Increase in long-chain polyunsaturated fatty acid $n-6/n-3$ ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease

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ABSTRACT

Hepatic steatosis is a major feature associated with NAFLD (non-alcoholic fatty liver disease). The aims of the present study were to assess the levels of PUFA (polyunsaturated fatty acids) in liver total lipids, triacylglycerols (triglycerides) and phospholipids of NAFLD patients in relation to those in adipose tissue and hepatic indexes related to oxidative stress as factors contributing to hepatic steatosis. Eleven control subjects and 19 patients with NAFLD were studied. Analysis of liver and abdominal adipose tissue fatty acids was carried out by GLC. The liver content of protein carbonyl groups and malondialdehyde were taken as indexes related to oxidative stress. NAFLD patients had a depletion in LCPUFA (long-chain PUFA) of the $n-6$ and $n-3$ series in liver triacylglycerols, with decreased 20:4,$n-6$/18:2,$n-6$ and (20:5,$n-3$ + 22:6,$n-3$)/18:3,$n-3$ ratios, whereas liver phospholipids contained higher $n-6$ and lower $n-3$ LCPUFA. These findings were accompanied by an enhancement of (i) $n-6/n-3$ ratio in liver and adipose tissue, (ii) 18:1,$n-9$ *trans* levels in adipose tissue, and (iii) hepatic lipid peroxidation and protein oxidation indexes. It is concluded that a marked enhancement in LCPUFA $n-6/n-3$ ratio occurs in the liver of NAFLD patients, a condition that may favour lipid synthesis over oxidation and secretion, thereby leading to steatosis. Depletion of hepatic LCPUFA may result from both defective desaturation of PUFA, due to inadequate intake of precursors, such as 18:3,$n-3$, and higher intake of the 18:1,$n-9$ *trans* isomer leading to desaturase inhibition, and from an increased peroxidation of LCPUFA due to oxidative stress.

Key words: lipid peroxidation, long-chain polyunsaturated fatty acid, obesity, oxidative stress, protein carbonylation, steatosis, *trans* fatty acid.

Abbreviations: apoB-100, apolipoprotein B-100; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHT, butylated hydroxytoluene; BMI, body mass index; FAME, fatty acid methyl esters; HDL, high-density lipoprotein; HOMA, homoeostasis model assessment; LDL, low-density lipoprotein; LFABP, liver fatty acid-binding protein; MDA, malondialdehyde; MUFA, monounsaturated fatty acid(s); NAFLD, non-alcoholic fatty liver disease; PPAR-α, peroxisome proliferator-activated receptor-α; PUFA, polyunsaturated fatty acid(s); LCPUFA, long-chain PUFA; SAFA, unsaturated fatty acid(s); SREBP-1, sterol regulatory element binding protein-1.

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INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is a syndrome frequently associated with obesity, diabetes mellitus and dyslipidaemia [1] that resembles alcoholic liver disease in patients who do not consume significant amounts of alcohol [2], with a prevalence of 25–75 % in subjects with obesity and Type II diabetes mellitus [3]. Although the pre-existing metabolic abnormalities may be multifactorial and/or polymorphogenetic, NAFLD is frequently associated with deranged fatty acid metabolism in obese patients [4–6]. Alterations in lipid metabolism give rise to hepatic steatosis, due to an increased lipogenesis, defective peroxisomal and mitochondrial β-oxidation [7], and/or a lower ability of the liver to export lipids [5]. In the latter case, accumulation of lipids in the liver impairs the turnover of apoB-100 (apolipoprotein B-100), the main structural protein of very-LDL (low-density lipoprotein) [8]. The assembly and secretion of very-LDL is influenced by several factors, including LFABP (liver fatty acid-binding protein) [9], a cytosolic protein that binds long-chain fatty acids with high affinity and is up-regulated by long-chain fatty acids, including LCPUFA (long-chain polyunsaturated fatty acids) [10]. The regulation of cellular lipid metabolism by dietary fats can occur at two levels, namely, (i) modulation of the signal transduction through manipulation of membrane fatty acid composition, and (ii) modification of gene transcription [11,12]. Recent studies suggest that LCPUFA and their eicosanoid products may direct fatty acids away from triacylglycerol (triglyceride) storage, favouring their oxidation, and enhancing glucose flux to glycogen [13,14]. In agreement with this view, variations in the content of arachidonic acid in serum, liver and muscle lipid fractions are correlated with alterations in lipogenesis, implying a role for arachidonic acid in fuel partitioning [15]. In human liver, enhancement in the size of fat droplets in hepatocytes is associated with a decrease in the percentage of eicosapentaenoic acid (20:5,$n-3$) present in the triacylglycerol moiety [16]. Furthermore, decreased hepatic triacylglycerol contents were observed after a diet containing fish oil [17], which is rich in $n-3$ fatty acids that displace fuel partitioning towards oxidation [18].

The aim of the present study was to test the hypothesis that depletion of hepatic LCPUFA is a major mechanism contributing to the pathogenesis of fatty liver in NAFLD patients. For this purpose, contents of long-chain fatty acid $n-3$ and $n-6$ series were determined in liver total lipids, triacylglycerols and phospholipids of control subjects and obese NAFLD patients. Results obtained in the liver were correlated with the content of PUFA (polyunsaturated fatty acids) in adipose tissue, which represent a suitable biomarker of dietary fatty acid intake, particularly for $n-6$ and $n-3$ *cis* PUFA and *trans* fatty acids [19].

