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BIOLOGICAL PROPERTIES OF CITRUS LIMON DERIVATES: INSIGHTS OF THE HEPATOPROTECTIVE EFFECTS OF LEMON ESSENTIAL OIL FRACTIONS AND OF LEMON-DERIVED NANOVESICLES.

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Summary:

The nutraceutical industry is increasingly focused on identifying natural compounds that offer both health benefits and appealing sensory qualities, aiming to develop foods that can be incorporated into a health-conscious diet. Citrus fruits, particularly Citrus limon, are widely regarded as essential in daily nutrition, containing beneficial compounds such as flavonoids, minerals, essential oils, and carotenoids. Nowadays their derivates are seen as a key player in preventive healthcare. In detail, Lemon essential oil (LEO) is renowned for its aromatic and health-enhancing qualities, yet less focus has been placed on the biological properties of the fractions derived from LEO. In the first part of the project, I aim to explore the capacity of a citral-enriched fraction of LEO (Cfr-LEO) to counteract inflammation, oxidative stress, and epithelial-mesenchymal transition (EMT) caused by lipopolysaccharide (LPS) in healthy human liver cells. Immortalized human hepatocytes (THLE-2 cell line) were pretreated with Cfr-LEO and subsequently exposed to LPS over varying periods. Our findings demonstrate that Cfr-LEO pretreatment inhibits LPS-induced inflammation, oxidative stress, and EMT in THLE-2 cells by reducing NF-κB activation, proinflammatory cytokine release, ROS production, and the expression of NRF2 and p53. Furthermore, Cfr-LEO exhibits a protective effect against LPS-induced EMT. These findings highlight the potential of Cfr-LEO in the nutraceutical field, offering both aromatic benefits and biological activity, with the possibility of developing food and beverage products enriched with Cfr-LEO to prevent or alleviate liver-related conditions.

Furthermore, numerous investigations have explored the interaction between plant-derived nanovesicles (PDNVs) and mammalian cells, underlining the capability of these natural nanovesicles to regulate several molecular signaling pathways. Apart from their enhanced bioavailability, stability, and low toxicity, PDNVs possess biological proprieties that make them applicable against pathological conditions, such as hepatic diseases. In the second part of the project, I aim to investigate the antioxidant properties of lemon-derived nanovesicles, produced at laboratory (LNVs) and industrial scale (iLNVs), stemming from an *in vitro* study in human healthy hepatocytes (THLE-2) to a functional *in vivo* experimentation in a rat model of hepatic dysmetabolism, i.e. metabolic syndrome induced by a high-fat diet (HFD). Our findings demonstrate that in THLE-2 cells LNVs and iLNVs decrease ROS production and upregulate the expression of antioxidant mediators, Nrf2 and HO1. Furthermore, the *in vivo* assessment reveals that the oral administration of iLNVs in HFD-fed rats improves glucose tolerance and lipid dysmetabolism, alongside amelioration in biometric parameters and systemic redox homeostasis. In addition to this, iLNVs

administration upregulates Nrf2/HO-1 signaling in HFD rat's liver. In light of our comprehensive outcomes, we support the potential of LNVs/iLNVs as a promising approach for managing hepatic and dysmetabolic disorders.

In the end, I preliminarily investigate the antimicrobial properties of LNVs isolated via Size Exclusion Chromatography (SEC) and their potential to stimulate the human immune system. The research determinates whether LNVs can enhance the innate immune response by promoting the activity of macrophages, which are crucial for detecting and eliminating pathogens. In particular, we established a protocol for evaluating if THP1 M0 differentiated cells, pre-treated with LNVs, influence bacterial growth and colony formation. This research holds promise for developing LNV-based treatments that target pathogens while enhancing the body's natural defenses, providing a dual strategy for combating infectious diseases.

In conclusion, this work paves the way for creating specialized products, formulated with scientifically tested citrus essential oils and LNVs, designed to prevent or alleviate chronic conditions related to liver dysfunction or to enhance the immune response against pathogens.

CHAPTER 1: INTRODUCTION

1.1 Citrus Fruits

The genus Citrus (*Rutaceae*) is one of the oldest, most widely traded, and beloved crops in history. Dating back to 2100 BC, its cultivation traces are among the earliest recorded [1] and though its exact origins remain debated, the consensus leans towards Southeast Asia [2]. The long natural evolution process and artificial selection formed various types of citrus, which had bred abundant citrus germplasm resources [3]. Citrus includes approximately 17 species of plants that produce some of the most widely cultivated fruits in the world. To date, the Citrus used for eating or processing mainly includes pomelos (*Citrus maxima*), sweet oranges (*Citrus sinensis*), sour oranges (*Citrus aurantium*), mandarins (*Citrus reticulata*), lemons (*Citrus limon*), limes (Citrus aurantiifolia), citrons (*Citrus medica*), grapefruits (*Citrus paradisi*), kumquat (*Citrus japonica*), and hybrids. Citrus fruits are globally appreciated for their numerous health benefits. Renowned for their enticing aromas and flavors, they are consumed extensively worldwide, thanks to their essential nutritional profiles and diverse health-promoting properties stemming from their abundance of nutrients and bioactive compounds. In addition to this, Citrus fruits are rich sources of useful phytochemicals, such as flavonoids, vitamins A, C and E, carotenoids, limonoids, coumarins, mineral elements, pectins, and other different compounds [4]. The phytochemicals, consumed through fresh fruits or their derived products, have been suggested to have a wide variety of biological functions including antioxidant, antiinflammation, antimutagenicity, anticarcinogenicity and anti-aging to human health [5], [6], [7]. Consequently, Citrus fruits are an abundant and unique source of bioactive compounds that can reduce inflammatory mediators and reactive oxygen species (ROS) in the body, lowering the risk of metabolic syndrome, neurodegenerative and cardiovascular disease and cancer [8].

1.2 Citrus limon

The lemon tree (Citrus limon (L.) typically grows to a height of 2.5–3 m and is recognized by its evergreen lanceolate leaves. Its bisexual flowers are white with a purple hue along the edges of the petals, arranged in small clusters or appearing individually within leaf axils. The fruit is an elongated, oval-shaped green berry that transitions to yellow as it ripens. The outer layer of the lemon's peel, known as the pericarp, consists of a thin, wax-coated exocarp enclosing the outer mesocarp, or flavedo, which contains oil vesicles and carotenoid pigments. The inner mesocarp, called the albedo, comprises a spongy, white parenchyma tissue. Segments within the lemon's flesh are separated by this spongy, white mesocarp tissue

[2] It is not known the location of the original natural habitat of C. *limon*, but there is a big probability it is native to North-Western or North-Eastern India [9, 10]. Recognized mainly as a cultivated species, C. *limon* has been grown in southern Italy since the 3rd century AD and reached Iraq and Egypt by 700 AD. The Arabs introduced it to Spain by 1150, and Marco Polo brought it to China in 1297. By the 19th century, its commercial production had expanded globally, particularly in Florida and California. Currently, the USA is the top producer of *C. limon*, with Italy, Spain, Argentina, and Brazil also playing key roles. Generally, in regions where it is cultivated, C. *limon* is the third most common Citrus fruit and it is well known for its dietary, medicinal, and agricultural benefits because of its valuable source of bioactive compounds. Studies have shown that the juice of C. *limon* has anti-inflammatory and anticancer properties [11]. Moreover, the juice is cytotoxic to the promyelocytic leukemia cell line HL60 [12] and generally exerts antiproliferative effects against several cancer cell lines *in vitro* [7].

Most studies on C. *limon* have concentrated on its juice, seeds, leaves, and flowers, often neglecting the peel. Notably, the peels of *C. limon* are rich in diverse secondary plant metabolites with anti-inflammatory and anticancer properties, including flavonoids and other phenolic compounds. In addition to this, it is known to contain phytochemicals, ranging from fatty acids to phenolics and flavonoids (catechin, rutin, hesperidin, and naringin), pectin, and compounds such as limonene, α-terpineol, γ-terpinene, 4-terpineol, α-phellandrene, β-myrcene, α-pinene, β-linalool, and α-selinene [13, 14, 15, 16, 17, 18]. Consequently, the significant biological importance of Citrus limon has had a profound impact on the nutraceutical industry, driving substantial growth in research focused on its derivatives to evaluate and validate their functional effects on human health. In the following sections, the biological importance of Citrus derivatives will be discussed.

1.3 Citrus limon derivates

To date, Citrus *limon* derivatives play a crucial role in the food industry. With the development of scientific research, it became possible to fully describe the potential of Citrus derivatives as new ingredients for a lineup of new and enhanced foods and beverages, making them applicable in the nutraceutical industry. Many nutriment supplements can be also formulated for a general enhancement of health and prevention of diseases. For example, the inclusion of citrus limon-derived beverages in the diet is essential due to their nutritional value and health benefits. These beverages are fortified with bioactive compounds which offer several advantages such as antioxidant properties, immune system support, hydration and detoxification, digestive health, cardiovascular health, and skin

health. By incorporating these nutrient-rich beverages into the diet, the nutraceutical industry aims to enhance overall well-being and support long-term health. Their versatility and health-promoting properties make them a valuable addition to a balanced diet. Despite this, beverages are not the only citrus derivates largely applied to promote health prevention. Nowadays, essential oils and plant-derived nanovesicles are paving the way for innovative scientific studies that potentially make them applicable in the nutraceutical fields as crucial elements derived from citrus. Their potential is relevant thanks to the strong biological and beneficial properties possessed by them. In the following paragraphs, their functional properties will be largely described, as well as the techniques used to extract them.

1.3.1 Essential oils

Comprised of natural, volatile, and aromatic compounds with distinctive odors, essential oils (EO) are secondary metabolites generated by aromatic plants. These oils are in plant organs, including buds, flowers, seeds, leaves, roots, fruits, wood, twigs, or bark. EOs represent a complex blend of natural compounds with multiple biological activities. In general, the Citrus species most used to produce essential oils are lemons (Citrus limon), oranges (Citrus sinensis), grapefruits (Citrus paradisi), mandarins (Citrus reticulata) and limes (Citrus Aurantifolia) [19]. The essential oils differ in odor and flavor based on the number of their different constituents and they also possess antioxidant and antimicrobial activities, thus acting as natural additives in foods or more generally in food products[20, 21]. In detail, citrus essential oils are made up of a mixture of monoterpenes, aldehydes, alcohols, esters, sesquiterpenes, and coumarins [22]. These substances have specific and different chemical-physical and aromatic properties, of great interest in nutraceutical and health purposes. Being rich sources of dietary fiber, vitamin C, phenols, and flavonoids, citrus fruits are believed to have potential health-promoting properties [23, 24]. Consequently, the essential oils of citrus species have long been used for medicinal, insecticidal, and cosmetic applications, and as flavoring agents in foods, beverages, and jams. Recent studies indicate the possibility of using these essential oils or their active ingredients to prevent or treat various pathological conditions, where they can be used as antimicrobials, antifungals, neuroprotective, anxiolytic, anticonvulsant, sedative, antinociceptive, anti-inflammatory and antioxidant [25, 26, 27].

In detail, EOs encompass terpenoids, shikimates, polyketides, and alkaloids, synthesized through three primary pathways: methyl-erythritol, mevalonate, and shikimic acid pathways. A significant portion of these compounds are monoterpenes, particularly d-limonene, which is the main optical isomer of limonene and a key ingredient in EOs. Monoterpenes like

limonene and α-pinene are known for their antiseptic and some anti-tumoral effects while linalool, caryophyllene, and limonene are known for their anti-inflammatory effects [28]. On the other hand, α-pinene and β-pinene possess antioxidant properties able to reduce the production of nitric oxide [29]. Specifically, the monoterpene D-limonene is an effective inhibitor of lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E (2) production and exerts an effect on some cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF-α). Furthermore, D-limonene can reduce the expression of these cytokines in a dose-dependent manner [30]. Due to their pleasant scent and antioxidant, antimicrobial, antibacterial, and anti-insect properties, EOs are broadly utilized in products like perfumes, cosmetics, food and beverages, household items, essences, pesticides, and pharmaceuticals.

To date, two main traditional methods of extraction are generally used: cold pressing (CP) and steam distillation (SD). CP involves using mechanical methods to extract oils from the peel and cuticles, producing a watery emulsion that is then centrifuged to separate and recover the EOs. In contrast, SD involves exposing Citrus peels to boiling water or steam to release the oils, which are then collected as vapors during distillation. Modern extraction methods like ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) offer many advantages over traditional techniques, including shorter extraction times, higher extraction rates, energy efficiency, and superior product quality at lower costs. Microwave steam distillation (MSD) further enhances efficiency compared to conventional SD, expediting the extraction process without altering the oil's composition. On the other hand, SFE utilizing carbon dioxide (CO2) is considered one of the most effective methods for EO extraction due to the non-polar nature of supercritical CO2 [31, 20, 21].

1.3.1.1 Lemon essential oil

Lemon essential oil (LEO), obtained from the Citrus *limon*, is known from a health point of view for its biological properties. The main components of this essential oil extracted from the pericarp of *C. limon* are mainly represented by monoterpenoids. LEO is indeed a complex mixture of limonene, γ-terpinene, citral, linalool, β-caryophyllene, α-pinene, and β-pinene. Interestingly, a recent study demonstrated the antioxidant and anti-inflammatory effect of a mix of four fractions of LEO enriched in citral in a model of human and murine macrophages [22]. Citral, a mixture of the two geranial and neral aldehydes, represents one of the main bioactive components of LEO and it is known for its role in the inhibition of oxidant activity, linked to the modulation of NF-kB and cyclooxygenase-2 (COX-2) [32]. In

addition to this, citral also has a significant effect on the prevention and treatment of cancer. In detail, it is known that hepatocarcinogenesis in rats can be inhibited by essential oil with a high citral content [33]. Furthermore, citral mainly contributes to the aroma of lemon, and for this reason, it finds application in the nutraceutical industry, which is today extremely interested in finding innovative solutions to obtain plant derivatives with beneficial properties and add them to foods and drinks. Citral has also been shown to significantly suppress LPS-induced NO production in a concentration-dependent manner. It can also inhibit the transcriptional activity and expression of iNOS and suppress the DNA binding activity, nuclear translocation of NF-κB, and phosphorylation of IκB, suggesting that the mechanism of action of this compound may determine the inhibition of NO production through the inhibition of NF-κB activation [34].

According to recent studies, LEO also has versatile therapeutic activities on the digestive system and the cardiovascular, nervous, and immune systems [30, 27, 24, 35, 36, 37, 38, 39, 40, 41, 42, 43]. To date, most studies are focused on the functional analysis of whole LEO, linked to its antioxidant and anti-inflammatory properties specifically associated with the presence of linalool [44] and limonene [30]. Less consideration has instead been given to the biological properties of the fractions obtained from the whole essential oil and the little information available focuses mainly on isolation techniques and the description of the molecular profile rather than on the functional role. Despite this, the essential oil's fractions can represent the starting point for the nutraceutical integration of these components of plant origin in the commercial production of innovative solutions of biofunctional compounds. For this reason, studies on specific fractions of essential oils are increasingly gaining ground in the scientific field.

1.3.2 Plant-derived nanovesicles

Extracellular vesicles (EVs) are lipoproteic carriers, delimitated by a lipid bilayer and released by prokaryotic and eukaryotic cells, heterogeneous in terms of size, origin, and content. They are secreted by various cell types under both normal physiological circumstances and pathological conditions. EVs are categorized based on their physical characteristics into small (diameter <100nm), medium (diameter <200nm), and large (diameter >200nm). These vesicles play a crucial role in intercellular communication by transporting molecules such as RNAs (miRNAs, lncRNAs, and circRNAs) and proteins from their cells of origin to target cells. Today, EVs are recognized as part of the body's cell-cell communication system; released EVs can be internalized by other cells, influencing the phenotype of the recipient cell.

While interest in cellular vesicles has primarily focused on biomedical research, recent attention has turned to vesicles introduced into our daily diet, including those found in vegetables. In-depth, the discovery of plant-derived nanovesicles (PDNVs) can be traced back to Jensen in 1965, who found multivesicular bodies (MVBs) and intraluminal vesicles in cotton [45]. In 1967, Halperin and Jensen examined the ultrastructure of clumps in wild carrot suspension cell cultures and identified MVBs that could fuse with the plasmalemma and release their contents into the wall space [46] while the first successful isolation of PDNVs from apoplastic fluid was carried out by Regente *et al*. in 2009 [47]. Nowadays, existing nanovesicle isolation protocols are predominantly designed for animal-derived material, but to date, several are also used for isolation from plant material. Despite its drawbacks, including contamination and being time-intensive, ultracentrifugation coupled with density gradient remains the gold standard for vesicle isolation, particularly for small particles. Other most-used techniques include precipitation-based and immunoaffinity-based protocols and size-exclusion chromatography (SEC), which emerged as a viable method for isolating vesicles, particularly for larger volumes than those typically processed by traditional methods. In particular, SEC separates particles by size using a porous matrix within a column, allowing quicker vesicle purification and eliminating soluble proteins that usually precipitate during ultracentrifugation. In addition to this, in the isolation process, material from different plant species can be used. PDNVs can be indeed sourced from various fruits and vegetables, including lemons [48, 49], blueberries [50], tomatoes [51], grapefruits [52], and garlic [53] (Figure 1).

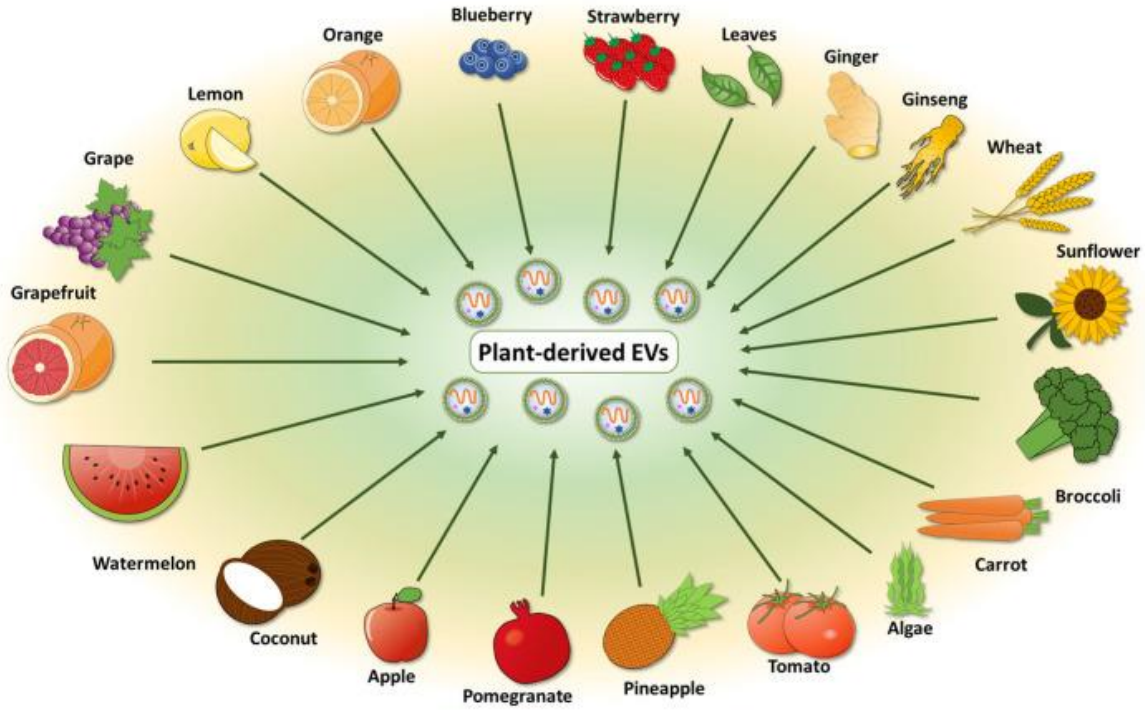

Figure 1: A schematic representation of different PDNVs sources, based on current scientific literature [54].

Moreover, these plant-derived nanovesicles are characterized by a variegate composition. They carry lipids [55], proteins [48], nucleic acids [56], and metabolites [57]. In particular, the major lipid species found in PDNVs are phosphatidic acid, phosphatidylethanolamine, and phosphatidylcholine [58]. Vegetable nanovesicles also showed significant enrichment in sphingolipids [55] while metabolomic analyses have revealed various bioactive compounds, such as shogaol [59], sulforaphane [57], and naringenin [60]. Instead, the specific protein composition depends on their origin in terms of secretory pathways and matrices, however, some protein families have been identified in PDNVs from different species (HSP70, HSP60 [61], HSP80, HSP90 [48], lipoxygenase, ATPases [62], Aquaporins [63], proteins involved in the cell wall remodeling such as 1,3-β-glucosidases, pectinesterases, polygalacturonases, β-galactosidases, and β-xylosidase/α-L-arabinofuranosidase 2-like) [58]. On the other hand, the presence of RNAs in PDNVs is one of the most interesting findings regarding PDNVs content due to their capability to regulate the expression of mammalian genes associated with different biological responses. For example, the anti-inflammatory activities of apple nanovesicles could be explained by their ability to upregulate some miRNAs in target cells, such as miR-146a-5p, which is involved in NF-κB regulation [64] while miRNAs derived from *Moringa oleifera* nanovesicles have proapoptotic effects in tumor cell lines, thanks to the regulation of BCL2 protein (Figure 2) [65].

