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C1. Replace the colon after "table 1" with a bold period.
C2. Make "values of the main plasma lips from animals (mean +/- standard error)" lightface.
C3. Add a period after "(31)"
C4. Rebreak "reproducibility" re-pro-duc-ibil-i-ty

Hyperlipidemic Chicken as a Model of Non-Alcoholic Steatohepatitis

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Non-alcoholic steatohepatitis (NASH) is part of the spectrum of non-alcoholic fatty liver disease (NAFLD), currently the most common cause of abnormal liver tests. Given the difficulty of studying all the factors involved in it in human populations, studies in animal models might provide crucial insights in the pathogenesis of steatohepatitis. Several physiological features predispose birds to fat deposition in the liver. The present study was conceived to explore the possibilities of the chicken fed a cholesterol and fat enriched diet as a model for steatohepatitis. We used two different diets: a standard growing mash (control group) and a standard growing mash enriched with 2% cholesterol and 20% palm oil (hyperlipidemic group). We investigated the effect of feeding a cholesterol and fat enriched diet, on plasma lipid levels, liver enzymes and hepatic histopathology. Semiquantitative and quantitative assessment by image analysis was performed to determine changes in lipid deposits and inflammatory infiltration. Statistically significant increases were observed in all plasma lipid parameters, liver macroscopic features, fat deposits and cell-ballooning of hepatocytes between control and hyperlipidemic animals. Significant differences were also observed in the inflammatory infiltration parameters (number of foci, density, area and maximal diameter). Results show that diet-induced hypercholesterolemia and hypertriglyceridemia are associated with severe impairment of liver histology (fat accumulation, inflammation and cell-ballooning), reproducing histological features of human NAFLD. This model, which is easy and reproducible, offers economic and technical advantages. Furthermore, the reversibility of the pathologic changes makes it suitable for drug intervention studies of steatohepatitis. Exp Biol Med 234:000–000, 2009

Key words: non-alcoholic steatohepatitis (NASH); chicken; experimental animal model

Introduction

Non-alcoholic steatohepatitis (NASH) is part of the spectrum of non-alcoholic fatty liver disease (NAFLD), currently the most common cause of abnormal liver tests. NAFLD is a continuum of diseases that include simple steatosis (non alcoholic fatty liver, NAFL), and the sometimes progressive, inflammatory form termed non-alcoholic steatohepatitis (NASH), ultimately leading to cirrhosis and hepatocellular carcinoma, which develops in the absence of excessive alcohol intake. NAFLD is the most common liver disorder in affluent societies, representing the hepatic metabolic consequence of relative overnutrition and altered diet composition in the setting of reduced physical activity and sedentary behaviours (1, 2). In fact, some studies reveal that the prevalence of NAFLD may be as high as 30%. Estimates for NASH are lower and the figures less reliable, mainly due to sample size considerations, but vary between 5.7% and 17% (3).

NAFLD is a complex disorder involving environmental factors and genetic predisposition. As a result of this complexity, animal models of the spectrum of NAFLD provide the necessary tools to overcome confounding variables, such as genetic heterogeneity, gender differences, and environmental factors, including diet and lifestyle (4). Much is still unknown about the pathophysiology of steatohepatitis in humans. Given the difficulty of studying all the factors involved in NAFLD in human populations, studies in animal models might provide crucial insights in the pathogenesis of steatohepatitis.

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In chickens, the lymphatic system is rudimentary, and the liver is the first organ to be exposed to dietary lipids. The major route of fat absorption is by mixed micelle formations. The chylomicrons are absorbed directly into the portal blood for transport to the liver before further synthesis and subsequent tissue deposition. Such a feature predisposes birds to fat deposition in the liver (5). Furthermore, the chicken is small and suitable for prolonged laboratory investigations. Therefore, the chicken model offers economic and technical advantages over mammalian models. In fact, we have previously used the chicken for the study of atherosclerosis (6, 7), which develops as a result of a combination of lipid storage and inflammation through mechanisms that are not completely understood (8). We observed that these animals also developed hepatic alterations that were consistent with steatohepatitis when they were fed a hyperlipidemic diet.

Therefore, the present study was conceived to explore the hepatic consequences in chickens fed a cholesterol and fat enriched diet as a model for non-alcoholic steatohepatitis. We used two different diets: a standard growing mash (control group) and a standard growing mash enriched with 2% cholesterol and 20% palm oil (hyperlipidemic group). We investigated the effect of feeding this cholesterol and fat enriched diet, on plasma lipid levels, liver enzymes and hepatic histopathology. Semiquantitative and quantitative assessment by image analysis was performed to determine changes in lipid deposits and inflammatory infiltration.