METHODS

Patients

Nineteen patients with a mean BMI (body mass index) of 45.6 ± 8.3 kg/m^2 and an age range of 39–45 years, who underwent voluntary therapeutic gastroplasty or gastrectomy with a gastro-jejunal anastomosis, were considered for inclusion in this study. Eleven patients (BMI, 27.8 ± 1.6 kg/m^2; age range, 38–41 years), who underwent voluntary anti-reflux surgery, were taken as the control group. The protocol was explained in detail to the subjects, who then gave their written informed consent to participate before any procedures were undertaken. All patients were non-smoking subjects. Nutritional and alcohol consumption histories with anthropometric measurements were obtained. Exclusion criteria included positive hepatitis B or C serology or positive antibodies (antinuclear, anti-mitochondrial and anti-smooth muscle antibodies), serum liver enzymes [AST (aspartate aminotransferase) and ALT (alanine aminotransferase)] exceeding five times normal values, fasting blood glucose levels over 200 mg/dl, and consumption of two or more alcoholic drinks/week. Insulin resistance was calculated from the fasting insulin and glucose values by HOMA (homoeostasis model assessment) analysis [20].

Design

Selected patients were subjected to a diet of 25 kcal/kg of body weight (where 1 kcal $\equiv$ 4.184 kJ), with 30 % of the energy given as lipids and 15 % as proteins, for at least 2 days prior to surgery, and liver biopsies of approx. 2 cm^3 were taken for histological diagnoses, immunohistochemistry, lipid composition and parameters related to oxidative stress. Liver samples were fixed in 10 % formaldehyde, paraffin embedded and sections were stained with either haematoxylin/eosin and Van Gieson's stain. Sections of each liver were observed in a blinded manner and evaluated for histological alterations by means of a previously defined code [21]. Patients were grouped according to liver histology as: normal (controls; Figure 1A), the presence of macrovesicular steatosis alone (steatosis group; Figure 1B) or steatosis and lobular inflammation with hepatocyte ballooning (steatohepatitis group; Figure 1C). The histology score was defined as the sum of the steatosis and inflammation scores, both graded as absent (0), mild (1), moderate (2) and severe (3) (Figure 1).

The Ethics Committee of the University of Chile Clinical Hospital approved the study protocol, according to Helsinki criteria.

Chemicals

The reagents were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany) and Riedel-de Haën (Germany) and were of the highest commercial grade available.

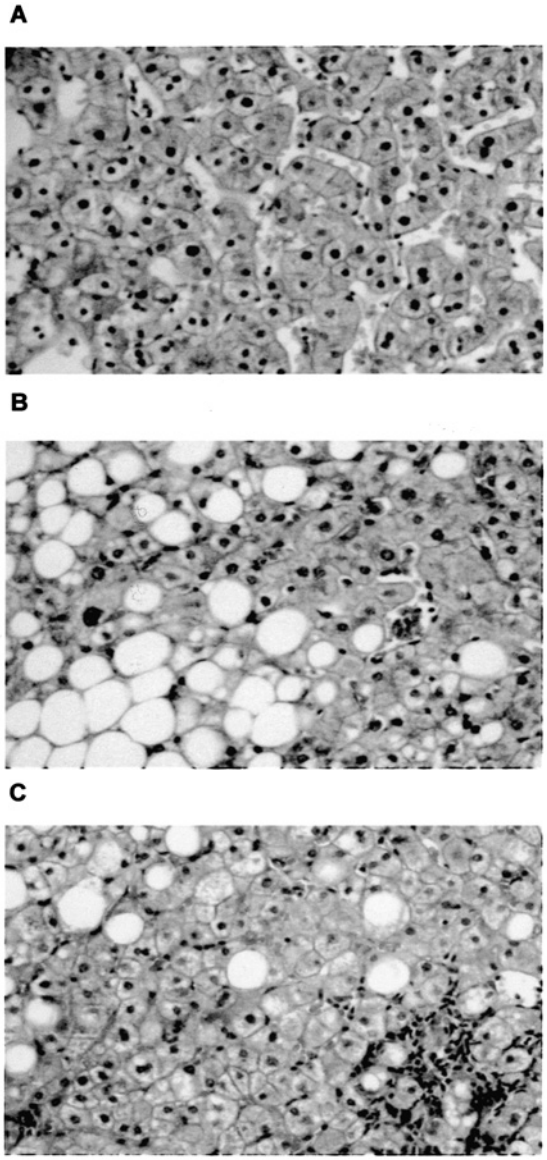

Figure 1 Haematoxylin/eosin-stained liver sections from a control patient (A) and from NAFLD patients with steatosis (B) or steatohepatitis (C)

Histology score (means ± S.E.M.): controls, 0 ± 0 ($n = 11$); NAFLD patients with steatosis, 1.8 ± 0.3 ($n = 10$); NAFLD patients with steatohepatitis, 3.4 ± 0.6 ($n = 9$), determined as described in the Methods section. Magnification, × 70.

