Treatment of preestablished diet-induced fatty liver by oral fatty acid–bile acid conjugates in rodents

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Background Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in industrialized countries. It has no accepted medical therapy. Fatty acid–bile acid conjugates (FABACs) were proven to prevent diet-induced NAFLD in rodents.

Aim This study was undertaken to test whether oral FABACs are also effective in reducing liver fat in preestablished diet-induced NAFLD.

Methods NAFLD was induced in mice and rats by a high-fat diet and maintained by various proportions thereof. The FABACs used were conjugates of cholic acid with either arachidic or stearic acids.

Results FABAC therapy reduced liver fat in all four series of experiments. The rapidity of the effect was inversely proportional to the concentration of fat in the maintenance diet. In mice on a 25% maintenance diet FABACs decreased total liver lipids by about 30% in 4 weeks ($P < 0.03$). Diglycerides ($P < 0.003$) and triglycerides ($P < 0.01$) were the main neutral liver lipids that decreased during FABAC therapy. Both FABACs tested reduced liver fat in NAFLD at doses of 25 and 150 mg/kg/day. High-fat diet increased, whereas FABAC therapy decreased plasma $16:1/(16:0+16:1)$ fatty acid ratio – a marker of stearoyl CoA desaturase activity. In HepG$_2$ cells FABACs decreased de-novo fatty acid synthesis dose dependently.

Conclusion Oral FABAC therapy decreased liver fat in preestablished NAFLD in mice and rats. Inhibition of stearoyl CoA desaturase activity and fatty acid synthesis are mechanisms that may contribute to this decrease. FABACs may be potential therapeutic agents for human NAFLD. *Eur J Gastroenterol Hepatol* 20:1205–1213 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease today in many western countries [1–3]. In population studies, mostly in adults, a prevalence of 20–25% was found. As some studies were based only on unexplained elevations of serum transaminases and as asymptomatic patients with fatty liver mostly have normal transaminases [3], the actual prevalence of NAFLD may be even higher.

NAFLD progresses to nonalcoholic steatohepatitis (NASH), cirrhosis and its complications in about 25% of cases [4,5]. It has recently been shown that reduction of liver fat by weight loss, induced by bariatric surgery or by fat malabsorption, can also reverse coexisting inflammation and fibrosis [6–8]. Thus, even the progressive form of the disease is potentially reversible. In practice, significant reduction of overweight by diet is, however, difficult to achieve and even more difficult to maintain. Medical treatment is therefore needed and to date no proven medical therapy exists [9].

It is relatively easy to induce NAFLD by dietary manipulation in rodents. Maintenance of NAFLD at a steady state, when testing the effect of therapeutic agents, is, however, more problematic and was addressed in this study.

In an earlier study we showed that fatty acid–bile acid conjugates (FABACs) prevented the accumulation of liver fat in rodents on different high-fat diets (HFDs) [10]. The clinical need is, however, for treatment of established NAFLD rather than its prevention. The aim of this study was to test whether FABACs would be effective in reducing liver fat in preestablished fat-induced NAFLD.

Materials and methods
Animals and diets
Male C57BL6 and BalbC mice (20–25 g, 4 weeks old) and male Fisher rats (80–100 g, 1 month old) were studied. The animals were purchased from Harlan Co. (Rehovot, Israel).

The regular diet (RD) was a standard chow diet (Koffolk, Israel) containing 5% of fat (w/w) and 3950 kcal/kg (11.4% of calories from fat). The HFD used for induction was composed of butter 15 g, cholesterol 1 g, cholic acid 0.5 g, and corn oil 2 g, added to 81.5 g of the RD to produce the final HFD [10]. It contained 4794 kcal/kg (39.9% from fat). The HFD used for maintenance contained 25, 50 or 100% of the fat added to the chow diet. The diets, animals, and duration of trial periods are specified in the text and legends to each figure. Water and the various diets were given to all animals *ad libitum*.

The FABAC was administered daily by gavage at a dose of 25 or 150 mg/kg as a suspension in saline (0.1–0.5 ml, depending on animal weight). The controls received an equal volume of saline by gavage. The studies were approved by the Institutional Animal Care and Use Committee of our institutions.

Experimental procedure
The animals were randomly distributed into separate groups fed the various diets. All animals had free access to food and water and were kept in temperature-controlled rooms (22–24°C), 40–70% humidity, under a 14 h (05 : 00–19 : 00 h) light–dark cycle. At sacrifice, between 10 : 00 and 11 : 00 h, the animals received an anesthetic (10% ketamine, 5% xylazine in saline) at a dose of 0.01 ml/g, intraperitoneally. Liver samples were immediately processed under liquid nitrogen and kept frozen (–70°C) until analysis and in 4% buffered formalin for histology.

