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Metabolic characterization of two different non-alcoholic fatty liver disease pre-clinical mouse models

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ABSTRACT

Introduction: non-alcoholic fatty liver disease is one of the most prevalent liver disorders in the developed world. Currently, there is no approved pharmacological therapy except for lifestyle intervention. Therefore, there is a need to increase the knowledge of preclinical models in order to boost novel discoveries that could lead to a better therapeutic management.

Material and methods: this study characterized the effects of two different diets, a long-term high-fat high-fructose diet (HF-HFD) and a choline-deficient, methionine supplemented high-fat diet (CDA-HFD) in C57BL/6J mice for 52 weeks or 16 weeks, respectively. Body weight, lipid and hepatic profile were analyzed and liver histology was subsequently evaluated.

Results: HF-HFD animals showed an increased body weight and total cholesterol levels, whereas the opposite occurred in CDA-HFD. Both HF-HFD and CDA-HFD animals had higher ALT and AST levels. With regard to histology findings, HF-HFD and CDA-HFD diets induced an increased collagen deposit and intrahepatic steatosis accumulation.

Conclusion: in conclusion, the comparison of these models helped us to decide if it is better to select a long-term but more physiological model for physiopathology studies or either a more rapid NASH model for novel molecules testing.

Key words: NAFLD. NASH. Animal models. Liver fibrosis.

Author´s contribution: Rocío Gallego-Durán y Leticia Álvarez-Amor contributed equally to the work and share co-first authorship. Francisco Martín and Manuel Romero-Gómez contributed equally to the work and share co-senior authorship.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a pandemic disorder with multifactorial etiology (1). The current prevalence of NAFLD in the EU ranges between 23.6% in France to 29.5% in Italy. In Spain, the estimated prevalence according to population studies reaches 25.8% (2), which is expected to increase (3). This disease encompasses a broad spectrum of conditions from simple steatosis, which is initially benign, to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis that may end up in hepatocellular carcinoma. NAFLD is currently the second leading cause of liver transplantation and is expected to be the primary cause within the next decade (4). Despite its high socioeconomic impact, there is still no pharmacological strategy for NAFLD treatment.

Animal models constitute one of the most powerful tools to study this disease. Taking into account the interspecies modifications, pre-clinical models are useful to identify novel routes related to its pathophysiology. Furthermore, the effects of certain compounds or molecules with potential beneficial properties for patients suffering from NASH can also be tested (6). To date, there are different experimental conditions able to trigger NAFLD in animal models, but none of them completely replicates the human phenotype. The main disturbances include spontaneous mutations *(ob/ob,*

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db/db), genetic modifications and toxic insults or formulated diets (5). An ideal preclinical NAFLD model should ideally present metabolic complications, such as obesity, insulin resistance and adipose tissue inflammation. Another central requirement is a time-dependent NAFLD progression, including liver steatosis, ballooning degeneration and lobular inflammation, features of NASH, preferably accompanied by fibrosis. Finally, systemic and intestinal inflammation is also desirable, as dysbiosis is a common feature within NAFLD populations (7). Similarly, interventions enriched in different compounds, such as extra virgin olive oil, seem to be protective against NASH, acting via inflammatory routes in adipose tissue and liver lipid composition (1).

Methionine and choline are essential methyl group donors. Methylation plays an indispensable role in the normal functioning of hepatocytes, as the final products of methylation result in the prevention of triacylglycerol accumulation in the liver. Therefore, the methionine and choline deficient model might be representative of intrahepatic triglyceride impairment. A major advantage of this model is that these animals rapidly develop liver steatosis, inflammation and fibrosis, which is usually accompanied by weight loss, lowered blood glucose levels and insulin resistance. Furthermore, there are differences among mice strains with regard to their susceptibility to diet-induced NASH. In fact, it has been shown that C57BL/6J mice maintained or gained weight when fed with a methionine and a no-added choline high fat diet (CDA-HFD), whereas A/J mice showed a steady body weight decline and liver fibrosis was identified earlier (8).

