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COIMBRA

Beatriz Maria Lemos Ormonde

ANTIATHEROGENIC PROPERTIES FROM OLEACEIN EXTRACTED FROM OLIVE TREE AND OLIVE OIL – EXPERIMENTAL STUDY IN ANIMAL MODEL

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Doutor Flávio Nelson Fernandes Reis e pelo Professor Doutor Carlos Manuel Marques Palmeira e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro de 2021

Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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Abbreviations

ACAT – Acyl-coenzyme A-Cholesterol Acyltransferase

ALD – Alcoholic Liver Disease

ALT – Aminotransferase

Ang II – Angiotensin II

Apo – Apolipoprotein

ApoE – Apolipoprotein E

ApoE KO – Apolipoprotein E knockout

AST – Aspartate Aminotransferase

ATD – Atherogenic Diet

BW – Body Weight

CAT – Catalase

cDNA – Complementary Deoxyribonucleic Acid

Chol – Cholesterol

CVD – Cardiovascular Disease

DNA – Deoxyribonucleic Acid

EFSA –European Food Safety Authority

ET-1 – Endothelin-1

EASL – European Association for the Study of the Liver

EU – European Union

EVOO – Extra Virgin Olive Oil

GADPH – Glyceraldehyde-3-Phosphate Dehydrogenase

GSH – Glutathione

GSR – Glutathione Reductase

GST – Glutathione S-transferase

GTT– Glucose Tolerance Test

HCC – Hepatocellular Carcinoma

HDL – High-Density Lipoprotein

H&E – Hematoxylin and Eosin

HFD – High Fat Diet

HMG-CoA – Hydroxy-Methylglutaryl-Coenzyme A

HPRT – Hypoxanthine Phosphoribosyltransferase

HSD – High Sugar Diet

HTy – Hydroxytyrosol

ICAM – Intercellular Adhesion Molecule

IDL – Intermediate Density Lipoprotein
INE – Instituto Nacional de Estatística
IOC – International Olive Council
IPGTT – Intraperitoneal Glucose Tolerance Test
ITT – Insulin Tolerance Test
LCAT – Lecithin-Cholesterol Acyltransferase
LDL – Low-Density Lipoprotein
LFD – Low Fat Diet
LPL – Lipoprotein Lipase
LPS – Lipopolysaccharide
MDiet – Mediterranean Diet
NAFLD – Non-Alcoholic Fatty Liver Disease
NASH – Non-Alcoholic Steatohepatitis
NO – Nitric Oxide
OL – Oleuropein
OLEA – Oleacein
OLEO – Oleocanthal
OO – Olive Oil
PGI2 – Prostaglandin 2
RCT – Reverse Cholesterol Transport
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species
RT-PCR – Real-Time Polymerase Chain Reaction
S.E.M. – Standard Errors of the Mean
SEM – Scanning Electron Microscope
SOD – Superoxide Dismutase
STD – Standard Diet
TBAR – Thiobarbituric Acid Reactive Substances
TC – Total Cholesterol
T2DM – Type 2 Diabetes Mellitus
TG – Triglyceride
TXA2 – Thromboxane A2
USD – United States Dollars
USA – United States of America
VCAM – Vascular Cell Adhesion Molecule
VLDL – Very-Low-Density Lipoprotein

VSMC – Vascular Smooth Muscle Cells

WHO – World Health Organization

WT – Wild Type

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Resumo

A aterosclerose é o principal substrato para a maioria das doenças cardiovasculares (DCVs), que são a maior causa de mortalidade a nível mundial. Os fatores de risco para o desenvolvimento de aterosclerose e de outras DCVs, além dos não modificáveis, relacionam-se com estilos de vida pouco saudáveis, incluindo dietas hipercalóricas e hiperlipídicas, bem como sedentarismo. Estes fatores estão associados a outras doenças, tais como obesidade e esteatose hepática. Abordagens não farmacológicas, nomeadamente intervenções nutracêuticas, poderão servir como terapias adjuvantes às terapêuticas farmacológicas atualmente disponíveis. A Dieta Mediterrânica (MDiet), baseada numa grande variedade de alimentos pouco refinados, tem sido considerada como um modelo dietético por várias organizações internacionais. O azeite, uma das principais fontes de gordura da MDiet, composto essencialmente por ácidos gordos monoinsaturados, apresenta uma pequena pequena fração de compostos fenólicos, que têm sido vistos como uma opção nutracêutica, exibindo efeitos marcantes na prevenção destas doenças. Um dos mais promissores é a oleaceína (OLEA), um polifenol do grupo dos secoroidoides que já tem mostrado *in vitro* efeitos antioxidantes, anti-inflamatórios, sensibilizadores de insulina e ainda potencial para propriedades hipolipemiantes e antiateroscleróticas.

Neste sentido, formulámos a hipótese de que uma intervenção nutracêutica com OLEA poderia exercer efeitos hepato e ateroprotetores num modelo animal susceptível ao desenvolvimento de aterosclerose, o murganho *knockout* para a apolipoproteina E (APOE KO) alimentado com uma dieta aterogénica (ATD). Desta forma, definimos duas abordagens experimentais. A primeira usando murganhos C57BL/6J wild-type (WT) com o objetivo de comparar os efeitos metabólicos de três regimes alimentares – i) dieta controlo padronizada não refinada (STD, n=8), ii) dieta refinada com baixo teor de gordura (LF, n=8) e iii) dieta refinada aterogénica (ATD, n=8) - ao nível dos perfis glicémico, insulinémico e lipídico. A segunda avaliando o potencial efeito hepatoprotetor, antidislipidémico e anti-aterosclerótico da OLEA no murganho APOE KO, onde se definiram dois grupos - com e sem tratamento com 50 mg/kg/dia de OLEA (n=8 cada) – comparados com um grupo WT alimentado com ATD. Todos os grupos experimentais tiveram alimento e água fornecidos *ad libitum*.

Os perfis glicémicos e insulinémicos foram avaliados nos tempos inicial e final e o perfil lipídico foi caracterizado em termos de valores séricos de colesterol total (c-Total), TGs, c-LDL e c-HDL, bem como de conteúdos hepáticos de TGs. Utilizou-se

marcações histomorfológicas e observações de microscopia eletrónica de varrimento (SEM) para caracterizar as lesões hepáticas e vasculares e a deposição lipídica. Avaliou-se também os níveis séricos e hepáticos de SOD e quantificou-se a expressão hepática de SOD-1 e SOD-2 por RT-PCR.

Os animais WT alimentados com ATD apresentaram um aumento significativo do peso corporal, sem alterações de tolerância à glicose ou de sensibilidade à insulina. Constatou-se existir no mesmo grupo um aumento da concentração sérica de c-Total, c-LDL e c-HDL, acompanhado de acumulação de TGs no fígado. Além disso, exibiram reduzida expressão hepática de ambas as isoformas de SOD, o que pode ser indicativo de um processo de stress oxidativo característico destas doenças.

Os animais alimentados com a dieta LF apresentaram alterações metabólicas mais moderadas do que os submetidos a ATD, incluindo uma tendência para valores superiores de glucose circulante após 6 horas em jejum, bem como um aumento da concentração sérica de c-Total, c-LDL e c-HDL, e de TGs hepáticos, quando comparados com os animais alimentados com STD, sugerindo que a presença de ingredientes refinados nesta dieta, embora que com baixo teor de gordura, não impede um impacto negativo no metabolismo, comparativamente à dieta STD não refinada.

Em relação à segunda abordagem experimental, constatou-se (por H&E e SEM) existirem lesões vasculares nos animais APOE KO compatíveis com a literatura. A OLEA apresentou um efeito marcante no perfil lipídico dos murganhos APOE KO, causando uma redução marcada dos valores séricos de c-Total, c-LDL, c-HDL e TGs, bem como uma forte atenuação da concentração hepática de TGs. Estes dados foram corroborados pelas marcações hepáticas com Oil Red O e H&E, verificando-se um efeito marcado anti-dislipidémico nos murganhos APOE KO. Não se observaram diferenças significativas nos perfis glicémicos e insulinémicos entre os 3 grupos.

Globalmente, verifica-se que a suplementação com OLEA melhorou significativamente o perfil lipídico dos murganhos APOE KO, com forte proteção contra a deposição lipídica hepática e eventual efeito antioxidante. Mais estudos serão necessários para clarificar os mecanismos celulares e moleculares subjacentes à proteção contra o desenvolvimento de esteatose hepática neste modelo de hiperlipidema e aterosclerose.

Palavras chave: Aterosclerose; Esteatose hepática; Murganho APOE KO; Dietas refinadas e não refinadas; Oleaceína

Abstract

Atherosclerosis is the main substrate for most cardiovascular diseases (CVDs) which are the main cause of mortality worldwide. The risk factors for the development of atherosclerosis and CVDs, in addiction to non-modifiable are related to unhealthy lifestyles, including hypercaloric and hyperlipidic diets, as well as sedentary lifestyle. These factors are associated with other diseases, such as obesity and hepatic steatosis. The non-pharmacological approaches, namely nutraceutical interventions, can serve as adjuvant approaches to the currently available pharmacologic therapies. The Mediterranean Diet (MDiet), based on a wide variety of unrefined foods, has been considered a dietary model by several international organizations. Olive oil, one of MDiet's main sources of fat, composed essentially of monounsaturated fatty acids, has a small fraction of phenolic compounds, which have been seen as a nutraceutical option, exhibiting marked effects in preventing these diseases. One of the most promising is oleacein (OLEA), a polyphenol from the secoiridoids group that has already shown *in vitro* antioxidant, anti-inflammatory, insulin sensitizing effects and even potential for lipid-lowering and anti-atherosclerotic properties.

In this sense, we hypothesized that a nutraceutical intervention with OLEA could exert hepato- and atheroprotective effects in an animal model susceptible to the development of atherosclerosis, the apolipoprotein E (APOE KO) knockout mouse fed an atherogenic diet (ATD). In this way, we define two experimental approaches. The first using C57BL/6J wild-type (WT) mice to compare the metabolic effects of three diets – i) unrefined standardized control diet (STD, n=8), ii) refined low-fat diet (LF, n=8) and iii) atherogenic refined diet (ATD, n=8) - at the level of glycemic, insulinemic and lipid profiles. The second evaluating the potential hepatoprotective, anti-dyslipidemic and anti-atherosclerotic effect of OLEA in the APOE KO mouse, where two groups were defined - with and without treatment with 50 mg/kg/day of OLEA (n=8 each) - compared with a WT group fed with ATD. All experimental groups had food and water provided *ad libitum.*

The glycemic and insulinemic profiles were evaluated in the initial and final times and the lipid profile was characterized in terms of serum values of total cholesterol (c-Total), TGs, c-LDL and c-HDL, as well as hepatic contents of TGs. Histomorphological markings and scanning electron microscopy (SEM) observations were used to characterize liver and vascular lesions and lipid deposition. Serum and hepatic SOD

levels were also evaluated and the hepatic expression of SOD-1 and SOD-2 was quantified by RT-PCR.

WT animals fed with ATD showed a significant increase in body weight, without changes in glucose tolerance or insulin sensitivity. It was found that in the same group there was an increase in the serum concentration of c-Total, c-LDL and c-HDL, accompanied by accumulation of TGs in the liver. In addition, they exhibited reduced hepatic expression of both SOD isoforms, which may be indicative of an oxidative stress process characteristic of these diseases.

The animals fed the LF diet showed more moderate metabolic alterations than those submitted to ATD, including a trend towards higher values of circulating glucose after 6 hours of fasting, as well as an increase in the serum concentration of c-Total, c-LDL and c-HDL, and liver TGs, when compared to animals fed with STD, suggesting that the presence of refined ingredients in this diet, although low in fat, does not prevent a negative impact on metabolism, compared to the unrefined STD diet.

Regarding the second experimental approach, it was found (by H&E and SEM) that there were vascular lesions in APOE KO animals compatible with the literature. OLEA had a marked effect on the lipid profile of APOE KO mice, causing a marked reduction in the serum values of c-Total, c-LDL, c-HDL and TGs, as well as a strong attenuation of the hepatic concentration of TGs. These data were corroborated by the liver markings with Oil Red O and H&E, showing a marked anti-dyslipidemic effect in the APOE KO mice. There were no significant differences in glycemic and insulinemic profiles between the 3 groups.

Overall, it appears that supplementation with OLEA significantly improved the lipid profile of APOE KO mice, with strong protection against hepatic lipid deposition and an eventual antioxidant effect. Further studies will be needed to clarify the cellular and molecular mechanisms underlying protection against the development of hepatic steatosis in this model of hyperlipidema and atherosclerosis.

Keywords: Atherosclerosis; Hepatic steatosis; APOE KO mouse; Refined and unrefined diets; Oleacein.

|Chapter I – Introduction

1. Cardiovascular diseases – atherosclerosis and beyond

1.1. Definition, epidemiology and risk factors

Cardiovascular diseases (CVD) can be defined as broad group of disease that can affect the circulatory system - heart and/or blood vessels – and can include coronary artery disease, stroke, heart failure, hypertensive heart disease, rheumatic fever, among others (USDHHS, 2003). CVDs are the main cause of mortality worldwide, representing about 50% of all deaths in Europe (Townsend *et al*., 2016). However, from the second half of XX century, there was a significantly drop on mortality rate due CVDs, mainly ischemic heart diseases and stroke (Herrington *et al*, 2016). Recent data, from World Health Organization (WHO) and European Cardiovascular Disease Statistics, shows that these numbers continue to be highly worrying, representing about 18 million deaths due to CVDs worldwide and 3.9 million in Europe (WHO, 2021; Wilkins *et al*., 2017). Regarding Portugal, CVDs are responsible for 29.4% of all deaths (Costa, J. *et al*, 2017).

Furthermore, according to European Cardiovascular Disease Statistics, CVDs has major economic impact that affects individuals, health systems and societies, representing a burden cost to families and a significant proportion of total healthcare expenditure around the world. It is estimated that the total global cost of CVD is set to rise from approximately 863 billion USD in 2010 to a staggering 1044 billion. Only in 2015, it was estimated to cost the European Union (EU) economy 210 billion euros (Timmis *et al*., 2019).

Thus, it is necessary to continue to invest in scientific research to establish early and effective therapeutic interventions. In this sense, an approach centered on specific and determinant risk factors, which leads to the emergence and/or progression of CVDs, is imperative.

CVDs' risk factors can be divided into non-modifiable, which mainly include genetic aspects, age and gender, and modifiable risk factors, such as unhealthy lifestyle habits, particularly sedentarism and unbalanced (especially hypercaloric) diets, which can contribute to the emergence of dyslipidemia, obesity, diabetes, arterial hypertension, among others (**Fig. 1**).

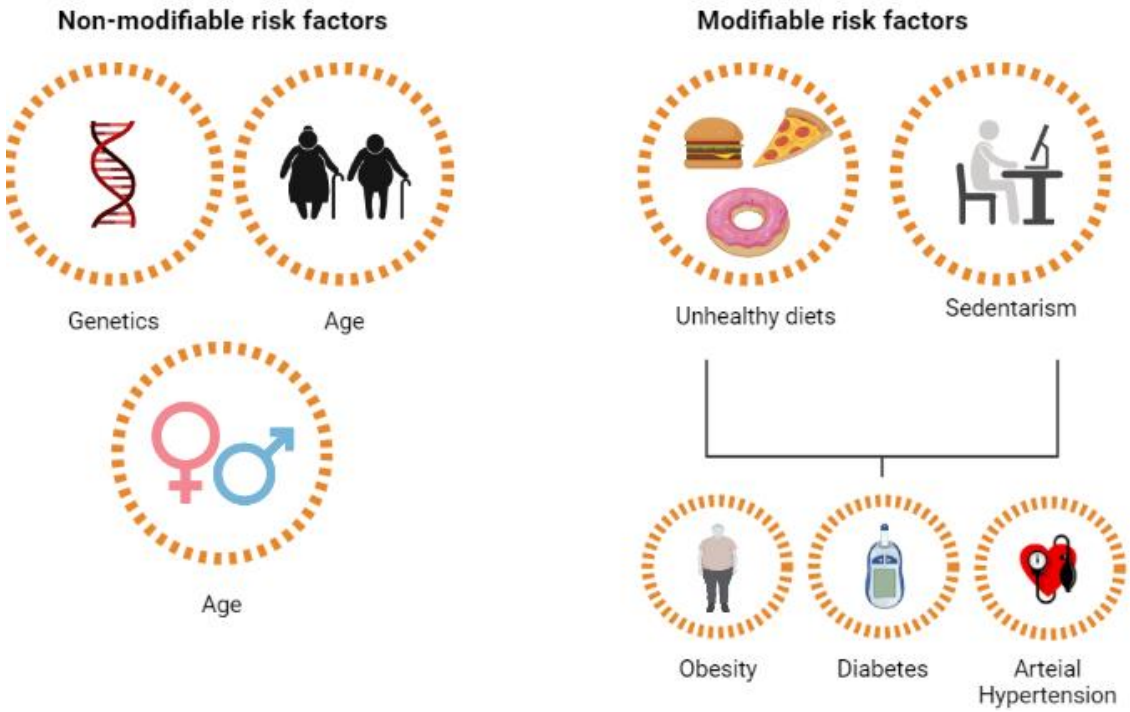

Figure 1 - Main non-modifiable and modifiable risk factors for CVD.

The correlation between an unbalanced diet and CVDs has been reported in several studies (Chandran *et al*., 2014; Slattery *et al*., 1998; Albanes, 1987; Babey *et al*., 2008; Bodicoat *et al*., 2015; Polsky *et al*., 2016; Ditzhuijzen *et al.*, 2016; Alter and Eny, 2005; Bahadoran *et al*., 2015; Singh *et al*., 2006). This connection has become more evident with the continued growth of fast-food industries and the rapid transition to western diets observed in United States of America (USA) and western societies, leading to an increase of CVD's prevalence and incidence. It is noted that, changes in lifestyle habits (exercise and/or diet) may not be enough to overcome these diseases, due to the contribution of non-modifiable risk factors (Moore, 2009; Wilson *et al*., 1998; Tuomistto *et al*., 2005). However, non-pharmacologic interventions have high potential to prevent, retard and event revert CVD's progression, especially when early implemented.

1.2. Atherosclerosis

1.2.1. Definition and physiopathology

The main substrate of most CVDs is atherosclerosis, a disease of a lipidic and inflammatory nature, predominantly asymptomatic and of slow progression, which makes it difficult to correctly estimate its overall incidence (Barquera *et al*., 2015). However, it is considered the main cause of cardiovascular death and morbidity in Occidental Europe, USA and other regions around the world (NHLITFA, 1971; Libby and Ridker, 2002; Libby, 2012). This disease is strongly related to cardio and cerebrovascular accidents, such as myocardial infarctions and strokes (Ross and Glomset, 1976; Lusis, 2000). WHO estimates that atherosclerosis is nowadays the leading cause of vascular diseases worldwide. According to the European Society of Cardiology, only in 2016, atherosclerosis was responsible for 14.5% of overall deaths in Portugal mainland (Costa *et al*, 2021).

Atherosclerosis is a complex and multifactorial disease, which involves several mediators and cell types, including vascular cells (endothelial and muscles cells) and inflammatory cells (monocytes and macrophages, among others) (Ross, 1999). The development of genetically modified or transgenic animal models has allowed the knowledge about the disease to evolve, namely regarding some of the mechanisms involved in its progression, including at the genetic level (Jawieñ *et al*., 2004). It is known that the early stages of atherosclerotic lesions can be influenced by different genes or environmental changes, becoming an even more complex and dynamic process (Lusis *et al.*, 2004).

By definition, atherosclerosis is a hardening and narrowing of arteries, associated to the formation of lipidic plaques in arteries walls, which lose flexibility resulting in a small lumen, causing the reduction and/or block of blood flow (Gregory *et al*., 2009). The increase in the thickness of the intima, is derived from the deposition and accumulation of lipids, originating atheromatous plaques. These plaques can induce the asymmetric thickness of inner tunica, leading to an obstruction of blood flow, which in worst case scenario can be completely blocked and causing cardio/cerebrovascular events (Hansson *et al*., 2006; Brown and Golstein, 1984).

Traditionally, this disease was categorized as a lipidic accumulation; however, due to animal's model use, it become possible to comprehend that atherosclerosis formation results on dynamic, gradual and complex process, involving lipids and other

mediators, including inflammatory, proliferative and migratory, respectively from vascular smooth muscles cells (VSMC) to inner tunica (Ross and Glomset, 1976).

Atherosclerotic lesions can be divided in three categories: *Fatty Streak* **(Fig. 2B)**, intermediate or fibrous lesion (**Fig 2C**) and fibrous plaque (**Fig 2D**). In the first case, it can be defined as any lesion in the inner tunica that is stained directly with dye, for example Sudan IV, and that has no other underlying lesion (Ross, 1993; Strong, 1969; Mendis *et al*., 2005). These are normally identified by the presence of yellowish layers at the inner tunica surface, which consists in an accumulation of lipids, monocytes and lymphocytes T (CD8+, CD4+ and T cells), originating *foam cells*, which has inflammatory character and its accumulation forms layers designed by *fatty streak* (Faggiotto *et al*., 1984; Insull and Bartsch, 1966). The intermediate or fibrous lesion is characterized by the formation of alternating layers of macrophages and T-cells with VSMC. The worsening of this injury can lead to the formation of fibrous plaque, the most advanced stage of atherosclerotic injury (Mendis *et al*., 2005). According to Strong (1969), the atherosclerotic plaque presents a pale gray, bright and translucent color, characterized by an increase in the number of VSMC and the density of the layers (Ross, 1995). However, these atherosclerotic fibrous plaques can originate even more complex condition, such as hemorrhages, ulcerations, necrosis, thrombosis and calcification (Strong, 1969). In extreme situations, the plaque may disintegrate and induce hemorrhagic/thromboembolic events (**Fig. 2E**) (Ross, 1993).

