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Original Article

Expression of fatty acid synthase in nonalcoholic fatty liver disease

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Abstract: Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation which starts with simple hepatic steatosis and may progress toward inflammation (nonalcoholic steatohepatitis [NASH]). Fatty acid synthase (FASN) catalyzes the last step in fatty acid biosynthesis, and thus, it is believed to be a major determinant of the maximal hepatic capacity to generate fatty acids by *de novo* lipogenesis. The aim of this study was to analyze the correlation between hepatic steatosis and inflammation with FASN expression. *In vitro* incubation of primary human hepatocytes with fatty acids dose-dependently induced cellular lipid-accumulation and FASN expression, while stimulation with TNF did not affect FASN levels. Further, hepatic FASN expression was significantly increased *in vivo* in a murine model of hepatic steatosis without significant inflammation but not in a murine NASH model as compared to control mice. Also, FASN expression was not increased in mice subjected to bile duct ligation, an experimental model characterized by severe hepatocellular damage and inflammation. Furthermore, FASN expression was analyzed in 102 human control or NAFLD livers applying tissue micro array technology and immunohistochemistry, and correlated significantly with the degree of hepatic steatosis, but not with inflammation or ballooning of hepatocytes. Quantification of FASN mRNA expression in human liver samples confirmed significantly higher FASN levels in hepatic steatosis but not in NASH, and expression of SREBP1, which is the main transcriptional regulator of FASN, paralleled FASN expression levels in human and experimental NAFLD. In conclusion, the transcriptional induction of FASN expression in hepatic steatosis is impaired in NASH, while hepatic inflammation in the absence of steatosis does not affect FASN expression, suggesting that FASN may serve as a new diagnostic marker or therapeutic target for the progression of NAFLD.

Keywords: Nonalcoholic fatty liver disease (NAFLD), Fatty acid synthase (FASN) , nonalcoholic steatohepatitis (NASH), SREBP1, expression, immunohistochemistry

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most frequent cause of liver damage and the most common reason for abnormal hepatic enzymes worldwide [1-4]. NAFLD is characterized by fat accumulation in the liver that starts with simple hepatic steatosis and may progress toward inflammation (nonalcoholic steatohepatitis [NASH]) with progressive fibrosis [4-6]. Major advances have been made in understanding the pathogenesis of NAFLD but the mechanisms that lead from benign steatosis to NASH are still poorly understood.

NAFLD is strongly associated with the metabolic syndrome and its defining pathophysiological components obesity, diabetes and dislipidemia. Thus, overeating delivers an excess of triglycerides to the liver. Further, peripheral lipolysis in obese and insulin resistant states leads to an increased hepatic influx of fatty acids (FA). In addition, hepatic FA synthesis is increased secondary to elevated glucose and insulin levels. Together, both uptake of exogenously derived FA and *de novo* hepatic synthesis of FA lead to an increase of hepatic lipid content, e.g. hepatic steatosis [3,7,8].

Fatty acid synthase (FASN) catalyzes the last step in fatty acid biosynthesis, and thus, it is believed to be a major determinant of the maxi-

mal hepatic capacity to generate FA by *de novo* lipogenesis. FASN synthesizes long chain FAs by using acetyl-CoA as a primer, malonyl-CoA as a carbon donor, and NADPH as a reducing equivalent [9-11]. Thus under normal conditions, excess carbohydrates are converted into FA, followed by esterification to triacylglycerols, which when necessary, provide energy through beta-oxidation.

In NAFLD increased mitochondrial oxidation of FA takes place as a compensatory adaptation. However, this homeostatic response leads to increased mitochondrial reactive oxygen products (ROS), and in a chronic stage, results in lipid peroxidation, inflammation and fibrogenesis [7,12,13].

Sterol regulatory element-binding proteins (SREBPs) are the major transcriptional factors in lipogenic gene expression including FASN [14]. SREBP1 is induced by high insulin levels, and feeding a high carbohydrate diet rapidly induced hepatic FASN expression in rats [15]. Furthermore, SREBP1 mRNA expression levels have been found to be increased in animal models of NASH [16]. However, information regarding FASN expression and function in NAFLD is sparse and studies in man are almost exclusively based on mRNA expression analysis and small patient numbers.