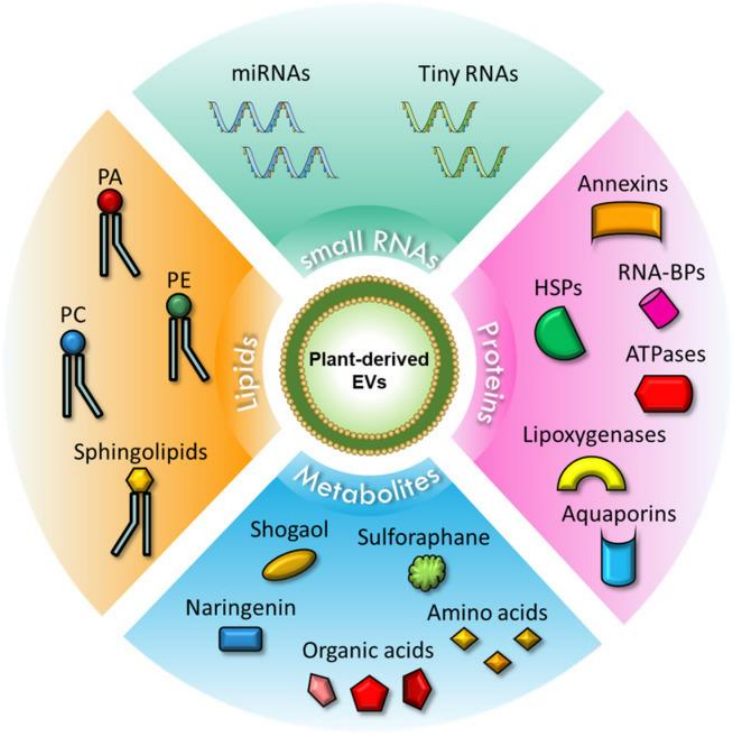

Figure 2: Schematic representation of PDNVs content. PDNVs are rich in lipids such as phosphatidic acid and phosphatidylcholine, along with proteins, nucleic acids, and metabolites like shogaol and naringenin. Protein composition varies by origin, though certain protein families (e.g., HSPs, ATPases) are consistently found. RNA presence in PDNVs is notable for its role in gene regulation [66].

Consequently, recent researchers have shown a growing interest in the potential roles of these vesicles in cross-kingdom communication, particularly focusing on the interactions between plant-derived nanovesicles and mammalian cells. PDNVs have been indeed recognized for their capability to regulate biological processes like inflammation and oxidative stress. Furthermore, the biological effects of these vesicles are often the result of multiple molecules working simultaneously, rather than a single molecule. Numerous studies have indeed documented the biological properties of PDNVs, demonstrating anti-cancer [48], anti-inflammatory [67], and antioxidant activities [68]. It is well known that PDNVs diverse contents indeed enable them to mitigate conditions that, if prolonged, could lead to pathological states. For example, garlic-derived exosomes have shown anti-inflammatory effects in THP-1 macrophages and mitigated lipid droplet accumulation in the livers of mice on a high-fat diet [53]. Garlic-derived nanovesicles also inhibit the migration and infiltration of macrophages into the liver, preventing hepatocyte apoptosis and inflammasome activation [69]. Moreover, Mu et colleagues discovered that the uptake of grape and grapefruit-derived nanovesicles by intestinal macrophages in mice induces anti-inflammatory and antioxidant effects [67]. In the gastrointestinal tract, where food is digested, PDNVs are indeed absorbed and enter the bloodstream; thus, reaching the final

recipient organs, such as the brain, liver, and kidney. Then, these nanovesicles release their content in target organs where they may exert their biological properties. In addition to this, they can potentially cross the BBB and reach the cells of the central nervous system, or they can be found in the urine of plant-eating humans (Figure 3) [54].

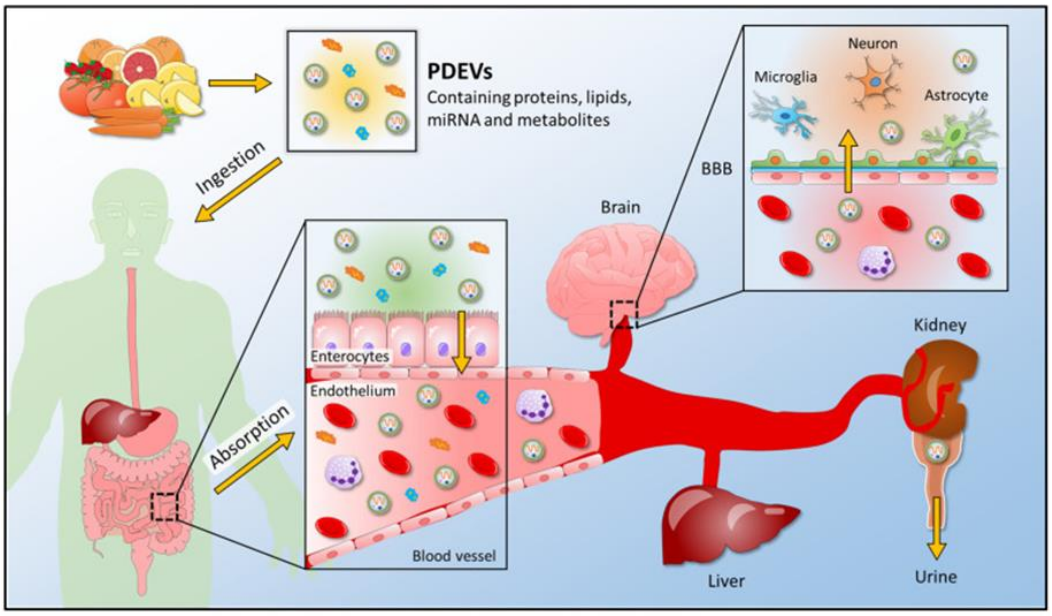

Figure 3: A schematic view for the uptake of plant-derived extracellular vesicles into the human body. After being absorbed in the digestive system, PDNVs circulate through the blood, delivering their contents to several organs. They may also cross the blood-brain barrier or appear in the urine. [54].

1.3.2.1 Lemon-derived nanovesicles

Lemon-derived nanovesicles (LNVs) represent a promising area of research due to their exceptional properties. LNVs, like other PDNVs, carry bioactive compounds, including proteins, lipids, and various types of RNA. These vesicles have been shown to exert a range of beneficial effects, including antibacterial, antifungal, anti-inflammatory, antioxidant, and anticancer activities. In particular, in cancer research, LNVs have demonstrated the ability to inhibit tumor growth, inducing apoptosis in chronic myeloid leukemia (CML) cells. This anti-tumor effect is largely due to the ability of LNVs to specifically target cancer cells, sparing normal cells from their cytotoxic effects. They achieve this by promoting the expression of pro-apoptotic genes like Bad and Bax while suppressing anti-apoptotic genes such as Survivin and Bcl-xL, thus inducing cell death via the TRAIL-mediated pathway [48]. Interestingly, Xiao et colleagues also demonstrate that these lemon-derived nanovesicles can be used as efficient drug delivery carrier. These nanovesicles marked with heparin-cRGD-EVs-doxorubicin were internalized by cancer cells through endocytosis, a

process by which cells engulf external particles and, once inside the cells, the nanovesicles induce energy dissipation, disrupting the cells' metabolic processes. Specifically, they reduce the production of ATP, weakening the cancer cells and making them more susceptible to chemotherapy drugs [70].

Furthermore, in a recent study, LNVs showed their antioxidant properties in human dermal fibroblasts and zebrafish by activating the AhR/Nrf2 signaling pathway [49]. In studies, LNVs have been shown to promote the healing process by enhancing the production of extracellular matrix molecules and reducing reactive oxygen species (ROS) *in vitro*. Furthermore, LNVs exhibit anti-inflammatory properties both *in vitro* and ex vivo by the regulation of NF-κB-/ERK1-2 signaling pathways. The anti-inflammatory effects of LNVs may be explained by the presence of different compounds such as flavonoids and limonoids which act synergistically and are packaged into a lipid bilayer that makes them stable from degradation and easy to be absorbed by target cells [71].

Additionally, LNVs have been investigated for their function in preventing kidney stone formation. They appear to mitigate the progression of kidney stones by counteracting endoplasmic reticulum stress in renal tubular cells, providing another potential therapeutic application [72]. Moreover, LNVs play an essential role in the chondrogenic differentiation of adipose-derived stem cells due to their capability of downregulating chondrogenic differentiation, representing an interesting future application for cartilage regeneration [73].

Consequently, these diverse biomedical applications underline the significant potential of LNVs, positioning them as valuable tools in both preventive and therapeutic medicine. Furthermore, LNVs, as well as lemon essential oils and other derivates, can positively impact human health by benefiting multiple organs, including the liver which is continuously exposed to various stimuli.

1.4 Liver

The liver is the largest and most active metabolic organ of the human body, crucial for managing nutrition, processing drugs and foreign substances, and detoxification. The liver features several types of cells, each with a specific role. In detail, hepatocytes are the main functional cells, handling many liver processes, while cholangiocytes are found in the bile ducts. On the other hand, stellate cells, which store vitamin A, are essential cells that become active during liver injury, leading to collagen production and scarring. Kupffer cells, as liver resident macrophages, handle immune responses, and sinusoidal endothelial cells form fenestrated plates crucial for blood-cell exchange. Additionally, the liver is structured into

lobules, which are hexagonal units with a central vein and portal triads at the vertices. Blood from different sources mixes in these lobules, creating gradients that support various metabolic functions. This organization allows for flexible adaptation in response to liver damage, highlighting the liver's dynamic nature [74]. Consequently, the liver is a versatile and complex organ, intricately regulated by physiological mechanisms and which can exert different functions. Key liver functions include producing bile to help eliminate waste and break down fats in the small intestine during digestion; synthesizing specific proteins needed for blood plasma; generating cholesterol and specialized proteins to transport fats throughout the body; transforming surplus glucose into glycogen for storage, which can later be converted back into glucose for energy; regulating the levels of amino acids in the blood, which are necessary for protein construction; processing hemoglobin to use its iron content; removing toxic substances from the blood, regulating the blood clotting process and defending against infections by producing immune factors and clearing bacteria from the bloodstream [74].

1.4.1 Hepatic Inflammation

Hepatocytes, the primary cells of the liver, are not only responsible for metabolic and energy functions but also play a significant role in the inflammatory response. However, recent studies have emphasized the role of hepatocytes as active drivers in liver inflammation and fibrosis through intercellular communication [75]. Organelle damage, including mitochondria, lysosome, and endoplasmic reticulum may determine the severity of hepatocyte injury [76]. Inflammation is indeed a fundamental process in almost all acute and chronic liver disorders. Immune mediators, particularly pro-inflammatory cytokines, can control several key features of liver diseases, such as acute liver failure, acute phase response, steatosis, cholestasis, hypergammaglobulinemia, and also the development of fibrosis. Among different cytokines, IL-6, IL-1β, and TNFα have emerged as factors related to the onset of liver disease [77]. In detail, IL-1β is not expressed in the healthy liver but acts only on the inflamed liver via specific receptors (IL-1R1/2), which are involved in all hepatic inflammatory processes. TNFα and IL-6 are instead the main players in the pathogenesis of many autoinflammatory, autoimmune, and infectious diseases and exert their effects through specific receptors [78]. Furthermore, TNFα is expressed only in the inflamed liver and regulates hepatic inflammatory processes including cellular infiltration and the induction of other mediators involved in the development of the disease [77, 78, 79, 80].

In general, hepatocytes can be activated by various stimuli such as toxins, infections, and metabolic stress, leading to the release of pro-inflammatory cytokines and chemokines. The role of alcohol in the induction of inflammation is deeply studied. The consumption of alcohol can promote the translocation of endotoxins from the gut to the portal bloodstream, thereby activating Kupffer cells through the LPS/Toll-like receptor (TLR) 4 pathways. This process triggers an imbalanced immune response, leading to elevated levels of proinflammatory cytokines and chemokines, which can result in hepatic diseases, including alcohol-related liver disease (ALD) [81, 82]. This pathological condition is the leading cause of alcohol-related deaths worldwide. Prolonged and excessive use of alcohol can induce inflammatory changes in the liver, leading to more serious damage known as alcoholic steatohepatitis or alcoholic hepatitis [83]. The pathogenesis of alcoholic liver disease involves multiple factors including alcohol-induced hepatocyte damage, cholestasis, and recruitment and activation of innate immune cells by gut-derived pro-inflammatory signals [84]. In detail, cytokines linked to ALD are IL-6, which has hepatoprotective properties, and IL-10, which serves as an anti-inflammatory agent [85]. These cytokines, generated by ethanol-stimulated LPS-activated Kupffer cells, can help reduce the severity of alcohol-induced liver damage.

Despite this, alcohol is not the only factor that can cause liver inflammation. Inappropriate eating habits can indeed be the cause of hepatic inflammation and non-alcoholic fatty liver disease (NAFLD) development. In detail, NAFLD is the main cause of loss of liver function in the Western world and is often associated with obesity and type 2 diabetes. NAFLD is characterized by the accumulation of fat in the liver exceeding 5% of its weight, without the influence of significant alcohol intake. It develops through the interaction of various genetic and environmental factors and includes a collaboration between the intestinal microbiota and the innate immune system [86]. In obese individuals, the accumulation of fat in the abdominal region affects both lipid and glucose metabolism, resulting in a fat-laden and insulin-resistant liver. Obesity is commonly associated with liver inflammation and increased expression of various pro-inflammatory cytokines such as TNFα, IL-1, and IL-6. The pathological progression of NAFLD follows a three-step process: steatosis, lipotoxicity and inflammation [87]. Steatosis leads to increased signaling of the transcription factor NF-κB and the production of inflammatory mediators such as TNFα, IL-6, and IL-1β.

On the other hand, other contributors to liver inflammation include viral infections, such as hepatitis B and C, which directly attack liver cells and trigger an immune response. Furthermore, certain medications, toxins, and autoimmune disorders can cause liver

inflammation by disrupting normal liver function and initiating immune reactions. Each of these factors can independently or collectively contribute to the development of hepatic inflammation and, if left unchecked, can progress to more severe liver diseases. Because of this, several studies have focused their attention on the development of inhibitors derived from natural resources to prevent or alleviate chronic inflammatory conditions.

1.4.1.1 Lipopolysaccharide and Toll-Like Receptor 4 signaling

The liver can be constantly exposed to pro-inflammatory components such as Lipopolysaccharide (LPS), a microbial endotoxin known for its ability to activate inflammation and oxidative stress. LPS is indeed an important component in the outer membrane monolayer of most Gram-negative bacteria. It consists of lipid A, an oligosaccharide core, and the O-specific oligosaccharide chain (Figure 4). Lipid A induces the activation of inflammatory response through the regulation of specific signaling pathways [88, 89]. The O-specific chain is instead made up of 20-40 repeated saccharides, with a different composition for each bacterial species. This variable region causes the production of different antibodies [90].

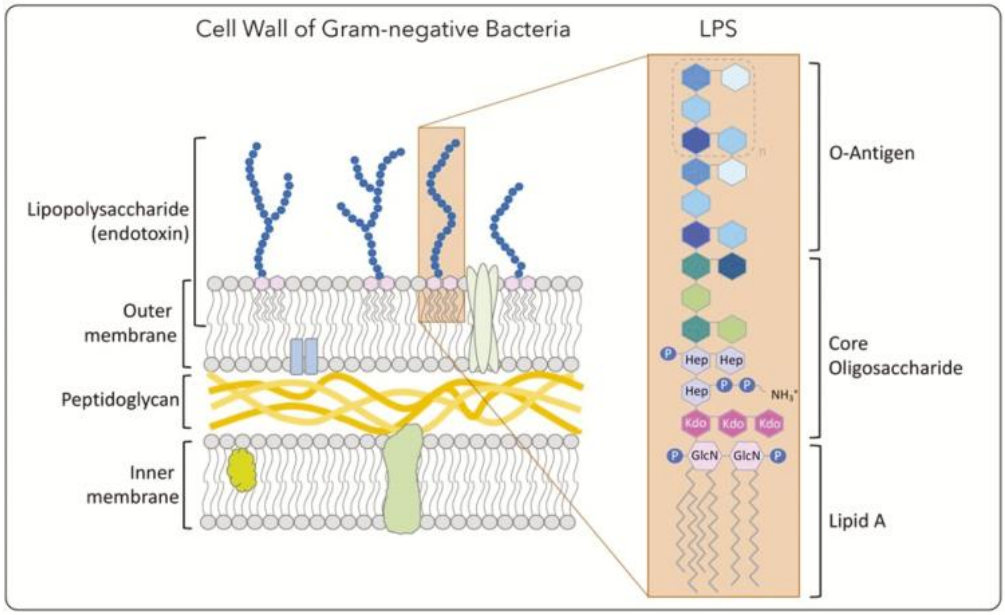

Figure 4: Gram-negative bacterial cell-wall structure with emphasis on the lipopolysaccharide (LPS) presence in the outer membrane. LPS consist of lipid A, a core polysaccharide, and an O-antigen. Lipid A is responsible for eliciting inflammatory responses and toxicity, while the O-antigen contributes to the diversity and immune evasion of different bacterial species. [91].

In detail, according to information already known in the literature, LPS can exert its role in the inflammatory process via the Toll-like Receptor 4 (TLR4) signaling. Toll-Like Receptors (TLRs) are a family of PRRs and transmembrane proteins originally identified in mammals based on their homology with Toll, a Drosophila receptor that contributes to the production of antimicrobial peptides that act against the invasion of the microorganism in the fly. They recognize molecules derived from pathogens, such as structural components unique to bacteria, viruses and fungi, and activate inflammatory cytokines and the production of type I interferon (IFN I). These receptors are expressed on the surface of macrophages, dendritic cells and epithelial cells [92]. In detail, TLR4 is a functional receptor characterized by two domains: an extracellular one consisting of 608 residues and an intracellular one consisting of 187 residues. In the liver, TLR4 is expressed not only on innate immune cells such as Kupffer cells but also on hepatocytes. For the signaling to be activated, several factors must come into play. In detail, CD14, an innate immune system protein, binds to LPS and subsequently presents it to TLR4 and MD-2. This determines the activation of the intracellular signaling pathway via myeloid differentiation factor 88 (MyD88), which then can activate NF-κB [93]. The recruitment of the adapter TRIF instead activates the phosphorylation of interferon regulatory factor 3 (IRF3) which consequently induces the production of IFN I. The LPS/TLR4 signaling pathway consists of the activation of transcription factors, such as NF-κB, capable of inducing the expression of pro-inflammatory cytokines.

Comprehensively, a wide range of studies available in the scientific literature have extensively examined TLR4 signaling pathways, including MyD88-dependent and MyD88-independent pathways [94, 95, 96]. Four adapter proteins are involved in these two intracellular signaling pathways: MyD88, TIRAP, TRIF and TRAM [97, 98]. Appropriate ligand binding to the TLR4 receptor causes its homodimerization through interaction between its intracellular TIR domains, followed by conformational changes that result in the activation of a cascade of downstream events. TLR4 recruits specific adapters capable of activating various transcription factors, giving rise to the appropriate inflammatory responses. Intracellular TLR4 signaling is initiated through at least two major pathways:

- the MYD88-TIRAP pathway (also known as MyD88-dependent pathway), in which TIRAP mediates the activation of the MyD88-dependent pathway downstream of TLR4 [99, 100], regulates early NF-κB activation and triggers transcription of inflammatory cytokines [101];

- the TRIF-TRAM pathway (or MyD88-independent pathway), involved in the induction of IFN I and interferon-inducible genes via the activation of IRF3 and other inflammatory mediators (Figure 5).

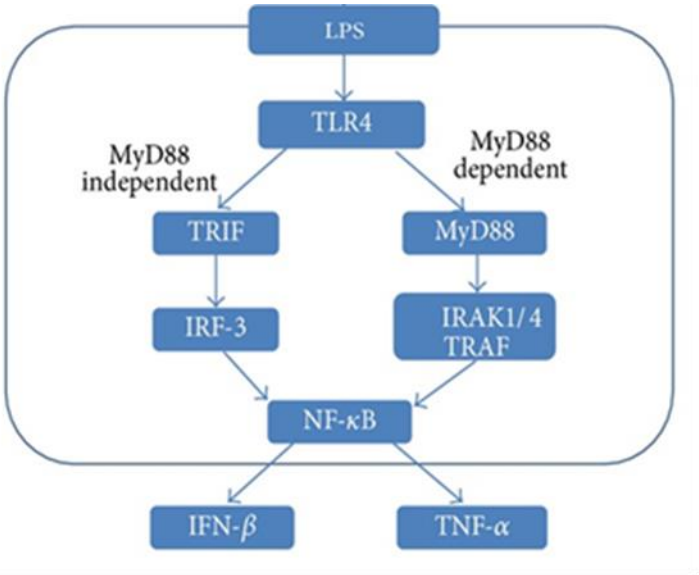

Figure 5: Intracellular TLR4 signaling occurs through two main pathways: the MyD88-TIRAP pathway, which activates NF-κB and promotes inflammatory cytokine production, and the TRIF-TRAM pathway, which triggers type I interferon and related genes via IRF3 activation [102]

In particular, MyD88 forms the Myddosome, a complex involving IRAK-1. This is associated with the polyubiquitinated TRAF6 complex and the protein kinase TAK1. In turn, TAK1 can activate the IKK-NF-κB complex and MAPKs. In detail, the IKK complex is made up of two catalytic subunits (IKKα and IKKβ) and the regulatory subunit NEMO (also known as IKKγ). The IKK complex indeed phosphorylates IκBα (NF-κB inhibitory protein), which undergoes degradation by the proteasome, allowing NF-κB to translocate into the nucleus and induce the expression of proinflammatory genes [103]. As mentioned above, TAK1 activates MAPK family members such as JNK, ERK1/2 and p38, which in turn mediate the activation of AP-1 family transcription factors [104].

The TRIF-TRAM pathway, also known as the MyD88-independent pathway, involves the activation of IRF3, which subsequently regulates the expression levels of some genes encoding IFN I and co-stimulatory factors. This pathway increases the production of TNFα and regulates its secretion. Consequently, TNFα binds to its specific receptors leading to the activation of NF-κB. Therefore, the TRIF-TRAM pathway is responsible for late-phase activation of NF-κB via secretion of TNFα and IRF3 and subsequent production of IFN I (Figure 6) [105].

