

Early effects of high-fat diet, extra-virgin olive oil and vitamin D in a sedentary rat model of non-alcoholic fatty liver disease

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Summary. Background and Aim. Western high-fat diet is related to metabolic syndrome and non-alcoholic fatty liver disease (NAFLD). Decreased levels of Vitamin D (VitD) and IGF-1 and their mutual relationship were also reported. We aimed to evaluate whether different dietary profiles, containing or not VitD, may exert different effects on liver tissue.

Methods. Twenty-eight male rats were fed for 10 weeks by different dietary regimens: R, regular diet; R-DS and R-DR, regular diet with respectively VitD supplementation (DS) and restriction (DR); HFB-DS and HFB-DR (41% energy from fat), high fat (butter) diet; HFEVO-DS and HFEVO-DR (41% energy from fat), high fat (Extra-virgin olive oil-EVO) diet. Severity of NAFLD was assessed by NAFLD Activity Score. Collagen type I, IL-1beta, VitD-receptor, DKK-1 and IGF1 expressions were evaluated by immunohistochemistry.

Results. All samples showed a NAS between 0 and 2 considered not diagnostic of steatohepatitis. Collagen I, although weakly expressed, was statistically greater in HFB-DS and HFB-DR groups. IL-1 was mostly expressed in rats fed with HFBs and HFEVOs and R-DR, and almost absent in R and R-DS diets. IGF-1 and DKK-1 were reduced in HFBs and HFEVOs diets and in particular in DR groups.

Conclusions. A short-term high-fat diet could

damage liver tissue in terms of inflammation and collagen I deposition, setting the basis for the subsequent steatohepatitis, still not identifiable anatomopathologically. Vitamin D restriction increases inflammation and reduces the expression of IGF-1 in the liver, worsening the fat-induced changing. EVOO seems to be protective against the collagen I production.

Key words: Non-Alcoholic Fatty Liver Disease, Western Diet, Mediterranean Diet, Histology, Immunohistochemistry.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in Western countries with prevalence of 30% (Lazo and Clark, 2008). Metabolic syndrome and insulin resistance are strictly related with the pathogenesis of NAFLD (Priore et al., 2015), involving intrahepatic triglyceride accumulation and inflammatory processes (Priore et al., 2015; Berzigotti et al., 2016). Calory restriction, in combination with physical activity, is widely suggested to decrease intrahepatic lipid content and improve NAFLD (Catalano et al., 2008; Berzigotti et al., 2016). In particular, great attention is focused on the Mediterranean Diet (MedD), which seems to help a gradual but significant improvement of the severity of NAFLD (Trovato et al., 2015; Baratta et al., 2017; Della Corte et al., 2017; Suarez et al., 2017).

Extra-virgin olive oil (EVOO) is the principal fat of

MedD. The beneficial effects of EVOO are attributed to its phenolic compounds that interact with the inflammatory cascade (Priore et al., 2015; Szychlinska et al., 2018), preventing cellular injury and oxidative stress (Musumeci et al., 2014a,b). Recent studies have demonstrated that EVOO and its phenols could regulate hepatic lipid metabolism by reducing the lipogenic pathway, and thus attenuating liver steatosis (Jurado-Ruiz et al., 2017).

Furthermore, vitamin D (VitD) is emerging as a possible player in the development and progression of NAFLD (Ju et al., 2014). VitD is metabolized in the liver and then in the kidney to the biologically active form that exerts its biological action via binding to VitD receptor (VDR) (Holick, 2007). Apart from the known physiological effect of VitD on mineral and skeletal homeostasis, over the last decades plenty of studies evaluated its pleiotropic functions (Verstuyf et al., 2010). VitD deficiency is strongly associated with features of metabolic syndrome and may play a role in modifying the cardio-metabolic risk in type 2 diabetes and cardiovascular diseases (Eliades and Spyrou, 2015; Zagami et al., 2015; Della Corte et al., 2016), moreover NASH patients are less prone to respond to VitD supplementation (Dasarathy et al., 2017). This is supported by the findings of various animal studies, showing that lack of VDR or VitD deficiency impairs insulin secretion (Zeit et al., 2003; Eliades and Spyrou, 2015).

Given the strong association of NAFLD with metabolic syndrome, there is a significant scientific interest in the potential role of VitD in NAFLD. Although VitD levels were found lower in subjects with NAFLD or NASH than in controls (Wang et al., 2015), some authors disagree (Ha et al., 2017) and limited evidence proves the effectiveness of VitD supplementation in NAFLD patients (Barchetta et al., 2016; Foroughi et al., 2016).

Our central hypothesis was that VitD depletion contributes to oxidative stress and hepatic necro-inflammation, setting the basis for NAFLD progression and fibrosis induced by high-fat diet and sedentary lifestyle. In particular we focused on the detection of early injuries. Indeed, the aim of the present study was to define whether different dietary regimens could influence liver tissue in sedentary rats. We tried to mimic dietary exposure to Western and Mediterranean diet, feeding rats respectively high-fat butter based diet (HFB) and high-fat EVOO based diet (HFEVO), combined with VitD restriction (DR) or supplementation (DS).

Materials and methods

Breeding and Housing of Animals

Twenty-eight healthy Sprague/Dawley male rats (Envigo RMS S.r.l., Udine Italy), (7-9 weeks-old; average body weight 271 ± 25 g), were housed in polycarbonate cages at standard temperature (20-23°C) and humidity for 10 weeks (experimental period), with photoperiod of 12 h light/dark and free access to water and food at the “Center for Advanced Preclinical In Vivo Research (CAPIR)” of our University. Before the start of the experiment, rats were allowed to adapt one week to their environment. Body weights, food and drink consumptions were monitored three days per week during the whole experiment. At the end of the 10 weeks, the animals were sacrificed by exposure to a chamber filled with carbon dioxide until one minute after breathing stops and then were decapitated. After sacrifice, liver tissue was used for histological analyses and immunohistochemical evaluation. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania (Protocol n. 2112015-PR of the 14.01.2015, Italian Ministry of Health). The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

Experimental design

Origin of extra-virgin olive oil

Extra-virgin olive oil (EVOO) derived from the variety of Tonda Iblea (Oleificio Guccione di Divita Vito e G. SAS, Chiaramonte Gulfi, Sicily, Italy). The composition of fatty acids in EVOO is reported in Table 1.

Diets

We sent the EVOO to Mucedola s.r.l. (Settimo Milanese- Milan - ITALY) for the preparation of the rat chow, according to the seven different diets used for the experiment. The composition of experimental diets is reported in Table 2.

We divided the 28 rats in 7 groups: R, control rats, regular diet (n=4); R-DS, regular diet with vitamin D supplementation (4000 U.I./Kg) (n=4); R-DR, regular diet with vitamin D restriction (0 U.I./Kg) (n=4); HFB-DS, high fat (butter) diet with vitamin D supplementation (4000 U.I./Kg) (n=4); HFB-DR, high

Table 1. Composition %m/m of fatty acids in EVOO.