The aim of this study was to analyze FASN protein expression in a large number of patients with normal liver histology or NAFLD without or with hepatic inflammation (e.g. NASH). In addition, SREBP1 and FASN expression were investigated in experimental murine models of nonalcoholic hepatic steatosis or steatohepatitis, and in an *in vitro* model of lipid accumulation in primary human hepatocytes.

Materials and methods

Chemicals and reagents

Palmitic acid (Cat#P0500) and recombinant human tumor necrosis factor alpha (TNF) (Cat#T0157) were obtained from Sigma pharmaceuticals (Hamburg, Germany).

Intracellular triglyceride assay

Total triglycerides were extracted using the method of Bligh and Dyer with slight modifications [17,18] and quantified with the GPO-triglyceride kit (Sigma, Deisenhofen, Germany) as described [19].

Murine models of hepatic steatosis and hepatic inflammation

Male BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at 6 weeks of age and housed in a 22°C controlled room under a 12 h light-dark cycle with free access to food and water. After 1 week of acclimatization mice were divided into three groups (n=6 each) and fed either with control diet, a high fat diet (HFD) containing 30% lard or a NASH inducing diet containing 30% lard, 1.25% cholesterol and 0.5% sodium cholate [20]. All chows were prepared by Ssniff (Soest, Germany). After 12 weeks feeding animals were sacrificed. Liver tissue was immediately snap-frozen and stored at -80°C for subsequent analysis.

Bile duct ligation (BDL) or sham operations were performed as described [21].

Human cell, tissues and tissue microarray (TMA)

Primary human hepatocytes (PHH) were isolated and cultured as described [23]. Formalin-fixed, paraffin-embedded human liver tissues for construction of a tissue microarray (TMA) were retrieved from the surgical pathology files of the Institute of Pathology of the University of Regensburg, covering a period of 11 years (1995 to 2006). The following exclusion criteria were applied: 1. chronic alcohol abuse (more than 30 g/day for men and 20g/day for women), 2. medications known to cause hepatic steatosis (at present or within the last 2 years), 3. significant weight loss (more than 3 kg within the last 3 months), 4. hepatobiliary diseases, 5. ascites in ultrasound, 6. inflammatory bowel disease, 7. infection with the human immunodeficiency virus or Hepatitis B and C virus, and 8. chemotherapy prior to partial hepatectomy. Thus, a TMA could be constructed with liver tissue from 106 different patients as described [24]. All patients were of Caucasian origin, 50 (46.7%) were male, and had undergone partial hepatectomy for metastatic liver tumors. The mean age was 60.8±12.0 years.

In addition to formalin-fixed liver tissue for TMA

construction, 16 corresponding liver specimens, which had been immediately snap frozen after surgical resection and stored at -80°C, were available.

Human liver tissue was obtained and experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR, with the informed patient's consent, and the study was approved by the local ethics committee of the University Regensburg.

Histological NAFLD/NASH score

A modified histological NAFLD/NASH Score according to the recommendations of the Nonalcoholic Steatohepatitis Clinical Research Network was used [25]. Briefly, four histological features were evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4). Steatohepatitis was diagnosed when the sum of the scores was 4 or more.

Immunohistochemical analysis

Immunohistochemical staining of 5-µm sections of the tissue microarray (TMA) blocks was performed using polyclonal anti-FASN antibody (Abnova (Taiwan), clone 3F2-1F3, dilution 1:2,000) with an automated platform (Bond; Labvision, Fremont, CA, USA) as described previously [22]. A surgical pathologist (M.O.R.) performed a blinded evaluation of the stained slides. For negative control, the primary antibody was omitted and IgG isotype control antibodies did not reveal any detectable staining. Protein expression of FASN was evaluated semi-quantitatively using a 2-tiered system for intensity when at least 10% of hepatocytes were positive. Liver specimens were scored as weakly positive, when a faint cytoplasmatic signal using the 100x magnification was barely detectable. Hepatic tissues were scored as strongly positive when a dark brown staining was detected using a 100x magnification.