Extraction and separation of tissue lipids

Quantitative extraction of total lipids from liver and adipose tissue was carried out following the method of Bligh and Dyer [22] in the presence of BHT (butylated hydroxytoluene) as an antioxidant. Tissue dissociation was achieved by homogenization in ice-cold chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) BHT using an Ultraturrax homogenizer (Janke & Kunkel, Stufen, Germany). Lipids from liver were separated by TLC aluminium sheets (20 × 20 cm silica gel 60 F-254; Merck), using a solvent system of hexane/diethyl ether/acetic acid (80:20:1, by vol.). After development of the plate, the solvent was allowed to evaporate and lipid bands were visualized by exposing the plates to a Camag UV (250 nm) lamp designed for use in the TLC laboratory. This solvent system separates phospholipids, cholesterol, triacylglycerols and cholesteryl esters in increasing order of relative mobility. Individual lipid zones were scraped from TLC plates and eluted from the silica gel with either diethyl ether or chloroform/methanol, according to the individual lipid [23].

Preparation of FAME (fatty acid methyl esters)

Fatty acids from total lipids of liver and adipose tissue and from phospholipids and triacylglycerols of liver were methylated. The phospholipids were eluted from silica gel with two 15 ml portions of chloroform/methanol/water (10:10:1, by vol.). Triacylglycerols were eluted with two 10 ml portions of chloroform/methanol (2:1, v/v). The solvent was evaporated in a stream of nitrogen, and 10 mg of tricosaenoic acid (23:0, internal standard) was added prior to esterification with 0.2 M NaOH/methanol for 30 min at 40 °C and then, with 0.2 M H_2SO_4/methanol as described for alkaline methylation. After the sample was cooled, FAME were extracted with 0.5 ml of hexane.

Analysis of FAME

FAME of all samples were analysed by GLC. A Hewlett-Packard gas chromatograph [model 6890, equipped with an apolar capillary column (50 m × 0.22 mm; BPX70)] was employed to separate FAME. The temperature was programmed from 180 to 230 °C at 2 °C/min with a final hold, separating 12:0 to 22:6,$n-3$. The temperature of both detector and injector was 240 °C. Hydrogen was used as carrier gas at a flow rate of 1.5 ml/min and split ratio of 1:80. FAME were identified by comparison of their retention times with those of individual purified standards and quantified using a Hewlett-Packard integrator (HP 3396 Series III) [24].

Isolation and quantification of total liver triacylglycerols

Total liver triacylglycerols were isolated as described by Sigfusson and Hultin [25], and were determined using kits coupling enzymic reactions and spectrophotometric detection of end products, as described by Daubioul et al. [26].

Biochemical parameters related to oxidative stress

Protein carbonylation was determined by a spectrophotometric method, based on the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls [27], and the results were expressed as nmol carbonyl/mg of protein. The assay for lipid peroxidation was performed by the

Table 1 Biochemical variables for patients with NAFLD and controls

Values represent means ± S.E.M. *$P < 0.05$ compared with control, †$P < 0.05$ compared with steatohepatitis, ‡$P < 0.05$ compared with steatosis, as assessed by one-way ANOVA and Bonferroni's test. HOMA is calculated as [fasting insulin (μunits/ml) × fasting glucose (mmol/l)/22.5]. IU, international units.

		NAFLD patients	
Parameters	Controls ($n = 11$)	With steatosis ($n = 10$)	With steatohepatitis ($n = 9$)
BMI (kg/m^2)	27.8 ± 1.6	41.7 ± 3.0*	49.9 ± 3.0*
Fasting glucose (mg/dl)	92 ± 2	100 ± 5	95 ± 3
Fasting insulin (μunits/ml)	12 ± 2	21 ± 4*	35 ± 8*
HOMA	2.7 ± 0.3	5.2 ± 0.8*†	8.2 ± 0.9*‡
Total cholesterol (mg/dl)	169 ± 6	224 ± 10*	201 ± 6*
HDL-cholesterol (mg/dl)	52 ± 4	42 ± 2*	40 ± 2*
LDL-cholesterol (mg/dl)	110 ± 9	140 ± 8	109 ± 6
Triacylglycerols (mg/dl)	109 ± 15	174 ± 22	178 ± 13
AST (IU/l)	26 ± 3	26 ± 3	28 ± 4
ALT (IU/l)	31 ± 3	37 ± 3	49 ± 8

thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of *n*-propanol/pyridine (15:1, v/v), as described by Ohkawa et al. [28], and the results were expressed as nmol MDA (malondialdehyde)/mg of protein.

