Diabetes Care In Press, published online March 2, 2007

Insulin resistance in liver cirrhosis is not associated with circulating Retinol Binding Protein 4

Received for publication 13 November 2006 and accepted in revised form 13 February 2007.

Eray Yagmur[1], M.D.; Ralf Weiskirchen[1], PhD; Axel M. Gressner[1], M.D.;
Christian Trautwein[2], M.D.; Frank Tacke[2], M.D., PhD

[1]Institute of Clinical Chemistry and Pathobiochemistry, and [2]Medical Clinic III, RWTH-University Hospital Aachen, Germany

Short title: RBP4 in chronic liver disease

Corresponding author:
Frank Tacke, M.D., PhD
Medical Clinic III
RWTH-University Hospital Aachen
Pauwelsstraße 30
52074 Aachen
Germany
E-mail: frank.tacke@gmx.net

Copyright American Diabetes Association, Inc., 2007

Abstract

Objective: Retinol Binding Protein 4 (RBP4) has been identified as a novel adipokine mediating systemic insulin resistance, and elevated serum RBP4 indicated overt or impending insulin resistance in lean, obese and type 2 diabetic subjects. As insulin resistance is present in nearly all patients with liver cirrhosis, we evaluated RBP4 in patients with chronic liver diseases (CLD).
Research Design and Methods: Serum RBP4 was measured 111 CLD patients. 99 age- and sex-matched healthy blood donors served as controls. RBP4 gene expression was also quantified in normal and cirrhotic rat liver.
Results: In CLD patients, serum RBP4 was significantly reduced compared with healthy controls, and closely correlated with the stage of liver cirrhosis. CLD patients without cirrhosis showed normal RBP4 concentrations, which correlated with serum glucose, insulin secretion and inversely with insulin sensitivity. In patients with Child A-C liver cirrhosis, however, RBP4 was not correlated with glucose metabolism or other adipokines such as adiponectin or resistin, but closely linked to the hepatic biosynthetic capacity, fibrotic changes in liver histology or clinical complications such as portal hypertension. In an animal model of experimental cirrhosis, hepatic RBP4 gene expression decreased in cirrhotic liver.
Conclusions: RBP4 appears, unlike in obesity or type 2 diabetes, not to be a relevant systemic factor in the pathogenesis of insulin resistance in liver cirrhosis. Liver function has a tremendous impact on RBP4 levels, and future studies will need to take liver function into account when examining serum RBP4 levels.

Recent findings assigned Retinol Binding Protein 4 (RBP4) a key role in the pathogenesis of insulin resistance associated with type-2-diabetes and obesity. Adipose RBP4 expression and serum RBP4 levels were elevated in mouse models of insulin resistance, and elevated circulating RBP4 increased blood glucose by inhibiting insulin signalling in skeletal muscle and upregulating hepatic gluconeogenesis (1). These results have been translated into the pathogenesis of insulin resistance in humans. Elevated serum RBP4 concentrations were an independent predictive biomarker at early stages of insulin resistance and identified individuals at risk of developing diabetes (2,3). Serum RBP4 correlated positively with the presence of insulin resistance in individuals with obesity, impaired glucose tolerance, or type-2-diabetes and was even increased in healthy individuals with a strong family history of diabetes (2).

Hyperinsulinemia and glucose intolerance are present in nearly all patients with liver cirrhosis (4,5), and insulin resistance is an established risk factor for disease progression and survival in CLD (4-8). The aim of our study was to investigate RBP4's potential pathogenetic role in insulin resistance in patients with chronic liver diseases (CLD).

Research Design and Methods

Patients and healthy controls

Written informed consent was obtained from all participants, and the study protocol was approved by the local ethics committee. We studied 111 Caucasian CLD patients who were evaluated as inpatients for potential liver transplantation (9). Patients with overt diabetes mellitus were excluded; none of the patients received insulin or oral antidiabetic/insulin-sensitizing medication or had a history of taking these medications. Body-mass-index (BMI) for CLD patients were calculated subtracting the rated volume of ascites and pleural effusions (by ultrasound) from body weight (10).

18 patients (16%) suffered from CLD without having cirrhosis, according to Child-Pugh's score and clinical criteria (Table 1); among these patients, the majority was evaluated for liver transplantation due to malignancies or hereditary disorders. The Child-Pugh and the model for end stage liver disease (MELD) score were assessed as independent prognostic predictors in patients with cirrhosis (11). Liver biopsies were performed in 65/111 patients and semiquantitatively evaluated by a blinded and experienced pathologist (12).

