Supplementary Appendix

Manuscript title Altered gut-liver axis and hepatic adiponectin expression in OSAS: novel mediators of liver injury in paediatric non-alcoholic fatty liver

Authors: Valerio Nobili, Anna Alisi, Renato Cutrera, Guido Carpino, Cristiano De Stefanis, Valentina D'Oria, Rita De Vito, Salvatore Cucchiara, Eugenio Gaudio, Giovanni Musso

Supplementary Appendix content:

Supplementary Appendix Text

Methods

Exclusion of competing causes of steatosis. Exclusion criteria were hepatic virus infections (hepatitis A, B, C, D, E, and G; cytomegalovirus; and Epstein–Barr virus), excessive alcohol consumption (≥ 140 g/week), history of parenteral nutrition, and use of drugs known to induce steatosis (eg, valproate, amiodarone, or prednisone) or to affect body weight and carbohydrate metabolism. Autoimmune liver disease, metabolic liver disease, Wilson's disease, celiac disease, and alpha-1-antitrypsin deficiency were ruled out using standard clinical, laboratory, and histological criteria.

Proinflammatory markers and cytokines. Serum C-reactive protein (CRP) was determined via a high sensitivity latex agglutination method on HITACHI 911 Analyser (Sentinel Ch., Milan).

Serum adiponectin, TNF-α and IL-6 were measured by sandwich ELISA (R&D System Europe Ltd, Abingdon, UK). Sensitivity and intra-and inter-assay CVs of each kit are detailed in Online Data Supplement.

Markers of hepatocyte apoptosis and of fibrogenesis. Circulating cytokeratin-18 (CK-18) fragment levels, a validated marker of hepatocyte apoptosis in paediatric NAFLD, were measured by the M30- Apoptosense ELISA kit (PEVIVA) purchased from Li Starfish (Milan, Italy).

Markers of extracellular matrix deposition. Serum hyaluronic acid (HA), a marker of extracellular matrix deposition, was measured using an enzyme-linked binding protein assay (Hyaluronan; R&D Systems, Minneapolis, Minn)(detailed in Online Data Supplement).

Proinflammatory markers and cytokines. The kit for serum C-reactive protein (CRP) had a minimum detection of less than 0.05 mg/L, and a measurable concentration range up to 160 mg/L. The intra-assay and inter-assay variation coefficients were, respectively, 0.8–1.3 and 1.0–1.5%.

For TNF-α the kit has a sensitivity of 0.12 pg/mL in a 200-μL sample size and a range of 0.5 to 32 pg/mL. The intra-and inter-assay coefficients of variation were 5.9% and 12.6%, respectively.

For IL-6, the kit has a sensitivity of 0.25 pg/mL in a 50-μL sample size and a range of 3.9 to 250 ng/mL. The intra- and inter-assay coefficients of variation were 3.4% and 5.8%, respectively.

For adiponectin, the kit has a sensitivity of 2.47 ng/ml in a 200-μL sample size and a range of 0.1-1,000 ng/ml. The intra-and inter-assay coefficients of variation were 10% and 15%, respectively.

For leptin the kit has a sensitivity of 0.5 ng/ml in a 200-μL sample size and intra-and inter-assay coefficients of variation were 4.2% and 4.5%, respectively.

For resistin the kit has a sensitivity of 0.5 pg/ml in a 200-μL sample size and intra-and inter-assay coefficients of variation were 3.4% and 5.5%, respectively

For retinol binding protein(RBP)-4, the kit has a sensitivity of o.o1 mg/ml in a 100-μL sample size and a range of 0.1-1,000 mg/ml. The intra-and inter-assay coefficients of variation were 3.1% and 2.2%, respectively.

Markers of hepatocyte apoptosis. For all patients in our study, a blood sample was taken at the time of the liver biopsy. All samples were originally processed to yield plasma and stored at − 80° C . The samples were subsequently used for quantitative determination of CK18 levels by the M30-Apoptosense ELISA kit (PEVIVA) purchased from Li Starfish (Milan, Italy). All assays were performed in duplicates, and the absorbance was determined using a microplate reader (Molecular Bio-Rad, Milan, Italy).

Markers of extracellular matrix deposition. Serum hyaluronic acid (HA), a marker of extracellular matrix deposition, was collected at the time of liver biopsy and immediately stored at –80°C. HA was then measured using an enzyme-linked binding protein assay (Hyaluronan; R&D Systems, Minneapolis, Minn) and is reported as ng/mL. According to our previous published data [ref 9 of main text], a value of HA≥1200 ng/mL makes the absence of fibrosis unlikely (7%, 95% CI: 1% to 14%) and a value of HA≥ 2100 ng/mL makes significant fibrosis very likely (89%, 95% CI: 75% to 100%).

Plasma Lipopolysacchariide (LPS) measurement.

Part of collected blood was centrifuged at 3000 RPM for 12 minutes and plasma was stored at -80°C pending further analysis. Plasma LPS concentration was measured by a commercially available kit (Amebocyte Lysate [LAL] LAL Chromogenic Endpoint Assay.
Cambrex Limulus kit; Hycult Biotech, Uden, The Netherlands). This assay has a sensitivity range of 0.04–10.0 endotoxin unit (EU)/ml. All materials used for the assay were rendered LPS-free.

Intestinal permeability test. Patients followed a lactulose- and mannitol-free diet for 24 h before the test. After an overnight fast, they voided a pre-test urine sample and ingested a solution containing 5 g of lactulose and 1 g of mannitol in 120mL of deionised water. Urine was collected during the next 6 h, with 1 mL of chlorhexidine (1 mg/mL) added as preservative. One hour after

the test was started, patients were encouraged to drink 50–150 mL of tap water. Total urine volume was measured, and a 10-mL aliquot was stored at −20 °C until analysis. The fractional excretion of lactulose was calculated from the ratio mg lactulose excreted/mg lactulose assumed. The mg lactulose excreted were obtained from mg/L lactulose X litres of urine. The same was performed for mannitol. The values of lactulose and mannitol calculated in the pre-test urine as mg/L were subtracted from the same value obtained in the 6 h collected urine.