Single high fat diets or combination interventions with variable added sugar levels have also been shown to replicate some human NAFLD phenotypes in mice, which usually become obese. These animals display patterns of liver injury closer to NASH and tend to develop significant fibrosis that can eventually promote carcinogenesis after long periods of time (9).

Considering the aforementioned difficulties in recapitulating NAFLD in animal models, we launched two preclinical models under different circumstances, in terms of timing and diet. The main aim of the study was to evaluate the effect of two different dietary regimens in the liver and metabolic features in mice, in order to facilitate decision-making for the selection of a NAFLD preclinical model. The impact of two different diets, a long term high-fat high-fructose diet (HF-HFD) *versus* an L-amino acid diet with 60 kcal% fat, 0.1% methionine and no added choline (CDA-HFD) was characterized.

MATERIALS AND METHODS

Animals

Five-week old male C57BL6J mice were purchased from Charles River (Cedex, France) and used throughout the study. Mice were bred and maintained in the central animal house in both the Andalusian Center of Molecular Biology and Regenerative Medicine (CABIMER) and the Institute of Biomedicine of Seville (IBiS), which are both specific pathogen-free facilities. All animal care and experimental protocols were performed in accordance with the guidelines for the care and use of laboratory animals of both Institutions, as well as the European Community Policy for Experimental Animal Studies (permission numbers 06-10-14-138 and 19/02/2016/023). The study was approved by the Animal Ethics Committee. Mice were raised in a temperature-controlled room of 23 °C with constant humidity and maintained on a 12/12-hour light/dark cycle. After one week of acclimatization, the mice were fed with the experimental diets. All mice were allowed *ad libitum* access to the test diets and water throughout the entire research period. Fresh water and a fixed amount of feed were provided three times per week. Body weights (BW) were recorded once per week throughout the study period using a calibrated scale, by transferring the mice to a clean empty weighing cage.

Experimental design and dietary regimen

Two different experimental conditions were analyzed, a high-fat high-fructose diet (HF-HFD) and a choline-deficient high-fat amino-acid controlled diet, with 0.1% added methionine (CDA-HFD). The diets were followed for 52 or 16 weeks, respectively. Mice from the HF-HFD group were randomly divided into two groups, a control group (n = 4) and a HF-HFD group (n = 24). For this set of animals, the control group was fed with were fed with standard chow that consisted of a low-fat control diet for Western diet: 11.9% kcal from fat, 15.9% kcal from protein and 72.2% kcal from carbohydrates (AIN-93G, Envigo). The HF-HFD group received a high-cholesterol (1%) Western diet: 38.9% kcal from fat, 15.7% kcal from protein and 45.4% kcal from carbohydrates as well as high-glucose high-fructose in drinking water (42 g/l, 55% (23.1 g/l) fructose and 45% (18.9 g/l) glucose) (Table 1).

Furthermore, mice from the CDA-HFD group were randomly divided into two groups; control (n = 6) and CDA-HFD (n = 6). The control group was fed with standard chow, which consisted of a low fat diet: 13% kcal from fat, 20% kcal from protein and 67% kcal from carbohydrate (2014 Teklad Global Rodent Maintenance Diet, Harlan, Spain); whereas the CDA-HFD group received a high fat diet, deficient in choline and supplemented with 0.1% methionine: 60 kcal% from fat, 18% kcal from protein and 21% kcal from carbohydrates (L-amino acid diet).

Plasma collection and biochemical analyses

Animals were fasted overnight prior to blood collection for glucose measurements. Blood was collected from the tail

Table 1. *Caloric information and physiological fuel values of the HF-HFD and CDA-HFD treatment groups*

	HF-HFD	CDA-HFD
Protein	15.7% kcal	18% kcal
Fat	38.9% kcal	62% kcal
Carbohydrate	45.4% kcal	21% kcal
Energy density	4.53 kcal/g	5.21 kcal/g

HF-HFD: high-fat high-fructose diet; CDA-HFD: choline-deficient, methionine supplemented high-fat diet.

