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CLINICAL STUDY

Fetuin A in nonalcoholic fatty liver disease: *in vivo* and *in vitro* studies

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Abstract

Objective: Fetuin A has been associated with insulin resistance and the metabolic syndrome. We therefore explored the role of fetuin A in nonalcoholic fatty liver disease (NAFLD).
Design: Cross-sectional and intervention studies.
Methods: We included 111 subjects with histologically proven NAFLD of whom 44 participated in a randomized, controlled trial with metformin. One hundred and thirty-one healthy subjects and 13 subjects undergoing hepatic surgery for metastatic cancer served as controls. Main outcome variables were circulating levels of fetuin A according to the presence of NAFLD, hepatic gene expression of fetuin A and key enzymes in glucose and lipid metabolism, and the effect of metformin on fetuin A levels *in vivo* and *in vitro* (HepG2 cells).
Results: Fetuin A levels were significantly higher in NAFLD patients compared with controls (324 ± 98 vs 225 ± 75 mg/l, $P < 0.001$). NAFLD was a significant predictor of elevated fetuin A levels ($\beta = 174$ (95% confidence interval: 110–234)) independent of body mass index, age, sex, fasting glucose, and triglycerides. Hepatic fetuin A mRNA levels correlated significantly with hepatic mRNA levels of key enzymes in lipid (sterol regulatory element-binding protein 1c, carnitine palmitoyltransferase 1) and glucose (phosphoenol pyruvate kinase 1, glucose-6-phosphatase) metabolism. Plasma fetuin A levels decreased significantly after metformin treatment compared with placebo (-40 ± 47 vs 15 ± 82 mg/l, $P = 0.008$). Metformin induced a dose-dependent decrease in fetuin A secretion *in vitro*.
Conclusions: Fetuin A levels were elevated in NAFLD. Hepatic expression of fetuin A correlated with key enzymes in glucose and lipid metabolism. Metformin decreased fetuin A levels *in vitro*.

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Introduction

Fetuin A, also known as α2-Heremans–Schmid glycoprotein, is an abundant serum protein produced predominantly in the liver (1). Fetuin A was originally described as a growth factor in fetal calf serum (2) and was later identified as an important inhibitor of ectopic calcification (3). More recent studies, however, have shown that fetuin A could also play a role in the development of the metabolic syndrome. Fetuin A has been found to inhibit autophosphorylation of the insulin receptor (4), and it has been suggested that fetuin A may contribute to insulin resistance as shown by improved insulin sensitivity in fetuin A-deficient mice (5). Along with insulin resistance, persons with the metabolic syndrome often have a low-grade systemic inflammation. Notably, in a recent study combining *in vitro* and *in vivo* experiments, fetuin A was shown to induce the expression of inflammatory cytokines and inhibit the expression of adiponectin, an adipokine with anti-inflammatory effects (6). Thus, fetuin A may play a role in the development of the metabolic syndrome via at least two different mechanisms, and increased serum levels of fetuin A have indeed been observed in patients with the metabolic syndrome (7).

Nonalcoholic fatty liver disease (NAFLD) is associated with insulin resistance and is commonly recognized as the hepatic manifestation of the metabolic syndrome (8). NAFLD has emerged as one of the most common causes of abnormal liver function tests, and several surveys have estimated the prevalence of NAFLD in the general population to be around 20% (9). Histologically, NAFLD reflects a spectrum of disease stages ranging from simple fatty deposition (simple steatosis) to necroinflammation in

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association with ballooning degeneration (non-alcoholic steatohepatitis (NASH)) with or without perisinusoidal and/or periportal fibrosis and subsequently cirrhosis in the most advanced forms of NASH.