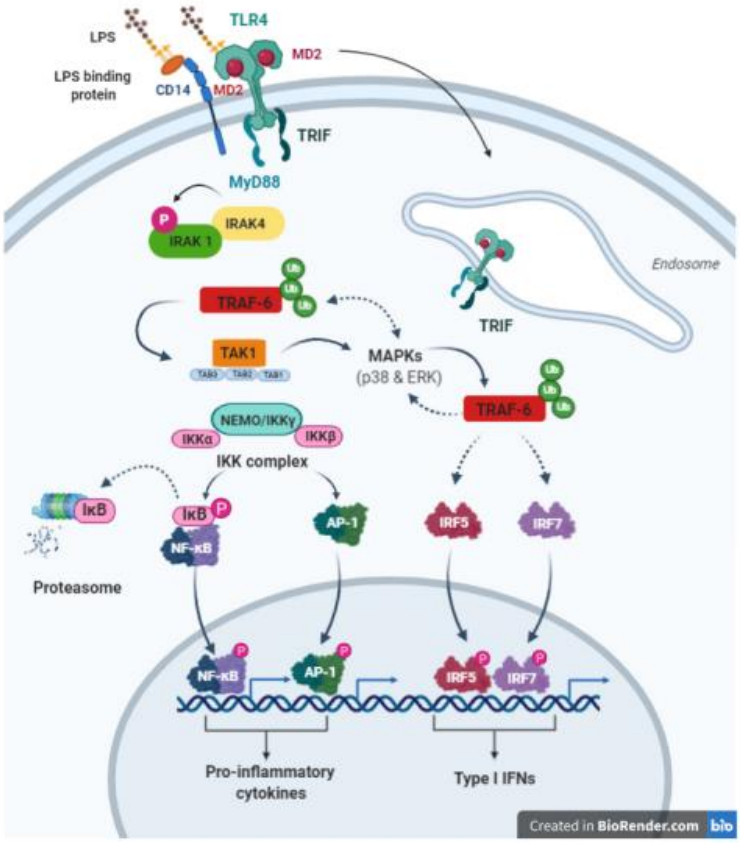

Figure 6: Comprehensive schematic overview of TLR4 signaling. MyD88 forms the Myddosome complex with IRAK-1, which activates TRAF6 and TAK1. TAK1 subsequently activates the IKK-NF-κB complex, leading to the phosphorylation and degradation of IκBα, allowing NF-κB to enter the nucleus and promote proinflammatory gene expression. The TRIF-TRAM pathway, or MyD88-independent pathway, activates IRF3, which regulates type I interferon and co-stimulatory factor expression, enhancing TNFα production. This TNFα then activates NF-κB, contributing to late-phase NF-κB activation [105].

1.4.2 Hepatic Oxidative stress

Oxidative stress is the result of an imbalance between reactive oxygen species (ROS) and antioxidant defenses. Normally, oxidants play pivotal roles in regulating various physiological and non-physiological processes, including cell division, inflammation, immune function, autophagy, and stress response. ROS are generated as byproducts of oxidative metabolism [106] and in response to external factors like pathogens [107, 108] or internal signals such as cytokines [109]. Uncontrolled ROS production can lead to cellular toxicity, contributing to the development of cancer and chronic diseases by damaging DNA, proteins, and lipids. To balance ROS production, cells possess all the resources necessary to exert the proper cellular antioxidant defense. One of the signaling involved in the reduction of oxidative stress is the Nrf2 pathway. Nrf2 is a positive regulator of the human Antioxidant

Response Element (ARE) that induces the expression of several antioxidant enzymes. The mechanism of Nrf2 activation involves Keap1, a suppressor protein anchored in the cytoplasm that physically binds Nrf2, preventing its translocation to the nucleus and its access to ARE-containing promoters [110]. After its translocation in the nucleus, Nrf2 promotes the expression of several antioxidant genes such as heme oxygenase 1 (HO-1) which regulates oxidative stress and inflammatory response and inhibits apoptosis by eliminating toxic heme [111, 112].

Generally, as mentioned above, the liver is the primary detoxifying organ that metabolizes various compounds associated with the production of free radicals. In addition to that, mammalians can metabolize various compounds associated with the production of free radicals, ROS and oxidative stress through the liver. Extrinsic (alcohol, drugs, environmental toxins, viruses, and smoking) and intrinsic (obesity and insulin resistance) sources can promote ROS production in the hepatic tissue. In particular, the mitochondria and Endoplasmic Reticulum (ER) are the main sites of ROS formation within the hepatocytes [113]. The electron and hydrogen transfer indeed within the mitochondrial transport chain, ultimately yielding water as a nontoxic product. Oxygen, instead of accepting two electrons, only takes in one, leading to the reduction of O_2 to O_2–, forming the superoxide, subsequently transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase [114]. ROS engage in reactions with other molecules, either gaining or losing electrons to achieve stability and transforming them into a free radical, which ultimately induces damage to the hepatocyte [115]. Furthermore, excessive ROS within hepatocytes can indeed induce hepatic structural and functional abnormalities that develop into various diseases, such as Non-Alcoholic Fatty Liver Disease (NAFLD), Alcoholic Fatty Liver Disease (AFLD), fibrosis and HCC [116]. In particular, concerning NAFLD, excess lipid in the liver causes lipotoxicity, leading to mitochondrial dysfunction and endoplasmic reticulum stress [117]. Consequently, a dysfunctional mitochondrion has a high capacity to produce ROS, leading to the onset of oxidative stress [118] The involvement of LPS in NAFLD is also known in the literature. LPS is capable of terminating the growth of triglyceride concentrations and the hepatic synthesis of VLDL [119]. The increase in LPS concentrations can therefore induce greater lipid peroxidation through oxidative stress, determining an association between the action of LPS and NAFLD. Not surprisingly, there is evidence indicating that elevated oxidative stress induces lipid accumulation in the liver, whereas reduced oxidative stress exerts a lipid-lowering effect in hepatocytes [120, 121, 122].

1.4.3 Hepatic Epithelial-mesenchymal Transition and Liver fibrosis

Epithelial-to-mesenchymal transition (EMT) is a physiological process necessary for embryonic development, mainly involved in mesoderm formation and cell morphogenesis. During EMT mechanisms, epithelial cells acquire the phenotype of motile mesenchymal cells, with a spindle-like morphology [123]. Apart from physiological conditions, EMT can be also involved in the process of tumor migration and invasion. Furthermore, EMT is the basis of the activation of the liver's attempt at regeneration following an insult or damage. This attempt at regeneration determines the activation of fibrogenesis and the achievement of a condition of fibrosis, correlated with unfortunate outcomes such as cirrhosis or liver cancer. In detail, the activation of hepatic stellate cells (HSC) and the origin of myofibroblasts (MFB) are the basis of chronic liver diseases. During activation, HSCs acquire a fibroblast-like shape and express large amounts of Alpha-Smooth Muscle Actin (α-SMA) and extracellular matrix (ECM) proteins (Figure 7) [124].

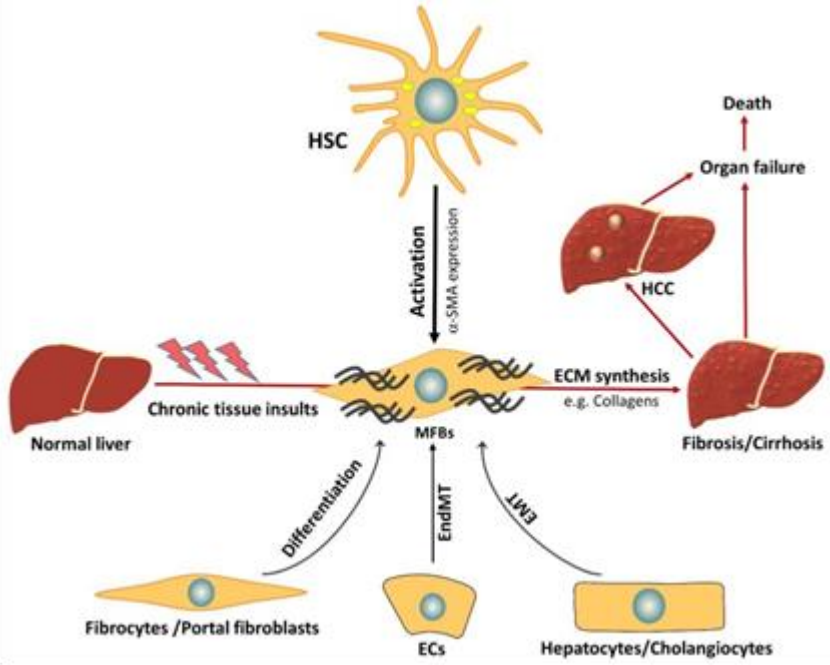

Figure 7: Representative image of HSCs, fibroblasts, hepatocytes and cholangiocytes in liver fibrosis. HSCs play a crucial role in liver fibrosis by transforming into myofibroblast-like cells that produce ECM, leading to scar formation. Fibroblasts also contribute to extracellular matrix production and tissue repair. Hepatocytes, the main liver cells, can show signs of injury and inflammation in a fibrotic environment, while cholangiocytes, the bile duct epithelial cells, may undergo pathological changes. [124]

Although hepatic stellate cell activation remains a central event in liver fibrosis, the epithelial-mesenchymal transition of hepatocytes and cholangiocytes is attracting great attention in the scientific community. *In vivo* studies have demonstrated that bile duct epithelial cells can undergo EMT, thus contributing to liver fibrosis [125, 126].

Transforming growth factor-beta 1 (TGF-β1) is also known to be the strongest inducer of EMT, capable of resulting in the loss of epithelial markers such as E-cadherin and the acquisition of mesenchymal markers such as vimentin, fibronectin and N-cadherin [127, 128]. In particular, E-cadherin and N-cadherin play a significant role in the mechanisms of EMT [129]. These proteins indeed shape a cadherin-catenin adhesion complex by binding β- and α-catenin via their cytoplasmic tails [130]. In particular, E-cadherin preserves epithelial phenotype and tissue homeostasis through targeting various molecular pathways. A decrease in E-cadherin can then trigger the EMT mechanism, leading to increased cell invasion and migration [131]. The N-cadherin protein, on the other hand, is minimally expressed in non-cancerous epithelial cells, while its expression is evident in tumor epithelial cells [132]. Up-regulation of N-cadherin in normal epithelial cells indicates impending EMT [133]. N-cadherin contributes to the formation of metastases and the invasion of epithelial cells during the mechanism of EMT. A further mesenchymal marker of the epithelial-mesenchymal transition is vimentin. Vimentin is one of the main constituents of the family of intermediate filament proteins, it is ubiquitously expressed in mesenchymal cells, and, being a structural protein of the cytoskeleton, it is known to have a function in maintaining cellular integrity. An increase in vimentin expression is associated with EMT, so much so that in recent years, this protein has acquired considerable importance as a marker of EMT [134].

Consequently, dysregulation of these markers and the redirection of cells towards a mesenchymal phenotype are the basis of the development of hepatic fibrosis. Furthermore, this pathological condition is also a necessary step in the progression of chronic liver disease to cirrhosis. The progression of chronic liver diseases involves the formation of parenchymal lesions, persistent activation of the inflammatory response and sustained activation of hepatic fibrogenesis. It is a dynamic molecular, cellular and tissue process, responsible for the excess accumulation of ECM components supported by a heterogeneous population of hepatic myofibroblasts [135]. The accumulation of ECM in fibrotic liver diseases is not a static or unidirectional event, but a dynamic process. In particular, fibrosis begins from a series of insults that lead to the death of hepatocytes, including viral infections, alcohol consumption and diet, which is the main cause of NAFLD. These insults induce the activation of HSCs, which are strongly involved in the mechanism underlying liver fibrosis [136]. Although fibrogenesis and fibrosis may represent an attempt to limit the consequences of chronic liver damage, they both underlie the pathological progression to liver cirrhosis

and Hepatocarcinoma. Furthermore, hepatic fibrogenesis is linked to persistent pathological angiogenesis that contributes to the expansion of tissue fibrosis [137, 138].

In addition to this, the involvement of LPS binding to TLR4 in the promotion of liver fibrosis is also known in the literature. TLR4 signaling is indeed capable of inducing liver inflammation, promoting fibrosis and progression to TLR4-dependent tumors [139]. Furthermore, it is essential to mention the role of TGF-β as an important risk factor for fibrogenesis. In fact, TGF-β stimulates ECM gene expression and reduces ECM degradation through down-regulation of metalloproteinases (MMPs) and up-regulation of metallopeptidases (TIMPs). Recent studies also show that TLR4 activation enhances TGF-β signaling in the development of liver fibrosis. Studies on mice have shown the regulation of TGF-β1 via the TLR4-MyD88-NF-κB-dependent pathway [140].

Liver damage and the induction of fibrosis can also be induced by the action of reactive oxygen species (ROS). These unstable compounds include superoxide, hydroxyl radicals, and hydrogen peroxide. They are generated through lipid peroxidation and can arise from hepatocytes, macrophages, HSCs and inflammatory cells [141].

Nowadays, it is known that many natural products are effective in preventing and treating liver fibrosis. Alkaloids, polysaccharides, flavonoids, polypeptides, terpenes and polyphenols are in fact known in the literature for their role in counteracting liver fibrosis [142]. Several studies have in fact demonstrated that treatment with natural compounds down-regulates TLR4/MyD88 expressions, even blocking downstream signaling through nuclear translocation and NF-κB phosphorylation. Some terpenes are in fact able to suppress liver fibrosis by blocking the activation of TLR4/MyD88/NF-κB signaling [143, 144].

1.4.4 Gut-Liver interaction

The gut microbiota (GM), an intricate ecosystem of bacteria, protozoa, archaea, fungi, and viruses, exists in a specific symbiotic relationship with the human body. Modern research highlights that GM is pivotal in human health, influencing physiological and pathological conditions by participating in digestion, vitamin B production, immune modulation, and the promotion of both angiogenesis and nerve function. In addition to this, the gut microbiota (GM) exists in a specific symbiosis with extraintestinal organs such as the kidneys, brain, cardiovascular system, and bones, and liver [145, 146]. In particular, the gut-liver axis depends on the anatomical and functional link between the gastrointestinal tract and liver through the portal circulation. In optimal health, the gut-liver axis enables dialogue between the host and microbiota, regulating immune homeostasis through a reciprocal mechanism

[147]. The GM-liver relationship is stabilized by metabolic, immune, and neuroendocrine interactions [148]. Tight junctions (TJ) in the gut epithelium create a barrier against bacteria, but antigens from pathogens or food can still pass through, triggering immune responses and inflammation. Low levels of pathogen-associated molecular patterns (PAMPs) like LPS activate inflammatory cytokines and chemokines in the liver, causing hepatocyte damage and engaging stellate and Kupffer cells in fibrosis [149].

Interestingly, the gut-liver axis's role in liver disease pathogenesis makes it a significant area of clinical research today. One of the causes of liver disease onset is dysbiosis, a condition characterized by an imbalance in the gut microbiota. Dysbiosis disrupts the normal symbiotic relationship between the gut and the liver, leading to a cascade of adverse effects. This imbalance can result in increased intestinal permeability, allowing harmful bacterial products and toxins to enter the bloodstream and reach the liver through the portal circulation. These substances can trigger inflammatory responses, immune system activation, and liver cell damage, contributing to the development and progression of liver diseases such as non-alcoholic fatty liver disease [146], alcoholic liver disease [150], cirrhosis and HCC [151]. Generally, increased intestinal permeability and bacterial translocation can allow microbial metabolites to reach the liver, disrupting bile acid metabolism and promoting gut dysmotility and systemic inflammation. These conditions can lead to gut dysbiosis, which, in turn, exacerbates liver damage.

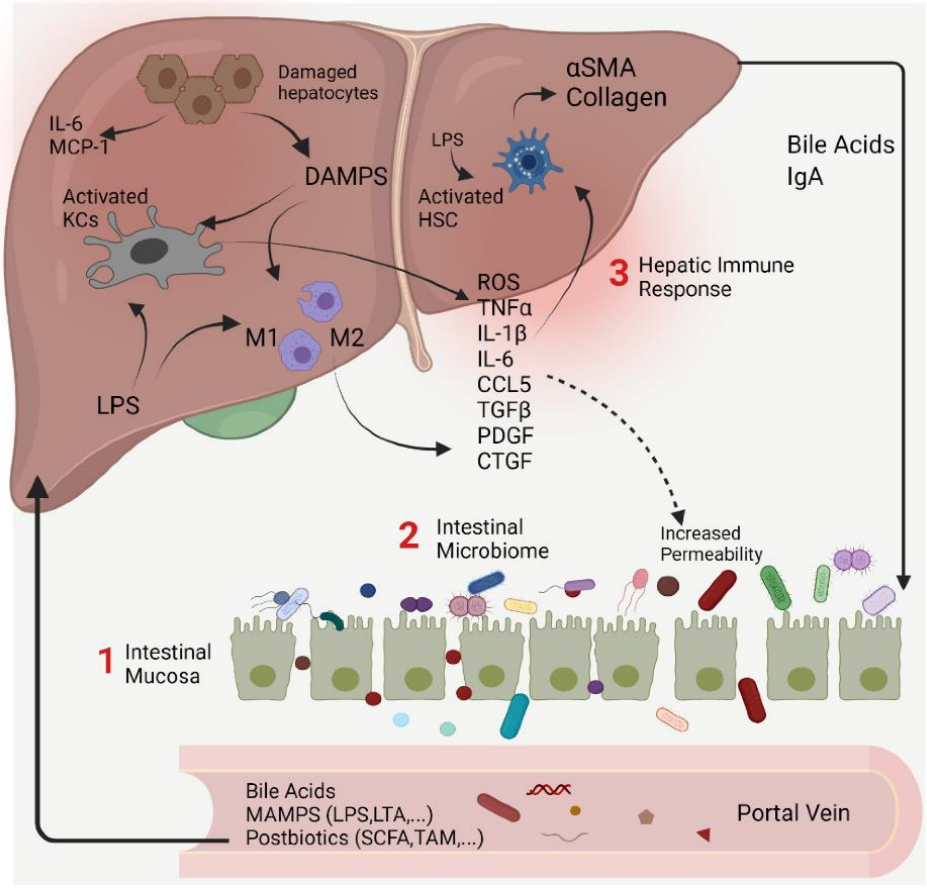

Figure 8: The gut–liver axis and its intersection with the intestinal microbiome. This bidirectional relationship operates through the portal vein, which transports immunogenic antigens from the gut to the liver, while the liver communicates back to the gut via bile and antibodies. Intestinal dysbiosis and impaired barrier function lead to the systemic spread of microbial components, triggering inflammatory responses in the liver [152].

1.4.5 Hepatoprotective effect of natural derivates

Currently, one of the open points in the scientific community is the search for effective preventive or therapeutic approaches against the main liver-related diseases. The substantial worldwide impact of chronic liver disease has spurred intensive research into alternative treatment options. Additionally, the scarcity of available organs for transplantation, which represents the definitive cure for end-stage liver disease, has underscored the urgent demand for alternative therapies [153]. To date, natural derivatives are gaining significant attention in biomedical research for their biological relevance and potential in treating liver dysfunction. In particular, grapefruit essential oil can mediate the suppression effect against the proliferation of liver cancer (HepG2) cells [154]. Furthermore, scientific results showed that the administration of rosemary essential oil, which exhibits free radical scavenging activity as determined by the DPPH assay, has beneficial effects in preventing CCl4-induced

liver toxicity in rats. This is achieved by reducing lipid peroxidation and, consequently, cell membrane damage [155]. Additionally, it is known that ginger essential oil and citral administration exert beneficial outcomes in the development of NAFLD in mice with obesity caused by a high-fat diet (HFD). These effects are likely due to the modulation of hepatic lipid synthesis, antioxidant enzymes, and inflammatory factors, involving the regulation of the hepatic SREBP-1c and CYP2E1 pathways [156]. Orange essential oil also counteracts obesity caused by HFD by reducing the levels of triglycerides, total cholesterol low-density lipoprotein cholesterol, and lipid accumulation in liver cells [157]. On the other hand, interestingly, Zhuang *et al.* demonstrated that nanoparticles derived from ginger can protect against liver damage caused by alcohol consumption. Their findings suggest that ginger-derived nanoparticles possess anti-inflammatory and antioxidant properties that help reduce liver damage, improve liver function, and potentially reverse some of the negative impacts of alcohol [59]. For instance, Zhao *et al*. discovered that blueberry-derived exosome-like nanoparticles, when administered, regulated the expression of antioxidant genes by activating Nrf2, enhanced liver function, prevented vacuole formation, and reduced lipid droplet accumulation in the livers of HFD-fed mice [50].

Notably, the use of lemon derivatives in preventing or treating liver diseases may potentially mark a significant advancement in the nutraceutical and therapeutic fields. While it is widely recognized that certain essential oils fractions and plant-derived nanovesicles can mitigate liver pathological conditions, their effects on hepatic health are not yet largely unexplored. Consequently, the following chapters will provide an in-depth exploration of the hepatoprotective properties of these two types of lemon derivatives.

CHAPTER 2: AIMS

2.1 General aims of the study

The following study aims to investigate the biological and hepatoprotective properties of Citrus limon derivatives, specifically of (i) a mix of lemon essential oils fractions enriched in Citral (Cfr-LEO) and (ii) lemon-derived nanovesicles (LNVs). In particular, this research explores the potential health benefits and therapeutic applications of these natural products and their role in modulating different molecular pathways deeply involved in some hepatic disorders. Lastly, we want to conduct a preliminary evaluation of the potential antimicrobial effect of LNVs.

By assessing their functional properties, the project seeks to provide scientific evidence supporting the use of lemon derivates in promoting liver health and treating liver-related diseases. Specifically, this study considers three main objectives.

- **Objective part I:** to examine in human healthy hepatocytes the anti-inflammatory and antioxidant capabilities of Cfr-LEO and its effects on counteracting the epithelial-mesenchymal transition (EMT) mechanism, a critical process involved in the development of hepatic fibrosis.
- **Objective part II:** to study the properties of LNVs produced both at laboratory and industrial scales and to assess their hepatoprotective effects *in vitro* and *in vivo*. In particular, LNVs are tested *in vitro* in human healthy hepatocytes and *in vivo*, in rats fed with High Fat Diet (HFD).
- **Objective part III:** to explore the antimicrobial capabilities of LNVs isolated by SEC, focusing on their potential to stimulate and activate the human immune system.

