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**Title:**

Fasting in healthy subjects is associated with intrahepatic accumulation of lipids as assessed by <sup>1</sup>H-Magnetic Resonance Spectroscopy

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**Key Words:**

Fasting, Fatty liver, <sup>1</sup>H-Magnetic Resonance Spectroscopy, Lipids, 3-hydroxybutyrate acids.

**Short title:**

Accumulation of hepatic lipids during fasting

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## Abstract

The impact of fasting on intrahepatic lipid content in human subjects has previously not been investigated, but data indicate that it may change rapidly in response to metabolic cues. We aimed to measure intrahepatic lipid content after fasting and to correlate this to circulating lipid intermediates.

Eight healthy non-obese young males were studied before and after 12 or 36 hours of fasting. Intrahepatic content of lipids were assessed by  $^1\text{H}$ -magnetic resonance spectroscopy and blood samples were drawn after the fasting period.

Intrahepatic lipid content increased significantly after the 36 h fasting period (median increase 156% (range: 4-252 %),  $P < 0.05$ ), furthermore a significant positive correlation between this increase and 3-hydroxybutyrate acid concentration was detected ( $p=0.03$ ). No significant change in intrahepatic content of lipids could be demonstrated after the 12 h fasting period. The baseline median inter-individual variation in intrahepatic lipids was 0.51% (range: 0.25-0.72%). The coefficient of variation of intrahepatic lipids measurements was 11.6%; 25-30% of the variation was of analytical origin and the remaining 70-75% could be attributed to repositioning.

Conclusion: Intrahepatic content of lipids increases in healthy male subjects during fasting, which demonstrates that nutritional status should be accounted for when assessing intrahepatic lipids in clinical studies. Moreover, the increase in intrahepatic lipids was positively correlated to the concentration of 3-hydroxybutyrate acids.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is prevalent in obese subjects and is by many considered an important feature of the metabolic syndrome. Although the majority of such cases are asymptomatic, several reports underscore an associations between NAFLD and dyslipidemia, hypertension, diabetes and cardiovascular disease[1]. The correlation between NAFLD and insulin resistance is close, and is independent of body mass index (BMI), gender and age [2-5]. Furthermore the morbidity and mortality in patients with NAFLD is increased [6], which is attributed to cardiovascular disease, diabetes, and liver cirrhosis [7-9].

Elevation of free fatty acids (FFAs) seems to be pivotal for the development of NAFLD, but the amount of intrahepatic lipids (IHL) is also determined by factors such as the capacity of the hepatocytes for uptake and turnover of FFAs, the release of triglyceride (TG) in very-low-density-lipoprotein particles, and TG storage in spherical droplets [10]. Donnelly et al. have calculated that in patients with NAFLD 59% of the TG in the liver derives from FFAs, 26.2% from de novo lipogenesis and 14.9% from the diet [11].

Intervention studies reveal that the pool of IHL may change rapidly, especially in response to dietary changes. In patients with type 2 diabetes, two weeks of hypocaloric diet or an 8% weight reduction reduce IHL by 27% and 81%, respectively [12;13]. Furthermore two weeks of low fat diet have been shown to decrease IHL by 20% in overweight non-diabetic subjects[14]. In rats as little as three days of high fat feeding causes a threefold increase in IHL [15].

It is well described that fasting induces an increase in circulating levels of FFAs and a decrease in both hepatic and peripheral insulin sensitivity. This is associated with an increase in both intramyocellular lipid content [16;17] and hepatic production of ketone bodies [18], both of which reflect mobilisation and oxidation of lipid intermediates. Therefore fasting is likely to be associated with significant changes in IHL in human subjects, but this remains to be experimentally verified.

The aim of the present study therefore was to measure changes in IHL during fasting in normal subjects by <sup>1</sup>H-MR Spectroscopy (<sup>1</sup>H-MRS) and to correlate this to circulating levels of ketone bodies.

