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Impact of Vitamin D intake and high-fat diets on liver and muscle: a rat model of Western and Mediterranean Diets

Final dissertation

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Abstract:

Background: The metabolic syndrome is associated with sarcopenia. Decreased serum levels of Vitamin D (VitD) and insulin-like growth factor (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 muscle and liver.

Methods: Twenty-eight male rats were fed for 10 weeks in order to detect early defects induced by different dietary regimens: regular diet (R); regular diet with vitamin D supplementation (R-DS) and regular diet with vitamin D restriction (R-DR); high-fat butter-based diets (HFB-DS and HFB-DR) with 41% energy from fat; high-fat extra-virgin olive oil-based diets (HFEVO-DS and HFEVO-DR) with 41% energy from fat. IL-1β, insulin-like growth factor (IGF)1, Dickkopf-1 (DKK-1), and VitD-receptor (VDR) expressions were evaluated by immunohistochemistry in both muscle and liver, Collagen type I expression was evaluated in the liver. Muscle fiber perimeter was measured by histology and morphometric analysis. Severity of NAFLD was assessed by NAFLD Activity Score (NAS).

Results: Muscle fibers of HEVO-DS rats were hypertrophic, comparable to those of the R-DS rats. An inverse correlation existed between the dietary fat content and the perimeter of the muscle fibers ($p < 0.01$). In HFB-DR rats, muscle fibers appeared hypotrophic with an increase of IL-1β and a dramatic decrease of IGF-1 expression. All liver 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 by 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: High-fat western diet could impair muscle and damage liver tissue in terms of inflammation and collagen I deposition, putting the basis for the

subsequent steatohepatitis, still not identifiable histopathologically. VitD associated with a Mediterranean diet showed trophic action on the muscle fibers. Vitamin D restriction increases inflammation and reduces the expression of IGF-1 in the liver, worsening the fat-induced changing. EVO seems be protective against liver collagen I production.

Keywords: Mediterranean diet; high-fat diet; skeletal muscle; vitamin D; extra-virgin olive oil

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1. Overview

We are witnessing a great increase in obesity and metabolic syndrome which are quickly reaching a global epidemic scale, affecting over 500 million people worldwide (Collaboration, 2016). This is critically associated with the development of cardio-metabolic disorders such as type-2 diabetes (T2DM) and non-alcoholic fatty liver disease (NAFLD) (Akhmedov & Berdeaux, 2013). NAFLD has become the most common chronic liver disease in Western countries with a prevalence of 30% (Lazo & 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).

Although undernutrition, impaired protein synthesis, and increased protein breakdown could lead to the progressive loss of muscle mass (sarcopenia), an important issue in the Western world is its association with obesity. This condition is called sarcopenic obesity and is characterized by high adiposity and low lean body mass (Poggiogalle *et al.*, 2016).

Dietary interventions, together with other important lifestyle changes, are widely suggested to achieve weight loss, decrease intrahepatic lipid content and modifying

the natural history of NAFLD (Catalano *et al.*, 2008, Berzigotti *et al.*, 2016, Trovato *et al.*, 2016, Sandouk & Lansang, 2017). In particular, great attention is focused on Mediterranean Diet (MedD), that 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 (EVO) is the principal fat and one of the cornerstones of the Mediterranean diet. The Mediterranean diet embodies the basics of healthy eating, coming from the traditional cooking style of countries bordering the Mediterranean Sea, including fruits, vegetables, fish, whole grains, red wine, and limited unhealthy fats (Trovato *et al.*, 2015). Research has shown that the traditional Mediterranean diet reduces the risk of different chronic diseases (Trovato *et al.*, 2016).

Vitamin D (VitD) deficiency is strictly related to sarcopenia, disability, and falls in the elderly (Ceglia, 2009, Kim *et al.*, 2011). In obesity, the increase of circulating fatty acids leads to ectopic lipid deposition in other tissues, including skeletal muscle, which represents the largest metabolically active tissue in the body (40% of body mass) (Turpin *et al.*, 2009). Several authors reported that VitD deficiency is strongly associated with features of the metabolic syndrome and may play an important role in modifying the cardio-metabolic risk (Zagami *et al.*, 2015, Lim *et al.*, 2017, Randhawa *et al.*, 2017).

Our central hypothesis is that the relationship between muscle, adipose tissue and liver begins earlier than obesity and metabolic syndrome develop.

The aim of the present study was to define whether different short-term dietary regimens could influence both skeletal muscle and liver in sedentary rats.

We tried to mimic short-term dietary exposure to Western and Mediterranean diet by feeding rats with, respectively, a high-fat butter-based diet (HFB) and a high-fat extra-virgin olive oil-based diet (HFEVO), combined with VitD restriction (DR) or supplementation (DS).

We hypothesized that high-fat butter-based diet could be detrimental for muscle and liver health, also when consumed for a short period of time, whereas even the same fat intake (41% of energy) could have beneficial effects when replacing the butter with EVO. The design included also the supplementation of VitD since the central hypothesis was that VitD depletion contributes to oxidative stress and hepatic necroinflammation, putting the basis for NAFLD progression and fibrosis induced by high-fat diet and sedentary lifestyle.

1.1 The relationship between liver and muscle

It is largely known that patients affected by liver cirrhosis suffer also from muscle wasting. But at which point of the disease progression, the relationship between liver and muscle starts, is not so clear.

Nonalcoholic fatty liver disease is one of the most common forms of chronic liver disease in developed countries and is characterized by an accumulation of fat in the liver. NAFLD is a global public health problem affecting approximately 20%-35% of adults in the general population in western countries and approximately 15% in Asian countries (Cimini *et al.*, 2017). This prevalence dramatically rises in obese populations to 74-91%, to 60% in diabetic individuals and to 90% in people affected by hyperlipidemia (Cimini *et al.*, 2017). In individuals who are of normal weight and who have no metabolic risk factors, the prevalence of NAFLD is about 16%. Moreover the number of patients affected is growing rapidly, and the disease has now reached epidemic proportions. Currently, nonalcoholic steatohepatitis (NASH) is the most rapidly growing indication for liver transplantation in the United States. Despite resurgence in alcoholic liver disease, NASH remains the second leading indication for liver transplantation (Cholankeril *et al.*, 2017).

NAFLD is characterized by lipid content of >5–10% of the weight of the liver, in the absence of excessive consumption of alcohol (< 30g/day for men and < 20g/day for

women) or other liver diseases. Histologically, NAFLD encompasses a spectrum of conditions characterized by simple steatosis to a more severe condition characterized by inflammation, NASH, which may in turn progresses to cirrhosis, hepatocarcinoma, and liver failure (Priore *et al.*, 2015). Authors tried to explain the pathophysiology of NAFLD with the two "hits" hypothesis in which the first hit consists of an intrahepatic triglyceride accumulation (due to either increased synthesis, decreased export, or both). Accumulation of lipids can exert toxic effects on the liver by inefficient oxidation or by activation of inflammatory pathway leading to the following "hit" that involves oxidative stress, cytochrome P450 activation, lipid peroxidation, mitochondrial dysfunction, cytokine production, and apoptosis (Adams *et al.*, 2005).

As mentioned above the origin of hepatic fat is various, the vast majority (59%) originates from increased lipolysis from the fat cells, the 26% comes from *de novo* lipogenesis in the liver and 15% originates from diet (Cimini *et al.*, 2017). Finally, reduction in lipid disposal through an impairment of fatty acid β-oxidation and triglyceride-rich lipoprotein secretion (very low density lipoprotein, VLDL) can also exacerbate this condition (Priore *et al.*, 2015). Hepatic steatosis results when the balance between delivery and synthesis of free fatty acids exceeds the liver capacity to oxidize or export them. Moreover conditions such as hyperglycemia and hyperinsulinemia seem to be associated with the high rate of lipogenesis, resulting in a shift of cellular metabolism from fatty acid β-oxidation to triglyceride esterification, and hence increasing hepatic fat accumulation (Adams *et al.*, 2005). Obesity is a major risk factor for the development of NAFLD, even if not all patients with obesity develop NAFLD (Cimini *et al.*, 2017). One reason for this incomplete overlap between obesity and NAFLD could be ascribed to the use of Body Mass Index (BMI) to define obesity. Indeed, although BMI cut-off points have good specificity for detecting excess adiposity, they lack sensitivity and also fail to provide information about the distribution, type and quality of body fat (Okorodudu *et al.*, 2010).

It is well known that, adipose tissue is an endocrine organ, that synthesizes and releases several hormones, cytokines, growth factors and vasoactive agents, collectively known as adipokines. Indeed, adipose tissue biology is much more complex than previously considered and visceral adipose tissue (VAT) dysfunction has been proposed as a major contributor to NAFLD (Cimini *et al.*, 2017) Adipocytes hypertrophy leads to increased adipokine and pro-inflammatory cytokines production and subsequently to hypoxia and adipocyte cell death (Cimini *et al.*, 2017). Moreover, different extracellular matrix (ECM) components, such as collagen VI, are expressed in dysfunctional VAT leading to progressive fibrosis, that limits the amount of fat stored in the adipocytes, thus promoting the deposition of ectopic fat in liver and muscle. (Szendroedi & Roden, 2009).

In normal conditon (i.e lean individuals) VAT homeostasis is maintained by adiponectin released by adipocytes and by M2 macrophages through the secretion of anti-inflammatory cytokines, such as interleukin (IL)-10 and arginase-1. In individuals affected by obesity (illustration 1), VAT undergoes excessive fibrosis and accumulation of inflammatory cells. Active macrophages surround dying adipocytes in typical "crown-like structures". Pro-M1 polarized macrophages secrete pro-inflammatory cytokines including TNFα, IL-1β and IL-6, which can promote chronic local and systemic inflammation (Cimini *et al.*, 2017).

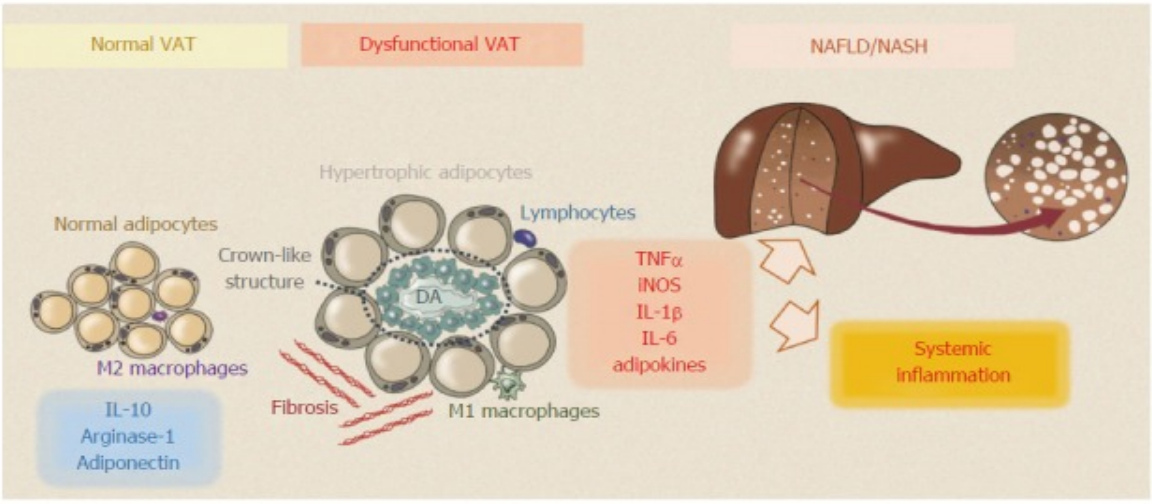


Illustration 1. The role of adipose tissue in NAFLD (Cimini *et al.*, 2017)

In adipose tissue, "classically activated" M1 macrophages and "alternatively activated" M2 macrophages can be found in various proportions. M2 macrophages maintain VAT homeostasis in lean individuals through the secretion of anti-inflammatory cytokines, such as IL-10, whereas in the case of obesity, pro-M1 polarized macrophages secrete pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, which can promote the proliferation of other inflammatory immune cells, chronic local and systemic inflammation, and can directly alter insulin receptor signaling in adipocytes, leading to insulin resistance (Cimini *et al.*, 2017)

The liver plays a critical role in the body's metabolic homeostasis and especially in the synthesis and storage of glycogen. The cirrhotic liver tissue loses the capacity for this physiological function with increasing decompensation, resulting in the breakdown of fat and muscle and finally in the promotion of gluconeogenesis from non-carbohydrate sources. This process leads to muscle wasting (Poggiogalle *et al.*, 2016). The decline in lean body mass, better known as sarcopenia, accompanies liver cirrhosis, likely related to malnutrition occurring in cirrhotic patients. Obesity and sarcopenia and are two faces of the health challenge of XXI century and sarcopenic obesity, characterized by high adiposity and low lean body mass is a frequent condition in Western Countries. The prevalence of this condition, also known as sarcopenic obesity is more frequent in women than in men, with a reported a range from 4% to 84% in men and from 4% to 94% in women. Obesity is strictly linked to fatty liver and insulin resistance is a key factor in the pathophysiology NAFLD (Poggiogalle *et al.*, 2016).

Both liver and muscle are target organs for insulin action and insulin resistance is also involved in age-related muscle protein loss, progressively leading to sarcopenia.

Conversely, the impairing and loss of skeletal muscle promotes insulin resistance, which could eventually lead to metabolic syndrome and NAFLD. Moreover, it is also understandable how decreased physical activity, induced by sarcopenia, can cause a reduction of energy expenditure, which may result in obesity and hepatic steatosis. It has also been demonstrated that individuals with sarcopenia have a higher risk of NAFLD than those without sarcopenia (Hong *et al.*, 2014). In an interesting study, sarcopenic patients had more body fat mass, and feature of metabolic syndrome, higher c-reactive protein (CRP) levels, and higher arterial stiffness compared to those without sarcopenia(Sanada *et al.*, 2012). Moreover the coexistence of sarcopenia and metabolic syndrome increases the risk of cardiovascular disease (CVD) in Japanese women (Sanada *et al.*, 2012).

Anyway, the role of potential co-factors in the development of fatty liver other than the impairment of insulin sensitivity need to be better clarified.

Indeed, NAFLD and sarcopenia seem to share the same pathophysiological backgrounds, such as chronic inflammation, VitD deficiency, oxidative stress, and sedentary lifestyle.

Authors have also demonstrated that reduced insulin sensitivity, or impaired in insulin signaling, can induce skeletal muscle loss via reduction of the synthesis of muscle protein and mitochondrial dysfunction, causing the vicious cycle between insulin insensitivity and sarcopenia (Wijarnpreecha *et al.*, 2018). Indeed, skeletal muscles secrete myokines such as irisin, IL-6, myostatin, adipocytokines and adiponectin that are involved in the regulation of glucose and fatty acid metabolism in the liver (Wijarnpreecha *et al.*, 2018).

The imbalance of myokine levels related to loss of muscle mass could affect glucose and fatty acid metabolism and lead to abnormal hepatic fat accumulation.

This association could be just the result of the same underlying factors that could predispose patients to both NAFLD and sarcopenia. Indeed, enlarged visceral fat is associated to increased oxidative stress and proinflammatory cytokines secretion,

that could promote catabolic state, resulting in loss of skeletal muscle (Wijarnpreecha *et al.*, 2018) as well as the development and progression of NAFLD (Poggiogalle *et al.*, 2016).

Another pivotal role in this context is played by the Growth hormone (GH)/ insulin-like growth factor 1 (IGF-1) axis that could partially explain the interrelationship between liver steatosis and sarcopenia (Poggiogalle *et al.*, 2016). It exerts significant effects on body composition, and lipid and glucose homeostasis. Several studies reported that in obese subjects GH secretion is blunted, and it has been demonstrated that GH and IGF- 1 are involved in fatty infiltration of the liver (Poggiogalle *et al.*, 2016). Ectopic fat deposition seems to be linked to reduced GH levels, as well as impairment in GH production occurring with aging. This is one of the causes of the changes in body composition leading to both excess adiposity and sarcopenia (Poggiogalle *et al.*, 2016).

Prolonged deficiency of VitD has been shown to be associated with a reduction in type 2 muscle fibers and lower serum 25-hydroxyvitamin D [25(OH)D)] levels increase the risk of sarcopenia in elderly (Visser *et al.*, 2003). VitD deficiency is also associated with an increased risk of NAFLD, possibly due to increased inflammatory cytokines (Wijarnpreecha *et al.*, 2018) and a strong association between NAFLD and low 25(OH)D levels was found also in an adult population with normal serum liver enzymes (Barchetta *et al.*, 2011) (see below for more details, paragraph 1.3).

