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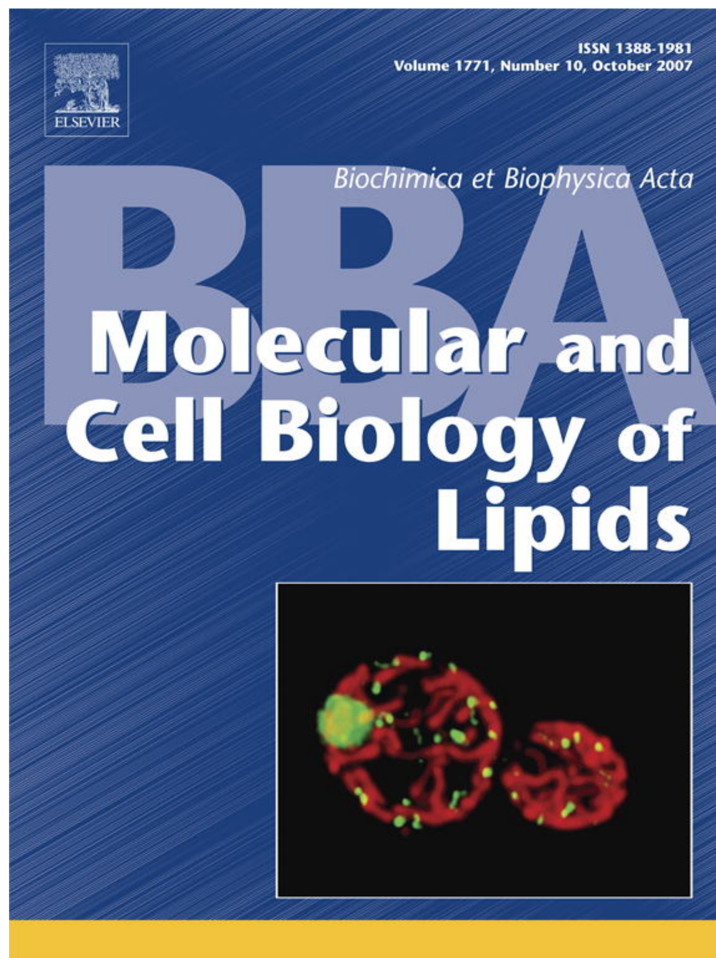

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Biochimica et Biophysica Acta 1771 (2007) 1263 – 1270

Biochimica et Biophysica Acta
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Metabolomics (liver and blood profiling) in a mouse model in response to fasting: A study of hepatic steatosis

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Received 16 February 2007; received in revised form 26 July 2007; accepted 27 July 2007
Available online 14 August 2007

Abstract

A metabolomic approach was applied to a mouse model of starvation-induced hepatic steatosis. After 24 h of fasting it appears that starvation reduced the phospholipids (PL), free cholesterol (FC), and cholesterol esters (CE) content of low-density lipoproteins (LDL). In liver lipid profiles major changes were observed using different techniques. High performance thin layer chromatography (HPTLC)-measurements of liver-homogenates indicated a significant rise of FC with 192%, triacylglycerols (TG) with 456% and cholesterol esters (CE) with 268% after 24 h of starvation in comparison with the control group. Reversed phase liquid chromatography coupled to mass spectrometry measurements (LC-MS) of liver homogenate indicated that the intensity of Phosphatidylcholine (PC) in the 24-h starvation group dropped to 90% of the value in the control group while the intensity of CE and TG increased to 157% and 331%, respectively, of the control group. Interestingly, a 49:4-TG with an odd number of C atoms appeared during starvation. This unique triacylglycerol has all characteristics of a biomarker for detection of hepatic steatosis. These observations indicate that in mammals liver lipid profiles are a dynamic system which are readily modulated by environmental factors like starvation.
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Keywords: Metabolomics; Lipids; Liver; Starvation; Triacylglycerols; Hepatic steatosis

1. Introduction

Hepatic steatosis has become a research area of interest due to its close association with obesity and metabolic syndrome (dyslipidemia, insulin resistance, type 2 diabetes mellitus). In the USA, nonalcoholic fatty liver disease (NAFLD) affects approximately 30 million Americans. The pathogenesis can be described by a two-hit model. First, there is a deposition of triacylglycerols (TG) in the cytoplasm of the hepatocyte (hepatic steatosis). The second step can be characterized by inflammation, cell death and fibrosis (steatohepatitis) [1].

While in our mouse model, hepatic steatosis is a surviving strategy to cope with low food conditions after a period of 24 h starvation, in humans, malnutrition, medicine exposure alcoholic abuse and exposure to toxic compounds (e.g. polychlorbiphenyls PCBs) may lead to fattening of the liver [1]. The major unresolved question in the research area of hepatic steatosis is the understanding of the transition from a benign survival strategy to the development of pathogenesis including inflammation, cell death and fibrosis (steatohepatitis) [1].

Under fed conditions, carbohydrates are burned to generate ATP and the surplus of carbohydrates are converted into fatty acids, which are stored as TG in adipose tissue [2]. When starvation occurs in an animal, there are many physiological changes as the animal attempts to satisfy its energy requirements. At the cellular level, catabolism continues to supply the substances required for anabolism and to sustain vital functions. Reserve stores of nutrients are utilized. Energy is generated from the utilization of proteins, fats, and carbohydrates [3]. The most readily usable material, the carbohydrate glycogen, is

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doi:10.1016/j.bbalip.2007.07.007

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utilized first. However, the energy derived from glycogen is stored in the liver and is exhausted within a few hours [3]. This is followed by stored fat from the various subcutaneous deposits, around the kidney, and in the mesentery and omental tissue [4]. Fat deposits in the parenchymatous organs are utilized next. The last area of the body to lose its fat deposits is the marrow of the bones. The final source of energy available is the protein comprising the cytoplasm of the cells [4].