Fatty acid–bile acid conjugate
FABACs were synthetized as described earlier [11]. In this study two different FABACs were used: (i) 3-β arachidyl amido 7α, 12α dihydroxy 5β cholan 24-oic acid (Aramchol; C20-FABAC) – a conjugate of arachidic and cholic acids and (ii) 3-β stearyl amido 7α, 12α dihydroxy 5β cholan 24-oic acid (Steamchol; C18-FABAC) – a conjugate of stearic and cholic acids. They were provided to us as a generous gift by Galmed Medical Research, Ltd., Tel-Aviv, Israel.

Analytical methods
Lipid extraction, thin-layer chromatography, and gas chromatography
The liver samples were weighed and homogenized with saline at a ratio of 1 : 5 (w : v) in plastic tubes on ice. Lipids were extracted from an aliquot of the liver homogenate according to the procedure of Folch *et al.*

[12]. The total amount (total liver lipids, TLL) was calculated after aliquot evaporation to constant weight. Neutral lipids were separated by thin-layer chromatography on silica gel 60 plates (Merck, Darmstadt, Germany). Neutral lipids were developed with a solvent system constituted by hexane, ethyl ether, and acetic acid, 70 : 30 : 1 (v : v : v). The lipids were identified by their Rf in comparison with known standards. They were detected by iodine vapors and quantified by densitometry (BIS 202D, Rhenium, Jerusalem, Israel) in comparison with calibration curves of the appropriate standards.

Fatty acids were analyzed by GC using the method of Batta *et al.* [13]. The retention times were compared to those of pure standards, and the quantification was achieved by the use of calibration curves

Analysis of fatty acid synthesis in vitro
De-novo fatty acid synthesis was measured in HepG2 calls by incubating them in DMEM + + + (Dulbeco minimal essential medium) supplemented with 5% L-glutamate, 5% PSN (penicillin, streptomycin, niacin) and 10% fetal calf serum. Different concentrations of FABAC were prepared by adding it in ethanol and diluting it with DMEM + + + to the desired final concentration. FABAC treatment was performed during 24 h on cell culture at 70–80% cell confluence.

Two hours before the end of the incubation period, the cells were pulse-labeled with [^{14}C] acetate at a final concentration of 35 mmol/l (2 mCi/l medium). After extraction of the medium, the cells were washed three times with phosphate buffered saline. Cell membranes were disrupted with hexane : isopropanol (3 : 2, v : v) at 37°C during 30 min. The solvent was evaporated. Cell lipids were extracted according to the procedure of Folch *et al.* [12] and applied on silica gel 60 plates (Merck). Neutral lipids were developed with a solvent system constituted by hexane, ethyl ether, and acetic acid, 70 : 30 : 1 (v : v : v). The lipids were identified by their Rf in comparison with known standards. They were detected by iodine vapors, identified by comparing their Rf to those of known standards, scraped and counted in a scintillation counter [14].

Glucose, insulin, and homeostasis model assessment
At the end of the experimental period, tail-bleed glucose levels were determined under fasting or nonfasting conditions. Animals were subjected to a glucose tolerance test after 4 h of fasting in the morning (11 : 30 h). Blood samples were collected from tail vein at time 0. Glucose (1 g/kg) was injected intraperitoneally, and additional blood samples were collected after 30, 60, 90, and 120 min. Blood glucose levels were immediately measured by Sugar Accu-Check Go sensor (Roche, Mannheim, Germany). Plasma insulin was determined using an

insulin immunoassay kit (MRC Mouse Insulin, Elisa 96T, Mercodia, Uppsala, Sweden). The homeostasis model assessment index [15] was calculated as plasma glucose (mmol/l) × plasma insulin (μU/ml)/22.5.

Light microscopic analysis of fatty liver
Formalin-fixed liver samples were stained with hematoxylin and eosin. Fat infiltration, inflammation, and fibrosis were scored as none (0), mild (1), moderate (2), and severe (3).

Statistical analysis
Values are expressed as mean ± SD. The differences among the different groups were analyzed by Student's *t*-test; $P < 0.05$ was considered significant.

Results
Animal weight
All animals continued to gain weight throughout the studies, irrespective of diet and FABAC supplementation (Table 1). Mice on the HFD gained more weight than animals on the RD, with the exception of those on the 50% HFD. Their induction started at a low body weight (ca 15 g) and at that stage the RD was better tolerated than the 100% HFD induction diet.

Liver fat
Reversion to regular diet
When animals were reverted to RD, after the induction of NAFLD, their liver fat returned rapidly to normal. In rats and mice this occurred within about 2 weeks, not permitting evaluation of the effects of therapy.

Maintenance on 25 and 50% high-fat diet
After induction of fatty liver in C57BL6 mice by HFD (100%), they were maintained on a diet containing 25% of the fat originally added. Test animals were given Aramchol (25 or 150 mg/kg/day) or Steamchol (25 mg/kg/day) in addition to the diet. Animals (seven per group) were sacrificed at 4 and 6.5 weeks from start of therapy. TLL are shown in Fig. 1. Despite the 25% maintenance diet liver lipids declined, but were still significantly higher than those on a RD at 4 weeks. This difference remained (NS) but declined further at 6.5 weeks.

