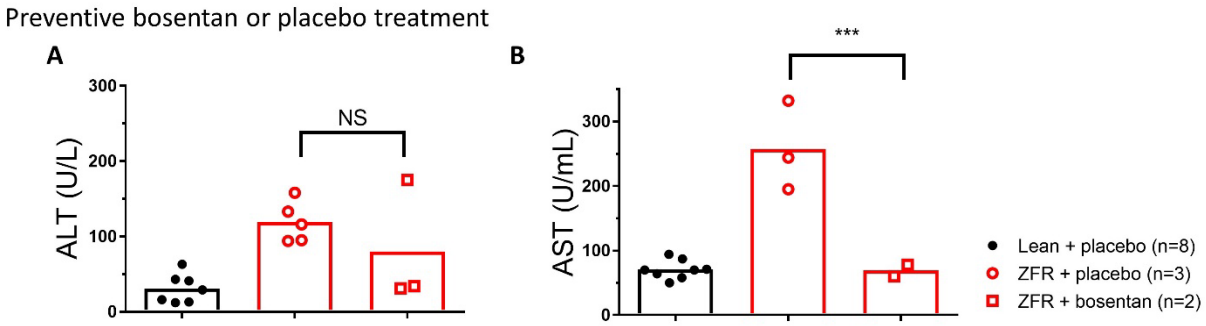


Fig. S8: Serum alanine and aspartate aminotransferases (ALT and AST, respectively) in lean Zucker rats fed a control diet and Zucker Fatty rats (ZFR) fed high fat high fructose diet during 4 weeks after preventive treatment with placebo or bosentan.

P-value compares ZFR with bosentan treatment to placebo treatment with $p<0.05$ considered statistically significant. Statistic comparison by two-way ANOVA and post-hoc Scheffé. NS = not significant, ***=$p<0.001$.

Table S1

Gene	CD vs. CD BOS	P value of: CD vs. CD BOS	Lower 95% CI of: CD vs. CD BOS	Upper 95% CI of: CD vs. CD BOS	CD vs. CD VAL	P value of: CD vs. CD VAL	Lower 95% CI of: CD vs. CD VAL	Upper 95% CI of: CD vs. CD VAL	CD vs. CD CEL	P value of: CD vs. CD CEL	Lower 95% CI of: CD vs. CD CEL	Upper 95% CI of: CD vs. CD CEL
Endothelin-related pathways												
Ece1	1,08	1,00	0,88	1,33	-1,03	1,00	0,81	1,16	1,05	1,00	0,90	1,22
Edn1	-1,03	1,00	0,91	1,04	-1,02	1,00	0,94	1,03	1,00	1,00	0,93	1,03
Edn2	1,10	1,00	0,77	1,55	-1,23	0,85	0,60	1,10	-1,11	0,99	0,63	1,28
Ednra	1,00	1,00	0,77	1,31	1,02	1,00	0,87	1,20	1,01	1,00	0,84	1,20
Ednrb	-1,06	1,00	0,80	1,13	-1,11	1,00	0,78	1,04	-1,10	1,00	0,79	1,04
Angiotensin-related pathways												
Ace	-1,15	1,00	0,69	1,09	-1,26	0,86	0,62	1,02	-1,21	0,95	0,64	1,07
Ace2	1,06	0,99	0,85	1,33	1,10	0,94	0,89	1,37	-1,02	1,00	0,77	1,26
Agt	1,06	0,99	0,91	1,25	1,05	1,00	0,93	1,18	1,05	0,99	0,94	1,17
Agtr1a	1,07	0,99	0,94	1,21	1,02	1,00	0,91	1,14	1,06	0,99	0,94	1,19
Cyclooxygenase-related pathways												
Lta4h	1,02	1,00	0,95	1,10	-1,07	0,87	0,85	1,03	-1,03	1,00	0,89	1,05
Ltc4s	1,28	0,86	0,75	2,19	1,81	0,07	1,06	3,08	1,46	0,45	0,85	2,51
Ptger3	1,06	1,00	0,86	1,32	1,15	0,92	0,91	1,45	1,00	1,00	0,79	1,27
Ptger4	1,04	1,00	0,88	1,24	-1,13	0,99	0,66	1,18	-1,09	1,00	0,73	1,17
Ptges	-1,16	0,96	0,66	1,13	-1,32	0,34	0,55	1,05	-1,17	0,96	0,66	1,10
Ptges3	1,15	0,91	0,93	1,43	1,12	0,96	0,92	1,36	1,14	0,89	0,96	1,35
Ptgfr	1,03	1,00	0,78	1,36	-1,00	1,00	0,77	1,30	-1,03	1,00	0,70	1,33
Ptgir	-1,09	0,99	0,73	1,15	1,02	1,00	0,81	1,27	1,04	1,00	0,75	1,45
Ptgis	1,04	1,00	0,69	1,56	-1,09	1,00	0,57	1,46	-1,24	1,00	0,51	1,28
Ptgs1	1,18	0,72	1,03	1,36	-1,06	0,99	0,77	1,15	1,07	1,00	0,94	1,21
Ptgs2	-1,01	1,00	0,72	1,38	-1,04	1,00	0,73	1,27	1,00	1,00	0,70	1,43
Tbxas1	1,18	0,99	0,91	1,54	-1,05	1,00	0,71	1,28	1,03	1,00	0,76	1,39