When glucose availability is low during periods of starvation, the TG stored in the adipose tissue are hydrolysed to free fatty acids (FFAs) and mobilized into plasma to reach the liver where they play a major role in energy production [5]. In the liver, the influxed fatty acids are oxidized by the β-oxidation system, leading to the production of acetyl coenzyme A (acetyl-CoA), which then condenses with itself to form ketone bodies [6]. Ketone bodies generated in the liver are transported out of the liver to serve as fuels for other tissues such as the skeletal, cardiac and brain tissue during starvation [5].

When not used for β-oxidation in mitochondria, FFAs can undergo re-esterification into TG, that can subsequently be deposited in the cytoplasm of the hepatocyte (hepatic steatosis). During starvation, the liver represents the major sink of fatty acids in the form of TG.

The fat constitution and composition of the liver after a period of starvation is largely unknown. We hypothesize that the concentration of lipid compounds in the liver has been changed, quantitatively and qualitatively, due to the rearrangement and repartitioning of the fat stores.

The aim of this study was therefore, using highly sensitive techniques like high-performance thin-layer chromatography (HPTLC) and reversed phase liquid chromatography coupled to mass spectrometry (LC-MS) to quantify and qualify the rearrangement and repartitioning of the hepatic fat stores. These are the fat stores (mainly triacylglycerols in the liver). Analysis of metabolites in liver tissue will facilitate the identification of novel biomarkers for hepatic steatosis.

2. Materials and methods

2.1. Animals

Mice were housed in a temperature-controlled room (23 °C) on a 10-h dark/14-h light cycle. Purebred male wild-type C57bl6 mice (age 8–12 weeks), obtained from Charles River (Maastricht, The Netherlands) were used. Animal experiments were approved by the animal experimentation committee of the Leiden University Medical Center (The Netherlands).

Table 1
Lipid compounds detected with High Performance Thin Layer Chromatography (HPTLC) determined in liver homogenates of a Control ($n=6$) and a 24 h starvation ($N=6$) mouse group

Compound	Control Mean±SD	Starvation Mean±SD	*P*-value	Percent %
Free cholesterol µg/mg protein	5.8±0.4	11.2±2.1	$P\leq0.001$**	+192%
Triacylglycerols µg/mg protein	12.9±2.4	58.7±15.5	$P\leq0.001$**	+456%
Cholesterol ester µg/mg protein	1.7±0.4	4.6±0.5	$P\leq0.001$**	+268%

Mean±SD is given. ** denotes significant difference $P\leq0.001$.

Table 2
Lipid compounds detected by liquid chromatography coupled to mass spectrometry (LC-MS) determined in liver homogenates of a Control ($n=6$) and a 24 h starvation ($N=6$) mouse group

Lipid resonances	Control Mean±SD	Starvation Mean±SD	*P*-value	% Change
Lisophosphatidyl choline (LPC)	0.32±0.11	0.32±0.07	$P\leq0.87$	100%
Phosphatidyl choline (PC)	0.96±0.06	0.86±0.07	$P\leq0.02$ *	−90%
Sphingomyelin (SPM)	0.03±0.001	0.02±0.001	$P\leq0.44$	67%
Cholesterol ester (ChE)	0.21±0.01	0.33±0.09	$P\leq0.01$ *	+157%
Triacylglycerols (TG)	0.42±0.07	1.39±0.35	$P\leq0.001$ **	+331%

Mean±SD is given.
* denotes significant difference $P\leq0.01$; ** denotes significant difference $P\leq0.05$.

2.2. Diet

Mice were fed a standard lab chow (RM3, Special Diet Services, Witham, UK) containing about 12 energy percent fat. Before the experiment started the animals were provided unrestricted amounts of food and water. During the experiment the Control mice were fed ad libitum. To standardize the metabolic rate of the Co-group they were fasted 4 h before the start of the experiment.

2.3. Fast performance liquid chromatography (FPLC)

The distribution of total cholesterol (TC), triacylglycerols (TG) and phospholipids (PL) over the different lipoproteins in plasma was analyzed by fractionation of 30 µl of pooled plasma of each mouse using a Superose 6 column (3.2×300 mm, Smart System; Pharmacia, Uppsala, Sweden). Total lipid content of the effluent was determined using enzymatic colorimetric assays taking the efficiency of recovery from the column into account. The concentrations of free cholesterol (FC) in serum were determined in the presence of 0.025 U/ml cholesterol oxidase (Sigma) and 0.065 U/ml peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% methanol). TC cholesterol content was determined after addition of 15 µg/ml cholesteryl esterase (Roche Diagnostics). In addition, the concentration of TG (Roche Diagnostics) and choline containing PL (Wako chemicals GmbH, Neuss, Germany) were determined.

2.4. High performance thin layer chromatography (HPTLC)

Approximately 1/5 portion of a whole liver was homogenized (∼10% wet weight/vol) in PBS (phosphate-buffered saline). Protein content of all samples was determined according to the Lowry assay [7]. Lipids were extracted according to Bligh and Dyer [8]. Briefly, a solution of 200 µg protein in 800 µl of MilliQ was mixed with 3 ml Methanol/Chloroform (2:1), after which 500 µl Chloroform, 100 µl Internal Standard and 1 ml MilliQ water was added. All were mixed and centrifuged for 10 min at 300 rpm. After the centrifugation the chloroform layer was collected and dried under nitrogen. The pellets were dissolved in 50 µl chloroform and transferred to a HPTLC plate for separation of TG, FC and CE [9]. The lipids were separated using high-performance thin-layer chromatography (HPTLC) on silica gel plates as described before [9] and subsequent analysis was performed by TINA2.09 software [10] (Rayest Isotopen Meβgeräte GmbH, Straubenhardt, Germany).