99 age- and sex-matched blood donors (Table 1) with normal aminotransferases, blood counts, fasting glucose and negative markers for viral hepatitis and HIV served as controls.

RBP4 serum concentrations

Serum RBP4 concentrations were measured by ELISA (ALPCO Diagnostics) (2), with coefficients of intra-/inter-assay variation of 5% and 9.8%. Serum resistin, adiponectin and C-peptide were measured as described (9,12). Insulin sensitivity was assessed by HOMA-S (homeostasis model assessment) index, calculated from fasting glucose and C-peptide (13).

Experimental model of liver fibrosis

The common biliary duct was double-ligated under halothane anesthesia in male Sprague-Dawley rats (14). Age- and sex-matched sham-operated animals served as controls. Rats were sacrificed after 14 days, and Sirius Red staining was performed on liver tissue sections to determine liver fibrosis (14). All animal experiments were approved by the German authorities.

RBP4 gene expression

RBP4 expression was quantified from normal and cirrhotic rat liver by real-time PCR (LightCycler, Roche) using two primer combinations (5'-TGCAGGGTGAGCAGCTTCAG-3', 5'-CACTTCCCAGTTGCTCAGAAG-3' or 5'-

TTAGCTCTCATCCAGTCTTC-3', 5'-GGAATCCCAAGCCTCAAACG-3'). The average of the measured crossing points (C_T values) was then normalized to GAPDH expression (15). The fold-increase in mRNA between the experimental groups was calculated by 2exp(delta-delta C_T).

Statistical analysis

Correlation analyses were done by Spearman rank correlation test. Comparisons between two groups were conducted with Mann-Whitney-U-test, multiple comparisons with Kruskal-Wallis analysis of variances (ANOVA) and Mann-Whitney-U-tests for post hoc analysis. Statistical analyses were performed using SPSS.

Results

Serum RBP4 is reduced in liver cirrhosis and directly related to disease severity and liver function

The median serum concentration of RBP4 in healthy controls was 35.5 mg/L (range 10.5-85.0), as anticipated from previous literature using the same assay (2). Lean controls (median BMI 22.8, RBP4 29.3 mg/L) had significantly lower RBP4 as compared to obese controls (median BMI 28.4, RBP4 37.5 mg/L, P<0.001). Patients with CLD showed significantly reduced RBP4 than controls (median 11.5 mg/L, range 1.0-117.0, P<0.001, Table 1), even if strictly matched to BMI. The decrease in RBP4 was directly related to the stage of liver cirrhosis, as defined by Child-Pugh score (r=-0.467, P<0.001; Fig.1A). Whereas CLD patients without liver cirrhosis (n=18, median 30.0 mg/L) did not differ from healthy controls, RBP4 significantly decreased between all stages of cirrhosis with the lowest level in Child C cirrhosis (median 4.3). RBP4 also inversely correlated with the MELD score (r=-0.264, P=0.005, Fig.1B), as an alternative assessment of disease severity. RBP4 was not associated with the patients' age or gender (data not shown).

In addition, serum RBP4 directly correlated with the liver's biosynthetic capacity (*all* P<0.001), e.g. cholinesterase activity (r=0.639, Fig.1C), serum albumin (r=0.482), coagulation factors II (r=0.641), V (r=0.633), VII (r=0.647) and XIII (0.495), and inversely with the prothrombin time (r=-0.546). No correlation was observed between RBP4 and renal function, e.g. serum creatinine or creatinine-clearance (not shown).

Serum RBP4 is not related with insulin resistance in liver cirrhosis

As we and others described previously (4,9), insulin resistance was common among CLD patients and clearly linked to the severity of liver cirrhosis. Hyperinsulinemia, reduced insulin sensitivity (HOMA-S) and elevated serum resistin were related to progression of cirrhosis (Table 1). In obese volunteers or diabetic patients with normal liver function, serum RBP4 was described to directly correlate with insulin resistance, impaired glucose tolerance or BMI (2). In the 18 CLD patients without cirrhosis, we also observed this relationship, as RBP4 correlated with fasting glucose, insulin secretion (C-peptide) and inversely with insulin sensitivity (HOMA-S, Fig.2). However, in the 93 patients with Child A-C cirrhosis, no correlation was found for RBP4 with fasting glucose, C-peptide, HOMA index, BMI or adipocytokines such as adiponectin or resistin (Fig.2, and data not shown).