Analysis of lipoproteins

Total plasma cholesterol, triacylglycerols and HDL (high-density lipoprotein)-cholesterol, expressed as mg/dl, were determined by an enzymic assay (Boehringer-Mannheim, Roche Diagnostics GmbH, Mannheim, Germany). LDL-cholesterol was calculated from the formula:

$$\text{LDL-cholesterol (mg/dl)} = \text{total cholesterol} - (\text{triacylglycerols}/5) - \text{HDL}$$

Statistical analysis

Results are expressed as means ± S.E.M. for the number of patients indicated. All statistical analysis of data was computed using Statistical Analysis System (software version 6.0; SAS Institute, Cary, NC, U.S.A.). The sources of variation for multiple comparisons were assessed by ANOVA, followed by Bonferroni's multiple comparison test. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Clinical and biochemical characteristics of NAFLD patients and control subjects

Patients characteristics are shown in Table 1. NAFLD patients were significantly more obese than control subjects, as assessed by BMI (150 and 179 % of control values for steatosis and steatohepatitis respectively; $P < 0.05$). Fasting blood glucose levels were not significantly different between the groups, but fasting insulin was higher in both groups of NAFLD patients ($P < 0.05$). Insulin resistance, calculated from the HOMA analysis, was found to be increased in the steatosis and steatohepatitis groups, the latter being significantly higher than the steatosis group ($P < 0.05$). Both groups of NAFLD patients exhibited significantly higher total cholesterol and lower HDL-cholesterol contents in plasma than those in the control group ($P < 0.05$). No significant differences were found in triacylglycerols and LDL-cholesterol contents and in the activities of AST and ALT enzymes between the groups.

Liver triacylglycerols content

The triacylglycerols content (mg/g of liver) of the liver in NAFLD patients with steatosis or steatohepatitis was significantly higher ($P < 0.05$) than that in control subjects [controls ($n = 10$), 48.4 ± 3.2; NAFLD patients with steatosis ($n = 8$), 255.6 ± 4.8 (5.3-fold higher than control); NAFLD patients with steatohepatitis ($n = 8$), 249.7 ± 4.1 (5.2-fold higher than control)], but no significant differences were detected between the two groups of NAFLD patients.

Fatty acid composition of liver lipids

When the fatty acid composition of liver total lipids was determined, a significant decrease ($P < 0.05$) in 14:0 (myristic acid; 63 and 53 % decrease) was observed, which was accompanied by a significant ($P < 0.05$) increase in 14:1,$n-7$ (myristoleic acid; 121 and 136 % increase), 16:0 (palmitic acid; 91 and 87 % increase), 16:1,$n-7$ (palmitoleic acid; 97 and 73 % increase) and 18:1,$n-9$ (oleic acid; 29 and 17 % increase) in steatosis and steatohepatitis groups respectively, compared with controls. No significant changes in 18:0 (stearic acid) were observed between the groups (Table 2). The contents of total saturated liver fatty acids were comparable among the groups studied (Table 2). In relation to SAFA (unsaturated fatty acids), total MUFA (mono-unsaturated fatty acids; 14:1,$n-7$, 16:1,$n-7$ and 18:1,$n-9$) were increased ($P < 0.05$) in NAFLD. Furthermore, both groups of NAFLD patients had no significant changes in hepatic 18:2,$n-6$ (linoleic acid) and 18:3,$n-3$ (α-linolenic acid), whereas the LCPUFA 20:4,$n-6$ (arachidonic acid; 71 and 84 % decrease), 20:5,$n-3$ (eicosapentaenoic acid; 59 and 67 % decrease) and 22:6,$n-3$ (docosahexaenoic acid; 77 and 86 % decrease) were significantly ($P < 0.05$) decreased in steatosis and steatohepatitis groups respectively (Table 2). The latter changes represented a significant 42 and 45 % decrease in total liver PUFA contents, particularly LCPUFA (73 and 84 % decrease), total $n-6$ PUFA (32 and 36 % decrease) and total $n-3$ PUFA (76 and 86 %

Table 2 Fatty acid composition of liver total lipids in control subjects and patients with NAFLD

Values represent means ± S.E.M. * $P < 0.05$ compared with controls, † $P < 0.05$ compared with steatohepatitis, ‡ $P < 0.05$ compared with steatosis, as assessed by one-way ANOVA and Bonferroni's test. SAFA are 14:0, 16:0 and 18:0; PUFA are 18:2,*n* − 6, 18:3,*n* − 3, 20:4,*n* − 6, 20:5,*n* − 3 and 22:6,*n* − 3; MUFA are 14:1,*n* − 7, 16:1,*n* − 7 and 18:1,*n* − 9; *n* − 6 LCPUFA are 20:4,*n* − 6; *n* − 3 LCPUFA are 20:5,*n* − 3 and 22:6,*n* − 3.