Liver histology. Liver biopsy was performed after an overnight fast, using an automatic core biopsy 18 gauge needle (Biopince, Amedic, Sweden) under general anesthesia and ultrasound guidance. A Sonoline Omnia ultrasound machine (Siemens, Munich, Germany) equipped with a 5-MHz probe (5.0 C 50, Siemens) and a biopsy adaptor was employed. The length of liver specimen was recorded: only samples with a length ≥15 mm and including at least 5–6 complete portal tracts were considered adequate for the purpose of the study. Biopsies were routinely processed (ie, formalin-fixed and paraffin-embedded) and sections of liver tissue were stained with hematoxylin-eosin, Van Gieson, Periodic acid-Schiff diastase, and Prussian blue stain. Biopsies were evaluated by a single hepato-pathologist, with a long-time experience in the field of liver pathology, who was blinded to clinical and laboratory data. Steatosis, inflammation, hepatocyte ballooning and fibrosis were scored using the NAFLD Clinical Research Network (CRN) criteria: Briefly, steatosis was graded on a 4-point scale: grade 0 = steatosis involving <5% of hepatocytes; grade 1 = steatosis involving up to 33% of hepatocytes; grade 2 = steatosis involving 33–66% of hepatocytes; and grade 3 = steatosis involving >66% of hepatocytes. Lobular inflammation was graded on a 4-point scale: grade 0 = no foci; grade 1 = <2 foci per 200×field; grade 2 = 2–4 foci per 200× field; and grade 3 = >4 foci per 200× field. Hepatocyte ballooning was graded from 0 to 2: 0 = none; 1= few balloon cells; and 2 =many/prominent balloon cells. The stage of fibrosis was quantified using a 5-point scale: stage 0 = no fibrosis; stage 1 = perisinusoidal or periportal (1a = mild, zone 3, perisinusoidal; 1b = moderate, zone 3, perisinusoidal; 1c = portal/periportal); stage 2

=perisinusoidal and portal/periportal; stage 3 = bringing; and stage 4 = cirrhosis. Additionally, the presence of Mallory bodies and portal fibrosis were noted.

Features of steatosis, lobular inflammation, and hepatocyte ballooning were combined to obtain the NAFLD activity score (NAS)**.** As recently recommended by NASH CRN, a microscopic diagnosis based on overall injury pattern (steatosis, hepatocyte ballooning, inflammation) as well as the presence of additional lesions (e.g. zonality of lesions, portal inflammation and fibrosis) has been assigned to each case. Accordingly, biopsies were subdivided into: not-NASH (not-NASH) and definite NASH subcategories.

Liver Immunofluorescence (IF).and immunohistochemistry (IHC)

IF staining for TLR4 was performed with anti-TLR4 incubated for 1 h (dilution 1:50; rabbit polyclonal, Novus Biological). After incubation with primary antibodies sections were revealed with Alexa Fluor 555-goat anti-rabbit (1:500), Alexa Fluor 488-goat anti mouse (1:500) purchased by Life Technologies, Carlsbad, CA, US). Nuclei were counterstained with DAPI for 5 min after extensive washing; sections were next mounted with PBS/glycerol (1:1) and covered with a coverslip.

The confocal microscopy imaging was performed by Olympus Fluoview FV1000 confocal microscope equipped with FV10-ASW version 2.0 software, using 20× and 40x objective. Optical single sections were acquired with a scanning mode format of 1024 × 1024 pixels, sampling speed of 40 μs/pixel, and 12 bits/pixel images. Fluorochromes unmixing was performed by acquisition of automated-sequential collection of multi-channel images, in order to reduce spectral crosstalk between channels.

Adipokine expression by HPCs.

For IHC, samples were then incubated for 20’ at room temperature (RT) with secondary biotinylated Abs and, successively, with streptavidin/horseradish peroxidase (LSAB+; code K0690; Dako Cytomation,Glostrup, Denmark). Diaminobenzidine (code K3468, Dako) was used as the

substrate, and the sections were counterstained with hematoxylin(the entire procedure was previously detailed) [ref 12 of main text].

HPC compartment was evaluated by counting the number of CK7-positive cells within the bile/reactive ductules by using a previously reported procedure [ref 12 of main text]. Solitary CK-7-positive HPCs or those in small clumps that were localized in the parenchyma or at the portal interface were included in these counts because they should be considered as a histological sectioning of bile/reactive ductules through a transverse plane without any unique IHC markers to distinguish them from cells within bile/reactive ductules . Cholangiocytes lining the interlobular bile ducts were excluded from the counts.

Supplementary Appendix Tables

Table 1. Primary antibodies used for liver immunohistochemistry and immunofluorescence .

Antibody	**Source**	**Vendor**	**Catalogue #**	**Dilution**
Cytokeratin-7	Mouse monoclonal	Dako, Glostrup, Denmark	M7018	1:100
Adiponectin	Mouse monoclonal	Abcam, Cambridge, United Kingdom	AB22554	1:100
Resistin	Mouse monoclonal	R&D Systems, Minneapolis, MN, USA	MAB1359	1:200
$p21^{waf1}$	Mouse monoclonal	Millipore Billerica (MA), USA,	MAB88058	1:20
Cleaved Caspase-3,	Mouse monoclonal	Cell Signaling Technology Boston (MA), USA	5A1E	1:100

Table 2. Main clinical, biochemical and histological features of study population, grouped according to obesity status (n=80).