Through these investigations, the project uncovers the preventive potential of lemon-derived products in promoting liver health, treating liver-related disorders, and enhancing immune function, thus contributing to the development of innovative nutraceutical products.

CHAPTER 3: PART I

Citral-Enriched Fraction of Lemon Essential Oil Mitigates LPS-Induced Hepatocyte Injuries

3.1 Objective part I

In the first part of this study, the anti-inflammatory and antioxidant properties of Cfr-LEO are explored as well as its effects on inhibiting the epithelial-mesenchymal transition (EMT) process, which is crucial in the development of hepatic fibrosis, in healthy human hepatocytes. In detail, we aim to demonstrate *in vitro* the multi-functional effects mediated by Cfr-LEO in regulating different processes strongly involved in the development of hepatic disease. To achieve this aim and to understand how Cfr-LEO can exert its beneficial properties, the TLR4 molecular signaling is investigated. In detail, hepatocytes are pre-treated with Cfr-LEO and then with LPS. In the following sections, the "Material and Methods" and the "Results" of this first part of the study will be deeply described.

3.2 Material and Methods part I

3.2.1 Cell Culture

THLE-2 (ATCC CRL-2706™, LGC Standards) human cells isolated from the left lobe of a healthy liver and immortalized with the catalytic subunit of human telomerase hTERT were maintained in RPMI 1640 medium (Euroclone, UK) supplemented with 10% fetal bovine serum (FBS, Euroclone, UK), 1% penicillin (100 U/mL) and streptomycin (100 µg/mL), 0.3 mL human recombinant epidermal growth factor (hEGF) (10 µg/mL), and 0.4 mL phosphoethanolamine (PEA) (100 µg/mL). The cell line was tested for Mycoplasma using the Hoechst staining and the N-GARDE Mycoplasma PCR reagent set (Euroclone), it was authenticated with a morphology check and cell proliferation rate evaluation, and bacteria contamination was excluded. Cells were grown on a coating made of 0.03 mg/mL bovine collagen type I (Advanced Biomatrix, San Diego region, CA, USA) and 0.01 mg/mL bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). Cfr-LEO was recovered by cold-pressed extraction mechanical process from the peels of winter fruits at the company Agrumaria Corleone S.P.A. (Palermo, Italy) and, after cold dewaxing at −20 °C for 48 h and subsequent filtration through a paper filter with 10-micron pores, lemon essential oil was fractionated by a newly developed adsorption column chromatography [22]. After purification, Cfr-LEO was solubilized in FBS and, for each treatment, directly diluted in RPMI 1640 medium. In particular, THLE-2 cells were consequently treated, as described in the graphical representation shown in Figure 9. Furthermore, two different batches of Cfr-LEO were used in this study, both provided by Azienda Agrumaria Corleone, which personally and thoroughly conducted multiple quality control assessments.

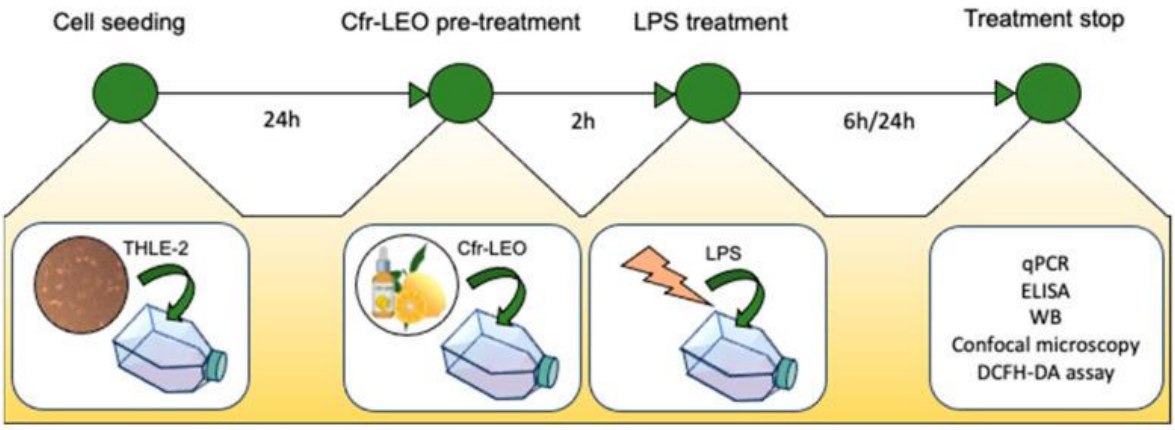

Figure 9: Graphical representation of the experimental design.

3.2.2 MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5 Diphenyl Tetrazolium Bromide) Assay

The MTT assay is used to measure cellular metabolic activity. Accordingly, MTT was performed to select the Cfr-LEO and LPS doses to use for treating the hepatocytes. THLE-2 were seeded in triplicate at 3×10^4 cells per well in 24-well plates; after 24 h and 48 h of seeding, cells were treated for further 24 and 48 h with different concentrations of Cfr-LEO (0.005%, 0.01%, 0.02%, 0.05%) prepared as described in the following. Cfr-LEO 100% was first solubilized in a solution of FBS 95% and DMSO 5% to bring it to a final concentration of 1%, then further diluted in cell culture medium to obtain the following final concentrations: 0.005%, 0.01%, 0.02%, and 0.05%. In the MTT assay, untreated cells and cells treated with the highest used concentration of DMSO (0.25%) were used as controls. The MTT solution was prepared as a 5 mg/mL stock solution in phosphate-buffered saline (pH 7.4) and filtered (0.22 μm, Millipore, Burlington, MA, USA). At the end of the treatment, the warmed (37 °C) MTT stock solution was added to each well according to the manufacturer's instruction. The plates were then incubated at 37 °C for 3 h and stopped with a solution of 0.4% HCl in isopropanol. The absorbance was measured with an ELISA reader at 540 nm (Microplate Reader, BioTek, Winooski, VT, USA). MTT assay was then performed to select LPS concentration (100 ng/mL, 250 ng/mL, 500 ng/mL, and 1000 ng/mL); 24 h after seeding, cells were treated with LPS for 24 h. After the treatment, the MTT stock solution was warmed at 37 °C and added to the cell according to the manufacturer's instruction. The plates were then incubated at 37 °C for 3 h and stopped with a solution of 0.4% HCl in isopropanol. The absorbance was measured with an ELISA reader at 540 nm (Microplate Reader, BioTek, Winooski, VT, USA). Values are expressed as a percentage of cell growth versus control (untreated cells).

3.2.3 RNA Isolation and Quantitative Real-Time PCR

Levels of interleukin-1β (IL-1β), interleukin (IL-6), and tumor necrosis factor (TNFα) were measured using quantitative real-time PCR (qRT-PCR). THLE-2 were seeded at 8×10^4 per well in 12-well plates and, after 24 h, treated with Cfr-LEO 0.01% following this protocol: pretreatment for 2 h with Cfr-LEO (0.01% and 0.02%) before their exposure to LPS (100 ng/mL) for 6 h, without essential oil removal. After the treatment, total RNA was isolated using the illustra TM RNAspin mini-RNA isolation kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Then, total RNA was reversely transcribed into cDNAs with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA was analyzed by performing qRT-PCR with SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA) and the primers reported in Table 1. The cycling qualifications were as follows: 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, then 55 °C for 30 s, and then 72 °C for 1 min. All reactions were performed in duplicate, and at least three independent experiments were analyzed. Quantitative analysis was performed calculating the expression of IL-1β, IL-6, and TNFα relative to the endogenous housekeeping gene GAPDH. Relative quantification was calculated using the comparative threshold cycle method (ΔΔCT).

Primers.	Forward	Reverse
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGTCATTGATGGCAACAATAT
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-1β	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT
TNFα	CCAGGCAGTCAGATCATCTTCTCTC	AGCTGGTTATCTCTCAGCTCCAC

Table 1: Oligonucleotides used in qRT-PCR (9); 60° is the temperature of the annealing.

3.2.4 ELISA (Enzyme Immunosorbent Assay)

The amount of IL6 released in the conditioned medium of THLE-2 cells was quantified using the specific ELISA kit (Thermo Fisher Scientific, Waltham, MA USA). THLE-2 cells were seeded at 8×10^4 per well in 12-well plates, and after 24 h, cells were pretreated for 2 h with Cfr-LEO (0.01%) before their exposure to LPS (100 ng/mL) for 6 h. The LPS treatment was carried out without Cfr-LEO removal, and, at the end of the experimental time, the conditioned medium was collected and centrifuged to remove cellular debris at 300 g for 5 min. The ELISA assay was performed according to the manufacturer's instructions.

The absorbance reading was executed with the spectrophotometer, using 450 nm as a wavelength.

3.2.5 Western Blot

THLE-2 were seeded at 3×10^5 cells on a coating of type I bovine collagen in the culture flasks (T25). Cells were pretreated for 2 h with Cfr-LEO (0.01% or 0.02%) and then with LPS (100 ng/mL or 1000 ng/mL) for 30 min, 6 h, and 24 h, depending on the experimental downstream investigations; THLE-2 were recovered from the culture flask using a cell scraper. Protein lysate was obtained by adding lysis buffer (Tris-HCl pH 7.6, 50 mM, NaCl 300 mM, TritonX-100 0.5%, PMSF 1X, leupeptin 1X, aprotinin 1X, phosphatase inhibitors 1X (phosphatase inhibitor cocktail 10X), and H2O milliQ). Protein quantification was carried out using Bradford assay, and the reading was executed on the biophotometer at a wavelength of 595 nm. H_2O, sample buffer (4X), and reducing agent (10X) were added to the quantized proteins, which were consequently loaded onto SDS-page Bolt TM 4–12% Bis-Tris Plus (Invitrogen). Proteins were transferred on a nitrocellulose membrane (MCE, Invitrogen, Life Technologies, Rockford, IL, USA) in the presence of the transfer buffer (10X Tris-glycine, 20% methanol, H_2O milliQ). The membrane was incubated in BSA blocking solution for 90 min and probed overnight with primary antibodies: anti-NRF2, anti-P53, anti-NF-κB p65 (Novus Biologicals, E. Briarwood Avenue Centennial, CO, USA), anti-p-NF-*κB* p65 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), anti-vimentin, anti-N-cadherin (Cell Signalling Technology, Lane Danvers, MA, USA), and anti-β-actin and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). The membrane was incubated with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:1000 dilution; Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescence was detected using the Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) chemiluminescence solution. The signal detection was performed with the "ChemiDocTMMP" (Imaging System) and the densitometric analysis was carried out with the "Image J" 1.48v software.

3.2.6 Confocal Microscopy

THLE-2 were grown and seeded at 1×10^5 cells into chambers of 4.2 cm^2 (Thermo Fisher Scientific, 155380), and after 24 h, cells were pretreated with Cfr-LEO for 2 h and then treated with 100 ng/mL of LPS for 30 min. At the end of the treatment, THLE-2 were fixed with PFA 4% for 10 min and then washed three times with PBS. Furthermore, THLE-2 were permeabilized with triton 0.1% for 2 min, and then they were incubated with BSA 1% for

30 min. After that, THLE-2 cells were labeled with anti-NF-κB p65 (Novus Biologicals) (dilution 1:100) for 1 h and then with the fluorescent secondary antibody Alexa fluor 594 (Life Technologies, Thermo Fisher Scientific) (dilution 1:500) for 1 h. Afterward, the nuclei were labeled with Hoechst 33342, trihydrochloride, trihydrate (Invitrogen, Thermo Fisher Scientific) (dilution 1:1000), and the cytoskeleton was labeled with Actin Green 488 Ready Probes reagent (Invitrogen, Thermo Fisher Scientific) for 30 min. At the end of the labeling method, cells were observed with confocal microscopy (Nikon A1).

3.2.7 Dichlorodihydrofluorescein Diacetate (DCFH-DA) Assay

Cell-permeable 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to detect reactive oxygen species (ROS) in cells. THLE-2 were seeded 3×10^4 per well in a 24-well plate. After 24 h, cells were pretreated for 2 h with Cfr-LEO (0.01%) and for 6 h with LPS (100 ng/mL). At the end of the treatment, the cell culture medium was removed, and DCFH-DA was added at a final concentration of 10 μM in the cell culture medium FBS-depleted. THLE-2 cells were incubated with DCFH-DA at 37 °C at 5% of CO_2 in the dark for 30 min and then were washed with phosphate-buffered saline (PBS) three times to completely remove excess DCFH-DA. ROS production was observed using fluorescence microscopy (Nikon Eclipse Ti) and quantified using ImageJ software.

3.2.8 Statistical Analysis

Data are reported as means ± SD. Comparisons were made by performing the one-way ANOVA multiple comparisons test with the Holm–Šídák method or the two-way ANOVA (or mixed model) multiple comparisons test with the Tukey method. Analyses were conducted using GraphPad Prism Version 10.1.0 (316). Values were considered statistically significant when $p \leq 0.05$.

3.3 Results part I

3.3.1 In Vitro Anti-Inflammatory Effects of Cfr-LEO

The effects of the Cfr-LEO treatment on THLE-2 cell viability were preliminarily evaluated by performing an MTT assay. The results, reported in Figure 10A, show that cell treatment with 0.005%, 0.01%, 0.02%, and 0.05% Cfr-LEO for both 24 h and 48 h did not affect THLE-2 cell viability. According to our previously published data [22], the Cfr-LEO concentrations ranging from 0.01% to 0.02% were employed for subsequent experiments.

We also previously tested various doses of LPS [22], selecting for the current study the doses 100 ng/mL and 1000 ng/mL, both of which did not affect cell viability.

To determine whether Cfr-LEO exhibited protective effects against LPS-induced inflammatory response in hepatocytes, we first demonstrated that the treatment of THLE-2 cell lines with LPS 100 ng/mL significantly upregulated the expression of the inflammatory mediators IL-6, IL-1β, and TNFα. Subsequently, we analyzed the effects of Cfr-LEO in counteracting LPS-induced upregulation of IL-6, IL-1β, and TNFα. As shown in Figure 10B, we found that the pretreatment of THLE-2 cells for 2 h with Cfr-LEO significantly inhibited the expression of TNFα induced by 6 h LPS 100 ng/mL treatment, while no effects were observed for IL-1β. A possible explanation for the different effects exerted by Cfr-LEO on IL-1β expression could be linked to a different LPS-mediated induction due to a diverse basal expression of the cytokine. Moreover, alternative pathways responsible for IL-1β transcription [158] could affect the cytokine expression.

Concerning IL-6, we found a significant decrease in the cytokine expression in the Cfr-LEO 0.02% pretreated condition when compared with LPS-treated cells and a not significative decrease trend for IL-6 expression in THLE-2 cells pretreated for 2 h with Cfr-LEO 0.01%. Despite this, we found, by performing ELISA assay, a significative decrease in IL-6 release in the Cfr-LEO 0.01% pretreated condition when compared with LPS-treated cells (Figure 10C). On the other hand, the TNFα expression at the protein level was not detectable in any analyzed condition. Overall, these results suggest that Cfr-LEO exerts an anti-inflammatory effect in LPS-stimulated hepatocytes, indicating its hepatoprotective role.

Furthermore, to understand the molecular mechanisms responsible for the anti-inflammatory effects mediated by Cfr-LEO, we investigated the consequences of the Cfr-LEO pretreatment on the LPS-induced activation of nuclear factor κB (NF-κB), a well-known target of the LPS/TLR4 signaling pathway [159]. As shown in Figure 10D, the pretreatment of THLE-2 for 2 h with 0.01% Cfr-LEO inhibited the phosphorylation of NF-κB induced by the LPS treatment. To confirm this data, confocal analysis was performed. It revealed that pretreatment of THLE-2 for 2 h with 0.01% Cfr-LEO clearly inhibited the nuclear translocation and the consequent activation of NF-κB compared with LPS-treated cells (Figure 10E).

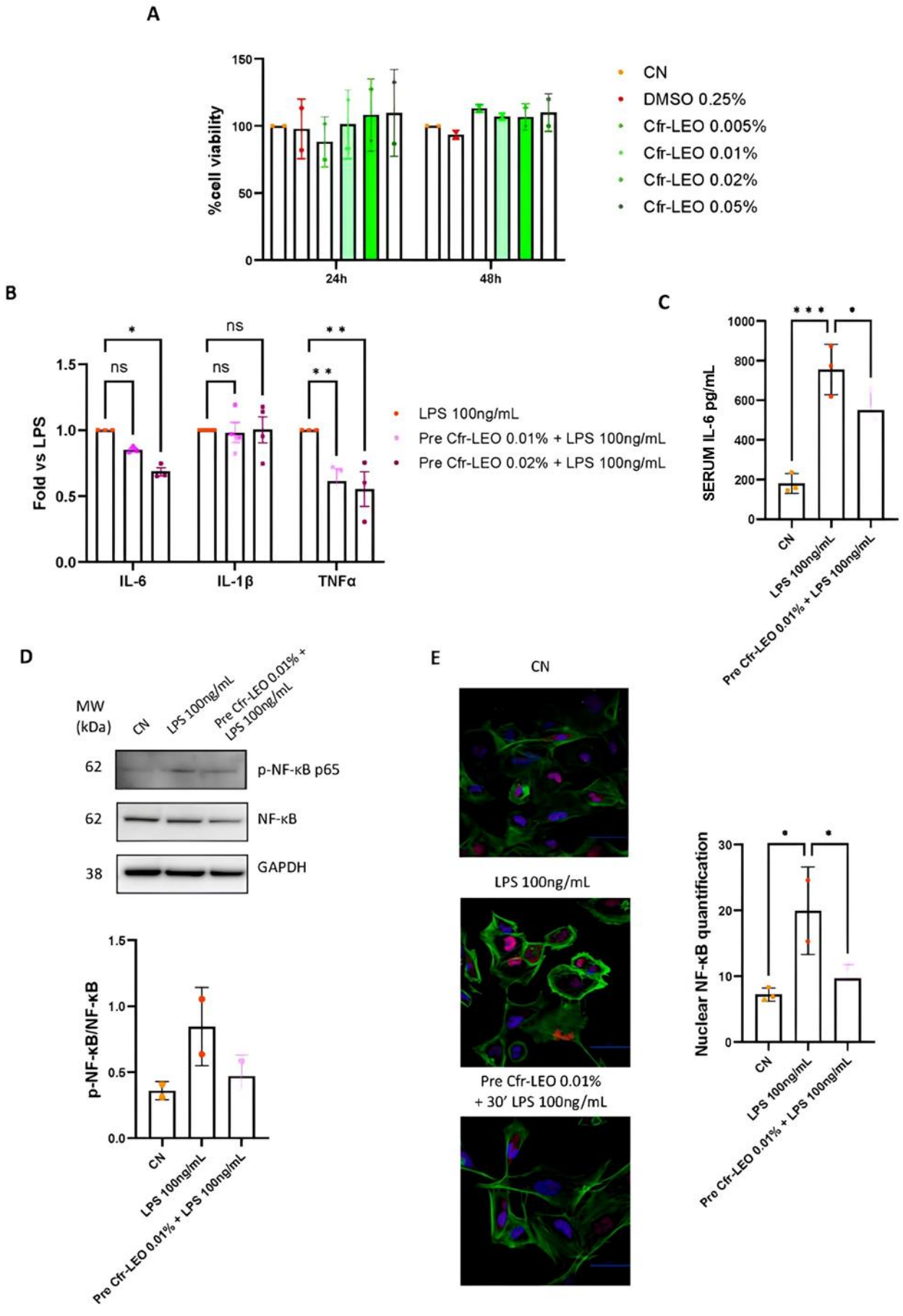
A
%cell viability
150
100
50
0
24h
48h
CN
DMSO 0.25%
Cfr-LEO 0.005%
Cfr-LEO 0.01%
Cfr-LEO 0.02%
Cfr-LEO 0.05%
B
Fold vs LPS
1.5
1.0
0.5
0.0
ns
ns
*
ns
**
**
IL-6
IL-1β
TNFα
LPS 100ng/mL
Pre Cfr-LEO 0.01% + LPS 100ng/mL
Pre Cfr-LEO 0.02% + LPS 100ng/mL
C
SERUM IL-6 pg/mL
1000
800
600
400
200
0
***
*
CN
LPS 100ng/mL
Pre Cfr-LEO 0.01% + LPS 100ng/mL
D
MW (kDa)
CN
LPS 100ng/mL
Pre Cfr-LEO 0.01% + LPS 100ng/mL
62
p-NF-κB p65
62
NF-κB
38
GAPDH
p-NF-κB/NF-κB
1.5
1.0
0.5
0.0
CN
LPS 100ng/mL
Pre Cfr-LEO 0.01% + LPS 100ng/mL
E
CN
LPS 100ng/mL
Pre Cfr-LEO 0.01%
+ 30′ LPS 100ng/mL
Nuclear NF-κB quantification
30
20
10
0
*
*
CN
LPS 100ng/mL
Pre Cfr-LEO 0.01% + LPS 100ng/mL

Figure 10: (A) Hepatocyte cell viability after exposure to Cfr-LEO. THLE-2 viability was measured with MTT assay after 24 and 48 h of treatment with different concentrations of Cfr-LEO (0.005%, 0.01%, 0.02%, and 0.05%). The values were plotted as the percentage of cell viability versus untreated cells (CN). Values are the mean ± SD of two biological replicates. (B,C) Anti-inflammatory effects of Cfr-LEO on LPS-stimulated THLE-2. (B) The effect of Cfr-LEO on inflammatory cytokines expression was assessed with qRT-PCR analysis. THLE-2 cells were treated for 2 h with 0.01% and 0.02% Cfr-LEO and then exposed to LPS 100 ng/mL for 6 h. Values are reported as fold change versus cells treated with LPS alone and are the mean ± SD of three biological replicates. (C) IL-6 protein level was measured with ELISA in the conditioned medium of THLE-2 cells treated for 2 h with 0.01% Cfr-LEO and then exposed to LPS 100 ng/mL for 6 h. Values are plotted as fold change versus cells treated with LPS alone (LPS). Values are the mean ± SD of three biological replicates. The statistical significance was calculated versus the LPS-treated cells (LPS) by using ANOVA test. (D,E) Effects of the Cfr-LEO pretreatment on the LPS-induced NF-κB activation. (D) Western blot showing the level of phosphorylated p65 subunit of NF-κB (p-NF-κB p65) and total p65 subunit of NF-κB (NF-κB p65) in THLE-2 cells treated for 30 min with 100 ng/mL LPS alone (LPS 100 ng/mL) or pretreated for 2 h with 0.01% Cfr-LEO (pre Cfr-LEO 0.01% + LPS 100 ng/mL). GAPDH was used as the loading control. CN: untreated cells used as control. The values reported in the densitometric analysis are the mean (±SD) of the analyzed protein normalized vs. loading control from at least two independent experiments. (E) Representative micrographs from confocal fluorescence microscopy of THLE-2 cells treated for 30 min with 100 ng/mL LPS alone (LPS 100 ng/mL) or pretreated for 2 h with 0.01% Cfr-LEO (pre Cfr-LEO 0.01% + LPS 100 ng/mL). THLE-2 cells were stained for NF-κB (red) and were labeled with Hoechst to observe the nucleus (blue) and with Actin Green for the cytoskeleton (green). CN: untreated cells used as control. The values reported in the densitometric analysis are the mean (±SD) of the analyzed micrographs from at least two observations. Colored dots represent the number of replicates for each condition. ns = not significant, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

3.3.2 Antioxidant Effects of Cfr-LEO

Since it is known that the LPS-induced TLR4/NF-κB signaling pathway also promotes the production of reactive oxygen species [160], we investigated whether Cfr-LEO could exhibit protective effects against LPS-induced oxidative stress. As shown in Figure 11A, we observed that the pretreatment of THLE-2 for 2 h with 0.01% Cfr-LEO inhibited the production of intracellular ROS compared with the cells treated with LPS alone. Interestingly, we found that the Cfr-LEO-pretreated hepatocytes did not respond to the pro-oxidant state promoted by LPS, as demonstrated by the expression levels of NRF2 and p53, which appeared comparable to control cells (Figure 11B, C).