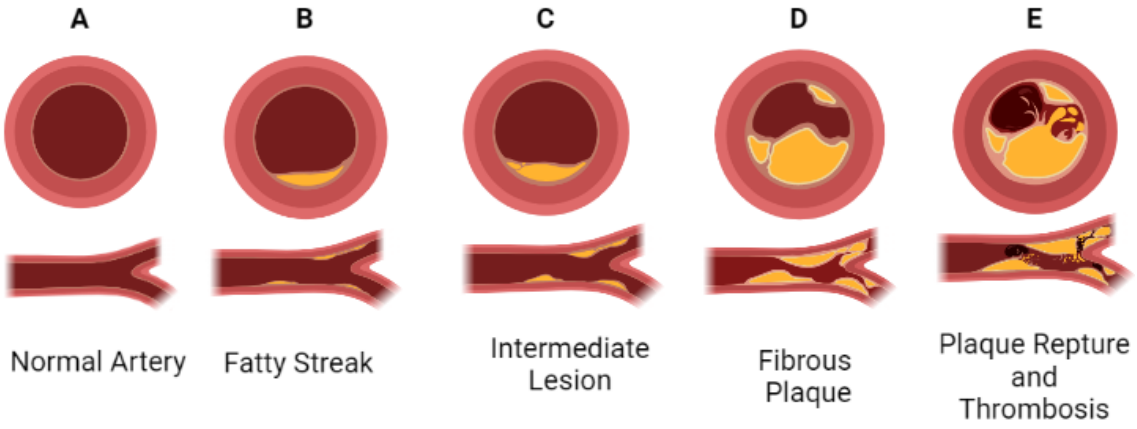

Figure 2 - Evolution of atherosclerosis disease. Cross and longitudinal section of an artery.

The arterial intima is lined by endothelial cells – endothelium (**Fig. 3A**) – that respond to mechanical and molecular stimuli, modulating muscle tone (Lüscher *et al*., 1991; Cines *et al*., 1998), homeostasis (Dahlbäck, 2000), inflammation (Luscinskas and Gimbrone, 1996) and thrombosis (Gross Aird, 2000; Bombeli *et al*., 1997). In the atherosclerotic lesion regions, the endothelium is exposed to agents that promote atherogenesis, resulting in endothelial dysfunction (Ross, 1993).

The endothelium dysregulation is caused by the concomitance of cardiovascular risk factors, such as hypercholesterolemia, arterial hypertension, smocking habits, among others (Drexler e Hornig, 1999). This process of endothelium dysregulation is reflected in a disturbance in the production of mediators that regulate muscle tone, resulting in vasoconstriction and platelet activation (Drexler *et al*., 1992). Thus, there is an increase in the production of vasoconstrictors such as endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2 (TXA2) and reactive oxygen species (ROS); on the other hand, less vasodilators are produced, such as nitric oxide (NO) and prostaglandin 2 (PGI2) (Endemann, 2004; Schiffrin, 2001).

In basal conditions, endothelium phenotype is characterized for being vasorelaxant, anticoagulant, anti-platelet and selectively permeable to molecular mediators (Drexler and Hornig, 1999). However, when there is endothelium dysfunction, the passage of low-density lipoproteins (LDL) into the intima occurs, originating an inflammatory response. LDL particles can suffer oxidation, due to the ROS production, displaying a role as promotors of atherogenesis. In these conditions, the inductions of VSMC, inflammatory mediators' secretion and an increase of cell adhesion molecules, for example, vascular cell adhesion molecule (VCAM-1) or intercellular adhesion molecule (ICAM-1) occurs (Witztum, 1991). The continuous secretion of inflammatory mediators results in an increase of LDL oxidation (Rocha, 2009). Consequently, circulating monocytes adhere to these adhesion molecules and (by a process called diapedesis) change their morphology, which allows passage through the endothelial lining. In the arterial intima, they mature in macrophages, that is, acquire phagocytic capacity, and internalize oxidized LDL, originating foam cells (**Fig. 3B**). These cells have a highly inflammatory character, which triggers a continuous recruitment of monocytes and consequent maturation in macrophages and LDL phagocytosis, forming more foam cells. This process leads to the formation of the first atherosclerotic lesion - *fatty streak* (Faggiotto *et al*., 1984), becoming the main promoter of the initiation of atherosclerosis and progression to subsequent phases (Behrendt and Ganz, 2002). Also, foam cells promote migration and proliferation of VCSM from tunica media to intima, which

heightens synthesis of collagen, leading to hardening of atherosclerotic plaque (**Fig. 3C**) During this process, foam cells will eventually die, releasing its lipid content, which will help the growth if the plaque. As the plaque grows it builds in pressure, causing the rupture of the plaque (**Fig**. **3D**).

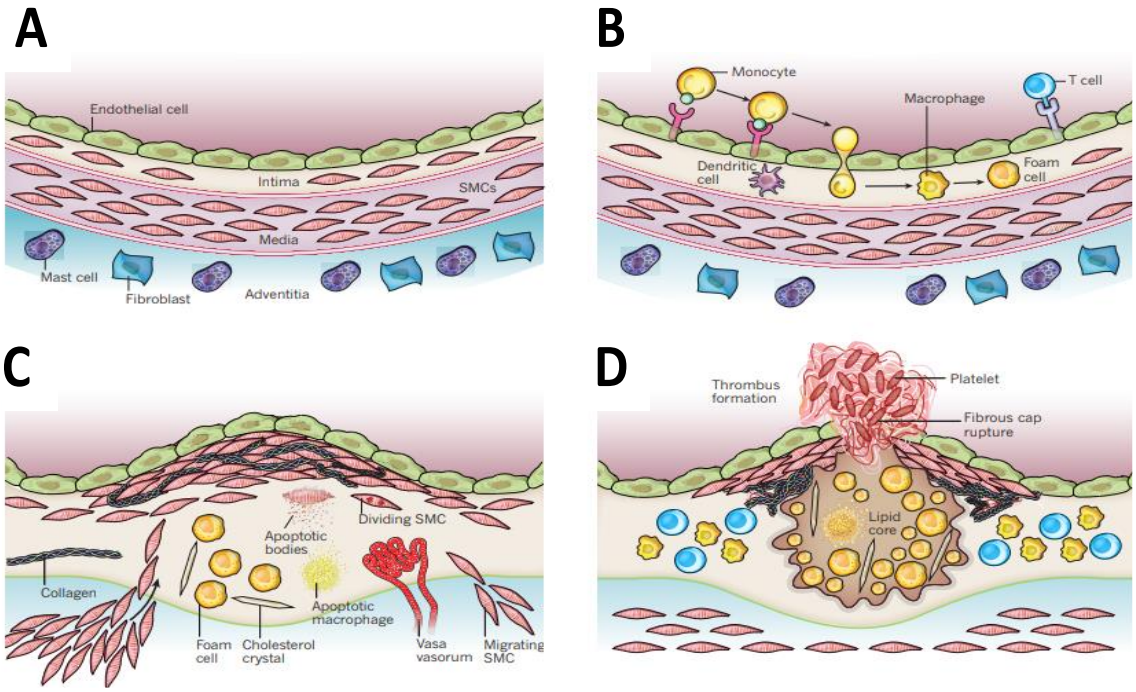

Figure 3 - Atherosclerosis progression. A - Healthy artery with 3 layers (intimate tunic, media tunic and adventitial tunic). B - First steps of atherosclerosis. Monocyte adhesion to VCAM and consequent alteration of its morphology - diapedesis - internalizing in the intima. Once inside, they mature in macrophages and phagocytize LDL giving rise to foam cells. C - Migration of smooth muscle cells from the tunica media to the tunica intima. D - Formation of a thrombus and consequent rupture of the plaque. Adapted from: Libby *et al*. (2011).

1.2.2. Metabolism of cholesterol and lipoproteins: the central role of liver

Some substances have an important role in the emerge/progression of atherosclerosis. As mention before, lipoproteins can contribute to this process. However, it is the cholesterol within lipoproteins that have a major impact in this disease. Cholesterol (Chol) together with triglycerides (TGs) and phospholipids are designated hydrophobic lipids and display important functions in animal cells. These lipids, as their name indicate, are non-polar substance, which makes them insoluble in water. This way, for its transportation, they need a vehicle to overcome this problem. Hence, lipoproteins play a crucial role in the transportation of these substances by making them polar, but most important, are responsible for cholesterol metabolism (Phan, 2001). This metabolism is highlighted in dyslipidemia, becoming a risk factor for atherosclerotic CVD. However, it should be noted that this specific lipid is relevant for some basic cellular functions, maintaining the fluidity of membrane cells, for example.

Generically, lipoproteins are a group of proteins synthetized in small intestine and liver. They are constituted by a hydrophobic core, mainly composed by cholesterol esters and triglycerides, and by a monolayer in the hydrophilic surface, constituted by phospholipids and fatty acids. It is in this outer layer that cholesterol is contained and apolipoproteins (apo) are incorporated (Durrington and Soran, 2014). Apolipoproteins are the protein component of lipoproteins; there are 4 main groups - apolipoprotein A, B, C, and E; within each group there are subgroups. Apolipoproteins play an important role in the regulation of lipoprotein metabolism, transport and redistribution of blood lipids to different tissues and cells, as they can serve as ligand for interaction with some specific lipoprotein receptors on cell surface (Liu *et al*, 2021).

According to the ratio between cholesterol and TGs, lipoprotein density will vary and different designations will be attributed. In this way, from lower to higher density: chylomicrons; chylomicron remnant; very-low-density lipoprotein (VLDL); intermediate-density lipoprotein (ILD); low-density lipoproteins (LDL) and high-density lipoprotein (HDL) (**Fig. 4**) (Cohen and Fisher, 2013).

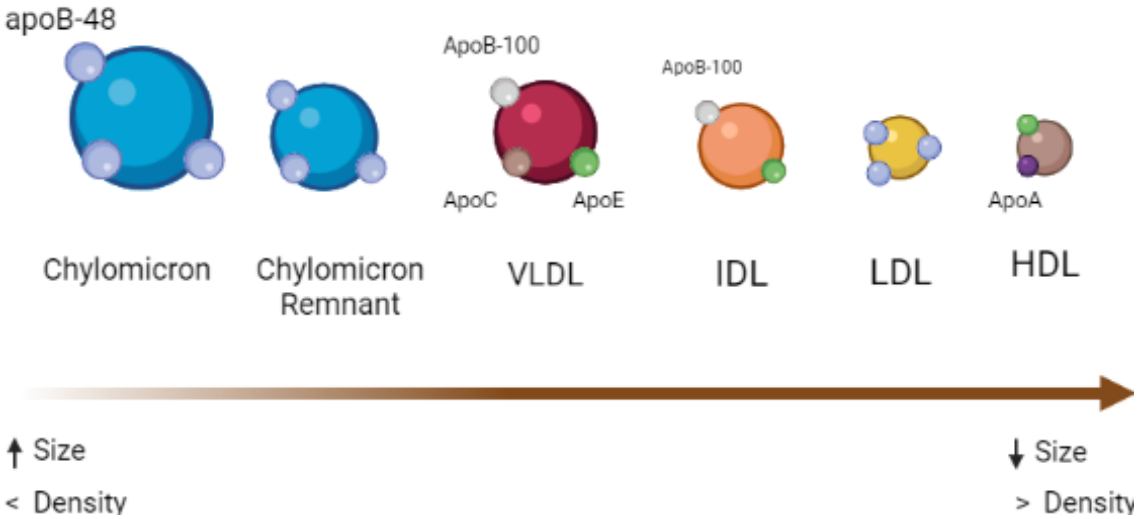

Figure 4 - Different lipoproteins based on their size/density. From lowest density (higher in size) to highest density (lowest in size).

The lipoproteins metabolism aims at production, transport and removal of cholesterol and TGs circulation. The lipidic transport includes direct and reverse cholesterol transport. The first transport involves two pathways (endogenous and exogenous) and the cholesterol transport back to the liver (**Fig. 5**). The endogenous pathway refers to the production of cholesterol by the liver while in the exogenous pathway cholesterol is obtained from diet. The second transport – reverse cholesterol transport (RCT) – refers to the cholesterol circulation in the opposite way, that means, the efflux from peripheral tissue back to the liver (Cohen and Fisher, 2013; Aguilar-Ballester *et al*., 2020).

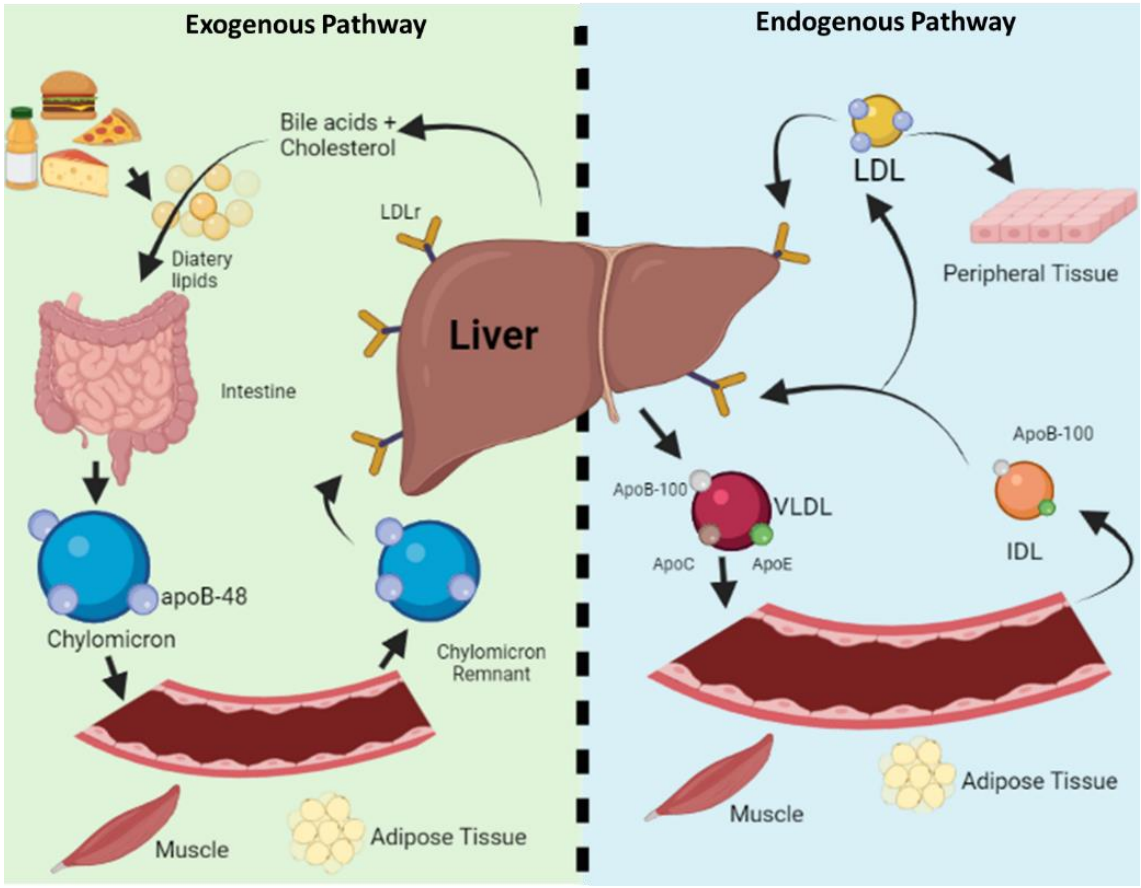

Figure 5 - Endogenous and exogenous pathway of lipoprotein metabolism.

Chylomicrons are the largest lipoproteins and are mainly constituted with TGs. These lipoproteins are secreted in small intestine epithelium cells, transporting cholesterol and TGs from diet (exogenous pathway) to the peripheral tissues, such as cardiac and skeletal muscle, as well as adipocytes. The main apolipoprotein in chylomicron is apoB-48. Once in the peripheral tissue, TGs are hydrolyzed in fatty acids and glycerol, by lipoprotein lipase (LPL), which can be used as energy for cellular metabolic functions or can be absorbed by adipocytes and stored in the form of TGs. The hydrolysis of TGs promotes a decrease in the size of the chylomicrons, having a higher percentage in terms of cholesterol ester (Durrington, 2007). These chylomicrons are called remaining chylomicrons, which will transport cholesterol to the liver, which can be used for various functions, such as the synthesis of bile acids or it can be reesterified by acylcoenzyme A-cholesterol acyltransferase (ACAT), becoming part of the constitution of VLDL together with TGs (Durrington, 2007; Critchley *et al*, 2004).

The endogenous pathway comprehends the hepatic secretion and VLDL, IDL and LDL metabolism. VLDL is a lipoprotein secreted by the liver and it comprises by TGs more that cholesterol. VLDL transports endogenously produced TGs from the liver to the peripheral tissues and it is converted to LDL by hydrolysis of fatty acids by LPL. Apo E, B-100, C-II are a part of the constitution of VLDL (Durrington and Soran, 2014). LPS hydrolyses TGs from VLDL to free fatty acids, originating small lipoproteins with a higher percentage of cholesterol. The VLDL decreases in size becoming IDL. IDL has around the same amount of cholesterol and TGs and has apo E and B-100 as its main apolipoproteins. This lipoprotein results from the degradation of VLDL. IDL transports cholesterol and TGs to liver from peripheral tissues and can have two different destinations: it can be either taken up by hepatocytes after binding to the LDL receptor in a process mediated by apoB-100 or apoE or it can be subject to increased activity of the lipase, losing TGs content and be released into the circulation as LDL. LDL, common refer to as “the bad cholesterol”, is a lipoprotein mainly composed with cholesterol rather than TGs. LDL is formed from IDL and it is endocytosed by target cells that contained LDL receptors, such as liver cells, supplying cholesterol to the tissues. After giving cholesterol to the tissues, LDL returns to the liver, by biding to LDL receptors. Once inside the liver cells, LDL are endocytosed, releasing cholesterol which decreases further uptake of cholesterol. Posteriorly, it can be recycled in the Golgi Apparatus to form more lipoproteins and excess cholesterol can be excreted through bile (Cohen and Fisher, 2013).

Due to their small size (22nm), they can pass through the endothelium and fluid tissue, transporting cholesterol to the peripheral tissues, such as arterial wall, contributing to the appearance of atherosclerosis. Once in the arteries, macrophages can phagocytize these lipoproteins and form foam cells. The continuing accumulation of LDL in the arterial walls, will eventually form the well-known atherosclerotic plaque (Durrington and Soran, 2014). The apolipoprotein associated with LDL is apoB-100 (Ogedegbe, H. and Brown, 2001).

HDL, the smallest lipoprotein, is mainly composed with cholesterol than TGs and it is secreted by intestinal epithelium and liver. It is referred to as “the good cholesterol”, because it receives excess cholesterol from peripheral tissue, such as cholesterol located in atherosclerotic arteries, and transports to back the liver (RCT) where it can be recycled or excreted via bile, becoming the principal route for the removal of cholesterol. The higher the concentration of HDL the lower the risk for coronary artery disease and CVD. ApoA-I and C-II are the main apolipoproteins associated with HDL. ApoA-I

component of HDL activates Lecithin-Cholesterol Acyltransferase (LCAT) to form cholesterol esters. It transfers apoC-II and apoE to nascent chylomicrons and VLDL (Durrington and Soran, 2014).

So, in part, this lipoprotein metabolism permits a continuous exchange of cholesterol which allows it to be recycled, migrate between cells or excreted, depending the body needs.

1.3. Hepatic steatosis – CDV crosstalk

1.3.1. Obesity and hyperlipidemia as drivers of hepatic steatosis

Over the years, liver diseases, in particularly steatosis, has been associated to atherosclerosis and CVDs. Most liver diseases derived from fatty liver (FL) and can be classified as non-alcoholic fatty liver disease (NAFLD) or as alcoholic liver disease (ALD). Together, they represent the most common and emerging causes of chronic liver disease. Even though, the accumulation of excess fat is the key to characterize these two conditions, it is the ethanol intake that can differentiate them (Bedogni *et al*., 2014; Mitra *et al*., 2020). According to European Association for the Study of the Liver (EASL), NAFLD is diagnosed when ethanol intake is less than or equal to 20 g/dl in women and less than or equal to 30 g/dl in men, above these limits it should be diagnosed as ALD (EASL, 2016). Most recent data indicate that NAFLD cases has increase significantly since 1990. Only in 2018, 1.7 billion cases were registered worldwide (Cai *et al*., 2020).