1.2 Mediterranean Diet and Metabolic Diseases

The term "Mediterranean diet" identifies the traditional dietary pattern found in the Mediterranean areas of Crete, Greece, and Southern Italy in the late 1950s and early 1960s (Willett *et al.*, 1995). This dietary profile consists of a high consumption of whole grains, legumes, nuts, vegetables, and fruits with a relatively high fat consumption (up to 40% of total energy intake), mostly from monounsaturated fatty acids (MUFA; up to 20% of energy) mainly provided by extra-virgin olive oil (EVO),

the principal source of culinary and dressing fat. Moderate to high consumption of fish, with poultry and dairy products (usually yogurt or cheese), consumed in moderate to small amounts, are also included. In addition there is low consumption of red meats, processed meat and meat products; and moderate alcohol intake, usually in the form of red wine consumed with meals. This dietary pattern was represented by a food pyramid (Willett *et al.*, 1995), that, with the due update, is still used (Bach-Faig *et al.*, 2011).

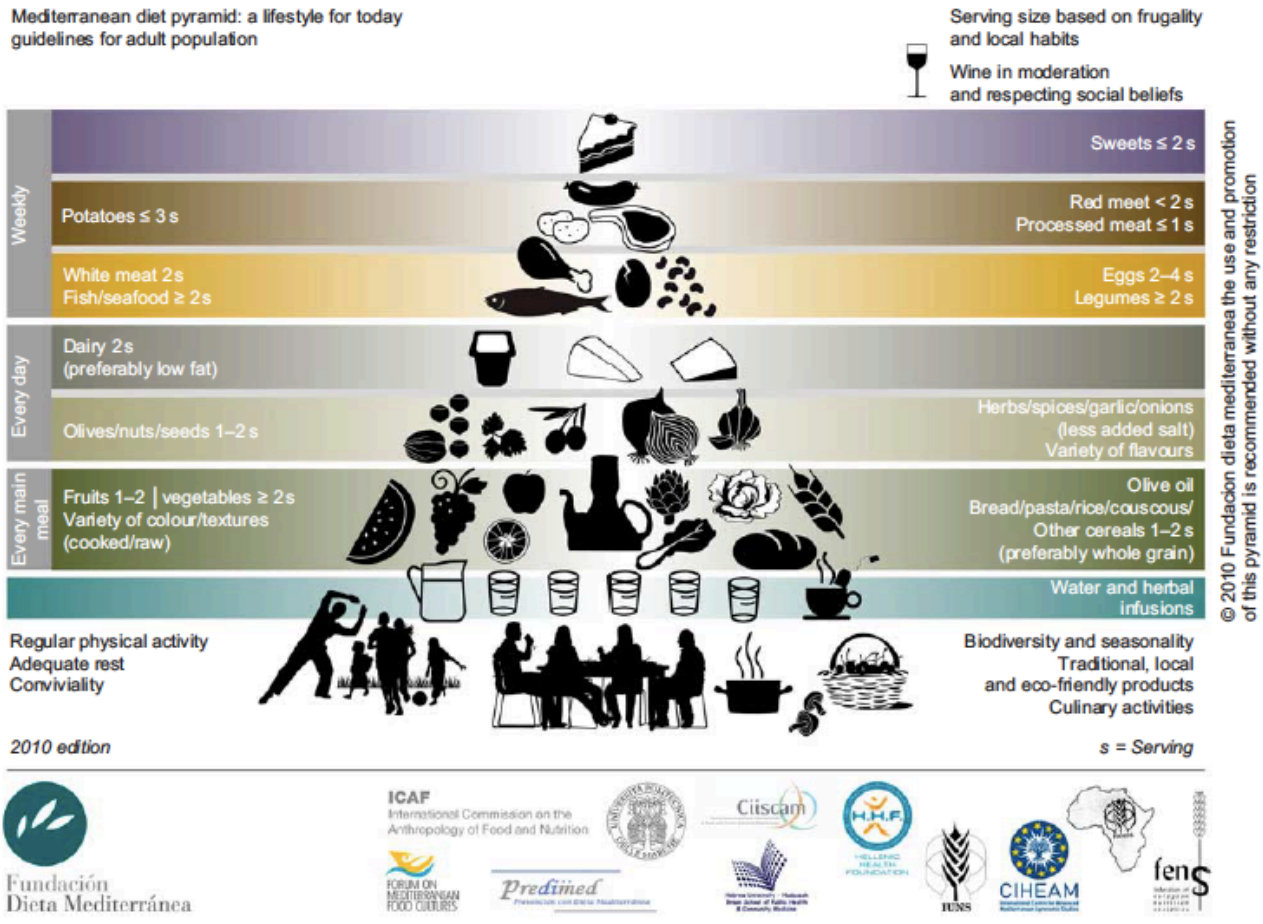


Illustration 2. The traditional Mediterranean diet (MD) pyramid has evolved to adopt the new way of life. The new pyramid follows the previous pattern: at the base, foods that should sustain the diet, and at the upper levels, foods to be eaten in moderate amounts. Moreover, social and cultural elements characteristic of the Mediterranean way of life are incorporated in

the graphic design. So, it is not just about prioritising some food groups from others, but also paying attention to the way of selecting, cooking and eating. It also reflects the composition and number of servings per meals. The pyramid establishes dietary daily, weekly and occasional guidelines in order to follow a healthy and balanced diet (Bach-Faig et al., 2011)

The biggest study on the benefits of Mediterranean Diet was the Seven Countries Study of Cardiovascular Diseases that started at the end of the 1950s and it continues to be run after >50 years. It enrolled, at entry, 16 population cohorts in eight nations of seven countries (Italy, USA, Japan, Netherlands, Greece, Yugoslavia and Finland) for a total of 12,763 middle-aged men. It was an epidemiological study seeking cultural contrasts and the first evaluate the incidence of CVD in relation to dietary differences (Menotti & Puddu, 2015). Higher incidence and mortality rates from CVD were found in North America and northern Europe, and lower rates in southern Europe - Mediterranean countries - and Japan. These differences in CVD rates were strongly associated with different saturated fat consumption and serum cholesterol levels, with lowest rates in Greece and Japan where the total fat intake was very different (Menotti & Puddu, 2015). Thus, the Mediterranean diet was assumed as a paradigm of healthy nutrition, and it is still studied, envisaging even pharmacological effects (Siniorakis *et al.*, 2013). This reduced incidence of CVD has been partially attributed to the regular intake (25–50 mL/day) of EVO, the principal fat and one of the cornerstones of the Mediterranean diet. Nowadays, thanks to the plenty of studies on its healthy properties, EVO is considered a functional food, which besides having a high level of MUFAs, in particular oleic acid, and contains multiple minor components with biological activities (Priore *et al.*, 2015), such as phenolic compounds, tocopherol, carotenoids.

They have been shown to possess antimicrobial, antioxidant and anti-inflammatory properties (Cicerale *et al.*, 2012, Priore *et al.*, 2015, Szychlinska *et al.*, 2018), preventing cellular injury and oxidative stress (Musumeci *et al.*, 2014) with subsequent positive effects on disease risk. Their abilities to scavenge free radicals and thus preventing cellular injury, are crucial since an excess of free radicals can cause oxidative damage to biomolecules (i.e. lipids and DNA), increasing the risk of developing various chronic diseases such as atherosclerosis, cardiovascular disease, cancer, chronic inflammation, stroke and other degenerative diseases (Musumeci *et al.*, 2013, Musumeci *et al.*, 2014). The phenolic fraction ranges from 50 to 800 mg/kg; depending on several factors, such as the cultivar, the climate, and the processing system employed to produce the different types of olive oil: extra-virgin, virgin, olive oil, or pomace olive oil (Priore *et al.*, 2015) . Virgin olive oils are those obtained from the fruit of the olive tree solely by mechanical or other physical means under the conditions that do not lead to alteration of the oil. It must be underlined that as extra-virgin olive oils (EVOs) are obtained from once cold-pressed unfermented olives, they are characterized by a free fatty acids content lower than 1% and the highest phenol levels (Priore *et al.*, 2015). The simple phenolic compounds include tyrosol, hydroxytyrosol (3,4-dihydroxyphenylethanol), and phenolic acids such as vanillic and caffeic acids. The complex phenolic compounds are mainly tyrosol and hydroxytyrosol esters (Jemai *et al.*, 2008b), oleuropein (Jemai *et al.*, 2008a), and its aglycone. The highest concentrations of olive oil phenolic compounds, widely noted for their antioxidant activities, are those of oleuropein, hydroxytyrosol, and tyrosol (Cicerale *et al.*, 2012).

Administration of both hydroxytyrosol and oleuropein decreased the serum levels of total cholesterol, triglycerides, and LDL-C significantly and increased the serum level of high-density lipoprotein cholesterol (HDL) (Jemai *et al.*, 2008a, Jemai *et al.*, 2008b) with antioxidant activity in several organs i.e. liver, heart, kidney, and aorta (Jemai *et al.*, 2008b). Oleuropein exerts anti-inflammatory and anti-atherogenic

effects which, at least in part, depend on its anti-oxidative activities (Barbaro *et al.*, 2014) and it has an additional beneficial effects on several aspects of cardiovascular disease via its vasodilatory, anti-platelet aggregation, anti-ischemic (Manna *et al.*, 2004) and hypotensive properties (Cicerale *et al.*, 2012, Bulotta *et al.*, 2014). In our previous study we demonstrated how rats submitted to exhaustive exercise had decreased parameters indicating oxidative stress, such as hydroperoxides and thiobarbituric acid-reactive substances, while parameters indicating antioxidant defenses of the body such as nonenzymatic antioxidant capacity and heat shock protein (Hsp)70 expression were increased, when fed with an experimental chow enriched with oleic acid, present in high percentage in extra-virgin olive oil (74–76%) (Musumeci *et al.*, 2014). Moreover EVO, in conjunction with physical activity, improves the recovery after knee injury, promoting lubricin expression in rats (Musumeci *et al.*, 2013).

Recent studies have demonstrated that EVO and its phenols could regulate hepatic lipid metabolism by reducing the lipogenic pathway, and thus attenuating liver steatosis (Jurado-Ruiz *et al.*, 2017). In particular oleuropein administration seems attenuating hepatic steatosis induced by high-fat diet in mice (Park *et al.*, 2011). HFD-fed mice showed drastic increases in the hepatic mRNA expression of α-smooth muscle actin (*α-SMA*) and collagen, that are markers of liver fibrosis, and this process is reversed by oleuropein (Park *et al.*, 2011).

Olive oil consumption seems to decrease the accumulation of liver triglycerides in rats with NAFLD. In particular, some authors suggested that olive oil may improve insulin resistance, increase the secretion of hepatic triglycerides as VLDL, and decrease the lipolytic flux from peripheral adipose tissue back to the liver (Hussein *et al.*, 2007). Moreover the polyphenol content increases HDL cholesterol levels and improve oxidative damage in addition to the benefits from monounsaturated fatty acid content (Covas *et al.*, 2006).

According to several evidences EVO should be included in the diet of the patients with NAFLD as it could decrease insulin resistance and blood triglycerides by increasing the expression of hepatic fatty acid β-oxidation enzymes. This occurs through the activation of their corresponding genes by the transcription factor peroxisome proliferator-activated receptor-a (PPARa) (Assy *et al.*, 2009).

Indeed in a cohort of patients we followed for lifestyle counseling and prescription of Mediterranean diet jointly with physical exercise for 6 months, we displayed a positive change of lifestyle, bodyweight and insulin resistance, assessed as HOMA-IR, concurrently with incresed adherence to Mediterranean diet (evaluated as AMDS: Adherence to Mediterranean Diet Score) and reduced sedentary habits. In particular, significant increase of adherence to Mediterranean Diet and increase of physical activity were observed at the first month, with further gradual increments in the following periods. A significant decrease of fatty liver (evaluated by ultrasonography as bright liver score) was observed after 6 months of intervention. The unfavorable factors associated with failure of NAFLD improvement were minor AMDS change, minor BMI decrease, minor physical exercise score increase and smaller HOMA-IR decrease (Trovato *et al.*, 2015).

1.3 Vitamin D in Metabolic Diseases

Vitamin D is a fat-soluble vitamin, found in nature under multiple forms. Vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) are the two major forms. VitD2 originates by phytoplankton, invertebrates and yeast in response to ultraviolet irradiation and is not produced by vertebrates. It is often used for supplementation. Conversely VitD3, is sintetized in the skin of most vertebrates including humans, after irradiation of 7-dehydrocholesterol with ultraviolet light (UVB). Dietary VitD2 is absorbed by the small intestine, incorporated into chylomicrons and transported to the liver bound to VitD-binding protein. Then VitD, from both the skin and diet, is

metabolized by hepatic 25-hydroxylase to 25-hydroxyvitamin D [25(OH)D], the major circulating metabolite and the most widely dosed indicator of VitD stores. In the kidney, 25(OH)D undergoes hydroxylation by 1a-hydroxylase to the biologically active form 1a,25-dihydroxyvitamin D [1,25(OH)2D]. VitD circulates in the blood bound to VitD-binding proteins, reaching its target tissues to exert endocrine actions. These latter are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor family of transcription factors, which is expressed in different tissues (Holick, 2007, Camperi *et al.*, 2017).

The amount of 1,25(OH)2D is regulated by the synthetic activity of 1a-hydroxylase and the catabolic activity of 24-hydroxylase which catabolizes 1,25(OH)2D to the water soluble and biologically inactive calcitoic acid which is then excreted in the bile. Parathyroid hormone, 1,25(OH)2D itself and Fibroblast growth factor 23 are the main regulators of these enzymes (Hong *et al.*, 2014).

The classical, and best known, functions of VitD include the regulation of calcium and phosphate metabolism, mainly acting on bone tissue, intestine and kidneys. More recently, VitD has been demonstrated to play a role in several physiological and pathological processes and a plenty of studies evaluated its pleiotropic functions, such as in immune response and cancer development (Li *et al.*, 1997, Verstuyf *et al.*, 2010). Indeed, VDR expression is found in a wide range of tissues including liver and muscles (skeletal, smooth and heart muscles), endocrine system (pancreas, pituitary, thyroid and adrenal cortex), immune system (T and B cells, macrophages, and monocytes), reproductive system (uterus, testis, ovary, prostate, placenta, and mammary glands), brain and skin (Bouillon, 2018). This finding raised considerable interest in understating the putative pleiotropic properties of VitD, introducing the idea of a paracrine/autocrine role in the regulation of cell proliferation, differentiation and apoptosis as well as immune-cells regulation (Bouillon, 2018).

The importance of VitD and the impact of its deficiency on muscle health have been highlighted in several recent studies. These trophic properties are crucial in

situations of loss of muscle mass, particularly in the context of chronic diseases (Domingues-Faria *et al.*, 2017). Moreover, VitD deficiency is strictly related to sarcopenia in the elderly (Ceglia, 2009, Kim *et al.*, 2011). Histological alterations of muscle fiber composition and diameter are associated with VitD deficiency (Ceglia, 2009). Despite this evidence, also the effectiveness of VitD administration in patients affected by muscle atrophy has not been assessed (Camperi *et al.*, 2017).

VitD3 supplementation increased intramyonuclear VDR concentration by 30% and increased muscle fiber size by 10% in mobility-limited women with VitD insufficiency (Ceglia *et al.*, 2013)

In disagreement with the above mentioned data Hong et al. found no significant association between 25(OH)D levels and skeletal muscle index or liver attenuation index in Korean men and women (Hong *et al.*, 2014).

Despite several epidemiological studies showing the existence of a close relationship between obesity and hypovitaminosis D, the mechanisms underlying this association are largely unknown (Cimini *et al.*, 2017). Some investigators have suggested that the increased uptake and sequestration of VitD by adipose tissue contributes to low circulating 25(OH)D concentrations in obese individuals in comparison to lean individuals (Earthman *et al.*, 2012, Luger *et al.*, 2015). Indeed, low serum 25(OH)D could affect lipogenesis and/or adipogenesis in the adipose tissue and contribute to obesity. Authors suggest that the adipose tissue could be a direct target of VitD and that may modulate adipose tissue formation and function (Luger *et al.*, 2015).

Moreover, in obesity, circulating fatty acids are increased, leading to ectopic lipid deposition in other tissues, including skeletal muscle, which represents the largest metabolically active tissue in the body (40% of body mass) (Turpin *et al.*, 2009). Muscle tissue maintenance is based on ongoing repair, regeneration, and growth (Akhmedov & Berdeaux, 2013). In obese individuals, insulin resistance and mitochondrial dysfunction negatively impact muscle metabolism and physical

performance (Turpin *et al.*, 2009). Low VitD levels were associated with higher risk of T2DM (Lim *et al.*, 2017), and a study showed that VitD treatment improved insulin resistance among patients with baseline glucose intolerance, although other authors contradicted those observations (Randhawa *et al.*, 2017).