2.5. Mass spectrometry

LC-MS analysis of lipids in liver homogenates was performed according to the previously published method of Verhoeckx et al. using a Thermo LTQ. A Thermo LTQ is a linear ion-trap LC-MS instrument (Thermo Electron, San Jose, USA) [11].

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2.6. Calculations and statistics

For all measured parameters given in Tables 1 and 2, the mean value of the control mice group was compared to the mean value of the starvation mice group. Statistics were performed via SAS (Statistical Analyzing Software) using an one-way ANOVA for differences between control and starvation groups. $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and F_{max} tests, respectively. Principal Component Analysis (PCA) was carried out on the parameters of lipid metabolism measured via reversed phase liquid chromatography coupled to mass spectrometry. This type of analysis allows one to simultaneously examine the relative state of individuals according to three or more variables. We used Principal Component Analysis (PCA) statistical methods, which are specially developed for application in biomedical research [12–14] using TNO IMPRESS, EQUEST and WINLIN software.

Principal components analysis (PCA) is a technique used to reduce multidimensional data sets to lower dimensions for analysis. The applications include exploratory data analysis data and for generating predictive models. PCA involves the computation of the eigenvalue decomposition or Singular value decomposition of a data set, usually after mean centering the data for each attribute. The results of a PCA are usually discussed in terms of scores and loadings. The score and loading vectors give a concise and simplified description of the variance present in the dataset [11].

A principal component is a linear combination of the original variables (lipid concentrations) and the magnitude of its eigenvalue is a measure of the explained variance. Typically only a few principal components are required to explain >90% of the total variance in the data. In other words PCA is a dimension reduction method, e.g. from >100 lipid attributes in the data to only a 4-principal component [11] which simplifies data visualization.

3. Results

Initial body weight of the mice was 24.4±0.6 g (mean±SD (n=6)), while the weight at the end of the experiment after 24 h

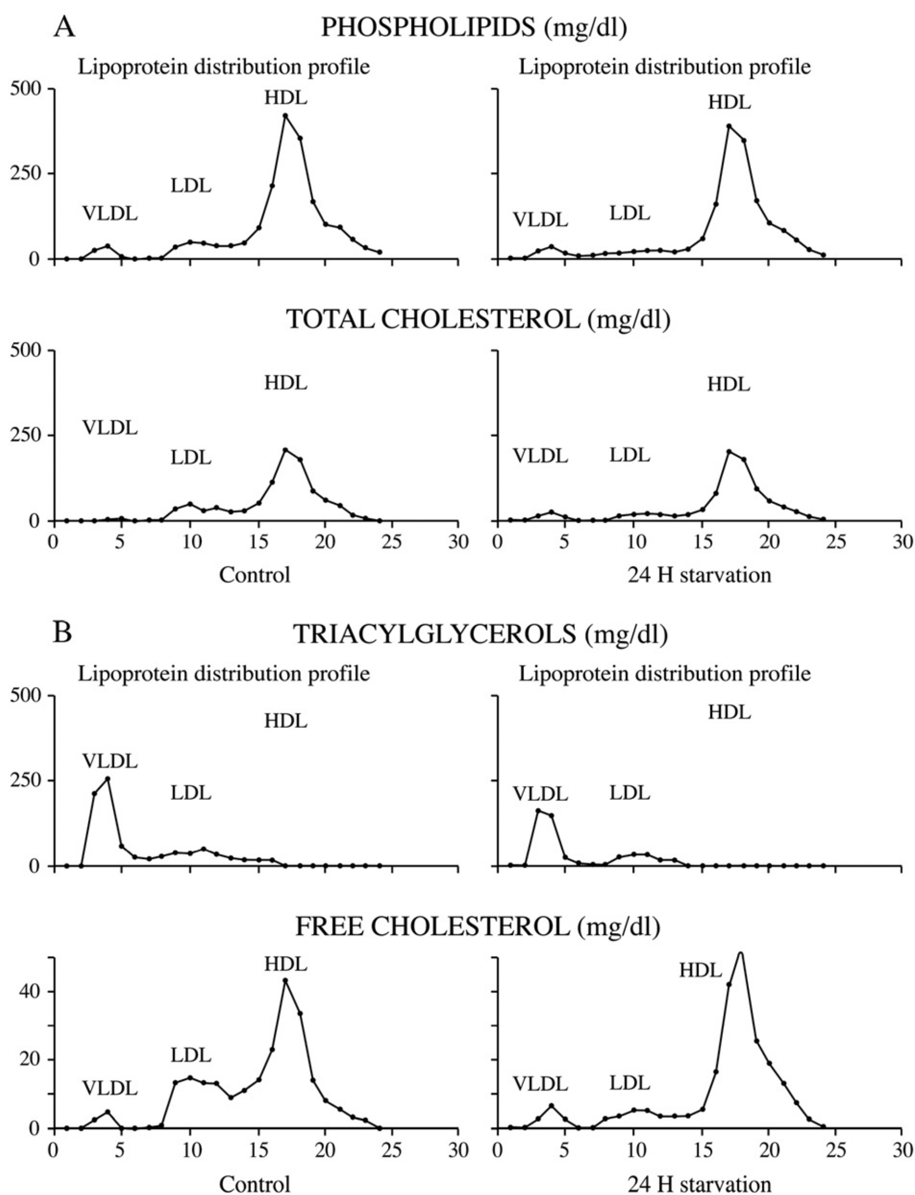

Fig. 1. The distribution of phospholipids, total cholesterol, triacylglycerols and free cholesterol over the different lipoproteins in a Control Mice group (left) and a group exposed for 24 h to starvation (right). Fractions 3–7 represent VLDL; fractions 8–14 represent LDL; fractions 15–19 represent HDL. Values represent a pooled blood sample of 6 mice.