All three FABAC-treated groups had significantly less liver lipids at 4 weeks than the 25% HFD control group (Fig. 1). At 6.5 weeks the findings were similar but statistical significance was lost (except for the Steamchol-treated group) because of the further decline in liver fat

Fig. 1
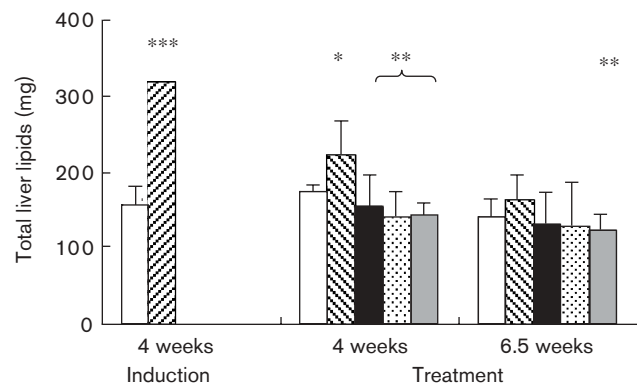
Total liver lipids in C57BL6 mice. Induction: at the end of 4 weeks on high-fat diet (HFD, bars with upward stripes) vs. regular diet (RD, white bars). Treatment: after 4 and 6.5 weeks on 25% HFD without fatty acid–bile acid conjugates (FABAC) (downward stripes) or with C-20 FABAC (Aramchol) 150 mg/kg/day (black bars), C-20 FABAC 25 mg/kg/day (dotted bars), C-18 FABAC (Steamchol) 25 mg/kg/day (grey bars). Mice were sacrificed ($n = 7$/group) and liver lipids measured at end of each period. (*$P < 0.05$ vs. RD, **$P < 0.03$ vs. 25% HFD, ***$P < 0.001$ vs. RD).

Table 1 Body weight gain (%)[a] at end of studies

	Induction		Treatment			
Diet	Duration (weeks)	Weight gain (%)	Diet	FABAC dose (mg/kg/day)	Duration (weeks)	Weight gain (%)
C57Bl6/J mice						
RD	7	35.4 ± 3.2	RD		13.5	59.6 ± 3.7
HFD (100%)		52.7 ± 14.4	HFD (100%)			71.3 ± 33.5
			HFD (100%)	Aramchol 150		82.0 ± 19.9
RD	4	80.4 ± 17.4	RD		8	95.4 ± 20.1
HFD (100%)		60.4 ± 25.4	HFD (50%)			91.3 ± 35.7
			HFD (50%)	Aramchol 150		89.5 ± 28.5
			HFD (50%)	Steamchol 150		93.7 ± 27.7
RD	4	27.2 ± 10.7	RD		6.5	35.3 ± 14.7
HFD (100%)		32.1 ± 10.3	HFD 25%			32.3 ± 9.3
			HFD 25%	Aramchol 25		18.9 ± 14.1
			HFD 25%	Aramchol 150		24.9 ± 15.8
			HFD 25%	Steamchol 25		35.0 ± 19.0
Fisher rats						
RD	3	ND	RD		12	64.8 ± 6.3
HFD (100%)		ND	HFD (100%)			79.6 ± 3.4
			HFD (100%)	Aramchol 150		68.9 ± 16.5

FABAC, fatty acid–bile acid conjugates; HFD, high-fat diet; ND, not determined; RD, regular diet.
[a](Final body weight−initial body weight)/initial body weight × 100.

in the control, 25% maintenance diet group. The findings were similar when expressed as liver lipid concentration (mg lipid/g of liver).

The effects of Aramchol and Steamchol at the same dose (25 mg/kg/day) were similar. At the decreased level of fat challenge (25% HFD) the effects of the two doses of Aramchol (25 and 150 mg/kg/day) were also similar (Fig. 1).

A steady-state fatty liver was achieved in C57BL6 mice using a maintenance 50% HFD after induction (Fig. 2). TLL on the 50% HFD at 4 and 8 weeks were equal and only slightly lower than after induction. The effect of FABACs was not seen at 4 weeks and became apparent only at 8 weeks. A reduction in TLL was seen with both Aramchol ($P < 0.05$) and Steamchol, but did not attain statistical significance with the latter.
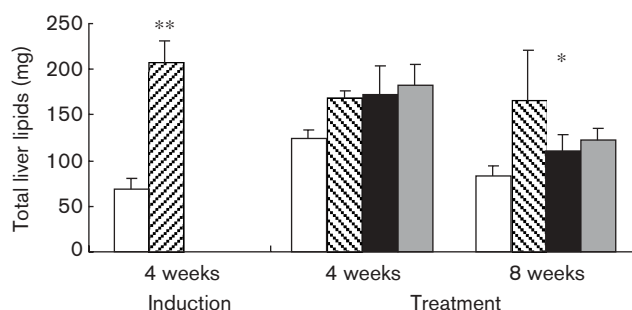
High-fat diet throughout
In the experiments depicted in Fig. 3a and b the HFD used for induction was continued throughout the study including the period of the therapeutic trial.