Fatty Acids	Linoleic Acid 18:2	Linolenic Acid 18:3	Oleic Acid 18:1	Palmitic Acid 16:0	Palmitoleic Acid 16:1	Stearic Acid 18:0
EVOO	9,69	0,84	70,38	14,41	1,31	2,18

Effects of extra-virgin olive oil and vitamin D on liver

fat (butter) diet with vitamin D restriction (0 U.I./Kg) (n=4); HFEVO-DS, high fat (EVOO) diet with vitamin D supplementation (4000 U.I./Kg) (n=4); HFEVO-DR, high fat (EVOO) diet with vitamin D restriction (0 U.I./Kg) (n=4).

Histology and histopathology

Liver explanted samples were rinsed in phosphate-buffered saline (PBS, Bio-Optica, Milano, Italy), fixed in 10% buffered-formalin (Bio-Optica, Milan, Italy) for 24h at room temperature. Following an overnight wash, specimens were processed for histology as previously described (Musumeci et al., 2011, 2014a).

The sections were stained with Hematoxylin and Eosin (H&E; Bio-Optica, Milan, Italy) for general morphological structure of the tissue and for histopathologic analysis. For histopathologic evaluations we used the NAFLD Activity Score (NAS) (Kleiner et al., 2005). Liver samples were processed also for immunohistochemical analysis as previously described (Musumeci et al., 2013). After blocking, the sections were incubated overnight at 4°C with the following antibodies: rat monoclonal anti-vitamin D receptor (ab115495; Abcam, Cambridge, UK), work dilution in PBS (Bio-Optica) 10 µg/ml; rabbit monoclonal anti-collagen 1 (ab138492; Abcam, Cambridge, UK), diluted 1/1500 in PBS (Bio-Optica); rabbit polyclonal anti-IL-1 beta (ab2105; Abcam, Cambridge, UK), diluted 1/100 in PBS (Bio-Optica); goat polyclonal anti-IGF-1 (sc-7144; Santa Cruz Biotechnology, Inc.), diluted 1/100 in PBS (Bio-Optica) and rabbit polyclonal anti-DKK-1 (sc-25516; Santa Cruz Biotechnology, Inc.), diluted 1/100 in PBS (Bio-Optica). Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-goat and anti-rabbit were used as secondary antibodies) and then detected with peroxidase labeled streptavidin, both incubated for 10 min at room temperature (LSAB+ System-HRP, K0690, Dako, Denmark). The samples were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRC5, Carl Zeiss).

Computerized densitometric measurements and image analysis

The antibodies staining status was identified as either negative or positive. Immunohistochemical positive staining was defined by the presence of light (low intensity) and dark brown (high intensity) chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described (Musumeci et al., 2011). Seven fields randomly selected from each section, area of which was about 150.000 µm², were analyzed to

quantify the percentage of the total immunostained area (immunolabelling extension) and the percentage of the high and/or low immunostained area (immunolabelling intensity) using image analysis software (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Data were expressed as mean±standard deviation (SD). Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, using objective lens of magnification ×20 i.e., total magnification 200) fitted with a digital camera (AxioCam MRC5, Carl Zeiss). Evaluations were made by three blinded investigators (2 anatomical morphologists and one histologist), whose evaluations were assumed to be correct if values were not significantly different. If disputes concerning interpretation occurred, unanimous agreement was reached after sample re-evaluation.

Statistical analysis

Statistical analysis (Musumeci et al., 2015) was performed using GraphPad Instat[®] Biostatistics version 3.0 software (GraphPad Software, Inc. La Jolla, CA, USA) and IBM SPSS Statistics (version 20, IBM corporation, Somers, NY, USA). Analysis of variance (ANOVA) - Tukey's multiple comparisons test was used for comparison between more than two groups.

Table 2. Composition of experimental diets.

	REGULAR (R) 9.0% of energy from fat	HIGH-FAT BUTTER (HFB) 41% of energy from fat	HIGH-FAT EVOO (HFEVO) 41% of energy from fat
Water [%m/m]	10.69	8.54	8.50
Protein [%m/m]	22.90	21.08	21.03
Fat [%m/m]	3.54	21.05	21.16
Fiber [%m/m]	3.63	3.23	3.23
Ash [%m/m]	7.55	7.26	7.26
FNE [%m/m]	51.44	38.58	38.58
Carbohydrates [%m/m]	55.07	41.81	41.81
M.E. [kcal/kg]	2757	3801	3801
Palmitic acid 16:0 [mg/kg]	6127	46149	30470
Palmitoleic acid 16:1 [mg/kg]	308	2170	2771
Stearic acid 18:0 [mg/kg]	1336	20139	4612
Oleic acid 18:1 [mg/kg]	8638	42976	148924
Linoleic acid 18:2 [mg/kg]	17300	12845	20504
Linolenic acid 18:3 [mg/kg]	2072	1672	1777

CONTENT IN VITAMIN D	DIETS
1400 U.I./kg	R
4000 U.I./kg	R-DS; HFB-DS; HFEVO-DS
0 U.I./kg	R-DR; HFB-DR; HFEVO-DR

R, Regular Diet; R-DS, Regular diet with Vitamin D Supplementation; R-DR, Regular diet with Vitamin D Restriction; HFB-DS, High-Fat Butter based diet with Vitamin D Supplementation; HFB-DR, High-Fat Butter based diet with Vitamin D Restriction; HFEVO-DS, High-Fat EVOO based diet with Vitamin D Supplementation; HFEVO-DR, High-Fat EVOO based diet with Vitamin D Restriction

Correlations between all variables were tested by Spearman's correlation coefficient. p-value of less than 0.05 was considered statistically significant; p-value of less than 0.01 was considered highly statistically significant. Data are presented as the mean \pm SD.

Results

Body weight and food intake

A physiological body weight increase was observed in all groups, even if the differences between groups at the end of the experiment (10th week) were not significant ($p > 0.05$). Considering the weight variation, a slight trend toward greater weight gain was detected in the DS groups in comparison to the corresponding DR groups, without reaching significance. Only HFEVO-DS rats had a weight gain significantly greater than HFB-DR rats ($p < 0.05$). Also, the food consumption was not significantly different between groups ($p > 0.05$). Data not

shown.

Histology and Histopathology

The H&E staining was used for general morphological structure of the tissue and for histopathologic analysis, using NAS, in all groups. All samples showed a NAS between 0 and 2 considered not diagnostic of steatohepatitis, according to Kleiner et al (Kleiner et al., 2005). Detailed description of the histopathological results are summarized in Fig. 1 and Table 3.