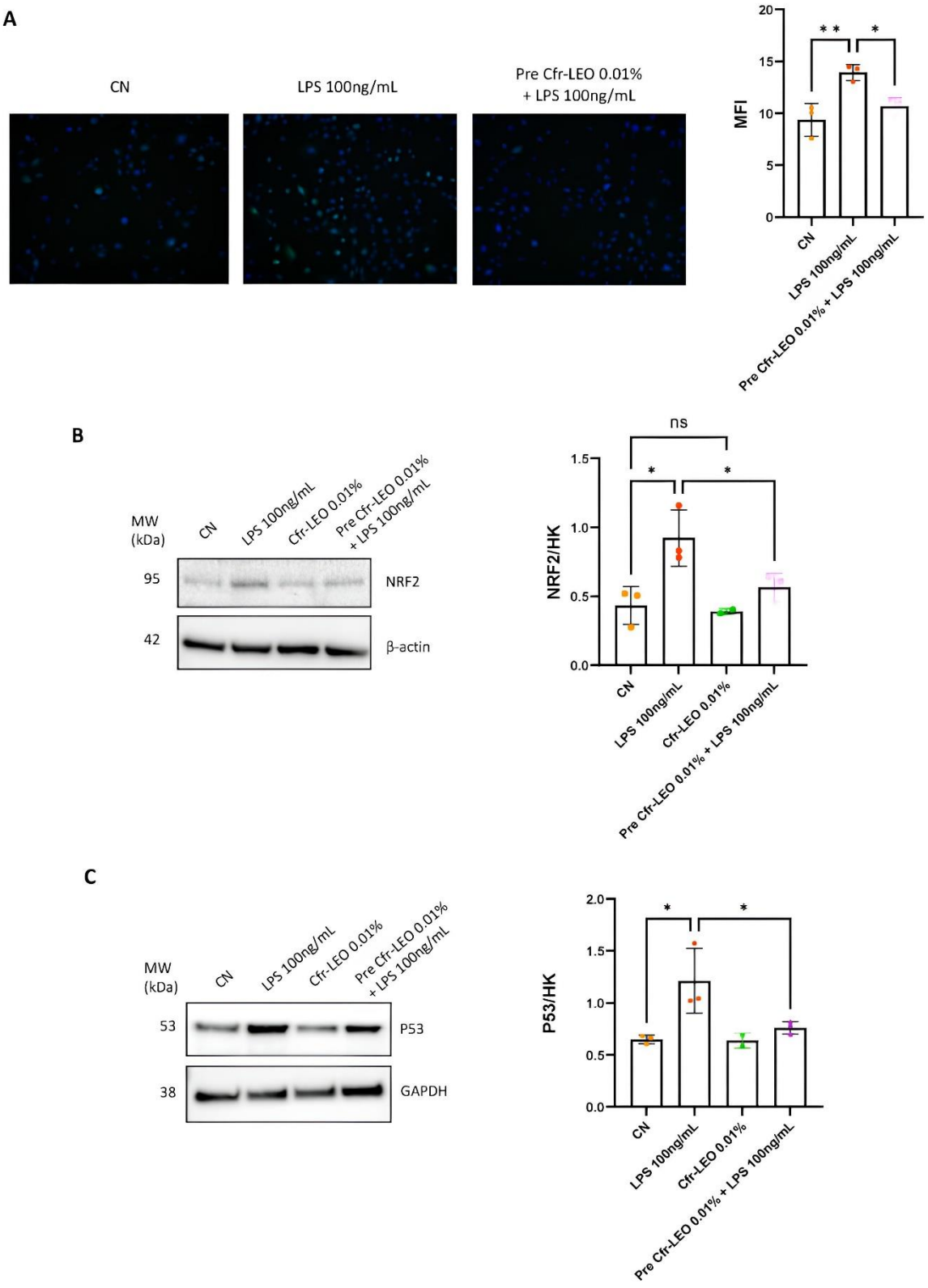

Figure 11: Antioxidant effects of Cfr-LEO on LPS-stimulated THLE-2. (A) The antioxidant effects of Cfr-LEO were evaluated with a DCFDA—Cellular ROS assay kit probe. The hepatocytes were treated for 6 h with 100 ng/mL LPS alone (LPS) or pretreated for 2 h with 0.01% Cfr-LEO (pre Cfr-LEO 0.01% + LPS 100 ng/mL). The intensity of the green fluorescence is proportional to the amount of ROS present in the sample. Values of

mean fluorescence intensity reported in the histogram were obtained by analyzing images with the Image J software and are the mean ± SD of three independent experiments. Values are plotted as fold change versus cells treated with LPS alone (LPS 100 ng/mL). Nuclei were labeled with Hoechst (blue). (B,C) Western blot analysis of NRF2 and p53 treated for 6 h with 100 ng/mL LPS alone (LPS 100 ng/mL) or pretreated for 2 h with 0.01% Cfr-LEO (pre Cfr-LEO 0.01% + LPS 100 ng/mL). GAPDH was used as the loading control. The values reported in the densitometric analysis are the mean (±SD) of the analyzed protein normalized vs. loading control from at least three independent experiments. CN: untreated cells used as control. Colored dots represent the number of replicates for each condition. ns = not significant * $p \leq 0.05$ and ** $p \leq 0.01$.

3.3.3 Cfr-LEO Protects Hepatocytes from the LPS-Induced EMT

Several studies have demonstrated that in the development and progression of chronic liver diseases, inflammation and fibrosis are often concomitant [161]. Even if most of the available data indicate that the activated hepatic stellate cells are the key players in the fibrogenic process, some evidence suggests that hepatocytes may also acquire a pro-fibrotic behavior through EMT, indicating this process as a potential target to develop new strategies to prevent liver fibrosis [162, 163]. Data in the literature report that the LPS induces EMT in epithelial target cells [164, 165, 166]. To evaluate the ability of Cfr-LEO to protect hepatocytes from the LPS-induced EMT, THLE-2 cells were treated for 2 h with 0.02% of Cfr-LEO and then for 24 h with 1000 ng/mL of LPS, a dose selected based on data reported in the literature [164, 165, 166]. The data reported in Figure 12A, and B show that the modulation of N-cadherin and vimentin induced by LPS was significantly counteracted by the pretreatment of THLE-2 with Cfr-LEO.

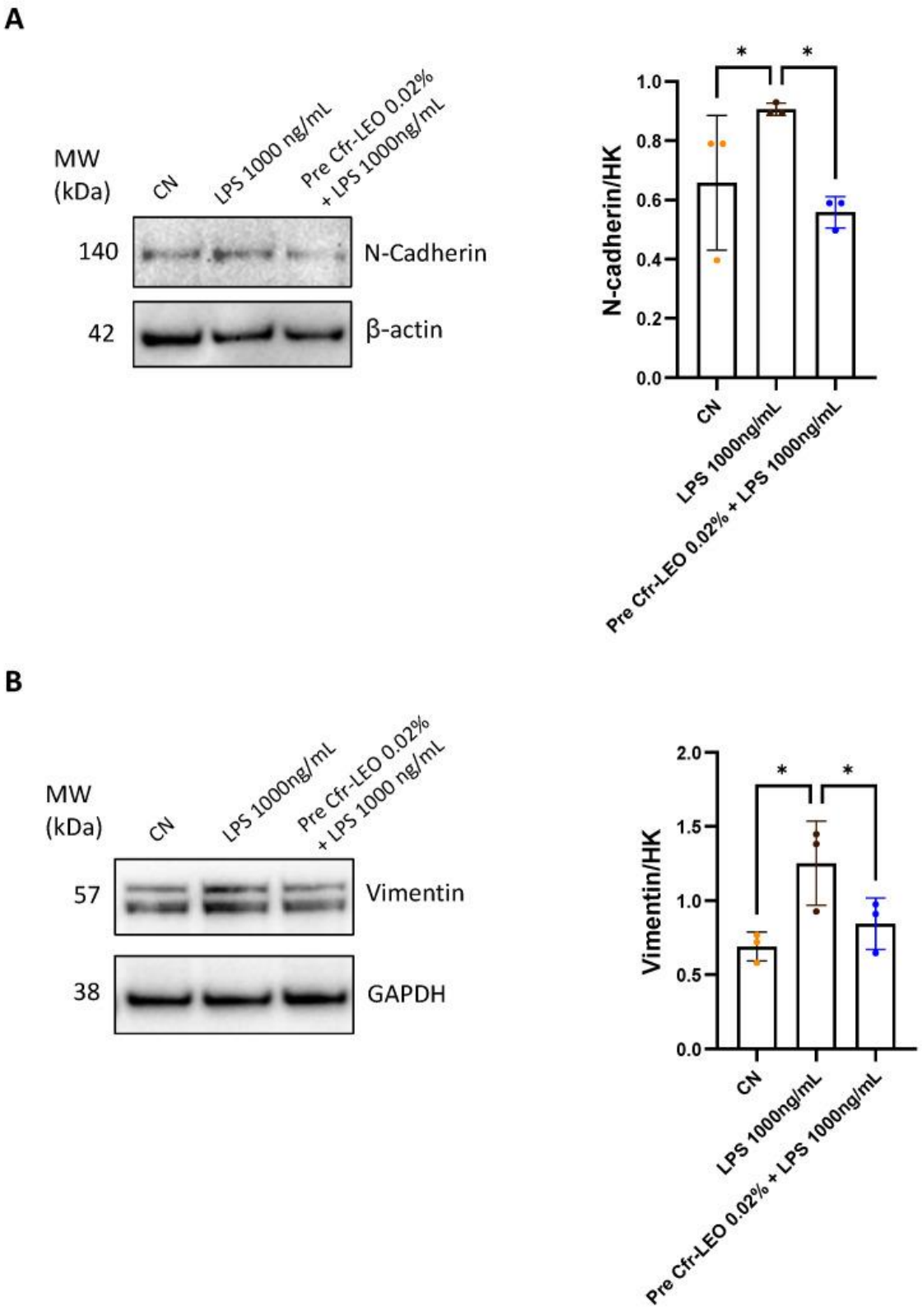

Figure 12: Analysis of the EMT inhibition properties of Cfr-LEO. (A,B) Western blot analysis of N-cadherin and Vimentin (double bands) in THLE-2 pretreated with Cfr-LEO (0.02%) and treated with LPS (1000 ng/mL) for 24 h. β-actin and GAPDH were used as the loading controls. The values reported in the densitometric analysis are the mean (±SD) of the analyzed protein normalized vs. loading control from at least three independent experiments. CN: untreated cells used as control. Colored dots represent the number of replicates for each condition. * $p \leq 0.05$.

CHAPTER 4: PART II

Protective effects of lemon nanovesicles: evidence of the Nrf2/HO-1 pathway contribution from *in vitro* hepatocytes and *in vivo* high-fat diet-fed rats

4.1 Objective part II

The second part of this research examines deeply the hepatoprotective properties of Lemon-derived nanovesicles produced at both laboratory (LNVs) and industrial scales (iLNVs). We aim to conduct a comprehensive analysis of these LNVs, examining their potential to safeguard liver function under oxidative stress and Metabolic Syndrome conditions. In the laboratory setting, we want to carry out *in vitro* experiments using human healthy hepatocytes (THLE-2) as a model system. This approach is essential to observe how these cells respond to LNV treatment at the molecular and cellular levels, providing insights into the mechanisms through which LNVs and m iLNVs can exert their protective effects against oxidative stress.

Concurrently, we extend our research into *in vivo* studies using a well-established animal model. Specifically, we administer iLNVs to rats fed with a high-fat diet (HFD), a regimen known to induce liver damage and metabolic dysfunction. This *in vivo* model allows us to assess the efficacy of iLNVs in a more complex biological system, observing not only their direct effects on liver tissue but also their influence on overall metabolic health.

These combined *in vitro* and *in vivo* studies aim to establish a robust understanding of how these nanovesicles, regardless of their scale of production, can contribute to liver health. By comparing the outcomes from laboratory-produced and industrial-scale LNVs, we also want to determine whether the scale of production affects their efficacy, thus providing valuable insights for the potential large-scale application of LNVs in clinical settings for liver protection and treatment.

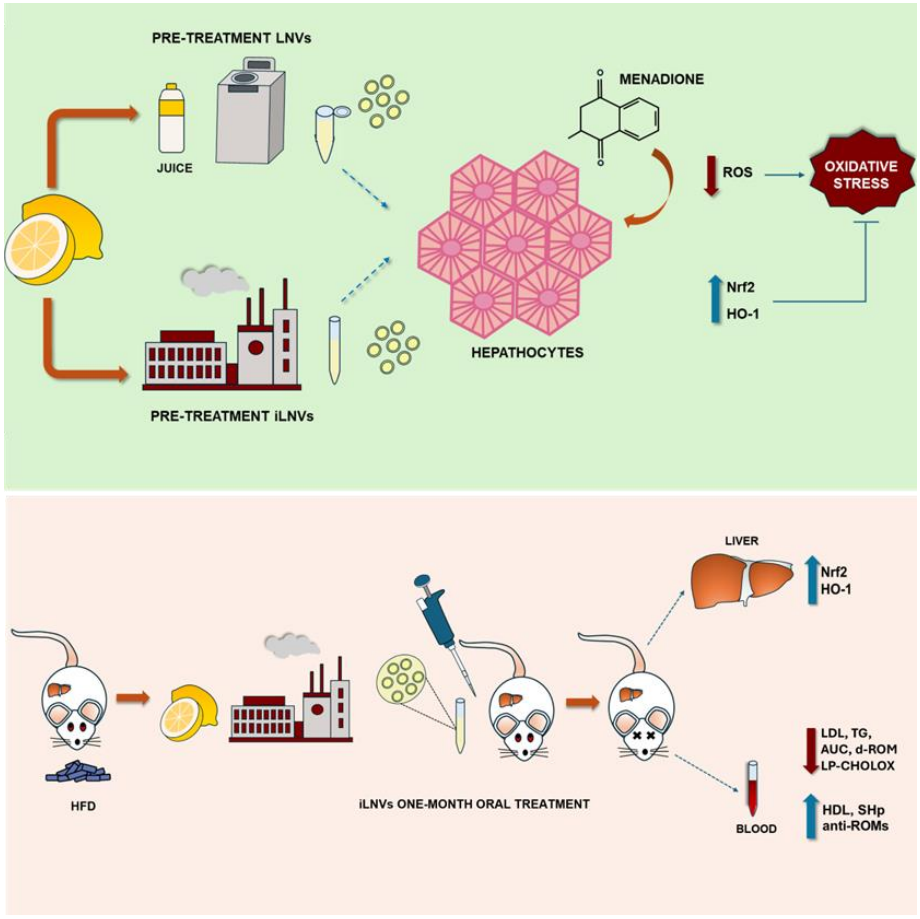

Figure 13: Graphical representation of the *in vitro* and *in vivo* experimental design.

4.2 Materials and Methods part II

4.2.1 LNVs and iLNVs isolation

LNVs were obtained from lemons coming from a private farmer as previously described [49, 71]. The juice was produced by manual squeezing and processed using the following differential centrifugations protocol: two centrifuges at 3000g for 15 minutes, two centrifuges at 10000g for 30 minutes, a filtration step with a 0.8 μm pores filter, a centrifuge at 16500g for 1h, followed by another filtration with 0.45 μm filters, and a final centrifuge at 16500g for 3h were performed. At the end of the process, the supernatant was collected and ultracentrifuged at 100.000 g for 1.45h. The pellet obtained was resuspended in PBS, which was the final buffer. Industrial-derived Nanovesicles (iLNVs) were produced using a patented process (IT patent n° 102019000005090, "Process for the production of vesicles from citrus juice") and were characterized as shown in our previous study [167]. Briefly, the lemon juice, obtained by Agrumaria Corleone s.p.a. (Palermo, Italy), was ultrafiltered with pore size 50,000 Dalton. The retentate was then microfiltrated at 0.45 μm, obtaining a

permeate containing the vesicles. Lemon juice matrix was the final buffer. Quantification of LNVs and iLNVs was performed by the Bradford protein assay (Pierce, Rockford, IL, USA) with Coomassie Brilliant Blue and the reading was finally performed using a biophotometer. Furthermore, various batches of LNVs and iLNVs were isolated and utilized in this study, and each was appropriately assessed for dimensional analysis.

4.2.2 Cell line and culture conditions

THLE-2 cell line (T-antigen immortalized Human Liver Epithelial cell – ATCC CRL-2706™, LGC Standards, Manassas, VA, USA) was used as an *in vitro* model of healthy human hepatocytes. Cells were cultured on 0.03 mg/mL bovine collagen type I coating (Advanced Biomatrix, San Diego region, CA, USA) and 0.01 mg/mL bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) and kept for 24h in an incubator at 37° at 5% CO_2. THLE-2 cell line was maintained in RPMI 1640 medium (Euroclone, UK), supplemented with 10% fetal bovine serum (FBS, Euroclone, UK), 1% penicillin (100 U/mL), and streptomycin (100µg/mL), 0.3 mL of human recombinant Epidermal Growth Factor (EGF) (10 µg/mL) and 0.4 mL of phosphoethanolamine (PEA) (100µg/mL). Cells were treated by diluting LNVs and iLNVs directly in RPMI 1640 medium.

4.2.3 Vesicles internalization assay

The internalization of LNVs and iLNVs in THLE-2 was evaluated by confocal microscopy. LNVs and iLNVs were incubated at room temperature with the lipophilic dye PKH26 (10 µM) for 20 minutes, considering a LNVs/PKH26 ratio concentration of 1:1. Once the incubation time was over, the labeled vesicles were centrifuged at 14000 rpm for 10 minutes to eliminate the excess of unbound dye. The pellet obtained was subjected to 4 washes in PBS and finally resuspended in RPMI medium 1640. THLE-2 cells were seeded at 3 x 10^4 cells per well in 8-well chamber (Thermo Scientific Nunc, Waltham, MA, USA). After 24h, cells were treated for 2h and 6h with the labeled LNVs (10 µg/mL and 25 µg/mL) or iLNVs (2.5µg/mL). After the treatment, cells were fixed with PFA 4%, and membranes were temporarily permeabilized with 0.1% Triton. Cell staining was performed by incubating for 30 minutes with Hoechst (Invitrogen Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) prepared 1:1000 in PBS and Actin Green (Invitrogen Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The internalization of LNVs and iLNVs in target cells was analyzed through confocal microscopy (Nikon A1).

4.2.4 Cell viability assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay was used as an indicator of cell viability. The MTT assay was performed to establish doses of nanovesicles that were not toxic to THLE-2; cells were seeded in triplicate, in a 96-well plate (1 x 10^4 cells per well) and after 24h were subjected to 24h treatment with LNVs (10 μg/mL, 25 μg/mL) or with iLNVs (2.5μg/mL). At the end of the treatment, the MTT solution (5 mg/mL stock) was added to each well in a 1:10 ratio. After 3 hours of incubation, the reaction was stopped with the addition of isopropanol HCl (1 +1/2 of the volume in each well). The absorbance was measured with an ELISA reader at 540 nm (Microplate Reader, BioTek, Winooski, VT, USA).

4.2.5 ROS quantification assay

The 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) probe was used to perform ROS quantification assay in THLE-2 pre-treated with LNVs (10 μg/mL and 25 μg/mL) or with iLNVs (2.5μg/mL) and exposed to menadione (5 μM and 10 μM). H2DCFDA can freely penetrate inside the cell where it is first deacetylated by cellular esterase and then oxidized by ROS to the fluorescent 2', 7'-dichlorofluorescein (DCF). The probe, excited at a wavelength of 485 nm, emits a signal at 535 nm (blue reading) detected by fluorescence spectroscopy. THLE-2 were seeded in triplicate, 1 x 10^4 per well, in a 96-well plate (Nunclon Delta Surface 96-well plate by Thermo Fisher Scientific, Waltham, MA, USA), and after 24h cells were pre-treated with LNVs or iLNVs. At the end of 24 hours, the medium containing the vesicles was removed and, in a medium FBS-free, the probe (10 μM) and the menadione (5 or 10 μM) were added at the same time. Using the Glomax Multi Detection Plate Reader (Promega, Madison, WI, USA), a reading of the fluorescence emitted was carried out to quantify the level of intracellular ROS.

