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HOW SEVERE IS THE LIPID ACCUMULATION IN FISH LIVER? A FRACTAL ANSWER

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Abstract

Background

Fractal dimension of a histologic image can be interpreted as a snapshot of an evolving process. In fish the liver represents the main source for lipid storage. The liver parenchyma involved in lipid accumulation acquires a pitted texture that resembles an irregular "network". The aims of the present study are: 1) to assess the Fractal Dimension of lipid accumulation in histologic sections of liver from reared *Solea senegalensis* 2) to compare Fractal Dimension with area involved by lipid accumulation obtained through histopathological image segmentation and histological scoring, and 3) to discuss functional meaning of the fractal pattern of lipid accumulation.

Methods

Liver histological sections of 36 soles were clustered into mild, moderate or severe, giving a score of 2, 3 and 4, respectively, to the lipid accumulation. When these changes were not present, the livers were considered to be normal (score 1). Digital image analysis, performed by a pathologist in blind, was carried out on five randomly selected fields of each section by means of a manual selection (segmentation) of two sets of pixel (cytoplasmic “empty area” and the remaining parenchyma). Computing of Fractal Dimension was performed with box counting method on the same images. Receiver Operating Characteristic (ROC) curve was used to *compare* the accuracy of the diagnostic *methods employed.*

Results

Histological scoring ranged from 2 to 4. No case had scoring equal to 1. Empty area ranged from 34,02 to 87,03%. Box counting Fractal Dimension ranged from 1.45 to 1.63. The histological scoring did not correlate with the Fractal Dimension. The comparison between Fractal Dimension computing and histological scoring showed a fail accuracy (area under the ROC curve - AUC was 0.545) while the comparison between segmentation method and histological scoring was highly accurate (area under the ROC curve - AUC was 0.925).

Conclusions

The box counting Fractal Dimension as presented can be useful in interpreting the process of lipid accumulation in the fish liver, although the measure of area by segmentation showed higher values

of sensitivity and specificity when compared to the histological scoring (gold standard), and can be proposed as a reliable digital image analysis method to assist the pathologist.

Background

Natural objects and processes show a complex, irregular and fractal behavior. The fractal dimension (FD) calculation is able to transduce shape complexity in analytical quantitative data, harmonizing the gap between structural features (typically qualitative) and functional quantitative measures [1,2]. The fractal theory has been widely used in physics, biology and medical fields [3,4] and particularly in histology and histopathology to assess the degree of morphological complexity of neurons [5,6] and to investigate the irregular shape of neoplasms related with prognosis [2,7,8,9]. FD of a pathologic image can be interpreted as a snapshot of an evolving process. The FD is usually fractional and increases with the object complexity. In Euclidean shapes a straight lines has FD 1, whereas fractal curve reveal values between 1 and 2 [10].

Concerning human hepatic histopathology, the FD calculation has been applied for the assessment of fibrosis only [11,12]. In studies of hepatic steatosis, on the contrary, digital image analysis has been proposed as reliable and robust method for quantifying liver fat content [13,14].

In fish the liver represents the main source for lipid storage: compared to mammals fish hepatocytes tend to be more vacuolated, corresponding to a relatively higher glycogen and/or lipid content. Such vacuolization, which tends to be uniformly distributed, is often especially apparent in the livers of captive animals [15,16]. Some scoring systems have been adopted for an objective assessment of hepatic histological changes in fish [17,18], including lipid accumulation [19]. The liver parenchyma acquires a pitted texture, that resembles an irregular "network" along with the progression of storage, consisting of optically empty cytoplasm and the nucleus pulled at the periphery (“signet ring” like).

The aims of the present study are: 1) to assess the FD of lipid accumulation in histologic sections of liver from reared *Solea senegalensis* 2) to compare FD with area obtained through histopathological image segmentation and histological scoring, and 3) to discuss functional meaning of the fractal pattern of lipid storage.

Methods

Liver histological sections of 36 soles were examined by two pathologists under a 40X lens with a light microscope; ten fields were selected and classified according to the scoring method applied to *Solea solea* [19,20]. The cases were clustered into mild, moderate or severe, giving a score of 2 (cytoplasmic filling of a clear, optically empty content forming microvesicular spaces), 3 (cytoplasmic filling of a clear, optically empty content pulling the nucleus at the cell periphery and forming macrovesicular spaces) and 4 (cytoplasmic filling of a clear, optically empty content and conferring to the cell a signet-ring like cell), respectively, to the lipid accumulation. When these changes were not present, the livers were considered to be normal (score 1) (Table 1).