Indeed, the results of a recent meta-analysis by Seida et al. showed no significant effect of VitD supplementation on the prevention of diabetes, or on the reduction of insulin resistance and hyperglycemia in individuals with pre-diabetes or established T2DM. However the presence of moderate heterogeneity between the studies and the short term of follow up limited the strenght of these results (Seida *et al.*, 2014).

Given the strong association of NAFLD with obesity and metabolic syndrome, VitD is emerging as possible player in the development and progression of NAFLD (Ju *et al.*, 2014).

Epidemiological data suggest that low levels of serum 25(OH)D are associated with NAFLD as diagnosed either by biochemistry, imaging or biopsy (Targher *et al.*, 2007). VitD deficiency is strongly associated with features of metabolic syndrome and may play a role in modifying the cardio-metabolic risk in T2DM and cardiovascular diseases (Eliades & Spyrou, 2015, Zagami *et al.*, 2015, Della Corte *et al.*, 2016). This is supported by the findings of various animal studies, showing that lack of VDR or VitD deficiency impairs insulin secretion (Zeitz *et al.*, 2003, Eliades & Spyrou, 2015). Patients with unexplained elevation in serum alanine aminotransferase (ALT) levels - a proxy of NAFLD - had lower 25(OH)D levels than those with normal ALT levels (Liangpunsakul & Chalasani, 2011).

VitD levels were found lower in subjects with NAFLD or NASH than in controls (Wang *et al.*, 2015), but also in this case the results of other studies are conflicting (Ha *et al.*, 2017).

The first to study showing the association between biopsy-proven NAFLD and VitD levels was by Targher et al (Targher *et al.*, 2007). Authors confirmed that 25(OH)D concentrations were lower in NAFLD subjects compared to controls.

Furthermore 25(OH)D levels predicted the histological severity of NAFLD, with NASH patients having lower 25(OH)D levels compared to those with isolated fatty liver. These findings have been confirmed by a recent meta-analysis, even if no association between serum 25(OH)D levels and disease severity as determined by NAFLD Activity Score (NAS) and fibrosis score among patients with NAFLD (Jaruvongvanich *et al.*, 2017).

Collagen deposition and fibrosis are attributed to hepatic stellate cell (HSC) activation, which induces increased cellular proliferation and transformation into a myofibroblast-like cell, which in turn leads to increased synthesis and deposition of extracellular matrix proteins, especially type I collagen (Wang *et al.*, 2015). Previous research has shown that the suppression of HSC proliferation by VitD was associated with antifibrotic effects in murine model (Abramovitch *et al.*, 2011). However, the therapeutic impact of VitD as an anti-fibrotic agent must be estimated. Moreover NASH patients are less prone to respond to VitD supplementation (Dasarathy *et al.*, 2017), indeed daily supplementation with cholecalciferol (2000 IU) for 6 months did not correct hypovitaminosis D in the majority of patients with NASH (Dasarathy *et al.*, 2017).

Limited evidences prove the effectiveness of VitD supplementation in the management of NAFLD patients (Barchetta *et al.*, 2016, Foroughi *et al.*, 2016). A randomized placebo-controlled double-blind clinical trial to evaluate the potential beneficial effects of oral calcium plus calcitriol supplementation versus calcitriol alone on liver enzymes and ultrasound-measured fat liver content in 120 patients with NAFLD, showed improved serum ALT and fasting plasma glucose, and increased HDL in the calcium plus calcitriol treated group (Lorvand Amiri *et al.*, 2017). Nevertheless a study investigating VitD effects on hepatic fat content (measured by MRI) in T2DM failed to show any benefits of 24-week oral VitD supplementation on hepatic steatosis or metabolic/cardiovascular parameters in T2D patients with NAFLD (Barchetta *et al.*, 2016)

2. The Model

Animal studies vary in the time of feeding, rat strain, sex and age of rats, the amount of energy derived from fats, the origin of fats; the ratio of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids; and the proportion of ω-3 and ω-6 PUFA.

Standard rat diets contain lower amounts of fat than the recommended human diet. In contrast to approximately 30% of total energy intake from fats in humans, the standard rat diets contain less than 10% of kcal fat, whereas high-fat diets (HFDs) and very high-fat diets contain 30%-50% and more than 50% of kcal fat, respectively (Kucera & Cervinkova, 2014). We chose a diet with 40% of kcal from fat (respectively butter or olive oil based).

In some experiments, HFD induced hepatic steatosis of varying degrees and patterns, while in others, HFD did not lead to the development of fat accumulation in the liver (Kucera & Cervinkova, 2014). Moreover, HFD led to histopathological hepatic changes similar to human NASH in several other experiments. Studies also differ in regards to the development of insulin resistance, obesity and dyslipidaemia, and other known pathogenic factors of human NAFLD.

We chose to fed rats *ad libitum*, even if in some papers, controlled feeding via intragastric cannula (oral gavage) was used (Kucera & Cervinkova, 2014). Inter-strain differences in the susceptibility of rats to HFD are also reported. We chosed Sprague-Dawley rats, since they showed diet-induced macrovescicular steatosis and moreover, the highest degree of fibrosis, hepatocyte damage, and reduced blood flow velocity in central veins compared to Lewis and Wistar strain (Rosenstengel *et al.*, 2011, Stoppeler *et al.*, 2013).

We examined the literature about VitD supplementation in animal model, in order to decide the best composition of diet.

The National Research Council (NRC)-nutrient requirement for a rat is 1000 IU/kg of diet (1995). An interesting study tested whether VitD could preserve or improve cognitive function with aging administrating three different dosages to mimick respectively low, medium and high supplementation (100 IU/kg of diet; 1000 IU/kg of diet; 10000 IU/kg of diet) (Latimer *et al.*, 2014). In another study on ovariectomized mice used as animal model for postmenopausal osteoporosis, 5000 IU/kg diet of vitamin D3 were administred in the study group compared to 1000 IU/kg in controls. This supplementation has been shown to prevent ovariectomy-induced bone loss in combination with virgin olive oil (Tagliaferri *et al.*, 2014).

We chose three different dosages: absolute restriction (0 IU/kg), recommended requirement (1400 IU/kg) and moderate/high supplementation (4000 IU/kg of diet).

Also in regard of the timing, in literature we found a certain heterogeneity. A study showed the development of diet-induced NAFLD in 3 weeks, when a very high-fat diet (71% of kcal form fat) was administered (Kucera & Cervinkova, 2014). Lipid accumulation in the liver was observed as early as 8 weeks up to 16 weeks, by both macroscopic and microscopic analysis in HFD-fed mice, in mouse model of diet-induced hepatic steatosis (Bose *et al.*, 2008, de Meijer *et al.*, 2010, Park *et al.*, 2011). A study comparing different HFDs (42% energy from fat), submitted rats to a 12 week dietary regimen, showing pronounced obesity, insulin resistance, and feature of steatosis in olive oil and lard based diet groups (Buettner *et al.*, 2006). A 10-week experiment on HFD and oleuropein supplementation showed significantly higher final body weight and cumulative body weight gain in HFD-fed mice compared control. Moreover oleuropein supplemented to HFD significantly reduced final body weight and body weight gain in mice (Park *et al.*, 2011). In another study 6 weeks of oral gavage with HF emulsion caused metabolic changed and hepatic steatosis in the study group (Zou *et al.*, 2006). Interestingly, in 16 weeks study evaluating both muscle and liver tissues, the phenotypic aspects of sarcopenia were observed prior to

the development of liver fibrosis, indicating that they could occur in early stages of NAFLD natural history (Cabrera *et al.*, 2016). Considering the above studies, in order to detect very early changes we chose a relatively short period of diet (10 weeks).

We tried to respect the Russell and Burch principles of replacement, reduction, and refinement for animal studies (Tannenbaum & Bennett, 2015). Replacement indicates minimizing research animal distress by substituting animals that can experience distress with insentient material that is incapable of feeling anything and therefore cannot experience distress. Reduction indicates minimizing research animal distress by decreasing the number of animals that can experience distress. Refinement is, by definition, diminution or elimination of distress (Tannenbaum & Bennett, 2015).

Sample size calculation was provided by 'resource equation' approach. Based on this approach, the acceptable range of degrees of freedom (DF) for the error term in an analysis of variance (ANOVA) is between 10 to 20. The resource equation approach is suitable for exploratory studies whenever it is not possible to assume the standard deviation and the effect size. This is applicable whenever the outcome is a quantitative variable and suitable for analysis by ANOVA (Arifin & Zahiruddin, 2017). With these parameters, the number of rats per groups can vary from 3 to 4. We chose 4 per group in order to have more power.

2.1 Investigated Molecules

2.1.1 IL-1beta

As inflammatory marker, we chose IL-1β, a member of the interleukin-1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase-1. This cytokine is an important mediator of the inflammatory response, and is involved in a

variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Del Campo *et al.*, 2018).

Hepatic inflammation is a common trigger of liver disease, and is considered the main driver of hepatic tissue damage, triggering the progression from NAFLD to severe fibrogenesis and, finally, hepatocellular carcinoma (HCC). Acute and chronic liver diseases are cytokine-driven diseases as several proinflammatory cytokines (IL-1α, IL-1β, tumor necrosis factor-alpha, and IL-6) are critically involved in inflammation, steatosis, fibrosis, and cancer development (Del Campo *et al.*, 2018)

In liver disease, saturation of fatty acids produces inflammation in the hepatocytes, which increases the induction of caspase-1 activation and the release of IL-1β (Del Campo *et al.*, 2018).

The mRNA levels of several pro-inflammatory genes were significantly higher in the liver of HFD-fed mice, including *IL-1β,* and this trend was reversed in oleuropein fed mice (Park *et al.*, 2011)

2.1.2 IGF-1

IGF-1, originally called somatomedin C, is a 70-amino acid polypeptide hormone. IGF-1 is the major mediator of prenatal and postnatal growth. It is produced primarily in liver and serves as an endocrine (as well as paracrine and autocrine) hormone mediating the action of GH in peripheral tissues including muscle, cartilage, bone, kidney, lungs, and the liver itself. The growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis is involved in protein metabolism in the skeletal muscle as well as bone growth and remodeling (Perrini *et al.*, 2010). The somatotropic axis activity declines with age, namely the somatopause, and represents an important determinant of the development of osteoporosis and sarcopenia. Although IGF-1 signaling, that is the major mediator of GH action, has a crucial role in the cross-talk linking striated muscle and bone, several studies have also reported decreased levels

of IGF-1, in subjects with NAFLD. Indeed, GH and IGF-1 influence carbohydrate and lipid metabolism. IGF-1 stimulates glucose uptake, favoring insulin signaling, whereas GH induces lipolysis, determining insulin resistance mediated by elevated free fatty acid levels (Takahashi, 2012, Aguirre *et al.*, 2016). Moreover, IGF-1 values were associated with the histological severity of NAFLD, independent of insulin resistance (Sumida *et al.*, 2015). Muscle fiber size and muscle strength, as well as IGF-1 levels decreased in animals with diet-induced NAFLD (Cabrera *et al.*, 2016).

2.1.3 Dickkopf-related protein 1 (DKK-1)

Another pathway we decided to explore, in order to explain how VitD could iterfere with liver steatosis, is Wnt/β-catenin. The Wnt signaling pathway has been implicated in various aspects of stem cell maintenance and tissue homeostasis, since it is a major regulator of cell function both in embryonic development and in adults. Alteration of signaling are linked to abnormal embryonic development and diseases (Ren *et al.*, 2013). The Wnt components have been described in all organs of the body with specific expression levels and distribution depending on the tissue evaluated (Cisternas *et al.*, 2014).

The Wnt family consists of 19 lipid modified secreted glycoproteins that are involved in two main pathways: canonical, or β –catenin dependent signaling (Wnt/β -catenin), and noncanonical or β -catenin-independent signaling (Chakkalakal & Brack, 2012, Yang *et al.*, 2016). In the canonical pathway, Wnt proteins bind to cell surface Frizzled receptors, which triggers the stabilization of the common downstream Wnt effector β-catenin. Normally, β–catenin levels and nuclear accumulation are kept low due to continuous degradation driven by a destruction complex composed of glycogen synthase kinase-3β (GSK-3), Adenomatous Polyposis Coli (APC) and Axin (Yang *et al.*, 2016). Stabilized β-catenin is then able to translocate to the nucleus and through interactions with the T-cell factor (Tcf)/lymphoid enhancer factor 1 (LEF-1), modulates the expression of specific genes. These genes

regulate cell proliferation, differentiation, adhesion, morphogenesis. Dickkopf (Dkk) family, which binds to the LRP subunit of the Wnt receptor complex, are Wnt extracellular antagonists.

In skeletal muscle, Wnt signaling participates in the myogenic process as well as in the regeneration process, modulating the activation of skeletal muscle stem cells in response to muscle injury (Brack *et al.*, 2007, Cisternas *et al.*, 2014).

Increasing data indicates the activation of Wnt/β-catenin signaling as a pro-fibrotic pathway, however, the molecular mechanisms is still under investigation, and the interactions with TGF-β signaling in muscle and angiotensin II signaling in the kidney have been postulated (Cisternas *et al.*, 2014)

Recent studies in vivo have shown that the activation of the canonical Wnt pathway is required for the action of TGF-β , since the presence of DKK-1 decreases the activity of TGF-β in a model of muscular fibrosis and renal fibrosis(Cisternas *et al.*, 2014). Moreover, the presence of DKK-1 decreased expression of fibronectin and collagen I (Ren *et al.*, 2013). Indeed, in a murine model for human dystrophies, the injection of DKK-1 decreased the expression of collagen and the progression of fibrosis (Trensz *et al.*, 2010).

Even more interesting is the fact that the Wnt coreceptor low-density lipoprotein receptor-related protein (LRP)5 is a target for VitD, and plays a key role in osteoblast proliferation and differentiation (Sankaralingam *et al.*, 2014). In fact, in some neoplasms, VitD increased dose-dependently the expression of DKK-1, that 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)

Ligand-dependent VDR signaling has been shown to antagonize the β-catenin signaling pathway through several mechanisms, including the increase of DKK-1 mRNA expression in human and murine colon cancer cells (Pendas-Franco *et al.*, 2008). Authors showed how VitD administration downregulates TGF-β signaling in chemically induced HCC in rats (Saad El-Din *et al.*, 2018).

Moreover, IGF-1 seems antagonize Wnt pathways in studies on tumorigenesis, while there are limited data on associations between the GH/IGF axis and Wnt signaling in metabolic disorders (Jin *et al.*, 2008, Schlupf & 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 the collagen production (Ozhan & 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, Ren *et al.*, 2013).

2.1.4 VDR

The vitamin D receptor (VDR) mediates the pleiotropic biological actions of 1,25-dihydroxyvitamin D_3. Expression of the VDR is regulated by external stimuli in a tissue-specific manner (Camperi *et al.*, 2017). VDR knock-out mice show the full phenotype of severe vitamin D deficiency, indicating that VDR is the major mediator of VitD action (Li *et al.*, 1997).

2.1.5 Collagen I

The principal extracellular matrix proteins produced in the progression of liver fibrogenesis are predominantly collagen types I, III, and IV (Mak *et al.*, 2012). These collagens are deposited in fibrotic foci, specifically in the walls of central veins, the

perisinusoidal space of Disse, the extracellular matrix surrounding hepatocytes (pericellular), portal tract stroma, and fibrous septa. Perisinusoidal hepatic stellate cells (HSCs) and myofibroblasts are fibrogenic cells involved in enhanced production of collagens in liver fibrosis. In addition to collagens, elastin production and the formation of elastic fibers have also been shown to occur in the presence of alcoholic liver disease and NASH (Mak *et al.*, 2012).

Findings from animal models revealed that VitD interferes with the activation of HSC (Abramovitch *et al.*, 2011). Clinical trials are needed to demonstrate that VitD supplementation could slow down the progression from NAFLD to NASH (Abramovitch *et al.*, 2011).

3. Materials and Methods

3.1. Breeding and Housing of Animals

Twenty-eight 7–9 weeks-old healthy Sprague/Dawley male rats (Envigo RMS S.r.l., Udine, Italy), with an average body weight of 271 ± 25 g, were housed in polycarbonate cages (cage dimensions: 10.25″ W × 18.75″ D × 8″ H) at controlled temperature (20–23 °C) and humidity during the whole period of the research, with free access to water and food and photoperiod of 12 h light/dark at the "Center for Advanced Preclinical In Vivo Research (CAPIR)". Rats were allowed to adapt one week to their environment before the experiments began. Body weights, food and drink consumptions were monitored three days per week throughout the experiment. At the end of the experimental period (10 weeks), the animals were humanely sacrificed by exposure to a chamber filled with carbon dioxide until one minute after breathing stopped and then were decapitated. After euthanasia, skeletal muscle (anterior tibial of leg of right hind limb) and liver tissue were used to perform 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).