Lowest RBP4 in patients with histological cirrhosis and typical clinical complications

Decreasing serum RBP4 levels were closely associated with the histological degree of fibrosis or cirrhosis (Fig. 3A), but not to hepatic steatosis (not shown). Furthermore, clinical complications typically found in patients with advanced cirrhosis were also associated with reduced RBP4 levels, e.g. ascites (median RBP4 5.5 mg/L vs. 14.5, P<0.001, Fig.3B), splenomegaly (9.5 mg/L vs. 21, P<0.001) or esophageal varices (8.0 mg/L vs. 17.3, P<0.001,

Fig.3B). No difference was found in patients with (n=15) or without (n=96) hepatocellular carcinoma (not shown).

Reduced hepatic RBP4 expression in experimental cirrhosis

As these data collectively indicated that serum RBP4 is linked to the hepatic function, we analyzed RBP4 gene expression by real-time PCR from normal and cirrhotic rat liver, 14 days after bile-duct ligation (Fig.3C). RBP4 mRNA expression was approximately 3.5-fold higher in normal compared with cirrhotic liver (P=0.006, Fig.3C).

Discussion

Recent data from mouse models, healthy human volunteers and patients with obesity, impaired glucose tolerance or type-2-diabetes described a novel function for RBP4 in the development of insulin resistance (1-3). In our controls and the subset of patients without cirrhosis and normal liver function, our observation of correlations between RBP4 and glucose, insulin secretion or reduced insulin sensitivity is generally in agreement with the recent literature (2,3).

However, in patients with liver cirrhosis and reduced hepatic biosynthetic capacity, no correlation between RBP4 serum levels and insulin resistance, a common finding in advanced disease (4,5,9), exists. In contrast, RBP4 is then closely linked to biomarkers of liver synthesis function and decreases with the progression of disease. This is line with previous results showing that the liver is the primary source of RBP4 synthesis (1,16,17), despite the reported correlation between adipose RBP4 expression and serum RBP4 (2). Our analysis of an animal model corroborated high gene expression of RBP4 in hepatic tissue and a reduction after induction of experimental cirrhosis.

These results may indicate that RBP4 is, unlike in obesity or type-2-diabetes, not a relevant factor in the pathogenesis of insulin resistance in liver cirrhosis. In contrast to RBP4, several ‘classical’ mediators of insulin resistance were also relevant in patients with liver cirrhosis, e.g. hyperinsulinemia or alterations in the adipose-derived adipokines adiponectin and resistin (4,5,9,12). However, whether *reduced* RBP4, due to reduced hepatic expression by the cirrhotic liver, has any consequences in regulating glucose metabolism in CLD patients is currently unclear. It could potentially render skeletal muscle more susceptible for insulin signals and reduce hepatic gluconeogenesis (1), thereby counteracting factors promoting insulin-resistance in cirrhosis. Moreover, the vitamin A status, likely reduced in cirrhotic patients (16,17), would influence RBP4’s effects on insulin resistance, as RBP4 needs vitamin A to regulate liver enzymes, such as hepatic PEPCK (1). Further investigations are needed to determine the relevance of RBP4 for insulin resistance in liver cirrhosis.

Our findings further imply that RBP4 may not be a clinically useful marker indicating overt or the risk for developing insulin resistance, in the presence of a concomitant liver dysfunction. The recent characterization of RBP4 as a ‘reliable’ biomarker for overt or impending insulin resistance in humans (2,3) is based on the correlation of adipose RBP4 expression and subsequent changes in the muscular and hepatic glucose metabolism (1). However, our study now emphasizes that the liver function tremendously impacts serum RBP4. Future studies will need to take liver function into account when examining serum RBP4 levels.

Acknowledgements

This research project is supported by the START-Program of the Faculty of Medicine, RWTH Aachen (to F.T.). The authors state that they have no conflict of interest.