While previous data have suggested a link between fetuin A and the metabolic syndrome, the role of fetuin A in patients with NAFLD is largely unknown. However, a significant correlation between plasma levels of fetuin A and liver fat in subjects with increased risk of the metabolic syndrome has been reported (10), and recently, a modest elevation of fetuin A serum levels was found in 99 adult patients with biopsy-proven NAFLD (11). Based on this finding as well as the role of fetuin A in inflammation and insulin resistance, we hypothesized that fetuin A could be involved in the pathogenesis of NAFLD. Here, we investigated this hypothesis by i) comparing circulating levels of fetuin A in patients with histologically proven NAFLD and healthy controls, ii) measuring hepatic expression of fetuin A mRNA and assessing its correlations with key enzymes in glucose and lipid metabolism, and iii) exploring whether metformin can suppress plasma fetuin A levels *in vivo* in patients with NAFLD and *in vitro* in hepatocytes (HepG2 cells).

Subjects and methods

Ethics

The studies were carried out in accordance with the Helsinki Declaration and were approved by the Regional Ethics Committee and the Norwegian Medicines Agency. Written informed consent was obtained from all participants.

Patients

The majority of the patients were recruited at one university hospital during 2003–2007. At this hospital, all 123 patients consecutively scheduled for a liver biopsy because of suspected NAFLD accepted participation in the study. NAFLD was verified in 118 subjects; however, serum or plasma was not available in 16 subjects, leaving 102 subjects eligible for this study. In addition, nine patients with histologically proven NAFLD (participating in a multicenter intervention trial) from three different university hospitals were also included. No other liver diseases (e.g. chronic hepatitis B or C, hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and malignancy) were present. None of the patients were using any known hepatotoxic medications or herbal products. Average alcohol consumption was <24 g/day in all. Forty-four of the patients participated in a randomized trial comparing metformin with placebo (12).

Control subjects

One hundred and thirty-one subjects, reporting to be without any chronic disease, were used as controls. All these subjects had serum alanine aminotransferase (ALT) within the normal range; however, ultrasonography was not performed to rule out presence of liver fat. For the assessment of hepatic gene transcription, we included a second control group consisting of six patients (five males, 62.7 ± 7.5 years, 26.5 ± 3.3 kg, ALT 23 ± 7 U/l), who underwent liver resection because of liver metastases from colorectal cancer ($n=5$) or carcinoid tumors ($n=1$). Apart from their liver metastases, these patients had no chronic liver disease (confirmed histologically).

Biochemical analyses

Serum or plasma from NAFLD patients and control subjects was stored at -80 °C. Blood samples were drawn at the time of liver biopsy ($n=67$) or within 6 months (median 3 months) after liver biopsy ($n=44$). Details of the blood sampling protocol have previously been reported (13). Fetuin A levels in serum, plasma, or in conditioned cell medium were analyzed by enzyme immunoassay (Biovendor, Modrice, Czech Republic). Inter-assay coefficient of variation was $<6\%$. Lower level of detection was 0.35 ng/ml. All samples were analyzed in duplicate, random order, and blinded to the clinical status of the participants. All other biochemical variables were analyzed using standard methods at the local hospital laboratories.

Measures of insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the modified HOMA calculator from the University of Oxford (http://www.dtu.ox.ac.uk/homacalculator/index.php). In patients without previously known type 2 diabetes mellitus (T2DM), an oral glucose tolerance test with 75 g glucose was performed in all but nine patients. Abnormal glucose tolerance was defined as fasting glucose ≥6.1 mmol/l or 2 h glucose ≥7.8 mmol/l or known T2DM.

Liver biopsy and histological examinations

All biopsies were obtained using a biopsy gun. In all 44 patients participating in the intervention trial (metformin vs placebo), a second biopsy was obtained after 6 months of treatment. In 42 biopsies (23 biopsies from 12 patients in the intervention trial and 19 biopsies from 13 NAFLD patients and six control subjects), a minor part was snap–frozen in liquid nitrogen and stored at -80 °C for mRNA analyses, and the remaining part was fixed in formalin. Slides were routinely stained with hematoxylin–eosin, Masson

Trichrome or AFOG, Gomori and Pearls' staining for iron and examined by two liver pathologists blinded for clinical data. Steatosis was defined when fat droplets were present in >5% of hepatocytes. NASH was defined when either ballooning and lobular inflammation or typical lobular fibrosis was present in addition to steatosis. Fibrosis was staged as follows: 0=no fibrosis; 1=pericellular fibrosis or isolated portal fibrosis; 2=combined pericellular and portal fibrosis; 3=bridging fibrosis; and 4=cirrhosis.