	Non-obese NAFLD			Obese NAFLD		
Parameter	**Non-OSAS subjects (n=10)**	**OSAS subjects (n=10)**	**P**	**Non-OSAS (n=18)**	**OSAS (n=42)**	**P**
Age (yr)	11.7±1.4	12.0±1.6	0.650	11.5±2.3	11.2±2.3	0.656
Gender(% males)	60%	60%	0.999	61%	52%	0.347
BMI (kg/m^2)	21.8±1.5†	22.1±1.8†	0.738	29.2±4.6	29.8±4.3	0.735
BMI *z score*	1.18±0.39†	1.18±0.42†	0.957	2.16±0.42	2.23±0.30	0.501
Waist circumference (cm)	87.5±5.0†	88.1±3.2†	0.617	89.9±4.9	90.1±3.9	0.873
Waist circumference *z score*	1.71±0.77†	1.55±0.48†	0.384	2.11±0.86	2.11±0.79	0.992
Systolic BP(mmHg)	113±18	113±13	0.664	113±15	111±10	0.552
Diastolic BP(mmHg)	68±10	66±10	0.671	68±6	68±7	0.632
Insulin (μU/mL)	10.0±3.7	13.2±6.6	0.931	17.4±10.6	13.8±9.6	0.148
Glucose (mg/dL)	91±12	91±11	0.931	86±16	83±15	0.621
HOMA-IR	1.97±0.90∗	3.00±1.54	0.169	3.06±2.05	3.10±2.11	0.682
ISI	3.81±0.79 #	2.54±1.10	**0.013**	3.04±1.31	2.66±1.56	0.371
Triglyceride(mg/dL)	102±62	119±65	0.843	98±49	115±64	0.252
Total cholesterol (mg/dL)	158±20	148±31	0.430	155±29	168±39	0.167
HDL-cholesterol (mg/dL)	42±6	40±9	0.706	44±7	42±7	0.469
Subjects with diabetes(%)	0%	0%	0.999	6%	2%	0.479
Subjects with Met Sy (%)	10%	30%	0.371	11%	17%	0.513
Subjects with abdominal obesity(%)	30%	29%	0.982	88%	86%	0.712
Subjects with hypertension(%)	20%	20%	0.999	25%	17%	0.391
Subjects with hypertriglyceridemia (%)	30%	30%	0.999	30%	40%	0.479
Subjects with low HDL-C(%)	25%	40%	0.289	47%	45%	0.876
Subjects with IFG/diabetes(%)	20%	40%	0.275	11%	22%	0.394

Adiponectin(ng/mL)	23.2±2.4*	21.8±2.0	**0.029**	22.6±2.3	20.6±1.6	**0.010**
Resistin (pg/mL)	22294±5982	28196±6894	0.427	31024±10168	27308±12407	0.423
RBP(mg/mL)	3.2±0.5	3.1±0.8	0.704	2.8±1.3	3.1±1.2	0.214
C-reactive protein (mg/L)	1.4±0.4 #	1.9±0.3	**0.028**	1.6±0.4	1.9±0.4	**0.028**
IL-6(pg/mL)	12.2±4.2	11.9±5.7	0.875	10.4±6.4	11.2±5.8	0.695
Leptin(ng/mL)	21.4±5.9	19.1±7.9	0.389	17.7±8.1	20.1±9.5	0.406
TNF-α(pg/mL)	6.7±2.0	7.4±1.9	0.476	6.9±2.2	7.1±2.3	0.585
L/M ratio	0.021±0.018#	0.045±0.027	**0.031**	0.022±0.022	0.061±0.041	**<0.001**
Pathologic L/M ratio n(%)	0	4	**0.043**	3	22	**0.011**
LPS(EU/mL)	1.78±0.46‡	2.57±0.51*	**0.002**	2.06±0.49	2.89±0.34	**<0.001**
AHI(events/hr)	0.50±0.27†	4.54±2.03*	**<0.001**	0.55±0.33	5.00±1.13	**<0.001**
Oxygen desaturation index (ODI)	0.60±0.42 #	1.45±0.64§	**0.019**	0.60±0.39	1.60±0.44	**0.001**
TST (hr)	7.8±0.9	8.1±0.9	0.198	7.91±0.71	9.03±0.64	0.523
Mean SaO2(%)	95.3±0.8#	93.5±0.5	**0.011**	95.5±1.0	92.5±1.0	**0.003**
Nadir SaO2 (%)	92.2±1.3#	87.5±1.1	**0.021**	91.6±1.1	84.6±1.8	**<0.001**
SaO2<90% (%)	14±12‡	29±18	**<0.001**	15±9	41±31	**<0.001**
ETpCO2(mmHg)	42.4±2.4‡	44.9±2.2	**0.044**	44.2±3.00	49.8±7.5	**<0.001**
Subjects with severe OSAS(%)	0% #	4(40%)	0.087	0%	19(45%)	**<0.001**
AST(IU/L)	30±12#	36±17	0.146	30±19	44±21	0.676
ALT(IU/L)	30±16#	36±17	0.231	33±16	42±18	0.111
GGT(IU/L)	16±5#	17±5	0.913	19±9	22±18	0.328
CK18 (U/L)	179±44*	270±66	**0.009**	232±61	321±68	**<0.001**
Hyaluronic acid (ng/mL)	893±479†	1003±546#	0.351	1102±461	1532±397	**0.040**
Subjects with NASH (%)	0%†	60%§	**0.004**	11%	98%	**<0.001**
NAS score	3.2±0.9 #	4.6±1.1 #	**0.012**	3.9±0.8	5.7±0.9	**<0.001**
Subjects with fibrosis stage (%)						
0	50%	0%	**0.033**	34%	0%	**<0.001**
1	50%	60%	0.859	53%	44%	0.439
1a	0%	0%	0.999	0%	0%	0.999

1b **1c** **2** **3** **4**	50% 0% 0% 0% 0%	0% 60% 40% 0% 0%	**0.033** **0.011** 0.087 0.999 0.999	20% 33% 13% 0% 0%	5% 39% 50% 6% 0%	0.293 0.714 **0.032** 0.612 0.999
Fibrosis any stage(%)	50%	100%	**0.033**	66%	100%	**0.010**
Significant (F≥2) fibrosis (%)	0%	40%§	0.087	13%	50%	**0.011**
Portal fibrosis n(%)	0%	100%§	**<0.001**	46%	89%	**0.007**

Data are reported as mean±SD, unless otherwise specified. Differences were considered statistically significant at $p<0.05$. Statistically significant differences are written in bold characters.

