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ORIGINAL ARTICLE

A critical analysis of three quantitative methods of assessment of hepatic steatosis in liver biopsies

Mariana Catta-Preta · Leonardo Souza Mendonca · Julio Fraulob-Aquino · Marcia Barbosa Aguila · Carlos Alberto Mandarim-de-Lacerda

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Abstract The issue of adequately quantitatively evaluating hepatic steatosis is still unresolved. Therefore, we compared three methods of quantitative assessment. Two groups of mice (n=10 each) were fed standard chow (10% fat, SC group) or a high-fat diet (60% fat, HF group) for 16 weeks, and hepatic triglyceride (HT) and liver tissue were then studied. Paraplast-embedded tissues stained by hematoxylin and eosin (H-E) were compared to frozen sections stained by Oil Red-O (ORO). In addition, the volume density of steatosis (Vv[steatosis, liver]) was measured by point counting (P-C, sections H-E or ORO) or by image analysis (I-A, sections ORO). HT was significantly higher in the HF group (104% greater, P= 0.0004) than in the SC group. With P-C and H-E, Vv [steatosis, liver] was 4.80±0.90% in the SC group and 33.50±3.17% in the HF group (600% greater, P<0.0001). With P-C and ORO, Vv[steatosis, liver] was 4.86±0.89% in the SC group and 25.21±1.27% in the HF group (420% greater, P<0.0001). With I-A and ORO, Vv[steatosis, liver] was 4.17±0.85% in the SC group and 23.35±1.58% in the HF group (460% greater, P<0.0001). Correlations between Vv[steatosis, liver] and HT were strong and significant in all methods. In conclusion, all methods were appropriate and reproducible. In P-C and H-E, there is a slight overestimation of steatosis in the HF animals in comparison to frozen sections and ORO; in frozen sections, differences between P-C and I-A are insignificant.

M. Catta-Preta · L. S. Mendonca · J. Fraulob-Aquino · M. B. Aguila · C. A. Mandarim-de-Lacerda (✉)
Laboratory of Morphometry, Metabolism and Cardiovascular Diseases, Biomedical Center, State University of Rio de Janeiro, Av 28 de Setembro 87 fds, 20551-030, Rio de Janeiro, RJ, Brazil
e-mail: mandarim@uerj.br
URL: www.lmmc.uerj.br

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Introduction

Non-alcoholic fatty liver disease (or NAFLD) is the most common liver disease in Western countries [1], affecting approximately 30% of the population of the USA and 75–100% of obese and morbidly obese people [2, 3]. Although initial characteristics are benign, NAFLD is the starting point of more serious diseases such as non-alcoholic steatohepatitis (or NASH), liver cirrhosis, and hepatocellular carcinoma [4].

Steatosis can take either of two forms of pathologic anatomy, depending on the size of the lipid vesicles present. These are microvesicular steatosis, where fat is stored in multiple small vesicles, and macrovesicular steatosis, where fat is stored in a single large vesicle. In microvesicular steatosis, hepatocytes appear with a cytoplasm rich in small vesicles with multiple cores [5]. Not much is known about the pathogenesis of microvesicular steatosis, but, in general, it has a worse prognosis than does macrovesicular steatosis [6].

Non-invasive assessment of hepatic steatosis is possible and should be standardized [7]; both T1-weighted dual-echo magnetic resonance (MR) imaging and one-proton MR spectroscopy have excellent diagnostic accuracy as compared to ultrasonography and computed tomography [8]. Still, a liver biopsy is used to establish or confirm the diagnosis of a particular type of liver disease, as well as for staging disease severity. Although a liver biopsy is considered the "gold standard" to assess hepatic steatosis and steatohepatitis, it is not without practical difficulties and controversies [7, 9]. Overall, a biopsy is still very useful because it provides information about the prognosis of the disease its proper clinical management [10, 11].

Semi-quantitative quantification of hepatic steatosis is considered strongly observer-dependent and poorly reproducible [9], and is known to overestimate the values of steatosis in liver biopsies and lead to misdiagnoses [12]. New automatic methods have promising diagnostic and prognostic perspectives [13]. Therefore, current standards of assessment, previously published data, and the clinical relevance of hepatic steatosis as a criteria for liver surgery and transplantation must be challenged [9].