Immunohistochemistry Observations

Collagen I

Collagen I-immunostaining was expressed both at cytoplasmic and nuclear levels in hepatocytes; it was barely perceptible in the cells of the bile ducts. The

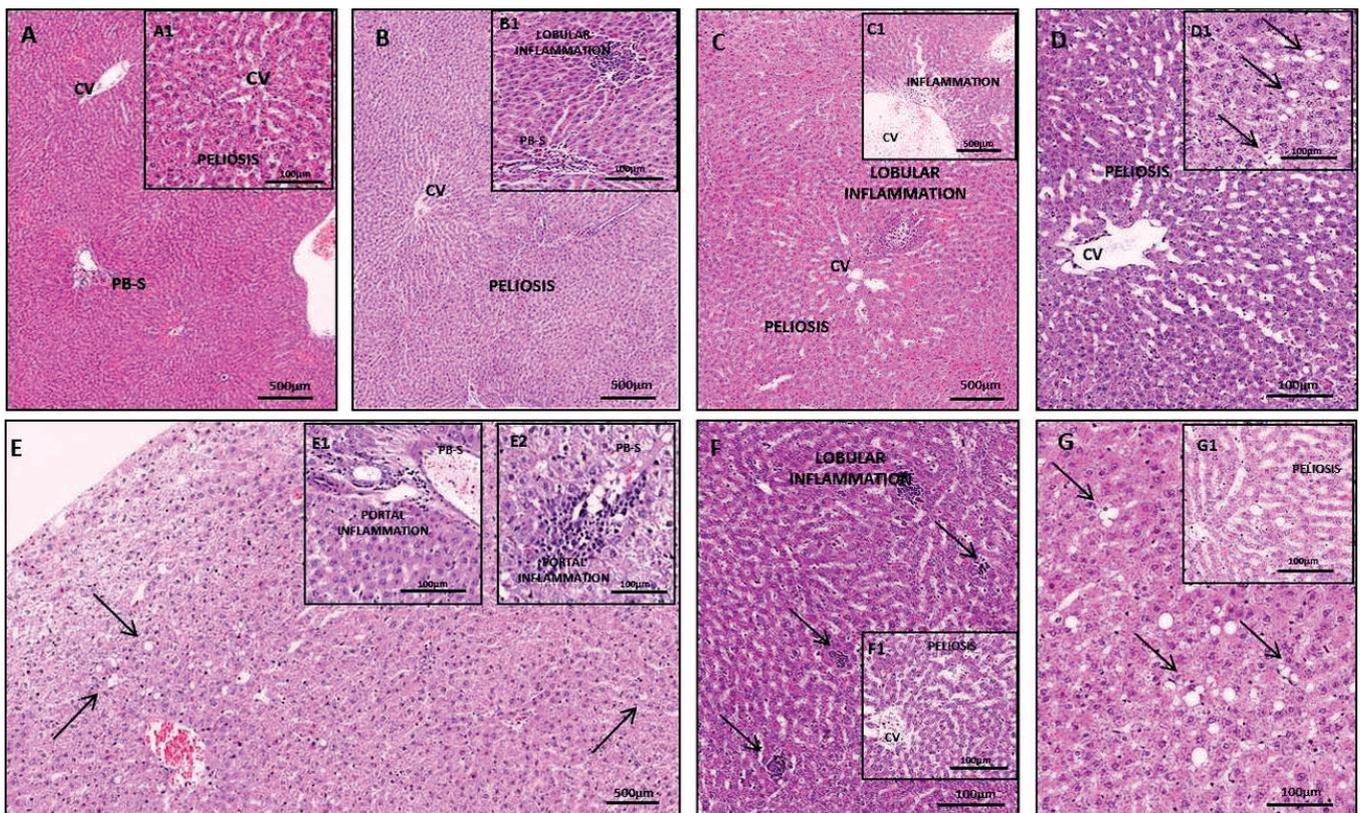


Fig. 1. Hematoxylin & Eosin staining and histopathologic analysis of groups. **A:** R group, normal structure of liver; **A1:** R group, focal and mild peliosis in normal liver. **B:** R-DS group, the liver structure is maintained with only mild peliosis hepatitis; **B1:** R-DS group, a focal lobular inflammation above of a portobiliary space (PB-S). **C:** R-DR group, histological section shows a focal and mild peliosis and a focus of lobular inflammation; **C1:** R-DR group, a central vein (CV) surrounded by inflammatory cells. **D:** HFB-DS group, histological examination exhibits focal peliosis; **D1:** HFB-DS group, single vacuoles of triglyceride fat () in liver cells (steatosis) are appreciable. **E:** HFB-DR group, focal and isolated vacuoles of triglyceride fat () in liver cells (steatosis); **E1** and **E2:** HFB-DR group, mild portal inflammation. **F:** HFEVO-DS group, multiple foci of lobular inflammation () on histological examination; **F1:** HFEVO-DS group, diffuse and prominent peliosis was evident. **G:** HFEVO-DR group, focal steatosis (); **G1:** HFEVO-DR group, focal steatosis and marked peliosis. Scale bars: A, B, C, C1, E, 500 μ m; A1, B1, D, D1, E1, E2, F, F1, G, G1, 100 μ m.

Effects of extra-virgin olive oil and vitamin D on liver

Table 3. NAFLD activity score.

Groups	Steatosis	Fibrosis	Inflammation	Livel cell injury ballooning
R	0.11±0.19	0	0	0.22±0.38
R-DS	0	0	0.88±0.52 (lobular)	0.11±0.19
R-Dr	0	0.11±0.19	1.33±0.38 (lobular)	0
HFB-DS	0.77±0.38	0	0.88±0.19 (lobular)	0
HFB-DR	0.88±0.57	1±0.38	1±0.0 (portal)	0.11±0.19
HFEVO-DS	0	0.22±0.38	1.88±0.19 (lobular)	0
HFEVO-DR	1±0.57	0	0	0

The score is defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8. Fibrosis is not included as a component of the activity score. Cases with NAS of 0 to 2 were largely considered not diagnostic of steatohepatitis, scores >5 were considered diagnostic of steatohepatitis according to Kleiner et al. (2005).