Immunostaining for fetuin A

The localization of fetuin A within the liver was examined in biopsies from six patients with NASH, six patients with simple steatosis, and six patients with normal liver tissue. Formalin-fixed paraffin-embedded sections were deparaffinized, rehydrated, and demasked in a microwave oven for 20 min in Target Retrieval Solution (pH 6.00–6.20; Dako Glostrup, Denmark). A polyclonal anti-fetuin A, titer 1:2500, was used as a primary antibody (obtained from Dr W Jahnen-Dechent, Aachen University Hospital, Germany). The antigen–antibody complex was visualized with avidin–biotin HRP system (Vectastain ABC KIT, peroxidase Rabbit IgG; Vector Laboratories, Burlingame, CA, USA) using 3,3-diaminobenzidin as the chromogen. The sections were counterstained with hematoxylin. Omission of the primary antibody served as a negative control.

Real-time quantitative RT-PCR

RNA was isolated from frozen liver specimens using RNeasy Mini Columns (Qiagen). DNase was treated and stored at −80 °C. cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantification of mRNA was performed using the SYBR green PCR Master mix (Applied Biosystems, Warrington, UK) or TaqMan Gene expression Master Mix (Applied Biosystems). The following gene transcripts were quantified: i) fetuin A; ii) sterol regulatory element-binding protein 1c (SREBP1c); iii) carnitine palmitoyltransferase 1 (CPT-1); iv) fatty acid synthase (FAS); v) phosphoenol pyruvate kinase 1 (PEPCK-1); and vi) glucose-6-phosphatase (Glu-6-P). TaqMan assay ID and primer sequences could be provided on request. Gene expression of the housekeeping gene *18S* RNA was used for normalization.

Cell culture studies

HepG2 cells, obtained from European Collection of Cell Cultures (Wiltshire, UK), were cultured in minimum essential medium (MEM; Invitrogen) containing penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), MEM nonessential amino acid solution (1×), and 10% FCS (all from Sigma–Aldrich). All experiments were performed in a humidified atmosphere (37 °C, 5% CO_2, and 95% air) at 75% confluence. Cell-free supernatants were collected and stored at −80 °C until analysis. The toxicity in cell cultures was examined routinely for lactate dehydrogenase leakage (Cytotoxity Detection Kit; Roche Applied Science).

Statistical analyses

Comparison of continuous variables between groups was performed by *t*-tests or one-way ANOVA. Pearson's correlation coefficients were calculated to explore associations between circulating fetuin A levels and continuous variables. Multivariable linear regression was performed to identify factors associated with circulating fetuin A levels. β-Estimates are presented with 95% confidence interval (CI_{95}). Variables correlating with fetuin A in univariate analyses were identified. Among intercorrelating variables, the strongest variable was introduced in the initial model together with age. Due to interaction between serum ALT and disease state (NAFLD vs control), ALT was split into separate variables for patients and controls. Using a backward stepwise approach, a final model with P value <0.10 for each variable was obtained. Multivariable linear regression was also performed to adjust for weight change when we explored the effect of metformin on circulating fetuin A levels. The primary endpoint in the intervention trial with metformin was histological changes. Accordingly, no formal sample size calculation was performed regarding the effect on fetuin A levels. A two-sided $P<0.05$ was considered statistically significant. Calculations were made using PASW version 18.

Results

Baseline characteristics of the study populations are given in Table 1. There were more males and the mean age was slightly higher in patients compared with controls. Body mass index (BMI) and serum ALT were higher, and variables related to the metabolic syndrome were more abnormal in patients than in controls. One half of the patients were diagnosed with abnormal glucose tolerance. Among the patients, median degree of liver fat (percentage of hepatocytes with fat droplet) was 45% (range: 5–90%); 53% were classified as having NASH, and fibrosis was seen in 45% (stage 1 ($n=35$), stage 2 ($n=6$), stage 3 ($n=8$), and stage 4 ($n=1$)).