The issue of adequately quantitatively evaluating hepatic steatosis remains unresolved. Therefore, the present study aimed to quantify steatosis and compare two methods of staining and two methods of quantitative assessment. The staining methods used were H-E on paraffin-embedded sections and Oil Red-O on frozen sections. The quantitative evaluations of steatosis used were the "point counting" and computerized "image analysis" methods.

Material and methods

Animal model

All procedures were conducted according to conventional standards for animal testing (Publication No. NIH 85-23, revised 1996). The care of the animals also followed the standards imposed by the State University of Rio de Janeiro and was approved by the local ethics committee.

In this study, two groups of ten C57BL/6 mice at 3 months of age were divided into two groups ($n=10$ for each group). One group was fed standard rodent chow containing 10% fat (standard chow or SC group) according to AIN93M (American Institute of Nutrition recommendation for laboratory rodents) [14]. Another group was fed a diet containing 60% fat (high-fat or HF group), which is a model already tested in our laboratory to induce steatosis [15, 16]. The diets were prepared specifically for this study by Pragsolucoes (www.pragsolucoes.com.br, Jau, SP, Brazil) and were given to the animals for 16 weeks. More information about the composition of the diets is shown in Table 1.

Table 1 Composition of the experimental diets

Content (g/kg)	Diets	
	SC	HF
Casein (≥85% protein)	140.0	190.0
Cornstarch	620.7	250.7
Sucrose	100.0	100.0
Soybean oil	40.0	40.0
Lard	–	320.0
Fiber	50.0	50.0
Vitamin mix[a]	10.0	10.0
Mineral mix[a]	35.0	35.0
L-Cystin	1.8	1.8
Choline	2.5	2.5
Antioxidant	0.008	0.008
Total grams	1,000	1,000
Energy content (kcal/kg)	3,573	5,404
Carbohydrates (%)	76	26
Protein (%)	14	14
Lipids (%)	10	60

HF high-fat diet, *SC* standard chow

[a] Vitamin mix and mineral mix are in accordance with the AIN-93 guidelines

Euthanasia

After 16 weeks of their respective diets, the animals were deeply anesthetized (sodium pentobarbital, 150 mg/kg), and livers were quickly removed and prepared for the different methods.

Determination of hepatic triglycerides

Several fragments of the liver of each animal were frozen at −80°C for biochemical analysis. Hepatic triglyceride levels were measured according to previously published protocol [17]. This determination was then used as a comparison with the results of quantitative histopathology. Briefly, 50 mg of frozen liver tissue was placed in the ultrasonic processor with 1 ml of isopropanol. The homogenate was centrifuged at 2,000×*g* and 5 μl of the supernatant was used with a kit for measuring triglycerides in a semi-automatic biochemical analyzer (K55; Bioclin, Belo Horizonte, MG, Brazil).

Paraplast-embedded material stained with hematoxylin and eosin (H-E)

Some fragments of the liver of each animal were instantly fixed [freshly prepared formaldehyde 4% (wt/vol) in 0.1 M phosphate buffer, pH 7.2] for 48 h at room temperature. Random fragments were dehydrated in graded alcohols of increasing concentration to absolute alcohol and were then cleared in xylene and embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA). The material was sectioned at 5 μm and then stained with H-E.

Frozen material stained with Oil Red-O (ORO)

Other fragments of the liver of each animal with volumes of approximately 1 cm^3 were embedded in Tissue-Tek OCT (Finetechnical Sakura, Tokyo, Japan) in aluminum molds, frozen quickly in liquid nitrogen, and stored at −80°C until microtomy. Frozen sections with 10-μm thickness were

obtained in a cryostat (SLEE cryostat SEM, Mainz, Germany), dried at room temperature for 60 min, fixed in 10% formaldehyde for 10 min, and then frozen and again dehydrated for 60 min. Afterward, the sections were placed in 100% propylene glycol for 3 min, stained with a solution of ORO pre-heated for 8 min at 60°C, differentiated in 85% propylene glycol for 3 min, washed in tap water for another 3 min, and mounted with glycerin.