3.2. Experimental Design

Diets and origin of the extra-virgin olive oil: the extra-virgin olive oil (EVO) used for the experiment was a protected designation of origin (PDO) Monti Iblei Sottozona Gulfi (Oleificio Guccione di Divita Vito e G. SAS, Chiaramonte Gulfi, Sicily, Italy, obtained from the olive variety of Tonda Iblea. EVO was obtained by extraction through a continuous cold cycle and natural decantation. The acidity of the EVO was 0.18%. The composition of fatty acids was analyzed by an external chemical laboratory using Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC) (EN ISO 9936:2006), and Spectrophotometry. The composition of fatty acids in the EVO is reported in Table 1.

Table 1. Composition % m/m of fatty acids in extra-virgin olive oil (EVO).

Fatty Acids	EVO
Palmitic acid 16:0	**14.41**
Palmitoleic acid 16:1	**1.31**
Stearic acid 18:0	**2.18**
Oleic acid 18:1	**70.38**
Linoleic acid 18:2	**9.69**
Linolenic acid 18:3	**0.84**

Seven different diets were used for the experiment, provided by Mucedola s.r.l. (Settimo Milanese, Milan, Italy) to which we sent the EVO for the specific preparation of rat chow. The composition of the experimental diets is reported in Table 2.

Table 2. Composition of the experimental diets.

Compound	Regular (R) 9.0% of Energy from Fat	High-Fat Butter (HFB) 41% of Energy from Fat	High-Fat EVO (HFEVO) 41% of Energy from Fat
Water (% *w*/*w*)	**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**	**46,149**	**30,470**
Palmitoleic acid 16:1 (mg/kg)	**308**	**2170**	**2771**
Stearic acid 18:0 (mg/kg)	**1336**	**20,139**	**4612**
Oleic acid 18:1 (mg/kg)	**8638**	**42,976**	**148,924**
Linoleic acid 18:2 (mg/kg)	**17,300**	**12,845**	**20,504**
Linolenic acid 18:3 (mg/kg)	**2072**	**1672**	**1777**
DIET		**Vitamin D**	
R		**1400 IU/kg**	
R-DS		**4000 IU/kg**	

HFB-DS	
HFEVO-DS	
R-DR	**0 IU/kg**
HFB-DR	
HFEVO-DR	

M.E. : Metabolizable Energy; 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 EVO-based diet with vitamin D supplementation; HFEVO-DR: High-fat EVO-based diet with vitamin D restriction.

The 28 animals were divided into seven groups: R, control rats, fed with regular diet (n = 4); R-DS, rats fed with regular diet with vitamin D supplementation (4000 IU/Kg) (n = 4); R-DR, rats fed with common diet with vitamin D restriction (0 IU/Kg) (n = 4); HFB-DS, rats fed with high-fat (butter) diet with vitamin D supplementation (4000 IU/Kg) (n = 4); HFB-DR, rats fed with high-fat (butter) diet with vitamin D restriction (0 IU/Kg) (n = 4); HFEVO-DS, rats fed with high-fat (EVO) diet with vitamin D supplementation (4000 IU/Kg) (n = 4); HFEVO-DR, rats fed with high-fat (EVO) diet with vitamin D restriction (0 IU/Kg) (n = 4).

3.3. Histology

3.3.1 Muscle

Skeletal muscle samples were fixed in 10% neutral buffered formalin (Bio-Optica, Milan, Italy), and, after overnight washing, were embedded in paraffin as previously described (Trovato *et al.*, 2018b). The samples were placed in the cassettes in longitudinal and cross directions after wax infiltration. Tissue samples (4–5 μm) were cut from paraffin blocks by a rotary manual microtome (Leica RM2235, Milan, Italy) and then mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and preserved at room temperature. Afterwards, the sections were dewaxed in xylene, hydrated by graded ethanol, and stained by Hematoxylin and Eosin staining

for histological evaluation, muscle fibers identification, detection of structural alterations, and histomorphometric measurements. The slides were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany), and pictures were taken with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

3.3.2 Liver

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).

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. Three sections from different segments were analysed from each liver. For histopathologic evaluations we used the NAFLD Activity Score (NAS) (Kleiner *et al.*, 2005).

3.4. Histomorphometric Analysis

3.4.1 Muscle

Seven fields, the total area of which was about 150,000 μm^2, randomly selected from each muscle (proximal area of anterior tibial of leg of right hind limb) cross section, were analyzed for morphometric analysis. The perimeter of the muscle fibers was considered and calculated using a software for image acquisition (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Negative images were used for a better software performance in the morphometric analysis. The data were expressed as mean ± standard deviation (SD). The statistical significance of the results was then evaluated. A Zeiss Axioplan light microscope

(Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany) was used to take digital micrographs.

3.5. Immunohistochemistry (IHC)

3.5.1 Muscle

Skeletal muscle samples were processed for immunohistochemical analysis as previously described (Trovato *et al.*, 2018b). In detail, the slides were dewaxed in xylene, hydrated by graded ethanol, incubated for 30 min in 0.3% hydroperoxyl (HO_2)/methanol to remove endogenous peroxidase activity and then rinsed in phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy) for 20 min. In order to unmask the antigenic sites, the samples were stored in capped polypropylene slide holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy) and heated for 5 min for three times through a microwave oven (750 W). In order to prevent non-specific binding of the antibodied, a blocking step with 5% bovine serum albumin (BSA, Sigma, Milan, Italy) in PBS for 1 h in a moist chamber was performed before the application of the primary antibodies. The sections were then 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, Milan, Italy) 10 μg/mL; rabbit polyclonal anti-IL-1β (ab2105; Abcam, Cambridge, UK), diluted 1/100 in PBS (Bio-Optica, Milan, Italy); goat polyclonal anti-insulin-like growth factor (IGF)-1 (sc-7144; Santa Cruz Biotecnology, Inc., Dallas, Texas, U.S.A), diluted 1/100 in PBS (Bio-Optica, Milan, Italy), and rabbit polyclonal anti-Dickkopf-1 (DKK-1) (sc-25516; Santa Cruz Biotecnology, Inc., Dallas, Texas, U.S.A), diluted 1/100 in PBS (Bio-Optica, Milan, Italy). The samples were then coated with a biotinylated antibody (horseradish peroxidase (HRP)-conjugated anti-goat and anti-rabbit were used as secondary antibodies), and the immune complexes were detected with peroxidase-labeled streptavidin, after incubation for 10 min at room

temperature (labeled streptavidin-biotin (LSAB) + System-HRP, K0690, Dako, Denmark). The immunoreaction was detected by incubating the sections for 2 min in a 0.1% 3,3 -diaminobenzidine, 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The slides were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) and mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA).

3.5.2 Liver

Liver samples were processed for immunohistochemical analysis as previously described (Musumeci *et al.*, 2013). After blocking, the sections were incubated overnight at 4°C with 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 Biotecnology, Inc.), diluted 1/100 in PBS (Bio-Optica) and rabbit polyclonal anti-DKK-1 (sc-25516; Santa Cruz Biotecnology, 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 streptavin, 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).

3.6. Computerized Densitometric Measurements and Image Analysis

3.6.1 Muscle

An image analysis software (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining of positive anti-IL-1β, anti-vitamin D receptor, anti-IGF-1, anti-DKK-1 antibodies immunolabelling, was used to calculate the densitometric count (pixel2) (immunolabelling intensity) and the percentage of the immunostained area (immunolabelling extension) in seven fields, the area of which was about 150,000 μm^2, randomly selected from each muscle (proximal area of anterior tibial of leg of right hind limb) cross section. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany), using a lens with a magnification of × 20, i.e., total magnification 200) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

3.6.2 Liver

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 μm^2, were analyzed to quantifies the percentage of the total immunostained area (immunolabelling extension) and the percentage of the high and/or low immunostained area (immunolabelling intensity) using an 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).

3.7 Statistical Analysis

The statistical analysis 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, Armonk, NY, USA). The analysis of variance (ANOVA)-Tukey's multiple comparisons test was used for comparisons between more than two groups. The correlations between all variables were tested by Pearson's or Spearman's correlation coefficients. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant; p-values of less than 0.01 ($p < 0.01$) were considered highly statistically significant. The data are presented as the mean ± SD.

4. Results

4.1 Body Weight

Body weights were monitored for all groups, once a week throughout the experiment. We observed a physiological increase in all groups (Figure 1A), but the differences between groups at the end of the experiment (10th week) were not significant ($p > 0.05$) (Figure 1B). When analyzing the weight variation, a slight trend toward a greater weight gain was seen in the DS experimental groups in comparison to the corresponding DR groups, without reaching significance. The only statistically significant difference was between HFB-DR and HFEVO-DS ($p < 0.05$) (Figure 1C).

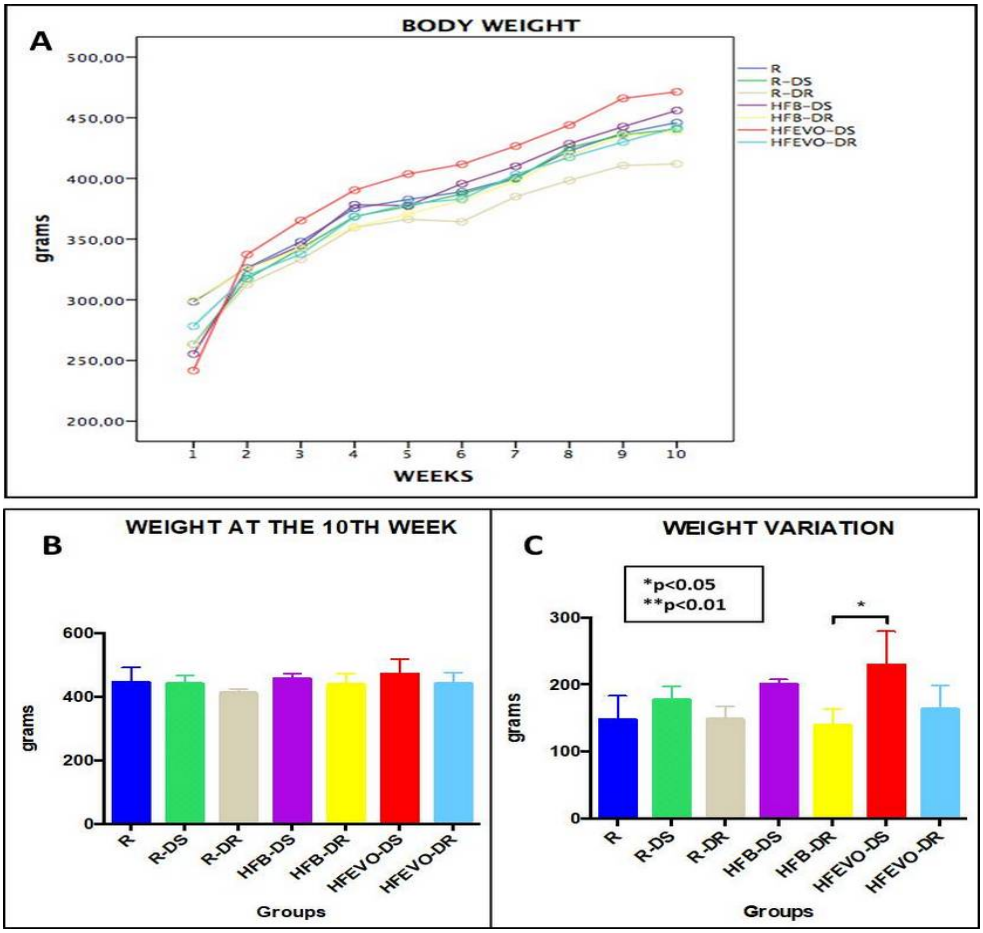


Figure 1. *Graphs: (**A**) and (**B**): body weight over 10 weeks, the differences between groups were not significant (p > 0.05). (**C**): body weight variation over 10 weeks, a slight trend toward a greater weight gain in the DS experimental groups is evident; the only statistically significant difference was between HFB-DR and HFEVO-DS (p < 0.05). 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 extra-virgin olive oil-based diet with vitamin D supplementation; HFEVO-DR: High-fat EVO-based diet with vitamin D restriction.*

4.2 Histology

4.2.1 Muscle

Hematoxylin & Eosin staining was performed to detect structural alterations in the muscle tissue of the experimental groups. None of the groups showed damage to the histological structure of the muscle fibers. However, muscle fiber hypertrophy

was observed in groups R-DS and HFEVO-DS, and hypotrophy in the HFB-DR group, as better reported in the histomorphometric analysis of muscle fibers.

4.2.2 Liver

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). Briefly he degree of steatosis was graded using the following four-point scale: grade 0, steatosis involving <5% of hepatocytes; grade 1, steatosis involving up to 33% of hepatocytes; grade 2, steatosis involving 33–66% of hepatocytes; and grade 3, steatosis involving >66% of hepatocytes. Lobular inflammation was also graded on a four-point scale: grade 0, no foci; grade 1, fewer than two foci per × 20 field; grade 2, two to four foci per × 20 field; and grade 3, more than four foci per × 20 field. Hepatocyte ballooning was graded on a three-point scale: 0, none; 1, a few balloon cells; and 2, many/prominent balloon cells. For the NAFLD activity score (NAS), features of steatosis, lobular inflammation and hepatocyte ballooning were combined, and the range of values were from 0 to 8. Cases with scores $\geqslant$5.0 were considered to exhibit NASH, whereas cases with values $\leqslant$2 were diagnosed as simple steatosis. The stage of fibrosis was evaluated on a seven-point scale: stage 0, no fibrosis; stage 1a, mild zone 3 perisinusoidal fibrosis; stage 1b, moderate zone 3 perisinusoidal fibrosis; stage 1c, portal fibrosis only; stage 2, zone 3 perisinusoidal fibrosis and periportal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis(Kleiner *et al.*, 2005). Detailed description of the histopathological results are summarized in Table 3 and Figure 2.

Table 3. NAFLD Activity Score (NAS)

Groups	Steatosis	Fibrosis	Inflammation	Livel cell injury balloning
R	0	0	0	0
R-DS	0	0	1	0

			(lobular)	
R-DR	0	0	1 (lobular)	0
HFB-DS	1	0	1 (lobular)	0
HFB-DR	1	1	1 (portal)	0
HFEVO-DS	0	0	2 (lobular)	0
HFEVO-DR	1	0	0	0

NAFLD Activity Score: 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 (Kleiner et al., 2005).

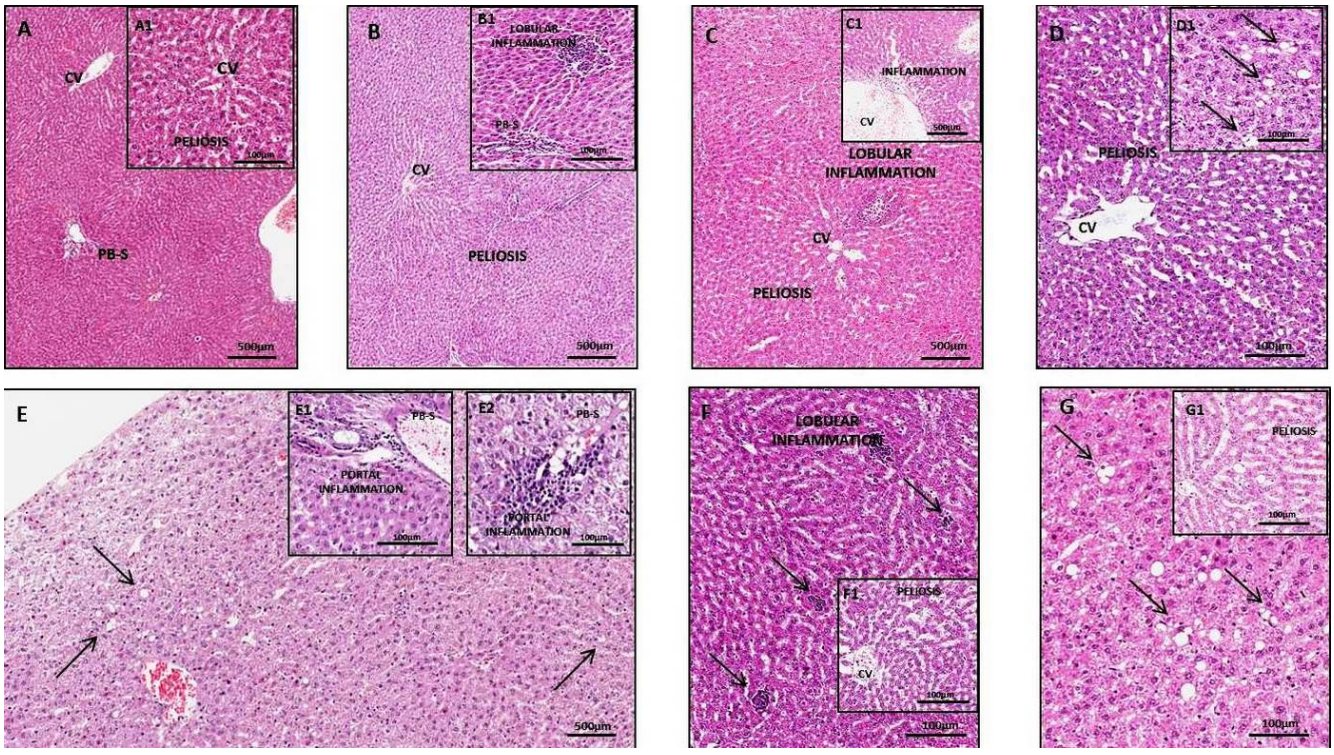


Figure 2: Hematoxylin & Eosin staining and histopathologic analysis of groups. A: R group, normal structure of liver; A1: R group, focal and mild peliosis in normal liver. B: R-DS group, the liver structure is maintained with only mild peliosis hepatis; B1: R-DS group, a focal lobular inflammation above of a portobiliar 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. A, B, C, C1 and E: scale bars: 500 μm; A1, B1, D, D1, E1, E2, F, F1, G and G1: scale bars: 100 μm.