According to histological lesion progression, NAFLD is spectrum of liver damage that can be classified as isolated steatosis, nonalcoholic steatohepatitis (NASH) with gradual progression that can culminate in one cirrhosis process and in worst case scenario can lead to hepatocellular carcinoma (HCC) (**Fig 6**). Steatosis is designed as the accumulation of at least 5% of fat (mainly triglycerides, cholesterol and fatty acids) in the liver (EASL, 2016).

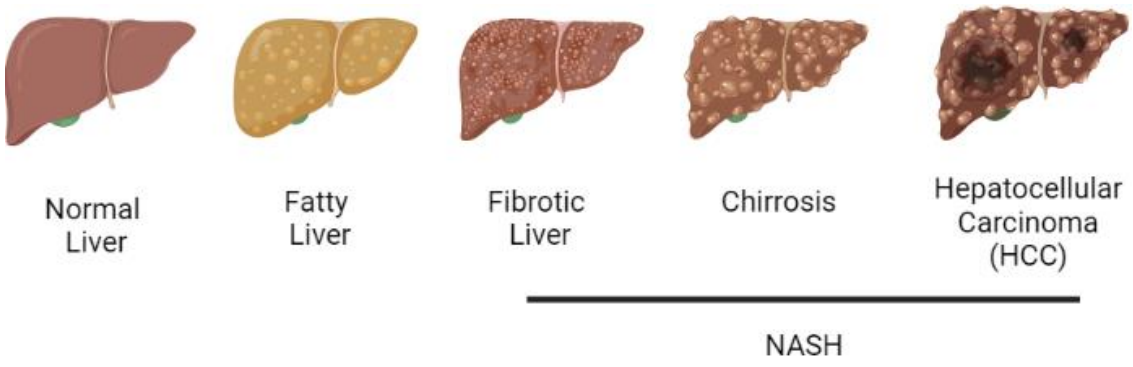

Figure 6 - Evolution of NAFLD.

The pathogenesis of hepatic steatosis involves multiple pathways, including fatty acid uptake, de novo lipogenesis, mitochondrial fatty acid oxidation and lipoprotein secretion as mention above. In normal condition, liver does not store TGs; however, when the caloric intake increases, it can lead to such stressed settings like obesity, where abnormal lipid metabolism occurs and ectopic hepatic lipid accumulation arises (Nassir *et al*., 2015; Cai *et al*., 2020). Classically, patients diagnosed with NAFLD, more specific with NASH, have slightly elevated liver enzyme values such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and it has been associated with intrahepatic fat content and insulin resistance (Rinella, 2015).

The storage of lipids, in TGs format in the liver, can also trigger lipotoxicity, which could lead to other cardiometabolic dysfunctions analogous to atherosclerosis and CVDs such as insulin resistance, dyslipidemia, derived from higher level of c-LDL and low levels of c-HDL, CV events (Nassir *et al*, 2015). These risk factors, once again, may derived not only from genetics, age or gender causes, but also due to the increase of unhealthy diet patterns and lack of physical exercise.

1.3.2. Hepatic steatosis as a risk factor for CVD

As mention previously, hepatic steatosis itself contributes for CVDs. In fact, people who suffer from NAFLD disease are most likely to die from CVDs. In this sense, NAFLD has a significant impact on CVD and its complications. Furthermore, NAFLD has a rapidly growth rate as others epidemics such as obesity, diabetes and metabolic syndrome. However, it was recently that NAFLD was recognized as an independent disease apart from the other mention previously, mainly due to the fact they have in

common some pathological parameters. Thus, studies have shown that less than 20% of nonobese people can develop NAFLD, while about 30% of diabetic patients do not have FL (Cai *et al*., 2020). Nevertheless, it should be note it that NAFLD may be a “road” for the development of those diseases.

Based on this recognition, EASL recommends that a modification on CVDs risk factors could be a major approach to prevent NAFLD emerging (EASL, 2016). Additionally, NAFLD is asymptomatic and in most cases people who suffer from this disease only get diagnosed when undergo testing for unrelated causes. Techniques based on histopathologic analysis from hepatic tissue collected by biopsies and biomedical imaging technologies (namely resonance) are the golden standard to for detecting and staging NAFLD (Serfaty and Lemoine, 2008; Rinella, 2015). The current therapeutic approaches for NAFLD, and particularly steatosis, are focused on life-style changes. Once again, healthy dietary regiments with significant levels of phenolic compounds could be a step forward on the prevention of NAFLD. Studies have shown that antioxidants can prevent several CVDs and NAFLD (Mehta *et al*., 2002; Lotfi *et al*., 2019; Mosca *et al*, 2020).

1.4. Animal models of atherosclerosis

For several years, atherosclerosis has been a topic of great interest by researchers around the world, as it is the main substrate for CVD’s, being the deathliest disease worldwide. In this way, to prevent and/or to retard the progression of atherosclerosis, firstly we have to comprehend its etiology. In order to do so, over the years, it has been goal to create better animal models to study lipoprotein disorders related to atherosclerosis and to identify the genes that are responsible for the development of atherogenesis and lesion progression. In this sense, animal models have been a crucial tool, to replicate the conditions associated with the disease and to translate the results for clinical research.

1.4.1 Overview of animal models

Pioneering atherosclerosis studies have been done using rabbits. Indeed, Ignatowski (1908), and later Anitschkow and Chalatow in 1913, administered a cholesterol-rich diet to rabbits and observed that it induced a disease similar to atherosclerosis in humans. Other larger animals, such as pig and non-human primates, have already been used occasionally to study the disease. However, they have some

limitation, such as associated costs, hard to maintain in laboratories and sometimes risk of extinction (Wissler and Vesselinovitch, 1976; Gerrity *et al*., 2001). The use of rodents, since the end of last century, it has been increasing, as they are small, easy to maintain in the laboratories, have a rapid reproduction cycle and, as a consequence, breeding becomes much simpler and easier to handle. In the late 80s, and early 90s more specifically, genetically modified mice began to be developed to replicate the etiology of atherosclerosis. These new models were quickly considered to be the most versatile for studying atherosclerosis and started to be widely applied in the study of the disease. The use of animal models in atherosclerosis studies aims at the capacity of replicating the phenotype of the lesion observed in humans, thus improving the translation of results (Daugherty and Whitman, 2003).

These biogenetic engineering approaches have become essential to better understand the functioning of genes and how they are related to the development of certain diseases. Generically, wild-type mouse from different species is very resistant to the development of atherosclerosis (Jawieñ *et al*., 2004), due to its high percentage of HDL and low percentage of LDL and for the lack of the cholesteryl ester transfer protein, an enzyme that transfers cholesterol ester from HDL to VLDL and LDL. In contrast, humans have high levels of LDL and low levels of HDL, which makes them more susceptible to develop atherosclerosis. The only exception is C57BL/6 strain, which was first observed in Paigen *et al* (1987) study. Giving this first observation, throughout the years, atherosclerosis studies have been using genetically modified mice with C57BL/6 background.

Genetic modifications in this strain that allows the development of atherosclerotic lesion to become more “severe” or more notable. In some cases, these lesions can be similar to the ones observed in humans. In 1993, LDL receptor deficient mice (LDLr-/-) was created by targeting the gene responsible for the expression of LDL receptor. This mutation induces a state of hypercholesterolemia up to 275mg/dl, three times higher than control mice. The lipid profile of LDL-/- mice - with a higher percentage of cholesterol carried in IDL/LDL particles – resembles that in dyslipidemic humans. As for the development of spontaneous lesions or plaque in this mouse model it is not possible. However, when submitted with a high cholesterol diet, medium plaques can occur as early as 12 weeks (Ishibashi *et al*., 1993). Other animal models have been developed to study this disease, such as apoE and LDL - receptor (LDLr) double - knockout (apoE/LDLr-DKO) mice, apoB transgenic mice, apoE/eNOS double - knockout mice, among others.

1.4.2 The apoE knockout mouse model

The apoE knockout (apoE KO) mouse is a genetically modified animal model created in 1992 by two different groups, using homologous recombination in embryonic stem cells. This model presents as a modification the complete deactivation or repression (knockout) of the gene encoding apolipoprotein E (apoE).

As above mentioned, apoE is part of the constitution of some lipoproteins, namely chylomicron, VLDL and HDL and it plays a preventive role in atherosclerosis, remaining a crucial element in the removal of some atherogenic lipoproteins. This apolipoprotein is synthesized in the liver, brain and other tissues in both humans and mice. It can be used as ligand to some receptors on the extracellular surface of liver cell membranes, such as LDL receptors. Moreover, apoE has the ability to influence immunological activation, suppressing T-cell proliferation required for normal innate immune function. This immunomodulatory capacity is highlighted in atherosclerosis, which is also defined by immune/inflammatory activation in addition to lipoprotein buildup. Also, apolipoprotein participates in the production of foam cells and RCT (Zadelaar *et al*., 2007).

So, the absence of apoE, makes it impossible to clear lipoproteins rich in cholesterol from the blood and arteries. In other words, the inactivation of the apoE coding gene induces a state of hypercholesterolemia in rodents higher than wild type mice and LDLr (-/-) mice, for instance, as well as the consequent early development of atherosclerotic lesions. In fact, apoE KO mouse has hypercholesterolemia values of around 400 mg/dl, approximately five times higher than those of control animals (Greenow *et al*., 2005; Linton *et al*., 1998; Zhang *et al*., 1992).

As for the development of the lesion phenotype in genetical modified animals is not intrinsically dependent on aging. Indeed, apoE KO mice manage to develop spontaneous plaques (i.e. *fatty streak*) in just 3 months, that is, under normal dietary conditions. However, when submitted with an atherogenic diet, that is rich in cholesterol and fat, they can develop abundant, large plaques as early as 14 weeks.

The apoE KO model is particularly important because the mechanism that causes the lesions, their progression and the cells involved show an extraordinary similarity to the atherosclerosis phenotype observed in humans, including the presence of oxidized lipoproteins (Jawieñ *et al*., 2004). Thus, this versatile model is currently the widest utilized animal model for the study of this disease. In fact, it has a defined genetic

background which allows the study of the pathophysiology of atherosclerosis and therapeutic options.

In addition, this animal model of atherosclerosis presents other metabolic features also present in humans, such as hepatic steatosis. In fact, some studies reported that occasionally with a STD fat accumulation occurs, but on HFD is more evident where this can progress to steatosis (Zheng and Cai, 2019; Silva *et al*., 2013). Not only that, this animal model has been used in oxidative stress studies (Cannizzo *et al*., 2012; Butterfiel and Mattson, 2020; Wang *et al*., 2021).

1.4.3. The relevance of diet for animal models

As mentioned previously, the exposure to unhealthy diets is implicated in the development of cardiovascular and cardiometabolic diseases. In order to better understand the etiology of those diseases, more specifically atherosclerosis and steatosis progression, animal models have been an essential key for the translation of results to clinical studies in humans. However, even with the best animal models existing, the phenotype of these diseases can take months to develop spontaneously. To overcome this problem, a previously exposure to western type diets, namely with high levels of fat, cholesterol and sugar, can trigger this phenotype in short term. Analogous to humans that can also develop those same phenotypes trough diet. In this sense, lesions or plaques can be developed in weeks, as well as accumulation of lipids in the liver.

Generically, diets can be divided in two categories: unrefined and purified diets. Unrefined diets are usually referred to as “normal diet”, “chow diet” or “standard diet” and it is composed by cereal grain-based and animal by-products, including corn, wheat, oat, alfafa, among others. However, these diets formula are not divulge to the scientific community and over time the cereal grain nutritional values may vary. In contrast, purified diets are constituted by highly refined ingredients isolated and separated from the original cereal grains and animal by-products context, each of which essentially contains one main nutrient and as a result these types of diets are well defined, allowing a minimal batch variation. Furthermore, the formula is disclosed to the community, permitting the researchers to personalize their diet with specific ingredients to induce the study disease. By doing that, it allows different ingredients to be adjusted and to design different diets according with the levels of fat, cholesterol or sugar (HFD, LFD, HSD, among others). However, the data/results from each diet – unrefined and purified diet - should not be

comparable due the existence of these differences between them (Pellizzon and Ricci, 2018).

In this way, it is crucial to establish a more “authentic” control diet to be possible the comparison of this data. So, the questioned that still remains to be answered is: which is the best diet to serve as control diet for HFD: standard chow or refined LF?

2. Olive oil polyphenols to counteract CVD and associated hepatic steatosis

2.1. Healthy diets as complementary therapeutics to hypolipidemic drugs: focus on the Mediterranean Diet

Atherosclerosis is specifically related with dyslipidemia, namely with increased levels of total cholesterol (TC), LDL cholesterol (c-LDL) and/or TGs, as well as reduction of HDL cholesterol (c-HDL) (Brown and Golstein, 1984; Gofman, 1956). HDL particles are typically atheroprotective as they promote RCT, that is, from vascular tissue to the liver, where it is recycled and reused for endogenous synthesis through a complex enzymatic cascade.

Lipidic profile allows the classification of individuals according with the risk: low, moderate, high or very high risk. There are tabulated values for the different lipid profile parameters, and for a normal individual the values, according with European Society of Cardiology and other international Institutions, should be: <155mg/dl of cholesterol total (CT), <115mg/dl of c-LDL, >90 mg/dl of c-HDL and <150 mg/dl of TGs. Values above these concentrations determine an increased risk, whose strict definition varies between individuals, depending on the presence of additional risk factors, such as pre-existing CVD, smoking habits, high blood pressure, diabetes and age. Individual with a highly number of risk factors are the ones who need to revert cholesterol values to normal levels more quickly to avoid vascular events (Mach *et al.*, 2020).

Anti-dyslipidemic therapeutics aims at the regulation of TC, c-LDL, c-HDL and TG levels. There are several classes of anti-dyslipidemic drugs, including statins, bile acid scavengers, nicotinic acid and fibrates, among others. Statins are the most used and the most effective medicine to revert hyperlipidemia, especially by reducing c-LDL levels. The mechanism of action of statins involves the inhibition of hydroxy-

methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in the endogenous synthesis of cholesterol (Rodwell *et al*., 1976). (Rodwell *et al*., 1976). In addition, statins interfere with other signaling pathways and promote a set of other actions, known as pleiotropic effects, which include improving endothelial function, inflammatory response (Wang and Bennett, 2012) and cellular senescence, among others (Haendeler *et al.*, 2004; Assmus *et al*., 2003). Collectively, the effects of statins significantly reduce cholesterol levels and improve vascular function, preventing plaque progression and reducing cardiovascular risk and mortality caused by CV events (Maron *et al*., 2000).

Despite this impact of statins on CVD, it has been reported for several years that statin intolerant patients develop side effects related to muscle and liver toxicity (Kiortsis *et al*., 2007; Sirtori, 2014; Hirota *et al*., 2020). On the other hand, many patients who respond favorably and efficiently to intensive statin therapy continue to experience cardiovascular events. This persistent risk, known as residual cardiovascular risk, leaves a large margin for discovering effective alternatives that complement the use of statins (Cziraky *et al*., 2008). Due to this insufficient effectiveness of existing therapies, namely in high-risk populations and possible potentially serious side effects, there is a need to look for new approaches, including non-pharmacological strategies that may have an adjuvant action to the available drugs and that can be introduced in earlier stages of CVD.

The most recommended non-pharmacological approaches are physical exercise combined with a balanced diet with adequate calorie content. The importance of a varied and balanced diet in the prevention of atherosclerosis and cardiovascular diseases has been recognized for long time but remains a major topic regarding the management of CVD and metabolic disease (Man *et al*, 2020). Indeed, adopting a healthy and varied diet, rich in micronutrients, including phenolic compounds, reveals a prudent choice to help preserve human health, by preventing cardiovascular risks and inflammatory disease. These diets can have an atheroprotective role, namely due to its antioxidant and anti-inflammatory effects, as previously demonstrated in several studies in the past (Birben *et al*., 2012; Sies, 1993; Rice-Evans *et al*, 1997; Nimse and Pal, 2015; Antoniades *et al*., 2003; Keaney and Vita, 1995; Jialal *et al*., 1991; Björkhem *et al*., 1991).

Among the various diets commonly under investigation by the scientific community, the Mediterranean Diet (MDiet) has been considered a model diet. Since 2010, Mediterranean Diet has been part of the Intangible Cultural Heritage (Reguant-Aleix, 2009). The origin of this diet takes place in the countries bordered by the

Mediterranean Sea or influenced by it. In the middle of last century, Ancel Keys was responsible for the disclosure of MDiet to the world. However, it is considered not only a diet, but a lifestyle, gathering many aspects on the day-life of Mediterranean people, such as: having meals with family or friends, promoting coexistence among people; incorporation of a wide variety of foods (fish, meat, vegetables, fruit, nuts, cereals, wine), including fresh products, little processed and local, respecting their seasonality; low to moderate consumption of dietary products and the use of olive oil as the main fat for cooking (Hu, 2003; Willett, 1995; Nestle, 1995; Aboul-Enein *et al*., 2017).

Furthermore, studies such as PREDIMED, shows that Mediterranean diet has a preventive/protective role against many diseases, such as CVDs, type 2 diabetes mellitus (T2DM), NAFLD, among others (Martínez-González *et al*., 2019; Perez-Martinez *et al*., 2011; Estruch *et al*., 2018, Martínez-González *et al*, 2015, Ros *et* al., 2014; Bullón-Vela *et al*., 2020).

2.2. Polyphenols from olive oil: focus on oleacein

Olive oil (OO) is considered the healthiest oil recommended by internationals organizations. This oil is extracted from olives, the fruit of the olive tree (*Olea europaea* L.). According with the International Olive Council (IOC), it is estimated that 139 olive trees varieties grown in 23 countries (Gómez-Rodríguez *et al*., 2020).

The study of olive cultivation and OO production dates back to biblical times. In Europe, the cultivation of olive trees and the production of OO occurs predominantly in the Mediterranean Basin (Owen *et al*., 2000). The Mediterranean conditions are characterized by hot and dry summers and mild winters, becoming favorable to the growth of olive trees and production of olives. OO production has grown over time, assuming a prominent role in the Mediterranean diet over the years. In 2020/2021 its estimated that OO production reach over 3.2 million tons worldwide (IOC, 2021).

Regarding its beneficial effects, several studies infer that a balanced diet rich in olive oil may confer protection against different diseases, such as type 2 diabetes mellitus, cancer, cardiovascular and neurodegenerative diseases (Tresserra-Rimbau *et al*., 2014; Qosa *et al*., 2015; Martínez-González *et al*., 2015). Moreover, the European Food Safety Authority (EFSA) suggests that a daily dose of OO rich in hydroxytyrosol (HTy) and derivatives (5 mg of HTy and derivatives/20 g of olive oil) provides protection

against lipid oxidation, delaying the accumulation of oxidized LDL, characteristic of atherosclerosis disease (EFSA, 2011).

The OO exhibits a diverse chemical composition, characterized mainly by the presence of lipophilic components rich in monounsaturated fatty acids (e.g., oleic acid) alongside a small hydrophilic fraction rich in phenolic compounds, including phenylalcohols, phenolic acids, flavonoids and secoiridoides (Kanakis *et al*., 2013). Overall, these polyphenols are known for their antioxidant activity, responsible for the properties mention above. These phenolic compounds are the secondary metabolites produced by higher plants, including olive tree, displaying important function on the physiology of these plants. Moreover, for the longest time they have bring interest within scientific community. Studies infer that these polyphenols have high free-radical scavenging activity, which helps remove free radicals originated from oxidative stress, preventing or reducing the risk associated with CVDs, metabolic diseases, cancer, and neurodegenerative disorders (Serreli and Deiana, 2020).

The correlation between dietary regimen - with increased levels of fat, sugar and cholesterol - and oxidative stress is already known (Matsuzawa-Nagata *et al*., 2008; Noeman *et al*., 2011; Kesh *et al*., 2016; Lasker *et al*., 2019; Tan and Norhaizan, 2019). Briefly, oxidative stress results from an imbalance in homeostasis between the production and accumulation of oxygen and nitrogen reactive species (RONS) in cells and tissues and the ability to neutralize these reactive species by a biological system (Sies *et al*, 2017). Naturally, when oxidative stress occurs, free radicals are produced and could lead to lipidic and protein peroxidation as well as DNA damage. This phenomenon is related to the diseases above mentioned. In order to avoid this situation, our bodies have natural defenses, such as antioxidant species. These antioxidants can be classified as preventives or primary antioxidants, intersection or secondary antioxidants and repair antioxidants. However, they can either have an enzymatic nature – for example superoxide dismutase (SOD), catalase (CAT), glutathione reductase, among others - or a non-enzymatic nature – glutathione (GSH), α-tocopherol (also known as vitamin E), ascorbic acid (vitamin C), carotenoids, among others (Finaud and Filaire, 2006). In particularly, SOD - group of metalloenzymes - have such a scavenging activity. Within this “family” there are three different SOD isoforms: intracellular Cupper-Zinc Superoxide Dismutase (CuZn-SOD), referred to as SOD-1; Magnesium-Superoxide Dismutase (Mn-SOD), referred to as SOD-2, which is present in mitochondrial matrix and extracellular CuZn-SOD isoform, referred to as SOD-3, which is found on tissues extracellular matrix and on cell surfaces. Together, they form the front line of defense

against ROS-mediated injury. They can degrade superoxide (O_2^-), via metal medium and dismutase reaction, into normal diatomic oxygen (O_2) and hydrogen peroxide and this latter specie will be detoxified by glutathione peroxidase or CAT. Generally, a dismutase reaction is based on the withdraw of two identical molecules as reactants and originates two distinctly different products. The metal which facilitates this reaction is different in each enzyme. In particularly, stress oxidative is a common situation between NAFLD and atherosclerosis. In fact, low levels of SOD-2, can actually increase blood vessels plaque instability and low levels of SOD in general are associated with NAFDL and its complications (Vendrov *et al.*, 2017; Peng *et al*., 2018). SOD has been suggested as a putative therapeutic target to some diseases with a marked oxidative stress background (Yasui, and Baba, 2006; Younus, 2018; Tsai *et al*., 2021).