Sampling for evaluation of steatosis

Several non-consecutive sections were obtained from each fragment in both Paraplast-embedded material and frozen material. The intensity of hepatic steatosis was studied in the histological slices as described. Thus, two sets of slices were analyzed: slices stained with H-E (Paraplast-embedded material) and slices stained with ORO (frozen material). Using these slices, two procedures to assess hepatic steatosis were made: point counting (P-C) and image analysis (I-A). With H-E-stained material, it was only possible to use P-C, but both methods were used with slices stained with ORO. For each staining method, at least ten non-consecutive random digital images were obtained per animal (BX51 microscope, ×100 planachromatic objective, DP71 camera; Olympus Co., Tokyo, Japan). The images were taken in TIFF format, at 36-bit depth, and a resolution of 1,280× 1,024 pixels.

Evaluation of steatosis by point counting

Digital images were projected on a high-resolution LCD flat-panel monitor (LG Electronics, Seoul, South Korea) (Fig. 1). A test system consisting of 36 test points (P_T) was assembled on the monitor. The volume density of hepatic steatosis (Vv[steatosis, liver]) was then estimated as the ratio of the points hitting the vesicles of fat (Pp) compared to the number of test points [18]: Vv[steatosis, liver]=Pp [steatosis]/P_T. This method was performed with Paraplast-embedded material stained with H-E and with frozen sections stained with ORO.

Evaluation of steatosis by image analysis

Computer-based image analysis used the software Image Pro Plus, version 7.01 for Windows (Media Cybernetics, Silver Springs, MD, USA). The digital images (Figs. 2 and 3) were prepared for the analysis as follows [19]: (a) hepatic tissue was segmented in a pure black and white image, (b) the fat vesicles were selected and assigned to white color, (c) the remaining liver tissue was selected and assigned to black color, and (d) the percentage of area occupied by white color (steatosis) in the whole image was measured using the histogram tool.

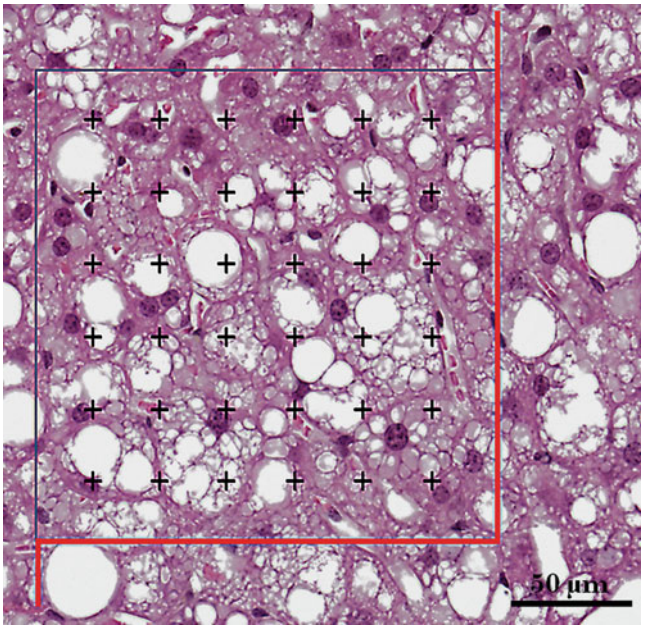

Fig. 1 Photomicrograph of the liver showing abundant macro- and microvesicular steatosis in an animal fed a high-fat diet (digital image of Paraplast-embedded material stained by hematoxylin and eosin). The volume density of steatosis was assessed by point counting after a test system containing 36 test points was superimposed on the photomicrograph (see "Materials and methods" for details)

The reproducibility and variability of the measures

Besides the evaluation made by the authors, other three different observers received basic training to assess the quantifications using the same set of images used by the authors. Therefore, the intra- and inter-observer variability and reproducibility were analyzed.

Data analysis

The normal distribution of the data was tested using the Kolmogorov–Smirnov test, and then the differences between the SC and HF groups were examined with Student's *t* test or among all groups with one-way ANOVA and post hoc Tukey's test. A P value <0.05 was considered statistically significant. In addition, a study of correlation and linear regression was performed, putting hepatic triglyceride levels on the abscissa (X axis) and Vv[steatosis, liver] on the ordinate (Y axis). The first-degree equations of the linear regressions were determined (fitted by the least squares method). The slopes of the equations were then compared (slopes test) (GraphPad Prism version 5.03 for Windows; GraphPad Software, San Diego, CA, USA). The measures obtained by the three additional observers were analyzed for the central tendency and variability. All values are given as mean ± SEM.