Materials and Methods

Experimental Animals and Study Design. Forty three-week-old male White Leghorn chickens (Pollos Pujante, Murcia, Spain) were housed under controlled conditions. Each room had air-conditioning and thermostatic control in order to minimize variations in temperature and humidity (approximately 23°C and 60%, respectively). The chickens were randomly assigned to one kind of diet (they received a standard growth diet during the first three weeks of their life). Water was given *ad libitum*.

SD (standard diet): A standard growing mash (fat content 4.8%). The weekly amount of this diet was increased with the age of the animals.

HD (hyperlipidemic diet): A standard growing mash with pure cholesterol (2% of the mixture) and 20% of the mixture of saturated oil (palm oil).

After a three-month induction period, ten chickens in each group were sacrificed to evaluate the hyperlipidemic effect. Afterwards, the remaining chickens were kept for another three-month period with the same diets. Thus, the groups of our study were as follows:

SD group ($n = 10$): SD for six months (healthy control).

HD group ($n = 10$): HD for six months (hyperlipidemic control).

Sampling. All animals were sacrificed by intraperitoneal administration of pentobarbital, after six months of receiving one of the above diets. Blood samples (1 ml) were extracted after an overnight fasting period from the axillary vein. In all cases, blood was collected into 10 mM trisodium citrate-containing tubes. Plasma was separated and analyzed for the determination of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl-transferase (γ-GT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), creatine kinase (CCK), C-reactive protein (CRP) and fibrinogen. Total cholesterol, LDL, HDL, triglycerides, AST, ALT, γ-GT, AP, LDH, and CCK were measured using a D-2400 and P800 analyzers (Hitachi Ltd., Tokyo, Japan) and commercially available assays from Roche Diagnostics (Manheim, Germany). The method described by Kostner *et al*. (9) was used for precipitation of HDL.

Livers were removed and cleaned of surrounding tissue for histological examinations.

All experimental procedures were approved by the University of Murcia institutional Animal Care Committee, in accordance with the guidelines for ethical care of experimental animals of the European Union.

Liver Macroscopic Analysis. Liver weights were recorded, and the relation to the total weight of the animal was calculated. Photographs of the organs were taken in the same light conditions, in order to compare gross histology and size with macroscopic parameters: colour, superficial roughness and liver weight. Thus, four levels of macroscopic alterations were established: Level 0 corresponds to a normal liver, of small size, smooth, brilliant and wine red coloured; 1 means a mildly, paler, slightly more granular and less brilliant than previous level; 2 or moderate, corresponds to a dark brown coloured, granular and size increased liver; and 3 or severe, is an ochre, without brilliance, swelling liver, with superficial yellowish striation. Four independent observers blinded to the study classified each sample in the described reference levels. Percentages of livers within each level of macroscopic alteration were evaluated for each of the experimental groups for statistical analysis ($n = 40$ for each group).

Histological Analysis of the Liver. Liver samples (always obtained from the same area, the internal border of the left liver lobe) were fixed in 10% formaldehyde (in PBS) (0.1 M phosphate-buffered saline, pH 7.4) for 10 hrs and embedded in paraffin; afterwards, 5μ-thick paraffin sections were cut and stained with haematoxylin and eosin (H&E) and Verhoeff Van Giesson staining techniques, dehydrated and mounted in Dpex mounting medium (Panreac, Spain). A histological assessment of the tissue was performed for each animal by a pathologist who was blinded to the study.

Semiquantitative and Quantitative Steatosis Analysis. Lipid deposits were evaluated in 10 microscopic fields (×400) in each animal (100 fields in each experimental group). Livers were semiquantitatively classified assigning a score relative to the level of lipid deposits in the sample, according to the histologic classification by Brunt *et*

C1 **Table 1: Values of the Main Plasma Lipids from Animals (Mean ± Standard Error)[1]** C2

Group	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
SD	95.11 ± 5.93[a]	79.55 ± 33.49[a]	53.11 ± 8.42[a]	26.00 ± 2.78[a]
HD	1136.00 ± 196.2[b]	454.00 ± 165.9[b]	233.80 ± 33.74[b]	811.3 ± 162.35[b]

[1] Statistical analysis was performed by Anova and Bonferroni tests. HDL, high density lipoprotein; LDL, low-density lipoprotein.
[a,b] Different lowercase letters show significant differences between groups ($P < 0.05$; $n = 10$ for each group).

al. (10) and modified by Angulo (11): 0 corresponds to normal, with absence of lipid deposits or a level lower than 5%; 1 or mild, with lipid deposits lower than 33%; 2 or moderate, with lipid deposits between 33% and 66%; and 3 or severe, with lipid deposit levels over 66%. Percentages of tissues within each histological classification were determined for each of the experimental groups for statistical analysis.