4.3 Histomorphometric Analyses

4.3.1 Muscle

In the morphometric analysis of the perimeter (μm) (mean ± SD) of the muscle fibers, the comparison between group R (health control) versus all other groups highlighted a statistically highly significant hypertrophy in groups R-DS and HFEVO-DS ($p < 0.01$) and a statistically highly significant hypotrophy in group HFB-DR ($p < 0.01$). In detail: R vs. R-DS, HFB-DR, HFEVO-DS had $p < 0.01$; R-DS vs. R-DR, HFB-DS, HFB-DR, HFEVO-DR had $p < 0.01$; R-DR vs. HFB-DR, HFEVO-DS had, respectively, $p < 0.05$ and $p < 0.01$; HFB-DS vs. HFB-DR, HFEVO-DR had $p < 0.01$; HFB-DR vs. HFEVO-DS had $p < 0.01$; HFEVO-DS vs. HFEVO-DR had $p < 0.01$ (Figure 3). Further analyses and comparisons between the groups are reported in the paragraph "Statistical analysis of the histomorphometric results".

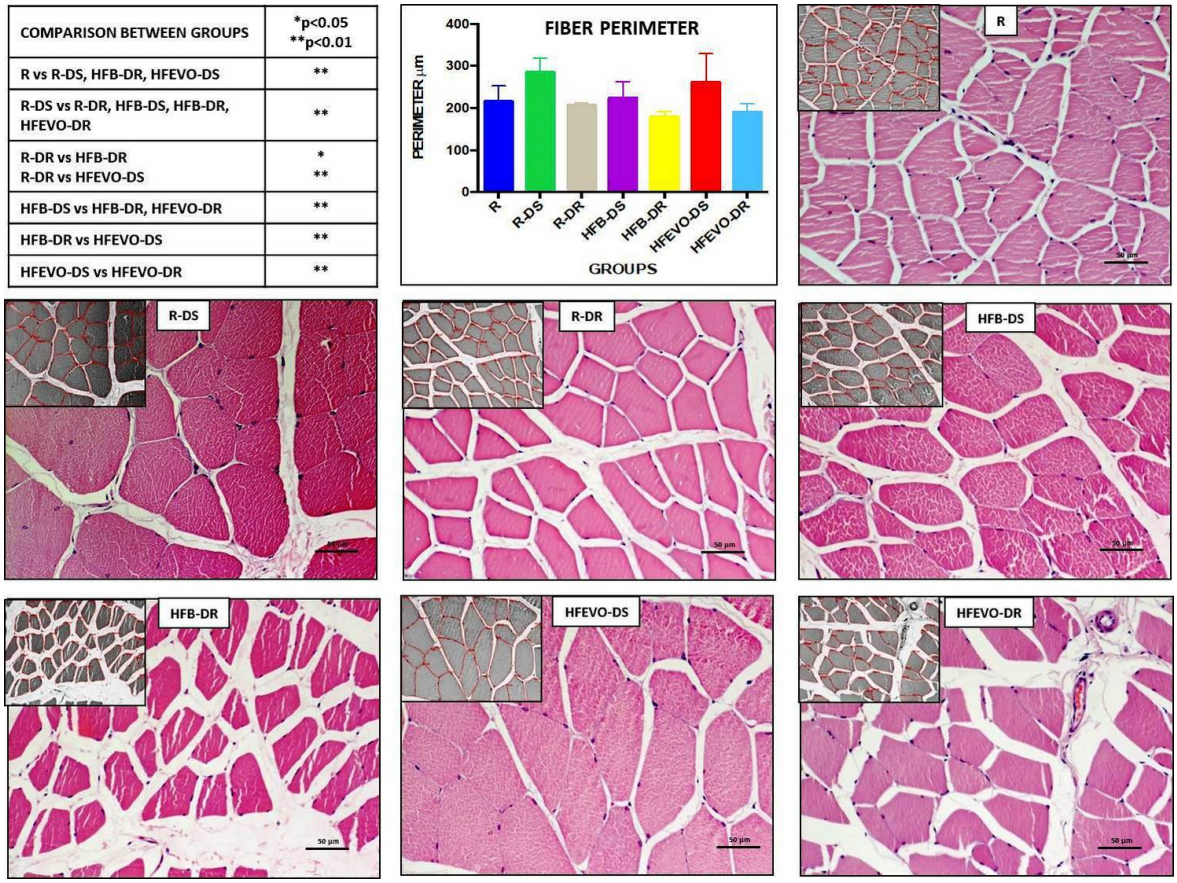


Figure 3. Hematoxylin & Eosin staining. Image analysis by software with morphometric analysis of the perimeter (µm) of the muscle fibers (inserts) and a graph representing the mean values of the perimeter (µm) in each group with statistical analysis (p-values in the table). For details, see the text. The data are presented as mean ± SD. Scale bars: 50 µm.

4.4 Statistical Analysis of the Histomorphometric Results

The fiber perimeters correlated positively with the dietary VitD content ($r = 0.603$; $p < 0.001$) and inversely with the dietary fat content ($r = -0.222$; $p < 0.05$). In our model, weight had no correlation with muscle fiber perimeter ($r = 0.003$). A multiple linear regression was calculated to predict muscle fiber perimeter in relation to weight at the end of the experiment, VitD, and fat content in diet. The results of the multiple linear regression indicated that there was a collective significant relationship between the fiber perimeter, VitD, and dietary fat ($F = 34.827$; $p < 0.001$, $r^2 = 363$). The individual predictors were examined further, and indicated that dietary VitD ($t =$

5.901; $p < 0.001$) and dietary fat ($t = -2.609$; $p < 0.05$) were significant predictors in the model.

4.5 Immunohistochemistry (IHC) Observations

4.5.1 Interleukin (IL)-1β

4.5.1.1 Muscle

IL-1β immunostaining in muscle fibers was mainly membranous and cytoplasmic and rarely nuclear; sometimes, it was detectable in the muscle satellite cells. The intensity of IL-1β immunostaining (densitometric count pixel2) was detected in many fields of the analyzed samples, albeit at different levels. In detail: the immunostaining in R was lower than in R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in R-DS, it was lower than in R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in R-DR, it was lower than in HFB-DS, HFB-DR ($p < 0.01$); in HFB-DS, it was higher than in HFEVO-DS ($p < 0.01$); in HFB-DR, it was higher than in HFEVO-DS ($p < 0.01$); in HFEVO-DS, it was lower than in HFEVO-DR ($p < 0.01$) (Figure 4). In relation to the immunostained area %, the statistical results were analogues to those of the intensity of immunostaining (data not shown).

COMPARISON BETWEEN GROUPS	*p<0,05 **p<0,01
R vs R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR	**
R-DS vs R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR	**
R-DR vs HFB-DS, HFB-DR	**
HFB-DS vs HFEVO-DS	**
HFB-DR vs HFEVO-DS	**
HFEVO-DS vs HFEVO-DR	**

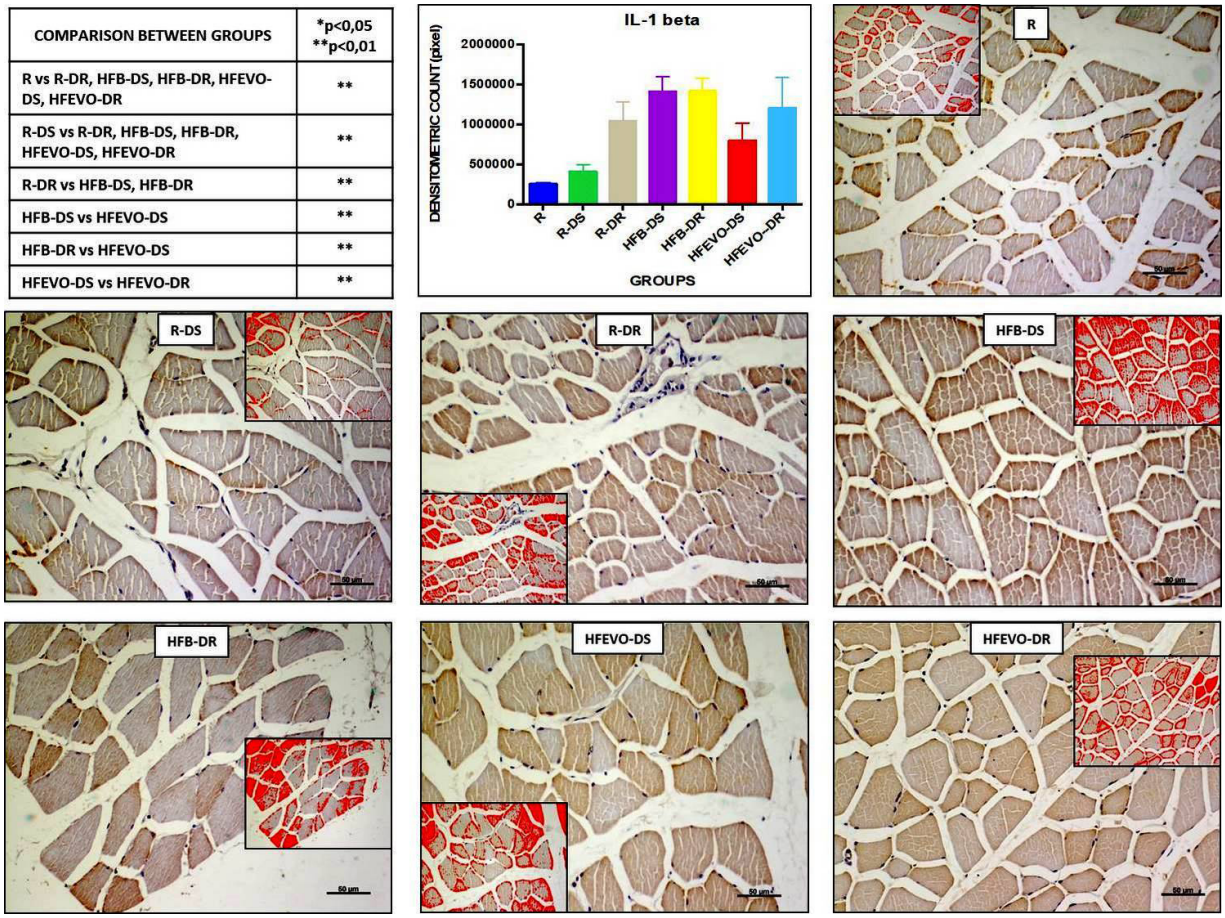


Figure 4. *IL-1β immunostaining, image analysis by software in which the red color represents the immunolabelling (inserts), and a graph representing the immunostained area % with statistical analysis (p-values in the table). For details, see the text. The data are presented as mean ± SD. Scale bars: 50 μm.*

4.5.1.2 Liver

In hepatocytes, IL-1 beta I-immunostaining was expressed primarily at cytoplasmic level even if in some field, 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 ($p<0,01$) (Figure 5). The IL-1 beta immunolabelling intensity distrubution is reported in Table 4.

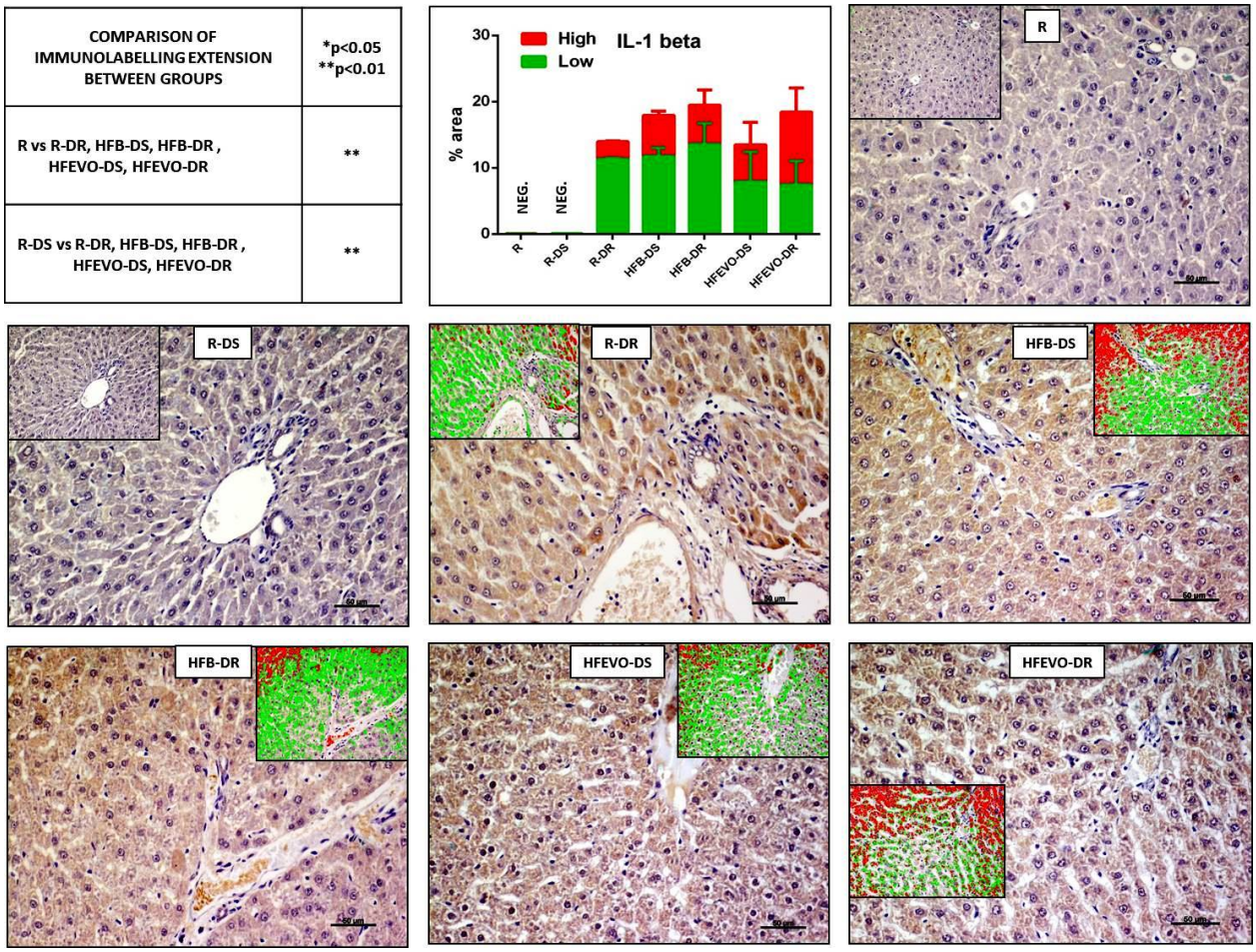


Figure 5. IL-1 beta-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained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

4.5.2 IGF-1

4.5.2.1 Muscle

In muscle tissue, IGF-1 immunostaining was mainly membranous and cytoplasmic and rarely nuclear. The intensity of IGF-1-immunostaining

(densitometric count pixel2) was detected at different degrees in all groups. In detail: in R, the immunostaining was higher than in HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in R-DS, it was higher than in HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in R-DR, it was higher than in HFB-DR ($p < 0.01$); in R-DR, it was higher than in HFEVO-DR ($p < 0.05$); in HFB-DS, it was higher than in HFB-DR ($p < 0.01$) (Figure 6).