	Fatty acid composition (g/100 g of FAME)		
		NAFLD patients	
Fatty acid	Controls (*n* = 11)	With steatosis (*n* = 10)	With steatohepatitis (*n* = 9)
14:0	1.9.4 ± 0.9	7.20 ± 0.70*	9.10 ± 0.50*
14:1,*n* − 7	0.94 ± 0.15	2.08 ± 0.21*	2.22 ± 0.20*
16:0	17.6 ± 0.4	33.7 ± 2.0*	32.9 ± 1.7*
16:1,*n* − 7	2.73 ± 0.15	5.38 ± 0.57*	4.71 ± 0.42*
18:0	0.10 ± 0.05	0.63 ± 0.47	0.61 ± 0.28
18:1,*n* − 9	25.3 ± 0.8	32.7 ± 1.4*	29.5 ± 2.0*
18:2,*n* − 6	17.4 ± 0.9	13.8 ± 1.4*	16.2 ± 1.5
18:3,*n* − 3	0.37 ± 0.08	0.38 ± 0.04	0.59 ± 0.06
20:4,*n* − 6	8.10 ± 0.80	2.33 ± 0.55*	1.29 ± 0.18*
20:5,*n* − 3	0.39 ± 0.09	0.16 ± 0.03*	0.13 ± 0.03*
22:6,*n* − 3	6.76 ± 0.82	1.56 ± 0.40*	0.98 ± 0.10*
Total SAFA	37.1 ± 0.9	41.5 ± 1.8	42.6 ± 1.6
Total MUFA	28.9 ± 1.3	40.2 ± 4.0	36.4 ± 2.7*
Total PUFA	33.0 ± 1.4	18.2 ± 1.9*	19.2 ± 1.6*
Total LCPUFA	15.2 ± 1.2	4.05 ± 0.76*	2.4 ± 0.25*
Total *n* − 6 PUFA	25.5 ± 1.2	16.1 ± 1.7*	17.5 ± 1.3*
Total *n* − 3 PUFA	7.15 ± 0.75	2.10 ± 0.46*	1.11 ± 0.16*
n − 6/*n* − 3 ratio	3.56 ± 0.51	7.66 ± 0.71*†	15.76 ± 0.92*‡

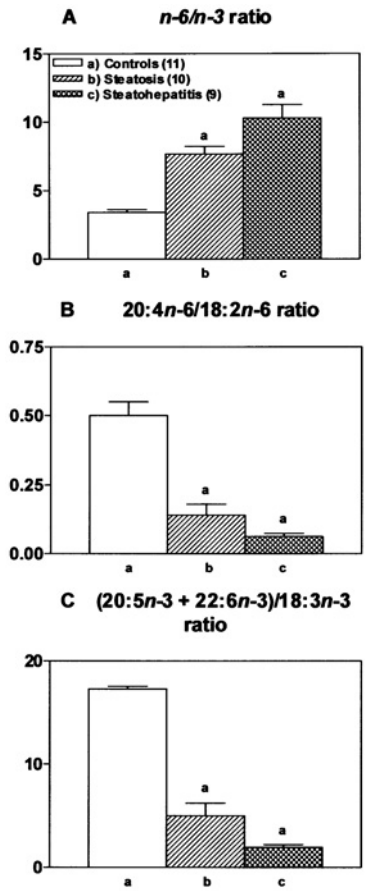

Figure 2 ***n* − 6/*n* − 3 ratios (A) and product/precursor ratios of *n* − 6 (B) and *n* − 3 (C) of PUFA in the liver of control subjects and patients with NAFLD showing steatosis or steatohepatitis**

Values are expressed as g/100 g of FAME and represent means ± S.E.M. for the number of subjects indicated in parenthesis. Significance differences between the groups are indicated by the letters identifying each group ($P < 0.05$; one-way ANOVA and Bonferroni's test).

decrease), with a 125 and 200 % increase in the *n* − 6/*n* − 3 ratio ($P < 0.05$; Figure 2A).

Pattern and product/precursor ratio of *n* − 6 and *n* − 3 series of liver PUFA

The product/precursor 20:4,*n* − 6/18:2,*n* − 6 (Figure 2B) and (20:5,*n* − 3 + 22:6,*n* − 3)/18:3,*n* − 3 (Figure 2C) ratios in the liver of control subjects and NAFLD patients indicated a substantial decrease in these relationships ($P < 0.05$), amounting to 66 and 83 % and 71 and 89 % decreases in patients with steatosis and steatohepatitis respectively.

Fatty acid composition of liver triacylglycerols

The fatty acid composition of liver triacylglycerols showed a lower proportion of 18:0 (stearic acid), 18:3, *n* − 3 (linolenic acid), 20:4,*n* − 6 (arachidonic acid), 20:5,*n* − 3 (eicosapentaenoic acid) and 22:6,*n* − 3 (docosahexaenoic acid) in NAFLD patients with steatosis and steatohepatitis compared with controls ($P < 0.05$), whereas the content of 14:0 (myristic acid), 16:0 (palmitic acid) and 18:1,*n* − 9 (oleic acid) was not different among the groups studied (Table 3).

Liver phospholipid fatty acid composition

The fatty acid composition of liver phospholipids showed no significant changes in the proportion of total SAFA, MUFA and PUFA in NAFLD patients with steatosis and steatohepatitis compared with controls (Table 4). However, the content of 20:4,*n* − 6 (arachidonic acid), one of the *n* − 6 LCPUFA, was significantly higher (245–250 %; $P < 0.05$) in NAFLD patients, whereas that of 20:5,*n* − 3 + 22:6,*n* − 3 was 40–50 % lower ($P < 0.05$) compared with control values, with a net 6.6-fold increase in the *n* − 6/*n* − 3 ratio.

Table 3 Fatty acid composition of liver triacylglycerols in control subjects and patients with NAFLD

Values represent means ± S.E.M. * $P < 0.05$ compared with controls, as assessed by one-way ANOVA and Bonferroni's test.