Quantitative real time-PCR analysis

Isolation of total cellular RNA from cultured cells and tissues and reverse transcription were performed as described [26]. Quantitative real time -PCR was performed applying LightCycler technology (Roche, Mannheim, Germany) as described [27] applying the following pairs of primers: murine MCP-1 (forward: 5'-TGGGCCTGCTGT TCACA; reverse: 5'-TCCGATCCAGGTTTTTAAGTA). Expression of murine and human FASN, SREBP1 and TNF mRNA was analyzed with the QuantiTect Primer Assays according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Statistical analysis

Statistical analyses were performed using SPSS version 10.0 (SPSS, Chicago, IL, USA) and GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA). Results are expressed as mean ± standard error (range) or percent. Comparisons between groups were made using the Mann-Whitney test. Contingency table analysis and the two-sided Fisher's exact test were used to study the statistical association between clinicopathological and immunohistochemical variables. P-values <0.05 were considered statistically significant.

Results

FASN expression in an in vitro model of hepatocellular lipid accumulation

First, we analyzed FASN expression in an *in vitro* model of hepatocellular lipid accumulation, which we have recently described [19]. Incubation of primary human hepatocytes (PHH) with palmitic acid induced a dose dependent intracellular triglyceride accumulation (**Figure 1A**), and notably, FASN mRNA expression (**Figure 1B**). In contrast, stimulation of PHH with TNF did not significantly change FASN expression (**Figure 1C**).

FASN expression in murine NAFLD models

Next, we investigate hepatic FASN expression in two different murine NAFLD models. In one model, which closely resembles (histo) pathophysiological changes observed in human NASH [20], mice received a high fat diet (30% lard) supplemented with cholesterol and cholate. In the second model, mice received a high fat diet with the identical lipid content as in the first model but without cholesterol and cholate. As it has been show before by others [20,28] and our group [29,30], twelve weeks feeding of both diets induced a significant and comparable steatosis in both groups **(Figure 2A** and data not

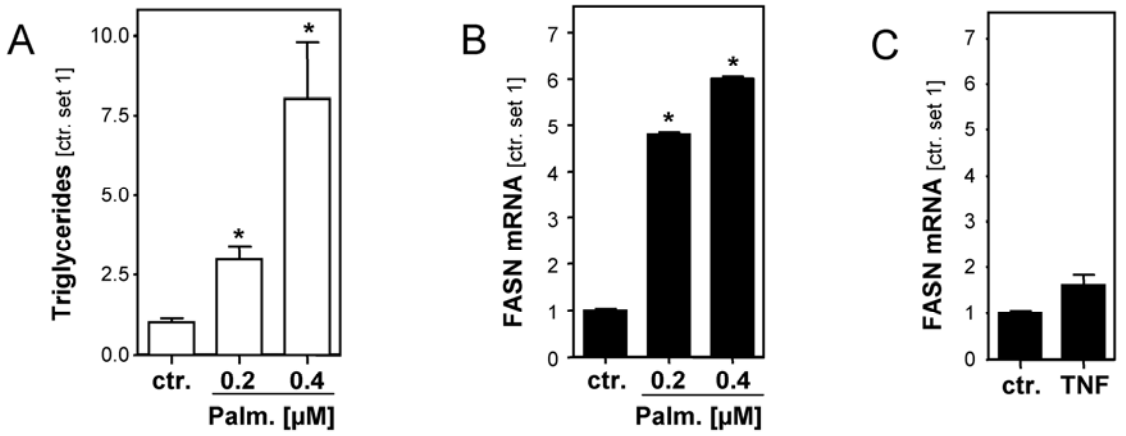

Figure 1. FASN expression in an *in vitro* model of hepatocellular lipid accumulation. Primary human hepatocytes were incubated with 0.2 mM and 0.4 mM palmitate (Palm.) or TNF (10 ng/ml) for 24 h. (A) Colorimetric assessment of intracellular triglyceride content. (B,C) Analysis of FASN mRNA expression by quantitative PCR. (*p<0.05 compared to control (ctr.)).