A more detailed evaluation of lipid deposits was carried out by quantification of the percentage of steatosis area in liver parenchyma: lobular and centrilobular zones in 10 microscopic fields (square fields of 134 μm^2), obtaining 100 determinations for experimental group and zone. Mean and standard error were determined for each group and zone, and a comparative statistical analysis was also carried out. These parameters were quantified by image analysis using the MIP 4.5 (Microm, Image Processing software, Consulting Image Digital, Barcelona). Briefly, the image analysis system consisted of a light microscope (Zeiss Axioskop, Madrid) connected to a video camera (Sony 151-AP) and a control computer. After obtaining a digital image, fat deposits were chosen interactively by a graphic line, and percentages of steatosis were measured.

Analysis of Inflammatory Infiltration. Number of inflammatory foci was assessed microscopically (×200) in 10 fields for each animal. Appearance of inflammatory foci was classified as 1, or low density; 2, moderate; 3, high density. Furthermore, area and maximal diameter of inflammatory foci were evaluated in 10 microscopic fields (square fields of 267 μm^2), obtaining 100 determinations for each experimental group, by image analysis using the MIP 4.5 (Microm, Image Processing software, Consulting Image Digital, Barcelona). Inflammatory density was calculated with the following ratio: area of inflammatory infiltration (obtained by image analysis)/area of the entire field. Measurements were made in 5 square fields of 267 μm^2 for each animal, obtaining 50 determinations for experimental group.

Hepatocyte Ballooning Analysis. Ballooning classification (0–2) was made following a histological scoring system (12): 0, none; 1, few balloon cells, i.e., rare but definite ballooned hepatocytes being present, as well as cases that are diagnostically borderline; 2, many cells or prominent ballooning. Measurements were made in 5 square fields of 134 μm^2 for each animal, obtaining 50 determinations for each experimental group.

Statistical Analysis. Results are expressed as mean ± standard error. Mann-Whitney *U* and Kruskal-Wallis non parametric tests were used for assessment of statistical significance in semiquantitative and macroscopic analysis, while statistical significance for quantitative analysis was evaluated by ANOVA, Bonferroni, Welch, or Games-Howell tests. Statistics were performed using SPSS v14. A P value < 0.05 was considered as statistically significant.

Results

Chickens fed on the hyperlipidemic diet for six months (HD group) showed an increase in all lipid parameters in the serum when compared to those of chickens fed the standard diet (SD). These included total cholesterol, tryglicerides, LDL and HDL ($P < 0.001$ in all cases; Table 1). No statistically significant differences were observed (Table 2) between concentrations of the analyzed enzymes (AST, ALT, GGT, AF and LDH). Animals fed on SD had comparatively lower levels of CRP ($P < 0.05$) than those fed on HD.

No statistically significant differences were observed for liver weight/body weight ratio between the two experimental groups. However, statistically significant differences ($P < 0.005$) were observed for macroscopic alterations between hyperlipidemic and control animals (Table 3). Healthy animals (SD group) showed mainly level 0 (78%), while a high percentage (55%) of livers with level

Table 2. Levels of Plasma Analyzed Enzymes (Mean ± Standard Error)[1]

Group	AST (IU/l)	ALT (IU/l)	GGT (IU/l)	AP (IU/l)	LDH (IU/l)
SD	136.22 ± 41.04	2.88 ± 0.59	8.88 ± 2.96	336.33 ± 138.66	393.77 ± 138.87
HD	217.60 ± 57.20	17.10 ± 6.74	8.40 ± 3.62	335.00 ± 60.27	1018.60 ± 214.7

[1] ANOVA and Bonferroni tests did not show significant differences between both groups ($n = 10$ for each group). AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; AP, alkaline phosphatase; LDH, lactic dehydrogenase; IU, international units.