In Fisher rats, fatty liver was induced by a HFD during 3 weeks. The same diet was continued for the subsequent 12 weeks without or with Aramchol (150 mg/kg/day) supplementation. Liver fat increased almost ten-fold during this long treatment period because of an increase in both liver lipid concentration and in liver weight. In FABAC-treated animals liver lipids decreased significantly compared with HFD controls, whether expressed as TLLs or lipid concentration (Fig. 3a and b).
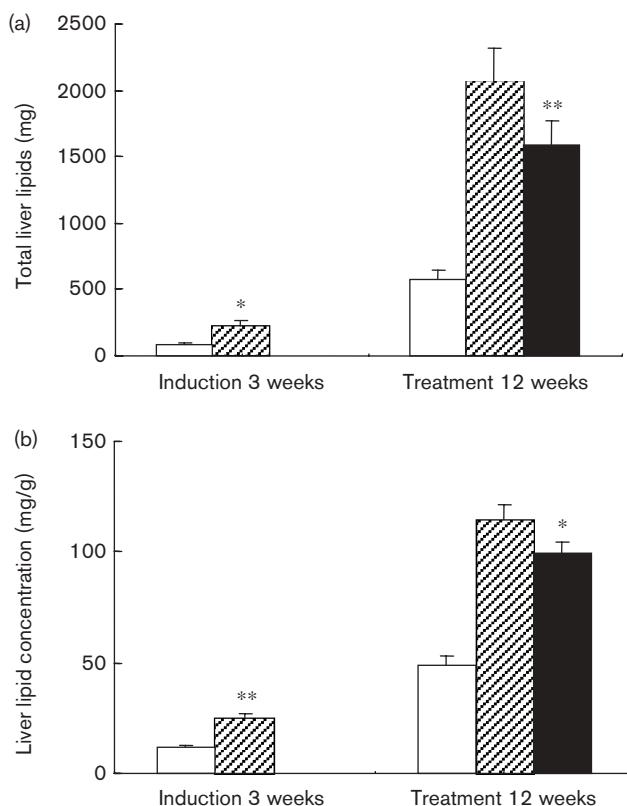
In C57Bl6 mice fatty liver was induced during 7 weeks (Fig. 4). The HFD was then continued throughout the

Fig. 3

Total liver lipids (a) and liver lipid concentration (b) in Fisher rats. Induction: at the end of 3 weeks on high-fat diet (HFD, $n=3$, striped bars) vs. regular diet (RD, $n=3$, white bars). Treatment: after 12 weeks on HFD without fatty acid–bile acid conjugates (FABAC) ($n=7$, striped bars) or with C-20 FABAC (Aramchol) 150 mg/kg/day ($n=8$, black bars). Rats were sacrificed and liver lipids measured at the end of each period. (Panel a: *$P<0.003$ vs. RD; **$P<0.0002$ vs. HFD. Panel b: *$P<0.01$ vs. RD; **$P<0.0008$ vs. HFD).
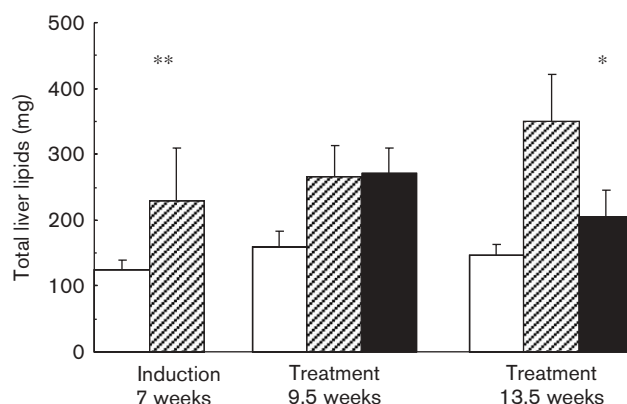
Fig. 2

Total liver lipids in C57BL6 mice. Induction: at the end of 4 weeks on high-fat diet (HFD, upward stripes) vs. regular diet (RD, white bars). Treatment: after 4 and 8 weeks on 50% HFD without fatty acid–bile acid conjugates (FABAC) (downward stripes) or with C-20 FABAC (Aramchol) 150 mg/kg/day (black bars), or C-18 FABAC (Steamchol) 150 mg/kg/day (grey bars). Mice were sacrificed ($n=6$/group) and liver lipids measured at end of each period. (**$P<0.0004$ vs. RD; *$P<0.05$ vs. 50% HFD 8 w).