## Materials and Methods

### Subjects

The study population comprised eight healthy, non-obese young males, age  $23.6 \pm 0.7$  yrs. [mean $\pm$ SE], BMI  $22.8 \pm 0.4$  kg/m<sup>2</sup>, fasting plasma glucose  $4.3 \pm 0.1$  mmol/l, glycosylated haemoglobin A (HbA1c)  $5.5 \pm 0.1\%$ , alanine aminotransferase (ALT)  $23.4 \pm 2.8$  U/l, TG  $0.73 \pm 0.08$  mmol/l, total cholesterol  $4.2 \pm 0.2$  mmol/l, high-density lipoproteins (HDL)  $1.5 \pm 0.1$  mmol/l, low-density lipoproteins (LDL)  $2.4 \pm 0.2$  mmol/l. All subjects were students from the local university.

The exclusion criteria included: a family history of type 2 diabetes, use of any medications, alcohol consumption above 21 units per week, known liver disease, claustrophobia and carriage of magnetic implant.

All subjects were instructed to consume a diet with no major deviations from the national recommendations (max 30% of the energy from fat, 50-60% from carbohydrates and 10-20% from protein), to avoid high fat meals and to abstain from alcohol 3 days before each study period. During the fasting period they were allowed to drink tap or mineral water and to perform normal ambulatory activities, excluding any kind of exercise.

### Design

The subjects were randomly assigned to one of two groups: 1) <sup>1</sup>H-MRS examination at baseline and after 36 hours of fasting (36h-fast) (n=6); 2) <sup>1</sup>H-MRS examination at baseline and after 12 hours of fasting (12h-fast) (n=3). Blood samples were drawn in both groups immediately after the fast. One of the subjects participated in both groups separated by two months.

The protocol was approved by the regional Ethics Committee, the nature and potential risks were explained, before participants gave written informed consent. The study was conducted according to the declaration of Helsinki (2000) of the World Medical Association.

### <sup>1</sup>H-MRS:

<sup>1</sup>H-MRS was performed using a 1.5 T scanner (Signa Excite 1.5 Tesla, Twin Speed, GE medical systems, Milwaukee, USA). The subjects were allowed to eat and drink until the beginning of the study period. The baseline examination was performed at 20.00h, group 1 was fasted for 36 hours, group 2 overnight (12 hours), before re-examination at 08.00h.

During each visit, three <sup>1</sup>H-MRS measurements (spectra 1-3) were made to enable calculations of the inter-individual variation in baseline IHL and the intra-day coefficient of

variation (CV). First, spectra 1 and 2 were obtained (with the same shimming and spatial position), then the subject was taken out of the magnet, allowed to move around a few minutes, and repositioned, before spectra 3 was made. Subjects were positioned feet first in the supine position. A belt was strapped around the lower part of the thorax and upper part of abdomen to minimize respiratory movement of the diaphragm and liver. To generate the spectra, a standard whole-body coil was used for radio frequency transmission and signal reception. The exact orientation of the liver was verified in a 3-plane T2 Localizer pulse sequence.

Oblique plane T1 Gradient Echo sequence using single-breath holding technique with TR 140 msec, TE 2.2/4.4, were made to enable identification of the area of interest.

The volume of interest (VOI  $2 * 2 * 3 \text{ cm}^3$ ) was carefully positioned in the lower posterior part of the liver (area 6), avoiding inclusion of costae, visible vessels and bile ducts. Autoshim was performed to optimize the magnetic field homogeneity. Water suppressed point resolved spectroscopy sequence during free breathing (TE: 30 msec, TR: 2000 msec, number of acquisitions: 128) was applied, using water as autocenter frequency. The full width at half maximum (FWHM) of the unsuppressed water peak was  $10.2 \pm 1.6 \text{ Hz}$ . Each session lasted approximately 40 min.

## Analytical Procedures and calculations

### Relative lipid content

The spectra were analysed using SAGE (GE medical systems, version 7). The height of the suppressed water signal intensity peak ( $S_{\text{water}}$ ) was measured at  $\sim 4.8 \text{ ppm}$ , the lipids/ (-CH<sub>2</sub>-)n signal intensity ( $S_{\text{lipid}}$ ) peak at  $\sim 1.4 \text{ ppm}$ , spectra from subject nr 4, before and after the 36h-fast are provided in Figure 1. We used peak height rather than the area under the curve (AUC), as our FWHM was relative low and constant ( $10.2 \pm 1.6$ ); moreover using AUC caused a higher CV.