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Table 3
Lipid profiles of lysophosphatidylcholines (LPC) in liver tissue determined with mass spectrometry

Compound	Control (Mean±SD)	24-h starvation (Mean±SD)	*P*-value	Change in (%)
14:0-LPC	0.014±0.0026	0.013±0.001	$P \leq 0.3371$	93%
16:0-LPC	0.655±0.235	0.744±0.180	$P \leq 0.47579$	114%
18:0-LPC	0.347±0.149	0.319±0.070	$P \leq 0.68728$	92%
18:1-LPC	0.132±0.048	0.102±0.028	$P \leq 0.21208$	77%
18:2-LPC	0.66±0.25	0.68±0.23	$P \leq 0.9220$	103%
20:3-LPC	0.074±0.025	0.022±0.0056	$P \leq 0.00061$ **	−30%
22:6-LPC	0.34±0.10	0.29±0.07	$P \leq 0.2981$	8%

**: denotes significant difference $P \leq 0.001$.

Table 5
Lipid profiles of sphingomyelin (SPM) and cholesterol esters (ChE) in liver tissue determined with mass spectrometry

Compound	Control (Mean±SD)	24-h starvation (Mean±SD)	*P*-value	Change in (%)
14:0-SPM	0.0212±0.0041	0.0182±0.0013	$P \leq 0.12039$	86%
18:0-SPM	0.030±0.0050	0.030±0.0065	$P \leq 0.92301$	100%
18:1-ChE	0.177±0.0181	0.555±0.1523	$P \leq 0.00013$ **	+314%
18:2-ChE	0.357±0.0358	0.532±0.1654	$P \leq 0.02949$ *	+149%
20:4-ChE	0.108±0.0148	0.071±0.0264	$P \leq 0.01388$ *	−66%
22:6-ChE	0.178±0.0159	0.149±0.0511	$P \leq 0.22406$	84%

*: denotes significant difference $P \leq 0.05$.
**: denotes significant difference $P \leq 0.001$.

of food deprivation had dropped to 21.1±0.7 g ($P \leq 0.0001$). The weight decline thus was 3.3±0.7 g which correspond to a decrease of 13.5% in comparison to the initial body weight.

After 24 h of fasting it appears that starvation reduced the phospholipids (PL), free cholesterol (FC), and cholesterol esters (CE) content of low-density lipoproteins (LDL). Very low-density lipoproteins, and high-density lipoproteins were unaffected in the 24-h starvation group for phospholipids (PL), total cholesterol (TC), triacylglycerols (TG) and free cholesterol (FC) (Fig. 1).

We observed major changes in liver lipid profiles. HPTLC measurements of mice liver-homogenates indicated a significant rise of free cholesterol with 192%, triacylglycerols 456% and cholesterol esters with 268% after 24 h of starvation (Table 1).

The overall response of the different lipid profiles measured by LC-MS is given in Table 2, while in Tables 3–6 for each lipid fraction, more in detail, the measured values for the individual molecules are given. Overall, the lysophosphatidyl choline (LPC) and the sphingomyelin fractions were unaffected, the phosphatidyl choline (PC) fraction dropped significantly ($P \leq$ 0.02) after starvation to 90%, while the CE and the TG fraction increased significantly to 157% ($P \leq 0.01$) and 331% ($P \leq 0.001$) of the control value, respectively.

Using reversed phase liquid chromatography coupled to mass spectrometry measurements (LC-MS) (for example of a

Table 4
Lipid profiles of phosphatidylcholines (PC) in liver tissue determined with mass spectrometry

Compound	Control (Mean±SD)	24-h starvation (Mean±SD)	*P*-value	Change in (%)
32:0-PC	0.274±0.047	0.298±0.036	$P \leq 0.35508$	109%
32:1-PC	0.256±0.069	0.175±0.033	$P \leq 0.02743$	68%
34:1-PC	2.093±0.381	1.919±0.160	$P \leq 0.32588$	92%
34:2-PC	3.463±0.170	3.934±0.399	$P \leq 0.02396$	114%
34:3-PC	0.472±0.025	0.384±0.063	$P \leq 0.00994$ **	−81%
36:1-PC	0.323±0.055	0.246±0.030	$P \leq 0.01266$ *	76%
36:2-PC	2.165±0.175	1.939±0.197	$P \leq 0.06275$	90%
36:3-PC	1.361±0.104	0.933±0.091	$P \leq 0.00002$ **	−69%
36:4-PC	1.07±0.073	0.965±0.100	$P \leq 0.06253$	90%
36:5-PC	0.467±0.044	0.231±0.029	$P \leq 0.000001$ ***	−50%
38:3-PC	0.393±0.041	0.133±0.016	$P \leq 0.000001$ ***	−34%
38:4-PC	0.867±0.0889	0.789±0.064	$P \leq 0.11450$	91%
38:5-PC	0.478±0.040	0.332±0.031	$P \leq 0.00003$ **	−70%
38:6-PC	1.680±0.109	1.481±0.093	$P \leq 0.00663$ **	−88%
40:6-PC	0.611±0.0733	0.621±0.040	$P \leq 0.77959$	102%
40:7-PC	0.216±0.0187	0.167±0.017	$P \leq 0.00072$ **	−77%
40:8-PC	0.102±0.0135	0.059±0.004	$P \leq 0.00002$ **	−58%