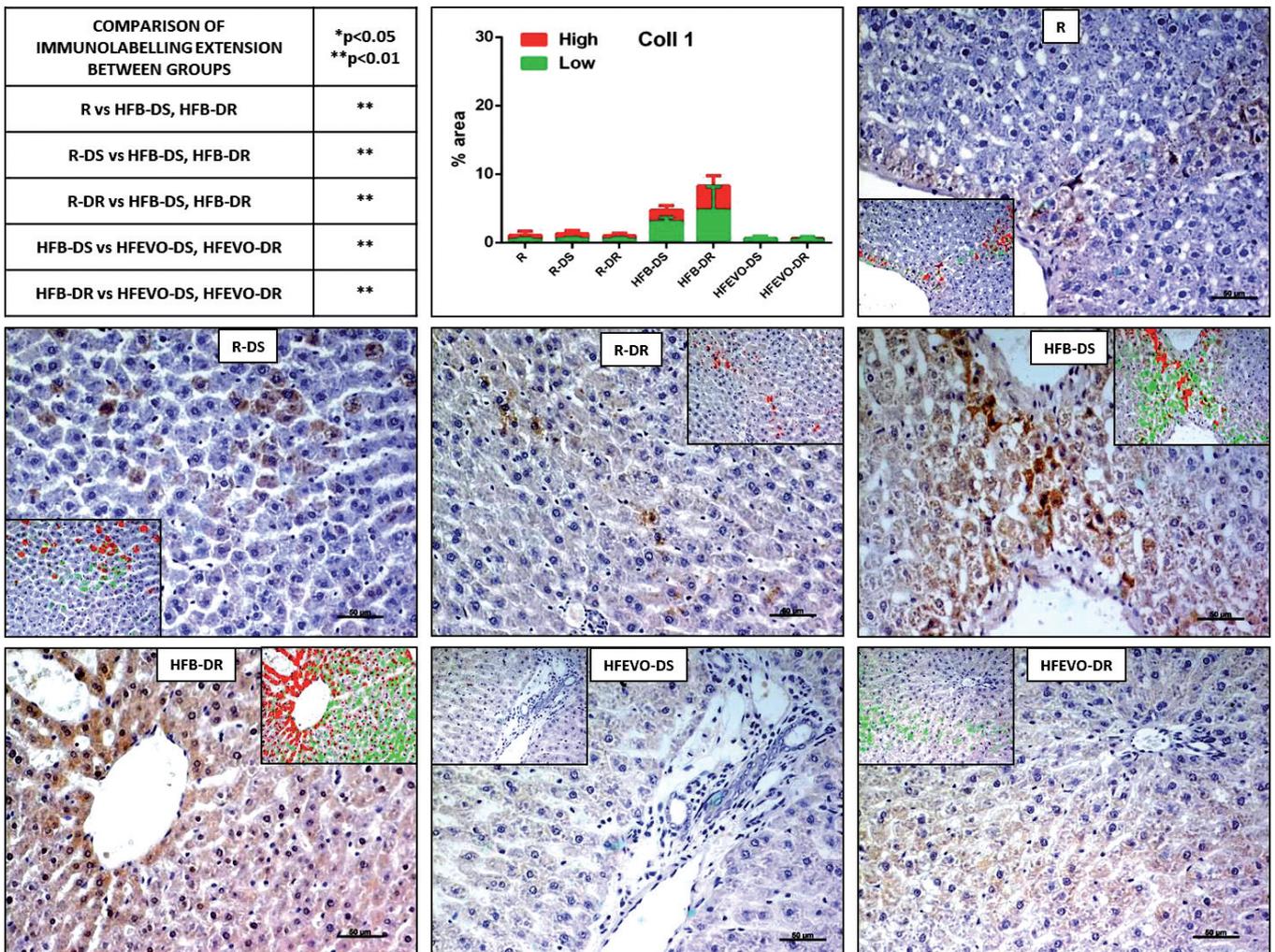


Fig. 2. Collagen 1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 µm.

immunolabelling extension (% of total immunostained area) of Collagen I was statistically greater in HFB-DS and HFB-DR groups, when compared to other groups in which it was very low. In detail: the immunostaining in R, R-DS, R-DR, HFEVO-DS and HFEVO-DR was lower than in HFB-DS, HFB-DR ($p<0.01$) (Fig. 2). The Collagen I immunolabelling intensity distribution is shown in Table 4.

Interleukin -1 beta

In hepatocytes, IL-1 beta I-immunostaining was expressed primarily at cytoplasmic level even if in some fields, it was detected also at nuclear level. Sometimes it was detected in the cells of the bile ducts and in the endothelial cells of vessels. The IL-1 beta immunolabelling extension was almost absent in R and R-DS groups, instead it was detected at different degrees in all the other groups. In detail: the immunostaining in R and R-DS was lower than in R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p<0.01$) (Fig. 3). The IL-1 beta immunolabelling intensity distribution is reported in Table 4.

VDR

VDR-immunostaining in hepatocytes was mainly cytoplasmic, although sometimes it was also in the nuclear membrane. Rarely, it was detected in the cells of the bile ducts and in the endothelial cells of large vessels. The VDR immunostaining extension was detected at different degrees in all groups, even if in

HFB-DR and HFEVO-DR it was very low. In detail: in R the immunostaining was lower than in R-DS and HFB-DS ($p<0.05$), HFEVO-DS ($p<0.01$); in R, R-DS and R-DR was greater than HFB-DR, HFEVO-DR ($p<0.01$); in R-DS the immunostaining was greater than in R-DR ($p<0.05$); in R-DR the immunostaining was lower than in HFEVO-DS ($p<0.05$); in HFB-DS and HFEVO-DS the immunostaining was higher than in HFB-DR, HFEVO-DR ($p<0.01$) (Fig. 4). The VDR immunolabelling intensity distribution is reported in Table 4.

DKK-1

In liver parenchyma, DKK-1-immunostaining was expressed mainly in the cytoplasm of cells, although sometimes it was also detected in the nuclei. In some cases, it was evidenced in the endothelial cells and in the cells of the bile ducts. The DKK-1 immunostaining extension was almost absent in HFB-DR group; in all the other groups, it was detected at different degrees. In detail: the immunostaining in R and R-DS was greater than in HFB-DS, HFB-DR, HFEVO-DR ($p<0.01$); in R-DR was greater than in HFB-DS, HFB-DR ($p<0.01$) and HFEVO-DR ($p<0.05$); in HFB-DR the immunostaining was lower than in HFB-DS, HFEVO-DR ($p<0.01$) and HFEVO-DS ($p<0.05$) (Fig. 5). The DKK-1 immunolabelling intensity distribution is reported in Table 4.

Insulin-like growth factor (IGF)-1

IGF-1-immunostaining in hepatocytes was mainly

Table 4. Evaluation of COLL I, IL-1 beta, VDR, DKK-1, IGF-1 intensity of immunostaining, expressed as % area of high/low immunostaining.