	Fatty acid composition (g/100 g of FAME)		
		NAFLD patients	
Fatty acid	Controls ($n = 11$)	With steatosis ($n = 10$)	With steatohepatitis ($n = 9$)
14:0	2.84 ± 0.23	3.15 ± 0.70	3.95 ± 0.50
14:1,$n-7$	0.92 ± 0.13	1.95 ± 0.21*	1.90 ± 0.20*
16:0	29.58 ± 0.40	31.0 ± 2.0	31.0 ± 1.7
16:1,$n-7$	5.20 ± 0.15	6.50 ± 0.57	5.95 ± 0.42
18:0	17.06 ± 0.32	5.56 ± 0.47*	5.59 ± 0.08*
18:1,$n-9$	30.2 ± 0.8	31.2 ± 1.4	30.8 ± 2.0
18:2,$n-6$	12.6 ± 1.8	20.4 ± 1.4*	20.5 ± 1.7*
18:3,$n-3$	0.21 ± 0.08	0.10 ± 0.04*	0.11 ± 0.06*
20:4,$n-6$	0.53 ± 0.02	0.03 ± 0.01*	0.08 ± 0.12*
20:5,$n-3$	0.32 ± 0.01	0.10 ± 0.03*	0.11 ± 0.03*
22:6,$n-3$	0.54 ± 0.03	0.01 ± 0.01*	0.01 ± 0.01*

Table 4 Fatty acid composition of liver phospholipids in control subjects and patients with NAFLD

Values represent means ± S.E.M. * $P < 0.05$ compared with controls, as assessed by one-way ANOVA and Bonferroni's test. SAFA are 14:0, 16:0, 18:0; MUFA are 14:1,$n-7$, 16:1,$n-7$ and 18:1,$n-9$; PUFA are 18:2,$n-6$, 18:3, $n-3$, 20:4,$n-6$, 20:5,$n-3$ and 22:6,$n-3$;$n-6$ LCPUFA are 20:4, $n-6$; $n-3$ LCPUFA are 20:5,$n-3$ and 22:6,$n-3$; $n-6/n-3$ ratio is 20:4,$n-6$/(20:5,$n-3$ + 22:6,$n-3$).

	Fatty acid composition (g/100 g of FAME)		
		NAFLD patients	
Fatty acid	Controls ($n = 11$)	With steatosis ($n = 10$)	With steatohepatitis ($n = 9$)
Total SAFA	63.8 ± 1.4	73.4 ± 2.5	72.0 ± 2.0
Total MUFA	16.6 ± 3.8	12.8 ± 2.1	13.1 ± 2.5
Total PUFA	14.9 ± 2.8	13.6 ± 3.6	13.4 ± 2.4
$n-6$ LCPUFA	2.0 ± 1.0	7.0 ± 1.1*	6.9 ± 1.0*
$n-3$ LCPUFA	12.9 ± 1.4	6.6 ± 1.0*	6.5 ± 1.4*
$n-6/n-3$ ratio	0.16 ± 0.01	1.06 ± 0.04*	1.06 ± 0.02*

Pattern, $n-6/n-3$ ratio and 18:1$n-9$ *trans* content of adipose tissue PUFA

The content of fatty acids in the abdominal adipose tissue, considered as a dietary biomarker [19], is shown in Table 5. Both 16:1, $n-7$, 18:2,$n-6$, 20:4,$n-6$, 22:6,$n-3$ and 18:1,$n-9$ *trans* (elaidic acid) were significantly increased in NAFLD patients compared with controls, whereas 18:3,$n-3$ was decreased ($P < 0.05$) and 20:5,$n-3$ remained unchanged. These changes represent a significant 53 % increase in total $n-6$ PUFA, a 49 % decrease in total $n-3$ PUFA and a 2-fold increase (200 %) in the $n-6/n-3$ ratio, a change that correlated ($r = 0.80$; $P < 0.05$) with that found in the liver (Figure 2A).

Table 5 Composition of PUFA of adipose tissue lipids in control subjects and patients with NAFLD

Values are means ± S.E.M. * $P < 0.05$ compared with controls, as assessed by one-way ANOVA and Bonferroni's test.

	Fatty acid composition (g/100 g of FAME)		
		NAFLD patients	
Fatty acid	Controls ($n = 11$)	With steatosis ($n = 10$)	With steatohepatitis ($n = 9$)
14:0	2.67 ± 0.41	2.25 ± 0.93	2.86 ± 0.51
16:0	27.5 ± 3.3	20.5 ± 4.0	24.3 ± 2.2
16:1,$n-7$	0.33 ± 0.12	3.58 ± 2.3*	3.71 ± 1.4*
18:0	38.9 ± 2.7	30.4 ± 9.6	37.1 ± 5.0
18:1,$n-9$ *cis*	13.2 ± 1.8	12.0 ± 3.4	13.9 ± 2.3
18:1,$n-9$ *trans*	5.96 ± 2.49	12.4 ± 1.0*	12.7 ± 2.3*
18:2,$n-6$	8.67 ± 1.06	13.1 ± 0.8*	12.0 ± 1.1*
18:3,$n-3$	1.88 ± 0.18	0.20 ± 0.05*	0.35 ± 0.10*
20:0	0.19 ± 0.05	0.48 ± 0.20*	0.52 ± 0.11*
20:4,$n-6$	0.34 ± 0.04	0.69 ± 0.06*	0.63 ± 0.09*
20:5,$n-3$	0.22 ± 0.01	0.31 ± 0.05	0.34 ± 0.08
22:0	0.07 ± 0.05	0.24 ± 0.10*	0.18 ± 0.06*
22:6,$n-3$	0.08 ± 0.01	0.47 ± 0.07*	0.44 ± 0.03*
Total $n-6$	9.01 ± 1.06	13.9 ± 0.8*	13.7 ± 0.9*
Total $n-3$	2.18 ± 0.20	1.11 ± 0.10*	1.19 ± 0.08*
$n-6/n-3$ ratio	4.13 ± 0.69	13.1 ± 0.12*	11.6 ± 0.25*