Once in a situation of oxidative stress, dietary antioxidants can assist our natural antioxidant enzymes in neutralizing these free radicals. Hence, Oleuropein (OL) and ligstroside are the two major compounds in secoiridoides class of polyphenols that can be found in olives and OO. OL when degraded by endogenous esterases originate demethylcarboxylated dialdehydic derivative, oleacein (Kanakis *et al*., 2013; Sarikaki *et al*., 2020). This compound has brought a great interest in the scientific community and is one of the most abundant components of Extra Virgin Olive Oil (EVOO) (Lozano-Castellón *et al*., 2019; Silva *et al*., 2018). Structurally, the presence of hydroxyl groups coupled to the aromatic ring is a key feature for the effects described above (**Fig. 7**). Although the presence of these compounds in olives and oil is well known it should be also noted that other parts of the plant, namely the leaves, have high amounts of oleacein (Kubola and Siriamornpun, 2008). This agricultural by-product gains particular prominence, namely from an economic point of view, in addition to the medical interest already mentioned

In vitro studies show an atheroprotective effect of oleacein in early and advanced lesions of the disease. In fact, the inhibition of foam cell formation has been described, resulting from a reduction in the deposition of macrophages and lipids, as well as a decrease in the expression of scavenger's receptors and an inhibition of plaque destabilization, resulting in a reduction in tissue factor and other biomarkers of cell damage (Filipek *et al*., 2020; Filipek *et al*., 2017). An anti-inflammatory effect of this compound has also been reported, changing the phenotype of macrophages from pro inflammatory to anti-inflammatory, by increasing the expression of certain receptors, such as IL-10 and CD136 (Filipek *et al*., 2015). Moreover, it has been reported the ability of oleacein to modulate several signaling pathways related to inflammatory (eg. inhibition

of cyclooxygenase enzymes, lipooxygenases, inducible NO, among others), oxidative (eg. inhibition of Nicotinamide and Adenine Oxidase Dinucleotide Phosphate) and infectious processes (eg. inhibition of *Escherichia Coli* growth) (Pang e Ching, 2018; Lozano-Castellón *et al*., 2019; Beaucham *et al*., 2005; Rosignoli *et al*., 2013; Naruszewicz *et al*., 2015) and reduce the proliferation, adhesion and migration of SH-SY5Y cells (Cirmi *et al*., 2020).

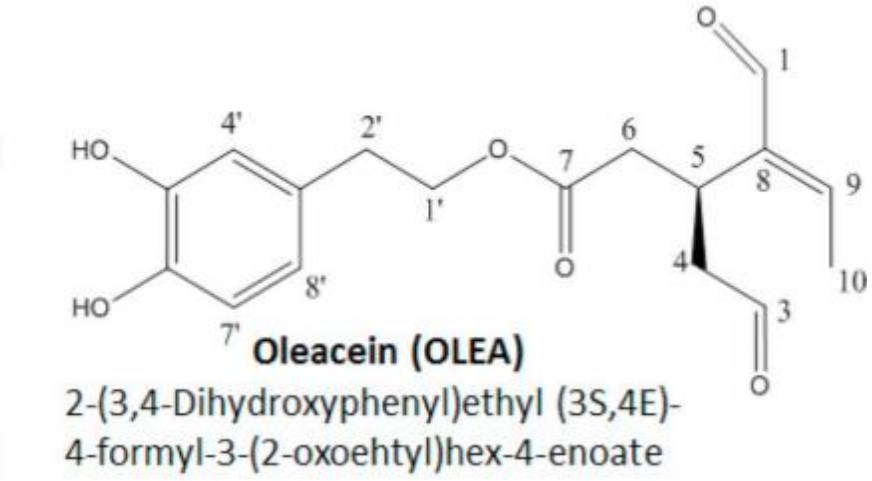

Figure 7 - Chemical formula of the oleacein derivative. Addapted from: Nikou *et al*., (2019).

Furthermore, *in vivo* studies using WT mice treated with oleacein showed a protective effect against weight gain and fat deposition in the liver, accompanied by reduced macrophage infiltration (Lombardo *et al*., 2018; Lepore *et al*., 2019). Moreover, it has been reported that oleacein presents neuroprotective effects in central nervous system in experimental autoimmune encephalomyelitis (EAE) mice (Gutiérrez-Miranda *et al*., 2020). However, the impact on animal models of cardiovascular and metabolic disease remains to be further elucidated, deserving further studies.

|Chapter II – Hypotheses and aims

It is widely recognized that lifestyle and dietary regimes can contribute to the development of human disorders. Changes in modifiable risk factors, namely in lifestyle habits, can be cornerstone strategies to prevent or delay progression of atherosclerosis and associated disorders, such as hepatic steatosis. Diets are not only important risk factors for cardiometabolic diseases but also key pieces for the development of several animal models. However, there is an ongoing scientific discussion regarding the most adequate diets to be used in animal models. We hypothesize that refined and unrefined control diets may promote distinct metabolic effects. So, we started by comparing three types of rodent diets: one unrefined diet (standard) and two refined diets (a low fat and an atherogenic one).

As mentioned above, oleacein has been tested, mainly *in vitro*, as a nutraceutical tool to overcome or retarding atherosclerosis disease and weight gain, due to its anti-inflammatory and antioxidant effects. However, the *in vivo* effects remain to be elucidated, namely in proper animal models of cardiovascular and metabolic disease. We hypothesize that oleacein may exert antidyslipidemic and hepatoprotective effects *in vivo* in the apoE-KO mouse fed with an atherogenic refined diet.

Accordingly, the aims of this study were:

1 – To characterize the metabolic effects of 3 distinct diets in wild type mice in order to correlate the absence/presence of atherosclerosis and steatosis with different dietary regimes.

Among the existing customized diets for animal research, three dietary regimens will be characterized: i) Refined HF diet (atherosclerotic/steatosis phenotype); ii) Refined Low Fat (LF) diet (nutrient-matched diet, control) and iii) Unrefined control diet (standard chow).

2 – To evaluate the potential beneficial effects of oleacein in an animal model of atherosclerosis (the apoE KO mouse), focusing on hepatoprotection.

To assess the putative antioxidant effect of oleacein, glycemic, insulinemic and lipid profile of three groups – i) control group WT with ATD; ii) APOE-KO mice with ATD and iii) preventive group receiving oleacein by oral administration – will be evaluated.

Chapter III – Materials and methods

1. Animals, diets and experimental study design

Two strains of male *Mus musculus* animals aged 11-week-old were used during this project: 24 animals C57BL/6J wt (control groups) and 16 apoE-KO mice (with C57BL/6J background) were purchased from Charles River Laboratory (Paris, France). The animals were maintained in a standard diet (4RF21, Mucedola) until they were used for the experimental procedure. The animals were given free access to water and food and were housed in racks with individually ventilated boxes (IVB) of dimensions 20/36/18 cm (four *per* cage), with temperature (~22 ° C), humidity (60%) and light (12h light / 12h dark cycle) controlled. All animal procedures were performed according to the National and European Communities Council Directives of Animal Care and received approval (22/2020) by the local (iCBR) Animal Welfare Body (ORBEA).

Two experimental settings were accomplished for the achievement of the aforementioned objectives:

1.1. Experimental setting 1: characterize the metabolic effects of 3 distinct diets in wild-type mice

It is known that the consumption of unhealthy diets represents the main risk for hepatic steatosis and atherosclerosis disease. In order to accomplish the purpose of this experimental setting, we assessed the impact on metabolic phenotype from a chronic intake (10 weeks) of three diets: unrefined Standard diet (STD), refined Low-Fat (LF) and Atherogenic Diet (ATD). Dietary nutritional compositions of commercially available diets adopted in the present study are presented in **Table 1.**

Table 1 - Composition and energetic profile of STD, LF and ATD dietary regiments.

Diet composition	Standard Diet (Mucedola 4RF21)	Low Fat Diet (TD.08485)	Atherogenic Diet (TD.88137)
Energy sources (% by weight)	18.5% Protein 53.5% Carbohydrate 3.0% Fat	17.3% Protein 61.3% Carbohydrate 5.2% Fat	17.3% Protein 48.5% Carbohydrate 21.2% Fat
Energy (Kcal/g)	Protein: 0.74 Carbohydrate: 2.14 Fat: 0.27 Total: 3.15	Protein: 0.69 Carbohydrate: 2.45 Fat: 0.47 Total: 3.6	Protein: 0.69 Carbohydrate: 1.94 Fat: 1.91 Total: 4.5
Proteins (% by weight)			
Casein	---	19.5	19.5
L-Cysteine	---	---	---
DL-Methionine	---	0.3	0.3
Carbohydrates (% by weight)			
Starch	53.5	---	---
Corn Starch	---	43.3	15.0
Maltodextrin	---	10.0	
Cellulose	---	5.0	5.0
Sucrose	---	12.0	34.1
Lipids (% by weight)			
Soybean oil	3.0	1.3	
Anhydrous Milkfat	---	3.7	21.0
Cholesterol	---	---	0.15
Vitamin/Mineral Mixes (% by weight)			
Mineral Mix, AIN-93G-MX (94046)	---	--	--
Mineral Mix, AIN-76 (170915)	---	3.5	3.5
Vitamin Mix, AIN-93-VX (94047)	---	--	--
Vitamin Mix, Teklad (40060)	---	1.0	1.0
Fatty acids (% by weight)			
Saturated fatty acids	22	---	61.8
Mono-unsaturated fatty acids	22	---	27.3
Poly-unsaturated fatty acids	56	---	4.7

Twenty-four male C57BL/6J wt mice (11 weeks-old) were randomly divided into three experimental groups (n=8 each).

Group 1: WT + STD - wt mice fed with standard diet (4RF21, Mucedola).

Group 2: WT + LF - wt mice fed with control diet (TD 08485, Teklad) - Low fat control diet - containing 19.1% protein, 67.9% carbohydrates and 13.0% fat. Normally, these atherogenic diets have their own control diets, commonly referred to as standard or chow diets. In this case, the TD 08485 diet will serve as a control. Control diets, and this one in particular, tend to differ from the study diet in terms of source, composition and levels of nutrient refinement.

Group 3: WT + ATD - wt mice fed with atherogenic diet (TD. 88137, Teklad). Atherogenic diet - containing 15.2% protein, 42.7% carbohydrates and 42% fat.

All animals were weekly monitored for BW evolution as well as for beverage and food intake. On the first week (T0), the following metabolic assays were performed: half of animals from each group were randomly divided to perform glucose tolerance test (GTT) and the other half insulin tolerance test (ITT), an experimental design's option aimed to avoid biased metabolic outcomes while upgrading the 3R's Refinement principle (a gold-standard pillar of Laboratory Animal Sciences). At weeks 9 and 10, all animals were submitted to a GTT and ITT. After 10 weeks (T10: Tf) blood and tissue were collected from the three groups.

In **Figure 8** it is schematized the in vivo assays performed in this experimental setting.

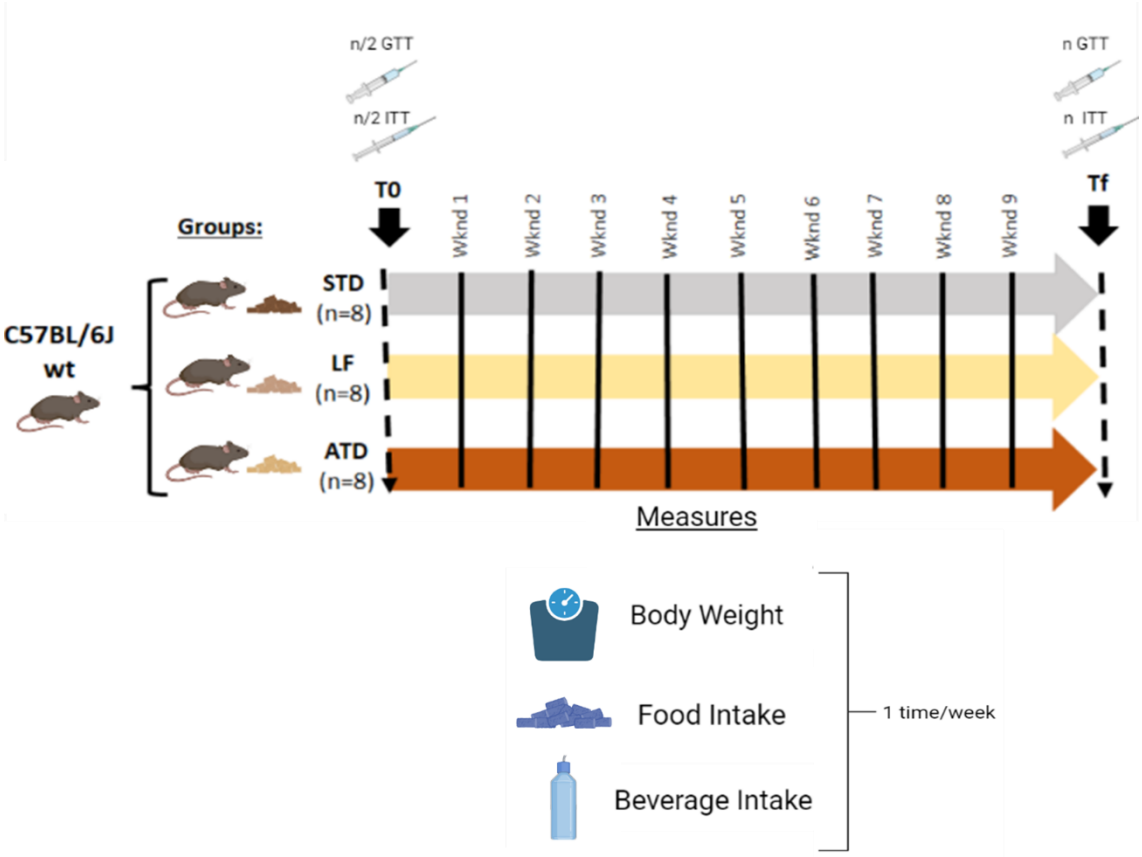

Figure 8 - Representative scheme of the Experimental Setting 1

1.2. Experimental setting 2: Evaluation of the potential beneficial effects of oleacein in the apoE-KO mouse

To assess the presumed hepatoprotection effect of oleacein, 8 male C57BL/6J wt (11 weeks-old) and 16 apoE-KO (with C57BL/6J background) mice were fed with ATD. The following 3 groups (n=8 each) were formed:

Group 1 – WT + ATD - WT mice fed with atherogenic diet (ATD: TD. 88137, Teklad);

Group 2 – APOE KO + ATD – apoE-KO mice fed atherogenic diet (TD.88137, Teklad);

Group 3 - APOE KO + ATD + OLEA – apoE-KO mice fed atherogenic diet (TD.88137, Teklad) and administered orally with oleacein (50 mg/kg), by a method of voluntary oral administration - semi-solid matrix form (Pill) developed and optimized for rodents (Pill, patent pending Nº PCT/IB2021/053124) - for 5 days a week for 10 weeks.

In vivo monitoring and assays were the same above described for the experimental setting 1, according to **Figure 9.**

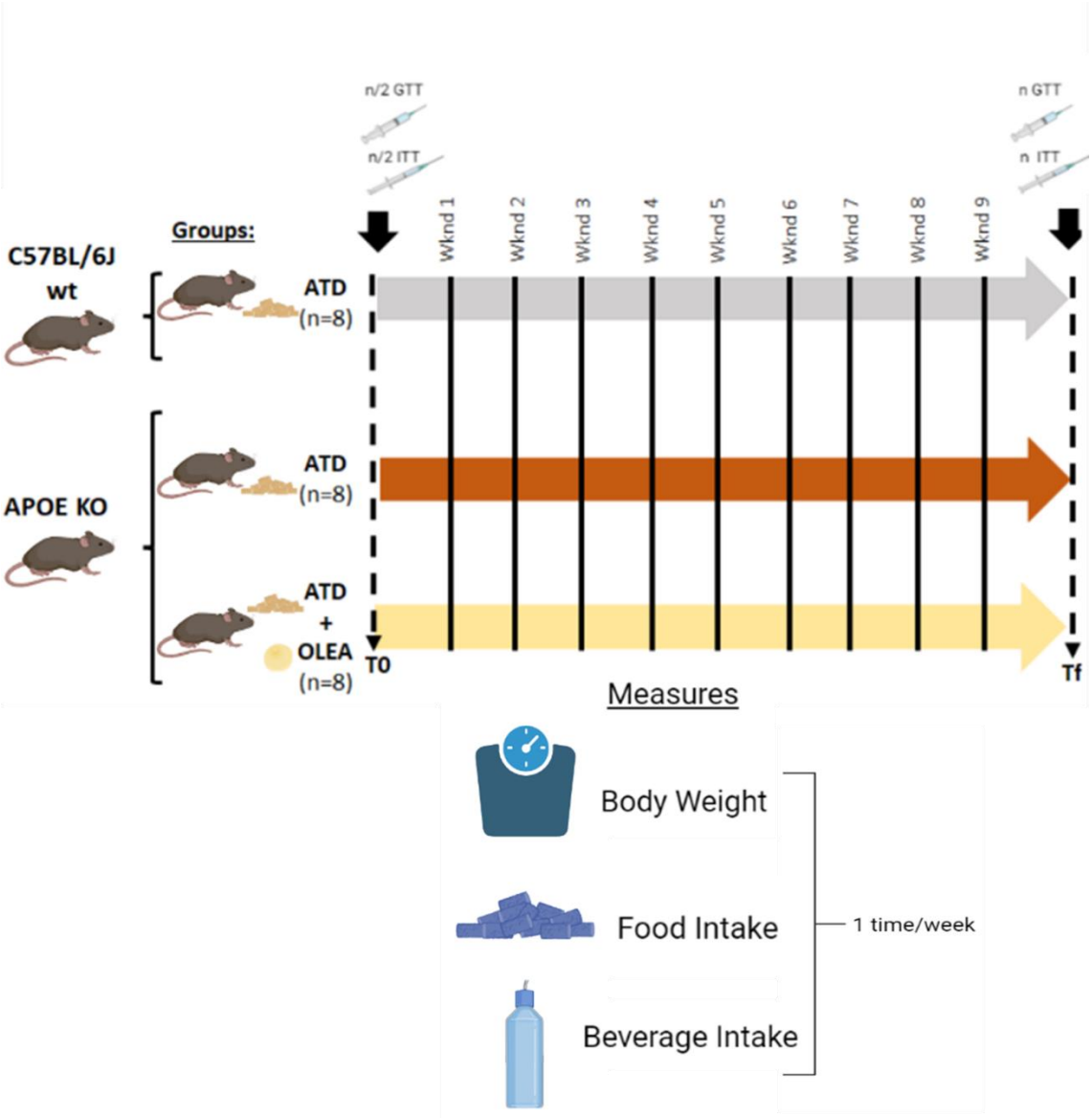

Figure 9 - Representative scheme of the experimental design 2

2. *In vivo* monitoring

2.I. Body weight, food and water consumption

BW were food and water consumption were measured once a week till the end of the study. BW were recorded using an analytical balance (CQT251 Core® Portable Compact Balance, Adam Equipment, USA) and food intake measurements with (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA). Food consumption was calculated by the difference in weight between the feed placed into the cage and the remaining feed at the end of each 7 days. Beverage consumption was followed by laboratory volumetric glassware.

2.2. Insulin Tolerance Test (ITT) and Glucose Tolerance Test (GTT)

On the first week of study (T0), four animals from each group were submitted an overnight fasting (12 hours) and a GTT test was performed. The other four animals from each group were fasted for 6 hours (morning fasting) and an ITT test was performed. Glucose solution (Sigma-Aldrich, Merch, 1.5 g/kg BW) and insulin solution (Novo Rapid, Novo Nordisk®, 0,5 U/kg BW) were administrated via intra peritoneal injection (i.p.), respectively. In order to measured glucose levels, blood samples were collected from the tail vein: the first drop of blood was discarded and the second drop was used to measure baseline glucose levels, through a portable commercial glucometer kit (GlucoMen® aero 2K, A. MENARINI diagnostics) at 0, 15, 30, 60, 90 and 120 minutes. On week 10 (T10) of the study, all animals were submitted to same tests. The AUC of GTT and of ITT curves were calculated using the trapezoidal method by Ghezzi *et al*., (2012).