Circulating levels of fetuin A

Circulating fetuin A levels were markedly elevated in patients (324±98 mg/l) compared with controls (225±75 mg/l) (Fig. 1A). This difference between

Table 1 Baseline characteristics of patients with nonalcoholic fatty liver disease (NAFLD; $n=111$) and healthy controls ($n=131$). Continuous data are given as mean (±S.D.) or median (range).

	NAFLD ($n=111$)	Controls ($n=131$)	*P* value
Age (years)	46.5 (±11.6)	43.3 (±10.3)	0.024
BMI (kg/m^2)	30.5 (±4.3)	23.9 (±3.0)	<0.001
Male/female	67/44	57/74	0.009
ALT (U/l)	89 (64, 121)	20 (15, 26)	<0.001
F-cholesterol (mmol/l)	5.7 (±1.4)	5.0 (±1.0)	<0.001
F-LDL-cholesterol (mmol/l)	3.5 (±1.1)	3.0 (±0.9)	<0.001
F-HDL-cholesterol (mmol/l)	1.2 (0.7–3.5)	1.6 (0.8–2.6)	<0.001
F-triglycerides (mmol/l)	1.8 (0.6–9.3)	0.8 (0.3–3.4)	<0.001
F-glucose (mmol/l)	5.5 (4.1–18.4)	4.9 (3.9–7.0)	<0.001
Abnormal glucose tolerance (%)[a]	51	3	<0.001
HOMA-IR[b]	2.21 (1.14)	1.40 (0.77)	<0.001

[a]Abnormal glucose tolerance indicates fasting glucose ≥6.1 mmol/l or 2 h glucose ≥7.8 mmol/l or known T2DM. An oral glucose tolerance test (OGTT) with 75 g glucose was performed in all but nine patients without previously known T2DM and in 37 out of 131 controls.
[b]Measured in 78% (87/111) of the patients and in 74% of the controls (97/131).

patients and controls was consistent across all BMI classes (Fig. 1B). Among NAFLD patients, fetuin A correlated negatively with fasting glucose ($r=-0.20$, $P=0.037$) and BMI ($r=-0.21$, $P=0.026$). No significant correlations with 2 h glucose, HbA1c, HOMA-IR, serum ALT, triglycerides, cholesterol, high-density lipoprotein (HDL)-cholesterol, or low-density lipoprotein (LDL)-cholesterol were observed (data not shown). There were no significant differences in fetuin A levels between patients with NASH compared with those with pure steatosis, and no significant differences were observed according to fibrosis stage, or according to degree of liver steatosis. Among controls, fetuin A levels correlated positively with serum ALT ($r=0.21$, $P=0.047$) but not with other measured variables (data not shown). In the multivariable regression model, NAFLD was a strong predictor of elevated fetuin A levels independent of BMI, age, sex, and several metabolic factors (Table 2). In controls, but not in patients, serum ALT was a significant predictor of fetuin A levels.

Fetuin A levels in patients treated with metformin

Forty-four of the patients participated in a placebo-controlled, double-blind, randomized study of metformin in patients with NAFLD (11). As previously reported, treatment with metformin for 6 months did not result in histological improvement of liver disease but was accompanied by a significant reduction in body weight (12). Plasma levels of fetuin A were significantly reduced in the metformin group (−40±47 mg/l) compared with placebo-treated patients (15±82 mg/l, $P=0.008$) (Fig. 1C). There was a significant correlation between change in body weight and change in fetuin A levels ($r=0.350$, $P=0.020$), and when using linear regression to adjust for change in body weight, the association between treatment with metformin and reduction in fetuin A levels did not reach statistical significance ($\beta=-47$ mg/l (CI_{95}: −98, 5), $P=0.076$).