4.2.6 RNA isolation, cDNA synthesis and Real-time PCR

8 x 10^4 cells were seeded in a 12-well plate. Cells were pre-treated for 24h with vesicles (10 μg/mL and 25 μg/mL) or with iLNVs (2.5μg/mL) and then treated for 30 minutes with menadione (5 μM), added without medium removal. HFD-fed rats were administered with iLNVs as reported in the "2.9 *In Vivo* Model" section. Total RNA was purified from cells using Nucleospin miRNA Kit (Macherey-Nagel, Düren, Germany) and from animal biopsies with NucleoSpin RNA Set for NucleoZOL (Macherey-Nagel, Düren, Germany). The total RNA was quantified with Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA) and Retrotranscription from RNA to cDNA was executed using the

High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with SYBR Green mix (Applied Biosystems, Foster City, CA, USA). Modulations in target genes were determined, relative to actin, using the ΔΔCt method.

Human	**Forward Sequence (5´to 3´)**	**Reverse Sequence (5´to 3´)**
ACTIN	TCCCTTGCCATCCTAAAAAGCCACCC	CTGGGCCATTCTTCCTTAGAGAGAAG
NRF2	CACATCCAGTCAGAAACCAGTGG	GGAATGTCTGCGCCAAAAGCTG
HO-1	CCAGGCAGAGAATGCTGAGTTC	AAGACTGGGCTCTCCTTGTTGC
Rat	**Forward Sequence (5´to 3´)**	**Reverse Sequence (5´to 3´)**
ACTIN	AAGGCCAACCGTGAAAAGAT	TGGTACGACCAGAGGCATAC
NRF2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
HO-1	ACAGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG

Table 2: Oligonucleotides used in qRT-PCR

4.2.7 Western blot

THLE-2 were seeded 3 x 10^5 per flask (T25), pre-treated for 24h with 10 μg/mL and 25 μg/mL of LNVs or with 2.5μg/mL of iLNVs, and treated with 5 μM menadione for 30 minutes. Once the treatment time was over, the cells were collected and resuspended in PBS. HFD-fed rats were administered with iLNVs as reported in the "2.9 *In Vivo* Model" section. The liver tissue was collected after the animals had been sacrificed and cut into smaller pieces. THLE-2 cells and hepatic biopsies were resuspended in a lysis buffer (consisting of 50mM Tris-HCl pH 7.6, 300mM NaCl, 0.5% TritonX-100, 1X PMSF, 1X leupeptin, 1X aprotinin, phosphatase inhibitors 1X (Phosphatase inhibitor cocktail 10X) and H_2O milliQ). Quantitation was performed using Coomassie Brilliant Blue and a biophotometer. The samples for the WB were then prepared: adding the H_2O, the sample buffer (4X) and the reducing agent (10X) to the proteins and denaturing them for 10 minutes at 70°. Samples were loaded onto an SDS-page BoltTM 4- 12% Bis-Tris Plus gel (Invitrogen, Waltham, MA, USA) and, in the presence of a running buffer consisting of MES and H_2O. After the electrophoretic run at 165V, the transfer of the proteins from the gel to a nitrocellulose membrane (Amersham, Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) inserted into transfer buffer (20% methanol, H_2O, 10X trisglycine) was completed at 50 V. The membrane was incubated at 4° for 1.30h with 1% BSA to block the non-specific antibody binding sites. The primary antibody (prepared in BSA 1%) was incubated overnight

at 4°C. The following day the secondary antibody was added (1:1000 in BSA 1%) at 4° for 1h. The primary antibodies used were anti-Nrf2 (Novus Biologicals, Centennial, CO, USA), anti-HO-1 (Bioworld Technology, Antibodies, St. Louis Park, MN, USA), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Amersham ECL Western Blotting Detection Reagent solution (Detection Reagent 1 and 2 in a 1:1 ratio) (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) allowed the detection of the chemiluminescence signal and the highlighting of the protein band using the "ChemiDocTMMP" (Bio-rad, Hercules, CA, USA). The densitometric analysis of the bands was performed with the "Image Lab" software.

4.2.8 Immunofluorescence assay

To evaluate the nuclear Nrf2 expression after the pre-treatment with LNVs/iLNVs and the following treatment with menadione, immunofluorescence protocol was performed, and the signal was detected by confocal microscopy. 25 x 10^3 cells per well were plated on an 8-well chamber (Thermo Scientific Nunc, Waltham, MA, USA) and these were pre-treated for 24 hours with LNVs (10 μg/mL and 25 μg/mL) or with iLNVs (2.5μg/mL). The day after, cells were treated for 30 minutes with menadione (5 μM). Once the treatment time with menadione was over, THLE-2 cells were fixed with PFA 4% for 10 minutes. After the permeabilization of membranes with Triton 0.1%, the incubation for 1h with the anti-Nrf2 primary antibody (Novus Biologicals, Centennial, CO, USA) diluted 1:200 in 1% BSA and then the incubation for 1h with the secondary antibody (Goat anti-Rabbit IgG H+L Secondary Antibody, DyLight™ 594, Invitrogen Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) prepared 1:500 in 1% BSA was performed. Nuclei and cytoskeleton were respectively labeled for 30 minutes with Hoechst (Invitrogen Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) prepared 1:1000 in PBS and Actin Green (Invitrogen Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence was observed using the confocal microscopy Nikon A1 (Amsterdam, Netherlands). The amount of nuclear and total Nrf2 was quantified using the NIS-Elements Software. The nuclear Nrf2 was calculated by measuring the media of fluorescence of the Region of interest (ROI) in the nuclei for each field analyzed (n=7-9). The total Nrf2 was calculated by measuring the media of total fluorescence associated with the ROI divided by the number of nuclei of every single field (n=7-9).

4.2.9 In Vivo Model

4.2.9.1 Animals and Diet Composition

Male Wistar rats (9 weeks old, n = 10) were obtained from Envigo S.r.l (Indianapolis, Indiana) and housed in pairs with a 12-hour light/dark cycle (8:00–20:00 h) with stable conditions of temperature (22–24 ◦C) and humidity (50 ± 10%). Once in the animal facility, rats were maintained in a 7-day acclimation period, initially receiving a standard chow diet providing 3.94 kcal/g. After this period, they were weighed to exclude eventual initial differences and included into two uniform groups that were both fed with a high-fat diet (HFD) with 60% of energy derived from fats (code PF4215-PELLET, Mucedola, Milan, Italy). This is a hypercaloric pelletized diet (5.5 Kcal/g) with 34% fat, 23% protein, 38% carbohydrates, and 5% fiber, that was provided for 10 weeks to induce MetS based on established criteria from previous literature [168, 169]. Throughout the experiment, all rats had unrestricted access to food and water. Animal care and procedures adhered to the ARRIVE guidelines and the European Directive (2010/63/EU). The University of Palermo's animal welfare committee approved the experimental protocols, and the Ministry of Health (Rome, Italy) granted authorization (Authorization Number n° 386/2024-PR).

4.2.9.2 Experimental Design and Nutritional supplementation

The experimental *in vivo* study is segmented into three distinct phases: T0 (initiation), T1 (Metabolic Syndrome induction), and T2 (experiment conclusion):

- Initial Group Allocation (T0): following the acclimatization phase (7 days), animals were fed with HFD for 10 weeks until the induction of Metabolic Syndrome.
- Metabolic Syndrome Induction and Group Stratification (T1): Verification of Metabolic Syndrome induction was performed at T1 after 10 weeks of HFD, employing established protocols [168, 169]. Animals were subsequently stratified into two groups relatively to the nutritional treatment received (iLNVs or vehicle) from T1 until T2, reached four weeks after T1. The HFD-iLNVs group (n=6) received daily iLNVs supplementation until T2, while the second group (HFD, n=4) representing the Metabolic Syndrome control was fed with the HFD and received daily gavage of vehicle (water) until T2 to make sure that they were under identical stress conditions as the HFD-iLNVs group.
- Experiment Conclusion (T2): This timepoint represents the end of the experimental study, occurring 14 weeks after the beginning (T0). Animals were sacrificed at T2 following authorized procedures to perform subsequent *ex-vivo* analyses.

Dietary supplementation with iLNVs involved oral gavage administration with a daily volume of 1 mL, corresponding to 1.2 mg/Kg for 4 weeks (T1-T2).

Figure 14 illustrates the experimental plan of the *in vivo* study.

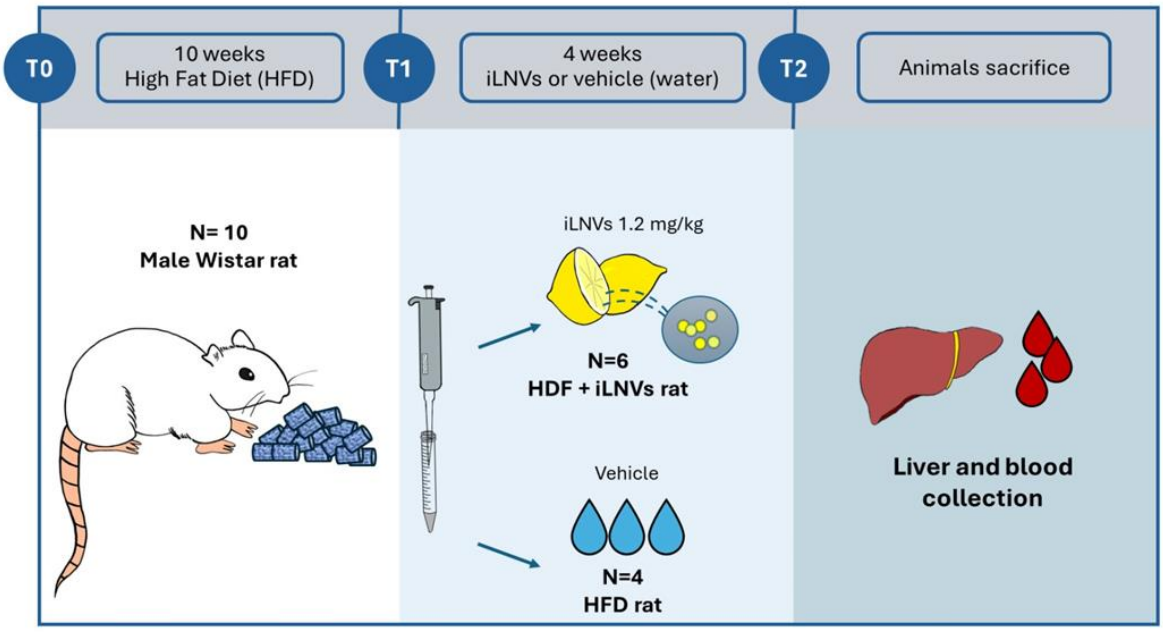

Figure 14: Representation illustrating the metabolic syndrome development in rats and the proposed treatment design.

4.2.9.3 Evaluation of Biometric, Biochemical, and Oxidative Homeostasis Parameters

The evaluation of *in vivo* parameters was performed at T2 to explore the *in vivo* effects of iLNVs on MetS-induced alterations via biometric, biochemical, and oxidative homeostasis assays, after euthanasia in adherence to authorized protocols. Plasma samples were gathered for subsequent analyses, encompassing assessments of glucose and lipid homeostasis, oxidative stress parameters, and plasma antioxidant status. Additionally, hepatic samples were collected for *ex-vivo* evaluations.

4.2.9.3.1 Body Weight Gain Evaluation

The evaluation of body weight of all the animals was performed throughout the experiment to evidence eventual differences between groups. In particular, we evaluated the final weight reached by all animals and we calculated the body weight gain, i.e. the Delta Body Weight (ΔBW), considering the final rat weight with respect to the initial weight recorded at T0.

4.2.9.3.2 Evaluation of Glucose and Lipid Homeostasis

Glucose Tolerance Test (GTT) was conducted at T2 following previously established methods [168] to assess the impact of nutritional treatments on glucose homeostasis in Metabolic Syndrome. In summary, after fasting overnight, a blood sample was collected from the tail vein and placed on a glucose test strip (Glucotest, Pic) to obtain a baseline

measurement using a Glucometer (Glucotest, Pic). Subsequently, a 20% glucose solution was injected intraperitoneally (2 g/kg body weight), and blood glucose levels were recorded at 30, 60 and 120 min post-injection. The area under the curve (AUC) was then calculated for the experimental groups based on blood glucose levels (mg/dL) over time. For lipid homeostasis analysis, blood samples were collected from each animal at T2 after sacrifice through cardiac puncture. Detailed methods, as outlined in our previous study [168] were used to assess the effect of iLNVs supplementation. Plasma concentrations of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL), and high-density lipoprotein cholesterol (HDL) were measured using commercial kits with the Free Carpe Diem device (FREE® Carpe Diem; Diacron International, Grosseto, Italy). Results are presented as mg/dL.

4.2.9.3.3 Oxidative Stress Parameters and Plasma Antioxidant Status

The evaluation of plasma redox balance at T2 was conducted using Diacron kits, as described in our earlier publications [168]. Prooxidant status was determined through the dROMs (Reactive Oxygen Metabolites) and LP-CHOLOX tests. Levels of hydroperoxides, lipoperoxides, and oxidized cholesterol were measured by commercial kits utilizing the Free Carpe Diem device (FREE® Carpe Diem; Diacron International, Grosseto, Italy). Data from dROMs tests are presented in Carratelli units (UCARR), with the normal range being 250–300 U. CARR, where 1 U. CARR is equivalent to 0.08 mg/dL of H_2O_2 [170]. The LP-CHOLOX test results, which indicates lipoperoxides and oxidized cholesterol levels, are expressed in mEq/L.

Regarding plasma antioxidant status, the SHp test assessed thiol groups' reducing properties, gauging the potential of iLNVs extracts to counteract thiol group oxidation and favor reduced forms. Furthermore, we investigated the levels of anti-ROMs, which is a colorimetric method based on the ability of plasma antioxidants to reduce ferric iron to ferrous iron which reacting with a specific chemical compound gives rise to reddish-purple solution [171]. The Anti-ROMs Test develops the reaction in two stages: in the first, it evaluates rapid exogenous antioxidant called “fast” such as Vitamin C and vitamin E (“exogenous anti-ROMs”) and after it reveals “slow” antioxidants such as uric acid, thiol group, bilirubin, and polyphenols (“endogenous anti-ROMs”). The analysis was performed on previously heparinized plasma samples using a photometer (FREE® Carpe Diem) and measurements were conducted by commercial kits (Diacron International, Grosseto, Italy). For the interpretation of the results, reference values were used according to data reported in the literature [171], which predict optimal values for exogenous antioxidant defenses greater than 200 µEq/L and for endogenous antioxidant defenses values greater than 1000

µEq/L. Otherwise, values of exogenous antioxidants below 100 µEq/L and endogenous antioxidants below 500 µEq/L are considered high deficiency.

4.2.10 Immunohistochemistry

The immunohistochemical reactions were carried out on 5 µm thick hepatic tissue sections, obtained from paraffin blocks, as previously described in detail [167]. The primary antibody used was anti-Nfr2 (rabbit polyclonal antibody, NOVUS BIO-Techne Abingdon, United Kingdom, NBP1-32822 dilution 1:200). Slides were observed with an optical microscope (Microscope Axioscope 5/7 KMAT, Carl Zeiss, Milan, Italy) coupled with a digital camera (Microscopy Camera Axiocam 208 color, Carl Zeiss, Milan, Italy). The immunopositivity was performed at high-power-field (HPF, magnification 400x), repeated for 10 HPFs, and the results were expressed as a percentage.

4.2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Version 10.1.0 (316), GraphPad Software, Inc., La Jolla, CA, USA). The normal distribution of the data was evaluated by the Shapiro–Wilk test. For datasets with a normal distribution, the statistical significance of differences between groups was assessed using an unpaired two-tailed Student's t-test. In cases where data do not pass the normality test, the comparisons were conducted using the non-parametric Mann-Whitney test. Differences were considered significant when $p < 0.05$. The results are presented as the mean ± standard deviation (SD).

4.3 Results part II

4.3.1 Internalization of LNVs and iLNVs in THLE-2 cells

To evaluate the ability of LNVs and iLNVS to interact with mammalian cells, nanovesicles were labeled with PKH26 (red signal) and incubated with THLE-2 cells. After 2h and 6h of treatment with 10µg/mL or 25µg/mL LNVs, and with 2.5 µg/mL iLNVs, immunofluorescence protocol was executed, and THLE-2 cells were observed at confocal microscopy. Figure 15 shows the time-dependent internalization of LNVs and iLNVs.

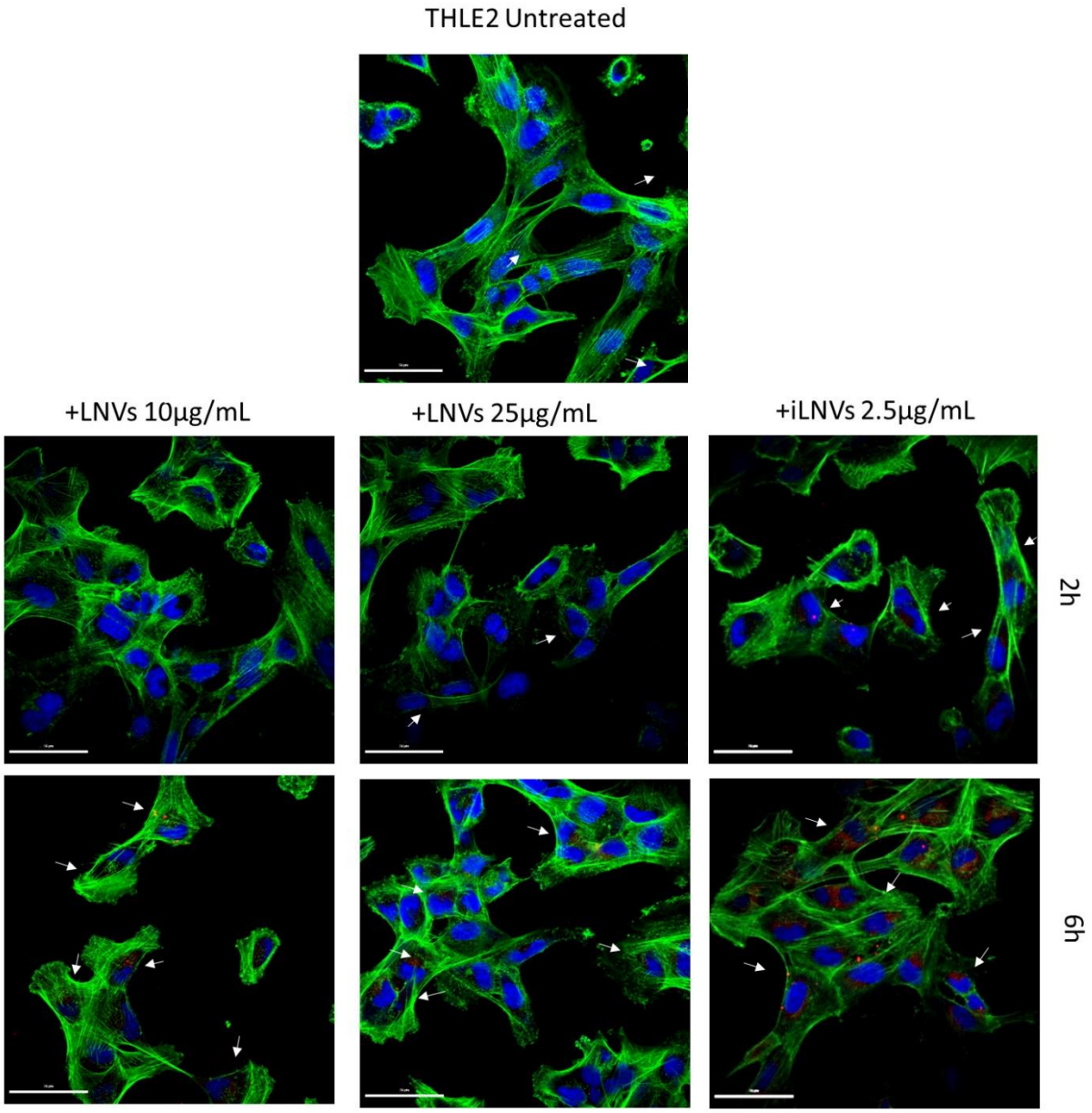

Figure 15: LNVs and iLNVs are internalized by THLE-2. Confocal microscopy images show the internalization of LNVs (10 µg/mL and 25 µg/mL) and iLNVs (2.5 µg/mL) by THLE-2 after 2h and 6h of treatment. The image of THLE2 untreated was captured after 6h. LNVs and iLNVs were labeled red with

PKH26, nuclei were stained blue with Hoechst, and cytoskeletons were stained green with Actin green. The association of PKH-26 with the vesicles was confirmed through confocal microscopy images, specifically from the red signal observed in treated cell conditions and absent in untreated cells.

4.3.2 Antioxidant effects of Lemon-derived nanovesicles on THLE-2 exposed to menadione

4.3.2.1 The treatment with LNVs and iLNVs does not alter cell viability and reduces ROS levels

To assess whether LNVs and iLNVs affect hepatocyte cell viability, the MTT assay was performed.

The doses have been selected according to our previous studies using vesicles obtained through two distinct approaches [49, 167]. As shown in Figure 16A, 24h of treatment with LNVs (10 μg/mL and 25 μg/mL) and iLNVs (2.5 μg/mL) does not induce a reduction in cell viability.

As mentioned above, different factors can induce ROS production in hepatocytes [113], potentially triggering liver disease. To explore the possible antioxidant properties of LNVs and iLNVs in human healthy hepatocytes, ROS production was quantified after exposure to menadione, a well-known pro-oxidant molecule. As shown in Figure 16B, we found a significant reduction of ROS levels in THLE-2 pre-treated for 24 hours with LNVs (10 and 25 μg/mL) or iLNVs (2.5 μg/mL) and subsequently subject for 30 and 60 minutes with menadione 5 μM (Figure 16B, upper panel) and 10 μM (Figure 16B, lower panel) compared to the cells treated with menadione alone. This effect is more evident in hepatocytes treated with the 5 μM dose of menadione compared to those treated with the 10 μM dose.