2.3. Sample collection

At week 10, animals were anaesthetized in a saturated chamber with isoflurane (IsoFlo®, Abbott) followed by intraperitoneal injection of 150 mg/kg of ketamine chloride (100 mg/kg; Imalgene®) in xylazine (10 mg/kg, Rompun®). Blood was immediately collected through heart puncture to serum tubes (BD Vacutainer SST II 47 Advance) and then centrifuged at 3500 rpm for 15 minutes (4 ºC) and stored at -20 ºC. Upon sacrifice, mice were transcardiacly perfused with ice-cold PBS1x and the aorta and the liver were isolated, collected, washed and weighted. The tissues' samples were divided into 3 sections for distinct purposes: the first one, for mRNA analyses, was immersed in RNA latter (R-0901, Sigma Aldrich); the second one was placed in OCT CryoMatrix (6769006, ThermoScientific) for fluorescence microscopy and the third section was snap frozen in liquid nitrogen for protein analysis. Samples were stored at -80 ºC until analyses were performed.

3. *Ex vivo* analysis

3.1. Oil Red O staining

Sections of fresh frozen tissue samples from liver and aorta were cut into 5 µm thickness, mounted on slides and allowed to dry for 30 minutes. The cryosections were placed in absolute propylene glycol for 2 minutes and transferred to 0.5% red oil in absolute propylene glycol solution for 10 minutes. The sections were differentiated in 85% propylene glycol solution for 2 minutes, washed in distilled water and counterstained in Hematoxylin Stain Solution Gill 1 (Sigma Aldrich; Missouri, USA) for 30 seconds. They were rinsed under running water for 3 minutes and mounted with CC/Mount aqueous mounting medium (Sigma Aldrich; Missouri, USA). The lipids were stained with bright red color and nuclei with a blue color.

3.2. H&E staining

Aorta and liver samples were formalin-fixed and embedded in paraffin wax. Cryosections (5 µm) from each block were reviewed. Briefly, tissue sections were deparaffinized in xylene and hydrated to a decrescent series of ethanol until distilled water. Thereafter, the tissue sections were immersed in hematoxylin stain Solution, Gill 1 (Sigma Aldrich, Saint Louis, MO, USA) for 2 min and washed in tap water. Then, they were counterstained with 0.5% aqueous eosin (Sigma Aldrich; MO, USA) for 30 s and after that dehydrated, cleared, and mounted. All samples from both groups (n = 8 for each) were examined by light microscopy using a Zeiss microscope Mod. Axioplan 2 (Göttingen, Germany).

3.3. TGs quantification

Following Oil Red-O sample analysis, TGs contents on liver samples were measured by an enzymatic colorimetric assay using a commercial kit (Ref.1155010, Triglycerides MR, Cromatest®, Linear Chemicals, Barcelona, Spain). Briefly, 50 mg of frozen tissue were homogenized in 1 mL of isopropanol. The homogenate was sonicated and then centrifuged at 3000 rpm for 5 min at 4 ºC, and the supernatant was analyzed following the manufacturer's instructions.

3.4. Gene expression analysis

3.4.1. RNA extraction

For liver samples, 35–50 mg of frozen liver tissue (preserved in RNA later Stabilization Solution, R-0901, Sigma Aldrich) were homogenized by mechanical dissociation using a Potter-Elvehjem (Thomas Scientific, USA) in 1 mL of Trizol (93289, Sigma) and stored overnight at -80 ºC. RNA was extracted using the RNeasy® Lipid Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

3.4.2. cDNA synthesis

Synthesis of complementary Deoxyribonucleic acid (cDNA) was performed using a Xpert cDNA Synthesis Mastermix (GK81.0100, Lot. 7E2709A, GRISP). For each tube, it was pipetted the volume corresponding to 2 µg RNA, 10 µL of Mastermix and water (to a final volume of 19 µL. Then, in the thermocycler (1861096, T100TM Thermal Cycler,

Bio-Rad) cDNA was synthesized following the Xpert cDNA Synthesis Mastermix protocol. Samples were stored at -20 ºC.

3.4.3. RT-PCR

A mixture was prepared containing 10 µL of Sybr Green (iTaq Universal SYBR Green Supermix 1725124, Bio-Rad), 0.4 µL of mix primers (**Table 2**) and 7.6 µL of autoclaved water. 18 µL of this mixture and 2 µL of the sample were transferred into each well. Realtime polymerase chain reaction (RT-PCR) protocol consisted of 1 cycle for initial denaturation (10 min at 95ºC), followed by 40 cycles comprising the following steps: 15 s, 95 ºC; 45s, 58 or 60 ºC; 30 s at 72 ºC. Standardization was achieved with GeNorm algorithm, where gene stability was attained with Hypoxanthine Phosphoribosyltransferase (HPRT) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression ratio of each of the target gene was computed on the basis of ΔΔCt ($2^{-\Delta\Delta}Cp$) values. Results are expressed as percentage of control.

Table 2 - Primer sequences and real-time PCR conditions.

Gene	Forward	Reverse	Temp (ºC)
GADPH	CGA CTT CAA CAG CAA CTC	TGT AGC CGT ATT CAT TGT	58
HPRT	TCC ATT CCT ATG ACT GTA	CAT CTC CAC CAA TAA CTT	58
SOD-1	AAC CAG TTG TGT TGT CAG GAC	CCA CCA TGT TTC TTA GAG TGA	60
SOD-2	CAG ACC TGC CTT ACG ACT ATG	CTC GGT GGC GTT GAG ATT GTT	60

3.5. Superoxide dismutase activity

Serum and liver SOD levels were determined by colorimetric methods, using the Superoxide Dismutase (SOD) Colorimetric Activity Kit (EIASODC, ThermoFisher Scientific), which is designed to measure all types of SOD activity (i.e., Cu/Zn, Mn and Fe superoxide dismutases). Briefly, blood samples were diluted 1:5 in PBS1X. The homogenate was centrifuged at 1500 g for 10 minutes at 4ºC, and the supernatant was analyzed following the manufacturer's instructions.

3.6. Statistical analysis

Results were expressed as means ± standard errors of the mean (S.E.M.) using GraphPad Prism® software, version 9.0.0 (GraphPad Software, Inc., La Jolla, CA, USA). The distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test to assess significant deviations from normality. Accordingly, comparisons between the 3 experimental groups were performed using the nonparametric Kruskal-Wallis test (followed by the Dunn's test for multiple comparisons) for non-normally distributed data or the parametric tests. One-way of variance (ANOVA, followed by Bonferroni's test for multiple comparisons) was used when appropriate. Repeated measures ANOVA, followed by Bonferroni post-hoc test, were used to compare BW evolution during the experimental period and glucose levels throughout the GTT and ITT assays. A p value < 0.05 was considered statistically significant.

|Chapter IV – Results

1. Experimental Setting 1: characterize the metabolic effects of 3 distinct diets in wild-type mice

1.1. Body weight, food and beverage monitoring

BW was monitored weekly during the 10 weeks of study (**Fig. 10).** ATD-fed animals displayed a statistically significant BW gain as early as two weeks ($p<0.05$), which was accentuated in the following weeks until the end of the study ($p<0.001$). Regarding the LF group, the animals displayed BW gain identical to that of the Sd-fed animals.

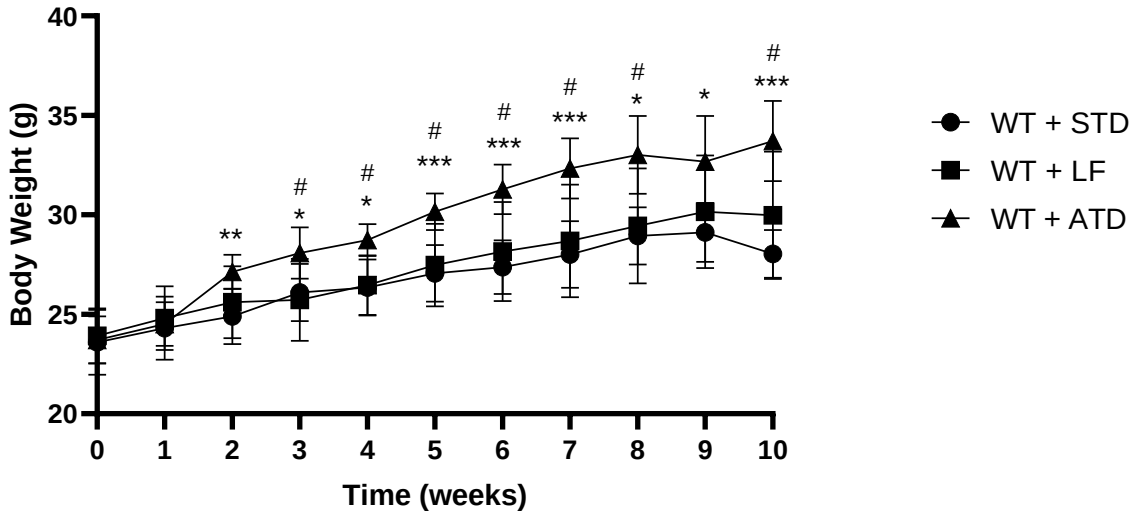

Figure 10 - Body weight evolution (Experimental Setting 1). Results are expressed as mean± S.E.M. of 6 - 8 animals per group. * p <0.05, ** p <0.01 and *** p <0.001 vs STD and # p<0.05 vs LF.

Food and beverage consumption and caloric intake were also evaluated during the entire study (**Table 3**). ATD-fed animals showed a lower food consumption ($p<0.001$ vs STD), but the highest caloric intake ($P<0.001$ vs STD and LF), which is explained by the caloric content of the diet. Additionally, LF group display a lower food and caloric intake when compared to STD group. Both refined groups (LF and ATD) exhibited a decrease in intake of carbohydrates, being more accentuated in ATD group ($p<0.001$). Regarding lipid caloric intake, ATD-fed animals had increased levels when compared to the other groups. Protein intake was lower in both LF and ATD, versus the STD-fed group.

Table 3 - Food, beverage and caloric intake (Experimental Setting 1).

Parameters	STD	LF	ATD
Food Intake (g/week)	24.038 ± 0.468	19.475 ± 0.251***	19.925 ± 0.237***
Beverage intake (ml/week)	39.275 ± 1.342	37.344 ± 0.838	36.563 ± 1.962
Calories from protein (kcal/week)	17.794 ± 0.347	13.391 ± 0.172***	13.629 ± 0.163***
Calories from carbohydrates (kcal/week)	51.441 ± 1.002	47.605 ± 0.6125***	38.286 ± 0.456***; ###
Calories from fat (kcal/week)	6.512 ± 0.127	9.114 ± 0.117***	37.748 ± 0.450***; ###
∑ Total calories (kcal/week)	75.718 ± 1.476	70.110 ± 0.902**	89.663 ± 1.068***; ###

Data are expressed as mean ± S.E.M. (n=8 per group). ** p <0.01 and *** p <0.001 vs STD and ### p<0.001 vs LF.

1.2. GTT and ITT

As for the glycemic profile, a GTT assay was performed on the first and last weeks of study (**Fig. 11**). On the first week (**Fig. 11A**) all groups present a similar capacity to manage the glucose bolus administered within the GTT test. The same pattern was observed in all groups after 10 weeks consuming the different diets (**Fig. 11B**). Accordingly, the AUC values for weeks 1 and 10 do not show statistical significance (**Fig**. **11C** and **11D**). Regarding fasting glucose levels no significance was achieved.

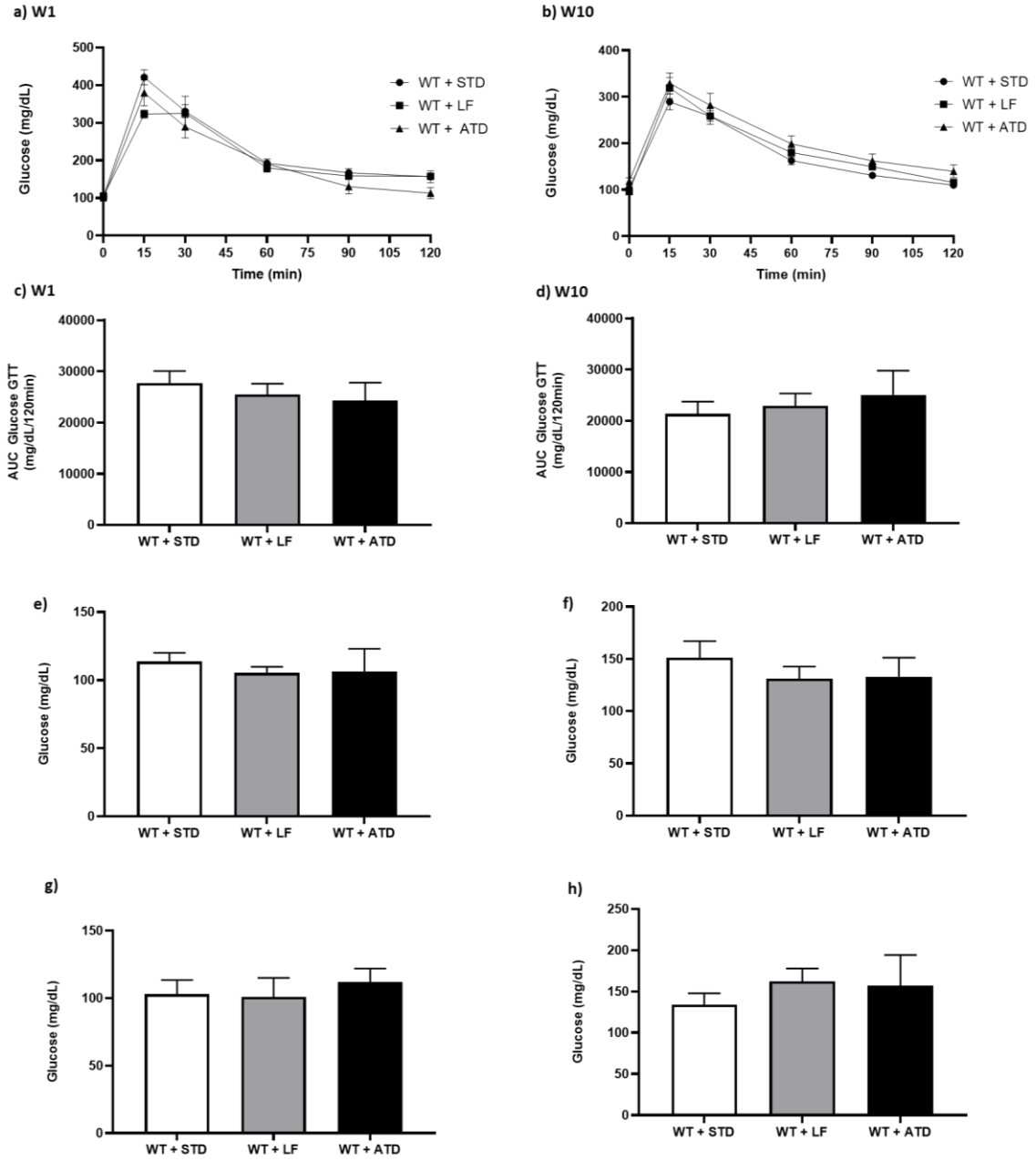

Figure 11 - Glycemic profile (Experimental Setting 1). A – b) Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of glucose solution (1.5 g/kg BW, GTT assay); c – d) AUC of GTT assay; e) Glucose values after 12h fasting (W1); f) Glucose values after 6h fasting (W1); g) Glucose values after 12h fasting (W10); h) Glucose values after 6h fasting (W10). Results are expressed as mean ± S.E.M. of 4 - 8 animals per group.

A similar pattern was obtained for the ITT assay. All groups manage to recover the glucose levels across the study (weeks 1 and 10) (**Fig. 12A and 12B**), confirmed by the ITT AUC (**Fig. 12C and 12D**).

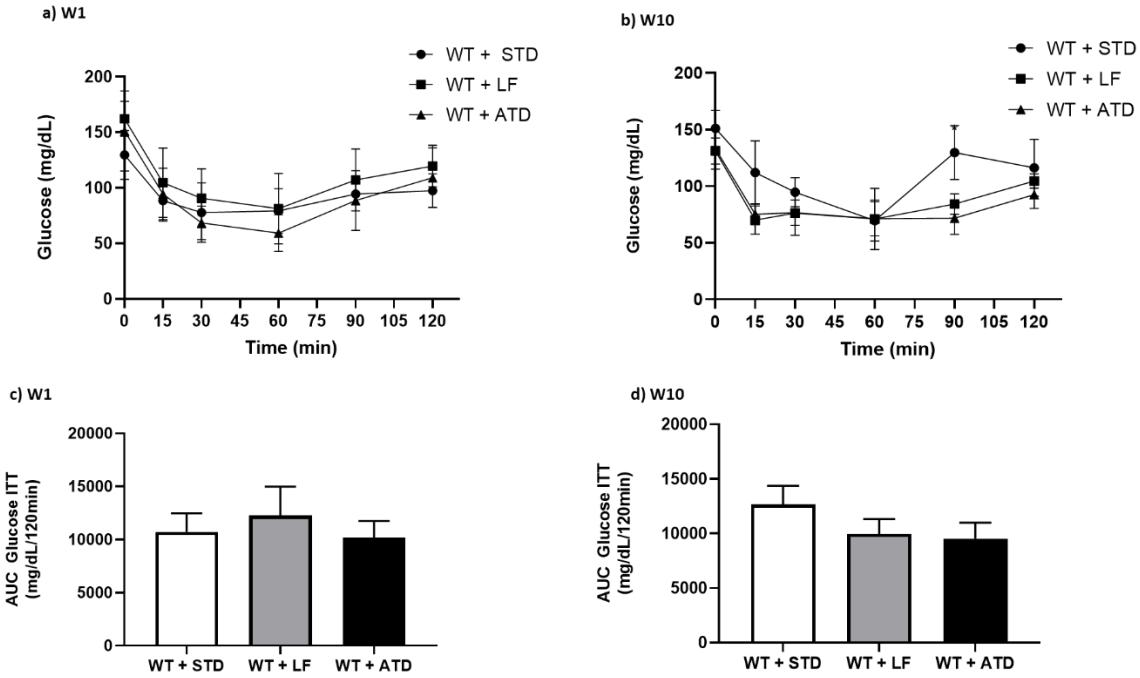

Figure 12 - Insulinemic profile (Experimental Setting 1). A – b) Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of insulin solution (0.5 U/kg BW, ITT assay); c - d) AUC values of ITT assay (W1 and W10, respectively). Results are expressed as mean ± S.E.M. of 4 - 8 animals per group.

1.3. Serum lipid profile and liver lipid deposition

To characterize the lipid profile, serum TGs, c-LDL, c-HDL and TC contents, as well as TGs content in the liver, were evaluated (**Fig. 13**). Regarding serum TGs, the ATD mice showed a trend to decreased levels, but no statistical significance was achieved (**Fig. 13D).** However, liver TGs content in were higher in the ATD group ($p<0.01$ compared with STD group and $p<0.05$ compared with LF group) (**Fig. 14**). WT + ATD group showed increased levels of c-HDL, c-LDL and c-Total (**Fig. 13A**, **13B** and **13C**) ($p<0.001$ vs STD). The results of liver Oil Red-O staining are in accordance with these findings (**Fig. 15**).

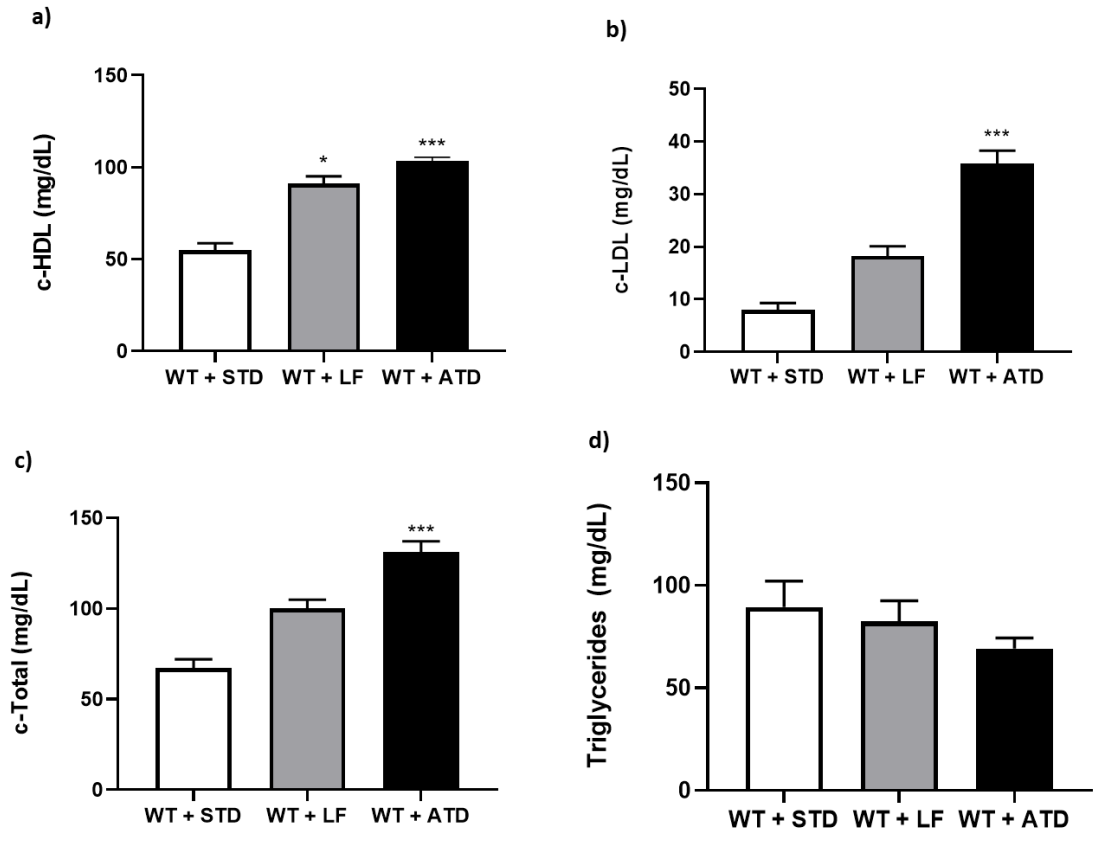

Figure 13 - Serum lipid profile (Experimental Setting 1). A) c-LDL levels, b) c-HDL levels; c) c-Total levels and d) Triglycerides levels. Results are expressed as mean ± S.E.M. (n=6-8 per group). * $p < 0.05$, *** $p < 0,001$ vs STD.