Hepatic oxidative stress-related parameters

The liver of NAFLD patients had a significantly ($P < 0.05$) higher lipid peroxidation index compared with that of control subjects, as shown by higher contents of MDA [controls ($n = 11$), 2.25 ± 0.11 nmol/mg of protein; NAFLD patients with steatosis ($n = 10$), 6.20 ± 0.20 nmol/mg of protein (175 % higher); NAFLD patients with steatohepatitis ($n = 9$), 6.66 ± 0.22 (196 % higher)] and a significantly augmented ($P < 0.05$) protein carbonylation [controls ($n = 10$), 0.98 ± 0.20 nmol carbonyls/mg of protein; NAFLD patients with steatosis ($n = 8$), 4.45 ± 0.80 nmol carbonyls/mg of protein (354 % higher); NAFLD patients with steatohepatitis ($n = 7$), 4.55 ± 0.90 nmol carbonyls/mg of protein (364 % higher)].

DISCUSSION

The data in the present study show that fatty liver from overweight patients exhibit a substantial triacylglycerol accumulation with a relative depletion in PUFA, particularly LCPUFA of the $n-6$ and $n-3$ series

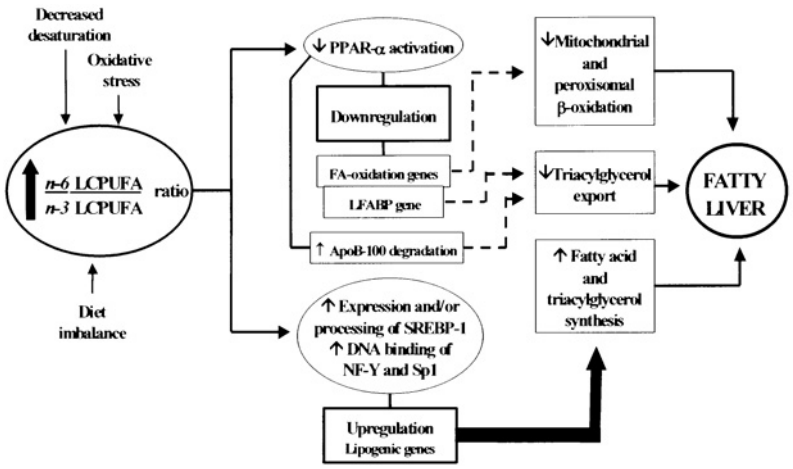

Figure 3 Factors influencing depletion of liver PUFA and consequent changes in the expression of genes coding for enzymes and proteins involved in lipid metabolism that promote fatty liver formation in NAFLD

Abbreviations: FA, fatty acid; NF-Y, nuclear factor Y; Sp1, stimulatory protein-1.

in hepatic triacylglycerols and of the $n-3$ series in liver phospholipids, with decreased 20:4,$n-6$/18:2,$n-6$ and (20:5,$n-3$ + 22:6,$n-3$)/18:3,$n-3$ ratios. These findings occur concomitantly with higher $n-6/n-3$ ratios in liver and adipose tissue, 18:1,$n-9$ *trans* contents in adipose tissue, and hepatic lipid peroxidation and protein oxidation indexes. These changes occurred in NAFLD patients exhibiting both liver steatosis or steatohepatitis.

Depletion of LCPUFA in the liver of NAFLD patients may contribute to the development of fatty liver, due to a derangement in the capacity to regulate lipid metabolism [13,14]. LCPUFA-induced fuel partitioning is achieved through different mechanisms, such as down-regulation of SREBP-1 (sterol regulatory element binding protein-1) expression and/or impairment of SREBP-1 processing, leading to inhibition of the transcription of lipogenic and glycolytic genes [29–31]. In addition, LCPUFA may induce transcription of genes encoding enzymes of fatty acid oxidation through their ability to act as ligand activators of PPAR-α (peroxisome proliferator-activated receptor-α) [32,33]. PPAR-α activation by LCPUFA also results in an increased secretion of apoB-100, due to decreased degradation [9], and up-regulation of LFABP expression [34]. Therefore increased liver triacylglycerol storage is expected in conditions involving depletion of LCPUFA, such as NAFLD, which would favour fatty acid and triacylglycerol synthesis over fatty acid oxidation and triacylglycerol export (Figure 3). Furthermore, the enhancement in liver $n-6/n-3$ ratios in total lipids, triacylglycerols and phospholipids observed in NAFLD patients compared with control subjects strengthens the view of a depressed hepatic lipid oxidation and secretion, considering that $n-3$ LCPUFA are more potent *in vivo* PPAR-α activators than $n-6$ LCPUFA [18,35]. In addition, hepatic steatosis could also be favoured by an increased peripheral lipolytic activity secondary to insulin resistance, a key pathophysiological abnormality that may concur in patients with NAFLD [36], in agreement with the present data obtained by the HOMA analysis (Table 1). The data in the present study show that the pattern of LCPUFA and $n-6/n-3$ ratio are comparable in the liver of patients with steatosis and steatohepatitis exhibiting normal function tests. However, the possibility that significant changes may occur with increased severity of hepatic lesions cannot be ruled out. In agreement with this suggestion, low plasma contents of LCPUFA were observed in patients with end-stage liver disease [37] or in cirrhotic patients with hepatitis B and C viruses [38]. Low contents of LCPUFA in plasma were also found in rats with carbon tetrachloride-induced liver damage, which correlates with the depletion of LCPUFA in the liver [39].