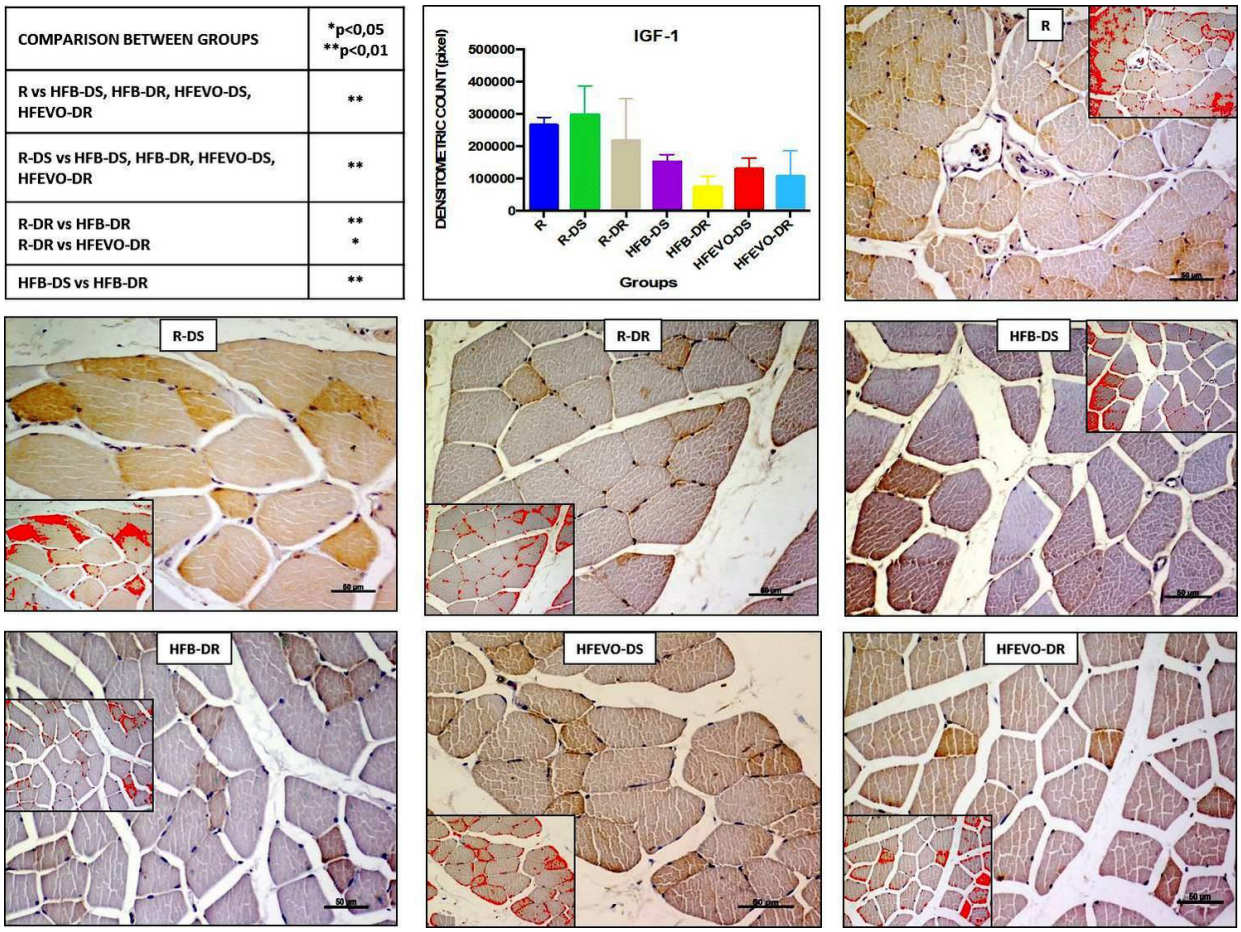


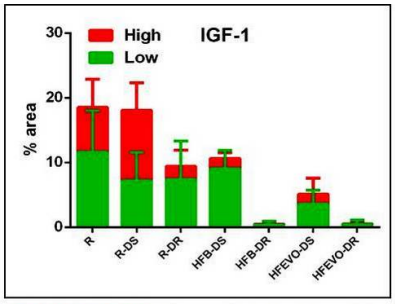
Figure 6. *IGF-1 immunostaining, image analysis by software in which the red color represents the immunolabelling (inserts), and a graph representing the intensity of immunostaining (densitometric count pixel2) with statistical analysis (p-values in the table). For details, see the text. The data are presented as mean ± SD. Scale bars: 50 µm.*

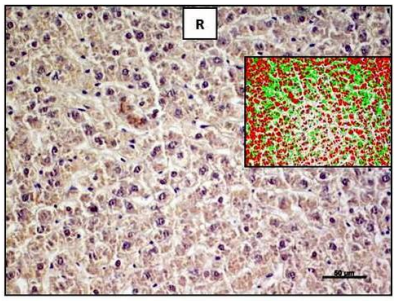
4.5.2.2 Liver

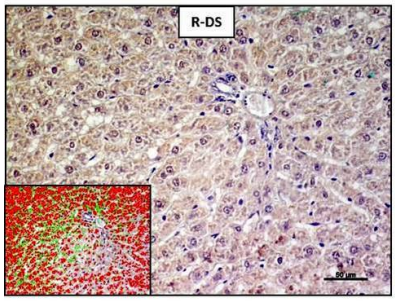
IGF-1-immunostaining in hepatocytes was mainly cytoplasmic, sometimes it was also nuclear. Rarely, it was detected in the cells of the bile ducts. The

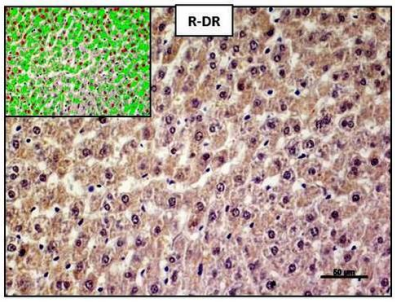
IFG-1-immunostaining extension was detected at different degrees in all groups, even if in HFB-DR and HFEVO-DR only traces of immunostaining were present. In detail: the immunostaining in R and R-DS was greater than in R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p<0,01$); in R-DR was greater than in HFB-DR, 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 ($p<0,05$) (Figure 7). The IGF-1 immunolabelling intensity distrubution is reported in Table 4.

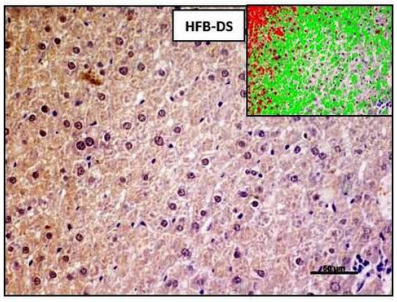
COMPARISON OF IMMUNOLABELLING EXTENSION BETWEEN GROUPS	*p<0.05 **p<0.01
R vs R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR	**
R-DS vs R-DR, HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR	**
R-DR vs HFB-DR, HFEVO-DR	**
HFB-DS vs HFB-DR, HFEVO-DR	**
HFB-DS vs HFEVO-DS	*
HFEVO-DS vs HFEVO-DR	**

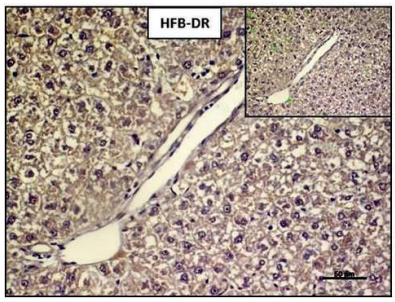


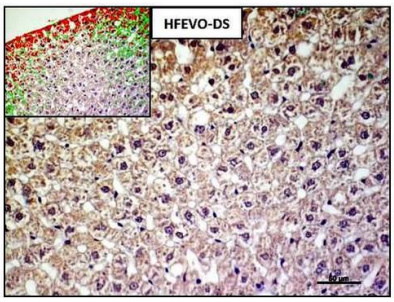












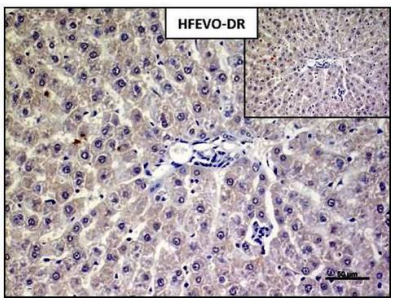


Figure 7: IGF-1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 μm.

4.5.3. Dickkopf (DKK) Wingless-type (WNT) Signaling Pathway Inhibitor 1

4.5.3.1 Muscle

DKK-1-immunostaining was mainly membranous and cytoplasmic and rarely nuclear in the muscle fibers. The intensity of DKK-1-immunostaining (densitometric count pixel2) was detected in all groups at different levels. In detail: the immunostaining in R was lower than in R-DS, HFB-DS, HFEVO-DS ($p < 0.01$); in R, it was lower than in R-DR, HFB-DR ($p < 0.05$); in R-DS, it was higher than in R-DR, HFB-DR, HFEVO-DR ($p < 0.01$); in R-DR, it was lower than in HFB-DS ($p < 0.01$) and HFEVO-DS ($p < 0.05$); in HFB-DS, it was higher than in HFB-DR and HFEVO-DR ($p < 0.01$); in HFB-DR, it was lower than in HFEVO-DS ($p < 0.05$); in HFEVO-DS, it was higher than in HFEVO-DR ($p < 0.01$) (Figure 8). DKK-1 immunostaining was highlighted in the sarcoplasm in groups in which it had a greater extension (immunostained area %), whereas it was mainly highlighted close to the plasma membrane in groups where the immunostained area % had a smaller extension. The statistical results of the immunostained area % were analogues to those of the intensity of immunostaining (data not shown).

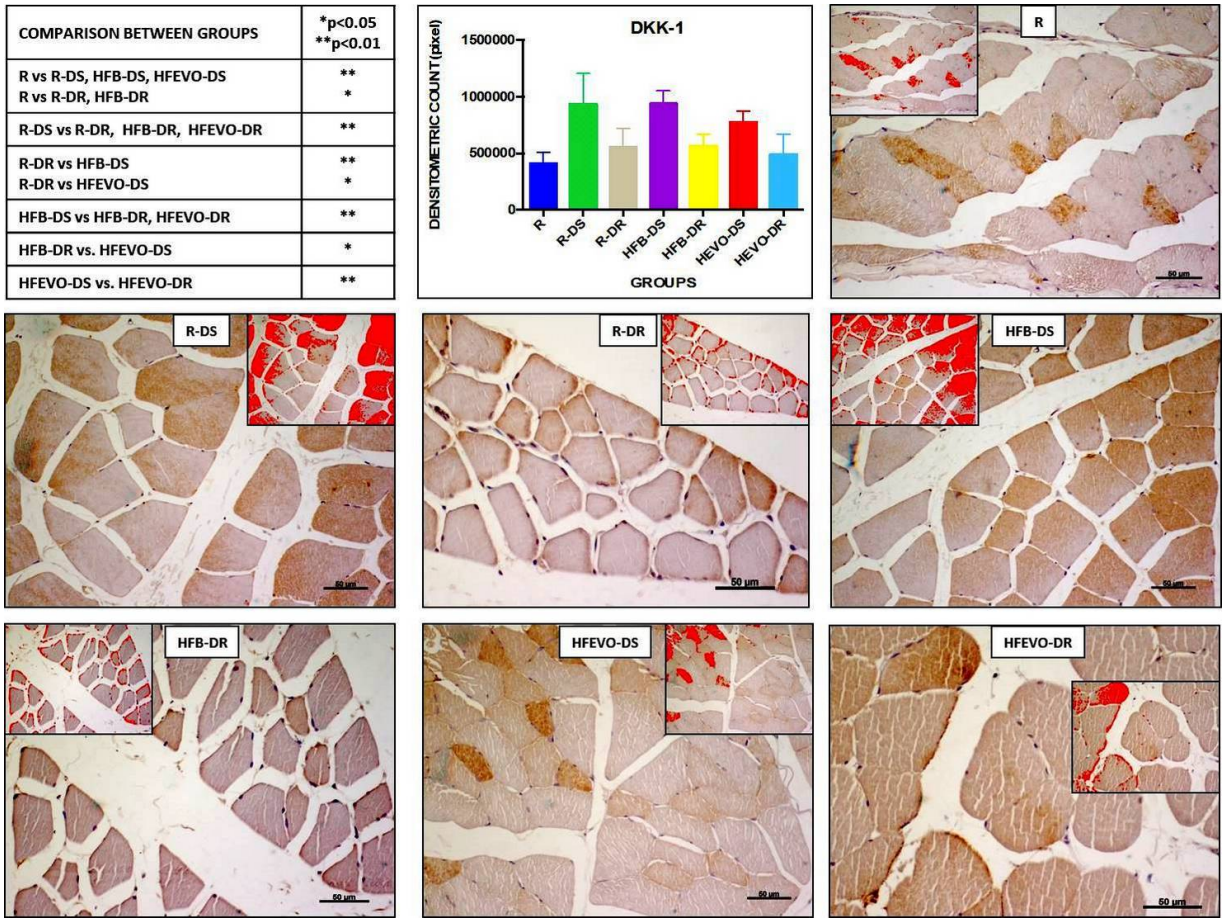


Figure 8. *DKK-1 immunostaining, image analysis by software in which the red color represents the immunolabelling (inserts), and a graph representing the intensity of immunostaining (densitometric count pixel2) with statistical analysis (p-values in the table). For details, see the text. The data are presented as mean ± SD. Scale bars: 50 µm.*

4.5.3.2 Liver

In liver parenchyma, DKK-1-immunostaining was expressed mainly in the cytoplasm of cells, even is 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 HBF-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 ($p<0,05$) (Figure 9). The DKK-1 immunolabelling intensity distribution is reported in Table 4.

COMPARISON OF IMMUNOLABELLING EXTENSION BETWEEN GROUPS	*p<0.05 **p<0.01
R vs HFB-DS, HFB-DR. HFEVO-DR	**
R-DS vs HFB-DS, HFB-DR, HFEVO-DR	**
R-DR vs HFB-DS, HFB-DR	**
R-DR vs HFEVO-DR	*
HFB-DR vs HFB-DS, HFEVO-DR	**
HFB-DR vs HFEVO-DS	**

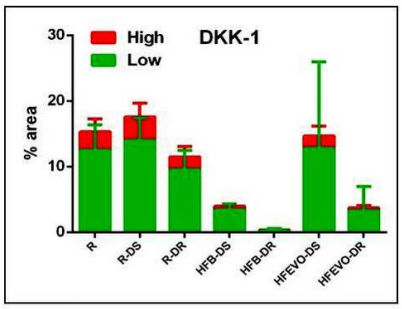


Figure 9. DKK-1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 μm.

4.5.4. VDR

4.5.4.1 Muscle

In muscle fibers, VDR immunostaining was mainly cytoplasmic and, in some samples, nuclear. The intensity of VDR immunostaining (densitometric count-pixel2) was higher in R, R-DS, HFB-DS, and HFEVO-DS groups. In detail: in R, the immunostaining was higher than in R-DR, HFB-DR, HFEVO-DR ($p < 0.01$); in R-DS, it was higher than in R-DR, HFB-DR, HFEVO-DR ($p < 0.01$); in R-DR, it was lower than in HFB-DS, HFB-DR, HFEVO-DS, HFEVO-DR ($p < 0.01$); in HFB-DS, it was higher than in HFB-DR, HFEVO-DR ($p < 0.01$); in HFB-DR, it was lower than in HFEVO-DS ($p < 0.01$); in HFEVO-DS, it was higher than in HFEVO-DR ($p < 0.01$) (Figure 10). In relation to the immunostained area %, the statistical results were analogues to those of the intensity of VDR immunostaining (data not shown).

