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ORIGINAL CONTRIBUTION

A moderate weight reduction through dietary intervention decreases hepatic fat content in patients with non-alcoholic fatty liver disease (NAFLD): a pilot study

Valentina Volynets · Jürgen Machann · Markus A. Küper · Ina B. Maier · Astrid Spruss · Alfred Königsrainer · Stephan C. Bischoff · Ina Bergheim

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Abstract
Purpose As a diet rich in fructose and an impaired intestinal barrier function have been proposed to be risk factors for the development of non-alcoholic fatty liver disease (NAFLD), the aim of the present pilot study was to determine whether a dietary intervention focusing on a reduction of fructose intake (−50 % in comparison with baseline) has a beneficial effect on liver status.
Methods A total of 15 patients with NAFLD were enrolled in the study of which 10 finished the study. Fructose and total nutrient intake were assessed using a diet history. At baseline and after 6 months liver status and markers of intestinal barrier function as well as plasminogen activator inhibitor (PAI-) 1 concentration were determined in plasma.
Results Hepatic lipid content and transaminases in plasma as well as body mass index and some parameters of glucose metabolism (e.g., fasting plasma insulin) were significantly lower at the end of the intervention when compared to baseline. Whereas the dietary intervention had no effect on the prevalence of bacterial overgrowth, orocecal transit time and the intestinal permeability or blood ethanol levels endotoxin and PAI-1 concentration in plasma were significantly lower at the end of 6 months intervention period than at baseline.
Conclusions Taken together, our results indicate that a dietary intervention focusing only on one dietary parameter like fructose may help to decrease intrahepatic fat content of NAFLD patients.

Keywords NAFLD · Endotoxin · Fructose · PAI-1 · Metabolic syndrome

Introduction

The prevalence of NAFLD (non-alcoholic fatty liver disease), comprising a disease continuum ranging from simple steatosis to cirrhosis, has increased dramatically throughout the last three decades. Bacterial overgrowth and an impaired function of the intestinal barrier have been suggested to be involved in the pathogenesis of NAFLD. Indeed, we [1] and others [2–5] reported that patients with different stages of NAFLD ranging from simple steatosis to non-alcoholic cirrhosis suffer from endotoxemia, a higher prevalence of bacterial overgrowth in the small intestine, a prolonged orocecal transit time and increased intestinal permeability but also exhale more ethanol in their breath even in the absence of ethanol ingestion [6]. However, causes of the alteration of the intestinal microbiota and increased intestinal permeability have not yet been clarified.

Results of studies of our own group [1] and those of other groups [7, 8] suggest that a diet rich in carbohydrates and herein particularly in fructose may be associated with

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V. Volynets · I. B. Maier · A. Spruss · S. C. Bischoff · I. Bergheim (✉)
Department of Nutritional Medicine (180 a), University of Hohenheim, Fruwirthstraße 12, 70599 Stuttgart, Germany
e-mail: ina.bergheim@uni-hohenheim.de

J. Machann
Section on Experimental Radiology, Department of Diagnostic and Interventional Radiology, Tuebingen University Hospital, Hoppe-Seyler-Straße 3, 72076 Tuebingen, Germany

M. A. Küper · A. Königsrainer
Department of General, Visceral and Transplant Surgery, Tuebingen University Hospital, Hoppe-Seyler-Straße 3, 72076 Tuebingen, Germany

the development of NAFLD and may increase the odds to develop the later stages of the disease (e.g., non-alcoholic steatohepatitis (NASH)). Somewhat contrary to these results, it was recently shown in patients with NAFLD that daily fructose ingestion is associated with a reduced risk to develop hepatic steatosis [9]; however, in this study fructose intake was related to an increased risk of hepatic fibrosis. In line with these findings results of several animal studies suggest that a diet rich in fructose may cause a significant accumulation of fat in the liver and that this is associated with an increased translocation of bacterial endotoxins [10–15]. However, whether a diet focusing on a reduction of dietary fructose intake has a beneficial effect on the liver has not yet been clarified. The aim of the present study was to assess the effect of a 6 months long dietary intervention focusing on a reduction of fructose intake on liver fat content of patients with NAFLD.