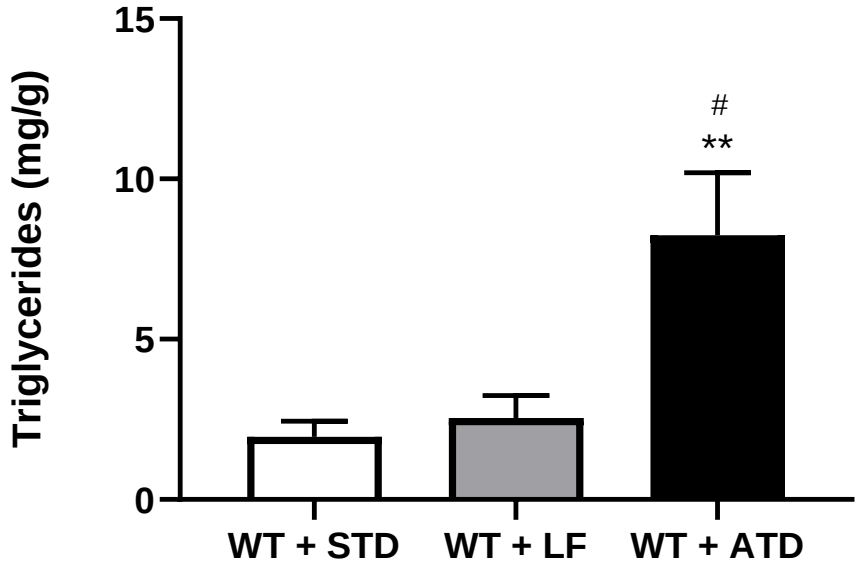

Figure 14 - Liver lipid deposition (Experimental Setting 1). Results are expressed as mean ± S.E.M. (n=6-8 per group). ** $p < 0.01$ vs STD; # $p < 0.05$ vs LF.

1.4. Liver enzymes and histomorphology

The activity of liver enzymes alanine aminotransferase (ALT) and alanine aminotransferase (AST) were quantified and presented in **Table 4**. No differences between groups were observed.

Table 4 - Activity of liver enzymes (Experimental Setting 1).

Enzyme	WT + STD	WT + LF	WT + ATD
ALT (U/L)	12.33 ± 2.55	10.00 ± 1.38	15.51 ± 2.94
AST (U/L)	90.29 ± 19.04	57.71 ± 10.00	78.57 ± 7.67

Data are expressed as mean ± S.E.M. (n=8 per group).

Representative images of H&E and Oil Red-O stained livers from all groups are shown in **Fig. 15**, respectively. Marked hepatic disturbance (fat vacuoles - black arrows) were observed in the WT animals fed with ATD, when compared with a normal profile in the other groups (H&E). This was accompanied by increased Oil Red O staining in this group.

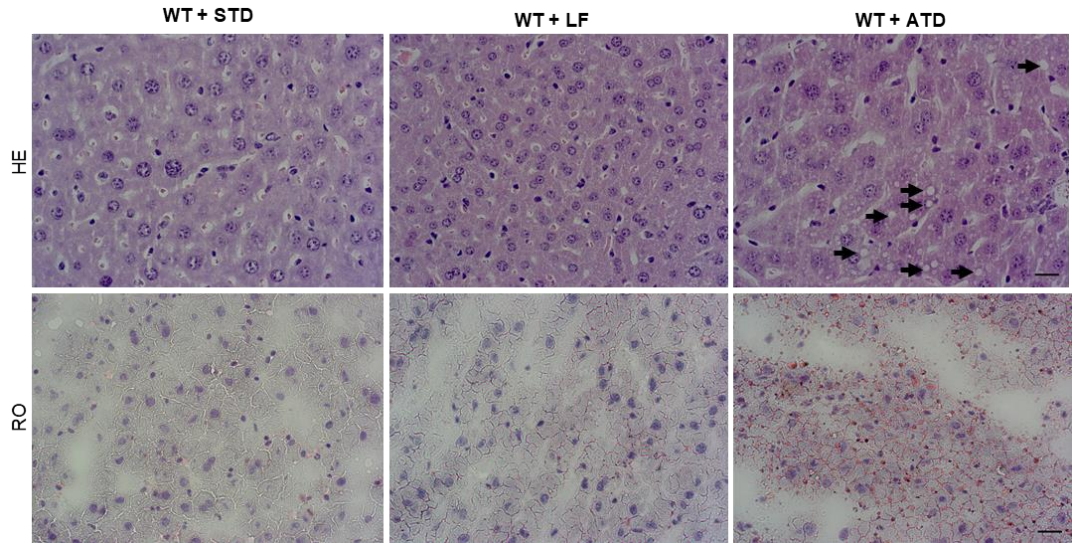

Figure 15 - Representative images of H&E and Oil Red-O staining in the liver (Experimental Setting 1). Black arrows depict hepatic liver deposition in fat vacuoles. Scale bar = 20 µm.

1.5. SOD gene expression and activity in the liver and serum

SOD 1/2 gene expression and SOD activity were evaluated in the serum and liver as a readout of antioxidant capacity. Results shows that LF and ATD groups have decreased hepatic SOD 1 and 2 gene expression (**Fig 16A** and **16B**) ($p<0.5$ vs STD). However, they show increased SOD activity in liver (**Fig 16C**), but no statistical differences were observed. Nevertheless, SOD activity in serum was higher in WT + ATD group ($p<0.05$ vs STD) (**Fig. 17**).

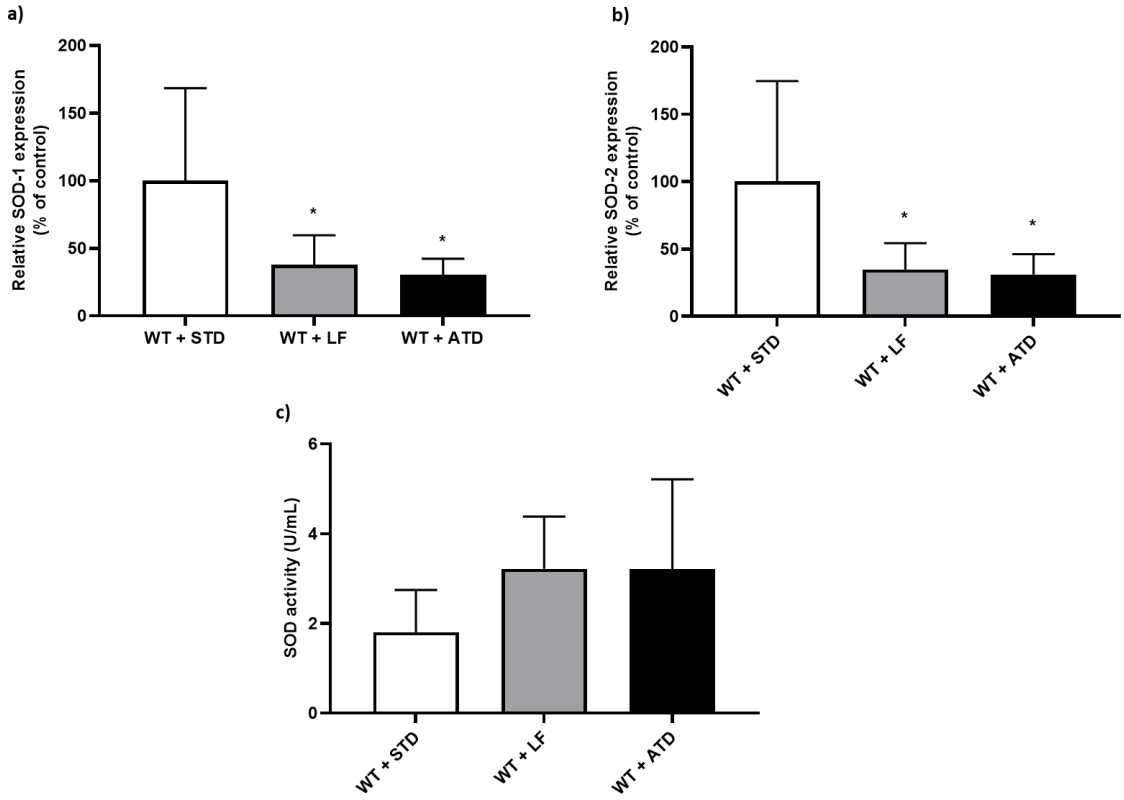

Figure 16 - SOD gene expression and activity in the liver (Experimental Setting 1). A) SOD-1 gene expression, b) SOD-2 gene expression and c) SOD activity in the liver. Results are expressed as mean ± S.E.M. of 6-8 animals per group. One-way ANOVA * $p<0.05$ vs STD.

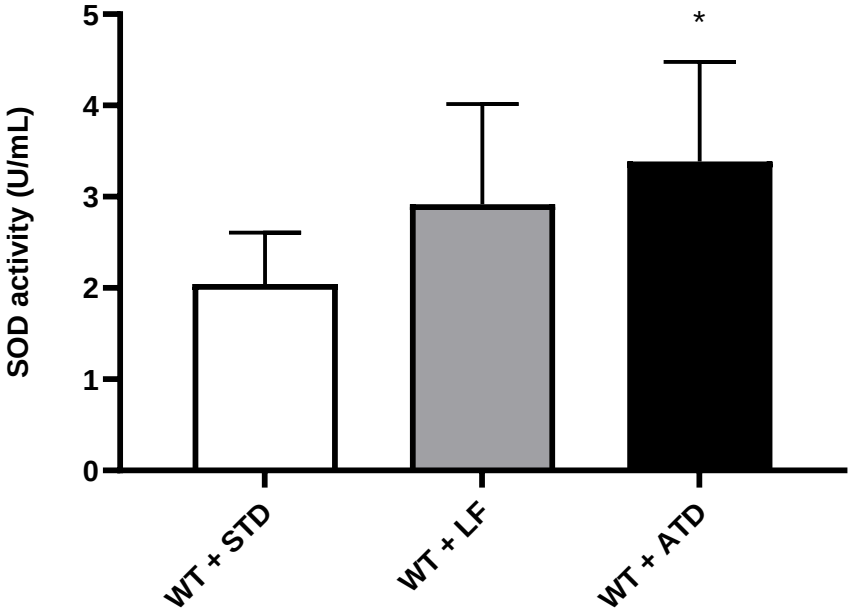

Figure 17 - SOD activity in the serum (Experimental Setting 1). Results are expressed as mean ± S.E.M. of 6-8 animals per group. One-way ANOVA * $p<0.05$ vs STD.

2. Experimental setting 2: Evaluation of the potential beneficial effects of oleacein in the apoE-KO mouse

2.1. Body weight, food and beverage monitoring

Both ApoE-KO groups presented unchanged BW evolution when compared with the control one (WT+ATD) (**Fig. 18**).

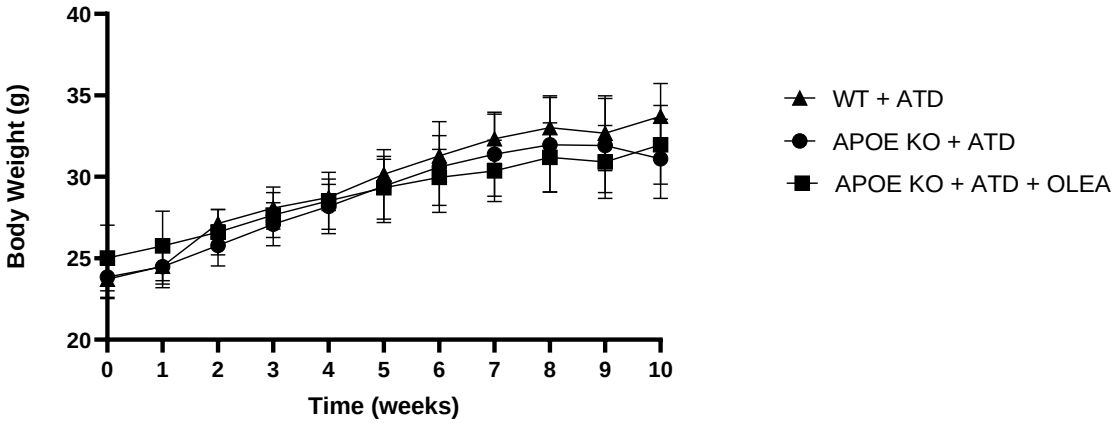

Figure 18 – **Body weight evolution (Experimental Setting 2).** Results are expressed as mean± S.E.M. of 8 animals per group. * p <0.05, ** p <0.01 and ***p <0.001.

As shown in **Table 5**, APOE KO + ATD + OLEA group had a higher beverage intake when compared to the other groups, while no changes between groups were observed for food and caloric intake.

Table 5 - Food, beverage and caloric intake (Experimental Setting 2).

Parameter	WT + ATD	APOE KO + ATD	APOE KO + ATD + OLEA
Food Intake (g/week)	19.925 ± 0.237	19.913 ± 0.238	20.388 ± 0.301
Beverage intake (ml/week)	36.563 ± 1.962	35.750 ± 0.811	43.875 ± 0.778***; ##
Calories from protein (kcal/week)	13.629 ± 0.163	13.629 0.163	13.945 0.206
Calories from carbohydrates (kcal/week)	38.286 ± 0.456	38.262 ± 0.457	39.259 ± 0.591
Calories from fat (kcal/week)	37.748 ± 0.450	37.724 ± 0.451	38.6624 ± 0.571
∑ Total calories (kcal/week)	89.663 ± 1.068	89.606 ± 1.071	91.744 ± 1.356

Data are expressed as mean ± S.E.M. (n= 8 per group). *** p <0.001 vs STD and ## p<0.01 vs LF.

2.2. GTT and ITT

On the first week (**Fig. 19A**) and in the last week (**Fig. 19B**) all groups present a similar capacity to manage the glucose bolus administered within the GTT test. Accordingly, the AUC values for weeks 1 and 10 were unchanged between groups (**Fig. 19C** and **19D**). Regarding fasting glucose levels no significant differences were observed (**Fig. 19E-19H**).

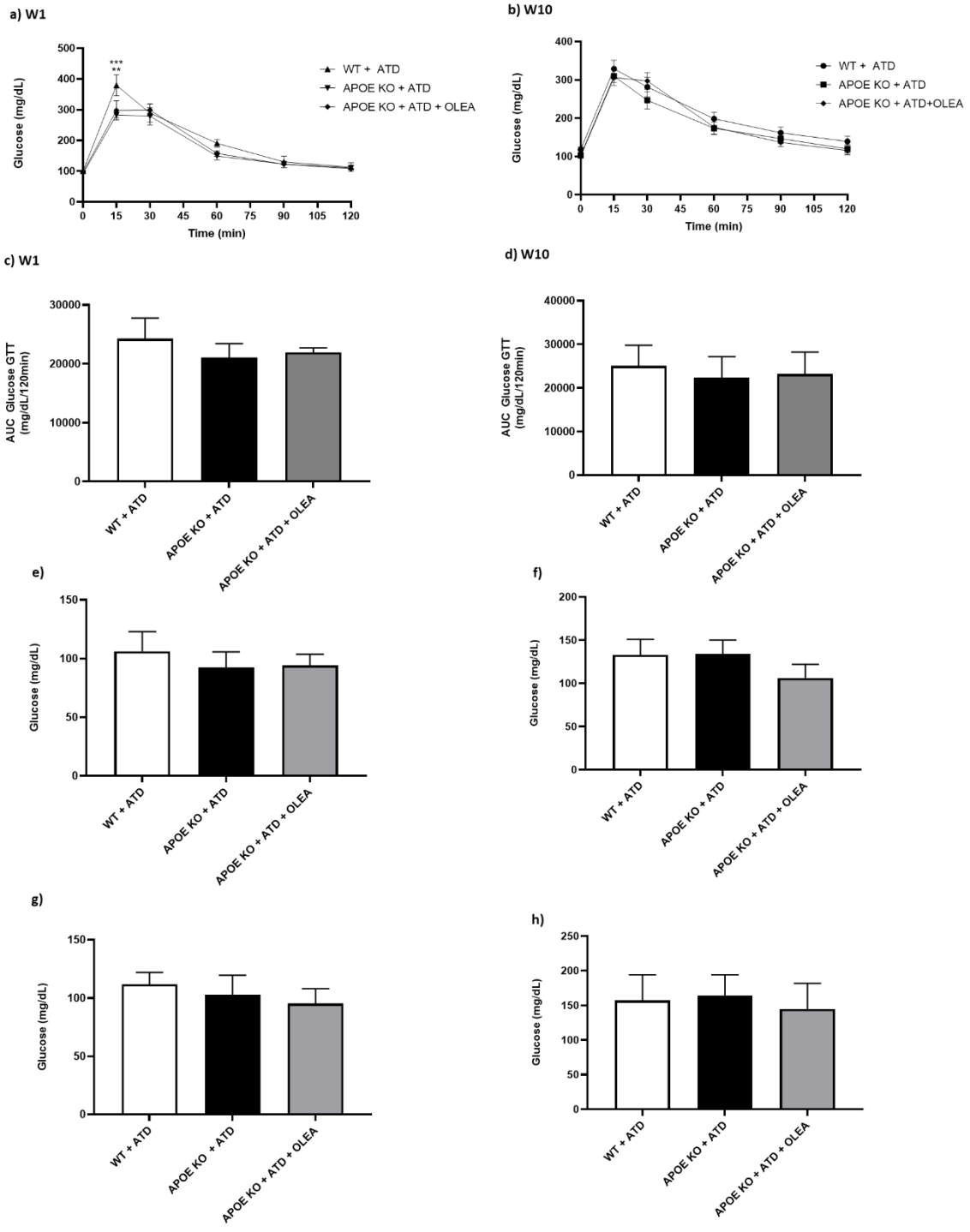

Figure 19 – Glycemic profile (Experimental Setting 2). A-b) Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of glucose solution (1.5 g/kg BW, GTT assay); c – d) AUC of GTT assay; e) Glucose values after 12h fasting (W1); f) Glucose values after 6h fasting (W1); g) Glucose values after 12h fasting (W10); h) Glucose values after 6h fasting (W10). Results are expressed as mean ± S.E.M. of 4 - 8 animals per group.

The same pattern observed in ITT assay. In both ITT curves and AUC all groups did not show statistical significance (**Fig 20**).

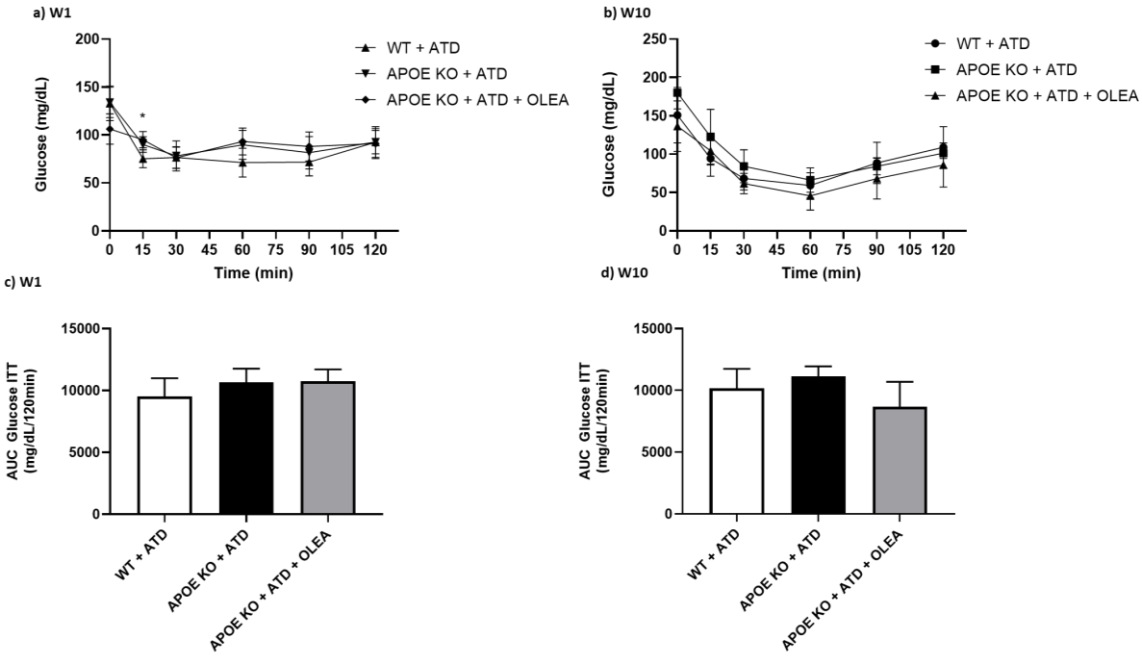

Figure 20 - Insulinemic profile (Experimental Setting 2). A – b) Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of insulin solution (0.5 U/kg BW, ITT assay); c - d) AUC values of ITT assay (W1 and W10, respectively). Results are expressed as mean ± S.E.M. of 4 - 8 animals per group.