This data validates that the pre-treatment of both LNVs and iLNVs can exert an antioxidant effect in THLE-2 cells stimulated with menadione.

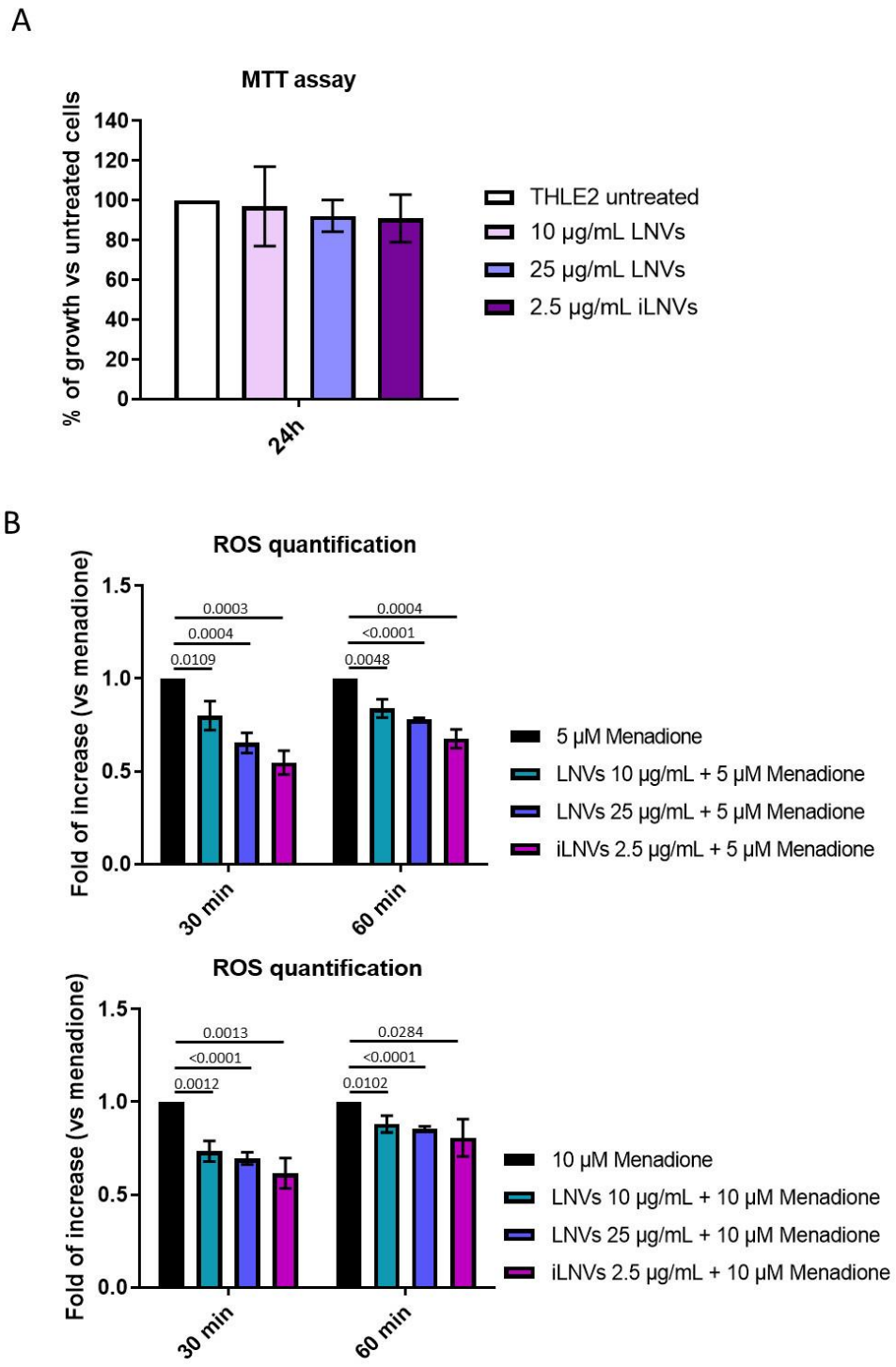

Figure 16: The treatment with LNVs and iLNVs does not alter cell viability and reduces intracellular ROS production. A) THLE-2 cell viability was evaluated by MTT assay after 24 h of treatment with different concentrations of LNVs (10 µg/mL and 25 µg/mL) and iLNVs (25 µg/mL). Histograms represent the percentage of cell viability versus untreated cells (cn). Values are reported as the mean ± SD (n=3-4). B) THLE-2 cells were pre-treated with LNVs (10 µg/mL and 25 µg/mL) or iLNVs (2.5 µg/mL) for 24h, followed by exposure to menadione 5 µM (upper panel) and 10 µM (lower panel) for 30 minutes and 60 minutes and to DCFDA 10µM. The intensity of the fluorescence was proportional to the amount of ROS present in the sample and was measured using a microplate reader. Values are reported as the mean ± SD (n=3). The statistical

significance of the differences between the two groups (cells treated with menadione Vs cells pre-treated with LNVs/iLNVs + menadione) was analyzed using a two-tailed Student's t-test.

4.3.2.2 The pre-treatment with LNVs and iLNVs regulates Nrf2/HO-1 pathway in vitro

After confirming LNVs and iLNVs' ability to decrease ROS levels in THLE-2 cells, we investigated the signaling involved in the antioxidant response mediated by the nanovesicles. Specifically, we studied the Nrf2/HO-1 signaling pathway, since previous studies demonstrated its pivotal role in the antioxidant response [49] and its involvement in the LNV-mediated antioxidant mechanism [50]. The protein expression of Nrf2 (Figure 17A) was evaluated in cells pre-treated for 24 hours with LNVs (10 μg/mL and 25 μg/mL) or iLNVs (2.5 μg/mL) and subsequently subjected to a 30-minute treatment with menadione (5 μM). When THLE-2 cells were pre-treated with LNVs or iLNVs, Nrf2 protein expression was upregulated compared to treatment with menadione alone; this upregulation is statistically significant in cells pre-treated with 10 and 25 μg/mL of LNVs.

Furthermore, as Nrf2 is a crucial antioxidant transcription factor capable of translocating in the nucleus, we demonstrated its activation by examining both the total and nuclear fractions. In-depth, through confocal analysis, we observed a significant increase in the total and nuclear levels of Nrf2 (Figure 17B) in LNVs/iLNVs pre-treated THLE-2, confirming the ability of nanovesicles to stimulate Nrf2 expression. This finding is pivotal since Nrf2 can determine the transcription of crucial antioxidant molecules such as HO-1. Consequently, we investigated the protein expression of HO-1 (Figure 17C) and we observed a non-significant trend of increase in HO-1 protein expression after 24-hour pre-treatment with LNVs or iLNVs. These data support the correlation between LNVs/iLNVs antioxidant effects and these two mediators of the antioxidant response.

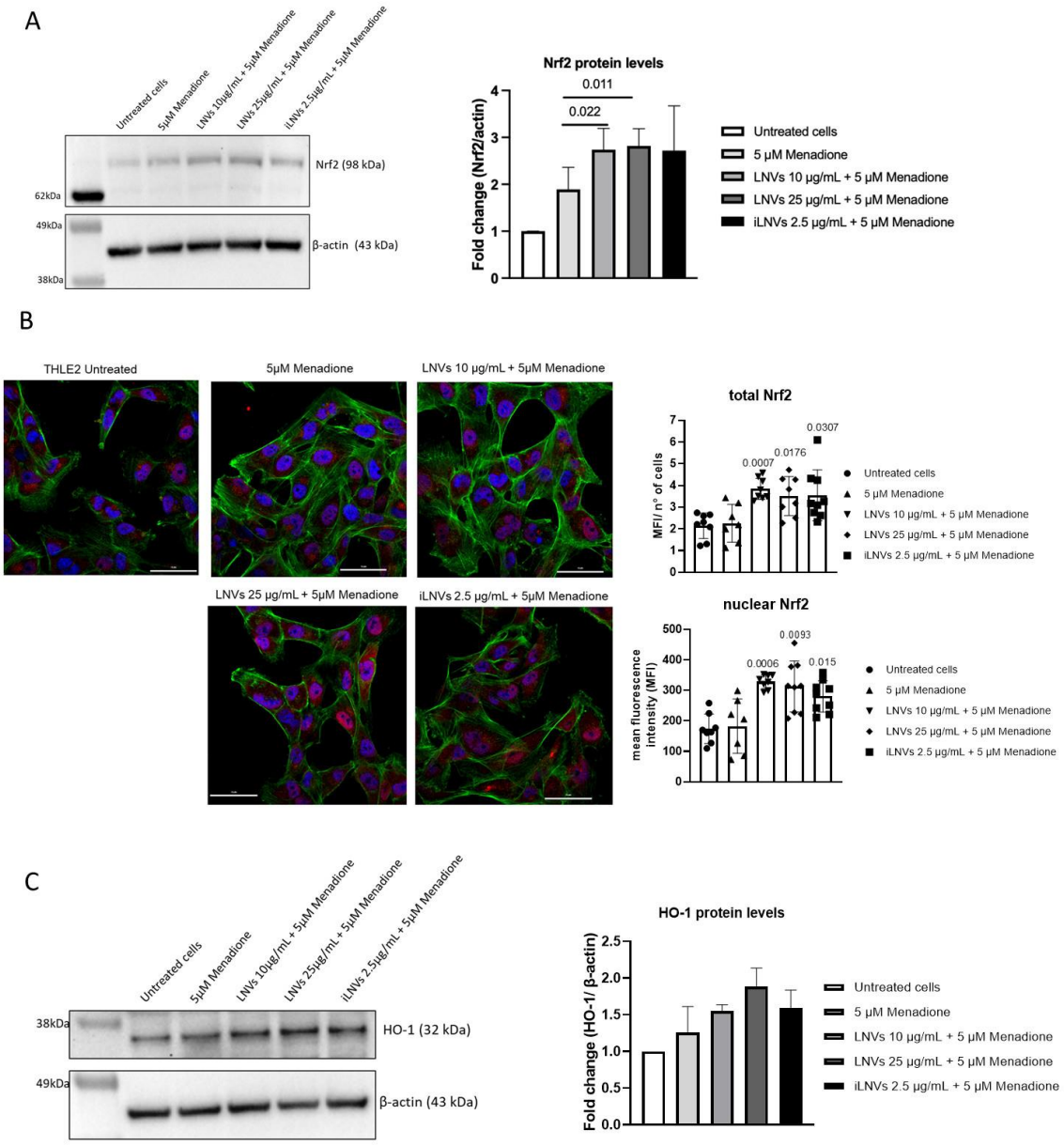

Figure 17: The pre-treatment with LNVs and iLNVs regulates the Nrf2/HO-1 pathway *in vitro*. A) Western Blot analysis of Nrf2 and β-actin in THLE-2 pre-treated with LNVs/iLNVs for 24h and with menadione (5 µM) for 30 minutes. B) Confocal microscopy of THLE-2 cells pre-treated with LNVs/iLNVs for 24h and with menadione (5 µM) for 30 minutes. THLE-2 cells were stained for Nrf2 and labeled with Hoechst to visualize the nucleus (blue) and with Actin Green for the cytoskeleton (green). The histograms show the quantification of the total (upper panel) and nuclear (lower panel) Nrf2; the data are the mean±SD (n=7-9). The statistical significance of the differences was analyzed using a two-tailed Student's t-test. C) Western Blot analysis of HO-1 and β-actin in THLE-2 pre-treated with LNVs/iLNVs for 24h and with menadione (5 µM) for 30 minutes. For both Nrf2 (A) and HO-1 (C), β-actin was used as the loading control. The values reported in the densitometric analysis are the mean (±SD) of the analyzed protein normalized vs. loading control (n=4).

4.3.3 Effects of iLNV supplementation on Body Weight

In the *in vivo* model, the animals were initially weighed under basal conditions with comparable average weights at T0. In order to induce MetS, rats were fed with HFD for 10 weeks (from T0 to T1) and then (from T1 to T2) they were supplemented daily with iLNVs or vehicles. At the end of the experiment (T2), final body weight was statistically compared using a two-tailed Student's t-test, that outlined significant differences between HFD and HFD-iLNVs (t = 2.359; df=8; p=0.046; Figure 18A). Also, body weight gains, calculated with respect to initial weight (T0), showed significant decreases in HFD-iLNVs versus HFD (t = 4.119; df=8; p=0.003; Figure 18B).

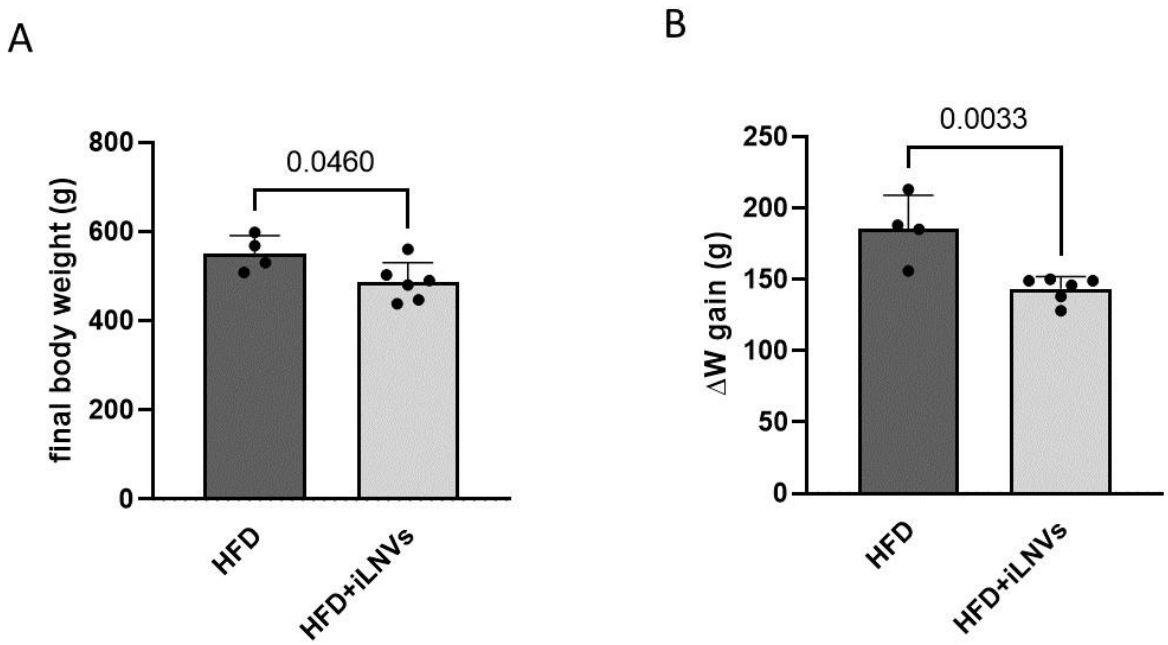

Figure 18: iLNVs reduce body weight in HFD-fed rats. A-B) After the HFD-fed rats were administered with 1.2 mg/Kg of iLNVs for 4 weeks, the final body weight and the weight gain T2-T0 were measured. The statistical differences between the two groups (iLNVs-HFD vs the HFD group) were analyzed using a two-tailed Student's t-test. Data are shown as the mean ± SD (n = 4-6).

4.3.4 Effects of iLNV supplementation on Glucose Tolerance and Lipid homeostasis

The GTT performed at T2 outlined significant differences between HFD and HFD-iLNVs in glucose homeostasis, by evaluating the AUC. The student's t-test highlighted a decrease in HFD-iLNVs vs. HFD group (t = 2.96, df = 8, p = 0.018, Figure 19A).

As for lipid homeostasis, plasma samples were collected from groups to observe eventual modifications in lipid profile in MetS following treatment with iLNVs at T2. Statistical analyses evidenced significant reductions of TG levels in HFD rats supplemented with iLNVs (U=2; p=0.038; Figure 19B). The parameters related to cholesterol levels showed a non-significant variation of total cholesterol levels, a marked decrease in LDL cholesterol (t

= 4.874, df = 8, p = 0.0012, Figure 19C), and a related increase in HDL cholesterol (t = 2.744, df = 8, p = 0.0253, Figure 19C) in HFD-iLNVs rats compared to HFD group.

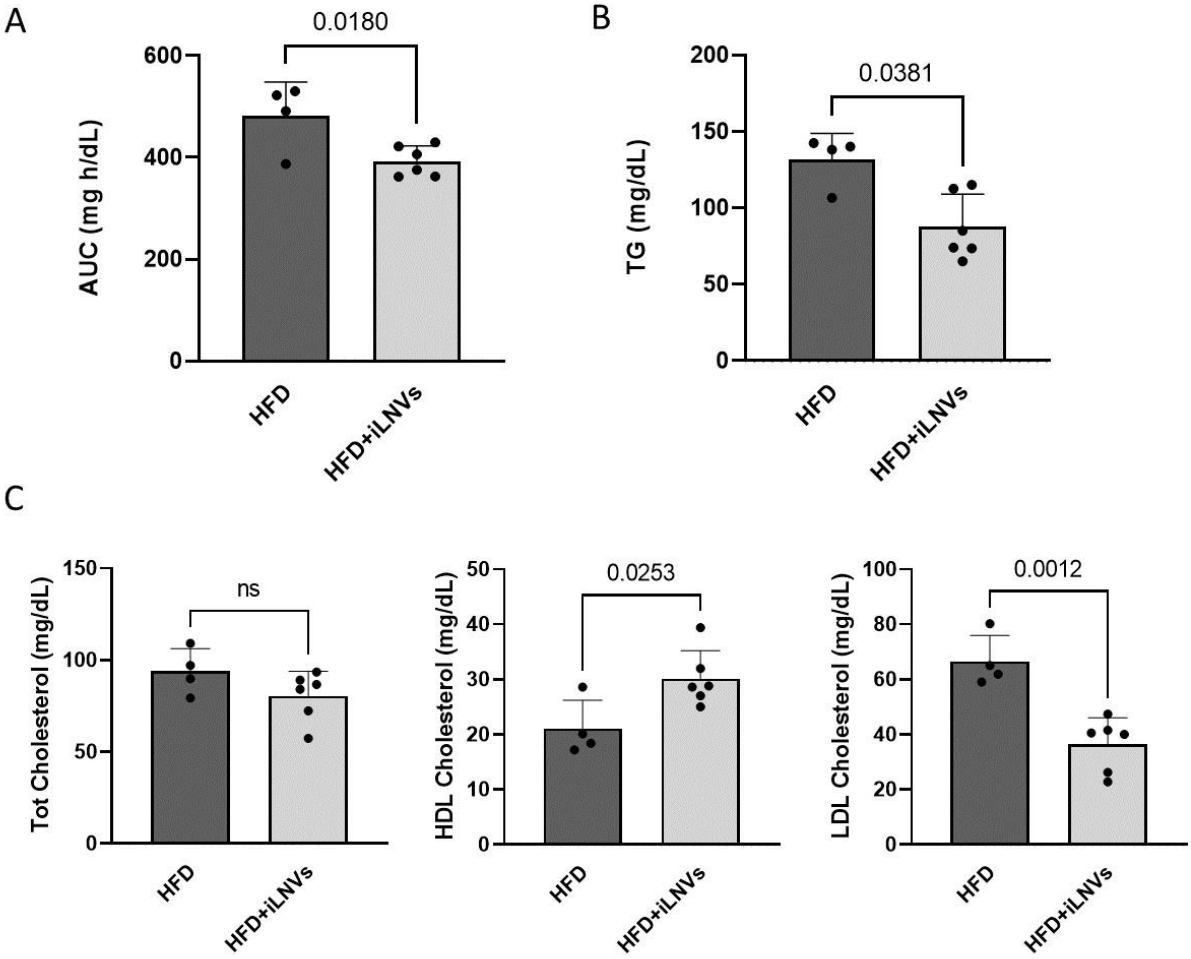

Figure 19: iLNVs improve glucidic and lipid homeostasis altered in HFD-fed rats. HFD-fed rats were orally administered for 4 weeks with 1.2 mg/Kg of iLNVs and we measured A) AUC, B) TG, C) Total Cholesterol, LDL and HDL were measured with commercial kits according to the suggestions from the suppliers. The statistical significance of the differences between the two groups (iLNVs-HFD vs the HFD group) was analyzed using a two-tailed Student's t-test. Data are shown as the mean ± SD (n = 4-6).

4.3.5 Impact of iLNV supplementation on Plasma Redox Homeostasis Biomarkers

At T2, we assessed antioxidant and pro-oxidant status in both groups of rats. Notably, statistical significance emerged from the analysis of plasma samples related to the systemic pro-oxidant status. Indeed, iLNVs supplementation in HFD rats reduced both dROMs and LP-Cholox levels compared to the HFD group (respectively: t = 3.73, df = 8, p = 0.0058 and t = 9.57, df = 8, p<0.0001, as illustrated in Figure 20A).

Furthermore, it was revealed a significant amelioration in the antioxidant capacity of animals in the HFD-iLNVs group compared to the HFD group. Specifically, an unpaired t-test was

conducted on the values of the SHp and endogenous Anti-ROMs test, indicating a significant increase in HFD-iLNVs compared to HFD (respectively: t = 3.22, df = 8, p = 0.012 and t = 2.864, df = 8, p = 0.021, as shown in Figure 20B). Non-significant differences were observed in the analysis of exogenous Anti-ROMs (Figure 20B).