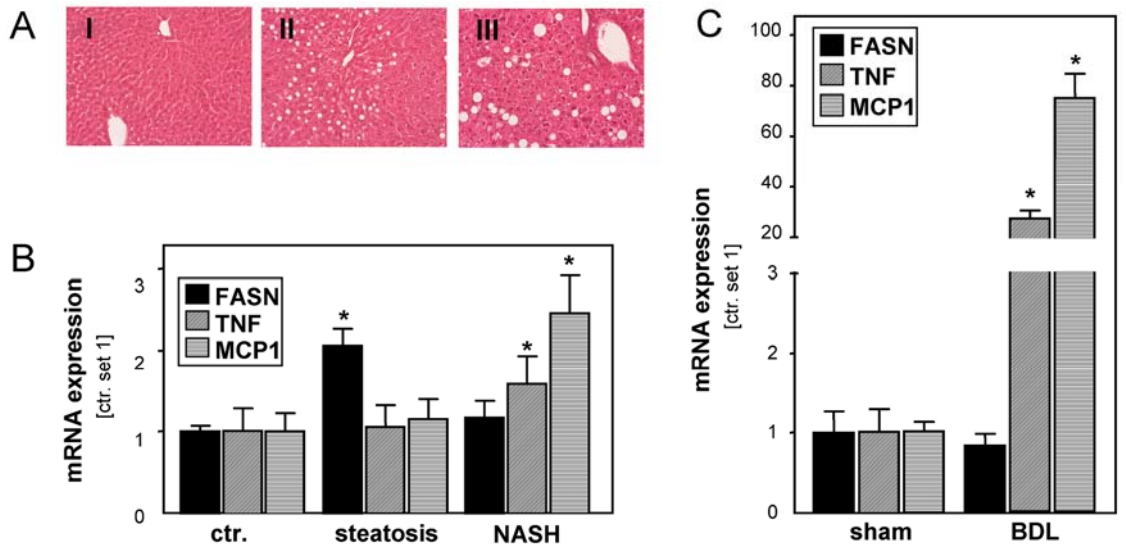

Figure 2. Hepatic FASN expression in two murine models of NAFLD and in a model of hepatic injury and inflammation not related to NAFLD. Mice were fed either a high fat diet leading to hepatic steatosis or a NASH inducing diet for 12 weeks. Mice receiving standard chow served as control (ctr.). In a second experimental setting mice underwent bile duct ligated (BDL) for 3 weeks to induce liver injury and hepatic inflammation (without steatosis). Sham-operated mice served as control (sham). (A) HE-staining of liver tissue sections from mice fed either high fat (II), NASH inducing (III) or control diet (I). (magnification 100X) (B,C) Analysis of hepatic FASN, TNF and MCP-1 mRNA expression by quantitative PCR. (*p<0.05 compared to control).

shown). However, in accordance with the literature [28-30], feeding of high fat only does not induce significant hepatic inflammation, while the additional application of cholesterol and cholate lead to manifest hepatic inflammation, as evidenced by increased expression of the proinflammatory cytokines tumor necrosis factor (TNF) and monocyte chemotactic protein-1 (MCP-1) **(Figure 2B)**, both known to play an important role in development and progression of