**: denotes significant difference $P \leq 0.001$.
***: denotes significant difference $P \leq 0.0001$.

Table 6
Lipid profiles of triacylglycerols (TG) in liver tissue determined with mass spectrometry

Compound	Control (Mean±SD)	24-h starvation (Mean±SD)	*P*-value	Change in (%)
42:0-TG	0.0142±0.0012	0.0188±0.0047	$P \leq 0.04020$	132%
44:0-TG	0.0228±0.0038	0.020±0.0025	$P \leq 0.20532$	88%
44:1-TG	0.0183±0.0022	0.088±0.0420	$P \leq 0.00228$ **	+481%
44:2-TG	0.0062±0.0029	0.1505±0.089	$P \leq 0.00273$ **	+2427%
46:0-TG	0.0178±0.0069	0.0080±0.0047	$P \leq 0.01611$	45%
46:1-TG	0.040±0.0081	0.118±0.0383	$P \leq 0.00060$ **	+295%
46:2-TG	0.036±0.0079	0.518±0.2385	$P \leq 0.00058$ **	+1439%
48:0-TG	0.0445±0.0241	0.0583±0.0122	$P \leq 0.23849$	131%
48:1-TG	0.095±0.0288	0.186±0.0689	$P \leq 0.01327$	196%
48:2-TG	0.133±0.0316	0.551±0.1982	$P \leq 0.00047$ **	+414%
48:3-TG	0.0607±0.0157	0.727±0.2969	$P \leq 0.00027$ **	+1198%
50:1-TG	0.403±0.109	1.254±0.351	$P \leq 0.00021$ **	+311%
50:2-TG	0.765±0.1382	2.047±0.605	$P \leq 0.00049$ **	+268%
50:3-TG	0.6242±0.1118	1.6487±0.6213	$P \leq 0.00262$ *	+264%
50:4-TG	0.177±0.039	1.146±0.4349	$P \leq 0.00029$ **	+648%
52:2-TG	1.959±0.247	5.807±1.473	$P \leq 0.00009$ ***	+296%
52:3-TG	2.823±0.3080	7.919±1.6316	$P \leq 0.00002$ ***	+281%
52:4-TG	1.7893±0.2653	6.1238±1.334	$P \leq 0.00001$ ***	+342%
52:5-TG	0.567±0.115	2.737±0.877	$P \leq 0.00013$ **	+483%
54:2-TG	0.289±0.076	1.249±0.384	$P \leq 0.00013$ **	+432%
54:3-TG	0.7543±0.1960	2.877±0.7484	$P \leq 0.00005$ ***	+381%
54:4-TG	0.9143±0.2037	2.955±0.6657	$P \leq 0.00003$ **	+323%
54:5-TG	0.772±0.1633	2.724±0.5628	$P \leq 0.00001$ ***	+353%
54:6-TG	0.5417±0.091	2.2985±0.5011	$P \leq 0.00001$ **	+424%
56:2-TG	0.046±0.013	0.153±0.060	$P \leq 0.00166$ **	+333%
56:3-TG	0.1252±0.0406	0.466±0.1617	$P \leq 0.00053$ **	+372%
56:4-TG	0.1588±0.0438	0.5128±0.1389	$P \leq 0.00014$ **	+323%
56:5-TG	0.2028±0.0342	0.4428±0.064	$P \leq 0.00001$ ***	+218%
56:6-TG	0.283±0.0195	0.6035±0.059	$P \leq 0.000001$ ***	+213%
58:3-TG	0.0155±0.0065	0.0583±0.024 1	$P \leq 0.00179$ **	+376%
58:4-TG	0.0228±0.0064	0.0663±0.0242	$P \leq 0.00167$ **	+291%
58:5-TG	0.0297±0.0063	0.0655±0.0129	$P \leq 0.00011$ **	+221%
58:6-TG	0.0565±0.0071	0.1107±0.0132	$P \leq 0.000001$ ***	+196%

*: denotes significant difference $P \leq 0.05$.
**: denotes significant difference $P \leq 0.001$.
***: denotes significant difference $P \leq 0.0001$.

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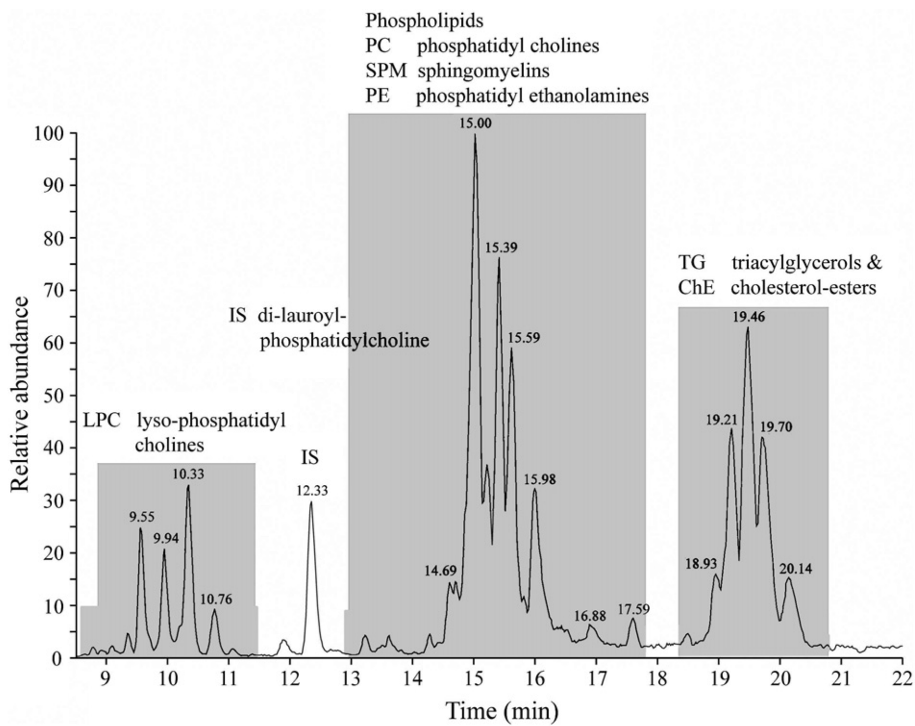