Materials and methods

Subjects

The study protocol was approved by the ethics committee of the Tübingen University Hospital (Tübingen, Germany) and was carried out in accordance with the Helsinki Declaration as revised in 1983. Written informed consent was obtained from all patients before the study. Patients included had (a) no history of taking lipid-lowering drugs or drugs affecting lipid metabolism, (b) no known medical conditions affecting lipid and glucose metabolism (e.g., diabetes), (c) no medical records of alcohol abuse and an alcohol intake <15 g ethanol/day, (d) no drug-induced hepatotoxicity, (e) no infection with hepatitis B or C virus, and (f) no clinical indication of impaired nutritional status and (g) a "normal" diet (e.g., no vegetarians). A total of 15 patients with NAFLD were enrolled in the study between December 2008 and May 2010. Patients were recruited through general practitioners and hospitals. For ethical reasons it was not possible to obtain biopsy specimen from the patients before and after the study; however, NAFLD was diagnosed by experienced hepatologists through ultrasound examination and assessing blood parameters. Before and after the study liver fat content was assessed by magnetic resonance imaging (MRI), and general health status as well as blood parameters was determined (e.g., transaminases, γ-GT). Details of MRI assessment are given below.

Magnetic resonance imaging and spectroscopy

All magnetic resonance examinations were performed on a 1.5 T whole-body imager (Magnetom Sonata, Siemens Healthcare, Erlangen, Germany) in the early afternoon.

Whole-body magnetic resonance imaging

Whole-body adipose tissue was quantified applying a T1-weighted fast spin-echo sequence [16]. Measurement parameters were as follows: echo time = 12 ms, repetition time = 490 ms, slice thickness 10 mm, interslice gap 100 %, field of view between 480 and 550 mm, depending on the breadth of the subject. Body coil was used as transmit/receive coil. Five slices were recorded per sequence, and table shift between the measurements was 10 cm. In total, 100–120 images were recorded and transferred for post-processing, which was performed by applying a threshold-based semiautomatic procedure based on Matlab (Matlab 7.5, the Mathworks). The number of pixels containing lean tissue and adipose tissue was summed up and multiplied by in-plane resolution, that is, pixel dimensions taken from the image header. From this, the volume of lean tissue and adipose tissue in each slice was determined and summed over the entire body for quantification of total adipose tissue (TAT). Visceral adipose tissue (VAT) was manually selected and quantified between head of femur and diaphragm.

Volume selective proton magnetic resonance spectroscopy (^{1}H-MRS) of the liver

For determination of intrahepatic lipid content (IHL) a single voxel STEAM technique (STimulated Echo Acquisition Mode) was applied [17]. T1-weigthed images were recorded, and the voxel for spectroscopic measurement was placed in the posterior part of segment seven of the liver, avoiding inclusion of macroscopic vessels. Measurement parameters are echo time = 10 ms, repetition time = 4 s, mixing time = 15 ms, voxel size $3.0 \times 3.0 \times 2.0$ cm^3. Thirty-two acquisitions were recorded in order to obtain a sufficient signal-to-noise ratio in a measuring time of 2 min 8 s. Shimming of the voxel was performed in the automatic mode, and the subjects were asked to breathe flatly in this time interval. Signal integrals of water (4.7 ppm) and lipids (methylene and methyl at 1.3 and 0.95 ppm) were manually quantified in fixed ppm-intervals (water: 3.1–6.2 ppm, lipids: 0.5–1.8 ppm). IHL values were calculated by the integral ratio of lipids and water.

Dietary intake, dietary counseling and physical activity

Dietary intake, alcohol consumption and leisure time activity of patients were assessed by an experienced nutritionist using a diet history before the study and after the 6 months intervention period using the software EBISpro©, version 8.0 (2007 Germany) used before successfully to assess dietary intake in various clinical settings [18]. Based on the daily fructose intake assessed at the

beginning of the study, NAFLD patients were advised to reduce their daily fructose intake by 50 %. Accordingly, using games, quizzes but also slidebased presentations in which specific situations like restaurant visits were shown, patients were trained to reduce the consumption of fructose-rich foods (e.g., to avoid sweets, lemonades, fruit juices) and to prefer foods with a lower content of fructose (e.g., diet-lemonades, fruit with low fructose-content). During the counseling, which took place every 2 weeks in small groups of 2–3 patients, NAFLD patients were also given the opportunity to exchange their individual experiences and techniques to avoid fructose intake. Furthermore, each study participant received a cook book with recipes in which sucrose was replaced with either artificial sweeteners or glucose. If a patient reduced fructose intake by less than 20 % in comparison with baseline, as assessed by a 24 h recall 3 months after the begin of the study and a diet history at the end of the intervention, the patient was defined as not compliant to the dietary intervention.