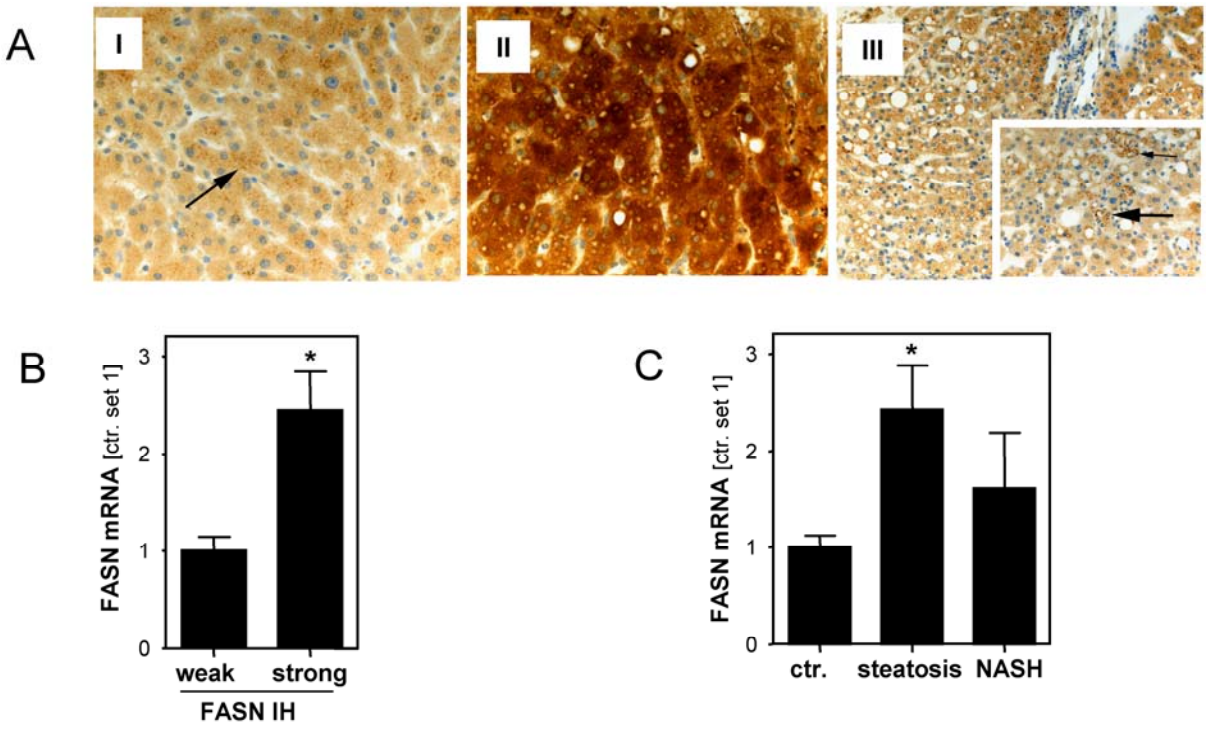

Figure 3. FASN expression in liver tissues of patients with and without NAFLD. (A) FASN immunohistochemical staining of a tissue micro array comprising 106 non-tumorous hepatic tissue specimens with different degree of steatosis and inflammation. Representative pictures of a (I) healthy liver, weakly positive for FASN (arrow: the coarse brown material is lipofuscin), (II) steatotic liver without inflammation showing strong cytoplasmatic positivity for FASN, and (III) NASH liver, weakly positive for FASN (bold arrow: lobular inflammation; thin arrow: the coarse brown material is lipofuscin). Magnification 200X and 400X in the insert, respectively. (B) Comparison of FASN mRNA expression levels in liver samples with high or low FASN protein expression as assessed by semiquantitative evaluation of FASN immunohistochemistry (IH). (C) Analysis of FASN mRNA expression levels in normal (ctr.), steatotic and NASH human liver tissues by quantitative PCR. (*$p<0.05$ compared to control).

hepatic inflammation. Of note, FASN expression was significantly increased in mice with hepatic steatosis without inflammation but not in NASH as compared to control mice (**Figure 2B**).

Furthermore, we analyzed FASN in a third murine model of liver damage, the bile-duct ligation (BDL) model, which is not related to NAFLD. This model is characterized by severe hepatocellular damage and inflammation, and while we observed markedly elevated expression of proinflammatory genes, hepatic FASN expression levels did not differ from those in sham operated control mice (**Figure 2C**).

FASN expression in patients with and without NAFLD

To study FASN expression in a large number of NAFLD patients in comparison to individuals with normal liver histology, we performed FASN immunohistochemical staining of a TMA comprising 106 non-tumorous hepatic tissue specimens with different degree of steatosis and inflammation. Exclusion criteria ruled out alcoholic steatohepatitis, and hepatic steatosis secondary to medication, rapid weight loss or infection with hepatitis C virus. Immunohistochemical staining was informative in 102 cases, and revealed a homogenous cytoplasmatic staining pattern of hepatocytes in all patients (**Figure 3A**). However, the intensity of the immunohistochemical FASN signal revealed significant differences in individual patients, and thus, was systematically categorized into weak or strong staining. Representative examples of weak and strong staining intensity are depicted in **Figure 3A**.