vein of conscious animals. Approximately 900 µl of blood was collected into heparinized tubes by cardiac puncture for plasma isolation. The blood was immediately processed by centrifugation for 15 minutes at 2,500 g at 4 °C and the plasma was subsequently isolated, aliquoted and the plasma was immediately at -80 °C until further analyses. Blood glucose was measured using an automatic glucometer (Accu-Chek® Aviva, Roche, Indianapolis, IN, USA). Insulin was measured using the Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL) and HOMA-IR was calculated as previously described by Matthews et al. (10). Finally, triglycerides, total cholesterol levels and liver transaminases (AST and ALT) in plasma were measured using the Cobas Integra® 400 (Roche), according to the manufacturer's instruction.

Histological evaluation

Mice were sacrificed by cervical dislocation. Liver tissue samples were extracted and directly fixed with 4% PFA for six hours at 4 °C. After fixation, the samples were washed three times with PBS for ten minutes and part of the tissue kept in 70% alcohol until it was paraffin embedded.

Six µm sections of paraffin-embedded liver tissues were prepared using the Leica Monitored Microtome DM6000B (Leica microsystems, Barcelona, Spain). Liver paraffin sections were stained with Sirius Red to estimate the percentage of the surface area that stained positive for collagen. Furthermore, tissue was stained with hematoxylin and eosin (H&E) to estimate the percentage of the surface area that stained positive for steatosis following standard protocols. Fibrotic and steatotic areas were evaluated by direct pixel counting on binary images captured under 10x magnification and the average was calculated from 10 images/liver using the ImageJ program (11). To minimize the bias, the color of veins or arteries was inverted in order to prevent their quantification.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA, USA). The groups were compared using the Mann-Whitney U test when the data was not normally distributed or there was an unequal variance. Statistical analyses for group comparisons were performed using a one-way ANOVA. Post-hoc tests were performed using a Tukey test when the variables were normally distributed and had equal variances. The Kruskal-Wallis test was used when the variables did not exhibit a normal distribution or did not have an equal variance. All values are expressed as the mean ± SEM and a *p* value of ≤ 0.05 was considered as statistically significant.

RESULTS

Body weight of HF-HFD and CDA-HFD animals

Changes in body weight during both studies are shown in figure 1A and B. Body weight was significantly higher in the HF-HFD group from week 15 compared to the control group (*p < 0.005). The opposite correlation was observed in the CDA-HFD animals for this parameter, which had a significantly lower body weight compared to the control group (*p < 0.005).

Hepatic and lipid profile

ALT levels in the HF-HFD group (Fig. 2A) were clearly higher than those of their control group at week 39 (p = 0.08), which only reached statistical significance at week 52 (395.8 ± 28.03 *vs* 60.80 ± 10.31 UI/ml; *p ≤ 0.0001). Similarly, ALT levels in CDA-HFD mice (Fig. 2B) were found to be significantly higher *vs* the control group from week 3 of dietary intervention until sacrifice at week 16 (182.5 ± 26.41 *vs* 27.6 ± 2.96 UI/ml; *p ≤ 0.0001). Furthermore, AST levels in the HF-HFD group (Fig. 2C) showed a similar behavior to the one described for ALT levels, with a significant increase at week 39 that reached statistical significance at week 52 (626.6 ± 57.9 *vs* 235.1 ± 104.7 IU/l, *p < 0.005). Finally, AST increased significantly from week 6 of diet in the CDA-HFD group and remained higher than the control mice until the end of the experiment (479.5 ± 48. 5 *vs* 90.1 ± 7.1 IU/l; *p ≤ 0.0001).