Intestinal permeability

To asses intestinal permeability a lactulose/mannitol test adapted from the methods published by Generoso et al. [19] was used. In brief, after an overnight fast patients drank a mixture of 5 g lactulose and 2 g mannitol (Ratiopharm GmbH, Ulm, Germany and Fagron GmbH & Co. KG, Barsbüttel, Germany, respectively) and collected urine for the next 6 h. The percentage of the orally administrated dose of the two sugars recovered in urine samples was determined using high-performance-anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex GmBH, Idstein, Germany). As described by others before [20] a ratio of lactulose/mannitol <0.030 was considered as normal.

Blood alcohol and PAI-1 ELISA

Alcohol levels were measured in heparinized plasma before and after the 6 months dietary intervention using the "ADH-enzymatic" method described by Bonnichsen et al. [21]. The concentration of functionally active PAI-1 in plasma was assessed using an ELISA kit (LOXO, Germany).

Endotoxin measurments

After incubating plasma samples at 70 °C for 20 min, endotoxin plasma levels were determined using a commercially available endpoint Limulus Amebocyte Lysate (LAL) assay (Charles River, L'Arbaesle, France) with a concentration range of 0.015–1.2 EU/mL.

Glucose hydrogen breath test

Using a breath gas analyzer (Electrochemical H_2 monitor, Stimotron medizinische Geräte, Wendelstein, Germany) a glucose hydrogen breath test was performed before and after the study. After ingesting 75 g of glucose, breath hydrogen exhalation was determined every 10 min for 120 min. A rise in breath hydrogen >10 ppm within the first 10–20 min after glucose ingestion was considered as an indication of small intestinal bacterial overgrowth (SIBO) [4, 22].

Lactulose hydrogen breath test

Lactulose hydrogen breath test was performed using a breath gas analyzer (Electrochemical H_2 monitor, Stimotron medizinische Geräte, Wendelstein, Germany). After ingesting 30 ml of lactulose syrup (Ratiopharm GmbH, Ulm, Germany) hydrogen concentration in breath of patients was then determined every 10 min for 180 min. Time until a rise in the hydrogen breath concentration >10 ppm in two subsequent measurements was determined and was taken as oroceocal transit time [23].

Statistical analysis

Results are reported as means ± SD or medians with interquartile ranges. Wilcoxon-test was used to compare data obtained at baseline and at the end of the intervention (software SPSS, Version 17.0, 2008). A $p \leq 0.05$ was selected before the study as the level of significance.

Results

Characteristics of NAFLD patients

Five patients dropped out of the study as they were claustrophobe ($n = 2$), became pregnant ($n = 1$), were found to suffer from a genetic dysfunction of lipid metabolism ($n = 1$) or were non-compliance with the intervention program ($n = 1$).

Fasting insulin levels were all within the normal range; however, one patient had elevated fasting glucose levels (Table 2). Plasma ALT and AST levels were in 6 and 3 patients, respectively, above the normal range, whereas γ-GT levels were within the normal range in all patients. All patients were diagnosed to have steatosis as assessed by MRI and ultrasound.

Nutritional intake

Results of the assessment of the nutritional intake of patients at baseline and at the end of the dietary intervention are summarized in Table 1 and Supplemental Table 1.