Abbreviations:

AHI (Apnea/hypopnea index): number of mixed/obstructive apnea/hypopneas per hour of sleep;

HOMA-IR: Homeostasis model assessment of insulin resistance;

ISI Insulin sensitivity index;

ODI (Oxygen desaturation index): number of haemoglobin desaturations (drop in Sa02 **≥4%** of baseline value) per hour of sleep.

TST: total sleep time(min);

SaO2<90%: total duration of desaturation, expressed as percentage of total sleep time (% TST);

ETpCO2: mean end-tidal carbon dioxide pressure

CK18: cytokeratin-18 fragments

NAS: NAFLD Activity Score;

Obesity: BMI≥95th percentile; abdominal obesity: waist circumference ≥90th percentile for age and sex; severe OSAS: an AHI≥5 events/hr.

∗ $p<0.05$ vs. obese subjects

†$p<0.01$ vs. obese subjects

§ $p<0.05$ vs. obese non-OSAS subjects

#$p<0.05$ vs. obese subjects with OSAS

‖ $p<0.01$ vs. obese non-OSAS subjects

‡ $p<0.01$ vs. obese subjects with OSAS

Table 3. Liver immunohistochemistry and immunofluorescence parameters of study population, grouped according to obesity status (n=80).

	Non-obese NAFLD			Obese NAFLD		
Parameter	**Non-OSAS subjects (n=10)**	**OSAS subjects (n=10)**	**P**	**Non-OSAS (n=18)**	**OSAS (n=42)**	**P**
Hepatocytes						
p21^{waf1} index‖	4.51±2.13∗	11.00±5.12§	**0.002**	8.13±5.58	15.93±8.12	**<0.001**
Apoptotic index ‖	5.89±5.12∗	12.02±5.31§	**0.028**	9.03±4.02	15.89±6.19	**<0.001**
Adiponectin-positive hepatocytes (%)	25.1±10.1#	9.0±5.7§	**<0.001**	23.1±9,5	8.5±5.5	**<0.001**
Resistin-positive hepatocytes(%)	18.9±12.1	16.4±12.1	0.412	13.3±12.1	15.9±11.4	0.782
TLR-4-positive hepatocytes†	38.6±4.9∗	49.0±8.4§	**0.003**	43.6±8.9	66.1±12.1	**<0.001**
Kupffer cells						
n-Kupffer cells †	7.5±1.3#	10.8±1.7§	**0.002**	7.7±2.4	11.4±2.0	**<0.001**
TLR-4-positive Kupffer cells						
n-cells	2.1±1.5∗	5.8±1.9§	**<0.001**	2.6±1.1	6.6±2.0	**<0.001**
% cells	27.9±13.4∗	53.8±17.1§	**<0.001**	33.5±11.5	58.1±11.7	**<0.001**
Hepatic stellate cells (HSCs)						
n-HSCs †	4.9±1.2#	6.4±1.5§	**0.024**	5.2±1.3	6.6±1.4	**<0.001**
TLR-4-positive HSCs						
n-HSC	1.5±1.1#	3.5±1.0§	**<0.001**	1.5±1.0	3.6±1.3	**<0.001**
% HSCs	27.0±10.2#	45.8±11.6§	**<0.001**	27.2±10.3	50.1±9.9	**<0.001**
Hepatic progenitor cells (HPCs)						
n-HPCs ‡	32.2±8.3#	46.8±7.1§	**<0.001**	39.6±8.0	47.8±8.3	**<0.001**
Adiponectin-positive HPCs						
n-HPCs	5.0±2.0#	3.2±1.8§	**0.032**	4.2±1.7	2.9±1.3	**<0.001**
% HPCs	13.4±6.1#	7.1±5.2§	**0.022**	9.9±4.1	6.0±3.7	**<0.001**
Resistin-positive HPCs						
n-HPCs	8.1±4.3	8.7±3.9	0.450	8.8±4.9	9.3±5.1	0.775
% HPCs	22.8±8.1	21.6±9.7	0.712	21.9±9.1	22.7±9.0	0.812
Intermediate Hepatocytes (% patients)	0%	2(20%)	0.475	0%	9(21%)	**0.047**

Data are reported as mean±SD, unless otherwise specified. Differences were considered statistically significant at $p<0.05$. Statistically significant differences are written in bold characters. Abbreviations: HSCs: hepatic stellate cells; HPC: hepatic progenitor cells; TLR-4: toll-like receptor-4

∗p<0.01 vs. obese subjects

§ p<0.05 vs. obese non-OSAS subjects

# p<0.05 vs. obese subjects with OSAS

† mean count in 5 different fields at a magnification of x20 under light microscopy (verificare)

‡ cells per HPF, i.e., at a magnification of x 20 under light microscopy

‖ at least 30 lobular fields at x40 magnification were analyzed (1,000 hepatocytes) for each section.

Supplementary Appendix Figures

Figure 1.Representative confocal immunofluorescence of TLR-4 co-receptor localization in liver-resident cells. The representative confocal immunofluorescence was performed on liver tissue sections. Co-staining of TLR-4 (red) with CD68, α-SMA or C8/18 (green) in children without NASH (**Not NASH**) and with NASH (**NASH**). The nuclei are revealed by specific DAPI staining, displayed in blue. Magnification x40.