Fig. 4

Total liver lipids in C57BL6 mice. Induction: at the end of 7 weeks on high-fat diet (HFD, striped bars) vs. regular diet (RD, white bars). Treatment: after 9.5 and 13.5 weeks on 100% HFD without fatty acid–bile acid conjugates (FABAC) (striped bars) or with C-20 FABAC (Aramchol) 150 mg/kg/day (black bars). Mice were sacrificed ($n=6$/group) and liver lipids measured at end of each period. (**$P<0.003$ vs. RD; *$P<0.009$ vs. HFD 13.5 w).

treatment period. TLL rose in the HFD controls during the study, but not as much as in the rats (Fig. 3). Concomitant Aramchol treatment (150 mg/kg/day) showed no effect at 9.5 weeks but reduced TLL significantly at 13.5 weeks ($P < 0.009$).

Liver lipid composition

Figure 5 shows liver lipid classes (neutral lipids) in Fisher rats at the end of 12 weeks of treatment. As can be seen, the major change occurred in triglycerides and

Fig. 5
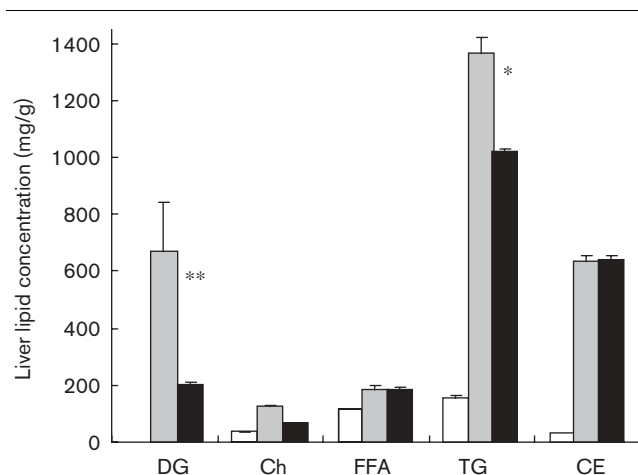
The distribution of neutral lipids in the liver at the end of 12 weeks of fatty acid–bile acid conjugates (FABAC) treatment in Fisher rats. The main lipid classes (CE, cholesterol esters; Ch, cholesterol; DG, diglycerides; FFA, free fatty acids; TG, triglycerides) are shown in control rats on regular diet (white bars) and on high-fat diet (HFD) without FABAC (grey bars) or with C-20 FABAC (Aramchol) 150 mg/kg/day (black bars). (*$P < 0.01$, **$P < 0.003$, HFD + Aramchol vs. HFD).

Fig. 6
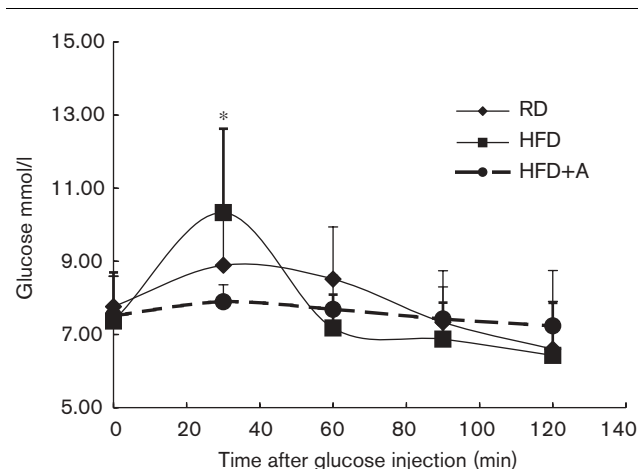
Glucose tolerance test in C57Bl6/J mice after 13.5 weeks on a regular diet (continuous line with diamonds), on a high-fat diet (HFD, dotted line with squares), and HFD + treatment with Aramchol, 150 mg/kg/day (broken line with circles). *$P < 0.05$ HFD + Aramchol vs. HFD.

diglycerides, which were markedly reduced ($P < 0.01$–0.003) after FABAC therapy.

Histology

Blinded histological analysis of hematoxylin and eosin-stained liver sections in rats receiving HFDs for 9.5 and 12 weeks with and without FABACs revealed steatosis, without inflammation or fibrosis. The average steatosis score was 0 in controls, whereas on HFD without FABAC it was 3.0 and with FABAC (Aramchol) 2.4. The morphologic distinction in liver fat concentration between FABAC-treated and untreated animals did not approach the precision provided by the chemical fat measurements (see Fig. 3), yet revealed the same beneficial effect. In mice induced for 4 weeks with HFD and maintained on a 25% HFD for another 6.5 weeks (with or without FABAC therapy), the mean steatosis score was 0 in controls and 0.86 in mice on HFD, whereas in mice supplemented with 25 and 150 mg/kg/d Aramchol or 25 mg/kg/d Steamchol it was 0, 0.67, and 1.0, respectively. In the mice HFD resulted in an inflammatory score of 1.29, which was decreased by these FABAC treatments to 1, 1.17, and 0.14, respectively. HFD-induced fibrosis (score 0.86) decreased by the FABAC treatments to 0.17, 0.17, and 0, respectively.