Table 1 Characteristics and nutritional intake of NAFLD patients

	Baseline	6 months
Sex (f/m)	6/4	6/4
Age	45.5 (34.5–51.5)	45.5 (35.0–52.0)
Body weight (kg)	91.7 (69.9–111.1)	89.8 (68.0–107.2)*
BMI (kg/m^2)	31.1 (25.6–40.6)	31.1 (24.6–39.3)*
Physical exercise (h/week)	1.5 (0.8–3.3)	1.1 (0.2–4.2)
Physical exercise (yes/no)	9/1	8/2
Energy (kJ/day)	12,820 (9,481–14,645)	8,151 (6,790–10,283)*
Fat (g/day)	125 (67–154)	86 (70–107)*
Saturated fatty acids (SFA) (g/day)	42.0 (25.7–54.3)	32.0 (20.8–38.8)*
Mono-unsaturated fatty acids (MUFA) (g/day)	38.5 (23.6–45.6)	33.4 (20.6–37.1)
Polyunsaturated fatty acids (PUFA) (g/day)	15.1 (10.3–22.6)	13.3 (9.4–18.2)
Protein (g/day)	131 (107–152)	91 (71–126)
Plant derived protein (g/day)	28.4 (13.7–39.9)	19.1 (15.7–22.3)*
Animal derived protein (g/day)	90.4 (45.9–104.4)	62.3 (41.7–93.6)
Carbohydrate (g/day)	333 (274–396)	186 (154–281)*
Complex carbohydrates (g/day)	138 (97–199)	119 (91–190)
Glucose (g/day)	53.4 (40.3–73.8)	20.3 (14.6–26.0)*
Sucrose (g/day)	72.0 (46.8–95.6)	22.0 (15.8–31.9)*
Fructose (g/day)	60.1 (38.0–67.4)	22.5 (12.4–28.0)*
Alcohol (g/day)	1.0 (0.1–5.0)	0.3 (0.0–3.3)

Data are medians with interquartile ranges in parentheses. * $p < 0.05$ compared to baseline

Patients had reduced their fructose intake significantly by $\sim$61 % $\pm$ 20 after the 6 months when compared to baseline, which was paralleled by an overall reduction of total carbohydrate intake (~ -37 % $\pm$ 14, $p < 0.05$) but also intake of glucose and sucrose (glucose: ~ -62 % $\pm$ 13 and sucrose: ~ -66 % $\pm$ 16, both $p < 0.05$), whereas intake of complex carbohydrates was not changed. Average daily intake of total calories, total fat and saturated fat was also significantly reduced at the end of the study in comparison with baseline. Patients also decreased their intake of protein (~ -22 % $\pm$ 18, n.s.).

Liver parameters

Mean liver fat content was significantly reduced (~ -36 % $\pm$ 26) at the end of the study (Fig. 1). After the 6 months of dietary intervention AST levels were within the normal range with the exception of one patient (F ig. 2). A similar effect was also found for ALT levels; however, only 2 patients reached the normal range for ALT levels. Whereas the median of γ-GT in plasma levels was not altered by the dietary intervention, γ-GT levels in plasma were lower in 7 of the 10 patients at the end of the study.

Parameters of the metabolic syndrome and visceral fat

Mean BMI, total and visceral fat contents were also significantly lower in patients at the end of the 6 months dietary intervention than at baseline (see Fig. 1). Mean fasting insulin, glucose, HOMA-IR Index and uric acid levels in plasma, despite not being above the normal range at baseline, were also found to be significantly lower at the end of the study in comparison with baseline. Furthermore, fasting glucose levels found to be elevated in one patient before the study were within the normal range at the end of the 6 months dietary intervention, whereas total cholesterol and low-density lipoprotein cholesterol levels in blood were not affected by the dietary intervention (see Table 2). Triglyceride and high-density lipoprotein (HDL) cholesterol levels in plasma were slightly increased in the due course of the study (triglycerides: $+\sim$25 %, $p = 0.28$, HDL cholesterol: +4 %, $p = 0.65$) (see Table 2; Fig. 2).

SIBO, orocecal transit time, intestinal permeability and ethanol as well as bacterial endotoxin and PAI-1 plasma levels

The dietary intervention had no evident effect on intestinal permeability, the prevalence of SIBO or plasma ethanol levels (see Table 2); however, both bacterial endotoxin and PAI-1 levels in plasma were significantly lower at the end of the 6 months of dietary intervention than at baseline (see Fig. 3).

Discussion

Results of recent dietary intervention studies performed in humans with NAFLD focusing on a reduction of