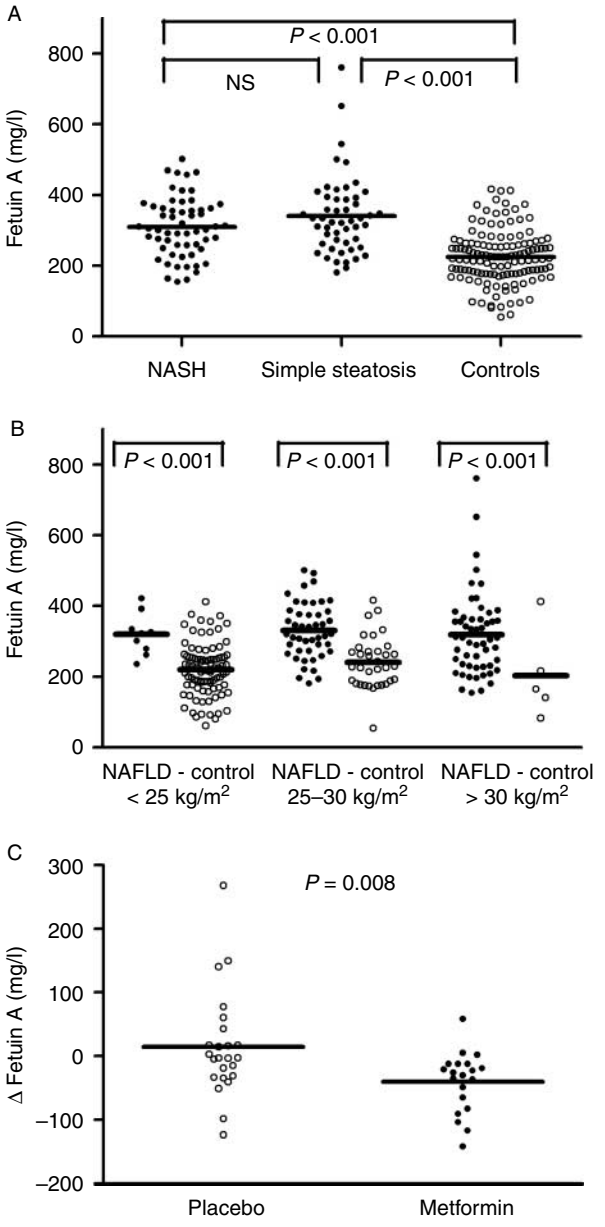

Figure 1 Circulating levels of fetuin A in 111 patients with NAFLD and 131 control subjects according to (A) disease state and (B) BMI class. (C) Change in circulating levels of fetuin A in patients treated with either metformin or placebo for 6 months. Horizontal lines represent mean values. *P* values were calculated by *t*-tests.

Table 2 Predictors of circulating fetuin A levels. Multivariable linear regression. The model was developed using a backward stepwise approach.

Dependent variable	Explanatory variable	β	95% CI for β	*P* value
Fetuin A (mg/l) (Adj.R^2=0.280)	NAFLD	174	110, 238	<0.001
	Sex (reference = male)	34	7, 61	0.013
	BMI (per kg/m^2)	−4	−7, −0.5	0.024
	F-glucose (per mmol/l)	−9	−16, −1	0.026
	Age (per year)	−1	−2, 0	0.072
	F-triglycerides (per mmol/l)	12	1, 23	0.034
	ALT in controls (per U/l)	3	0, 5	0.025

Hepatic expression of fetuin A in NAFLD and controls

Quantification of fetuin A mRNA in liver tissue by RT-PCR revealed a relatively strong expression, with no significant difference between 13 NAFLD patients and six controls. In patients with simultaneously obtained plasma and liver tissue specimens, we found no significant correlation between hepatic fetuin A mRNA levels and circulating fetuin A levels ($r=-0.036$, $P=0.87$). At the protein level, positive staining for fetuin A was observed within hepatocytes both in NAFLD patients and in controls, localized in a granular pattern on the canalicular side of hepatocytes, possibly reflecting its synthesis in the Golgi apparatus (Fig. 2).