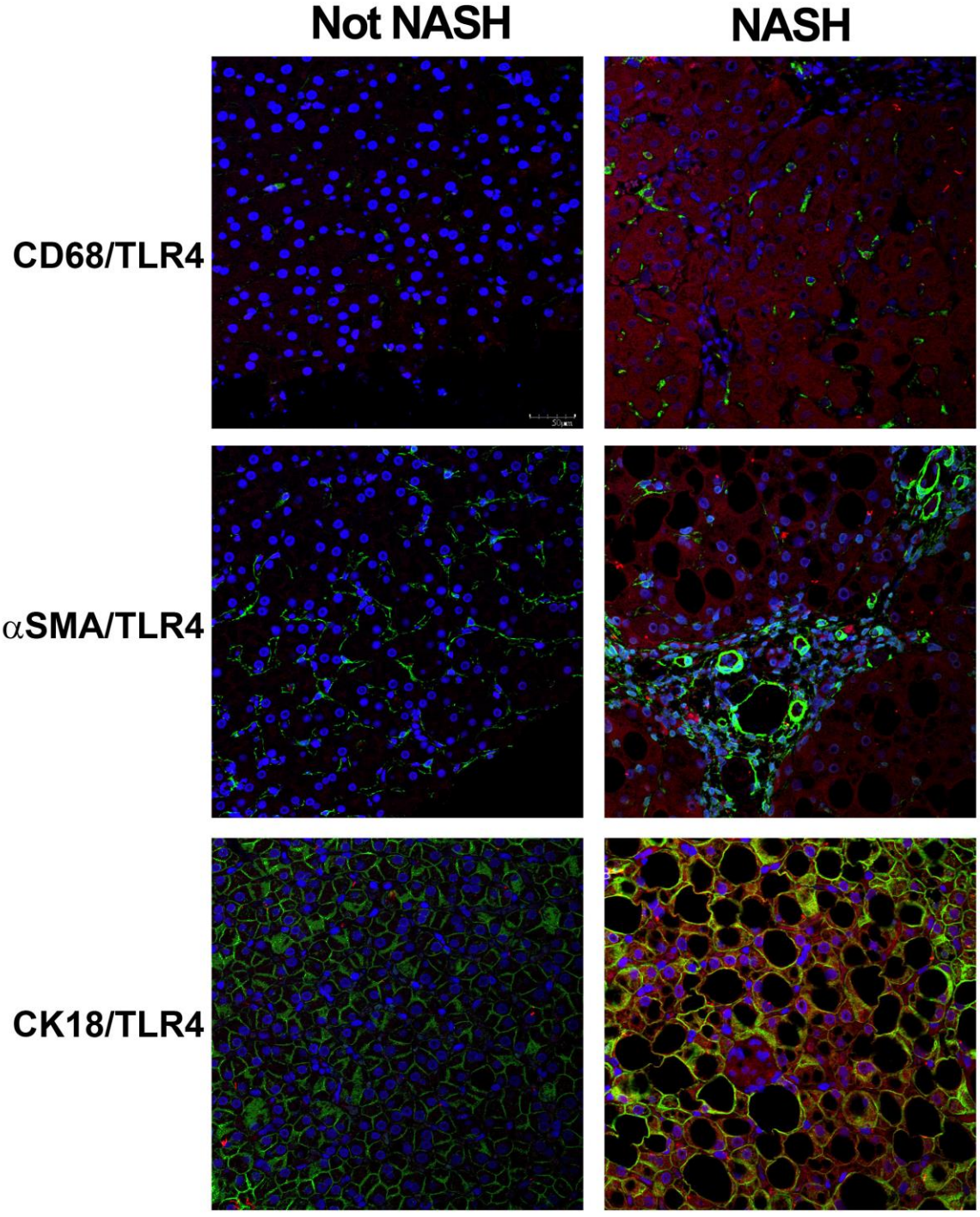
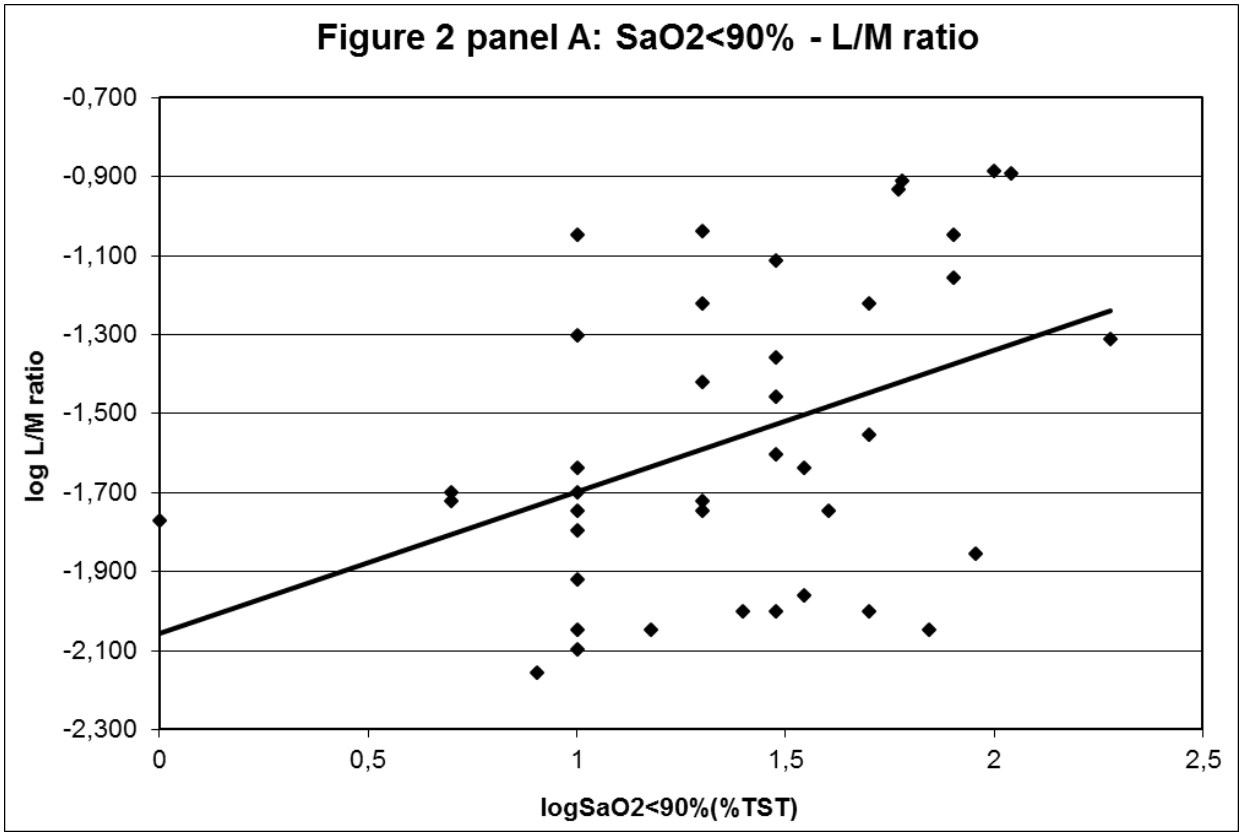