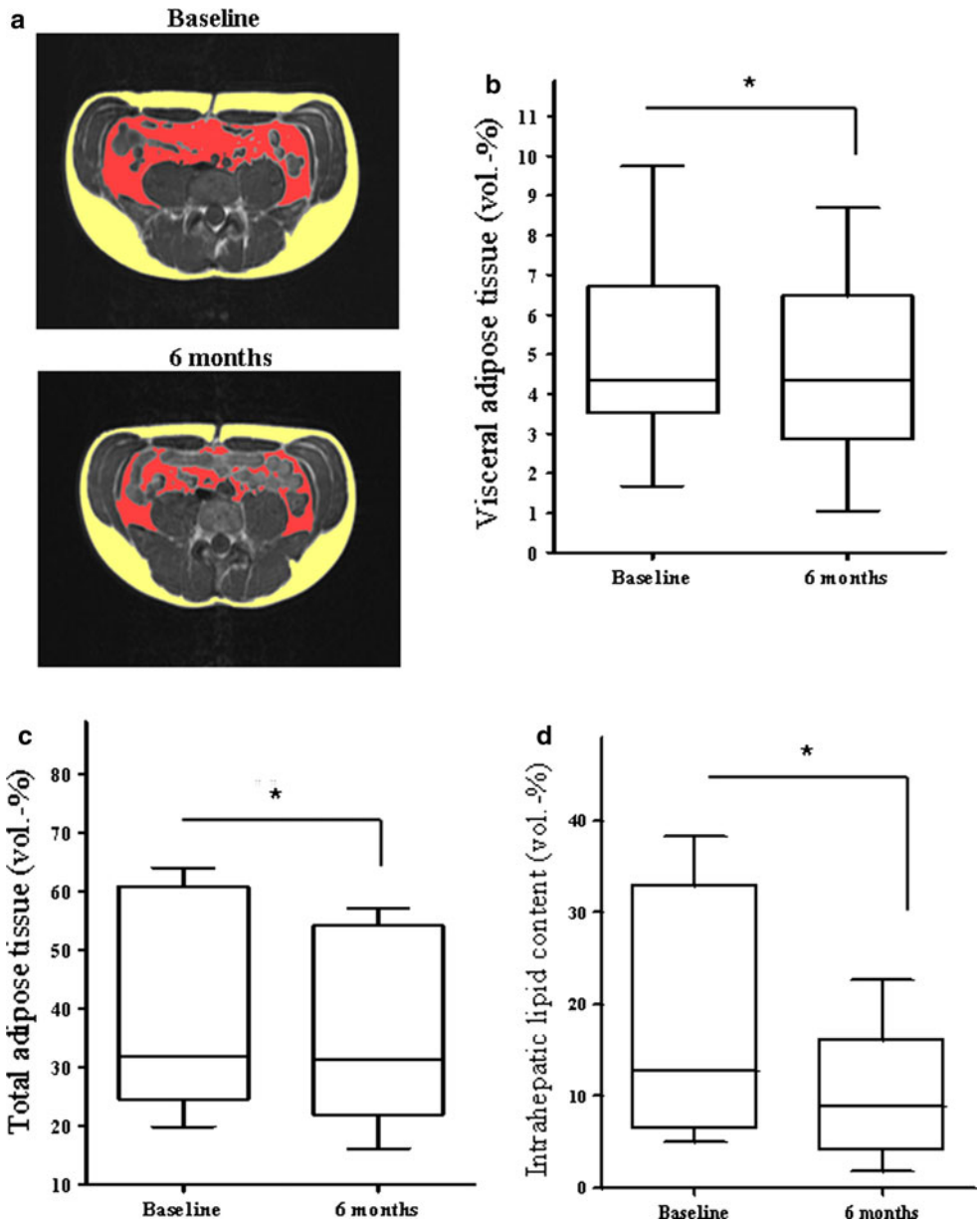


Fig. 1 Effect of the dietary intervention on total, visceral and intrahepatic fat contents of NAFLD patients. **a** Representative pictures of a visceral fat MRI scan. Subcutaneous adipose tissue is shown in *yellow*, visceral adipose tissue is shown in *red*. **b** Quantitation of visceral fat, **c** total body and **d** intrahepatic fat content at the beginning and at the end of the study. Data are medians with interquartile ranges. $*p < 0.05$

carbohydrate intake ("low carb diet") and herein particularly when combined with a reduction of the general caloric intake suggest that a reduction of carbohydrate intake may be beneficial for the liver (for review see York et al. [24] and Centis et al. [25]). However, whether a diet intervention in which the counseling focuses only on a reduction of fructose without an explicit caloric restriction and/or an increase of physical activity has a positive effect on liver status and disease progression has to our knowledge so far not been clarified. In the present study we assessed the effect of a 6 months dietary intervention, in which the counseling focused on a reduction of fructose intake by ~50 % on liver status of patients with NAFLD. Patients decreased their intake of fructose according to the study design. However, along with the reduction of fructose patients also reduced total caloric intake as well as glucose, fat and protein consumption as they tried to avoid sugar rich foods rather than to substitute fructose with glucose. These data suggest that the approach to only advice patients to avoid fructose in their daily diet and to leave them a free choice of foods is not a feasible way to test the question whether fructose intake is causally involved in the development and progression of NAFLD in humans. Rather, our results suggest that this question should either be addressed in a more controlled study setting where patients are not left a choice of food and beverages or where dietary intake is more closely monitored (e.g., through food dairies). However, despite these limitations