## Stearoyl CoA desaturase marker and fatty acid synthesis

The changes in serum $16:1/(16:0 + 16:1)$ fatty acid ratio are shown in Figs 7 and 8. This ratio is a reliable surrogate marker of stearoyl CoA desaturase (SCD) activity [16]. It is also influenced by dietary fat composition. During the HFD in mice, this ratio increased both during induction and maintenance (25% HFD) periods (Fig. 7a). Treatment with Aramchol (25 and 150 mg/kg/day) or Steamchol (25 mg/kg/day) significantly reduced ($P < 0.04$–0.01) this ratio despite the 25% HFD in mice (Fig. 6b). The same was found in rats receiving Aramchol (150 mg/kg/day) (Fig. 8).

In HepG2 cells *in vitro* the addition of Aramchol to the medium at increasing concentrations rapidly reduced de-novo fatty acid synthesis from labeled acetate moieties (Fig. 9). This effect was apparent already at a concentration of 0.2 µg/ml.

Carbohydrate metabolism

Fasting glucose levels in C57BL6 mice on a 100% HFD for 20 weeks with or without Aramchol (150 mg/kg/d) for 13.5 weeks were similar and not different from the RD controls (Table 2). The same was found for postprandial blood glucose. On glucose tolerance testing (in the same mice) the levels at 30 min were, however, significantly ($P < 0.03$) higher in the mice on HFD versus those on RD. The FABAC-treated mice demonstrated a significant ($P < 0.04$) reduction in glucose levels at 30 min, as compared with those on HFD only (Fig. 6). In mice, the HFD elevated the insulin and homeostasis model assessment

Fig. 7
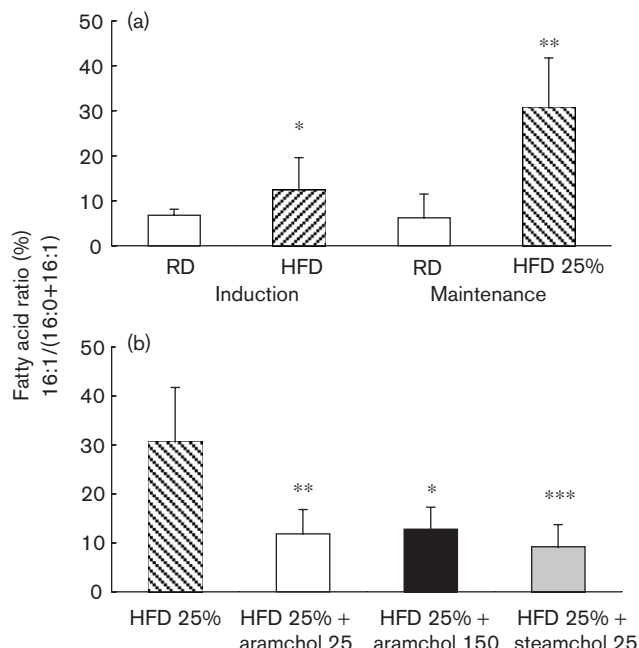
Plasma $16:1/(16:0+16:1)$ fatty acid ratio in C57BL6 mice. Upper panel (a): at the end of the 4 weeks induction period and after an additional 4 weeks of maintenance diet. [regular diet (RD), high-fat diet (HFD), maintenance diet-25%, HFD). *$P<0.04$ RD vs. 100% HFD **$P<0.01$ RD vs. 25% HFD. Lower panel (b): after 4 weeks of maintenance diet with 25% HFD without fatty acid–bile acid conjugates (FABAC) and with FABAC supplementation. *$P<0.05$, **$P<0.004$, ***$P<0.001$ all vs. 25% maintenance diet only.

Fig. 8
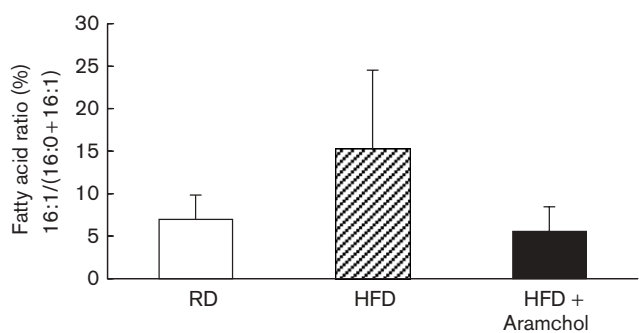
Plasma $16:1/(16:0+16:1)$ fatty acid ratio in Fisher rats after 12 weeks on regular diet (RD), 100% high-fat diet (HFD) with and without FABAC (Aramchol), 150 mg/kg/day. FABAC, fatty acid–bile acid conjugates.
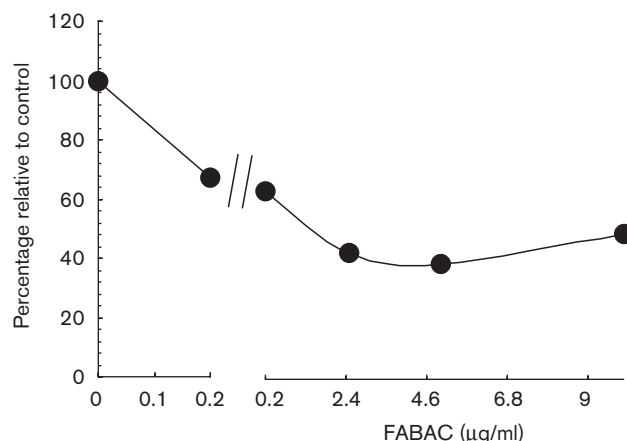
levels significantly ($P<0.03$) compared with the RD controls. FABAC treatment reduced ($P<0.04$) these elevated levels back to normal (Table 2).