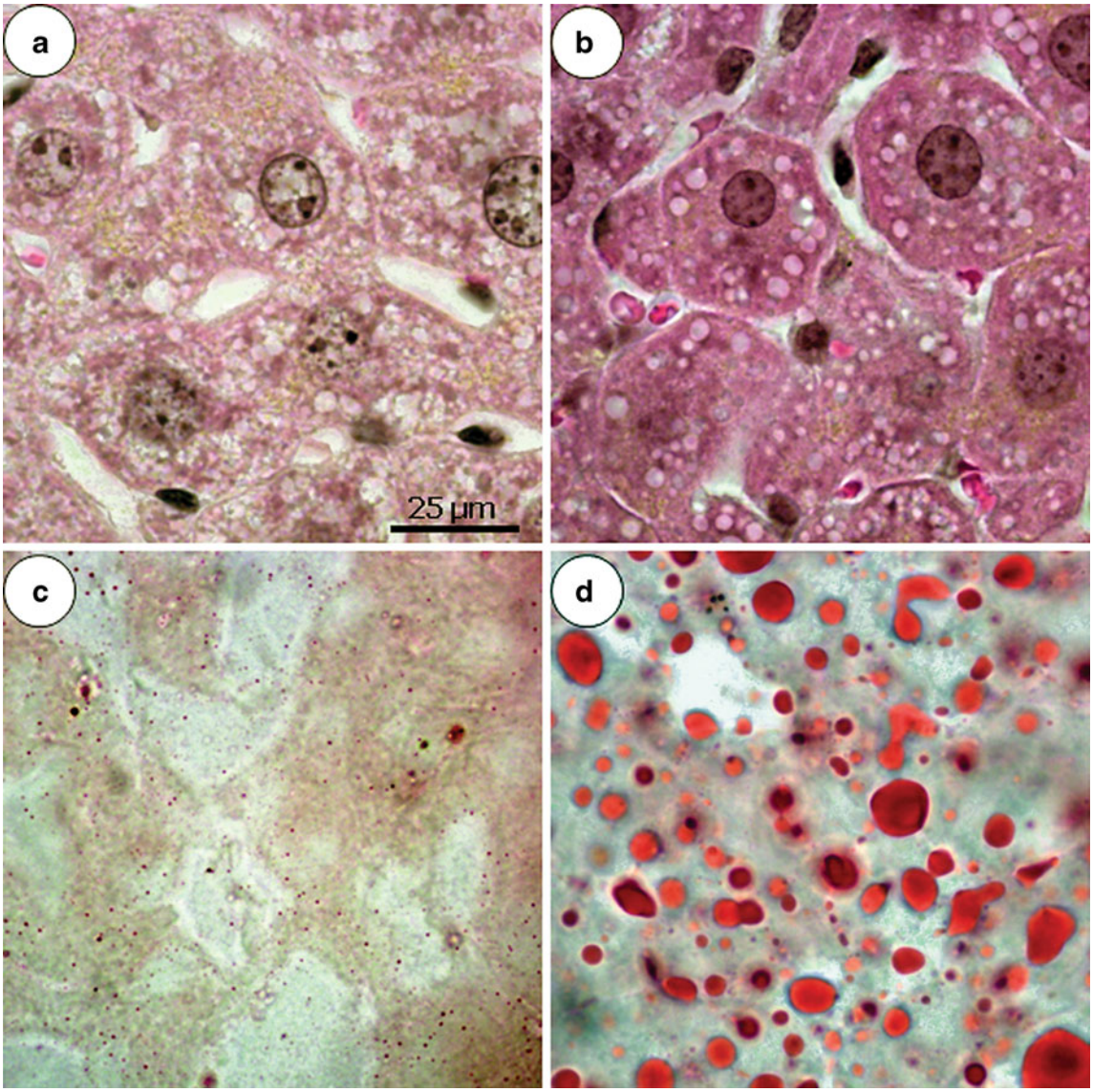

Fig. 2 Photomicrographs of liver sections (same magnification for all sections). Digital images of Paraplast-embedded material stained by hematoxylin and eosin: **a** SC group, **b** HF group. Digital images of frozen sections stained by Oil Red-O: **c** SC group, **d** HF group. Macro- and microvesicular steatosis can easily be identified in the HF group with both embedding and staining methods. However, in Paraplast-embedded material stained by hematoxylin and eosin, we observe that the intra-hepatic spaces were formerly occupied by both fat and glycogen, while in the frozen sections stained by Oil Red-O, the fat itself is stained. *HF* high-fat diet, *SC* standard chow

Results

Hepatic triglycerides

The hepatic triglyceride content was significantly higher in the HF group (104% greater, $P=0.0004$) than in the SC group (Table 2). The photomicrographs of liver tissue from the two groups show this difference clearly (Fig. 2). It was evident that the HF diet induced the formation of much more developed intra-hepatic fat vesicles than those produced by the SC diet.