Table 2 Parameters of glucose metabolism, lipid levels, intestinal permeability, orocecal transit time (OCTT) and ethanol concentration in plasma of NAFLD patients

	Baseline	6 months
n	10	10
Fasting glucose (mg/dL)[a]	95.5 (90.5–107.0)	93.0 (88.5–96.5)*
Insulin (pmol/L)[b]	58.9 (27.0–121.0)	33.9 (20.5–100.0)*
HOMA-IR Index	2.7 (2.0–4.8)	1.9 (1.4–4.2)**
Triglycerides (mg/dL)	125.5 (73.0–198.5)	158.5 (80.5–249.5)
Cholesterol (mg/dL)	228.5 (169.0–259.0)	225.5 (160.5–265.0)
HDL–Cholesterol (mg/dL)	41.5 (37.5–53.5)	44.0 (35.0–54.0)
LDL Cholesterol (mg/dL)	159.5 (119.0–181.5)	155.5 (113.5–172.0)
Lactulose (mg/L)	5.2 (3.5–12.7)	6.4 (5.1–10.2)
Mannitol (mg/L)	235 (181–289)	244 (159–395)
Ratio lactulose/mannitol	0.027 (0.02–0.038)	0.031 (0.019–0.038)
OCTT (min)	60.0 (45.0–90.0)	47.5 (35.0–75.0)
Ethanol (μmol/μL)	0.08 (0.06–0.15)	0.09 (0.06–0.10)

Data are medians with interquartile ranges in parentheses

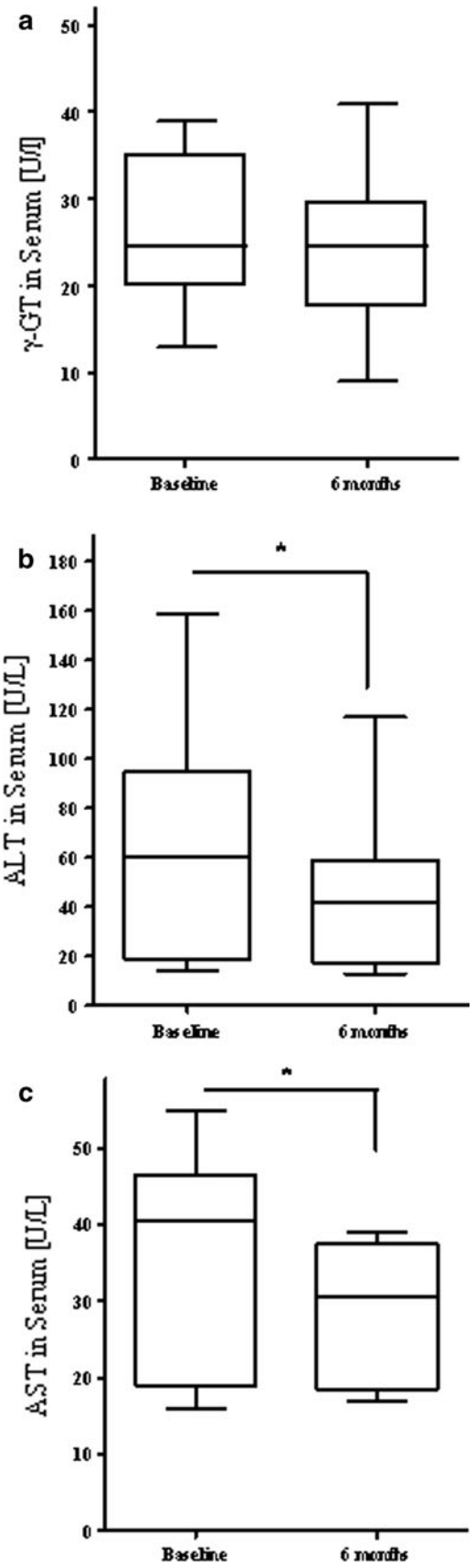
* $p < 0.05$ compared to baseline; [a] normal range <110 mg/dL; [b] normal range <175 pg/L