Total cholesterol (Fig. 3A and B) showed the opposite behavior in the two diet groups. Mice fed on the HF-HFD had increased cholesterol levels from week 39, which was significantly higher than the controls at week 52 (350.3 ± 17.9 *vs* 121.2 ± 13.5 mg/dl, *p < 0.0001). On the other hand, the CDA-HFD diet mice had lower levels of total cholesterol

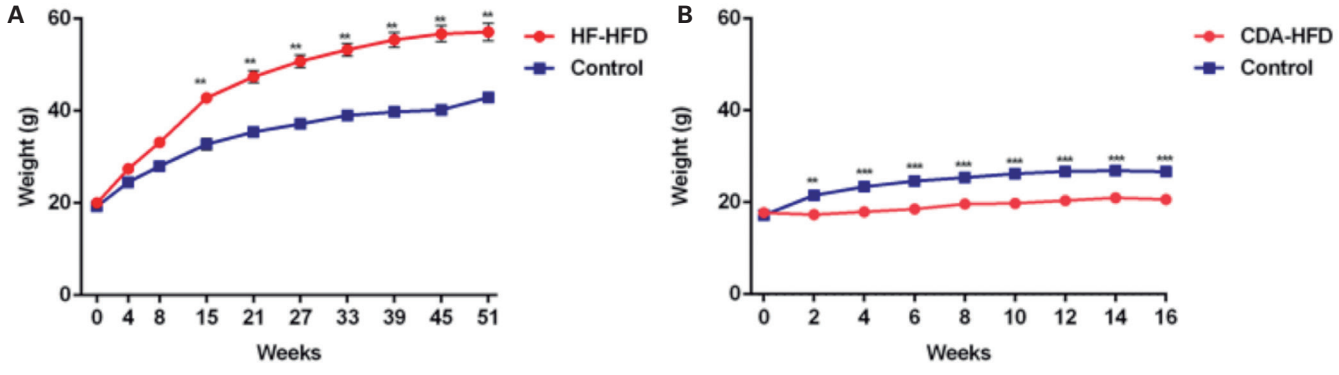

Fig. 1. *Weight over time in HF-HFD (1A) and CDA-HFD animals (1B). Data shown as mean ± SEM (**p ≤ 0.01 and ***p ≤ 0.005).*