2.3. Serum lipid profile and liver lipid deposition

Analogous to Experimental Setting 1, serum concentrations of TGs, c-LDL, c-HDL and TC, as well as liver TGs content were also evaluated. Regarding serum TGs, WT + ATD-group showed a decreased content, but no statistical significance was achieved (**Fig. 21A**). As for liver TGs' content, APOE KO + ATD + OLEA showed a decreased level ($p<0.05$ compared with APOE KO + ATD group) (**Fig. 22**). APOE KO + ATD + OLEA showed decrease serum c-Total and triglycerides levels (**Fig. 21C** and

22D) (p<0.001 vs STD, p<0.05 vs APOE KO + ATD and p <0,001 vs APOE KO+ ATD, respectively). Liver Oil Red-O coloration is in accordance with these findings (**Fig 25**).

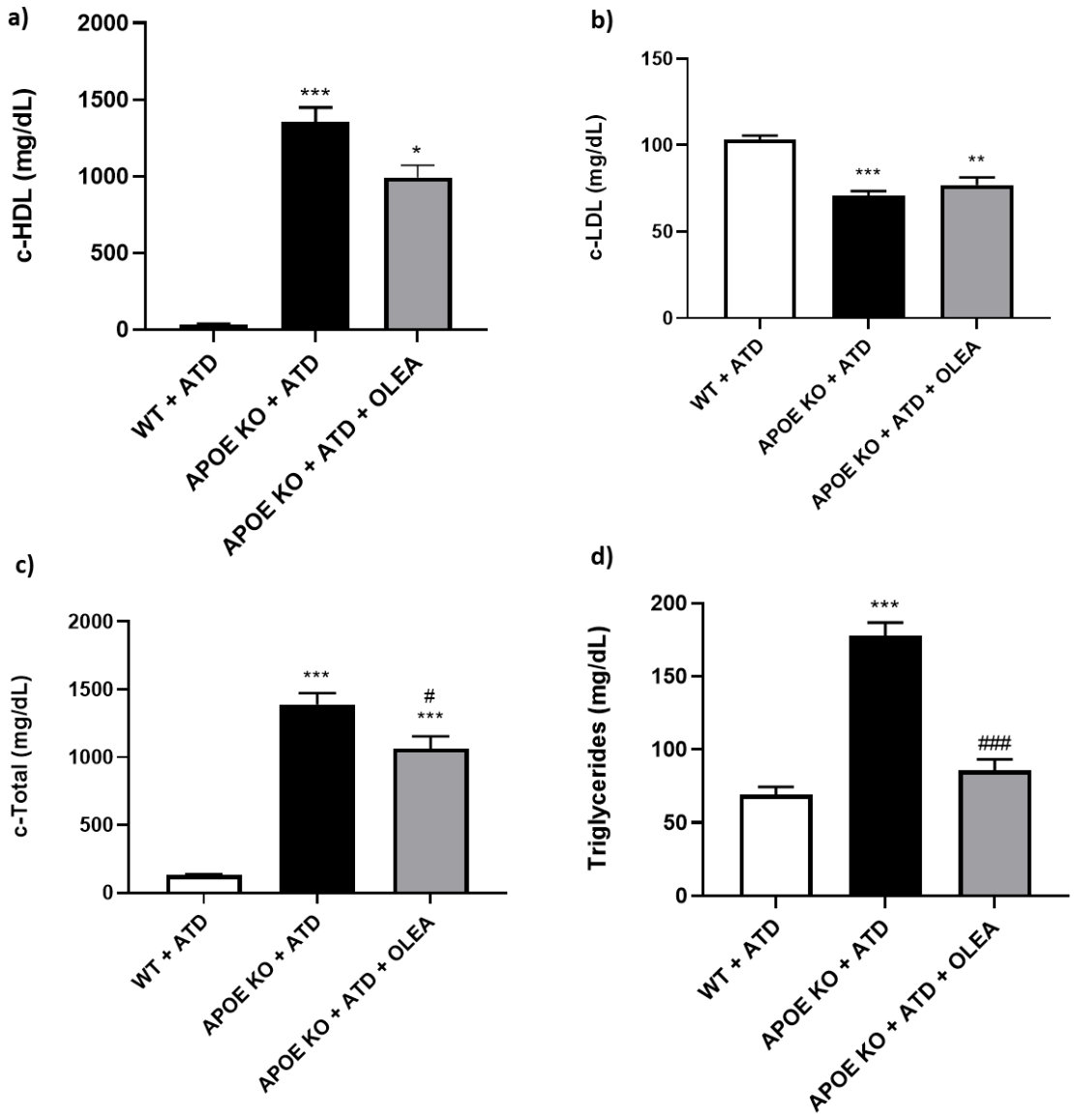

Figure 21 - Serum lipid profile (Experimental Setting 2). A) c-LDL levels, b) c-HDL levels; c) c-Total levels and d) Triglycerides levels. Results are expressed as mean ± S.E.M. (n=6- 8 per group). * p < 0.05, ** p<0,01, *** p < 0,001 vs STD and $^{\#}$ p<0.01, $^{\#\#\#}$ p<0.001 vs APOE KO + ATD.

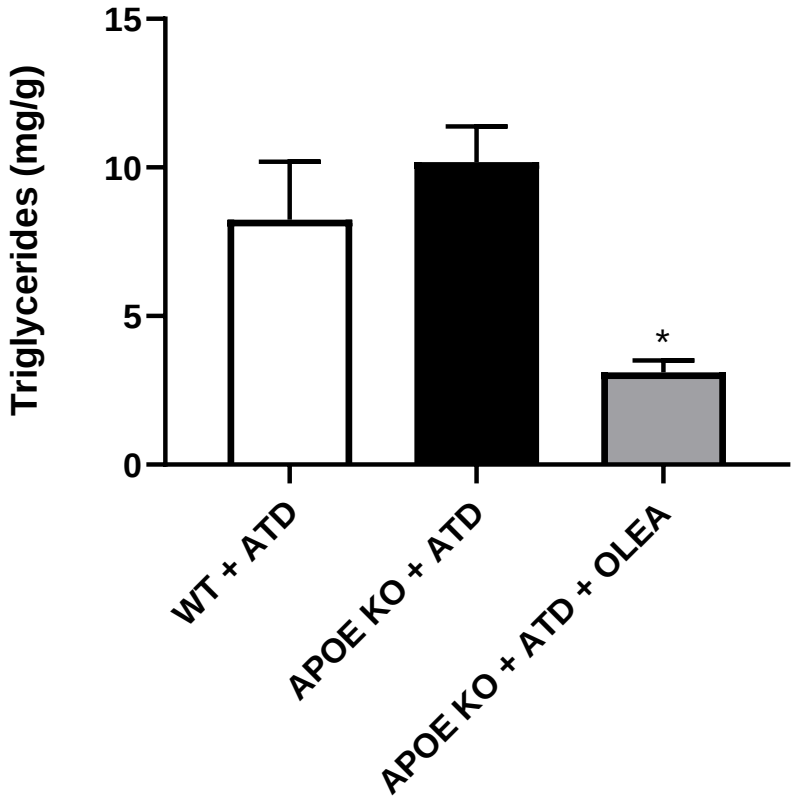

Figure 22 - Liver lipid deposition (Experimental Setting 2). Results are expressed as mean ± S.E.M. (n= 6-8 per group). * p < 0.01 vs STD.

2.4. Aorta histomorphology

Images of H&E staining in aorta rings of WT + ATD, APOE KO + ATD and APOE KO + ATD + OLEA groups are shown in **Fig. 23**. WT + ATD group did not show any lesion or significant change in vessel wall (**Fig. 23A**). Both APOE + ATD groups, with and without OLEA, were able to show changes on aorta structure, including necrotic core and cholesterol cleft (**Fig. 23B and 23C**). **Fig. 24** shows SEM images of the lesions found in the APOE + ATD group, exposing the disorganization of endothelium and the formation of foam cells.

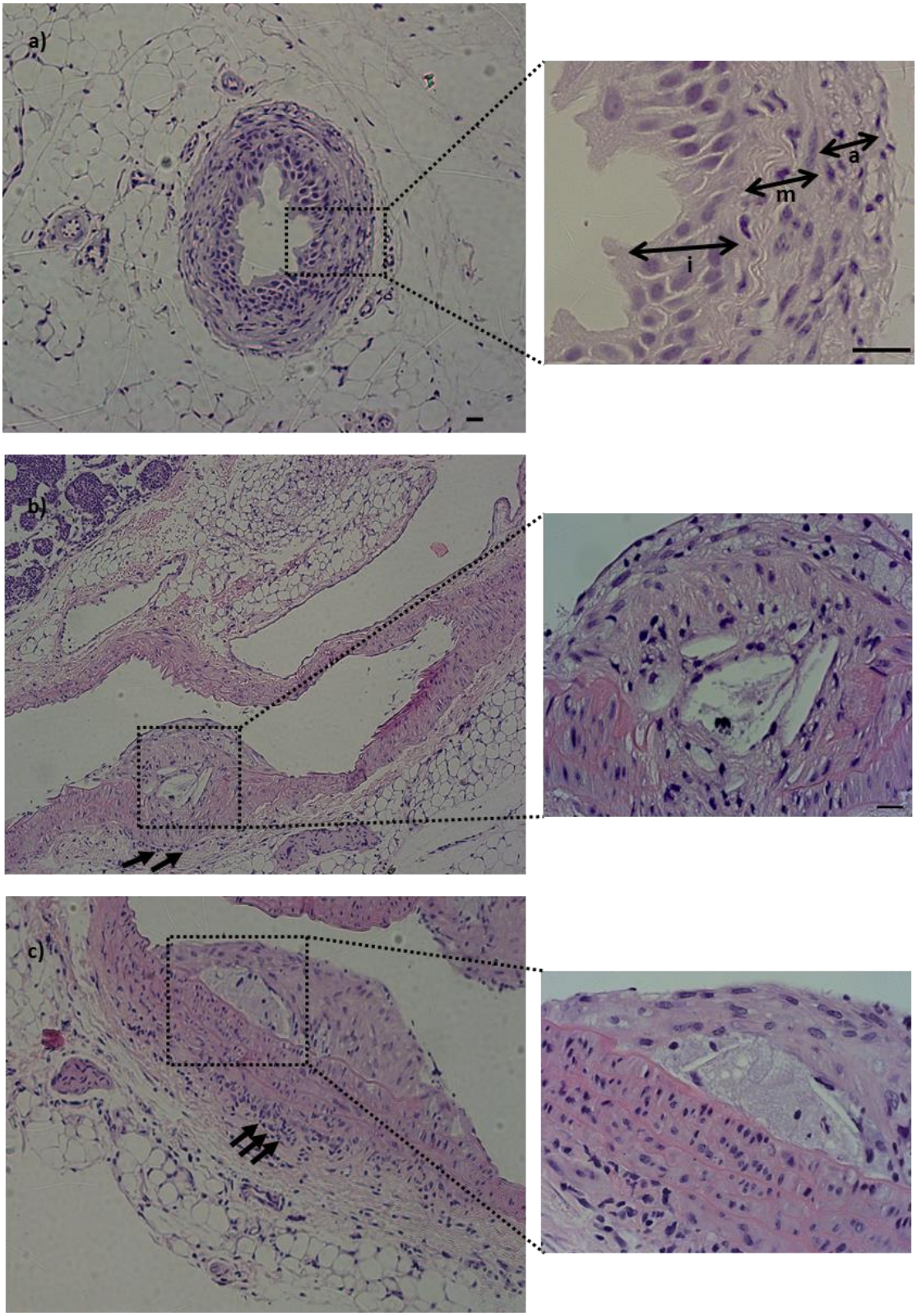

Figure 23 - Images of H&E staining in aorta rings (Experimental Setting 2). a) Image of aorta ring of the WT+ATD group, with adventitia (a), media (m) and intima (i) layers identified; b) Image of aorta ring of the APOE + ATD group with atherosclerotic lesions: square indicates a necrotic core and cholesterol cleft; black arrows indicate inflammatory cell infiltration; c) Image of aorta ring of the APOE + ATD group: square indicates a necrotic core and cholesterol cleft; black arrows indicate inflammatory cell infiltration. Scale bar = 20 µm.

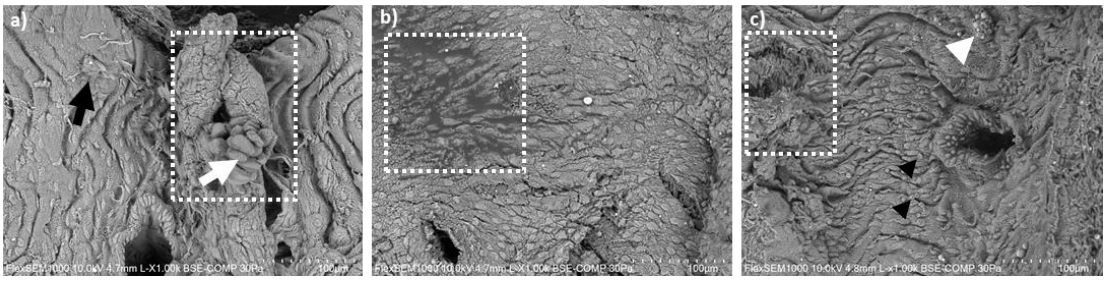

Figure 24 - SEM images of aorta of the APOE + ATD group. a) Square region shows perturbance within endothelium region. White and black arrows depict erythrocytes and leukocytes, respectively; b) Square region shows an area of impaired endothelium surface (denudation); c) Square region shows an area of endothelium disorganization. White arrow shows foam cell formation. Black arrows show erythrocytes.

2.5. Liver enzymes and histomorphology

Table 6 presents the activity of liver enzymes ALT and AST. There was a trend to increased activity of both ALT and AST in the APOE KO + ATD group, despite the values have not reached statistical significance.

Table 6 – Activity of liver enzymes (Experimental Setting 2).

Enzyme	WT + ATD	APOE KO + ATD	APOE KO + ATD + OLEA
ALT (U/L)	15.51 ± 2.94	44.50 ± 12.41	36.13 ± 8.47
AST (U/L)	78.57 ± 7.67	90.67 ± 11.32	72.25 ± 7.71

Data are expressed as mean ± SEM (n=8 per group).

Representative images of H&E and Oil Red-O stained livers from all groups are shown in **Fig. 25**, respectively. We can observe that APOE KO + ATD + OLEA showed a decrease in size of fat rich vacuoles in accordance with Oil Red-O staining, which shows tenuous marking. Accordingly, Oil Red-O staining was more intense in APOE KO + ATD, followed by WT+ ATD group, which can be comparable to H&E staining.

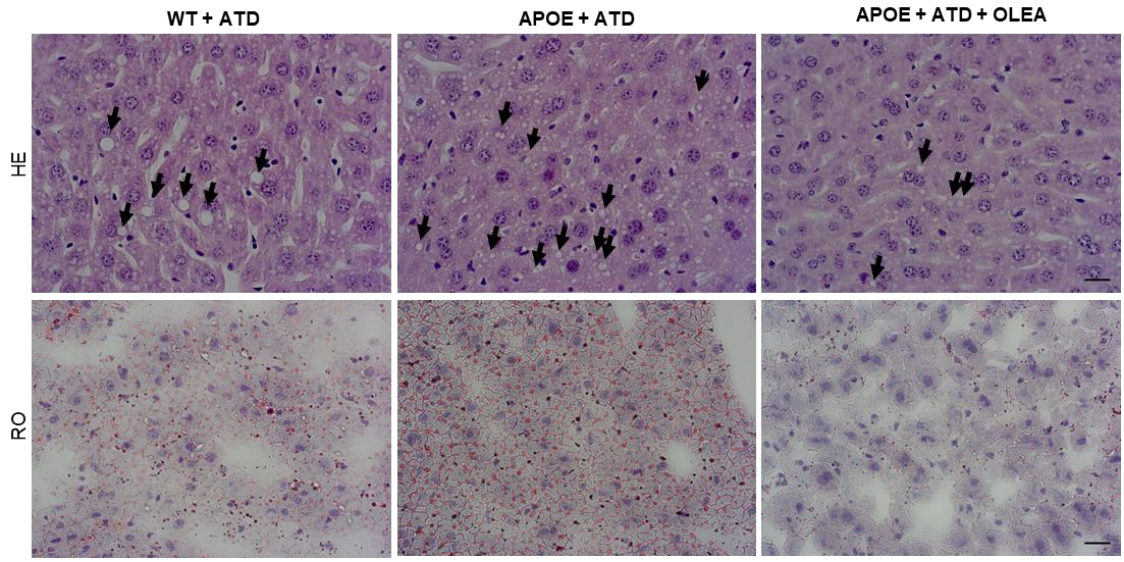

Figure 25 - Representative images of H&E and Oil Red-O staining in liver (Experimental Setting 2). Black arrows depict hepatic liver deposition in fat vacuoles. Scale bar = 20 µm.

2.5. SOD gene expression and activity in the liver and serum

Similar to Experimental Setting 2, SOD 1 and 2 gene expression and SOD activity in the serum and liver were also evaluated. Results shows that APOE KO + ATD group had a higher expression of SOD 1 and 2 compared with both groups, being statistical different (**Fig 27A** and **27B**) ($p<0.05$ vs WT + ATD). However, no significance was observed in SOD activity in the liver. However, APOE KO + ATD group showed a decreased levels of SOD activity in serum (**Fig. 28**) ($p<0.01$ vs WT + ATD).

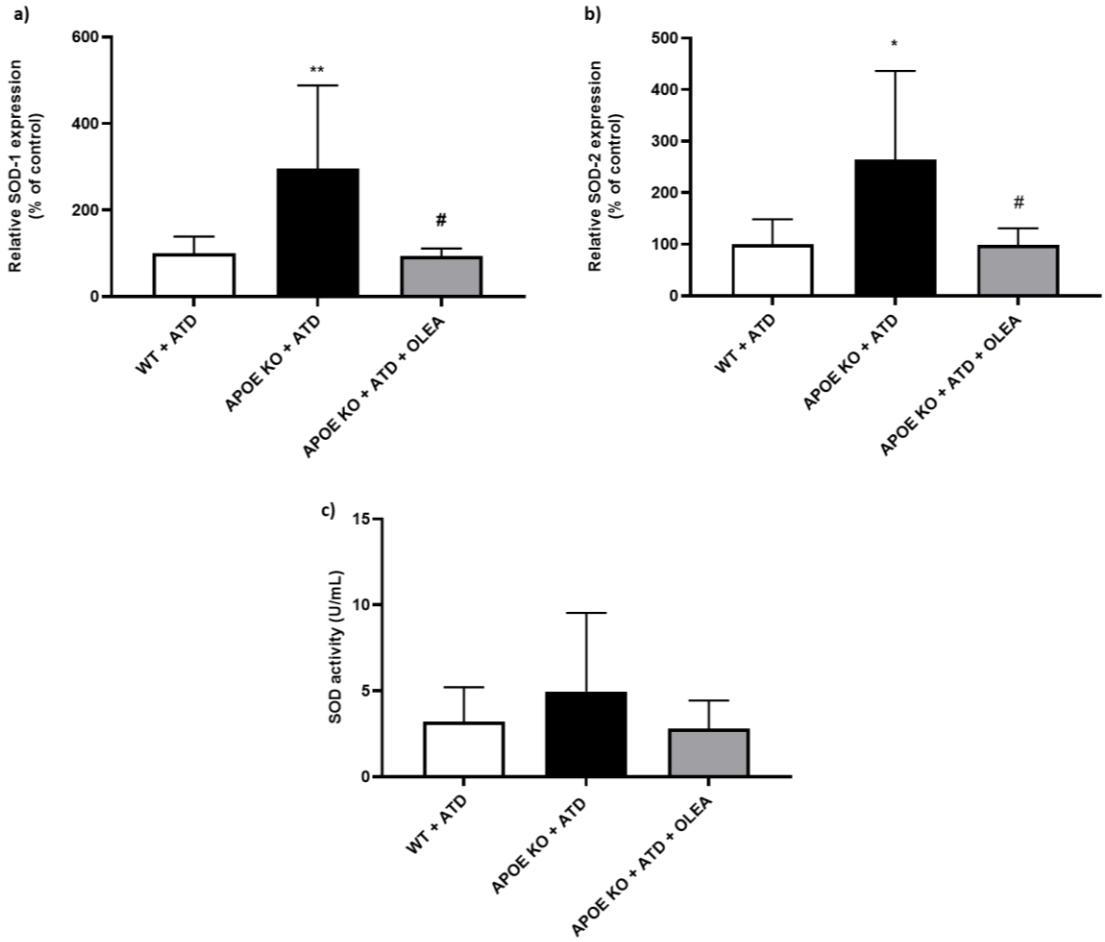

Figure 26 - SOD gene expression and activity in the liver (Experimental Setting 2). A) SOD-1 gene expression, b) SOD-2 gene expression, c) SOD activity in the liver. Results are expressed as mean ± S.E.M. of 6-8 animals per group. One-way ANOVA * $p<0.05$ vs STD and $^{\#}$ $p<0.05$ vs APOE KO + ATD.