** $p < 0.01$

HOMA-IR Index = [fasting insulin (μlU/ml) × fasting glucose (mmol/l)]/22.5 [31]

and in line with the finding of the reduced caloric intake, BMI and total body fat as well as visceral fat were also reduced in the due course of the intervention. Furthermore, liver fat content of patients improved markedly as assessed by MRI and transaminases. Glucose metabolism also improved considerably, whereas blood lipid levels (e.g., total and LDL cholesterol) were only slightly altered. The impact of this dietary regimen used in the present study on blood lipid levels will have to be determined in future studies in a larger cohort. Despite the rather moderate body weight loss (~ -3 % body weight reduction) the magnitude of changes in regard to liver parameters is in line with that reported before by others for low (800–1,800 kcal/day) and very low-calorie diets (<800 kcal/day) as well as those for carbohydrate restriction (20–50 g/day) (for overview see [26]). Taken together, the results of the present pilot study suggest that a dietary counseling focusing only on one dietary parameter like the reduction of fructose intake without a caloric restriction may not only improve liver status but also other parameters of the metabolic syndrome and add to a reduction of body weight. However, as patients not only decreased their intake of fructose but also their total caloric intake as well as fat and—to a lesser extend—protein intake and through the avoidance of fructose-rich foods probably changed their overall dietary pattern, the beneficial effects found in the present study may not be attributed solely to the reduction of fructose intake.

Results of several human studies suggest that an impaired intestinal barrier function and increased translocation of bacterial endotoxin may be a critical factor in the development of NAFLD in humans [1–4]. In the present study, despite not finding any apparent effects on intestinal permeability, the prevalence of SIBO, intestinal motility or plasma ethanol concentration, plasma endotoxin levels were found to be significantly reduced after the 6 months dietary intervention when compared to baseline. This apparent discrepancy of data may be due to the fact that the sensitivity, but also the test used to determine SIBO (e.g., glucose ingestion) was not sufficient to determine intestinal overgrowth in the upper parts of the gastrointestinal tract. The relative molecular mass of endotoxins ranges from $\sim$10 to 1,000, whereas the relative molecular mass of lactulose and mannitol is 342 and 182, respectively. It may be that the dietary intervention decreased the permeability for larger molecules (e.g., endotoxins) but had no effect on the permeability of small molecules like the two test sugars. This needs to be addressed in future studies using either other markers to determine intestinal permeability (e.g., polyethylene glycol (PEG) as described by Parlesak et al. [27]) or by determining concentration of tight junction proteins in biopsy specimen obtained from the gut. It also could be that the clearance of endotoxin by the liver from the blood was improved in the due course of the study. Indeed, it has been shown before in animal studies that in fatty livers clearance of endotoxin may be impaired (for overview see [28]). However, molecular mechanisms responsible for the decrease in endotoxin levels remain to be determined. In line with the findings for endotoxin levels and liver status, levels of active PAI-1 in plasma were also found to be decreased at the end of the dietary intervention. PAI-1 plasma levels have been shown before to be related to endotoxin levels in humans [1]. Furthermore, recent data obtained in animal studies suggest that an increased translocation of endotoxin subsequently may lead to an induction of PAI-1 expression in the liver, which in turn has been shown to modulate hepatic lipid export and add to the development of fatty liver [13]. Taken together, these data suggest that a lifestyle intervention focusing on a reduction of fructose intake may protect the liver not only as patients tend to take in less calories but may also diminish bacterial endotoxin levels and the release of



◀ **Fig. 2** Effect of the dietary intervention on **a** γ-GT and **b** ALT levels and **c** AST levels. Data are medians with interquartile ranges. $^{*}p < 0.05$

active PAI-1. However, whether the decreased endotoxin levels are a direct effect of the reduced intake of fructose or other dietary compounds on the intestinal barrier function or are indirectly caused (e.g., through alterations of bacterial flora) remains to be determined. It also remains to be determined whether the decrease in active PAI-1 levels in plasma found in the present study resulted from the decreased bacterial endotoxin levels or whether other molecular mechanisms are involved.