Paraplast-embedded material stained with H-E

Analysis by point counting

The slides of liver tissue stained by H-E were not difficult to identify as having hepatic steatosis, but we knew beforehand that not all spaces observed correspond to regions previously occupied by fat. The results are shown in Table 2; the Vv[steatosis, liver] was 4.80±0.90% in the SC group and 33.50±3.17% in the HF group (600% greater, $P<0.0001$).

Table 2 Hepatic triglycerides (TG) and volume density of hepatic steatosis (Vv[steatosis, liver]) determined by different methods

Data analysis	Hepatic TG (mg/dl)/mg		Vv[steatosis, liver] %					
			P-C and H-E		P-C and ORO		I-A and ORO	
	SC	HF	SC	HF	SC	HF	SC	HF
Mean	2.78	5.68*	4.80	33.50*	4.86	25.21*, **	4.17	23.35*,**
SD	0.95	1.43	2.86	10.04	2.81	4.03	2.69	4.99
SEM	0.30	0.45	0.90	3.17	0.89	1.27	0.85	1.58
CV %	34	25	60	30	58	16	65	21
CE %	11	8	19	9	18	5	20	7

Significant differences are denoted by a single asterisk, when different from SC counterpart (*t* test, $P<0.05$), or by double asterisks, when different from HF group determined by P-C and H-E method (one-way ANOVA and post hoc Tukey's test, $P<0.05$)

CE coefficient of error, *CV* coefficient of variation, *H-E* hematoxylin and eosin stain, *HF* high-fat group, *I-A* image analysis method, *ORO* Oil Red-O stain, *P-C* point-counting method, *SC* standard chow group, *SD* standard deviation, *SEM* standard error of the mean

Analysis by image analysis

As stated earlier, this method of tissue preparation is not adequate for study by I-A, and so it was not performed.

Frozen material stained with ORO

Analysis by point counting

ORO staining was excellent in terms of both intensity and accuracy for identifying intra-hepatic fat vesicles. This facilitated the use of the P-C method to estimate Vv[steatosis, liver] in both groups. The results are shown in Table 2; Vv [steatosis, liver] was 4.86±0.89% in the SC group and 25.21±1.27% in the HF group (420% greater, $P<0.0001$).

Comparing Vv[steatosis, liver], as evaluated by P-C with the two staining methods, with the H-E stain, no difference was observed in the SC group, but Vv[steatosis, liver] was overestimated by 25% in the HF group in H-E sections in comparison to the ORO sections (Table 2).

Analysis by image analysis

Using the same slices that were studied by P-C, I-A was performed to assess hepatic steatosis in both the SC and HF groups. The ORO stain was essential to this analysis because of its good contrast between fat vesicles and the remaining liver tissue. The results of this analysis are detailed in Table 2; Vv[steatosis, liver] was 4.17±0.85% in the SC group and 23.35±1.58% in the HF group (460% greater, $P<0.0001$).

Using the ORO stain, no significant differences were observed in Vv[steatosis, liver] comparing the P-C and I-A methods across the same group (SC or HF). However, Vv [steatosis, liver] was overestimated by 33% in the HF group using H-E sections evaluated by P-C in comparison to I-A and ORO sections.

Linear regressions: comparison of slopes

Figure 4 shows the linear regression lines obtained for each different method. Each line represents the analysis of data obtained by a staining method and/or its assessment method. No significant difference was found comparing the slopes, and all the correlations were strong and significant. The strongest coefficient of correlation was observed with I-A and ORO ($R=0.95$, $P<0.0001$), followed by P-C and ORO ($R=0.88$, $P=0.0009$) and finally P-C and H-E ($R=0.82$, $P=0.004$).

Intra- and inter-observer variability

The results are shown in Table 3. Considering the three assessments of the Vv[steatosis, liver] obtained by the additional observers, the calculated coefficient of variation (CV) was astonishingly low for the method P-C and H-E, but it was progressively higher for the methods P-C and ORO, and I-A and ORO. In all cases, the HF group had lower CV than SC group for all methods.