Hepatic gene expression of fetuin A and key metabolic enzymes

The hepatic mRNA levels of fetuin A correlated strongly with the expression of several key enzymes in glucose and lipid metabolism, both when analyzing baseline values and changes over time. In the first analyses based on 12 patients with NAFLD, significant correlations were observed between mRNA levels of fetuin A and mRNA levels of SREBP1c, CPT1, PEPCK1 and Glu-6-P, but not with FAS (Fig. 3A). Notably, when analyzing Δ-values (post-treatment minus pre-treatment levels) in 11 patients participating in the metformin study (metformin ($n=4$) and placebo ($n=7$)), we also found strong correlations between changes in fetuin A mRNA levels and changes in mRNA levels of SREBP1c, CPT1, and PEPCK1, but not FAS and Glu-6-P (Fig. 3B). Finally, in a separate analyses based on 13 different NAFLD patients and six control subjects, we again found significant correlations between fetuin A mRNA and mRNA levels of SREBP1c and PEPCK1, but not CPT1 and FAS, with the same pattern observed in NAFLD patients and controls (Fig. 3C).

Effects of metformin on fetuin A protein secretion in HepG2 cells

Our findings suggest that metformin may decrease fetuin A levels. As immunohistochemistry showed strong staining in hepatocytes, we further explored the interaction between metformin and fetuin A by exposing HepG2 hepatocytes to metformin for 48 h. As shown in Fig. 4, metformin dose dependently decreased the release of fetuin A, suggesting that the decrease in fetuin A during metformin therapy could, at least in part, reflect direct effects on the liver. The effect of metformin was not influenced by changing the insulin and glucose concentrations in the medium (data not shown).

Discussion

Accumulating evidence indicates an association between fetuin A, insulin resistance and the metabolic syndrome. Fetuin A has also been found to correlate with liver fat in patients at risk of T2DM (14), and our data show that circulating fetuin A levels were markedly elevated in patients with NAFLD, a disorder closely linked with the metabolic syndrome. Furthermore, in liver biopsies, we found strong correlations between the expression of the fetuin A gene and key enzymes in lipid and glucose metabolism, indicating that these share common regulations. To our knowledge, only two previous reports about fetuin A in NAFLD have been published. Reinehr & Roth (15) found elevated serum levels of fetuin A in 12 obese children with NAFLD and a decrease that paralleled weight loss during follow-up, and in a recent study among adult patients with biopsy-proven NAFLD, fetuin A levels were moderately increased compared with the control group (11). Our findings provide further support for the association between elevated fetuin A and NAFLD. Although the controls and NAFLD patients were not matched for BMI, the separate analysis according to BMI class and the multivariable analysis indicate that the elevated fetuin A levels in NAFLD, at least in part, are independent of

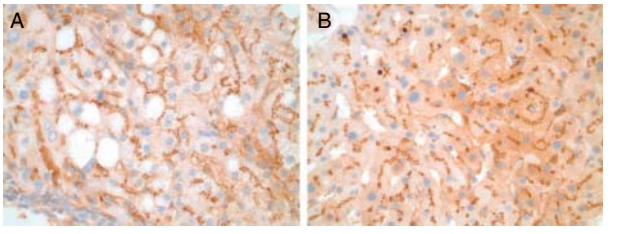

Figure 2 Immunostaining for fetuin A protein from a patient with (A) NASH and (B) normal liver tissue in a patient with focal liver disease. Fetuin A is localized in a granular pattern on the canalicular side of hepatocytes, possibly within the Golgi apparatus. Original magnification ×400.