Table 3. Results of Liver Macroscopic Analysis (%)[1]

Group	Level 0	Level I	Level II	Level III
SD[a]	78	19	3	0
HD[b]	0	15	30	55

[1] Kruskal-Wallis and Mann-Whitney *U* tests showed significant differences between groups, indicated by different lowercase letters ($P < 0.05$; $n = 40$ for each group).

3 morphology was observed in hyperlipidemic animals (HD group).

Histological analysis showed that the liver samples of hyperlipidemic chickens presented numerous fat deposits, both macro- and microvesicular. However, fatty change was predominantly macrovesicular. Also, a widespread and disperse inflammatory infiltration, mainly due to the increased presence of mononuclear cells (monocytic/macrophagic and lymphocytic cells) was observed, both in liver parenchyma and the portal zone. Lipogranulomas and cell-ballooning of hepatocytes were commonly observed throughout the entire liver parenchyma. In contrast, control animals showed neither liver steatosis nor inflammation. Verhoeff Van Giesson staining showed no fibrosis either in the control or HD group.

Semiquantitative analysis of steatosis (Table 4) showed a score 0 in the control group (SD), while a statistically significant percentage (66%, $P < 0.005$)) of samples in the hyperlipidemic animals had a score of 3 (i.e., severe steatosis). Percentages of lipid deposits in lobular and centrilobular zones (see Fig. 1) for each experimental group are shown in Table 4. A significantly high level of steatosis was observed in animals fed the hyperlipidemic diet, while no fat deposits were detectable in those fed the standard diet ($P < 0.005$). No differences existed between lobular and centrilobular zones.

Significant differences were observed between normal and hyperlipidemic animals for inflammatory infiltration parameters (number of foci, appearance, density, area and maximal diameter) (Table 5). No inflammatory foci were observed in the SD group (Fig. 1).

Statistically significant differences were observed in cell-ballooning analysis between normal and hyperlipidemic animals (Table 5).

Discussion

Non-alcoholic steatohepatitis has become a focus of increasing medical attention since obesity, hyperlipidemia, and disturbances related to excessive or unbalanced food ingestion have reached epidemic proportions in the developed world (13, 14).

Avian models have contributed greatly to our understanding of vertebrate lipid metabolism. Birds generally have a very high capacity for lipid biosynthesis and, unlike other vertebrates, the liver, rather than adipose tissue, is the principal site for de novo fatty acid synthesis (15). Various aspects of lipid transport also differ between avians and mammals. For example, in birds, exogenous lipids are absorbed in the small intestine and transported as portomicrons directly via the portal system to the liver (15), whereas in mammals chylomicron particles are secreted into the central lacteal of the villus, which drains into the lymph vessels and enters the general circulation via the thoracic duct at the right atrium. Therefore, avians may represent a suitable model for the study of the development of hepatic steatosis and its pathogenesis (16). Saadoun A, Leclercq, B. In vivo lipogenesis of genetically lean and fat chickens: effects of nutritional state and dietary treatment. J Nutr 117:428–435, 1987. View Record in Scopus | Cited By in Scopus (31)

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Our results show that diet-induced hypercholesterolemia and hypertriglyceridemia are associated with severe impairment of liver histology (fat accumulation, inflammation and cell-ballooning). No fibrosis was observed by Verhoeff van Giesson histological staining. We note that a severe level of disease was developed in our experimental model, and a longer duration of the study could have led to a great number of animals failing to complete the study.The available data suggests that excess dietary cholesterol may induce hepatic alterations and that there is an association between liver abnormalities and atherosclerosis in humans (17). In fact, we have previously used the chicken model to study atherosclerosis (6, 7), and the associated liver steatosis. Similarly, Simpson and Harms (18) have described attendant aortic atherosclerosis in nonlaying hens with fatty liver syndrome. It was suggested that the endogenous hypercholesterolemia and cessation of egg production, characteristic of severe fatty liver syndrome, were due to the reabsorption of involuted egg yolks and that

Table 4. Semiquantitative and Quantitative Steatosis Analysis

	Semiquantitative analysis (%)[1]				Quantitative analysis (%)[2] (Mean ± standard error)	
Group	Level 0 < 5%	Level I 5–33%	Level II 33–66%	Level III > 66%	Lobular	Centrilobular
SD	100[a]	0[a]	0[a]	0[a]	0.0 ± 0.0[a]	0.01 ± 0.01[a]
HD	0[a]	0[b]	33[b]	67[b]	55.11 ± 1.21[b]	53.76 ± 2.10[b]

[1] Kruskal-Wallis and Mann-Whitney *U* tests.
[2] ANOVA and Bonferroni tests.
[a,b] Different lowercase letters show significant differences between groups ($P < 0.05$; $n = 100$ for each experimental group).