For quantification of area involved by lipid accumulation digital image analysis was performed by ImageJ 1.46 plugin segmentation (http://bigwww.epfl.ch/sage/soft/colorsegmentation/). Briefly, each image was manually segmented and partitioned in two pixel clusters, the area involved by lipid accumulation (empty area=EA) and the remaining area, both expressed as a percentage (Table 1). These images were those used in the paper by Mandrioli *et al*. [19].

To determine the FD a second pathologist processed the same images independently as follows: images were subjected to a segmentation process and minimally manipulated to obtain the outline of the lipid droplets. Briefly, contrast was enhanced to emphasize the difference between lipid droplets and the remaining parenchyma and binarization was then performed. Computing of FD was performed with box counting method with ImageJ 1.46 (http://rsb.info.nih.gov/ij/) using boxes of 2,3,4,6,8,12,16,32,64,128,256,512,1024,2048 pixel (see macro as appendix for details). Calculation of FD takes approximately 10 minutes for 180 photographs.

Statistical analysis

Mean values of hepatocyte EA and mean values of five FD values of the 36 animals were subjected to statistical analysis using SPSS 19 software. Preliminary, Kolmogorov–Smirnov test for goodness of adaptation was used to verify distribution normality. The Receiver Operating Characteristic curve (ROC) was plotted. It is a plot of the true positive rate against the false positive rate for the different possible cut-points of a diagnostic test. For the construction of the ROC curve the cut-off value for histological scoring was ≥ 4; histological scoring 2 and 3 were considered together (both

for EA and FD) for evaluating if proposed morphometric measures are able to discriminate severe from mild and moderate lipid accumulation. Histological scoring of liver lipid accumulation was considered the gold standard.

Results

Histological scoring ranged from 2 to 4. No case had score equal to 1. EA values ranged from 34.02 to 87.03%. The results of mean box counting FD ranged from 1.47 to 1.63 (see Table1 for details about values of EA and FD in respect to scoring 2,3,4).

A statistically significant correlation (Pearson' correlation coefficient = 0.69; $p<0.001$) was found between EA and histological scoring. No correlation was identified between FD and EA (Pearson' correlation coefficient = -0.04; p=0.79). The histological scoring did not correlate with the FD (Spearman'rho = -0.027; p=0.875). The lipid accumulation ranged from microvesicular to macrovesicular: the higher FD (1.63) corresponded to small lipid droplets (microvesicular lipid accumulation) that impart a jagged texture to the hepatic cords outline, while lower FD (1.45) characterized larger lipid droplets (macrovesicular lipid accumulation) (Table 1). Moreover, the intermediate class of lipid accumulation (score=3), which was predominant, shared highly variable FD. Area under the ROC curve (AUC) for FD showed a fail accuracy (AUC=0.545) while the AUC for EA was highly accurate (AUC=0,925). At EA value equal to 55%, sensitivity and specificity are 93.8% and 85%, respectively (Figure 1).

Discussion

In fish species, liver does not show the typical lobular architecture of mammals. Routinely stained histological sections can be interpreted as a monostrate of densely packed polyhedral cells arranged in cords as a dual-plated muralium, without a lobular arrangement and with scattered "diads" (a biliary tract and a vein or an arteriole) [21]; as a consequence the standard scheme for degenerative changes classification could not be used.

In this study hepatocytes expanded by large single lipid droplet displayed a less complex texture, simplifying the fractality, and showing as a consequence a lower FD. The main reason for this fact is that round Euclidean shape of large lipid droplets is a form that minimizes the interface between

triglycerides and polar cytoplasm causing a “loss of fractality”. Unexpectedly, the histological scoring did not correlate with the FD; this evidence could be explained by the fact that scoring classes are too scant (only four), so the “middle class” is overrepresented. In fact, the intermediate class of lipid accumulation (score=3) is indeed predominant, although the cases shared highly variable FD. To the best of our knowledge this is the first study that report the FD of hepatic lipid accumulation in fish. The higher values of FD can be interpreted as normal liver histology, as a mild lipid accumulation can be considered physiological in fish. Indeed, it is assumed that healthy biological processes follow fractal patterns, so a “loss of fractality” can be considered a deviation from a physiological to pathological state [22]. Applying this concept to hepatic lipid accumulation in *Solea senegalensis*, the moderate to diffuse lipid content of hepatocytes can be interpreted as a dynamic, physiological state that switches in some subjects to unhealthy less fractal condition.