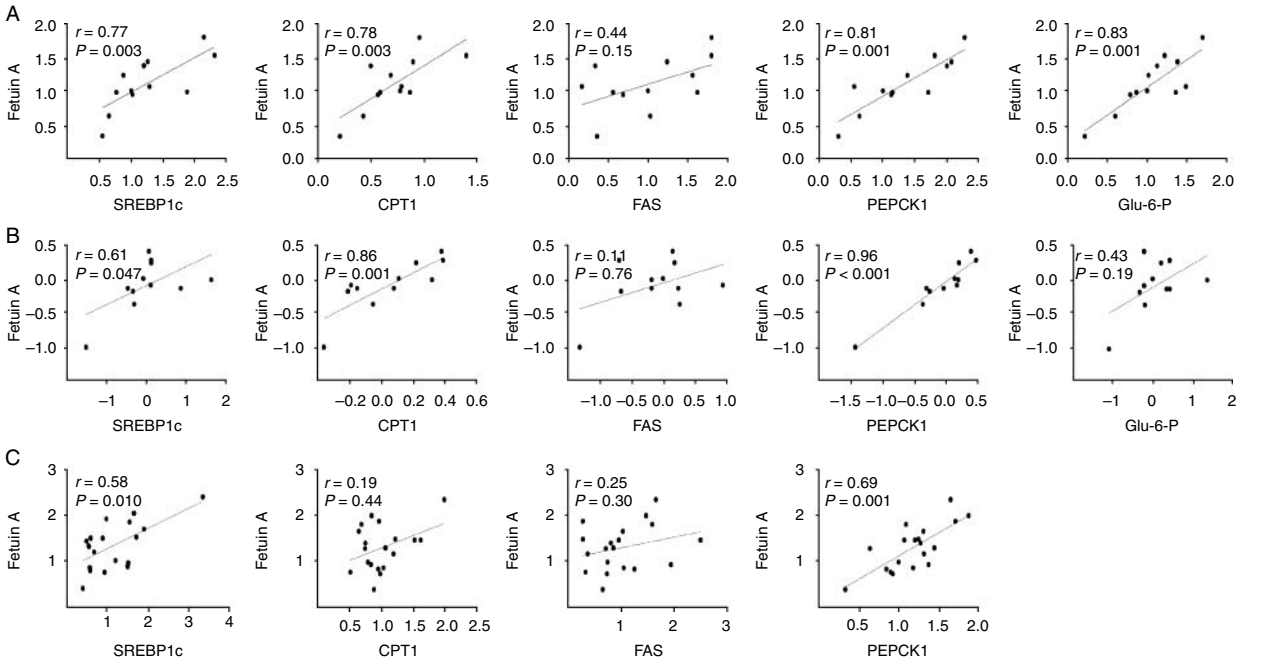

Figure 3 Correlation plots relating hepatic gene expressions of fetuin A to the key transcription factor SREBP1c and rate-limiting enzymes in glucose (PEPCK1 and Glu-6-P) and lipid (CPT1 and FAS) metabolism. (A) Baseline data from 12 NAFLD patients. (B) Δ-Values in 11 of these patients after 6-month treatment with metformin ($n=4$) or placebo ($n=7$). (C) Baseline data from another 13 patients with NAFLD and six controls. Gene expression of the housekeeping gene *18S* RNA was used for normalization. $r=$ Spearman's rho.

BMI and several metabolic variables, possibly linking fetuin A more directly to the pathophysiological events in NAFLD.

At variance with the study by Yilmaz *et al.* (11), we found no correlation between circulating fetuin A levels and fibrosis stage. Given the well-known association between insulin resistance and fibrosis stage in NAFLD patients (16), one could expect fetuin A, as a marker of insulin resistance, to increase in parallel with the degree of liver fibrosis. On the other hand, fetuin A is a known extracellular inhibitor of transforming growth factor β (17), the key profibrogenic stimuli in chronic liver disease (18). Accordingly, elevated fetuin A could represent a counteracting and protective mechanism against development of liver fibrosis. At present, no firm conclusions can be drawn about the role of fetuin A in liver fibrogenesis in NAFLD.