Fig. 2. Lipid profiles determined by Reversed phase liquid chromatography coupled to mass spectrometry (rp LC/MS) in liver homogenate of a mice exposed to 24 h of fasting. Principle of the method is separation based on mass and polarity.

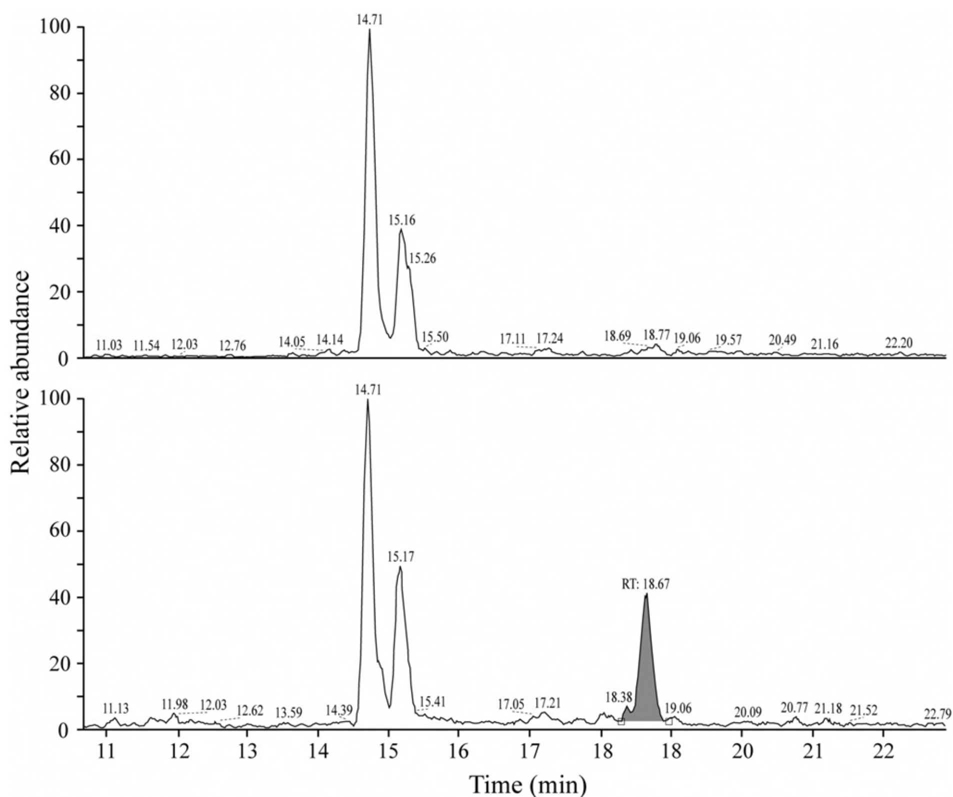

Fig. 3. Chromatogram of 49:4 triacylglycerols. Discovery of a biomarker in liver homogenate of a mice model after 24 h of starvation. A 49:4 triacylglycerol with an odd number of C atoms appeared after 24 h of starvation (RT: 18.67) while it was absent in the control group.

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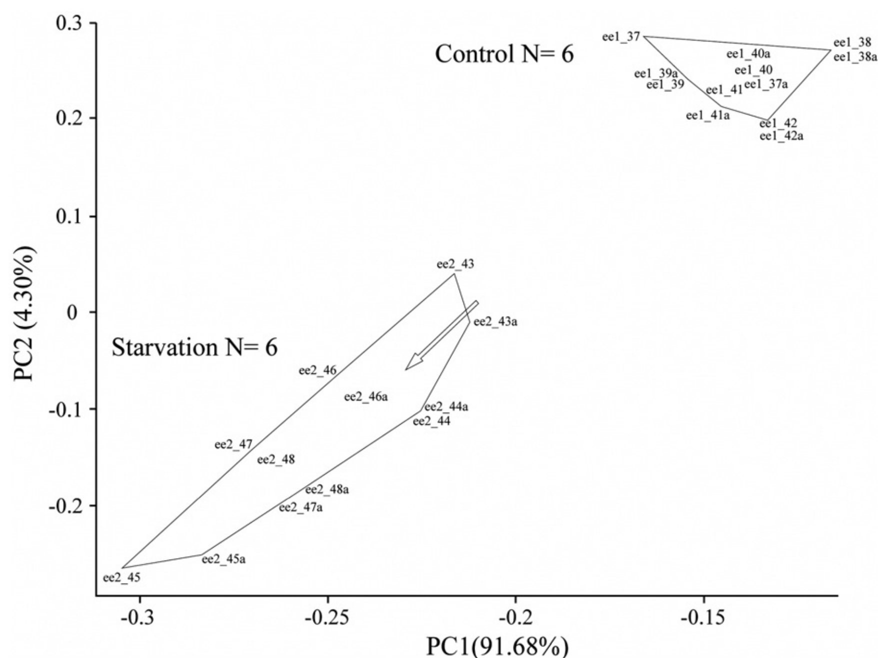

Fig. 4. Canonical correlation of LC-MS (ES) data in a study of the lipid analysis in liver homogenate in control and 24 h starved mice. Principal component analysis (PCA) was performed a) on liver homogenate of the 24 h starvation mouse group ($n=6$) versus Control ($n=6$). Each data point on the plot represents a separate measurement of metabolites in liver homogenate measured with LC-MS techniques.