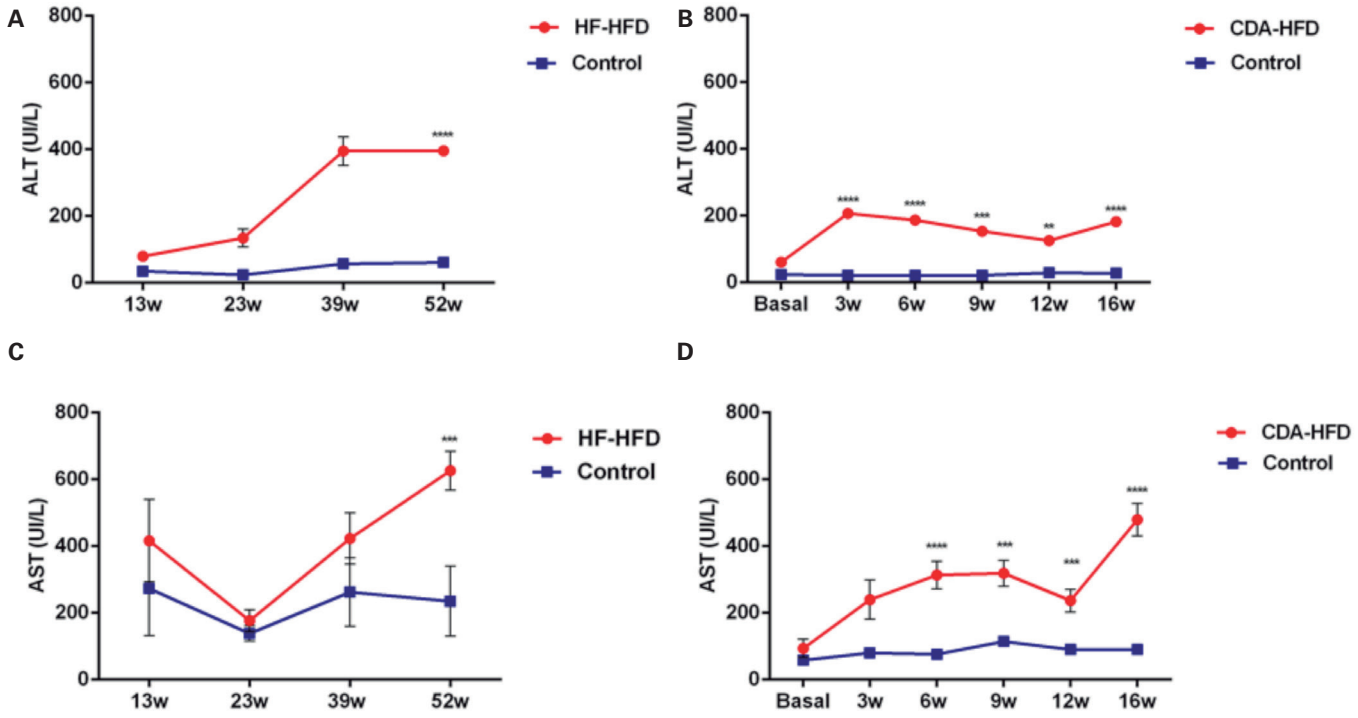

Fig. 2. *Liver enzymes panel: ALT evolution during intervention in both treatment groups (2A and 2B) and AST levels after the dietary regimen (2C and 2D). Data shown as mean ± SEM (**p ≤ 0.01; ***p ≤ 0.005 and ****p ≤ 0.0001).*

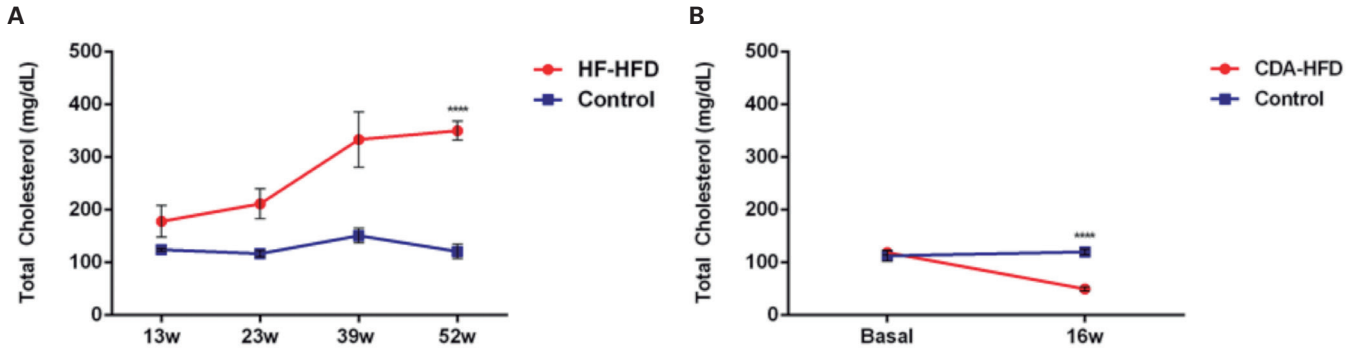

Fig. 3. *Total cholesterol levels in HF-HFD (3A) and CDA-HFD (3B) animals, pre and post interventional study. Data expressed as mean ± SEM (****p ≤ 0.0001).*