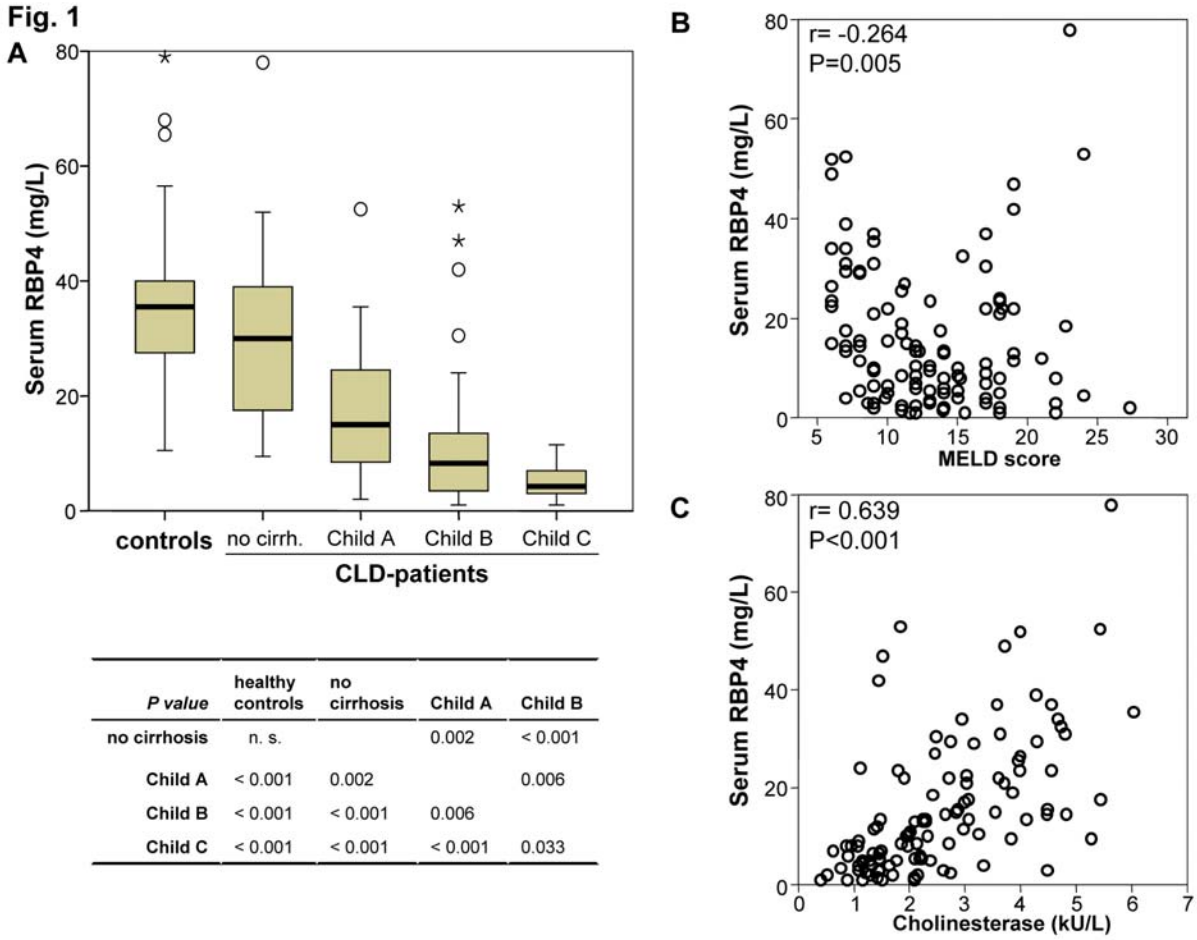
References

1. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 2005;436:356-362.
2. Graham TE, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 2006;354:2552-2563.
3. Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH, Lee HK, Park KS. Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. Diabetes Care 2006;29:2457-61.
4. Petrides AS. Liver disease and diabetes mellitus. Diabetes Rev 1994;2:2-18.
5. Bugianesi E, McCullough AJ, Marchesini G. Insulin resistance: A metabolic pathway to chronic liver disease. Hepatology 2005;42:987-1000.
6. Bianchi G, Marchesini G, Zoli M, Bugianesi E, Fabbri A, Pisi E. Prognostic significance of diabetes in patients with cirrhosis. Hepatology 1994;20:119-125.
7. Sud A, Hui JM, Farrell GC, Bandara P, Kench JG, Fung C, Lin R, Samarasinghe D, Liddle C, McCaughan GW, George J. Improved prediction of fibrosis in chronic hepatitis C using measures of insulin resistance in a probability index. Hepatology 2004;39:1239-1247.
8. Muzzi A, Leandro G, Rubbia-Brandt L, James R, Kaiser O, Malinverni R, Dufour JF, Helbling B, Hadenque A, Gonvers JJ, Mullhaupt B, Cerny A, Mondelli MU, Negro F, Swiss Hepatitis C Cohort Study. Insulin resistance is associated with liver fibrosis in non-diabetic chronic hepatitis C patients. J Hepatol 2005;42:41-46.
9. Yagmur E, Trautwein C, Gressner AM, Tacke F. Resistin serum levels are associated with insulin resistance, disease severity, clinical complications and prognosis in patients with chronic liver diseases. Am J Gastroenterol 2006;101:1244-1252.
10. Tacke F, Brabant G, Kruck E, Horn R, Schoffski P, Hecker H, Manns MP, Trautwein C. Ghrelin in chronic liver disease. J Hepatol 2003;38: 447-454.
11. Durand F, Valla D. Assessment of the prognosis of cirrhosis: Child-Pugh versus MELD. J Hepatol 2005;42:S100-S107.
12. Tacke F, Wustefeld T, Horn R, Luedde T, Srinivas Rao A, Manns MP, Trautwein C, Brabant G. High adiponectin in chronic liver disease and cholestasis suggests biliary route of adiponectin excretion in vivo. J Hepatol 2005;42:666-673.
13. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28: 412-9