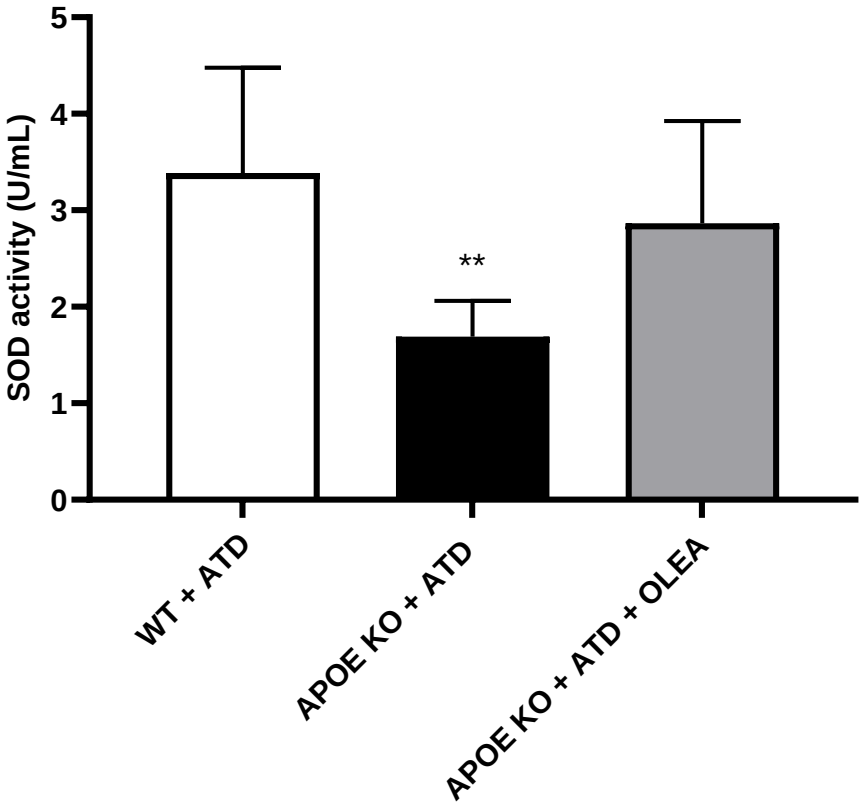

Figure 27 - SOD activity in the serum (Experimental Setting 2). Results are expressed as mean ± S.E.M. of 6-8 animals per group. One-way ANOVA ** p<0.01 vs STD

|Chapter V – Discussion and conclusions

1. Discussion

CVDs are the number one cause of death worldwide. Frequently, CVDs are associated with unhealthy lifestyle habits. It is known that the exposure to unhealthy diets with high levels of simple carbohydrates and saturated fatty acids for long-time can lead to the emerge of these diseases (Moore, 2009; Wilson *et al*., 1998; Tuomistto *et al*., 2005). This evidence become clear with the continuing consume of those diets and the rapid transition from traditional diets with fresh products – including locally-grown and harvested plants and animals – to western diets (as occurred in the USA and other western societies) together with the rapid growth of fast-food Industries (Bodicoat *et al*., 2015; Polsky *et al*., 2016; Ditzhuijzen *et al.*, 2016). Furthermore, the consumption of diets that are saturated with artificial flavors, preservatives, conservatives, is proportional to the increase of overweighting and obesity (Blaisdell *et al*., 2014). In order to better understand the etiology of these diseases, high fat diets have been essential to induce obesity and other metabolic diseases in animal models. In fact, diets must be specially developed to trigger a diseased phenotype and assure that specific disease will eventually emerge.

As mentioned previously, diets used in experimental animals can be defined as unrefined and/or purified/refined diets. Usually, the unrefined diets are used as control diets in metabolic studies and composed by grain-base and animal by-products, including corn, wheat, oat, alfafa, among others. In the literature are often referred to nebulously as “normal diet”, “chow diet” or “standard diet”. The formula of these “chow” diets is not only undisclosed to the scientific community but also, over time, variation on cereal grain nutritional levels may occur. Thus, it becomes difficult to replicate data and results from other studies (Blaisdell *et al.*, 2017; Almeida-Suhett *et al*., 2017).

Opposing to normal diets, study-diets that are representative of western societies consumption profile, such as the high fat diet or the atherogenic diets, are usually designated as purified diets. These diets comprise in their composition highly refined/purified ingredients, such as casein, sucrose, cellulose, soybean oil, among others. These ingredients are isolated and separated from the original cereal grains and animal by-products context, each of which essentially contains one main nutrient and as a result these types of diets are well defined, allowing a minimal batch variation. Additionally, the formula is disclosed to the community, permitting the researchers to personalize their diet with specific ingredients to induce a specific diseased phenotype.

By doing that, it allows different ingredients to be adjusted and to design different diets according with the levels of fat, cholesterol or sugar (HFD, LFD, HSD, among others). Within purified diets, there is a low-fat refined diet, that some companies advise to use as control to high fat refined diets. However, the data/results from each diet – unrefined and purified diet – should not be comparable due the existence of these differences between them (Almeida-Suhett *et al*., 2017; Pellizzon and Ricci, 2018).

In first place, we tried to analyze the metabolic effects of three different regiments, as a way to have more consistent data to choose the most adequate diets to be used in the Experimental Setting 2. We selected two purified diets, an ATD diet (TD. 88137) and a LF diet (TD 08485), to be compared with an unrefined diet (4RF21), in order to characterize the metabolic effects using the wild type mice (C57BL/6J). The results shown that the WT + ATD group had the higher caloric intake, mostly derived from lipidic component along with weight gain from week 2 till the end of the study. Regarding control groups, we can observe that the LF group presented a lower food and caloric intake, but a higher lipidic intake, possible due to the fact of the higher lipidic component of LFD composition, when compared to STD-fed animals. Thus, resulting in a slightly increase in body weight, even though it was not significant. This pattern was observed in a previous study using the same strain, where low fat showed a slightly increased in body weight (Almeida-Suhett *et al*., 2017). Moreover, studies done in Long Evans rats showed that the LF refined diets can augment body weight gain (Blaisdell *et al.*, 2017; Blaisdell *et al*., 2014).

In order to study the diet influence on the metabolism of WT mice, glycemic, insulinemic and lipidic profiles were evaluated. The results of our study regarding the impact of refined ATD on glycemic or insulinemic profiles of C57BL/6J mice contrast with previous studies. The animals of the WT + ATD group appear to have a trend to increase AUC curves ($p=0.1$) of GTT assay when compared to the mice of control group. Even though no statistical significance was observed, the LF and ATD groups showed slightly increase glucose values after 6h hours of fasting ($p= 0.1$ and $p= 0.2$, respectively). In fact, after 8 and 24 weeks on an HFD, WT mice can develop impaired fasting glucose (IFG) (Xu *et al*., 2019; Rendina-Ruedy *et al*., 2015, respectively). Also, other studies using rats showed that HF animals significantly upregulated fasting glucose and insulin levels (Xia *et al*., 2019). Actually, highly processed/refined diets tend to augment glycemic values, owing to unstructured/fractionated food matrices that modify nutrient bioavailability.

As their name indicate, low fat diets have lower levels of fat, when compared to refined HFD. However, in order to have energy for feeding animals, high levels of carbohydrates are added, as it is shown in LFD composition. In this sense, it was also expected that low fat diet had an impact on these profiles. In agreement with the results of our study, Agardh *et al*. (2012) observed that LF fed-animals did not achieved significance in AUC glucose values after an IPGTT. The present study was design for 10 weeks of a chronic ingestion of refined diets and it is possible that with a more prolongate exposure with these diets, the evidences would be clearer, and LF and ATD would have a more evident impact on glucose metabolism. Of course, different study diets will have different levels of nutrients, cholesterol, fat and carbohydrates and, once again, these differences within diets composition may often lead to different results. This reinforces the importance of choosing an adequate diet to be used as control on experimental studies' design. Moreover, C57BL/6J is an in-bred strain; it has been reported that differences within the strain may occur and can influence interpretations/translations of data and results of different experimental studies (Watkins-Chow and Pavan, 2007). Nevertheless, the results of these studies prove that the consumption of refined diets has an aggravate impact in glycemic and insulinemic profiles.

Next, lipid profile and lipid accumulation in the liver and serum were analyzed. Hepatic lipid accumulation results from an imbalance in homeostasis lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal (via fatty acid oxidation or triglyceride-rich lipoprotein secretion) (Ipsen *et al*., 2018). As mention before, this lipidic accumulation is enhanced when dietary caloric intake increases and as consequence abnormal lipid metabolism occurs, leading to hepatic injury. In this study, TG concentration was lower in serum on ATD-fed mice; however, this group showed higher levels in the liver tissue. In normal conditions, liver does not store TGs; yet, after a long exposure to these types of diets, which are enriched in fatty acids, there will be liver absorption of lipids (including TGs), leading to lipidic accumulation. Our results agree with those obtained by previous studies in mice fed with an HFD, which found an increased TG content in the liver tissue (Fjære *et al*., 2014; Sun *et al*., 2017; Zhang *et al*., 2018). The lipid accumulation was also evident in Oil Red O staining. In fact, ATD-fed mice liver presented higher color intensity and extension. Although the difference was not statistically significant in terms of TGs content in the liver of LF mice, the hepatic Oil Red-O staining was increased when compared with the STD diet animals.

Regarding H&E staining, the formation of fat vacuoles, as a result of lipid accumulation, was observed. In serum, the ATD group has the higher levels of TC ($p<0.001$), c-LDL and c-HDL (both $p<0.001$ vs STD group). Concerning liver enzymes, impaired serum ALT and AST levels have been used as indicators of liver dysfunction and eventually damage. While ALT in mainly presented in the liver, AST is also found in muscles (cardiac and skeletal), kidneys, lungs, brain and blood cells (Goorden *et al*., 2013). In our study, once again the ATD mice appear to have a trend to augment the ALT levels in serum, in agreement with other studies (Kim *et al*, 2012; Guo *et al*, 2016). However, serum AST content was higher in the STD group, even though no statistical significance was observed.

The composition of these diets has an impact on the results presented here. ATD and LFD are refined diets, where components within their composition (ex: fiber, vitamin, minerals, among others) have been separated from the original food content. Hence, the body processes these substances more quickly, which will reflect on the impairment of the metabolic profile. The LFD used in our study has a higher lipidic composition when compared with STD. This may explain the presence of Oil Red staining in liver samples, in contract to STD fed animals, where no staining was observed. Of course, ATD shows the higher lipidic composition when compared with the others, reflecting on the presence of higher levels on TGs and other parameters already mention as well as the Oil Red intensity color observed.

As mention previously, HFD can promote oxidative stress (Matsuzawa-Nagata *et al*., 2008; Noeman *et al*., 2011; Kesh *et al*., 2016; Lasker *et al*., 2019; Tan and Norhaizan, 2019), which is crucially involved in the connection between hypercholesterolemia, hepatic steatosis and atherosclerosis. Antioxidant systems, enzymatic and non-enzymatic, are the defense against reactive oxygen species, in order to avoid oxidative stress. SOD, in particular, eliminates superoxide anion, thus avoiding the formation of other radicals, such as the peroxynitrite, when superoxide anion is conjugated with NO. This system is usually deregulated in cardiometabolic disorders. In the present study, the hepatic mRNA expression of both SOD-1 and -2 was evaluated, as well as total SOD activity in serum and liver. The results showed that WT mice fed with atherogenic diet had lower liver expression of both SOD-1 and -2 mRNA, followed by the LF group. In accordance, Ishizuka *et al* (2020), showed that animals fed with HFD also have reduced mRNA expression of both SOD isoforms. However, in the present study, ATD group presented a trend to augmented SOD activity in the liver, accompanied by significantly increased serum activity ($p<0.05$ vs STD group). Previous studies have reported lower

activity in mice fed with high fat diet (Nemes *et al*., 2019; Lorizola *et al*., 2018), contrasting to our results. In our study, the LF-fed mice also presented a trend to increased activity in both liver and serum, although no statistical significance was achieved. Maybe the increase of this activity could be a response to oxidative stress, in order to compensate the ROS formation. Previous studies showed an increased serum and liver lipid peroxidation in animals fed high fat diets (Lee *et al*., 2006; Chen *et al*., 2012). Furthermore, the evaluation of others markers, such as GSH/GSSG, thiobarbituric acid reactive substances (TBAR), CAT, can be important to clarify the impact and the correlation of diets and oxidative stress.

The second aim of this thesis was to assess the putative anti-atherogenic and hepatoprotective effects upon oleacein consumption. Mediterranean Diet has been considered to be model dietary regiment to follow, not only because of their fresh and locally grown products, but also for the inclusion of a variety of antioxidant compounds, which reflects on the remarkable impact of human health, namely in the prevention against development of CVDs and metabolic diseases, such as atherosclerosis and NAFLD (Serreli and Deiana, 2020, Martínez-González *et al*., 2019). In particularly, olive oil is a major source of fat of MDiet. The composition of olive oil mainly characterized by the presence of lipophilic components rich in monounsaturated fatty acids, such as oleic acid. However, it comprises a small hydrophilic fraction rich in phenolic compounds, including phenylalcohols, phenolic acids, flavonoids and secoiridoides (Kanakis *et al*., 2013). Oleacein, derived from oleuropein, is an abundant component of EVOO. Previous studies have suggested that oleacein may exert atheroprotective and hepatoprotective effects (Filipek *et al*., 2020; Filipek *et al*., 2017; Lombardo *et al*., 2018) The protective effect of oleuropein against injuries caused HFD was already reported (Lepore *et al*., 2015).

In this study, we used the apoE-KO mouse fed with ATD as model of atherosclerosis with hepatic steatosis, with and without oleacein (APOE KO + ATD and APOE + ATD + OLEA, respectively), compared with WT mouse also fed with ATD (WT + ATD). OLEA was orally administered (50 mg/kg), by a method of voluntary oral administration - semi-solid matrix form (Pill) developed and optimized for rodents (Pill, patent pending Nº PCT/IB2021/053124) - for 5 days a week for 10 weeks. In has been reported that 20 mg/kg of OLEA through i.p. injection administration shows an improvement in lipid accumulation and BW reduction (Lombardo *et al*., 2018; Lepore *et al*., 2019). Different administration routes may lead to different processing mechanisms. Oral administration has been a preferred route to administrated substances, drugs and

other compounds. Due to digestion processes that may occur in stomach and even absorption on the gut, we decide a dosage substantially higher. However, it has been found that OLEA was stable at gastric acid and may be absorb in the small intestine by passive diffusion through the membrane due to its favorable partition coefficient, giving support to the beneficial effects of olive oil in diet (Naruszewicz *et al*., 2015; Castejón *et al*., 2020).

In our study, there was no difference in BW between the three groups, which is in contrast to previous studies. As mention before, oleacein was able to reduce BW in WT mice (Lombardo *et al*., 2018). In addition, Lepore *et al*. (2019), reported a protection effect of oleacein in adipogenesis, suggesting that may directly modulate the expression of important regulators of adipogenesis.

In line with what was done in Experimental Setting 1, we have evaluated the impact of oleacein treatment on glycemic, insulinemic and lipidic profile. Regarding the *in vivo* GTT assay, no significant differences were encountered among the groups. However, the group treated with OLEA showed a trend to reduce blood glucose levels after 12 hours of fasting ($p=0.09$). Regarding the *in vivo* ITT assay, the treated group showed a tendency to improve to recover glucose values ($p=0.1$), in agreement with a previous study (Lombardo *et al*., 2018).

ApoE KO mice has an inactivation on coding gene for apolipoprotein E. As mention before, this apolipoprotein helps the clearance of some lipoprotein rich in cholesterol. Because of that, this inactivation induces an elevated hypercholesterolemia state, higher than WT mice and even other mouse models (Greenow *et al*., 2005; Linton *et al*., 1998; Zhang *et al*., 1992). The results showed that APOE + ATD group had the higher serum levels of cholesterol, including c-Total, c-LDL and c-HDL. In addition, TGs content was higher in serum ($p<0.001$) and tend to be higher also in the liver, despite this last difference have not reached statistically significant. Regarding the OLEA treated group, the impact on lipid profile was remarkable. There was a clear improvement of the lipid profile, as viewed by the reduction of serum c-Total ($p<0.001$ vs control group; $p<0.05$ vs APOE + ATD group) and TGs ($p<0.001$ vs APOE + ATD group). Furthermore, TGs levels in the liver were also lower when compared with the other groups. This goes in agreement with Lombardo *et al* (2018) study. Our results agree with the data obtained for Oil Red-O staining; in fact, the APOE KO + ATD group showed increased staining when compared to the other groups. This result is also in accordance with the H&E staining, which clearly showed the presence of lipidic vacuoles. In addition, the OLEA-

treated group presented a lower staining intensity on Oil Red-O and with fewer and smaller fat vacuoles on H&E staining. Regarding serum ALT and AST activity, despite no significant changes between the 3 groups, the APOE + ATD + OLEA group showed a trend to prevent the higher values found in the APOE + ATD, which is in agreement with the literature (Silva *et al*., 2021). In fact, other studies suggest that phenolic compound from olive oil can decrease these levels (Zheng *et al*., 2021; Andreadou *et al*., 2007).

As previously mentioned, ATD has a higher lipidic composition, which once more reflects on the results presented previously. Also, ATD composition comprises higher levels of saturated fatty acids, which can increase cholesterol levels and as consequence increase the risk of atherosclerosis. The results from H&E staining and the scanning electron microscope (SEM) pictures showed that the APOE-KO mice were able to developed the atherosclerotic lesion reported in the literature. In fact, we found a profile of endothelium denudation and foam cell formation. Unfortunately, we were unable to analyze the aorta segments needed to confirm (or deny) an atheroprotective effect of oleacein. Further observations are required to robustly evaluate the effect. However, the major impact on dyslipidemia suggests the possibility of a beneficial effect against progression of atherosclerotic plaque.

Apart from the amelioration of lipid profile in serum and liver, the APOE KO + ATD group presented an increased SOD activity in the liver. It is important to bear in mind that apoE can serve as ligand to some receptor in cells. Other studies show a decreased SOD activity in the liver in APOE-KO mice fed with HFD (Han *et al*., 2017; Qian *et al*., 2021). Accordingly, Shea *et al.* (2002) observed other endogenous antioxidants levels (including SOD) increased in brain, suggesting that could be a response to the elevated oxidative stress to compensate for the lack of apoE. As mentioned previously, the absence of this apolipoprotein induces a state of hyperlipidemia which can trigger an oxidative stress situation. The apoE-KO mice fed with ATD show lower activity in serum ($p<0.001$ vs control group), which agrees with previous reports (Rong *et al*., 2017; Han *et al*.;2017). Other studies have shown a beneficial effect of others OO polyphenols in APOE KO. In fact, Sergio-Acín *et al* (2007) has demonstrated, that APOE KO mice fed with olive oil had decreased F2-isoprostanes levels – a marker of oxidative stress – in serum. Also, a study conducted by Ricón-Cervera, show that olive oil fed wt mice presented higher levels of SOD activity in liver, in contrast to the results present here. Maybe the supplementation of OLEA exerts a scavenging effect similar to SOD, lowering its activity in liver. However, SOD activity in

serum was higher when compared with APOE + ATD group. Moreover, other olive oil polyphenols derivates such as oleocanthal (OLEO) and HTy have the ability to enhance the activity of others antioxidant enzymes, like glutathione reductase (GSR), glutathione S-transferase (GST) and CAT, and lowering others oxidative stress markers levels (Cardeno *et al*., 2013; Montoya *et al*., 2021; Chiang *et al*., 2021). As previously mentioned, the results now obtained in our study deserve a broad analysis with other parameters and markers of redox status in order to confirm (or deny) an antioxidant effect involving SOD activity and other enzymatic and non-enzymatic players.

2. Conclusions

Our study confirmed the hyperlipidemic effect of a refined ATD diet on WT mice, significantly increasing the serum levels of c-Total, c-HDL and c-LDL. However, LFD-fed mice also presented an increment of lipids in liver, despite being referred as low-fat diet. No significant changes on glucose profile were observed for both diets when compared with the unrefined control diet (standard).

Regarding the effect of OLEA, our study showed that this phenolic compound is capable of exert an anti-dyslipidemic effect, viewed by the significantly reduced serum levels and lipid accumulation in the liver tissue of APOE KO mice. These effects were in accordance with H&E and Oil Red-O staining data, showing a preventive action of OLEA against hepatic steatosis. Although the putative anti-atherosclerotic effect of OLEA was not elucidated, the hypolipidemic properties pave the way for that possibility.

Further studies should be performed in order to clarify the expected antioxidant properties, as well as other mechanisms potentially involved in the protection against hyperlipidemia and hepatic steatosis.

|Chapter VI – References

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