Discussion
Animal model
Fatty liver can be induced by manipulating the composition (fat, sucrose, choline, etc.) [17,18] or amount

Fig. 9

De-novo fatty acid synthesis in HepG2 cells: effect of FABAC (Aramchol) concentration in the growth medium on ^{14}C-acetate incorporation into fatty acids in HepG2 cells. FABAC, fatty acid–bile acid conjugates.

Table 2 Blood glucose, insulin, and HOMA in C57Bl6/J mice

Treatment	Fed state			Fasting state
	Glucose (mmol/l)	Insulin (μIU/ml)	HOMA	Glucose (mmol/l)
RD	8.11 ± 0.57	24.66 ± 3.98	8.65 ± 1.09	6.69 ± 2.65
HFD	7.55 ± 0.74	59.29 ± 19.84*	19.96 ± 6.98*	7.44 ± 1.34
HFD + A[a]	7.92 ± 2.66	26.52 ± 7.56**	8.70 ± 4.04**	7.56 ± 0.90

HFD, high-fat diet; HOMA, homeostasis model assessment; RD, regular diet.
[a]Aramchol 150 mg/kg/day.
*$P<0.03$ vs. RD.
**$P<0.04$ vs. HFD.

(calories) of the diet. Overfeeding via a gastrostomy [19] or genetic manipulation leading to hyperphagia [20] will induce NAFLD or NASH, but are either too drastic or result in multiple interrelated metabolic alterations. We found that the HFD was more effective and useful in inducing NAFLD than sucrose overload (unpublished observations). On a HFD the animals are unrestrained (unlike with chronic gastrostomy), they eat *ad libidum* and reproducibly develop NAFLD. The problem (not much discussed in the literature), however, is the maintenance of a steady-state fatty liver throughout the test period to evaluate the effectiveness of potential therapeutic agents. If after induction of fatty liver the diet is switched to regular chow, the excess liver fat decreases rapidly, reaching normal levels within 2 weeks. This period is too short and the decrease is too rapid to test and compare the effects of potential therapeutic agents. If after induction of fatty liver the same HFD is continued, there is a steady, and in some animals, massive increase in liver fat content. In Fisher rats the increase was 1000% (!) over 12 weeks. Continuation of the HFD throughout the trial is not only unphysiologic, but is not pertinent to humans. A moderately obese person with

fatty liver who maintains a more or less steady weight and a sedentary lifestyle consumes daily about 2200 calories. As about 40% are usually from fat, this implies 880 calories or about 90 g fat per day. The usual person would not consume 360 g fat per day for any considerable length of time. It is also inherently illogical to treat a medical condition, while simultaneously administering an excessive dose of the offending agent.

Chow fed mice consume normally < 0.25 g fat/day, whereas on the HFDs they were given about 1.2 g fat/day. The ideal situation is to give a lower amount of fat during the experimental (treatment) period so as to keep liver fat in a steady-state condition. We did not find a suitable model in the literature. Deng *et al.* [19] used a chronic gastrostomy exteriorized through the skin and connected to a swivel in mice in microisolator cages to administer increasing amounts of diet to induce NASH. This is a complicated model, quite different from our unrestrained animals, eating *ad libidum*. Strain differences are also observed. In BalbC mice a 25% maintenance diet resulted in a continuing rise in liver fat (data not shown). In C57BL6 mice the same 25% diet resulted in slowly decreasing liver fat. This permitted demonstration of the effect of the various FABACs, particularly at 4 weeks. At a 50% maintenance HFD liver fat content was stable during the 8 weeks of therapy. These conditions are likely to be species, strain, and diet specific.

In our model the weight gain on RD and HFD, though not equal, was roughly similar (Table 1). Yet, NAFLD developed consistently in about 4 weeks in all animals on HFD. This was confirmed by actual measurement of liver fat, and supported by histological examination. It is thus a model of NAFLD induced by dietary fat content and not by obesity.

After several months on the diet, signs of impaired glucose tolerance and insulin resistance appeared (Table 2). Liver inflammation and fibrosis were also demonstrated in mice. Thus, the diet seems to lead to NASH albeit after several months. It is noteworthy that concomitant FABAC therapy abolished these manifestations of carbohydrate intolerance and reduced liver inflammation and fibrosis.