Discussion

In this study, we induced hepatic steatosis to compare quantification methods. The separate measurement of hepatic triglycerides was important for characterizing the fatty livers in animals fed the HF diet to compare the observed histopathologic features of steatosis (similar to previous observations using this model) [16, 20, 21]. The HF diet causes obesity and comorbidities such as dyslipi-

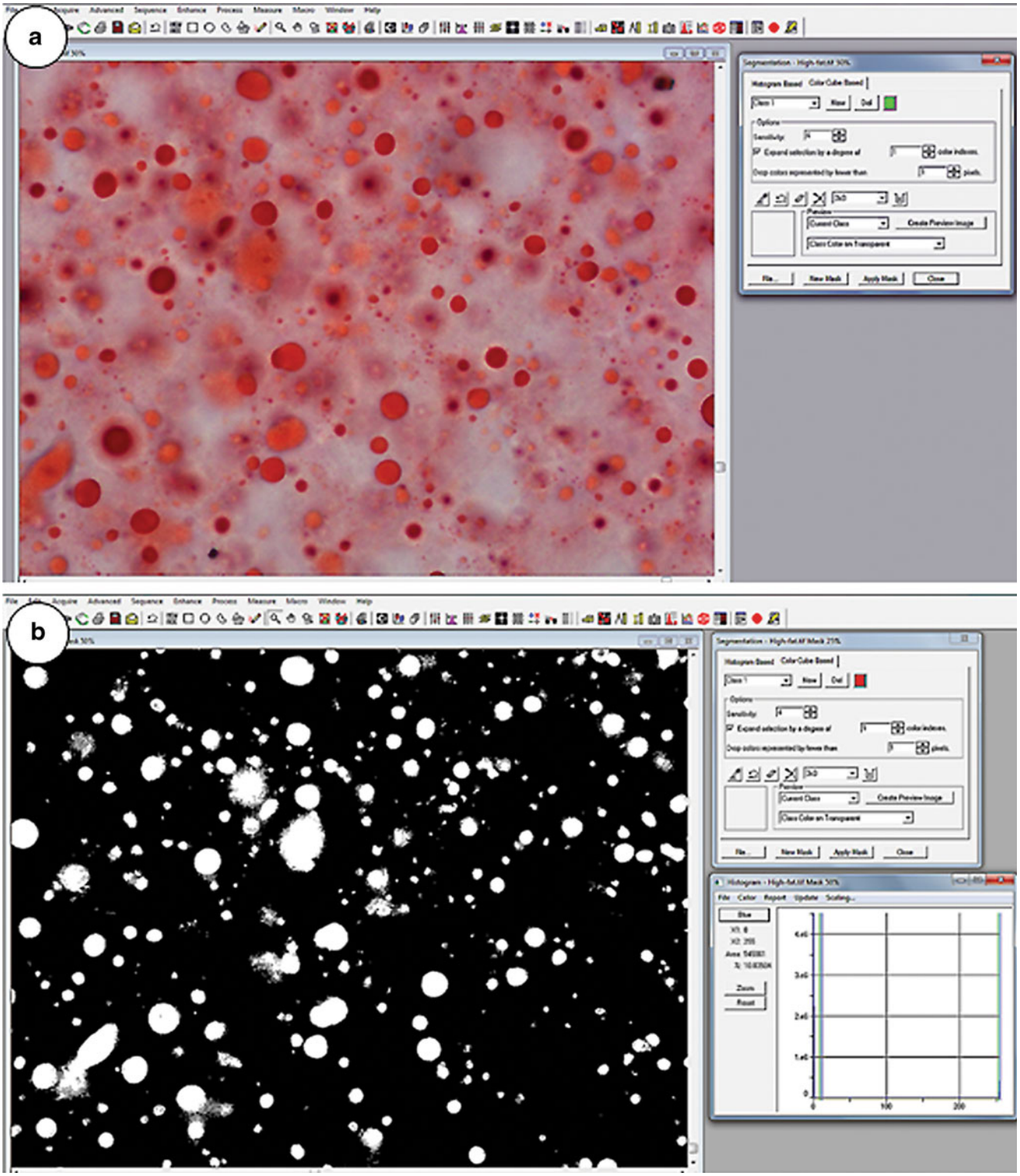

Fig. 3 The two steps of the Image Analysis method to assess hepatic steatosis (digital image of frozen section stained by Oil Red-O): **a** image taken by the software preparing the analysis; **b** after image segmentation (transformation to pure black and white image, white assigned to fat vesicles and black assigned to remaining liver tissue). The relative area fraction of "white" color was determined automatically with the histogram tool (*right bottom corner*)

demia and insulin resistance in C57BL/6 mice, and these animals showed increased liver mass and NAFLD [15]. C57BL/6 mice fed a HF diet also show hypertrophy of adipocytes, as well as hypertrophy of pancreatic islets