24 hr fasting animal see Fig. 2) the following patterns in lipid profiles were observed. 18:2-LPC and 22:6-LPC were the most abundant in liver homogenate of the control group. However, only 20:3-LPC dropped significantly ($P \leq 0.0006$) to 29.7% of its initial value after 24 h of starvation (Table 3).

From Table 4 we can conclude that in liver homogenate 34:2-PC was the most abundant in the control and starvation group and not 36:3-PC and 36:2-PC were the most abundant in liver homogenate of the control group. Nearly half (8) of the total amount of 17 PCs dropped significantly after 24 h of starvation to 34–88% of the initial control value (Table 4).

As compared to the abundant phospholipids, PC, the levels of sphingomyelin (SPM) were relatively low (0.02–0.03) in both groups and were not affected significantly by 24 h of food deprivation (Table 5). In contrast, the intensity of the CE was 5–10 times higher and showed a dichotomyous response after 24 h of food deprivation. 20:4-CE showed a significant decrease ($P \leq 0.014$) until a value of 66% of the initial control value while a 18:2-CE and a 18:1-CE both showed a significant rise of 149% and 314% respectively in comparison to the initial control value (Table 5).

The TG displayed an intensity in the range of 0.006–2.8. The most abundant two TG were 52:2-TG and 52:3-TG with a concentration of around 2 and 2.8 respectively in the control group. Of the 33 with LC-MS detected lipid compounds, 28 increased significantly in intensity after 24 h of starvation with intensities in the range of 196%–2430% in comparison to the control group. The TGs with the most significant increases after 24 h starvation were 44:2-TG (2427%), 46:2-TG (1439%) and 48:3-TG (1198%) (Table 6).

Interestingly, in liver homogenates of the 24 h starvation group a 49:4-TG with an odd number of C atoms appeared which was not present in the control group (Fig. 3). Odd triacylglycerols are rare and can possibly be used as biomarkers.

Principal Component Analysis (PCA) was carried out on the parameters of lipid metabolism measured via reversed phase liquid chromatography coupled to mass spectrometry. Relationships between factorial axes and variables are indicated in the factorial plane (Fig. 4). In this figure it is shown that the Control and the Starvation group are clearly separated in the factorial plane.

4. Discussion

Bioanalytical research into metabolites and small molecules encompasses many decades of active research [12,13]. The ‘Systems Biology’ approach aims to understand phenotypic variation to assemble comprehensive data and models of cellular organization and biochemical function, and to elucidate interactions and pathways [14].

The liver plays a pivotal role in mammalian lipid metabolism. Under conditions of food restriction, animals switch from a carbohydrate to a fat metabolism. Lipid compounds are highly concentrated stores of metabolic energy.

The aim of this study was to describe the rearrangement and repartitioning of the fat stores in the liver of a mouse model of starvation-induced hepatic steatosis by metabolic profiling.

After a period of 24 h of food deprivation, in this mouse model first a decline of the body weight by 13.5% was observed. These data are in agreement with Heijboer et al. [6], who showed in a mouse model a decrease of 11.1% after 16 h of food deprivation and Lawrence and Sobocinski [15] who found in a study with mice and rats exposed to 48 h of food deprivation a weight reduction of 21–23%. Weight reduction can be

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explained by the fact that there is no food intake which in addition will negatively influence the water intake of the mice. In this respect, it should also be mentioned that in a small mammal like the mouse, with its high metabolic rate [16], 24 h of food deprivation is already a relatively prolonged period.

A second important observation of this study was the excessive influx of fatty acids into the liver upon starvation. This is the result from hydrolysis of body fat stores under conditions of long-term fasting and predisposes the liver to hepatic steatosis. In the liver, there is a repartitioning of FFA, which can either be used for β-oxidation in mitochondria, or re-esterified into TG followed by secretion of the TG in the form of VLDL. In the circulation, TG transported by VLDL particles are lipolysed by lipoprotein lipase (LPL), thereby and delivering FFA to other tissues, like skeletal muscle [2], where FFA are used for β-oxidation. The capacity for secretion TGs via VLDL is limited, which may lead to accumulation of TG in the liver under conditions of an enlarged intracellular pool of fatty acids. Indeed we did not observe any changes in the VLDL, LDL and HDL of the various fractions (TG, FC, PL, TC) in the blood profiles after 24-h starvation. As a consequence of the limited VLDL TG export capacity, TGs can be deposited in the cytoplasm of the hepatocyte (hepatic steatosis). This fasting-induced phenomenon has been observed for dogs fasting overnight [17] and for rodents [5] and is probably characteristic for all mammals under conditions of starvation switching from a carbohydrate metabolism to a fat metabolism.

The processes of fat accumulation in the liver are regulated via processes at the 'input-side': hepatic TG uptake dependent on the influx of free fatty acids (FFA), fatty acid synthesis, and esterification. The three main sources of hepatic fatty acids available for TG formation are dietary fat, adipose fat, and fatty acid synthesis in the liver. Furthermore, removal of fat from the liver is also regulated via processes affecting the 'output' side: TG export as very low-density lipoprotein (VLDL) or its oxidation to CO_2, H_2O and/or ketones [18]. A major cause for hepatic steatosis is increased fatty acid flux to the liver caused by a high availability of plasma FFA in relation to peripheral oxidative requirements. Under two conditions this process can be observed. Firstly, an increase of exogenous fat, i.e. high-fat feeding, increases liver TG content [19].