All four series of experiments (Figs 1–4) showed a significant effect of FABACs in reducing liver fat, in preestablished fatty liver; that is, in a therapeutic setting. In two of the experiments the full 100% HFD was given throughout the maintenance (treatment) period. Even under these difficult experimental conditions the therapeutic effect of the FABACs was achieved, albeit after a prolonged period. Altogether, reduction of liver fat was demonstrated in three different situations: during decrease of liver fat, during steady-state liver fat, and

during increase of liver fat. It is clear that the timing of the therapeutic effect is inversely proportional to the concentration of the noxious agent (fat) in the diet. The 25% HFD produced the most rapid effect.

The effects were seen with C18-FABAC (Steamchol) and with C20-FABAC (Aramchol) at two dose levels. In our earlier study on the prevention of fatty liver, several different FABACs were also shown to be effective [10].

The lower FABAC dose (25 mg/kg/day) was tested in this study for the first time in the treatment of NAFLD and was found to be effective (Fig. 1). This dose is equivalent, on a molar basis, to the currently used dose of bile acid treatment (ursodeoxycholic acid, 15 mg/kg/day) in humans. Moreover, as a murine dose is reduced by more than ten-fold when applied to humans this would translate to less than 2.5 mg/kg/day if and when applied to patients.

Effects of fatty acid–bile acid conjugates
The FABACs have been shown to influence several metabolic processes. They were proven to prevent the formation of cholesterol gallstones [21] and dissolve existing stones [22]. They were subsequently shown to induce cholesterol efflux from human fibroblasts [23] and other cells. This effect on reverse cholesterol transport was strengthened by finding an increased neutral sterol and bile salt excretion in rats treated with FABAC [24]. An enhancement of CYP7A1 activity [25] further contributed to cholesterol catabolism and loss from the body. Not surprisingly, a reduction in arteriosclerosis was found in experiments in mice [26]. Prevention of diet-induced fatty liver, found in several animal species under various experimental conditions [10], however, seems to be different from the above effects.

Mechanism of action
Earlier studies have provided only circumstantial evidence of possible modes of action [10]. In this study we have, for the first time, identified potential mechanisms. The decrease in the $16:1/(16:0 + 16:1)$ fatty acid ratio in the serum occurring during the continuing consumption of the same HFD is highly suggestive of a decrease in SCD activity. This key enzyme has multiple activities [27–29]. Reduction of its activity, as in knockout mice, leads among other effects to a reduction of fatty acid synthesis and an increase in fatty acid oxidation. Both would have additive effects leading to a reduction in liver triglycerides, as found with FABAC therapy. The decrease in fatty acid synthesis by Aramchol in HepG2 cells could be part of the effect on SCD or it could be an SCD-independent effect. Reduction of SCD activity might explain most of the observed effects of FABACs in NAFLD. As FABACs were found to have multiple effects on cholesterol metabolism [29] it is, however, quite

possible that additional mechanisms will be found for the triglyceride-lowering effects of FABACs in the fatty liver.

Potential future medical therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

Ursodeoxycholic acid has recently, in a large multicenter trial, been shown to be ineffective [9]. The same has been shown for some vitamins/antioxidants [30]. Agents affecting carbohydrate metabolism and insulin resistance such as metformin [31], and recently pioglitazone [32] have shown promise. In conjunction with a calorie-restricted diet they were shown to reduce liver fat and improve carbohydrate metabolism in mice [31] and in humans [32]. They do, however, increase body weight and body fat content [32].

Fatty liver in our and other animal models (such as ob/ob mice) as well as in most but not all patients, is clearly associated with an increased calorie intake, or increased fat intake or both. The initial lesion is NAFLD, which may then progress to NASH and beyond. It is therefore important to note that recently several studies have proven in humans that removal of liver fat only, the primary offending agent, will reduce and reverse the process also at its early progressive stage – NASH. This was demonstrated with diet only [33], diet and a lipase inhibitor [8], as well as weight loss induced by bariatric gastric surgery [6,7]. This is similar to liver damage induced by other noxious agents such as iron or copper, where removal of the offending agent will reverse the liver disease, provided it is not too advanced. FABACs may act in a similar way. FABACs may thus represent a potential medical agent able to reduce liver fat even in individuals who overeat. They may be of potential use in NAFLD as well as in NASH.

In summary, we have proven, for the first time, that FABACs reduce liver fat in preestablished diet-induced NAFLD. The rapidity of this effect is inversely related to the fat concentration in the maintenance diet fed during treatment. This effect was demonstrated for two FABAC molecules, at two dose levels. We have also shown that FABACs reduce a surrogate marker of the SCD enzyme as well as fatty acid synthesis. These are potential mechanisms of the therapeutic effect. In future FABACs may be evaluated as therapeutic agents for human NAFLD.

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