In this study the histological scoring does not seem to be determined by the more or less jagged appearance of liver parenchyma (macrovesicular or microvesicular pattern) but by the amount of involved parenchyma (EA). The fractal dimension cannot quantify the amount of the lipid accumulation but it reflects the histological complexity (jagged appearance). On the other hand, the segmentation method, as the histological scoring, is able to define the amount of involved parenchyma and the two methods are closely related (Table 1).

The adoption of ROC curve allowed to evaluate and compare the performance and accuracy of used methods and to obtain a cutoff value, able to identify a threshold of measured values. The EA cutoff value of 55% was set to separate severe from mild to moderate lipid accumulation; this value was chosen for highly accurate sensitivity and specificity.

In human pathology special histochemical techniques (Oil-Red-O) and image analysis are recommended as the most reliable techniques for the correct quantification of hepatic lipid accumulation [14]; in this study the use of FD computing and EA calculation were performed on routinely H&E-stained sections without needing a histochemical technique.

Conclusions

The box counting FD here presented can be useful in interpreting the progression of lipid accumulation in the fish liver, although the measure of empty area involved by lipid accumulation

(EA) showed higher values of sensitivity and specificity when compared to the histological scoring (gold standard). The segmentation of histological images of *Solea senegalensis* liver can be proposed as a robust method in assisting the histological scoring, and the percentage of 55% of EA can be identified as the cutoff between severe accumulation (over 55%) and mild to moderate hepatic lipid accumulation (below 55%).

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Figure legends

Table1. Examples of processed photos (histological image, segmented image, outline, histological scoring, FD and EA) of three scored classes of lipid accumulation.

Figure 1. ROC curve representing accuracy of FD and EA methods. Area under the ROC curve is higher for EA than FD showing a fail accuracy of FD method.

Appendix

```
run("Enhance Contrast", "saturated=10 equalize");
run("Invert");
run("Make Binary");
run("Despeckle");
run("Smooth");
run("Smooth");
run("Make Binary");
run("Smooth");
run("Despeckle");
run("Find Edges");
run("Make Binary");
run("Fractal Box Count...", "box=2,3,4,6,8,12,16,32,64,128,256,512,1024,2048");
close();
```