Groups	COLL 1	IL-1 beta	VDR	DKK-1	IGF-1
R	0.682±0.56 0.411±0.20	0.009±0.002 0.040±0.006	4.784±0.53 3.543±0.25	2.784±1.94 12.533±3.84	5.817±2.71 10.251±3.97
R-DS	0.791±0.46 0.511±0.20	0.009±0.004 0.069±0.003	8.789±0.56 9.061±3.23	3.592±2.09 14.005±3.39	11.265±2.60 7.108±0.77
R-DR	0.541±0.37 0.478±0.24	2.622±0.11 11.34±0.13	3.336±0.47 4.288±0.64	1.906±1.58 9.582±2.89	2.145±1.73 8.418±3.16
HFB-DS	1.703±0.68 3.042±0.63	6.180±0.64 11.753±1.26	6.348±4.38 7.117±4.09	0.485±0.24 3.466±0.87	1.601±0.52 8.968±0.31
HFB-DR	3.568±1.48 4.706±3.45	5.911±2.30 13.569±3.14	0.352±0.37 1.410±1.20	0.068±0.03 0.309±0.26	0.070±0.03 0.314±0.30
HFEVO-DS	0.049±0.04 0.578±0.35	5.575±3.36 7.924±4.48	5.370±2.76 6.987±1.55	1.837±1.51 12.838±13.12	1.856±1.50 4.374±1.77
HFEVO-DR	0.201±0.06 0.415±0.47	10.953±3.65 7.472±3.57	0.019±0.007 1.323±0.27	0.363±0.37 3.344±3.61	0.063±0.03 0.555±0.24

Blue lines: High immunostaining; white lines: Low immunostaining. The Collagen 1 immunolabelling intensity distribution was similar in almost all groups; in HFB-DS, HFB-DR and HFEVO-DS the low immunostaining prevailed over the high immunostaining. In R-DR, HFB-DS and HFB-DR groups, the low IL-1 immunostaining prevailed over the high immunostaining, instead in HFEVO-DR the high immunostaining prevailed over the low immunostaining. High and low VDR immunostaining distribution was similar in most groups, except for HFEVO-DR group in which the high immunostaining was only in traces. In all groups, the DKK-1 low immunostaining prevailed over the high immunostaining. The IGF-1 low immunostaining was prevalent in all groups with the exception of R-DS group in which the high immunostaining prevailed over the low one.

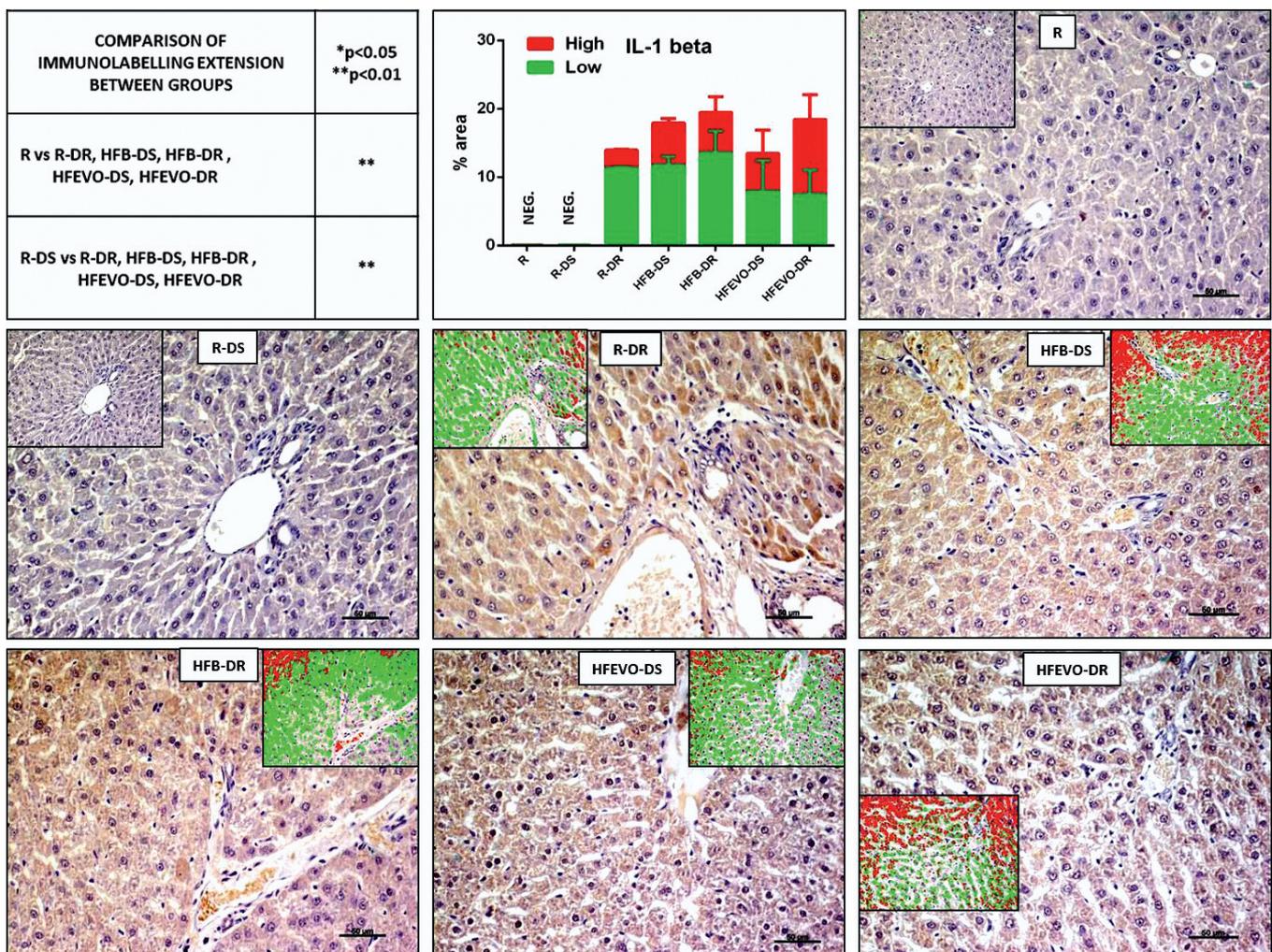
Effects of extra-virgin olive oil and vitamin D on liver

cytoplasmic, sometimes it was also nuclear. Rarely, it was detected in the cells of the bile ducts. The IFG-1-immunostaining extension was detected at different degrees in all groups, although in HFB-DR and HFEVO-DR only traces of immunostaining were present. In detail: the immunostaining in R and R-DS was greater than in R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in R-DR was greater than in HFB-DR, HFEVO-DR ($p < 0.01$); in HFB-DS and HFEVO-DS was greater than in HFB-DR, HFEVO-DR ($p < 0.01$); in HFB-DS was greater than in HFEVO-DS ($p < 0.05$) (Fig. 6). The IGF-1 immunolabelling intensity distribution is reported in Table 4.