14. Borkham-Kamphorst E, Herrmann J, Stoll D, Treptau J, Gressner AM, Weiskirchen R. Dominant-negative soluble PDGF-receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. Lab Invest 2004;84:766-777.
15. Tacke F, Trautwein C, Zhao S, Andreeff M, Manns MP, Ganser A, Schoffski P. Quantification of hepatic thrombopoietin mRNA transcripts in patients with chronic liver diseases shows maintained gene expression in different etiologies of liver cirrhosis. Liver 2002;22:205-212.
16. Smith FR, Goodman DS. The effects of diseases of the liver, thyroid and kidney, on transport of vitamin A in human plasma. J Clin Invest 1971;50:24-6.
17. Leo MA, Lieber CS. Hepatic vitamin A depletion in alcoholic liver injury. N Engl J Med 1982;307:597-601.

Table 1. Characteristics of the study population.
* median and range in parenthesis. n.a., not applicable / not assessed.

	controls	**all patients**	**stage of liver cirrhosis**			
			no cirrhosis	Child A	Child B	Child C
n	*99*	*111*	*18*	*35*	*44*	*14*
sex (male / female) *n*	59/40	66/45	11/7	18/17	30/14	7/7
age * [years]	43 (20-67)	46 (18-70)	46 (18-65)	41 (18-64)	48 (20-70)	40 (26-69)
etiology of liver disease n	*n.a.*					
virus hepatitis		32	1	9	17	5
biliary / autoimmune		27	1	14	10	2
alcohol		29	1	11	10	7
other		23	15	1	7	0
selected laboratory values *						
BMI [kg/m²]	24.9 (17.8-37.3)	23.0 (15.4-37.9)	22.9 (16.3-32.3)	22.6 (15.4-32.4)	23.3 (17.7-32.0)	23.7 (19.7-37.9)
RBP4 [mg/L]	35.5 (10.5-85)	11.5 (1-117)	30 (9.5-117)	15 (2-52.5)	8.3 (1-53)	4.3 (1-11.5)
C-peptide [ng/mL]	*n.a.*	4.0 (1.1-16.5)	2.9 (1.5-11.9)	4.0 (1.4-9.4)	4.9 (1.1-16.5)	5.9 (2.3-12.1)
HOMA-S [%]	*n.a.*	30 (7-105)	43 (11-83)	34 (13-63)	25 (7-105)	20 (10-61)
adiponectin [nmol/L]	*n.a.*	15.3 (2.3-98.5)	10.4 (2.3-65.5)	14.5 (7.6-83.1)	19.5 (7.3-98.5)	15.4 (8.9-45.6)
resistin [µg/L]	*n.a.*	5.2 (2.2-22.0)	3.9 (2.5-9.6)	4.4 (2.3-9.5)	6.2 (2.2-22.0)	9.2 (3.8-18.6)

Figure Legends



P value	healthy controls	no cirrhosis	Child A	Child B
no cirrhosis	n. s.		0.002	< 0.001
Child A	< 0.001	0.002		0.006
Child B	< 0.001	< 0.001	0.006	
Child C	< 0.001	< 0.001	< 0.001	0.033