Several factors may be involved in the depletion of LCPUFA in the liver of NAFLD patients, including impairment of the pathways for desaturation and elongation of the essential fatty acids 18:2,$n-6$ and 18:3, $n-3$ required for their synthesis. Considering that the content of $n-3$,$n-6$ *cis* PUFA and *trans* fatty acids in adipose tissue is a suitable biomarker of dietary fatty acid intake [19], enhancement in adipose tissue 18:2, $n-6$ content in NAFLD suggests an adequate intake for utilization in the liver, in agreement with the higher content of $n-6$ LCPUFA found in liver phospholipids. However, the content of 18:3,$n-3$ in adipose tissue is significantly decreased, probably due to a low-to-moderate intake. This may constitute a limiting factor for the production of 18:3,$n-3$-derived LCPUFA, such as 22:6,$n-3$, a minor product of hepatic biosynthesis in man [40], consistent with the lower levels of $n-3$ LCPUFA found in liver lipids of NAFLD patients. In

addition, the substantial decrease in product/precursor ratios of PUFA found in the liver of NAFLD patients is consistent with a major impairment in Δ-5 and Δ-6 fatty acid desaturases, key enzymes leading to the synthesis of $n-3$ and $n-6$ LCPUFA occurring mainly in the liver [40]. Supporting this view is the product/precursor ratio analysis showing an association between obesity and decreased Δ-5 desaturase activity [41]. Of particular interest is the finding of increased contents of the 18:1$n-9$ *trans* isomer in the adipose tissue of NAFLD patients, suggesting a defective desaturation activity in the liver of these patients. This suggestion is supported by the drastic inhibition of hepatic Δ-6 desaturase activity [40,42] and biosynthesis of PUFA [40] exerted by dietary *trans* fatty acids, which is in agreement with the product/precursor data reported. Although a decreased desaturation activity is likely to occur in the liver of NAFLD patients, due to diet imbalance (Figure 3), lower product/precursor ratios and *trans* fatty acid-dependent inhibition of desaturases, determination of the activity of these enzymes is required to ensure the contribution of defective desaturation in NAFLD.

Oxidative stress is a redox imbalance between the rate of production of reactive oxygen and nitrogen species and their consumption by antioxidant mechanisms favouring the generation of pro-oxidants [43] which, in NAFLD, is related to the induction of cytochrome P450 2E1 [44] exhibiting a high pro-oxidant activity [45]. Data reported in the present study indicate the enhancement of the oxidative stress status of the liver of NAFLD patients, as evidenced by the marked increases in lipid peroxidation and protein carbonylation. These processes result from enhanced free-radical reactions involving PUFA and amino acid residues in proteins [43]. The lipid peroxidation data reported in the present study are in agreement with previous studies in experimental animals [46] and NAFLD patients [47]. In the latter process, lipids containing PUFA are readily oxidized by free-radical chain mechanisms, as their allylic hydrogen atoms are easily removed to produce a radical site subject to O_2 addition, which is followed by decomposition reactions with loss of unsaturated lipids [43,48]. These processes are particularly important in relation to LCPUFA, as the length of a lipid radical chain reaction is increased by the degree of lipid unsaturation [48], thus highlighting oxidative stress-dependent lipid peroxidation as an alternative mechanism contributing to the depletion of liver LCPUFA in NAFLD (Figure 3). Finally, the decreased proportion of LCPUFA compared with PUFA could also involve the transformation of 20:4,$n-6$ into lipid mediators of inflammation, namely, eicosanoids.

In conclusion, enhancement in the ratio of $n-6/n-3$ LCPUFA occurs in the liver of NAFLD patients, a condition that may direct fatty acids away from oxidation and secretion and towards triacylglycerol storage, thus contributing to hepatic steatosis. The increase in hepatic $n-6/n-3$ ratios may result from different factors (Figure 3), including a defective desaturation of PUFA, due to diet imbalance comprising inadequate intake of precursors, such as 18:3,$n-3$, and higher intake of the 18:1,$n-9$ *trans* isomer, leading to desaturase inhibition. In addition, a higher consumption of LCPUFA is likely to occur through free-radical processes imposed by oxidative stress.

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