Limitations of the pilot study

Our pilot study has several limitations that need to be considered when interpreting the data. First, we had a small sample size with only 10 completers. However, our results are in line with the findings of others that reported that dietary interventions focusing on a reduction of total carbohydrate intake or high fructose corn syrup, respectively, with or without a restriction of caloric intake may have positive effect on liver status [8, 29, 30]. Second, it cannot be ruled out at the moment that positive effects found in the present study are also due to the decrease of dietary glucose intake or the overall decrease of caloric intake and the subsequent weight loss. Our results therefore may not be generalized to dietary fructose intake only. Third, patients included in the present study all only displayed the early stages of NAFLD. Our results may therefore not be generalized to later stages of the disease. Fourth, liver status of patients was assessed using MRI/MRS and transaminases levels. Of course the quality of the study would have been improved by assessing liver status using biopsies in addition; however, for ethical reasons this is not possible in Germany. Finally, although we detected a marked improvement in liver status and some parameters of glucose metabolism, the duration of our study (6 months) may not have been sufficiently long enough to determine a counterbalance in dietary pattern. Also, a follow-up was not performed. However, we thought that the study length would be long enough to test whether a diet focusing on a reduction of only one parameter in diet (e.g., fructose intake) may have a positive effect on the liver without resulting in a high drop-out rate. Long-term effects will have to be determined in larger studies with a longer duration.

Taken together, the results of the present study suggest that a lifestyle intervention focusing on a reduction of fructose intake may decrease liver fat content and transaminases of patients with NALFD but may also decrease

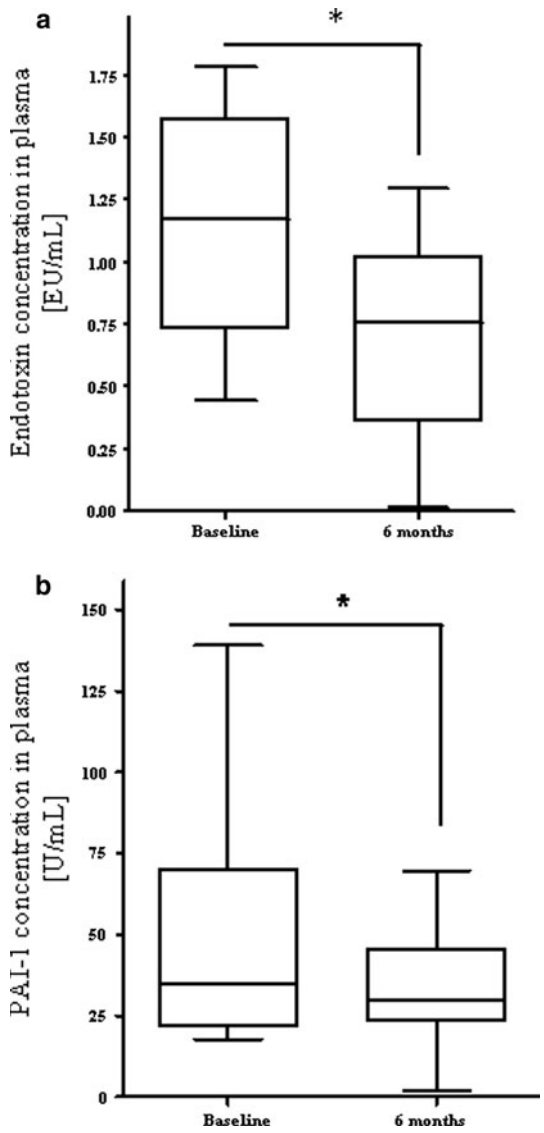


Fig. 3 Effect of the dietary intervention on plasma endotoxin and PAI-1 concentration of NAFLD patients. **a** Endotoxin and **b** PAI-1 concentration in plasma. Data are medians with interquartile ranges. $^{*}p < 0.05$

intestinal translocation of bacterial endotoxin and lower PAI-1 levels. However, further studies are needed to investigate the underlying molecular mechanisms of the protective effects of this dietary intervention and to delineate if other factors altered through this dietary intervention strategy (e.g., total caloric intake, changes in dietary pattern) also contributed to the protective effects found.

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Conflict of interest The authors declare that they have no conflict of interest.

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