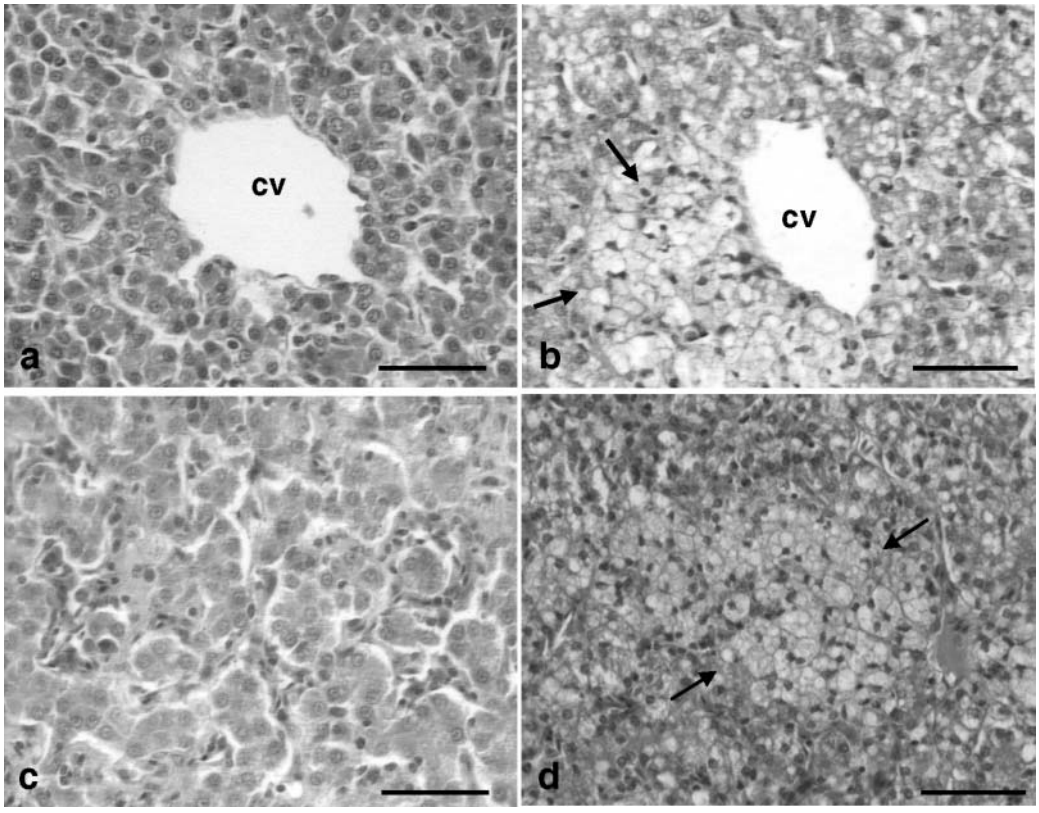

Figure 1. Representative examples of liver centrilobular zones in SD (a) and HD samples (b). Note the absence of lipid deposits in the healthy sample, whereas a big lipid deposit surrounded centrolobular vein in the hyperlipidemic sample. Liver parenchyma in SD (c) and HD (d) samples. Stained by Hematoxilyn-eosin. cv, centrilobular vein; arrows, lipid deposits; bar, 50 mm.

this in turn caused the development of aortic atherosclerosis and deposition of excess body fat (18). Therefore, the chicken may represent a suitable model in which to study both liver steatosis and atherosclerosis, and the potential overlap in their pathogenesis.

Several animal models have been proposed for the study of hepatic steatosis and steatohepatitis (4). In our model, chickens develop a reproducible steatohepatitis in 3–6 months, which is a reasonable experimental time with no need for special technical expertise and easy blood sampling procedure. This is a simple, cheap and readily available model. On the other hand, models, such as genetically modified mice, are much less readily available, and are associated with higher costs for the long-term maintenance of the colonies.