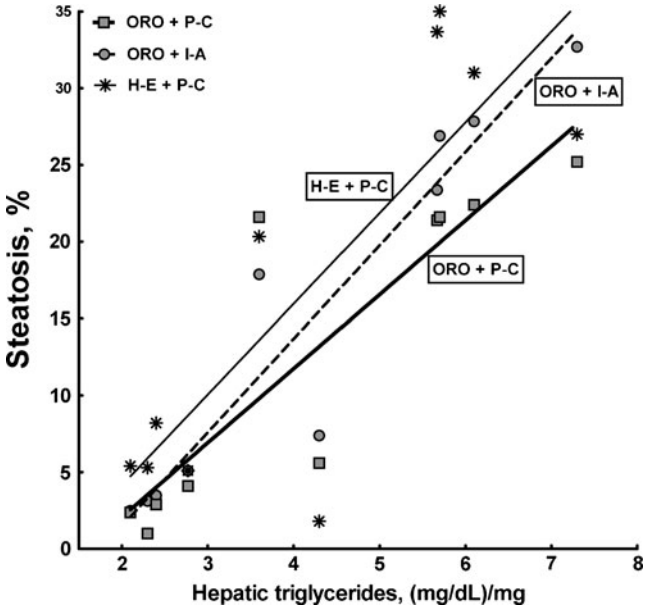

Fig. 4 Graphed linear regressions of the volume density of steatosis (*Y* axis) against hepatic triglycerides levels (*X* axis). No difference among the slopes of the lines was observed (slope test). The coefficients of correlation of these lines were as follows: I-A+ORO (R=0.95, P<0.0001); P-C+ORO (R=0.88, P=0.0009); and P-C+H-E (R=0.82, P=0.004)

concomitant with hyperinsulinemia and increased immunodensity for insulin and glucagon and reduced expression of GLUT-2 in the islets, indicating adverse morphological remodeling and impaired pancreatic tissue function [16].

The staining methods for microscopy in routine liver studies include H-E, trichrome (useful in assessing liver fibrosis), reticulin or its equivalent (for assessment of hepatic architecture), periodic acid-Schiff diastase (to evaluate glycogen, basement membrane, and vessel wall), and staining for iron [22]. One disadvantage of these staining methods is the loss of lipid droplets during processing for paraffin embedding, which, of course, compromises the evaluation of hepatic steatosis. To complicate matters, there are also spaces in the hepatocytes in places once occupied by glycogen. Thus, not all the spaces observed with H-E staining are unequivocally the remains of vesicles of steatosis [23, 24]. It is well understood that there are difficulties in detecting lipid microvesicles after paraffin embedding, and this can cause both an underestimation of the degree of fatty change and a misdiagnosis of "normal" liver that, in fact, contains high levels of microsteatosis [25]. The regular use of special stains such as ORO and Sudan III should be common to stain fat in the liver. However, these methods require frozen sections, which are not made routinely in most laboratories.

Table 3 The variability of the estimation of the volume density of hepatic steatosis (Vv[steatosis, liver]) as determined by three additional observers

Data analysis	Vv[steatosis, liver] %					
	P-C and H-E		P-C and ORO		I-A and ORO	
	SC	HF	SC	HF	SC	HF
Mean	5.0	33.6	3.3	21.3	3.1	22.3
SD	0.2	0.4	0.9	1.6	1.4	5.1
CV %	4.0	1.2	27.3	7.5	45.2	22.9

CV coefficient of variation, *H-E* hematoxylin and eosin stain, *HF* high-fat group, *I-A* image analysis method, *ORO* Oil Red-O stain, *P-C* point-counting method, *SC* standard chow group, *SD* standard deviation