Correlations

Weight at 10th week was directly related with the

VitD content in diet ($r_s: 0.340$; $p < 0.01$). Moreover, VitD content was directly related with IGF-1 ($r_s: 0.539$; $p < 0.01$) and DKK-1 ($r_s: 0.335$; $p < 0.05$) expression, and negatively with IL-1 beta ($r_s: -0.317$; $p < 0.05$). Conversely, the respective energy from fat, in each diet, was also directly related to weight at 10th week ($r_s: 0.284$; $p < 0.05$), but negatively related to IGF-1 ($r_s: -0.691$; $p < 0.01$) and DKK-1 ($r_s: -0.515$; $p < 0.01$) expressions, while the IL-1 beta expression increased linearly with the fat content ($r_s: 0.586$; $p < 0.01$). IGF-1 expression was directly related to DKK-1 ($r_s: 0.801$; $p < 0.01$) and VDR ($r_s: 0.514$; $p < 0.01$) and negatively with IL-1 beta ($r_s: -0.632$; $p < 0.01$). DKK-1 was inversely correlated with IL-1 beta expression ($r_s: -0.546$; $p < 0.01$). VDR expression was directly related with VitD content in diet ($r_s: 0.660$; $p < 0.01$).



Discussion

Obesity, sedentary lifestyle and Western diet are some of the major concerns of this century (Trovato et al., 2016). In Western countries, the prevalence of NAFLD in the general population is estimated to be 20%-30%, increasing to 57.5%-74% in obese population (Hong et al., 2014). The prevalence of VitD deficiency in patients with liver diseases is almost universal, despite the etiology (Arteh et al., 2010; Malham et al., 2011).

In our study, a physiological increase in body weight of all groups was observed without differences between groups at the end of the experiment, probably because the duration of dietary regimens was not enough to determine obesity. Only a slight, but not significant, trend toward greater weight gain was detected in the DS groups in comparison to the corresponding DR groups.

This could be explained by the trophic effect of VitD that has been shown on muscle (Trovato et al., 2018). It was interesting to notice how, although anatomopathological analysis showed only faint sign of liver fibrosis in HFB-DR, collagen I was overexpressed in both HFB groups. Moreover, in both HFEVO groups it was underexpressed, similarly to control. This is noteworthy, as the expression of collagen I could be an early sign of future fibrosis development, and EVOO seems to be protective. Conversely, VitD has been shown to have anti-inflammatory and antifibrotic properties and to inhibit the proliferation of hepatic stellate cells (HSC), which are responsible for collagen deposition and extracellular matrix remodeling (Firrincieli et al., 2014; Abramovitch et al., 2015; Jurado-Ruiz et al., 2017). However, in our early model, no difference between DS and DR groups were

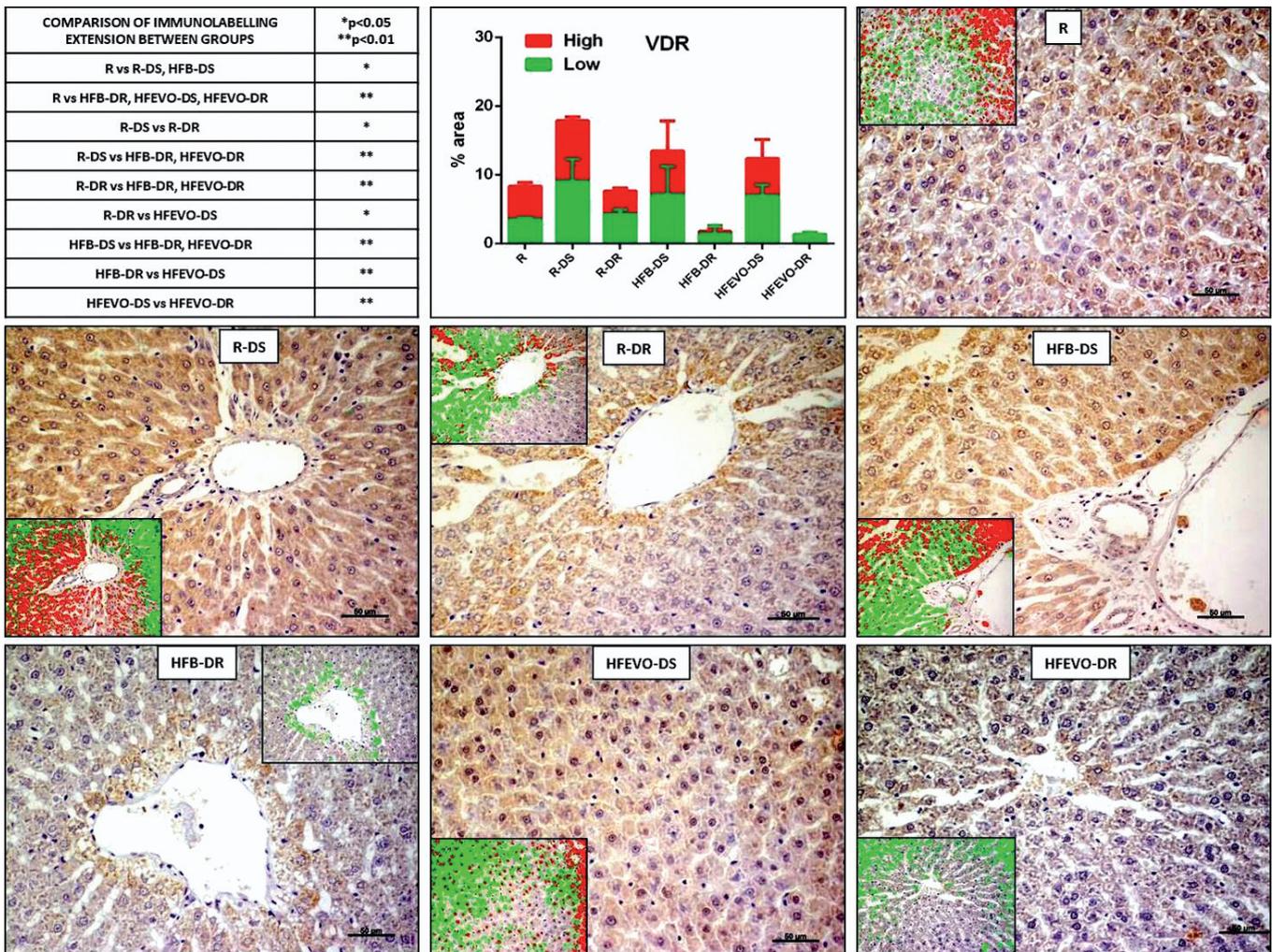


Fig. 4. VDR-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 μm.

Effects of extra-virgin olive oil and vitamin D on liver

detectable in terms of collagen I deposition, since the effect on collagen I expression seems only ascribable to the EVOO content. This is also confirmed by the fact that collagen I expression is independent of that of VDR.