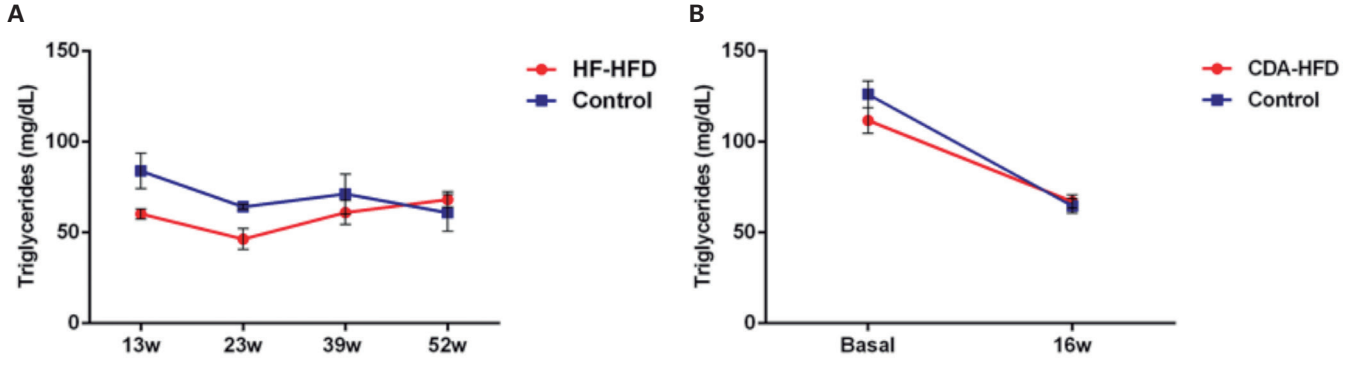

Fig. 4. *Triglycerides levels in the HF-HFD (4A) and CDA-HFD (4B) groups after dietary intervention. Data expressed as mean ± SEM.*

compared to the control group at the end of week 16 (49.7 ± 3.8 *vs* 120.2 ± 5.1 mg/dl, *p < 0.0001). Triglycerides at the end of treatment were not statistically different from control groups (p = ns) for both treatment groups (Fig. 4A and B).

Finally, HOMA-IR values (Fig. 5) were significantly increased in the HF-HFD compared to the control group at week 52 (28.37 ± 4.7 *vs* 2.26 ± 0.4; p = 0.03). Furthermore, CDA-HFD animals displayed significantly lower levels of HOMA-IR at week 16 when compared to the control group (1.84 ± 0.3 *vs* 0.92 ± 0.15; p = 0.01).

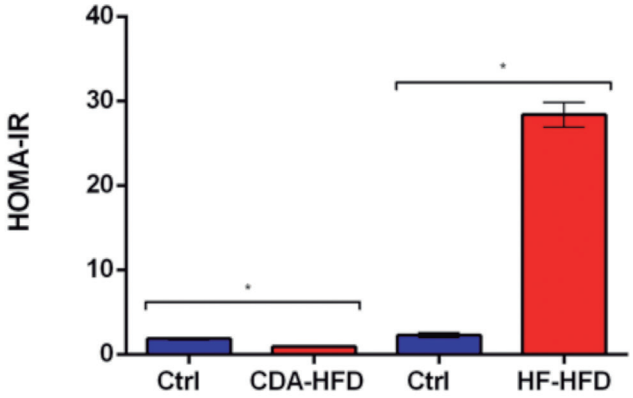

Fig. 5. *HOMA-IR in the CDA-HFD and HF-HFD compared to their control groups. Data expressed as mean ± SEM (*p ≤ 0.05).*

Histological results

Histological findings after 52 and 16 weeks of the diet are shown in figure 6. Mice that received the HF-HFD diet had a