The relative lipid content (RLC) was calculated using the following formula:

$$RLC = \frac{S_{\text{lipid}} \times 100}{S_{\text{water,corr}} + S_{\text{lipid}}} \quad [19]$$

$$S_{\text{water,corr}} = \frac{S_{\text{water}} \times 100}{WS\%}$$

WS%: percentage of water suppression.

### Validation of data

The CV was calculated by dividing the standard deviation with the mean (of the three RLC), and presented in terms of percentage. To demonstrate the intra-day CV the median CV of RLC was determined.

### Blood analysis

A blood sample was drawn at the end of the fasting period. Plasma glucose was measured in duplicate immediately after sampling on a Beckman Glucoanalyzer (Beckman Instruments, Palo Alto, CA, USA). Serum samples were frozen immediately and stored at -20 °C. Insulin, growth hormone and cortisol were analysed using time-resolved fluoroimmunoassay (AutoDELFIA, PerkinElmer, Wallac, Turku, Finland), C-peptide by ELISA (DakoCytomation, Cambridgeshire, United Kingdom), FFAs were analysed by a commercial kit (Wako Chemicals, Neuss, Germany). ALT was determined by a commercial method (Pyridoxal Phosphate Activated Cobas Integra 800, Roche, Mannheim, Germany). Glycerol, lactate, alanine and 3-hydroxybutyrates (3-OHB) were measured using a COBAS biocentrifugal analyzer with flurometric attachment (Roche Diagnostics, Welwyn Garden City, UK)[20].

### Statistical Analysis

The windows software Intercooled Stata 9.0 was used for the statistical analysis. To analyse the changes in IHL, the mean of the three RLC, at baseline and after the fasting period, were used as the best estimates of the “true” values. Student’s paired t-test was used. For estimation of the different components of variation in the RLC measurements two Bland-Altman analysis were made followed by a 2 component analysis of variance on the two standard deviations. For statistical significant results, a p-value < 0.05 was required. Since the distribution of the RLC data were skewed, data were log transformed before applying relevant statistical tests, and only medians are presented. Unless otherwise stated, data are presented as mean±SE.



## Results

### Baseline variation in RLC

The inter-individual variation in RLC before the fasting periods ranged from 0.25% to 0.72% (5th vs. 95th percentile) with a median of 0.51%. Since one subjects participated in both the 12 and 36h-fast, a mean of his baseline RLC was used.

### Validation of RLC measured by $^1\text{H-MRS}$

The median CV for RLC measured by  $^1\text{H-MRS}$  was 11.6%. None of the spectra were significantly different, as seen in Figure 3. After performing a 2 component analysis we found that 25-30% of the variation was due to differences between spectra 1 and 2, attributed to elements of uncertainty in the analysis of data, equipment errors and respiration movement, the remaining 70-75% was attributed to repositioning before the third spectra was made. This underlines the importance of precision when the voxel is placed.

### Changes in IHL during the fasting periods

The IHL increased significantly during the 36h-fast. Median RLC increased from 0.42% to 0.74%,  $p=0.009$  (CI: 1.34 – 3.54). The median percentage change in RLC during the 36h-fast was 156% (range: 4-252%,  $p<0.05$ ). (Table 1 and Figure 2)

No significant change in the IHL was observed after the 12h-fast [RLC: 0.72% (before) vs. 0.42% (after),  $p=0.45$ ].

### Analytes in blood

Mean $\pm$ SE levels of the measured analytes at the end of each fasting period are provided in Table 2. Due to the low sample size a statistical comparison between the levels after the 2 fasting studies was not performed, but, as expected, the concentrations of all lipid intermediates increased with more prolonged fasting. There was a significant positive correlation between the increase in RLC in terms of percentage (RLC %) and the 3-OHB concentration after the 36h-fast ( $r=0.85$ ,  $p=0.03$ ), as illustrated in Figure 4. The RLC% as well as RLC after the 36h-fast did not correlate to either levels of FFAs ( $p=0.31$ ,  $p=0.60$ ) or ALT levels ( $p=0.68$ ,  $p=0.20$ ) after the 36h-fast.



## Discussion

This study demonstrates for the first time that fasting is associated with an increase in IHL in human subjects. Apart from extending our knowledge about substrate metabolism in humans, the data also demonstrate that food intake must be taken into consideration when interpreting IHL results.