Otherwise, EVOO did not show the same beneficial effect on inflammation, indeed both HFB and HFEVO were strongly related to IL-1 expression, without any difference. One explanation could be that high-fat diet determines the accumulation of visceral fat leading to increased secretion of proinflammatory cytokines that may promote the development of NAFLD (Lim et al., 2015). It is also notable that the restriction of VitD determined a similar effect in rats fed with R diet. In fact, recent studies showed how VitD interferes with the increase in pro-inflammatory cytokine expressions in muscle following intensive physical exercise (Choi et al., 2013). Moreover, according to previous studies on

muscle, a similar effect was expected also in HFEVO groups. Therefore, EVOO improved diet-induced steatosis also in other models (Musumeci et al., 2014b; Jurado-Ruiz et al., 2017). Probably our finding could be attributed to the high-fat content (41% of energy intake) provided by EVOO, which could blunt the beneficial properties of the phenolic compounds and monounsaturated fatty acids (MUFA), such oleic acid, which have been described with a strong anti-inflammatory action (Calder, 2006; Carrillo et al., 2012). Moreover EVOO has a reduced concentration of polyunsaturated fatty acids (PUFA) in comparison to the regular diet content, and this could be a further explanation of the reduced anti-inflammatory action in HFEVO groups in comparison to R (Calder, 2006). Indeed, HFEVO-DR together with HFB-DS and HFB-DR groups showed initial signs of steatosis and this is in

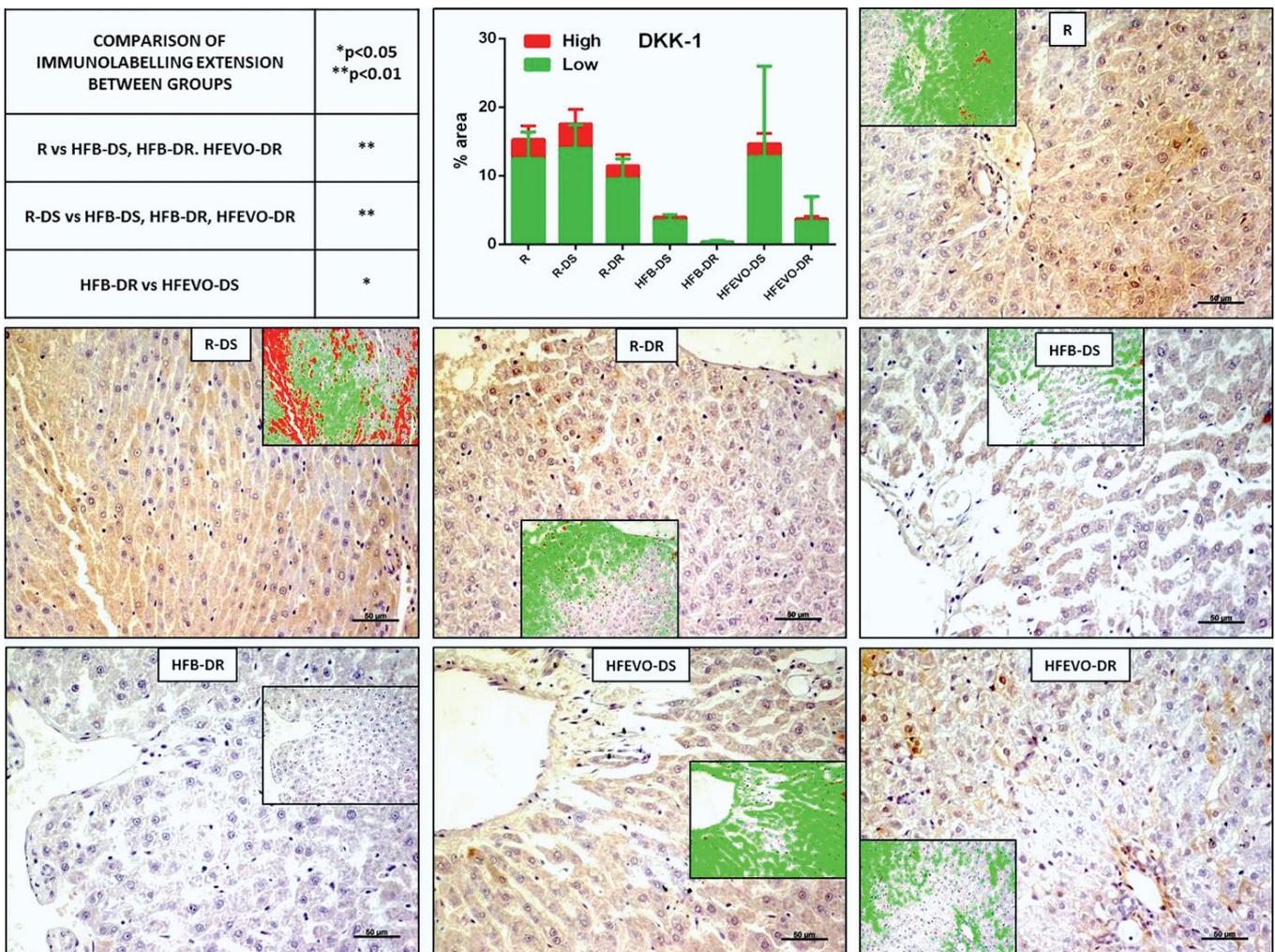


Fig. 5. DKK-1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 μm.

line with the IGF-1 expression that was strongly reduced in HFB and HFEVO compared to control. This difference was even more pronounced considering the DR groups, in which only traces of immunostaining were present.

In fact, Growth hormone (GH) and IGF-1 exert significant effects on body composition, and lipid and glucose metabolism. Several studies reported a reduced GH secretion in obese subjects, and GH and IGF-1 seem to be involved in fatty infiltration of the liver (Poggiogalle et al., 2016). IGF-1 induces insulin sensitivity and has been shown to have antifibrotic properties in rodent models of NASH (Dichtel et al., 2017). Moreover, VDR expression shows correlation to that of IGF-1. We can hypothesize that VitD restriction and high-fat diets, in general, are detrimental for IGF-1 expression with consequences for liver steatosis. The

liver is the major source of circulating IGF-1 and is also considered a target tissue for VitD (Ameri et al., 2013a). A small recent study demonstrated that concentrations of IGF-1 were significantly higher in patients treated with VitD supplementation (Ameri et al., 2013b). Conversely, data from several studies indicate that IGF-1 causes an increase in the circulating levels of 1,25-dihydroxyvitamin D by stimulating the expression and activity of the 1 α -hydroxylase in the kidney (Ameri et al., 2013b), whereas there seems to be no effect of IGF-1 on 25-hydroxylation (Nesbitt and Drezner, 1993).