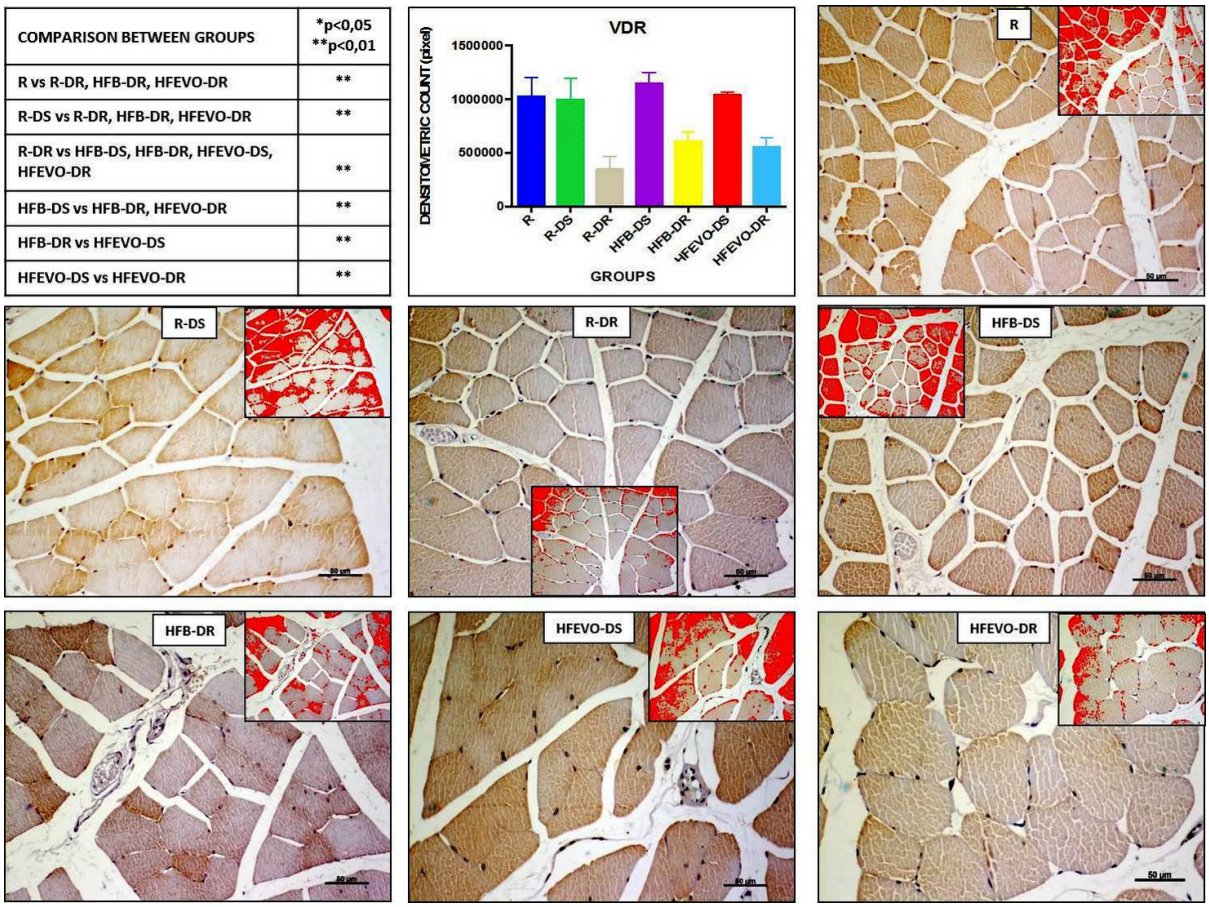


Figure 10. *VDR immunostaining, image analysis by software in which the red color represents the immunolabelling (inserts), and a graph representing the intensity of immunostaining (densitometric count pixel2) with statistical analysis (p-values in the table). For details, see the text. The data are presented as mean ± SD. Scale bars: 50 μm.*

4.5.4.2 Liver

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 ($p<0,01$) (Figure 11). The VDR immunolabelling intensity distribution is reported in Table 4.

COMPARISON OF IMMUNOLABELLING EXTENSION BETWEEN GROUPS	*p<0.05 **p<0.01
R vs R-DS, HFB-DS	*
R vs HFB-DR, HFEVO-DS, HFEVO-DR	**
R-DS vs R-DR	*
R-DS vs HFB-DR, HFEVO-DR	**
R-DR vs HFB-DR, HFEVO-DR	**
R-DR vs HFEVO-DS	*
HFB-DS vs HFB-DR, HFEVO-DR	**
HFB-DR vs HFEVO-DS	**
HFEVO-DS vs HFEVO-DR	**

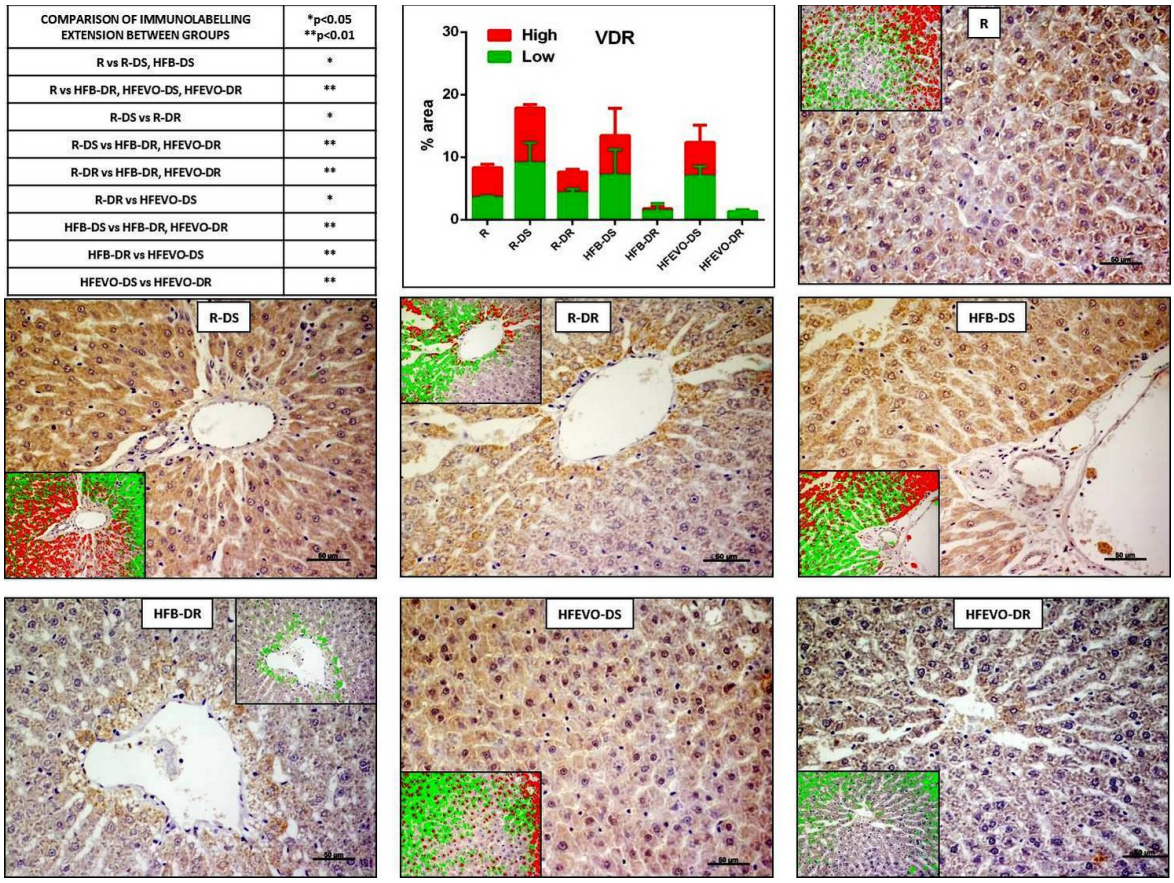


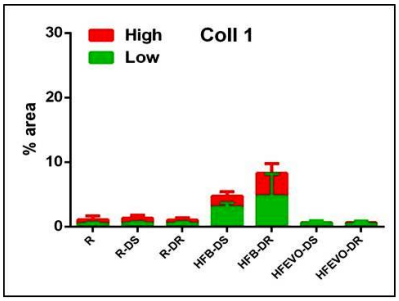
Figure 11. VDR-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area

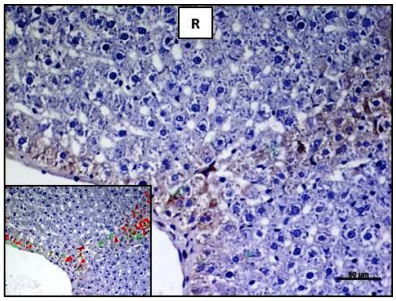
(immunolabelling intensity), and a graph representing the immunolabelling extension (% of total immunostained area) with statistical analysis (p-values in the table). For details, see the text. Data are presented as mean±SD. Scale bars: 50 μm.

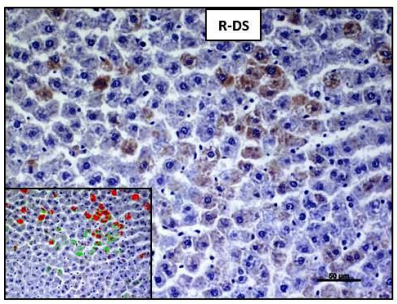
4.5.5 Collagen I (Liver)

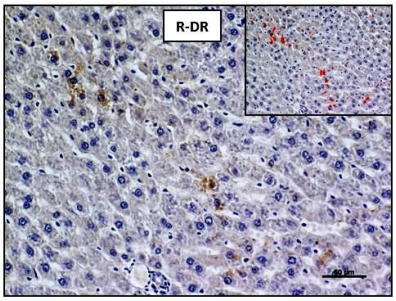
Collagen I-immunostaining was expressed both at cytoplasmic and nuclear levels in hepatocytes; it was barely perceptible in the cells of the bile ducts. The immunolabelling extension (% of total immunostained area) of Collagen I was statistically greater in HFB-DS and HFB-DR groups, when compared to 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 ($p<0,01$) (Figure 12). The Collagen 1 immunolabelling intensity distribution is showed in Table 4.

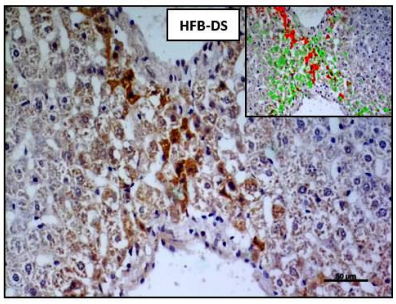
COMPARISON OF IMMUNOLABELLING EXTENSION BETWEEN GROUPS	*p<0.05 **p<0.01
R vs HFB-DS, HFB-DR	**
R-DS vs HFB-DS, HFB-DR	**
R-DR vs HFB-DS, HFB-DR	**
HFB-DS vs HFEVO-DS, HFEVO-DR	**
HFB-DR vs HFEVO-DS, HFEVO-DR	**

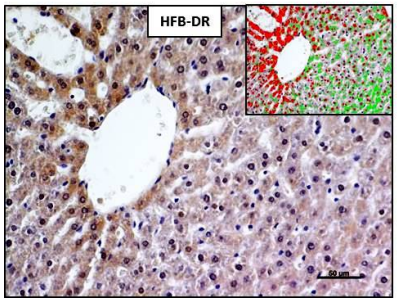


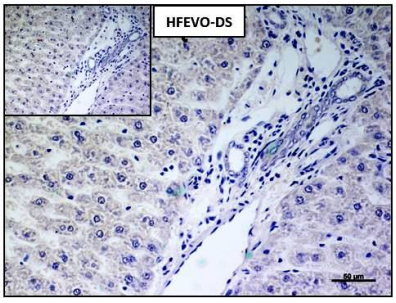












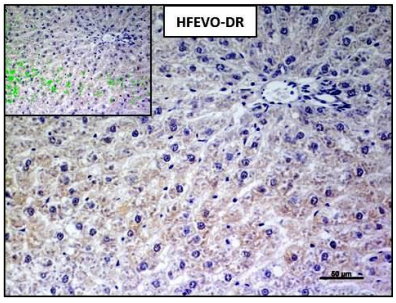


Figure 12. *Collagen 1-immunostaining and its image analysis by software (inserts) in which red color represents the % of high immunostained area and the green represents the % of low immunostained area (immunolabelling*

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.

Table 4. Liver immunostaining

Groups	COLL 1	IL-1 beta	VDR	DKK-1	IGF-1
R	+/-	+/-	+	+	+
	+/-	+/-	+	++	++
R-DS	+/-	+/-	+	+	++
	+/-	+/-	+	++	+
R-DR	+/-	+	+	+	+
	+/-	++	+	+	+
HFB-DS	+	+	+	+/-	+
	+	++	+	+	+
HFB-DR	+	+	+/-	+/-	+/-
	+	++	+	+/-	+/-
HFEVO-DS	+/-	+	+	+	+
	+/-	+	+	++	+
HFEVO-DR	+/-	++	+/-	+/-	+/-
	+/-	+	+	+	+/-

Table 4. Evaluation of COLL I, IL-1 beta, VDR, DKK-1, IGF-1 intensity of immunostaining in the liver, expressed as % area of high/low immunostaining. Intensity of immunostaining was scored as percentage of high or low immunostained areas in six categories: negative (-); <1% (+/-); 1<10% (+); 10<20% (++); 20<35% (+++), and >35% (++++). Blue lines: High immunostaining; white lines: Low immunostaining. The Collagen 1 immunolabelling intensity distribution was similar in every group. In R-DR, HFB-DS and HFB-DR groups, the low IL-1 immunostaining prevailed on the high immunostaining, instead in HFEVO-DR the high immunostaining prevailed on the low immunostaining. High and low VDR immunostaining distribution was similar in R, R-DS, R-DR, HFB-DS and HFEVO-DS groups, instead in HFB-DR and HFEVO-DR groups the low immunostaining prevailed on the high immunostaining. In R, R-DS, HFB-DS, HFEVO-DS and HFEVO-DR groups the

DKK-1 low immunostaining prevailed on the high immunostaining. The distribution between IGF-1 high and low immunostaining was similar in almost all groups and only in R and R-DS there was some difference, indeed in R group the low immunostaining was prevalent, on the contrary in R-DS group the high immunostaining prevailed on the low one.

4.6 Statistical Analysis of Immunohistochemistry

Weight at 10th week was directly related to the VitD content in diet ($p<0,01$). Moreover, VitD content was directly related to liver IGF-1 expression ($r=0,410$; $p<0,01$) and DKK-1 expression in both liver ($r= 0,283$; $p<0,05$) and muscle ($r=0,691$; $p<0,01$). Conversely, VitD content was negatively related to IL-1 beta in both liver *($r = -0.286$; $p < 0.05$)* and muscle *($r = -0.274$; $p < 0.05$)*. VDR expression was positively correlated to dietary VitD content ($r = 0.799$; $p < 0.01$), weight ($r = 0.327$; $p < 0.01$), muscle fiber perimeter ($r = 0.410$; $p < 0.01$), and DKK-1 ($r = 0.445$; $p < 0.01$) in muscle. Moreover IL-1β negatively correlated with muscle fiber perimeter ($r = −0.420$; $p < 0.01$), VDR ($r = 0.322$; $p < 0.05$), and dietary VitD ($r = −0.274$; $p < 0.05$). In muscle also DKK-1 expression was positively related to dietary VItD content ($r = 0.697$; $p < 0.01$), VDR, and fiber perimeter ($r = 0.493$; $p < 0.01$).

Conversely, the respective energy from fat, in each diet, was also directly related to weight at 10th week, but negatively related to IGF-1 in both tissues ($p<0,01$) and DKK-1 expressions in the liver ($r= -0,556$; $p<0,01$). While IL-1β in both tissues ($p<0,01$) and collagen I expressions in the liver ($r=0,281$; $p<0,05$) increased linearly with the fat content. Liver IGF-1 expression was directly related to DKK-1 ($r=0,721$; $p<0,01$) and VDR ($r=0,387$; $p<0,05$) and negatively with IL-1β ($r=-0,626$; $p<0,01$). Liver DKK-1 expression was inversely correlated with IL-1β expression ($r= -0,517$; $p<0,01$).

A multiple linear regression model was calculated to predict muscle fiber perimeter based on IL-1β, VDR, DKK-1 expressions. Results indicated that there was a collective significant relationship between the fiber perimeter, IL-1β, and DKK-1.

About 41.6% of the variance is explained by the model ($r^2 = 0.416$; $F = 21.330$; $p < 0.001$). The individual predictors were examined further and indicated that DKK-1 ($t = 4.95$; $p < 0.001$) and IL-1β ($t = -4.20$; $p < 0.001$) were significant predictors in the model.

5. 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 of 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 mirror the trophic effect of VitD. Nevertheless, weight was not a predictor of muscle fiber size, that in a multilinear regression model was instead explained by the VitD dietary content positively and dietary fat content negatively. It was interesting to notice how, although histopathological analysis showed only faint sign of liver fibrosis in HFB-DR, collagen I was expressed in both HFB groups. Moreover in both HFEVO groups it was underexpressed, similarly to control (Trovato *et al.*, 2018a). This is noteworthy since collagen I expression could be interpreted as early marker of liver fibrosis. Thus, although HFEVO diet consists of the 41% of energy from fat, the expression of collagen I is comparable with the controls, as if EVO had some protective effects on the liver against collagen I synthesis. This observation needs to be confirmed with longer term studies, since this finding could only be attributable to

the duration of dietary exposure. Conversely, VitD has been shown to have anti-inflammatory and antifibrotic properties and to inhibit the proliferation of HSC, which are responsible of 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 detectable in terms of collagen I deposition and the effect on collagen I expression seems only attributable to the EVO content. This is also confirmed by the fact that collagen I expression seems independent from that of VDR (Trovato *et al.*, 2018a).