The fatty acids in adipose tissue were not determined in this study but will be studies in future studies. However it is an interesting observation that 44:2-TG and 48:3-TG are most abundant in adipose tissue. We observe large increases in liver tissue of these compounds after starvation (relatively 2427% and 1198%) which may suggest that the primary source is adipose tissue and that there is a transportation to liver tissue [19].

The exact mechanism is unclear but in general Hepatic steatosis (NAFL) may progress to steatohepatitis (NASH), which leads to fibrosis and cirrhosis. This progression may be due to damage caused by lipid peroxidation and the production of reactive oxygen species leading [20,21] to insulin resistance.

Secondly, excessive influx of fatty acids into liver, resulting from hydrolysis of body adipose stores under conditions of overnight fasting, predisposes to hepatic steatosis.

Since in chronic liver disease (CLD), numerous aetiological, environmental, nutritional and metabolic factors are involved, and the term CLD embraces different stages of liver dysfunction the pathogenic mechanisms underlying the relationship between IR or diabetes and CLD remain to be elucidated [22].

At this moment, it is unclear if the new TG marker would be useful for the more pathogenic forms of hepatic steatosis.

Future studies with hepatic steatosis due to starvation or a fatty diet in combination with LC-MS data of liver homogenate have to give an answer to this question.

Other observations obtained with HPTLC techniques in this study and related to liver lipid metabolism and starvation included a significant rise of hepatic FC, and CE. The latter observation was confirmed with LC-MS techniques. Cholesterol is by far the most common member of a group of steroids in animal tissues where it has an essential role in maintaining membrane fluidity [23]. It is generally believed that this is a main function of cholesterol by interacting with their complex lipid components, specifically the PL such as PC and SPM [24]. So possibly, starvation will also have effects on membrane fluidity. However, it should be mentioned also that the presence of cytoplasmic lipid droplets might modify the intracellular partitioning of lipids [23].

Finally, LC-MS techniques indicated that the intensity of PC in the 24-h starvation group dropped to ≈90% of the control group. PC (once given the trivial name 'lecithin') is usually the most abundant PL in animal and plants, often amounting to almost 50% of the total. As such it is obviously the key building block of membrane lipid bilayers. In particular, it makes up a very high proportion of the outer leaflet of the plasma membrane. In animal tissues, some of its membrane functions appear to be shared with the structurally related sphingolipid–sphingomyelin [23]. In our study the latter shows, in contrast to PC, no significant changes in liver homogenates of starved mice (Table 3).

An important area in which metabolomics has great potential is the discovery of biomarkers related to a metabolic process like hepatic steatosis or a disease. Using LC-MS, molecular weight and retention time are useful analytical parameters to separate different compounds (for a detailed description of analytical and statistical techniques *vide* [11–13]. In this respect molecular mediators of hepatic steatosis and liver injury are important to study because they can serve as biomarkers to trace people vulnerable and susceptible for the pathogenesis of metabolic syndrome [1].

A possible biomarker is oleic acid (9-octadecenoic acid) which accumulates in humans and mice with hepatic steatosis [1]. A membrane protein known as CD36 is being hailed as a novel marker of insulin resistance and atherosclerosis that can be measured with a simple plasma assay. It was demonstrated that soluble CD36 levels were inversely correlated with insulin-stimulated growth disposal and directly correlated with fasting plasma glucose, fasting insulin and body mass index [25]. Also for non-alcoholic steato-hepatitis (NASH) alanine aminotransferase (ALAT) was used as a marker to diagnose or monitor treatment of NASH [26,27]. An interesting development is that breath biomarkers can be used to study liver disease, in particular non-alcoholic fatty liver disease (NAFLD). It was found that breath ethanol, ethane and acetone can be useful biomarkers in

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patients with NAFLD. In particular, breath ethanol can be associated with hepatic steatosis, and breath acetone can be associated with non-alcoholic steatohepatitis [28].

At this moment, no other biomarkers are available to predict the success of therapeutic interventions aimed at improving insulin sensitivity and to identify those individuals that will benefit most from distinct therapies.

In this study, we observed after starvation in a mouse model in liver homogenate a 49:4-TG with an odd number of C atoms which can be used as a biomarker. This is an important observation and possibly can be used in future studies in identifying biomarkers related to hepatic steatosis in other mammals, including man.

Hepatic steatosis can be measured with the non-invasive MRS technique (unpublished results). However MRS will not detect inflammation [29], which is considered as the marking point for steatohepatitis [30]. Therefore the invasive liver biopsy technique can still be considered as a valuable tool in clinical trials to obtain tissue samples from which a biochemical marker can be derived via other techniques like GCMS, like we demonstrated in this study for starvation.

In conclusion, in this study we demonstrated with research tools/methods like FPLC blood lipid-profiling, HPTLC techniques and sophisticated research tools like reversed phase liquid chromatography coupled to mass spectrometry (LC-MS) that the liver is a highly dynamic system which plays a central role in the adaptive response to fasting and the interconversions of metabolites in lipid metabolism. In the liver, there is a repartitioning and turnover of free fatty acids (FFA) resulting in the accumulation of triacylglycerol stores in hepatocytes, a process called hepatic steatosis. In addition, an odd 49:4-TG was identified as a new biomarker for starvation-induced hepatic steatosis.

Acknowledgments

This study was supported by a grant of Center for Medical Systems Biology (CMSB), Leiden University.

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