Figure 2: correlation of total duration of haemoglobin desaturation on PSG, expressed as SaO2<90%(%total sleep time, %TST), with intestinal permeability, expressed as L/M ratio (panel A), with plasma LPS (panel B), with % hepatocytes expression toll-like receptor 4(TLR4) on liver immunofluorescence (panel C), with the number of Kupffer cells expressing TLR4 (panel D) and with the number of hepatic stellate cells (HSCs) expressing TLR4 (panel E). Variables with skewed distribution were log-transformed.



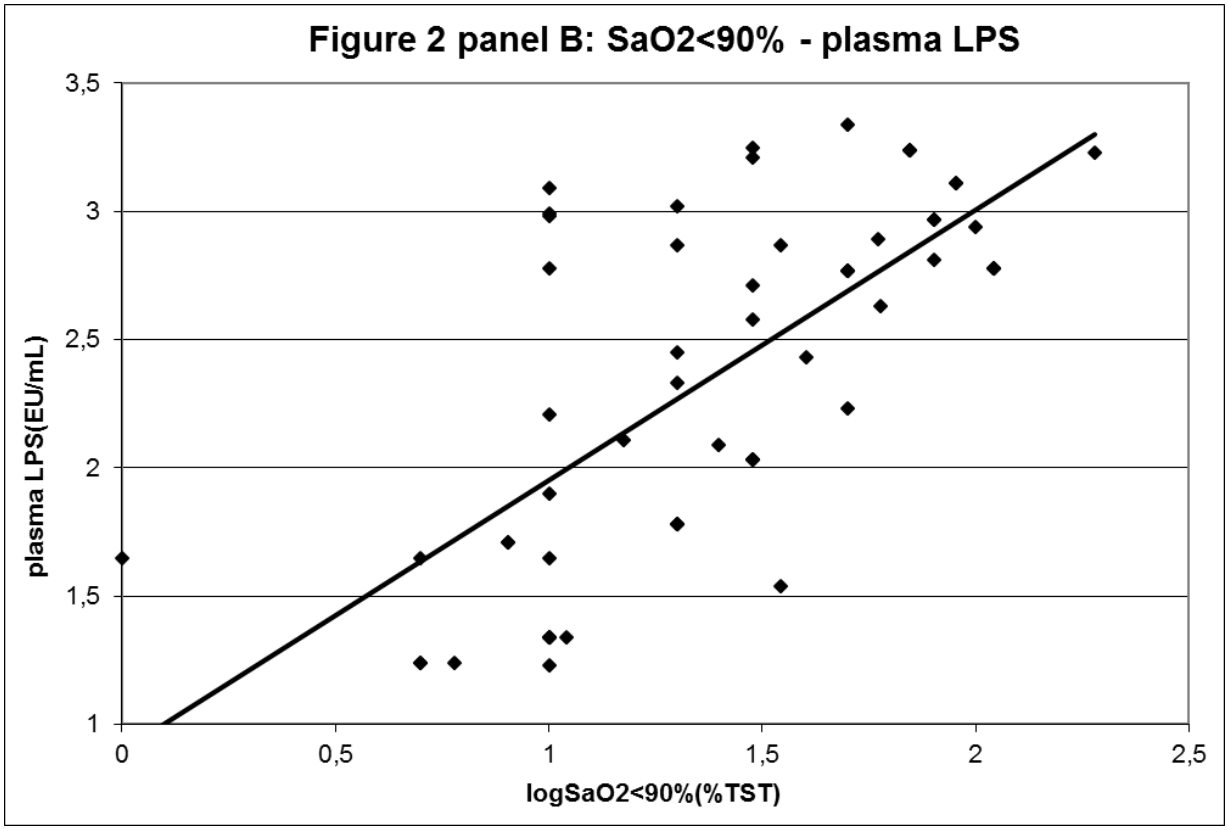


Figure 2 panel B: SaO2<90% - plasma LPS
plasma LPS(EU/mL)
3,5
3
2,5
2
1,5
1
0
0,5
1
1,5
2
2,5
logSaO2<90%(%TST)