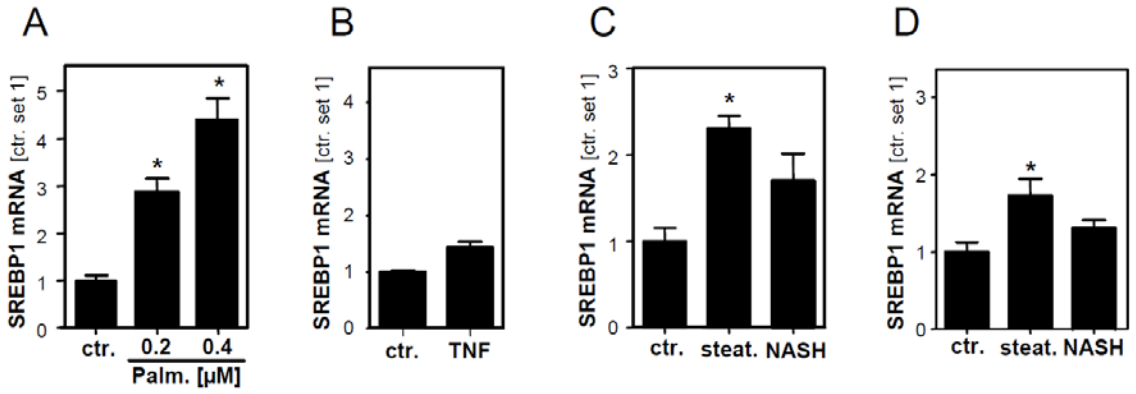

Figure 4. SREBP1 expression in *in vitro* and *in vivo* models of hepatic steatosis and human NAFLD tissue. Analysis of SREBP1 mRNA expression by quantitative PCR in (A) Primary human hepatocytes (PHH) incubated with 0.2 mM or 0.4 mM palmitate (Palm.) for 24 h, (B) PHH stimulated with TNF (10 ng/ml) for 24 h, (C) murine liver tissues from control mice (ctr.), mice fed a high fat diet leading to hepatic steatosis and mice fed a NASH inducing diet, and (D) liver tissue from patients with healthy livers (ctr.), steatosis without inflammation or NASH. (*p<0.05 compared to control (ctr.)).

Interestingly, the intensity of the FASN immunoreactivity correlated significantly with the degree of hepatic steatosis, while no association was found with hepatic inflammation or ballooning of hepatocytes (**Table 1**). Also, FASN staining intensity showed no correlation with age, gender, degree of obesity or presence of diabetes.

Matched frozen hepatic tissue samples for mRNA analysis and semiquantitative assessment of protein expression on the TMA were available from 16 patients. FASN mRNA expression was significantly higher in cases with strong FASN immunosignal (n=3) compared to cases, where FASN signal was weak (n=16; **Figure 3B**). This finding confirmed that FASN expression was accurately detected by immunohistochemistry and that the categorization into samples with low or high FASN immunosignal truly reflected the hepatic FASN expression levels.

Also in line with the immunohistochemical staining results, FASN mRNA expression was significantly higher in steatotic livers than in normal liver tissue **(Figure 3C)**. Interestingly, FASN mRNA levels were slightly lower in NASH compared to steatotic livers without inflammation.

Regulation of FASN expression in nonalcoholic hepatic steatosis and steatohepatitis

To get insight into the regulation of FASN expression in NAFLD, we investigated SREBP1, a known transcriptional regulator of FASN, in the *in vitro* model of hepatocellular lipid accumulation described above. Lipid accumulation in primary humane hepatocytes significantly induced SREBP1 expression (**Figure 4A**), while stimulation with TNF exhibited only a minimal effect on SREBP1 expression levels **(Figure 4B**).

Similarly as in hepatocytes *in vitro* and in line with FASN expression levels, SREBP1 expression was significantly increased in steatotic murine and human livers without inflammation compared in normal hepatic tissue **(Figure 4C, D**). In contrast, FASN mRNA levels were slightly lower in NASH and did not differ from control tissue.