Fasting is associated with distinct and dynamic changes in substrate metabolism, and Cahill reports that approximately 40% of FFAs are metabolised via hepatic ketogenesis after long term fasting, reflecting that ketone bodies becomes increasingly important as fuel for the brain [18]. Our study showed that the increase in IHL after 36 hours of fasting was positively correlated to the hepatic 3-OHB production. Whereas high levels of IHL in steady state conditions such as the metabolic syndrome seem to be predictive of disease activity, the observed increase during fasting is more likely to reflect a physiological adaptation to alteration in substrate metabolism in general and the increase in hepatic ketogenesis in particular.

In several publications ALT levels have been used as a surrogate marker of IHL, but in our study ALT did not correlate to any indices of IHL. This may reflect the short duration of our study, but it is also plausible that the mechanisms underlying the increased IHL during fasting differ from those of NAFLD.

Since our study population consisted of lean and healthy adult males, without any evidence of hepatic disease or steatosis, one would anticipate IHL to be low. We found a baseline median RLC of 0.51%, with inter-individual variation ranging from 0.25% to 0.72% (5th vs. 95th percentile). Compared to the median of 1.9 recorded by Szczepainak et al. in a group with low risk of steatosis [21], IHL in our subjects is indeed low. Several factors, including dietary habits, physical fitness, age and gender are known to influence IHL in otherwise healthy subjects. Westerbacka et al, who measured IHL by <sup>1</sup>H-MRS in obese non-diabetic women before and after 2 weeks of isocaloric high-fat or low-fat diet, recorded a 20% decrease in IHL in the low-fat diet group, and a 35% increase in the high-fat diet group [14], yet a single high fat meal does not seem to influence IHL [21]. A high level of habitual physical activity is associated with low IHL [22]. Cross-sectional studies indicate that age is inversely related to IHL [23;24], whereas the impact of gender remains controversial.

In conclusion, this study shows for the first time that IHL increases during fasting in healthy human subjects and that this increase correlates positively with circulating levels of 3-OHB. This contributes to our knowledge about the regulation of substrate metabolism during alterations in

nutritional supply. It also emphasises that nutritional status should be standardised when assessing IHL in patients.

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**TABLE 1**

Change in RLC (%) during 12 and 36 hours of fasting.

Subject	12h-fast	36h-fast
1		151.7
2		160.1
3		167.9
4		260.8
5		76.6
6	-8.7	-4.2
7	10.1	
8	-41.1	

**TABLE 2**

Blood samples drawn after 12 and 36 hours of fasting, respectively.

Values are given as mean±SE.

Hours of fasting	12h-fast [n=3]	36h-fast [n=6]
FFAs [μmol/l]	493.3 ± 59.5	1115.7 ± 87.1
Glucose [mmol/l]	4.9 ± 0.1	3.8 ± 0.1
Insulin [pmol/l]	13.6 ± 3.8	13.8 ± 1.7
C-peptide [pmol/l]	325.5 ± 36.9	207.8 ± 67.9
Glucagon [[pg/ml]	36.7 ± 7.1	88.7 ± 13.9
Cortisol [nmol/l]	447.7 ± 114.5	473.7 ± 56.8
Growth hormone [μg/l]	2.9 ± 1.6	8.3 ± 3.5
Alanine [μmol/l]	216.7 ± 40.5	636.7 ± 38.2
Lactate [μmol/l]	466.7 ± 53.4	194.2 ± 15.8
Glycerol [μmol/l]	53.3 ± 14.8	76.7 ± 2.5
3-hydroxybutyrate [μmol/l]	215 ± 87.6	2455 ± 233.9
ALT [U/l]	8.6 ± 3	9 ± 1.4