Otherwise, EVO did not show the same beneficial effect on liver inflammation, indeed both HFB and HFEVO were strongly related to IL-1β expression, without any difference. One explanation could be that high-fat diet determines the accumulation of visceral fat leading to increased secretion of pro-inflammatory cytokines that may promote the development of NAFLD (Lim *et al.*, 2015). It is also notable that the restriction of VitD determined a similar detrimental effect in rats fed with R diet. In fact, previous studies showed how VitD interferes with the increase in pro-inflammatory cytokine expressions in muscle following intensive physical exercise (Choi *et al.*, 2013). According to our previous study on muscle confirmed by the present one, a similar effect was expected also for HFEVO groups in the liver, especially because EVO in other models improved diet-induced steatosis (Musumeci *et al.*, 2014, Jurado-Ruiz *et al.*, 2017). Our finding could probably be attributed to the high-fat content in the HFEVO diet, which could blunt the beneficial properties of the phenolic compounds and MUFA, as oleic acid, that have been described with a strong anti-inflammatory action (Calder, 2006, Carrillo *et al.*, 2012). Moreover EVO has a reduced concentration of polyunsatured fatty acids (PUFA) in comparison to the regular diet content, and this could be a further explanation of the reduced antinflammatory action in HFEVO groups in comparison to R (Calder, 2006). Indeed, HFEVO-DR together with HFB groups showed initial signs of steatosis and this is in line with the IGF-1 expression that was strongly reduced in HFB and HFEVO

comparing to control. This difference was even more pronounced considering the DR groups, in which only traces of immunostaining were present.

In fact, GH and IGF-1 exert significant effects on body composition, and lipid and glucose metabolism and reduced 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 anti-fibrotic properties in rodent models of NASH (Dichtel *et al.*, 2017). Moreover VDR expression showed correlation to that of IGF-1. The liver is the major source of circulating IGF-1 and is also considered a target tissue for VitD (Ameri *et al.*, 2013a). We can hypothesize that VitD restriction and high-fat diets, in general, are detrimental for IGF-1 expression with consequence on liver steatosis. Moreover IGF-1 in both liver and muscle was negatively related to IL-1β expression. A small recent study demonstrated that concentrations of IGF-1 were significantly higher in patients treated with VitD supplementation (Ameri *et al.*, 2013b). Coversely, 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 1α-hydoxylase in the kidney (Ameri *et al.*, 2013b), whereas there seems to be no effect of IGF-1 on 25-hydroxylation (Nesbitt & Drezner, 1993).

Moreover, IGF-1 seems 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 & Steinbeisser, 2014).

We hypothesized that VitD could exert its action on liver through enhancing DKK-1 expression. Indeed, Wnt/β-catenin signaling promotes fibrosis in response to injury in different tissues, including liver (Ozhan & Weidinger, 2015), and 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 early stage, confirm the negative correlation between DKK-1 and collagen I expression in liver tissue. Moreover it is confirmed the

linear correlation between VitD administration VDR and DKK-1, that were both decreased in DR groups and most of all in HFB, indicating a potential involvement of VitD in Wnt/β-catenin signaling not only in cancer (Pendas-Franco *et al.*, 2008, Rawson *et al.*, 2012, Johnson *et al.*, 2015) but also in muscle pathophysiology. Conversely, both hepatic IGF-1 and DKK-1 expressions reduced as the percentage of dietary fat increased while in muscle this trend is confirmed only for IGF-1. On the other hand, DKK-1 role in liver diseases is still debated, since it was proposed by some authors as noninvasive marker of NAFLD and 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 (Vongsuvanh *et al.*, 2016). Unfortunately, this is a morphological study, so our conclusions are limited by the lack of serum dosages.

Our results on muscle follow the findings on liver tissue. We proved the original hypothesis, since the muscle fibers of rats fed with EVO-based high-fat diet (HEVO-DS) were hypertrophic, comparable to those of the regular diet (R-DS) group, confirming that EVO does not have the same detrimental effect on muscle fiber as other kinds of fat, in particular butter, as shown in HFB groups. In fact, we found an inverse correlation between the dietary fat content and the perimeter of muscle fibers. In HFB-DR, the muscle fibers appeared hypotrophic, probably because VitD action was lacking and the HFB diet led to the increase of IL-1β expression and to a dramatic decrease of IGF-1 expression. When the supplementation of VitD antagonized the fat effect, as in HFB-DS, the fibers were not different from the control. Surprisingly, VitD depletion, per se, did not show any effects on muscle fiber size when associated with the three dietary profiles, and this was probably due to the relatively short term of the restriction in young rats. In fact, VitD restriction determines increasing in IL-1β expression indicating that early inflammatory changes are occurring. Conversely, HFEVO-DS did not show detrimental effects on muscle

fiber size, although IL-1β was more expressed in comparison to control, but even significantly lower that HFB-DS diet. However, considering HFD-DR and HFEVO-DR, the expression of IL-1β becomes comparable. This could be interpreted by the fact that VitD restriction in some ways reduces the beneficial effect of EVO and concurs with the detrimental effects of high-fat diet, in general, on muscle and systemic inflammation.

Our results are in line with the recent literature. Several studies have reported that a Mediterranean dietary regimen has a favorable role in the prevention of sarcopenia (Hashemi *et al.*, 2015, Mohseni *et al.*, 2017). Moreover, in our previous study, we demonstrated that EVO possesses anti-oxidative properties and can improve the adaptive response of the body in conditions of oxidative stress (Musumeci *et al.*, 2014, Musumeci *et al.*, 2015), thanks to its ability to scavenge free radicals, thus preventing cellular injury. Obesity is a known state of chronic inflammation associated with a reduction in skeletal muscle regeneration (Akhmedov & Berdeaux, 2013). Indeed, obese mice displayed abnormal muscle fiber size, providing evidence for a cross-talk between adipocytes and muscle cells, leading to muscle atrophy (Pellegrinelli *et al.*, 2015). Interestingly, similar results on muscle fibers are showed in our high-fat diet model, in which rats were not obese compared to controls. VitD biological effect on muscle recovery raised considerable interest in literature (Holick, 2007, Ceglia, 2009). Moreover, acute inflammation triggers the process of muscle regeneration (Tidball & Villalta, 2010), with a critical role of the immune cells. Several observational studies have been performed on the role of VitD in skeletal muscle proliferation, differentiation, and apoptosis as well as in immune cells regulation (Verstuyf *et al.*, 2010). VDR knock-out mice have provided evidence for a role of VDR in the development and differentiation of skeletal muscle, showing smaller myofibers in knock-out mice than in wild-type mice (Endo *et al.*, 2003). Animal models suggested that the administration of VitD leads to an increase in proliferation and a decrease in apoptosis in injured muscle (Stratos *et al.*, 2013), and,

conversely, a high-fat diet with VitD deficiency would markedly impair bone and muscle metabolism (Oku *et al.*, 2016). A clinical trial highlighted that VitD supplementation in sedentary women increased VDR expression and muscle fiber size (Ceglia *et al.*, 2013). These results indicate a direct effect of VitD on myocites, leading to the enhancement of skeletal muscle recovery (Domingues-Faria *et al.*, 2016). VitD has effects not only on muscle cells (Ceglia, 2009), but also on immune cell functions (Hewison, 2012), so we decided to investigate the relationship between VitD and inflammation. Several recent studies have identified inflammatory mediators, such as IL-1, IL-6, and Tumor Necrosis Factor (TNF)-α, as contributing to the development of sarcopenia (Schaap *et al.*, 2006), since they may induce muscle atrophy promoting protein degradation and reactive oxygen species (ROS) accumulation (Arthur & Cooley, 2012, Imbesi *et al.*, 2014). Adipocytes secrete inflammatory cytokines, as well as adipokines, which promote inflammation and fat mass accumulation, impairing muscle mass formation (Arthur & Cooley, 2012). IL-1β is a major cytokine produced largely by adipose tissue macrophages. Its release is enhanced in obesity (Bing, 2015) and it is implicated in the development of insulin resistance (Bing, 2015). The blood concentration of anti-inflammatory cytokines IL-10 and IL-13 were found decreased in VitD-deficient subjects following physical training, compared to controls. Conversely, in a rat model, VitD supplementation modulated pro-inflammatory cytokine expression in muscle following intensive physical exercise (Choi *et al.*, 2013). Moreover, a recent research by our group demonstrated similar effects of the administration of EVO with diet on muscles undergoing exhaustive exercise in rats, supporting the view that EVO can improve the adaptive response of the body in conditions of oxidative stress (Musumeci *et al.*, 2013).

Insulin resistance has been associated to cases of both excess fat and sarcopenia, given that skeletal muscle is one of the major target tissues of insulin action (Cleasby *et al.*, 2016). GH and IGF-1 act on body composition and protein metabolism in the

skeletal muscle (Perrini *et al.*, 2010). At the molecular level, IL-6 and IL-1β downregulate IGF signaling, decreasing muscle protein synthesis (Glass, 2010). Fatty liver has been related to the reduction of insulin sensitivity at the level of skeletal muscle and adipose tissue (Gaggini *et al.*, 2013). Authors showed that VitD supplementation increased circulating IGF-1 (Ameri *et al.*, 2013b), and we confirmed this observation, but what was even clearer is the reduction of IGF-1 in both high-fat diets. Our results confirmed the findings of a recent study, in which muscle size and strength, as well as IGF-1 levels, decreased in mice with diet-induced NAFLD, and the morphological aspects of sarcopenia were observed in early stages, prior to the development of liver fibrosis (Cabrera *et al.*, 2016).

DKK-1 expression correlated directly with muscle fiber perimeter and VDR expression and, most importantly, with dietary VitD supplementation, without differences attributable to different fats. It is already known that the targets for VitD include LRP 5, the Wnt coreceptor, that plays a key role in osteoblast proliferation, differentiation, and function (Sankaralingam *et al.*, 2014). In breast and colon cancer, VitD increased dose dependently the expression of the extracellular canonical Wnt inhibitor, DKK-1, that is associated with growth inhibition, showing a protective role of VitD against breast cancer development, progression, and metastasis (Johnson *et al.*, 2015). DKK-1 is involved in many processes of bone metabolism, but exerts also action on muscle. This is well known in cardiomyocytes, where Wnt/β-catenin signaling has been implicated in the regulation of cardiac remodeling and injury responses (Ozhan & Weidinger, 2015). Wnt/β-catenin signaling promotes fibrosis in response to injury in order to prevent cardiac dilation (Ozhan & Weidinger, 2015) and, interestingly, it has also been reported in fibrotic diseases of other organs (liver, lung, and kidney), since it is crucial for the differentiation of fibroblasts and for collagen production (Ozhan & Weidinger, 2015). Increased Wnt signaling may inhibit myogenicity, impairing the muscle regenerative potential by promoting the transition of aged skeletal muscle to fibrogenic tissue, thereby accelerating aging (Arthur &

Cooley, 2012). This lineage conversion can be suppressed by Wnt inhibitors (Brack *et al.*, 2007), like DKK-1 that is upregulated by VitD (Fleet *et al.*, 2012). In our model VitD supplementation causes muscle fiber hypertrophy both in regular and HFEVO diet, probably through a pathway involving IGF-1 and DKK-1. Our results need to be strengthened by further studies, since the small sample size and the use of immunohistochemistry as unique technique are major limitations.

6. Conclusions

A short-term high-fat diet could damage liver tissue in terms of inflammation and collagen I deposition, putting the basis for the subsequent steatohepatitis, still not identifiable histopathologically. Vitamin D restriction increases inflammation and reduces the expression of IGF-1 in the liver, worsening the fat-induced changing. EVO seems protective against collagen I production. High-fat western diet could impair also muscle metabolism and create a basis for subsequent muscle damage. Vitamin D shows trophic action on muscle fibers, not only in rats fed with regular diet, but also in the case of a diet mimicking the Mediterranean diet. Otherwise, in butter-based diets there is no difference on IL-1β according to VitD administration, indicating that the dietary intervention with VitD does not counteract the detrimental effect of high-fat butter based diet. Even if the complex mechanism linking IGF-1, DKK-1 and VitD must be further elucidated, our research supports the hypothesis that the relationship between muscle and liver tissue starts earlier than NAFLD develops and that we can modify the natural history of the disease with a dietary intervention (illustration 3). However, this is a preliminary research, and further studies are needed to strengthen and confirm our data and provide dietary recommendation.

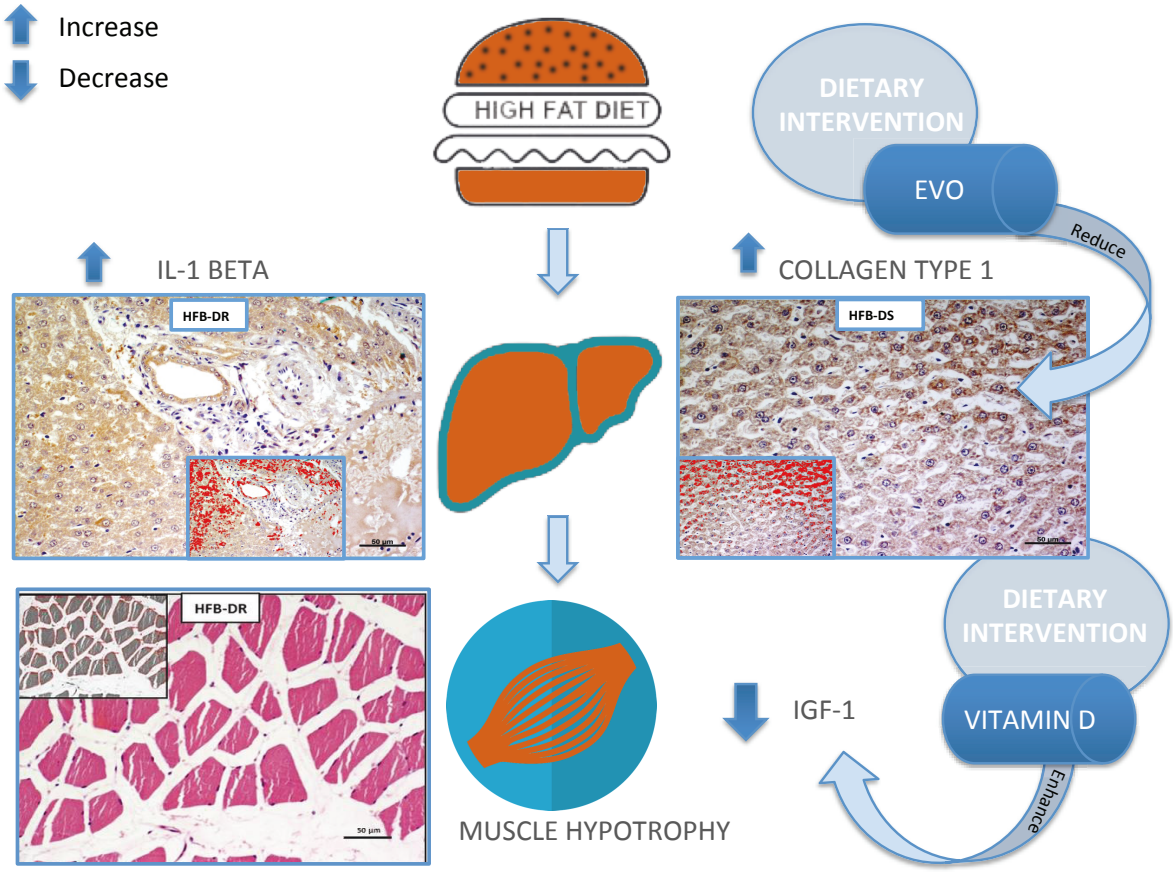


Illustration 3. High fat diet causes the increase of inflammation in both liver and muscle (IL-1 β), and increased collagen type I expression in the liver, this leads to a reduced expression of IGF-1 in muscle and muscle hypotrophy. Dietary interventions could modify the natural history of this process. Indeed when butter is substituted with extra-virgin olive oil (EVO), collagen I is less expressed in the liver. Moreover the supplementation of Vitamin D blunts the high-fat diet effect on hypotrophy and IGF-1 expression (Trovato et al. The interplay between liver and muscle begins much earlier than cirrhosis. A diet-induced animal model. Presented as a poster at International Liver Congress 2018).

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