The regulation of hepatic fetuin A secretion is largely unknown. *In vitro* studies in hepatocyte models have shown upregulation upon incubation with high levels of glucose and fructose (19) as well as with saturated fatty acids (20). These findings are highly relevant in insulin-resistant states, and extending on these studies, we found remarkably strong correlations between hepatic gene expression of fetuin A and several key enzymes in glucose and lipid metabolism, suggesting co-regulations of these proteins. In particular, fetuin A expression seems to parallel both the expression of the rate-limiting enzyme in gluconeogenesis (PEPCK1) and the expression of the key transcription factor in lipogenesis (SREB1c). Although less consistently, our findings further indicate possible co-regulations with the enzyme responsible for cellular export of glucose (Glu-6-P) and the rate-limiting transport factor (CPT-1) necessary for mitochondrial β-oxidation of fatty acids. Thus, our findings further suggest a link between fetuin A and glucose and lipid metabolism within the liver.

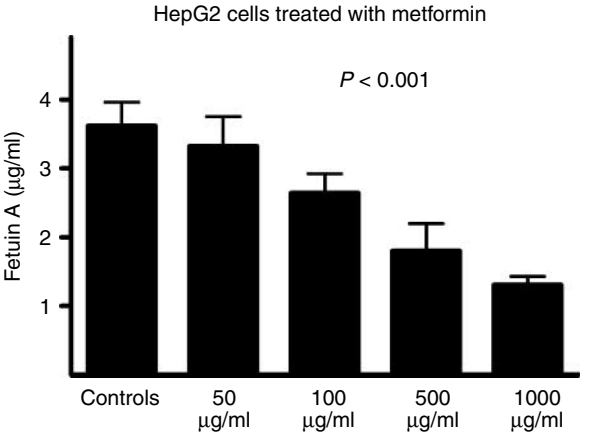

Figure 4 Fetuin A secretion by HepG2 hepatocytes after treatment with metformin at various doses for 48 h. Fetuin A secretion decreased in parallel with increasing metformin dose. Data are mean (S.E.M.).

In the current study, we observed a trend, although not statistically significant, toward a metformin-induced decrease in fetuin A levels *in vivo* in NAFLD patients after controlling for changes in body weight, and we found a direct effect of metformin when studying HepG2 hepatocytes. The mechanism of action for metformin is incompletely understood, but activation of the energy sensor AMP-activated protein kinase (AMPK) is suggested to be important (21). Hepatic AMPK activity is currently regarded as an important factor in the metabolic syndrome (22). Interestingly, AMPK is intimately involved in the regulation of all proteins that correlated with hepatic fetuin A expression in our study (22). Thus, it may be hypothesized that fetuin A synthesis is inhibited by AMPK stimulation, and that, conversely, in states of over-nutrition, fetuin A secretion increases secondary to decreased AMPK activity. As such, fetuin A can be regarded as a hepatic signal of energy excess, a relevant signal considering its role as a growth factor; but in states of over-nutrition, this signal may contribute to insulin resistance (4) and low-grade inflammation (6). However, these issues require further studies before drawing any firm conclusion.

NAFLD is associated with an increased risk of cardiovascular disease; interestingly, fetuin A was recently found to be a significant and independent predictor of myocardial infarction and ischemic stroke (23). The increased levels of fetuin A in NAFLD could therefore be of interest as a potential biomarker for cardiovascular disease in this population, potentially linking liver pathology, inflammation, and metabolic disturbances in NAFLD patients.

A limitation of our study was that controls were not matched for BMI. However, our analyses show clearly that fetuin A levels were elevated irrespective of BMI class. Other limitations of the study include the use of indirect measures of insulin resistance, not fully characterized glucose metabolism, and no assessment of possible presence of liver fat in the control groups, and finally, the limited number and type of control subjects for the biopsy studies, possibly explaining why hepatic fetuin A mRNA levels in NAFLD patients were not significantly different when compared with controls.

Conclusions

Fetuin A levels are significantly elevated in NAFLD and the hepatic gene expression of this protein seems to be co-regulated with key factors in glucose and lipid metabolism. Further studies are needed to explore the effects of metformin on fetuin levels *in vivo*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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