Discussion

The aim of this study was to analyze the expression of FASN, which is a central enzyme of hepatic lipogenesis, in NAFLD. We revealed a significant correlation of FASN expression with the degree of steatosis in primary human hepatocytes *in vitro*, as well as in experimental murine models and in livers of patients with NAFLD *in vivo*. Surprisingly, this association was only found in steatotic hepatic tissue without significant inflammation, while FASN expression in murine and human NASH did not differ significantly from levels in normal liver tissue. Similar data have been obtained by Ito et al. based on a

Table 1. FASN immunoreactivity (IR) in liver tissue of 102 NAFLD patients and controls in relation to clinicopathological characteristics

Variable	Categorization	n	%	FASN IR weak	FASN IR strong	P*
Age at diagnosis						0.810
	<60 years	48	*47.1*	37	11	
	≥60 years	54	*52.9*	44	10	
Gender						1.000
	female	56	*54.9*	44	12	
	male	46	*45.1*	37	9	
BMI class **						
	0	34	*33.3*	30	4	0.231
	1	43	*42.2*	34	9	
	2	22	*21.6*	15	7	
	3	3	*2.9*	2	1	
steatosis						**0.038**
	<5%	52	*51.0*	42	10	
	5-33%	40	*39.2*	34	6	
	34-66%	8	*7.8*	3	5	
	67-100%	2	*2.0*	2	0	
inflammation						0.169
	none	85	*83.3*	70	15	
	mild	7	*6.9*	5	2	
	middle	6	*6.9*	4	2	
	strong	4	*3.9*	2	2	
Ballooning						0.094
	none	88	*86.3*	72	16	
	rare	11	*10.8*	8	3	
	frequent	3	*2.9*	1	2	

* Fisher's exact test (2-sided); bold face representing P-value <0.05.
** class 0: <25 kg/m²; class 1: ≥25.0 and ≤29.9 kg/m²; class 2: >29.9 and ≤34.9 kg/m²; class 3 ≥35.0 kg/m²

different NASH model [31], and Mitsuyoshi et al. who found increased FASN mRNA expression in human steatotic liver tissue without histological signs of inflammation [32]. Noteworthy, we demonstrated that the expression levels of SREBP1, the main transcriptional regulator of FASN, paralleled the FASN mRNA expression levels *in vitro* and *in vivo*, and FASN mRNA and protein expression in human liver samples showed a significant correlation.

These findings suggested that the increased FASN expression upon hepatocellular lipid accumulation is regulated on the transcriptional level, while the observed steatosis induced induction seems to be impaired by hepatic inflammation. However, neither *in vitro* in primary human hepatocytes stimulated with TNF nor *in vivo* in an experimental model of liver inflammation, which is not related to NAFLD, FASN expression differed from control cells and tissues.

In line with this, hepatic FASN expression in patients with NAFLD revealed no correlation with histological inflammation or ballooning of hepatocytes as characteristic sign of advanced hepatocellular damage.

Thus, it appears that (hepatic) inflammation does not directly impair the increased FASN expression in response to hepatocellular lipid accumulation. Rather, one may speculate whether a lack of FASN increase in NAFLD actually predisposes to hepatic inflammation. Actually, while mice with liver specific FASN knock out reveal a similar phenotype as control mice when fed a control diet, they developed hepatic steatosis due to reduction of beta-oxidation upon feeding a low fat high carbohydrate diet [33]. Although such a feeding model is not directly related to the situation found in most patients with NAFLD, it appears that - at least under certain nutritional conditions - lack of FASN may aggravate liver injury. Conversely, effective and complete lipogenesis e.g. conversion of acetyl-CoA and malonyl-CoA into palmitate, which can subsequently be esterified into triglycerides, prevents the potentially harmful increase of mitochondrial oxidation and ROS generation, since triglycerides themselves are not toxic but buffer the accumulation of fatty acids [7,34]. Therefore, FASN expression in steatotic hepatocytes may be considered as a compensatory adaptation at early stages of NAFLD or NASH development, respectively. However, upon overriding the capacity of this homeostatic response FASN expression and lipogenesis, respectively, can no longer prevent increased lipid peroxidation and progression to inflammation and fibrogenesis.

Certainly, the regulation of hepatic lipogenesis is a highly coordinated process occurring on the transcriptional as well as post-transcriptional level. Further, despite the fact that FASN is considered as the key enzyme of hepatic lipogenesis, we did not assess whether all the lipogenic steps are coordinated affected. Still, our study revealed a clear correlation between hepatocellular lipid accumulation and FASN expression, which seems to be impaired in NASH. Further studies are required to unravel, whether FASN expression may serve as a diagnostic marker or therapeutic target for the progression from sole steatosis to steatohepatitis in patients with NAFLD.

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