We observed an increase in all lipid parameters measured in the plasma of chickens fed a cholesterol and fat enriched diet, when compared to those of chickens fed the standard diet. A similar dyslipidemia, with high increases of tryglicerides and LDL, which is associated with NAFLD, has been described in humans (19). Ho *et al.* (20) described a hereditary hyperlipidemia in non-laying hens, which is spontaneous, and does not require an induction diet.

Chickens fed on the hyperlipidemic diet showed increased systemic inflammation demonstrated by elevated CRP levels, when compared to chickens fed the standard

Table 5. Analysis of Inflammatory Infiltration and Cell-Ballooning (Mean ± Standard Error)

Group	Number of foci/field[1]	Appearance[2]	Foci area (μm^2)[1]	Maximal diameter (μm)[1]	Lobular density (%)[1]	Portal density (%)[1]	Cell ballooning[2]
SD	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0.44 ± 0.15^a	0 ± 0^a	0 ± 0^a
HD	3.71 ± 0.16^b	2.37 ± 0.78^b	8754.42 ± 643.32^b	130.27 ± 6.57^b	7.88 ± 0.37^b	6.25 ± 1.02^b	1.85 ± 0.02^b

Appearance of inflammatory foci was classified as 1, or low density; 2, moderate; 3, high density. Cell-ballooning classification: 0, none; 1, few balloon cells; 2, many cells or prominent ballooning.
[1] Welch and Games-Howell tests.
[2] Kruskal-Wallis and Mann-Whitney *U* tests.
[a,b] Different lowercase letters show significant differences between groups ($P < 0.05$; $n = 100$ for each experimental group).

diet. In humans, CRP is considered an inflammation biomarker, and indicates cardiovascular risk.

Hepatic enzyme concentrations were not significantly increased in the HD group, as previously shown for spontaneously hypertensive and hyperlipidemic rats (21), normal mice (22) and rabbits (23), fed on hyperlipidemic diets. ALT is the most specific enzyme for hepatocellular injury, and therefore serves as a marker of liver status in population studies. Increased ALT and AST levels are associated with NAFLD (24). However, it is also common to find NAFLD patients with normal levels of aminotransferases. Only a third of the patients who presented with histologic hepatic inflammation showed increased levels of ALT (25). Furthermore, Zelberg-Sagi *et al*. used comparative liver ultrasonography to show that serum ALT was not a reliable screening tool for NAFLD in the general population (26). Other plasma biochemical parameters could have a higher sensitivity in order to assess potential hepatic disorders in the chicken, such as biliary acid determination (27).

We agree with other authors (5, 28) who found pale and swollen livers in White Leghorn hens fed a fat-enriched diet. Furthermore, we have used a scoring system (1–3) in order to evaluate hepatic macroscopic alterations in a simple and fast way. Quantitative analysis of steatosis analysis did not show the highest degree of lipid deposits, as obtained by semiquantitative analysis (level III, $> 66\%$). Therefore, it seems that the semiquantitative analysis could overestimate the presence of lipid deposits. A similar finding was observed by Franzen *et al*. (29), who considered the semiquantitative analysis to be less reproducible. Therefore, quantitative morphometric steatosis assessment by image analysis shows higher accuracy.

A significantly increased number of inflammatory foci, as well as increased density, area and maximal diameter, were observed in hyperlipidemic chickens. Hübscher (30) observed inflammatory infiltration predominantly associated to lobular location, but we did not find differences between lobular and portal density of inflammatory foci. Therefore, chickens in the HD group developed steatohepatitis and not a simple steatosis. Although the number of inflammatory foci has been included for grading and staging of the histological lesions in NAFLD (10), to our knowledge, assessment of area, density and maximal diameter by image analysis is not currently being included in histological studies of NAFLD. However, we believe that quantification of the inflammatory infiltration parameters (especially density) in combination with the number of inflammatory foci provide more detailed information.

The most important diagnostic criterion for distinguishing steatohepatitis from simple steatosis is the presence of hepatocyte ballooning (30). However, this parameter is not systematically assessed in animal experimental studies of NAFLD, perhaps because it is difficult to distinguish and subject to variations between different pathologists (31). Significant hepatocyte ballooning was observed in our study, a fact which denotes a further stage of disease progression.

In summary, chickens fed a cholesterol and fat enriched diet develop severe impairment of liver histology (fat accumulation, inflammation and hepatocyte ballooning), which reproduces histological findings of human NAFLD. The chicken experimental model offers economic and technical advantages, including ready availability, reproducibility, as well as potential reversibility, hence possible suitability for drug intervention studies in steatohepatitis. C4

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