Gene	CD vs. MCD	P value of: CD vs. MCD	Lower 95% CI of: CD vs. MCD	Upper 95% CI of: CD vs. MCD BOS	CD vs. MCD BOS	P value of: CD vs. MCD BOS	Lower 95% CI of: CD vs. MCD BOS	Upper 95% CI of: CD vs. MCD BOS	CD vs. MCD VAL	P value of: CD vs. MCD VAL	Lower 95% CI of: CD vs. MCD VAL	Upper 95% CI of: CD vs. MCD VAL	CD vs. MCD CEL	P value of: CD vs. MCD CEL	Lower 95% CI of: CD vs. MCD CEL	Upper 95% CI of: CD vs. MCD CEL
Endothelin-related pathways																
Ece1	1,49	0,01	1,27	1,75	1,45	0,03	1,20	1,75	1,33	0,17	1,07	1,66	1,46	0,02	1,22	1,75
Edn1	-1,58	0,00	0,51	-1,05	-1,26	0,65	0,67	0,95	-1,40	0,10	0,57	-1,13	-2,01	0,00	0,43	-1,74
Edn2	1,49	0,30	1,14	1,79	1,36	0,57	1,03	1,79	1,26	0,85	0,93	1,71	1,26	0,86	0,93	1,69
Ednra	1,08	1,00	0,87	1,46	1,26	0,46	1,08	1,46	1,16	0,84	1,01	1,34	1,12	0,97	0,94	1,34
Ednrb	-1,38	0,19	0,62	-1,20	-1,52	0,01	0,52	0,83	-1,43	0,05	0,57	-1,16	-1,28	0,57	0,67	-1,09
Angiotensin-related pathways																
Ace	-1,56	0,04	0,50	-1,17	-1,50	0,11	0,52	0,85	-1,73	0,00	0,45	-1,36	-1,63	0,02	0,49	-1,29
Ace2	1,17	0,66	0,95	1,37	1,10	0,96	0,88	1,37	1,14	0,78	0,93	1,41	1,18	0,61	0,96	1,44
Agt	2,51	0,00	2,23	2,27	1,95	0,00	1,68	2,27	2,04	0,00	1,75	2,39	2,28	0,00	2,04	2,54
Agtr1a	1,54	0,00	1,37	1,71	1,45	0,00	1,23	1,71	1,16	0,69	0,96	1,39	1,70	0,00	1,51	1,90
Cyclooxygenase-related pathways																
Lta4h	1,06	0,96	0,97	1,08	-1,02	1,00	0,88	1,08	1,03	1,00	0,94	1,12	1,01	1,00	0,92	1,10
Ltc4s	8,25	0,00	5,49	12,42	8,25	0,00	5,49	12,42	8,25	0,00	5,49	12,42	8,25	0,00	5,49	12,42
Ptger3	1,14	0,94	0,89	1,56	1,23	0,65	0,97	1,56	1,22	0,71	0,95	1,58	1,11	0,98	0,88	1,40
Ptger4	-1,26	0,93	0,63	1,08	-1,15	1,00	0,71	1,08	-1,49	0,20	0,48	-1,07	-1,58	0,14	0,50	-1,25
Ptges	1,06	1,00	0,88	1,29	1,08	1,00	0,90	1,29	1,05	1,00	0,88	1,26	1,06	1,00	0,89	1,26
Ptges3	1,19	0,70	1,01	1,29	1,06	1,00	0,88	1,29	1,18	0,80	0,97	1,44	1,13	0,97	0,88	1,45
Ptgfr	1,19	0,94	0,85	1,65	1,22	0,87	0,90	1,65	-1,04	1,00	0,72	1,28	1,23	0,82	0,91	1,67
Ptgir	1,97	0,00	1,61	1,80	1,50	0,09	1,26	1,80	1,69	0,02	1,33	2,15	1,87	0,00	1,54	2,27
Ptgis	-1,86	0,84	0,34	-1,08	-1,86	0,76	0,31	0,93	-3,22	0,00	0,18	-1,90	-1,37	1,00	0,46	1,15
Ptgs1	1,17	0,81	0,99	1,31	1,11	0,98	0,93	1,31	1,18	0,77	1,00	1,40	1,05	1,00	0,90	1,21
Ptgs2	-1,15	1,00	0,65	1,11	-1,14	1,00	0,69	1,11	-1,44	0,42	0,48	1,01	1,04	1,00	0,81	1,34
Tbxas1	-1,55	0,09	0,51	1,16	-1,12	1,00	0,68	1,16	-1,28	0,74	0,58	1,06	-1,49	0,18	0,53	-1,18

Table S1: mRNA transcription of genes involved in endothelin-, angiotensin- and cyclooxygenase-related pathways.