The semi-quantitative evaluation of steatosis is characterized by the use of a subjective scale based on a multigrade proportional area occupied by fat in the hepatocytes or the percentage of hepatocytes in a given biopsy [26]. The histology of the liver is graded according to a number of histological factors, usually by a histopathologist, although there is little consistency in the scale used [26, 27]. Usually the scales have four or five degrees, with different boundaries between grades. For steatosis, a four-grade scale may be zero, mild, moderate, and intense; thus, empirically, we can accept that 0 means no steatosis, 1 is up 33%, 33–66% is between 1 and 2, and 3 is >66% [25]. This grading system is recommended by the American Gastroenterology Association to assess NAFLD [28]. More recently, it was proposed that hepatic steatosis should be evaluated on a five-grade scale: grade 0, no or minimal steatosis (<5%); grade 1, ≥5% but <25%; grade 2, ≥25% but <50%; grade 3, ≥50% but <75%; and grade 4, ≥75% [29].

In 2002, the National Institute of Diabetes and Digestive and Kidney Diseases of the United States sponsored the Pathology Committee of the Network of Clinical Research about NASH (NASH Clinical Research Network) to develop a scoring system for the entire spectrum of NAFLD. The system was developed and validated by nine different pathologists in the group after two double-blind trials of 32 biopsies of adults and 18 pediatric biopsies. In this scoring system, the components of steatosis, lobular inflammation, and hepatocellular ballooning are semi-quantified [30]. It was actually a modification of the existing Brunt scoring system for fibrosis, whereby a score for 1 was changed to include mild fibrosis (1b) and then dense fibrosis or perisinusoidal fibrosis (1c). Scores above 5 in this classification suggest a diagnosis of "steatohepatitis installed", while scores between 0 and 2 carry the diagnosis of "definitely not NASH" [31].

In the recent literature, there has been a demand for a more accurate and reproducible method for estimating hepatic steatosis in biopsies [32, 33]. Whatever the method used (semi-quantitative, P-C, or I-A), the problem arises from the method used to stain liver tissue. Thus, usually,

the evaluation is based on a quantitative "negative" image of fat, i.e., spaces that should have been occupied by fat that was removed during processing of the material.

The method of P-C was classically used to estimate volume densities in a series of studies [34, 35], including for hepatic steatosis [18, 36]. The method of I-A allows measurement of the area of steatosis in liver biopsy material and generates a continuous variable that facilitates statistical analysis [29]. In addition, digital quantification of steatosis can be consistently more precise and reproducible than manual assessment of steatosis in grades 1 (1% to <6%) and 2 (6% to <34%) (semi-quantitative analysis) and may prove to be especially preferable in clinical trials of pharmacotherapeutic agents [37].

Experimental studies have attempted to quantify liver fibrosis based on the number and size of lesions. A proposed scale was (−) not detected, (±) rare, (+) mild, (++) moderate, and (+++) intense [38]. Because increased steatosis has been associated with increased fibrosis, I-A may also be helpful in measuring fibrosis [39], mainly if slices are stained by picrosirius red to enhance the collagen fibers [40, 41]. The I-A is particularly important if we consider that today images are captured by digital cameras and image handling is done entirely within the computer [42].

It is very important to note that the variability of the estimate of Vv[steatosis, liver] was very low with the method "P-C and H-E", which can be an additional advantage of this method. It is natural that the variability is greater in the estimate of Vv[steatosis, liver] when there is no obvious steatosis (which was the case with the SC group). In the HF group (which had marked steatosis), the variability of the estimate of Vv[steatosis, liver] by three observers can be considered acceptable.

In conclusion, the present study compared three methods for assessing hepatic steatosis. All of the methods detected a far greater amount of steatosis in animals fed an HF diet than in animals fed a standard chow. Thus, from that standpoint, all methods were appropriate. In frozen sections stained with ORO, the small differences in the assessment of hepatic steatosis (by P-C or by I-A) were insignificant. In material embedded in Paraplast, stained with H-E, and then analyzed by P-C, there was a slight overestimation of steatosis in HF animals in comparison to frozen sections stained by ORO. Paraplast-embedded material stained by H-E, however, has the advantage of being already in use in the vast majority of routine histopathology laboratories, requiring no special attention. All three methods proved to be reproducible. Therefore, all are better than the subjective semi-quantitative scaling currently used to assess hepatic steatosis.

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

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