Figure 1

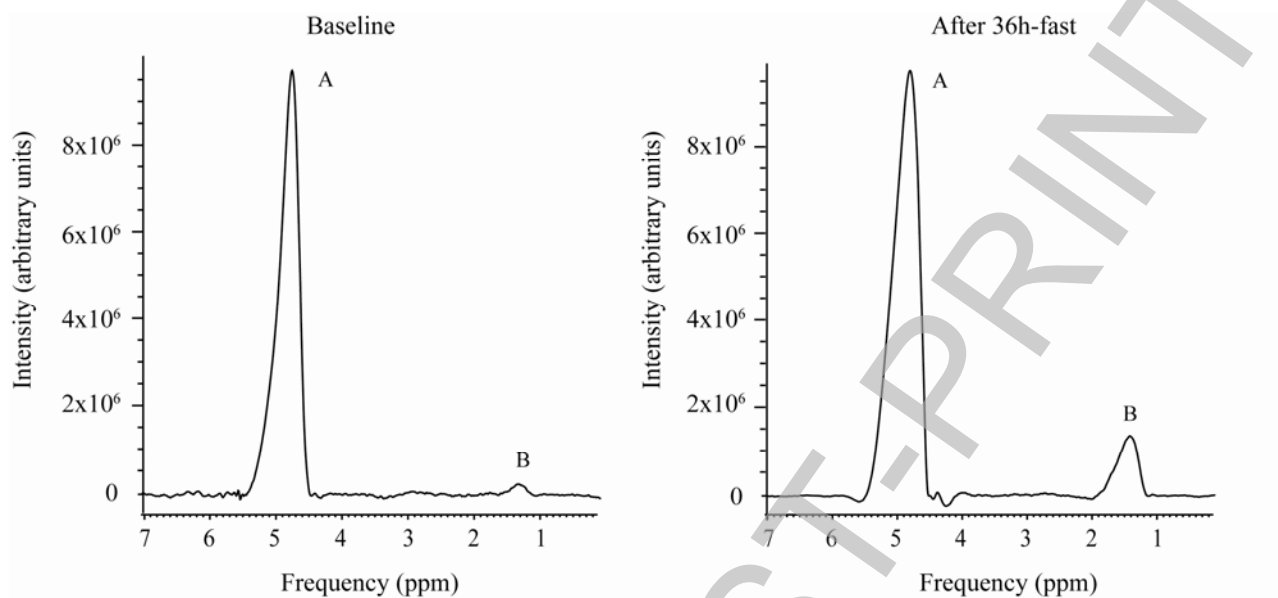
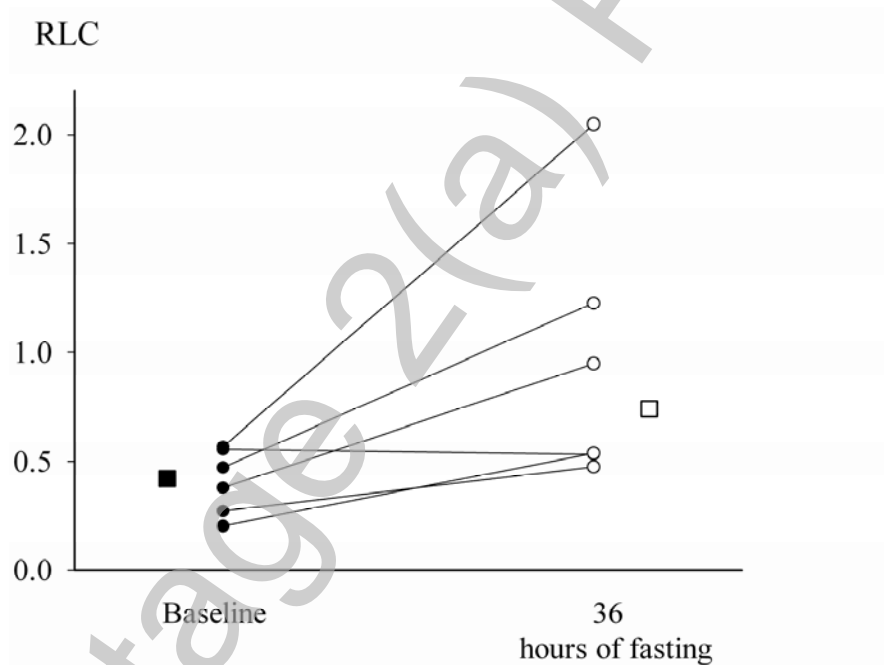