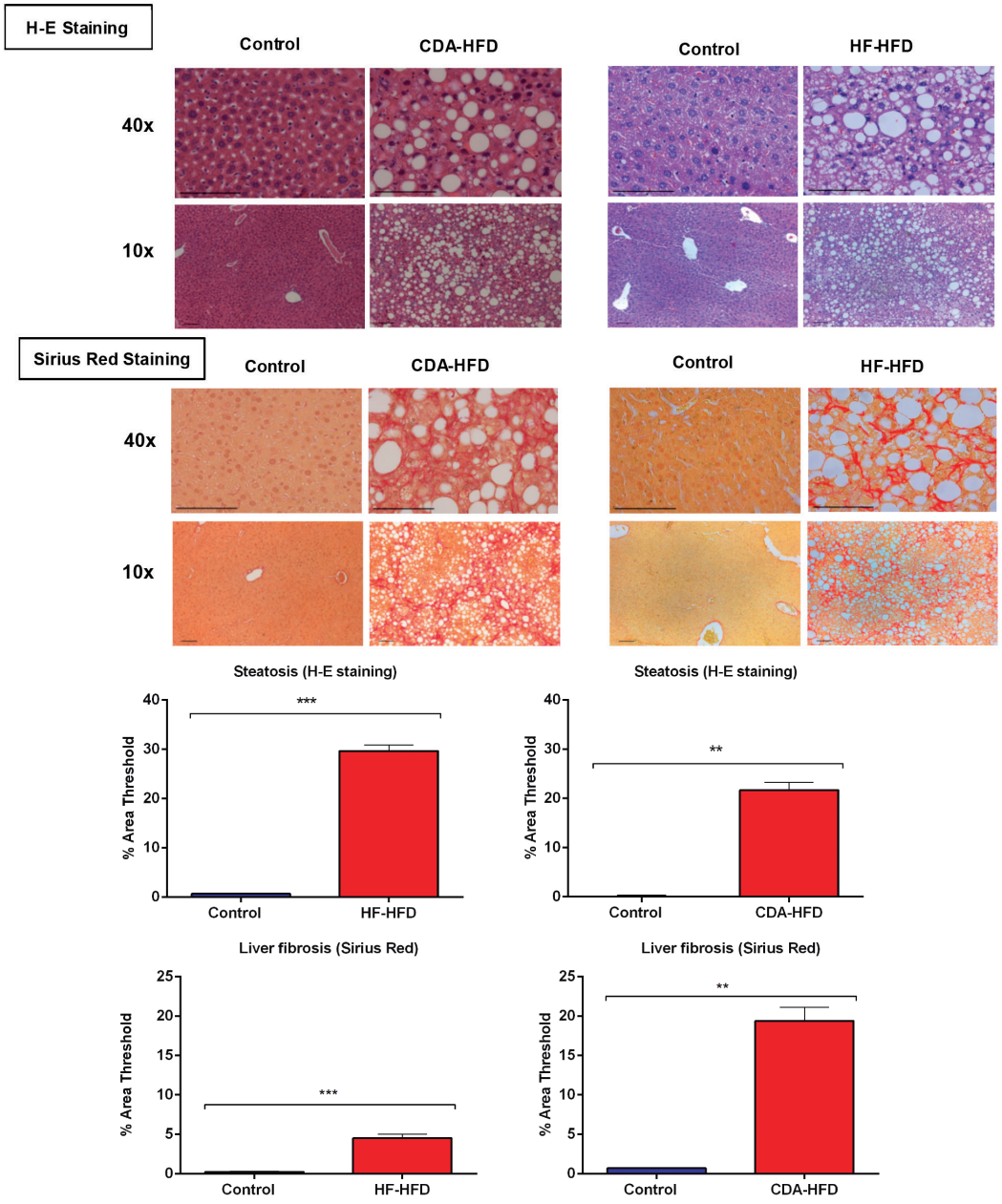

Fig. 6. *Hematoxylin and eosin (steatosis) and Sirius Red (fibrosis) staining comparing CDA-HFD after 16 weeks and HF-HFD after 52 weeks of diet. Scale bars = 100 μm.*

higher amount of collagen deposits than the control group, which was evaluated by Sirius Red stained areas (4.5 ± 0.5 *vs* 0.4 ± 0.2 area threshold) indicating the formation of fibrotic bands. Similarly, CDA-HFD animals showed a significant ($p < 0.002$) increase in the percentage of collagen deposits compared to controls after 16 weeks of diet (19.38 ± 1.76 *vs* 0.71 ± 0.05% of stained area) (Fig. 6, right panel). Hematoxylin and eosin staining showed an increase in hepatic steatosis in both groups when compared to their control groups. CDA-HFD animals showed a higher percentage of stained area than the control group (21.67 ± 3.9 *vs* 0.06 ± 0.01 % area, $p < 0.0000$), similar to that found in HF-HFD mice (29.65 ± 5.85 *vs* 0.67 ± 0.15 % area, $p = 0.0007$) (Fig. 6, left panel).

DISCUSSION

Despite advances, there is still no well-defined animal model that comprises entirely the human NAFLD phenotype and suited for the high variety of studies that this disease needs. Even though a preclinical model will obviously never be exactly the same as a patient, they are crucial to identify novel therapeutic targets and also to pave the way for further development of potential treatments. Therefore, it is essential to bear in mind the final goal of the study in order to select the most appropriate model according to several aspects as follows.