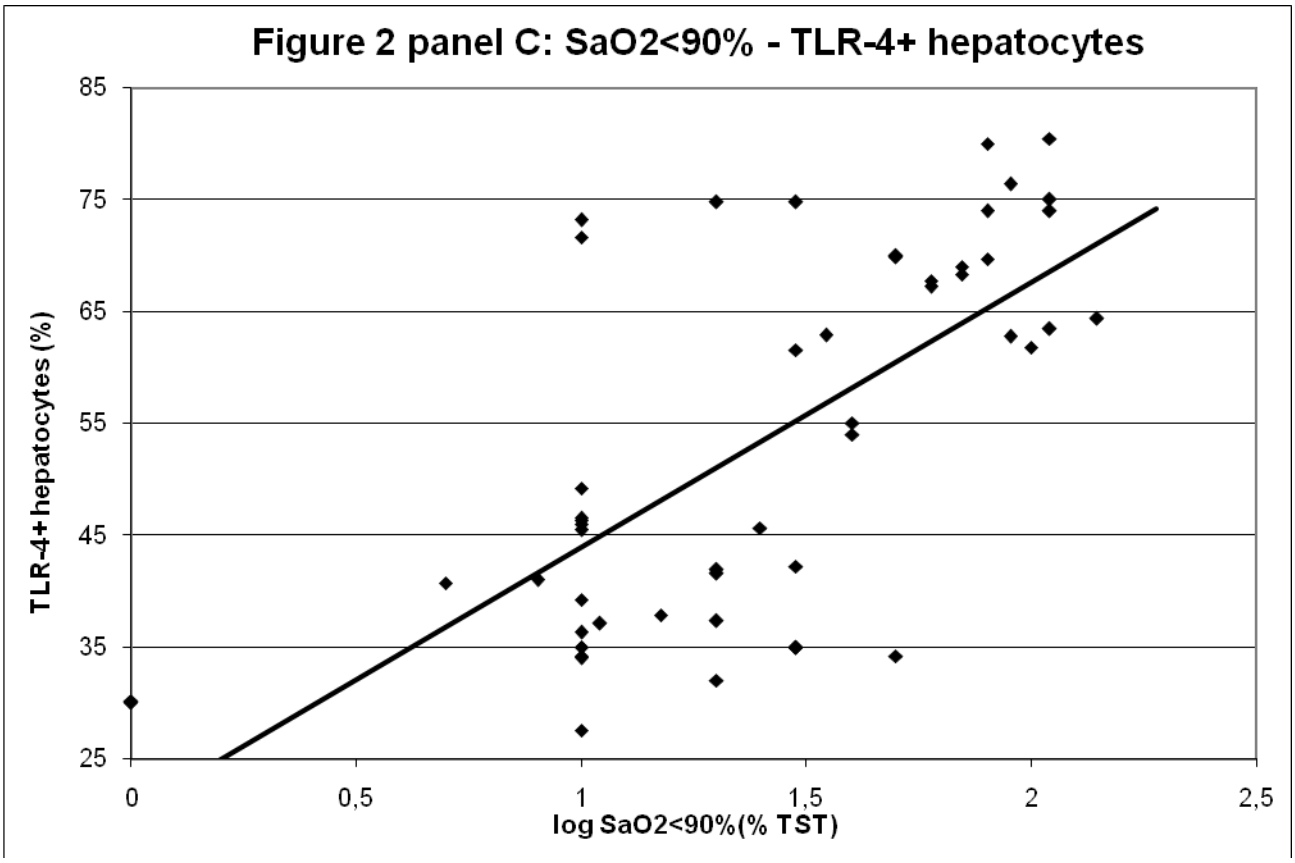


Figure 2 panel C: SaO2<90% - TLR-4+ hepatocytes
TLR-4+ hepatocytes (%)
85
75
65
55
45
35
25
0
0,5
1
1,5
2
2,5
log SaO2<90%(% TST)