Figure 2



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Figure 3

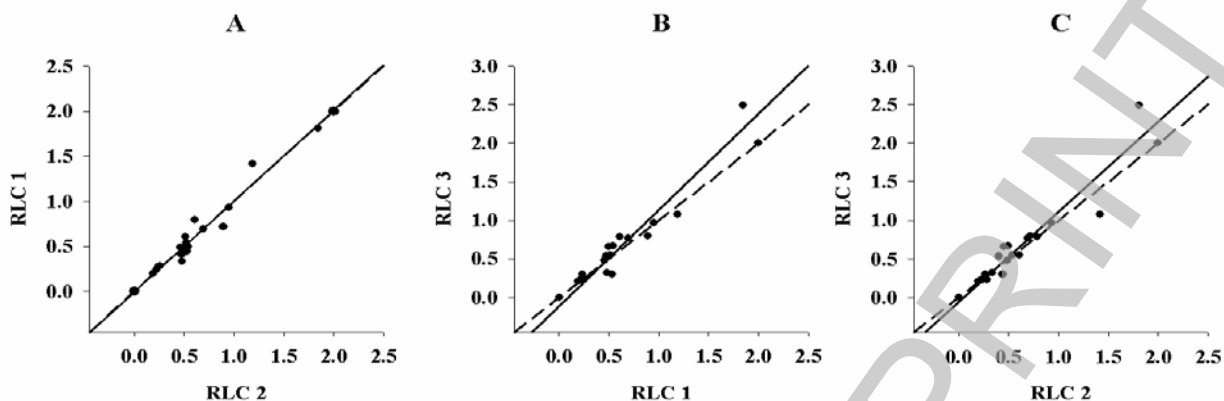
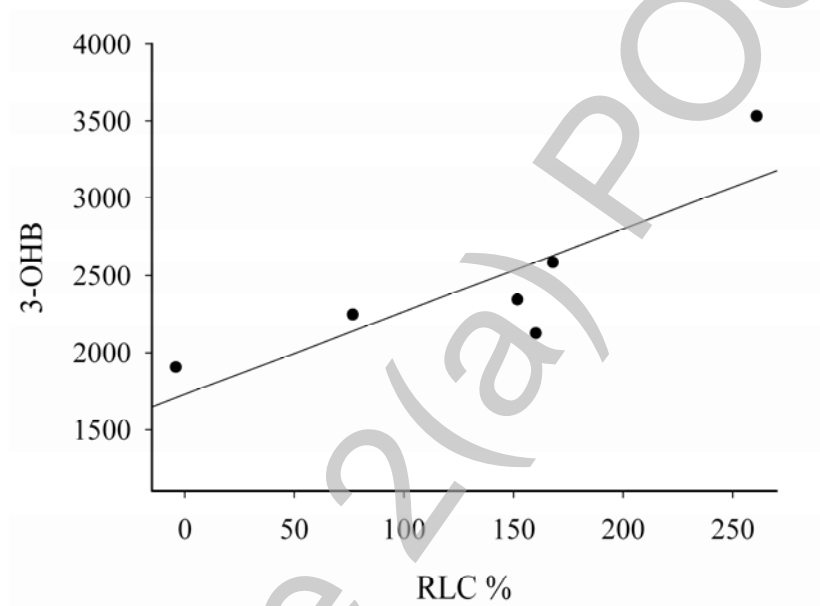


Figure 4





Legends

Figure 1

Spectra from subject nr 4, before and after the 36h-fast. A: suppressed water peak, B: lipid peak.

Figure 2

RLC at baseline (●) and after 36h-fast (○) from each subject in group 1(n=6). Median RLC at baseline (■) and after 36h-fast (□), (p=0.009).

Figure 3

Reproducibility of the three measurements of RLC obtained at the 18 MR sessions. The dotted line depict the optimal correlation (slope=1). A: The correlation between RLC 1 and 2,  $r=0.97$  ( $p<0.01$ ). B: correlation between RLC 1 and 3,  $r=0.95$  ( $p<0.01$ ). C: correlation between 2 and 3,  $r=0.93$  ( $p<0.01$ ).

Figure 4

Scatter plot illustrating the correlation between the change in RLC during the 36h-fast in terms of percentage and the 3-OHB immediately after the 36h-fast ( $r=0.85$ ,  $p=0.03$ ).