Table 1

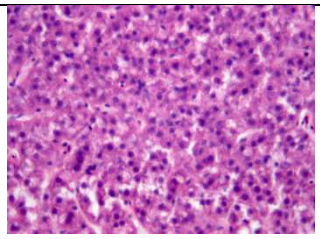
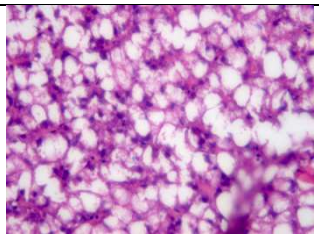
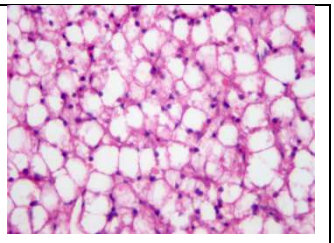
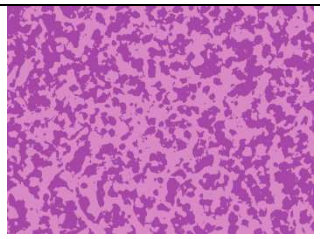
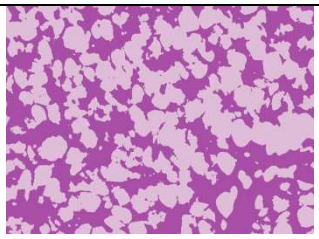
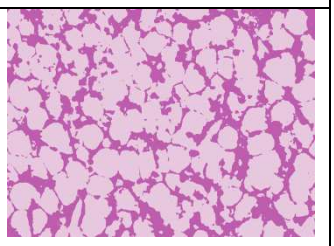
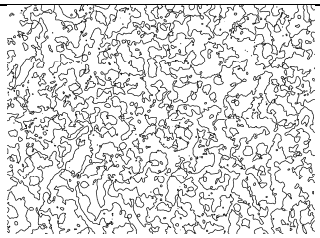
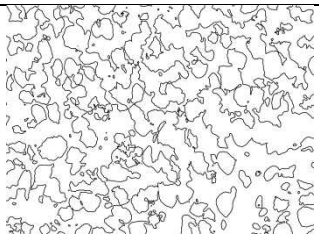
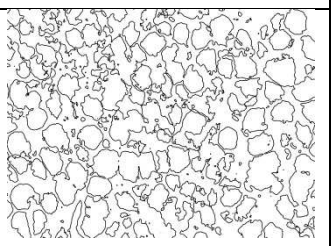
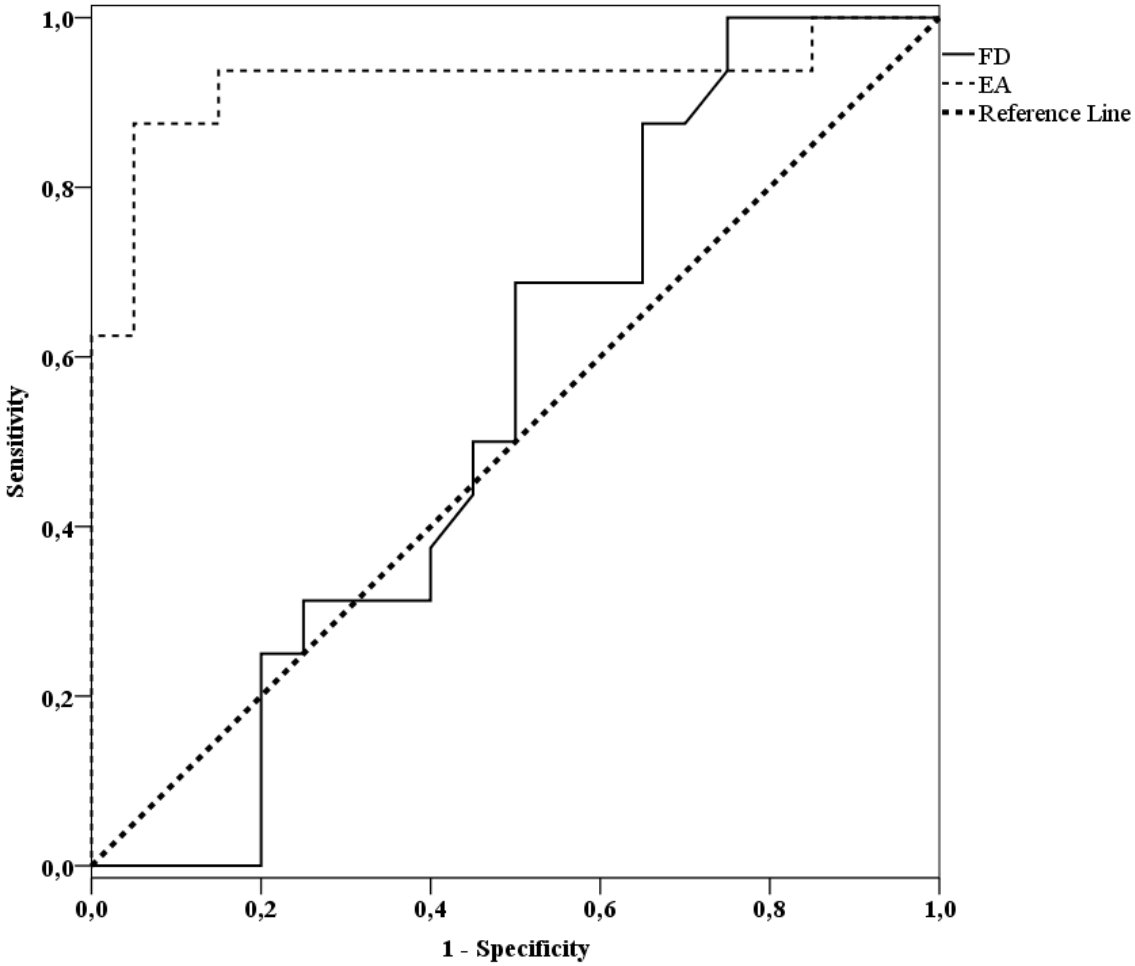
Histological image			
Segmented image			
Outline			
Histological scoring	2	3	4
FD	1.64	1.54	1.57
EA (percentage)	51.44%	53.94%	73.60%

Figure 1


1,0
0,8
0,6
0,4
0,2
0,0
Sensitivity
0,0
0,2
0,4
0,6
0,8
1,0
1 - Specificity
FD
EA
Reference Line