Bosentan-, celecoxib-, valsartan and placebo-treated rats after 4 weeks of methionine choline deficient diet (MCD) were compared to control rats with placebo treatment (CD). Results were normalised in the nSolver Analysis Software (NanoString Technologies) by the geometric mean of 5 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase [*Gadph*], beta actin [*Actb*], β2-microglobulin [*B2m*], ribosomal protein lateral stalk subunit P2 [*Rplp2*] and phosphoglycerate kinase 1 [*Pgk1*]). Fold changes and 95% confidence intervals are demonstrated.

Table S2

Preventive treatment in controls				
	Placebo (n=8)	Bosentan (n=8)	Valsartan (n=7)	Celecoxib (n=8)
Body weight (g) at W4	356.5±6.5	348.4±4.9 p=0.665	344.1±11.1 p=0.635	341.9±4.9 p=0.151
Liver weight (g) at W4	11.8±0.4	11.5±0.4 p=0.999	11.6±0.7 p=1.00	13.2±0.5 p=0.111
Liver/total body weight-ratio (%) at W4	3.2±0.1	3.3±0.1 p=0.996	3.4±0.4 p=0.970	3.9±0.1 p=0.111
Preventive treatment in steatosis				
	Placebo (n=8)	Bosentan (n=8)	Valsartan (n=8)	Celecoxib (n=8)
Body weight (g) at W4	195.9±2.4	200.3±3.3 p=0.926	181.9±4.3 p=0.509	187,1±1.5 p=0.570
Liver weight (g) at W4	10.5±0.5	9.2±0.3 p=0.228	8.3±0.5 p<0.05	10.0±0.4 p=0.886
Liver/total body weight-ratio (%) at W4	5.4±0.2	4.6±0.1 p<0.05	4.6±0.2 p<0.05	5.3±0.2 p=1.00

Table S2: Total body weight, liver weight and liver/total body weight-ratio after 4 weeks of methionine choline deficient diet (steatosis) or control diet after preventive placebo, bosentan, valsartan and celecoxib treatment.

Results are presented as mean ± standard error of the mean. P-value compares treatment to placebo with the same diet and treatment duration ($p<0.05$ considered

statistically significant). Statistic comparison by two-way ANOVA and post-hoc Scheffé.

Table S3

	Lean Zucker rats + placebo (n=16)	Zucker fatty rats + placebo (n=16)	Zucker fatty rats + bosentan (n=8)
Body weight (g) at W4	294.4±8.9	468.1±10.3 p<0.001	468.1±12.9 p<0.001
Liver weight (g) at W4	11.6±0.6	21.3±0.6 p<0.001	25.3±0.8 p<0.001
Liver/total body weight-ratio (%) at W4	3.9±0.2	4.6±0.1 p<0.05	5.4±0.3 p<0.001
Body weight (g) at W8	344.8±11.2	526.3±24.5 p<0.001	555.7±10.1 p<0.001
Liver weight (g) at W8	10.4±0.6	22.7±1.4 p<0.001	23.8±0.7 p<0.001
Liver/total body weight-ratio (%) at W8	3.0±0.2	4.4±0.3 p<0.05	4.3±0.1 p<0.05

Table S3: **total body weight, liver weight and liver/total body weight-ratio in lean Zucker rats and Zucker fatty rats after 4 or 8 weeks of high fat high fructose diet**

(HFHFD) and preventive treatment with placebo or bosentan. Results are presented as the mean ± standard error of the mean. P-value compares value to placebo treatment in lean Zucker rats with $p<0.05$ considered statistically significant. Statistic comparison by two-way ANOVA and post-hoc Scheffé.

Table S4

	Controls + placebo (n= 4)	Steatosis + placebo (n = 3)	Steatosis + bosentan (n = 10)
MABP (mmHg)	127.1 ± 6.5	120.5 ± 7.3 p=0.743	119.4 ± 3.2 p=0.522
PR (BPM)	264.2 ± 13.0	237.2 ± 11.5 p=0.423	223.7 ± 8.6 p=0.061
PP (mmHg)	4.4 ± 0.3	5.7 ± 0.7 p=0.112	4.8 ± 0.2 p=0.725
ICVP (mmHg)	0.7 ± 0.1	0.7 ± 0.2 p=0.999	0.7 ± 0.1 p=0.978
THPH (mmHg)	3.7 ± 0.3	5.0 ± 0.6 p=0.062	4.1 ± 0.2 p=0.610

Table S4: In vivo pressure measurements in rats fed a control diet (controls) treated with placebo during 4 weeks, rats fed a methionine choline deficient diet (steatosis) treated with bosentan or placebo during 4 weeks.

MABP: mean arterial blood pressure; PR: pulse rate; BPM: beats per minute; PP: portal pressure; ICVP: inferior caval vein pressure; THPG: transhepatic pressure gradient.

Results are presented as the mean ± standard error of the mean. P-value compares value to placebo treatment in controls. Statistic comparison by two-way ANOVA and post-hoc Scheffé.