Fig. 1. RBP4 in chronic liver disease. Serum RBP4 was determined in 111 patients with chronic liver diseases (CLD) and 99 age- and sex-matched healthy controls. (A) Serum RBP4 is significantly reduced in CLD patients and decreases with the Child's stage of liver cirrhosis. The box-and-whiskers plots display the median, quartiles, range and extreme values. The whiskers extend from the minimum to the maximum value excluding outside (>1.5 times upper/lower quartile, open circle) and "far out" (>3 time upper/lower quartile, asterixes) values P-values (not adjusted) are given in the table. n.s., not significant. (B) RBP4 is also inversely correlated with the MELD (model of end-stage liver disease) score. (C) RBP4 is closely correlated with biomarkers indicating the hepatic synthesis capacity, e.g. cholinesterase activity.

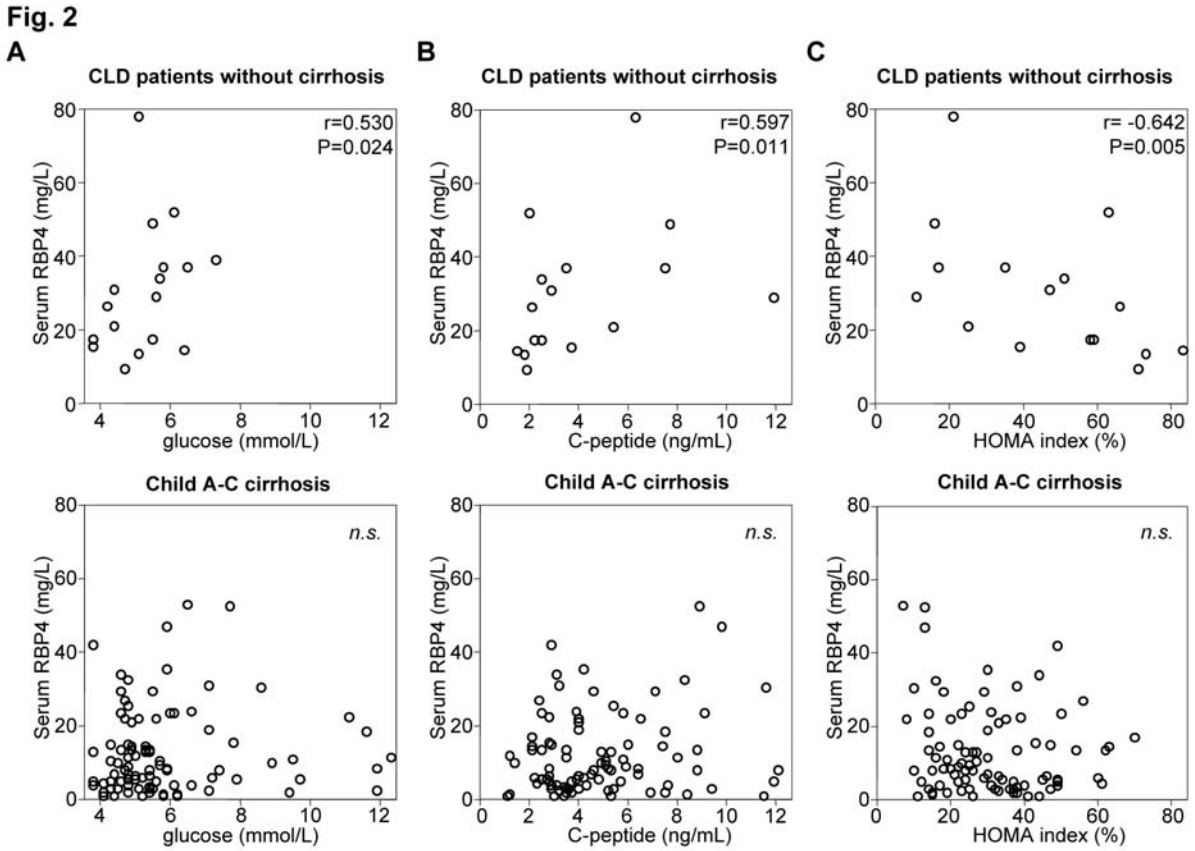
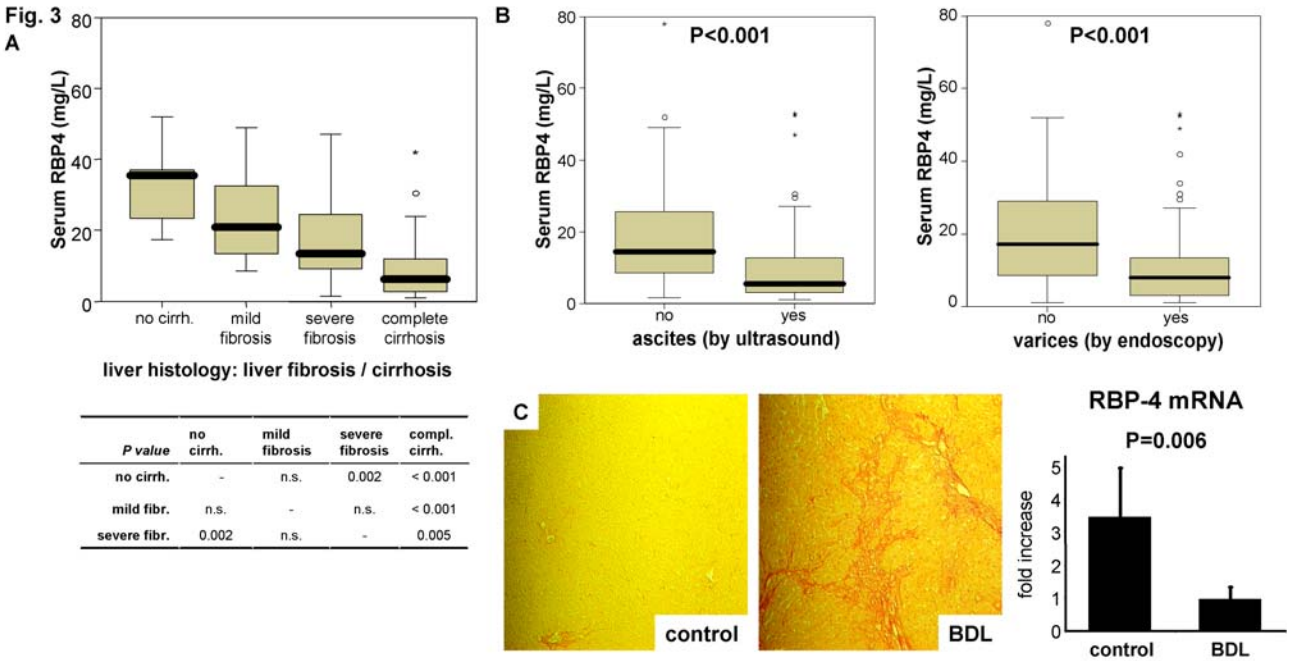