Another pathway we decided to explore, in order to explain how VitD could interfere with liver steatosis, is Wnt/ β -catenin. In fact, the Wnt coreceptor LRP5 is a target for VitD, and plays a key role in osteoblast proliferation and differentiation (Sankaralingam et al., 2014). In effect, in some neoplasms, VitD increased

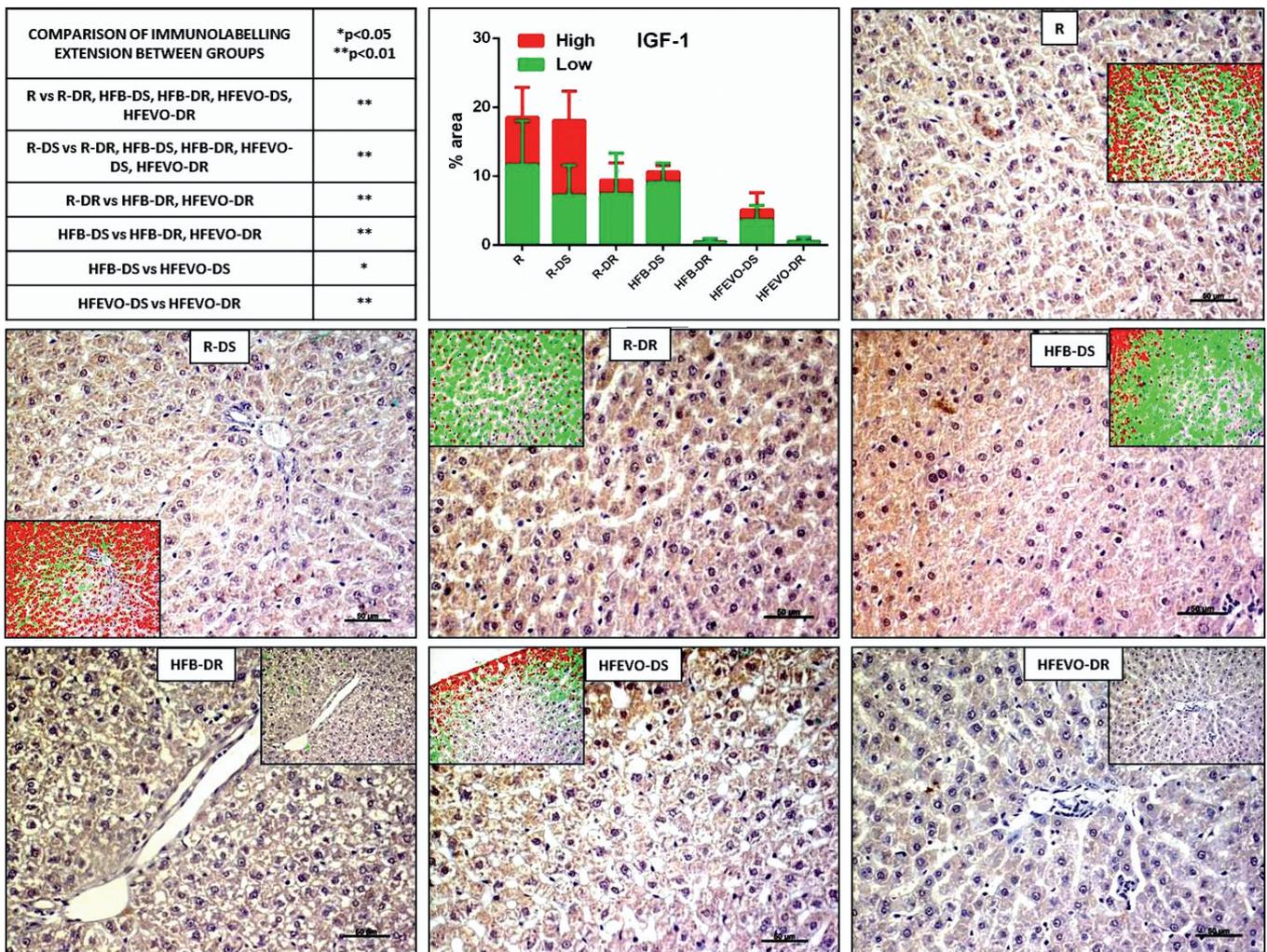


Fig. 6. IGF-1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean \pm SD. Scale bars: 50 μ m.

dose-dependently the expression of DKK-1, the extracellular canonical Wnt inhibitor, which is associated with growth inhibition, showing a protective role of VitD against cancer development, progression, and metastasis (Rawson et al., 2012; Johnson et al., 2015). Moreover, IGF-1 seems to antagonize Wnt pathways in studies on tumorigenesis, while there are limited data on associations between the GH/IGF system and Wnt signaling in metabolic disorders (Jin et al., 2008; Schlupf and Steinbeisser, 2014).

Knowing that, we hypothesized that VitD could exert its action on liver through the expression of DKK-1. Indeed, Wnt/ β -catenin signaling promotes fibrosis in response to injury in different tissues, including liver, and is crucial for the differentiation of fibroblasts and collagen production (Ozhan and Weidinger, 2015). Overexpression of DKK-1 prevented fibrosis in inflammation-driven models and increases apoptosis of cultured HSC (Cheng et al., 2008), indicating that the inhibition of the canonical Wnt pathway might be effective in fibrotic disease (Akhmetshina et al., 2012). Our results, although at an early stage, confirm the negative correlation between DKK-1 and collagen I expression in liver tissue. Moreover, both IGF-1 and DKK-1 expressions reduced as the percentage of dietary fat increased. Indeed, the expression of DKK-1 was greater in R, R-DS and HFEVO-DS groups with a dramatic reduction in the respective DR and in HFB groups, confirming that VitD administration has a role in the expression of DKK-1. This finding is opposite to collagen I expression that is more pronounced in HFB groups, the HFEVO groups being comparable with the control. The role of DKK-1 in liver diseases is debated, since it was proposed by some authors as a noninvasive marker of NAFLD and the DKK-1 gene is found overexpressed in HCC patients (Polyzos et al., 2016; Watany et al., 2017). Other authors, instead, showed that DKK-1 serum levels were not different between NASH-HCC, cirrhotic, chronic hepatitis and healthy control patients (Vongsuvanhan et al., 2016). Unfortunately, this is a morphological study, so our conclusions are limited by the lack of serum dosages. Moreover, the complex cross-link between VitD, IGF-1 and DKK-1 must be elucidated with further study, since the small sample size is a major limitation of this research, but our results could give a clue for the basis of the pathogenesis of diet-induced NAFLD.

Conclusion

A short-term high-fat diet could damage liver tissue in terms of inflammation and collagen I deposition, setting the basis for the subsequent steatohepatitis, still not identifiable anatomopathologically. Vitamin D restriction increases inflammation and reduces the expression of IGF-1 in the liver, worsening the fat-induced changing. EVOO seems protective against collagen I production. The complex mechanism linking IGF-1, DKK-1 and VitD must be further elucidated.

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