1. *Endpoint of the model:* the analysis of disease transition and natural history, phenotype reversion or the direct evaluation of the most aggressive features of the disease are not the same. Hence, hepatic characterization is crucial in order to be able to describe the model.
2. *Triggers*: the diets used should resemble human diets, ideally without unnatural toxins. This means that essential factors should be present, at least in small quantities. In this regard, the use of facilitators to speed up the process such as CCl4 injections (12) may be useful. However, these need to be carefully considered as the functional pathways of gene expression and immunity should closely resemble human disease.
3. *Reproducibility of the model*: the model should be reproducible in different centers under diverse conditions and this is one of the cornerstones when dealing with preclinical models. Robustness is also important, in terms of housing conditions, animal strains, financial aspects and the availability of the equipment, as increasing difficulty results in a higher probability of failure.
4. *Swiftness of the model*: optimization of an animal model is time consuming and requires a lot of resources. Therefore, the selected model should suit the specific endpoint.

This study compared a high fat, choline-deficient, 0.1% methionine and L-amino acid defined diet in an animal model that was maintained for 16 weeks *versus* a high-fat diet with high-fructose in drinking water for 52 weeks. Our results suggest that both models may be useful to evaluate NAFLD. This study had a variable sample size among both groups of animals and would require further characterization of liver damage, in terms of a deeper exploration of inflammation and apoptosis markers.

The CDA-HFD mice exhibited a NASH-like metabolic phenotype but did not completely recapitulate the physiology of the disease, as animals tended to lose weight, had lower cholesterol and glucose levels and did not develop insulin resistance. In fact, one of the main problems when using the methionine choline deficient (MCD) diet is the drastic weight loss than can lead to the early death of the animal. Methionine and choline are necessary for the hepatic secretion of triglycerides via very-low density lipoproteins (VLDL). Diets with a limited incorporation of both amino acids can compromise lipid export from the liver to peripheral tissues and this could explain the diminished levels of cholesterol found in the CDA-HFD model. Methionine is the precursor for choline synthesis and the controlled addition of this amino acid as a supplement to a choline deficient diet can correct the weight loss (8). Glucose homeostasis can be altered in mice treated with streptozotocin which is an anti-neoplasic agent that kills pancreatic-beta cells and is commonly used as a chemotherapy agent in pancreatic cancer and also to induce diabetes in rodent models (14). Therefore, CDA-HFD diets combined with streptozotocin could provide a way to promote NASH in a physiological context closer to the human condition. Harada et al. (15) recently showed that this combination exacerbated the liver injury in the early phase of NAFLD, which favors this idea. However, the addition of this compound needs to be carefully evaluated, because its effects might cope with other effects unrelated to the liver. The major advantage of this system probably relies on the fact that it is able to induce measurable hallmarks of the disease within a short period of time. This is highly desirable for some type of studies, especially those devoted to determine the efficacy and efficiency of novel drugs (13).

NAFLD models based on hypercaloric and/or hyperlipidic diets constitute a different approach to mimic NAFLD, especially in Western diets based on high fat and cholesterol levels. Features of NAFLD are not that marked in these animals. Thus, the addition of fructose to a high-fat diet could promote ballooning degeneration with progressive liver fibrosis (16). The pathophysiology of the disease was better reproduced in our model based on high fat high fructose diet maintained for 52 weeks, in terms of obesity and hypercholesterolemia. Therefore, it is more suitable for long-term studies to elucidate molecular events that drive the disease. The main drawback of this model is the fact that it is time-consuming and therefore more expensive as these diets are custom-made. From our experience, the HF-HFD model may satisfactorily mimic the human condition, mainly due to the fact that it is a chronic model with hypercholesterolemia, obesity and accompained by liver fibrosis.

In conclusion, the HF-HFD seems to be more physiological and therefore, may be ideal for natural history studies. The CDA-HFD may be more suitable for therapeutic approaches that target the liver and their potential side effects.

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