Fig. 2. RBP4 and glucose metabolism. Serum RBP4 was analyzed separately for CLD patients without liver cirrhosis (n=18) and for patients with a Child A-C liver cirrhosis (n=93). Whereas patients without cirrhosis show correlations between RBP4 and serum glucose (A) or insulin secretion (C-peptide, B) and an inverse correlation with the HOMA-S index (insulin sensitivity, C) as reported for healthy controls, no such correlations are found in patients with a liver cirrhosis. Significant correlation coefficients (r) and P-values are given in the figure. n.s., not significant.



P value	no cirrh.	mild fibrosis	severe fibrosis	compl. cirrh.
no cirrh.	-	n.s.	0.002	< 0.001
mild fibr.	n.s.	-	n.s.	< 0.001
severe fibr.	0.002	n.s.	-	0.005

Fig. 3. RBP4 and liver cirrhosis. (A) Serum RBP4 levels decreased with the histological degree of fibrosis or cirrhosis in liver biopsy. P-values (not adjusted) are given in the table. n.s., not significant. (B) Serum RBP4 also decreased in patients with typical clinical complications of liver cirrhosis, such as ascites (assessed by abdominal ultrasound) or esophageal varcies (assessed by upper gastrointestinal endoscopy). (C) Liver samples from sham-operated rats (control, left) and 14 days after bile duct ligation (BDL, right) were stained by Sirius Red to identify collagen expression and deposition in the fibrotic liver (right). RBP4 gene expression was analyzed by real-time PCR in normal and cirrhotic rat liver (n=5 per group) using two independent primer pairs and normalized to GAPDH. The bar graphs show fold-increase differences in RBP4-mRNA calculated from normalized crossing point analysis. P-value given in the figure (two-tailed t-test).