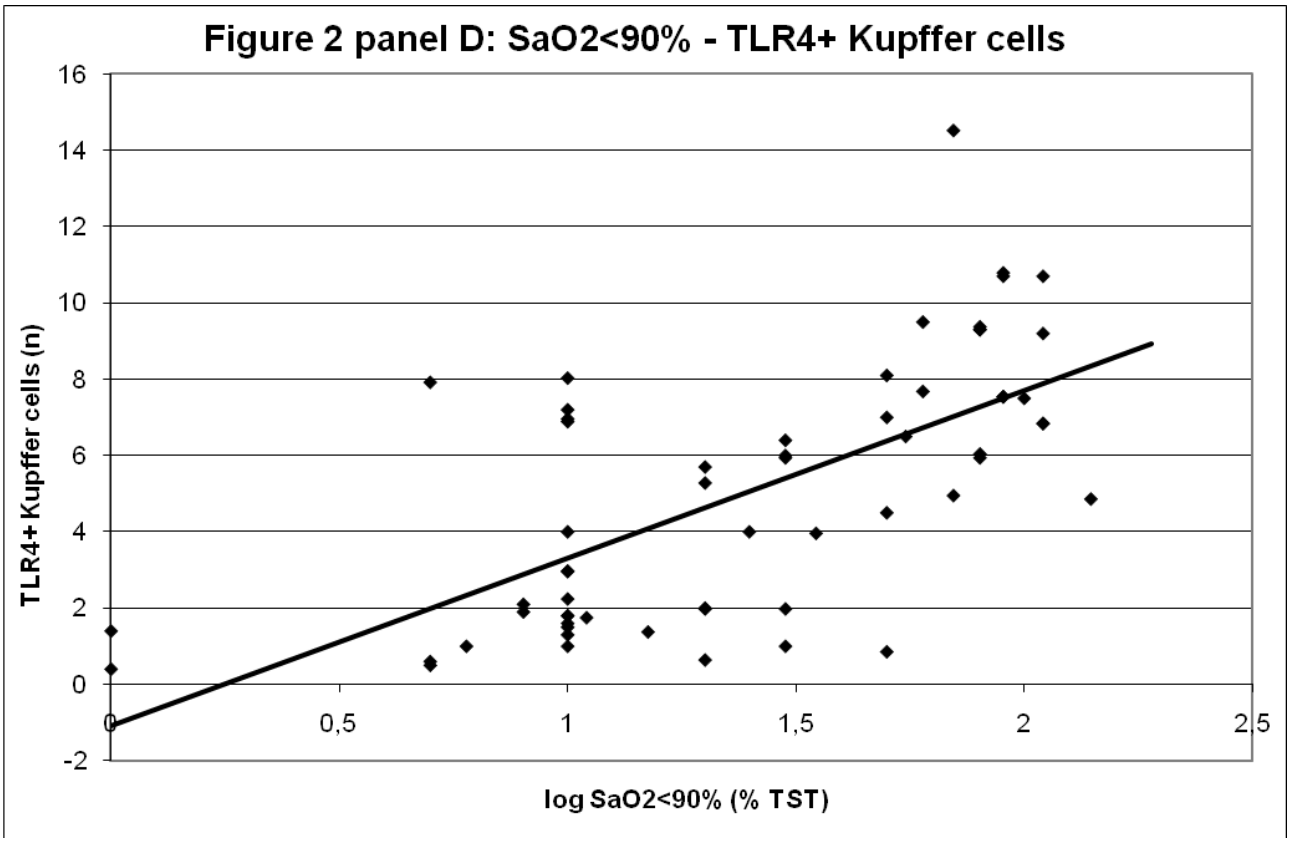


Figure 2 panel D: SaO2<90% - TLR4+ Kupffer cells
TLR4+ Kupffer cells (n)
16
14
12
10
8
6
4
2
0
-2
0
0,5
1
1,5
2
2,5
log SaO2<90% (% TST)

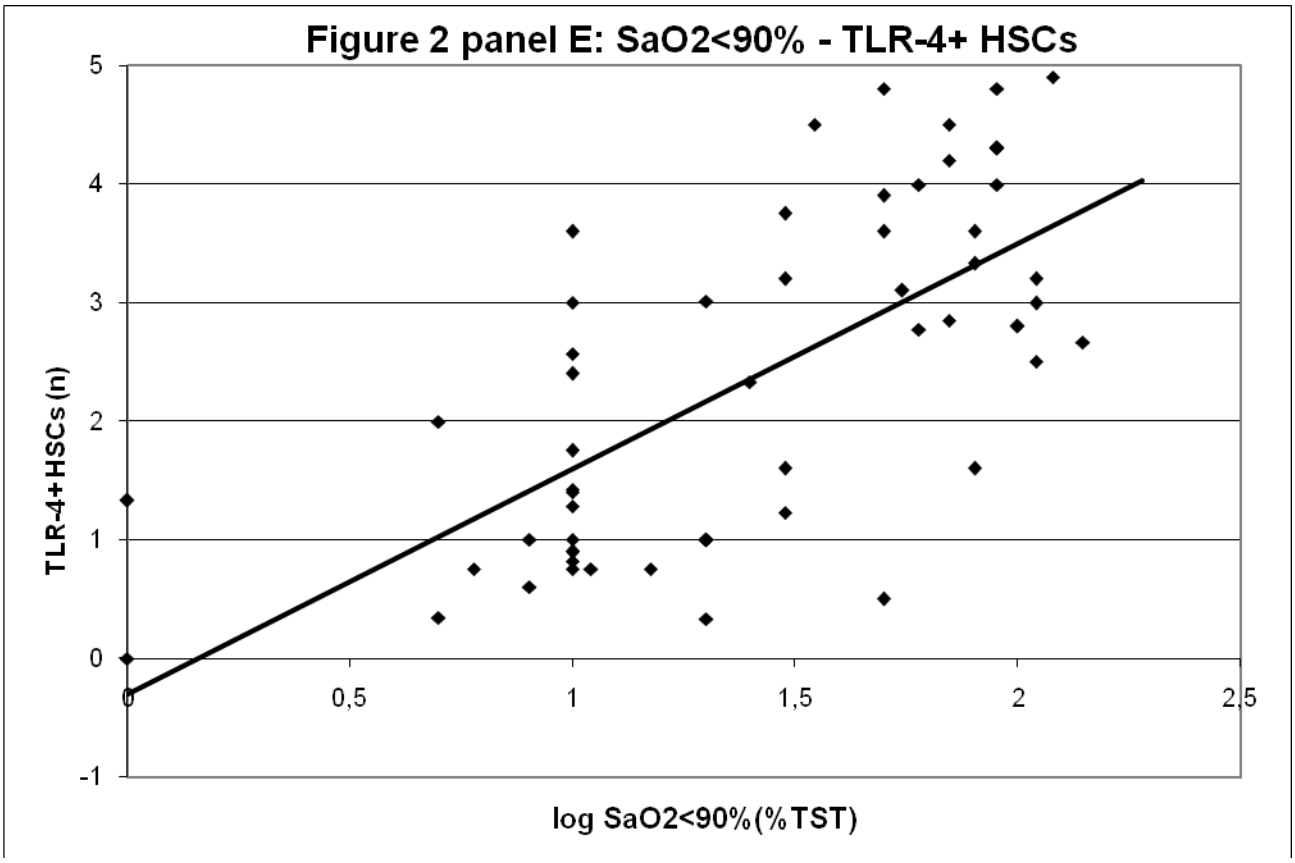


Figure 2 panel E: SaO2<90% - TLR-4+ HSCs
TLR-4+HSCs (n)
5
4
3
2
1
0
-1
0
0,5
1
1,5
2
2,5
log SaO2<90%(%TST)