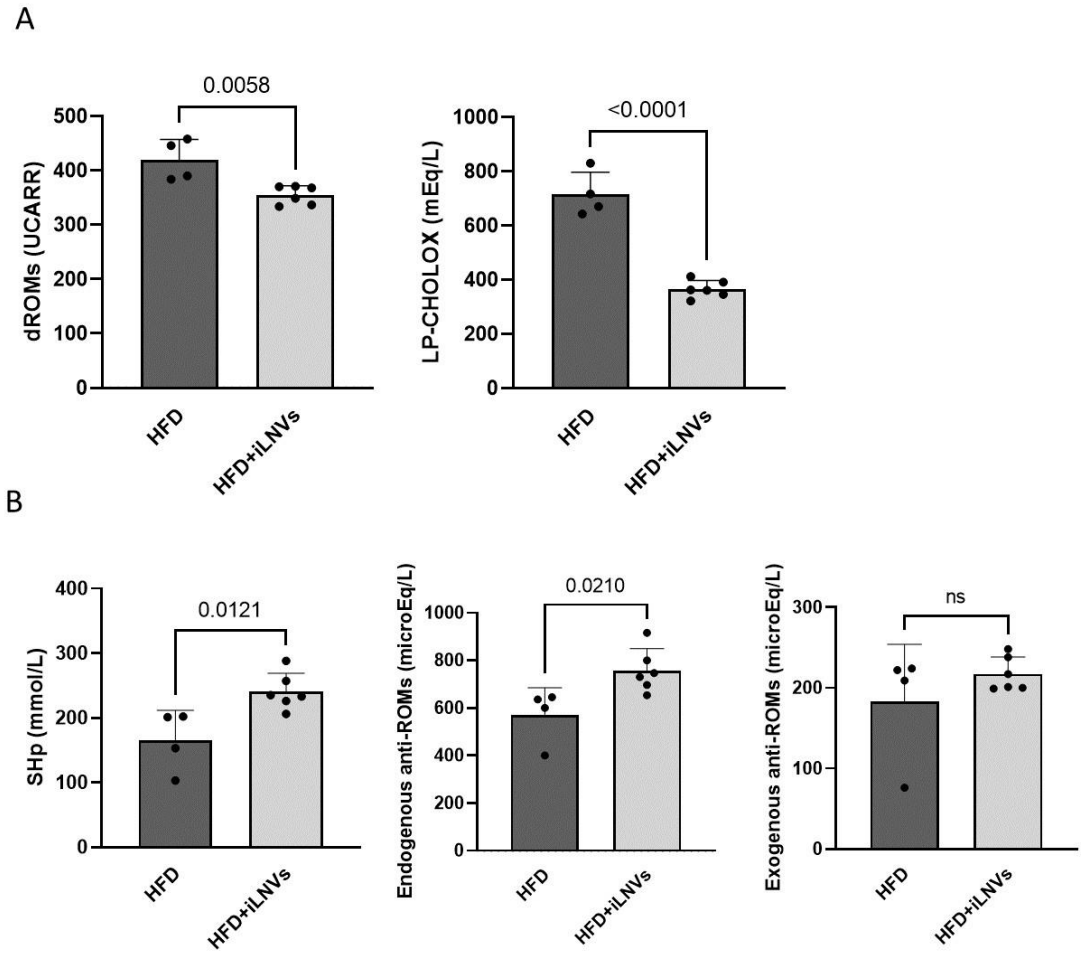

Figure 20: iLNVs reduce systemic oxidative stress and increase systemic antioxidants. After the HFD-fed rats were orally administered for 4 weeks with iLNVs (1.2 mg/Kg), A) systemic oxidative stress parameters d-ROM and LP-CHOLOX and B) systemic antioxidants SHp, endogenous anti-ROMs (SH, uric acid, polyphenols) and exogenous anti-ROMs (vitamin C, vitamin E). The statistical significance of the differences between the two groups (iLNVs-HFD vs the HFD group) was analyzed using a two-tailed Student's t-test. Data are shown as the mean ± SD (n = 4-6).

4.3.6 The administration of iLNVs regulates Nrf2/HO-1 signaling in HFD-fed rats liver

Also in the *in vivo* experiments, in accordance with the *in vitro* part of the study, Nrf2 and HO-1 modulation were investigated in the HFD rat's liver. In detail, we observed a significant increase of Nrf2 and HO-1 transcript levels in the HFD-iLNVs rat group

compared to the untreated HFD-fed rats (Figure 21A). Furthermore, the statistically significant upregulation of HO-1 was validated at the protein level, whereas for Nrf2, we observed a non-significant increasing trend in the HFD-iLNVs group (Figure 21B). Nevertheless, immunohistochemical analysis of the hepatic tissue strongly indicated that Nrf2 is predominantly cytoplasmic in untreated HFD-fed rats, but it is mostly nuclear in the iLNV-treated group (Figure 21C). As a result, the data reported herein confirm the activation of Nrf2/HO-1 antioxidant signaling in the livers of rats administrated with iLNVs.

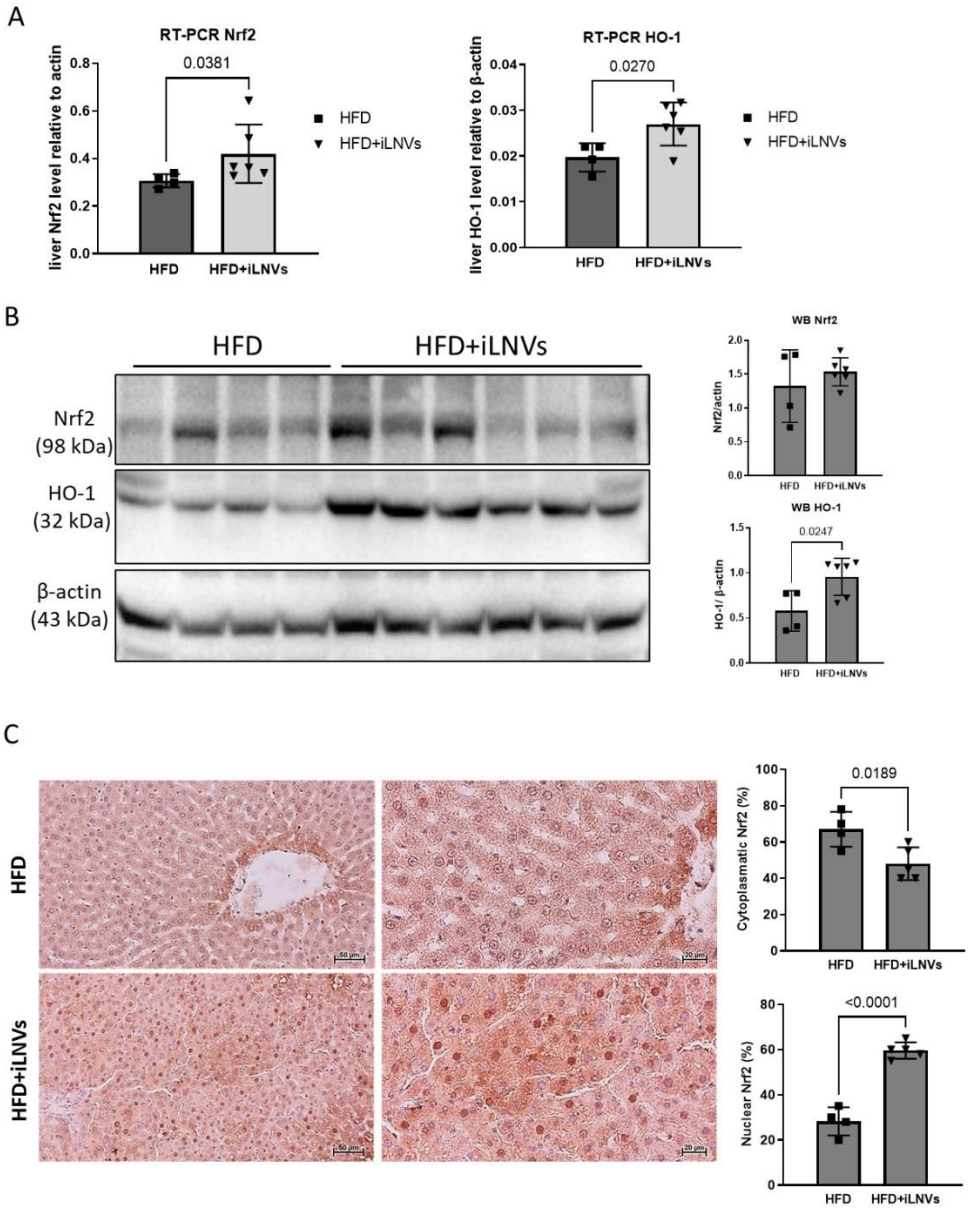

Figure 21: iLNVs regulate Nrf2/HO-1 signaling in HFD-fed rats liver**.** A) The iLNVs ex vivo-mediated modulation on Nrf2 and HO-1 transcription levels were assessed by qRT-PCR analyses. HFD rats were administrated for 4 weeks with iLNVs. The values were expressed as Nrf2 and HO-1 levels normalized to the housekeeping gene, actin (2^ ΔCt). Results are expressed as means±SD (n=4-6). The statistical significance of the differences between the two groups (HDF vs iLNVs-HFD) was analyzed using a two-tailed Student's t-

test. B) Western Blot analysis of Nrf2, HO-1, and β-actin in HFD and iLNVs-HFD rat's hepatic tissues. In sequence, the initial four samples correspond to Nrf2, HO-1, and β-actin in liver tissues of rats receiving only HFD. The remaining six samples reflect these proteins in liver tissues of rats fed with HFD and administered with iLNVs. The values reported in the densitometric analysis are the mean (±SD) of the analyzed protein normalized vs. loading control (n = 4-6). C) Immunohistochemical results for Nrf2 in the hepatic sections of the two groups. Magnification 200x, scale bar 50 μm for the images on the left, magnification 400x, scale bar 20 μm for the images on the right. The percentage of immunopositivity was calculated on the evaluations made at high-power-field (HPF, magnification 400x). The histogram reports the results of the immunohistochemistry. The data are means±SD of ten evaluations for each group. The statistical significance of the differences between the two groups (iLNVs-HFD vs the HFD group) was analyzed using a two-tailed Student's t-test.

CHAPTER 5: PART III

Impact of Lemon Nanovesicles on Macrophage-Mediated Immune Response to Bacterial Pathogens

5.1 Objective part III

The last objective of this study focuses on the preliminary investigation of the antimicrobial capabilities of LNVs isolated through Size Exclusion Chromatography (SEC) and specifically their potential to stimulate and activate the human immune system. Consequently, our research wants to study if LNVs can enhance the innate immune response by promoting the activity of key immune cells such as macrophages, which play crucial roles in detecting and eliminating pathogens. By interacting with these immune cells, LNVs can boost their phagocytic activity, leading to more efficient bacterial clearance. Our goal is specifically to establish a protocol that would allow us to investigate how THP1 M0 differentiated cells and pre-treated with LNVs may influence bacterial colony formation and exert their effect against bacteria growth. This goal can potentially hold significant promise for the development of LNV-based treatments that not only target pathogens but also enhance the body's natural defenses, offering a dual approach to managing infectious diseases. In detail, the methods and results presented in the following sections were produced during my time abroad in Valencia, Spain, in collaboration with Professor Marcilla's research group.

5.2 Materials and Methods part III

5.2.1 Size Exclusion Chromatography (SEC)

The LNVs were isolated from the lemon juice, which was processed through a series of differential centrifugation steps as follows: two centrifugations at 3000g for 15 minutes each, followed by two centrifugations at 10000g for 30 minutes each. The supernatant was then filtered using a 0.8 μm pore filter and centrifuged again at 16500g for 1 hour. Before the isolation, columns containing 10 mL of stacked Sepharose-CL2B (Sigma-Aldrich) were equilibrated by washing them with 30 mL of PBS previously filtered using 0.22 μm filters. Samples were passed through manual Tangential Flow Filtration (TFF) to concentrate them 50 times more and then through the Sepharose column. Filtered PBS was used as the elution buffer and 1 mL of concentrated LNVs was loaded onto the column. Consequently, a total of 20 fractions of 500 μL each were collected. LNVs were eluted in fractions 6–10 and then concentrated using Amicon Ultra-4 filter devices (Merck Millipore) at 3,200g for 20 min at 4°C. MicroBCA assay was carried out to measure the protein concentration.

5.2.2 Co-plating of THP-1 M0 and bacteria

The human monocyte THP-1 cell line was obtained from ATCC (Manassas, VA, USA). Cells were cultures in RPMI-1640 medium (ATCC, Manassas, VA, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Euroclone, UK). THP-1 monocytes were differentiated into M0 macrophages (THP-1 M0) according to our previous study [22]. Specifically, cells were plated at 3×10^4 cells/mL and incubated at 37°C with 5% CO2 for 72 hours in the presence of 50 ng/mL of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Saint Luis, MO, USA); subsequently, the medium was discarded and replaced with fresh medium. The cells were then treated with different doses of LNVs as described below.

Escherichia coli and *Enterococcus faecalis* were freshly cultured overnight and resuspended in LB media at an OD600 of 0.10 corresponding volume. They were then opsonized and diluted 1:100 for 90 minutes at 37˚C in RPMI media with 10% FBS and without antibiotics. Opsonization is specifically enabled by the presence of FBS and its associated factors within the medium. The THP-1 M0 cells were first pre-treated for 24h with LNVs (10 µg/mL) and, after media remotion, subjected for 3h at 37˚C to bacteria resuspended in RPMI at a ratio of 10 bacteria for every cell (1:10). Control group cells were pre-treated with PBS only. To lyse the cells, autoclaved H_2O was added, and diluted aliquots (1:50) for *Enterococcus faecalis* and not diluted aliquots for *Escherichia coli,* were spread on LB agar. The plates were then incubated overnight at 37˚C, and the colony-forming units (CFU) were counted. A bacterial suspension without any cells was used as an input control. The number of colonies was counted using the ImageJ software.

5.3 Results part III

5.3.1 LNVs improve the effect of THP1 M0 against bacteria

To study if LNVs isolated by SEC have an antibacterial effect, their activity was assessed through an indirect evaluation of immune system modulation. Specifically, their potential to enhance and increase the macrophage-mediated immune response against both gram-positive and gram-negative bacteria was investigated. In particular, SEC-isolated LNVs have already been characterized by our group of research, consequently, this section of the study will focus just on their functional effects. In particular, THP-1 M0 cells were pre-treated for 24h with 10 µg/mL of LNVs and then treated for 3h with *Enterococcus faecalis* or *E. coli*,

respectively a gram-positive and a gram-negative bacteria. After the co-plating of cells and bacteria in LB agar and the incubation overnight, the number of colonies was counted and analyzed. As shown in Figure 22, THP-1 M0 pre-treated for 24h with LNVs significantly reduced the *Enterococcus faecalis*'s colony forming unit (CFU) compared to the cells pre-treated with PBS. This significant reduction of CFU was also confirmed in THP-1 M0 pre-treated with LNVs and exposed for 3h with *E. coli*. Consequently, these results represent an interesting starting point to understand how the LNVs can modulate the activity of the macrophages and improve their response against the bacteria. The 24-hour pre-treatment with LPS (10 µg/mL) was used as a positive control for the activation of THP-1 M0 cells.

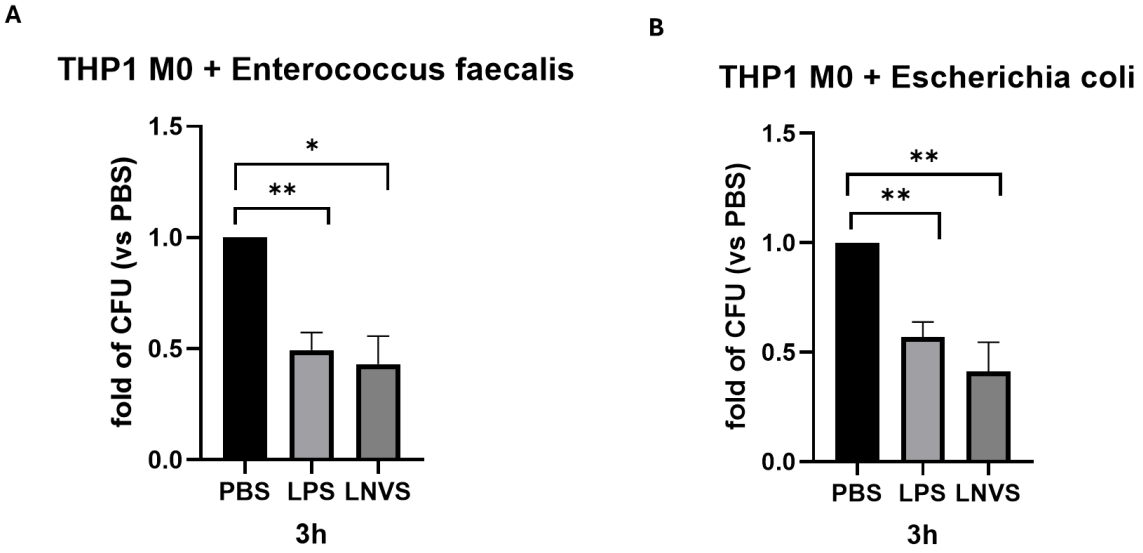

Figure 22: LNVs improve the effect of THP-1 M0 against bacteria. A-B) THP-1 M0 were pre-treated for 24h with LNVs and with *Enterococcus faecalis* or *E. Coli* for 3h. LPS 10 µg/mL was used as a positive control. After the collection of cells and bacteria, both were seeded together in LB AGAR plates. The number of colonies were counted using ImageJ software and the values were expressed as fold of CFU (vs PBS). Results are expressed as means ± SD of four biological replicates.

CHAPTER 6: DISCUSSION AND CONCLUSION

6.1 Discussion

The interest of the nutraceutical industry is focused on discovering natural compounds that possess both biological and organoleptic properties, thus introducing food that can be included in a health-promoting diet. Medicinal plants and their derivatives contain a wide variety of bioactive compounds that possess beneficial properties such as antioxidant and anti-inflammatory activities [172, 173]. Citrus fruits are worldwide recognized to be essential elements in the everyday diet. Within Citrus limon, there are several essential natural compounds, including flavonoids, minerals, essential oils, and carotenoids, all known for their positive impact on health, due to their natural anti-inflammatory and antioxidant characteristics [3]. To date, nutraceuticals are expected to play a central role in preventive healthcare, representing an exciting new opportunity to converge food and pharma. One of the most studied citrus derivates is essential oil. In detail, several essential oils obtained from different plants have hepatoprotective effects and can contrast the pro-inflammatory, pro-oxidant, and pro-fibrotic activities of hepatotoxic molecules [174, 175]. Recently, Pucci *et al*. reported the biological properties of Cfr-LEO on LPS-activated macrophages, thus highlighting its application not only in healthcare for its beneficial properties but also in the nutraceutical industry, as a natural food additive for its organoleptic properties, conferred by citral enrichment.

Here, we confirmed the biological properties of Cfr-LEO on a model of healthy human hepatocytes. We demonstrated, first, that Cfr-LEO counteracts the expression of pro-inflammatory cytokines such as IL-6 and TNFα in LPS-stimulated hepatocytes by preventing the LPS-induced NF-κB phosphorylation and nuclear translocation. In particular, we hypothesized that these effects could be due to Cfr-LEO's ability to inhibit the TLR4/NF-κB pathway. Indeed, to date, several molecules from plants and herbs of traditional Chinese medicine, such as berberine, atractylenolide I, and zhankuic acid A, have been described as TLR4 antagonist molecules. Moreover, curcumin from Curcuma longa, sulforaphane and iberin from cruciferous vegetables, xanthohumol from hops and beer, and celastrol from Tripterygium wilfordii have already also been identified as TLR4 antagonists [176]. Our results suggest that Cfr-LEO, similarly to other natural compounds, could act as a TLR4 antagonist, thus hindering LPS binding to the receptor and therefore counteracting the downstream targets of LPS/TLR4 signaling, among which are NF-κB and its target genes involved in the inflammatory response. These data lay the basis for further studies aimed at demonstrating the role of Cfr-LEO in preventing TLR4-associated inflammatory response. Moreover, we demonstrated Cfr-LEO's protective effect on hepatocytes' oxidative stress.

Oxidative stress is at the basis of the establishment of lipid peroxidation and the accumulation of lipid droplets, the main causes of the development of liver diseases such as metabolic syndromes and NAFLD, fibrosis, and HCC [177]. We found that pretreatment with Cfr-LEO counteracts LPS-induced oxidative stress by reducing the ROS level. Moreover, we found that NRF2 and p53, factors involved in oxidative stress defense, showed comparable expression levels in both control and Cfr-LEO-pretreated cells. Taken together, these results support the hypothesis that Cfr-LEO could act as a TLR4 antagonist by an upstream blocking of the TLR4 signaling cascade, thus making cells unresponsive to LPS-induced oxidative stress. Further studies will be necessary to confirm our preliminary hypothesis.

Lastly, we demonstrated that Cfr-LEO counteracts the expression of EMT markers in LPS-stimulated hepatocytes [178], thus highlighting the ability of Cfr-LEO to prevent the establishment of a fibrotic environment in the liver. Liver fibrosis and cirrhosis are the ultimate consequences of chronic hepatic injury induced by various etiological agents, and they are associated with significant morbidity and mortality in the world [179, 180]. In detail, liver fibrosis is characterized by abnormally enhanced tissue deposition of extracellular matrix (ECM) components and oxidative stress, inflammation, and EMT play a key role in fibrogenesis induction [163, 181, 182]. Although liver fibrosis has been reported to be reversible at early stages, it becomes irreversible with advanced disease, leading to the malignant transformation of cells toward a “cancer phenotype” [165, 183]. To date, the mechanism of liver fibrosis has been extensively studied, and in recent years, many strategies have emerged as crucial to inhibit the occurrence and development of liver fibrosis, including inhibition of hepatic stellate cells (HSCs) activation and proliferation, reduction in ECM overproduction, and acceleration of ECM degradation. However, to date, effective antifibrotic therapies are lacking [184]. On the other hand, in the current study, we found that the mesenchymal markers vimentin and N-cadherin show comparable expression levels in both controls and Cfr-LEO-pretreated cells, thus further supporting the ability of Cfr-LEO to protect cells from hepatotoxic LPS stimuli.

However, one of the major problems with the dietary intake of natural compounds is their low stability and bioavailability caused by their reduced absorption and the digestive activity of the organism. From this premise arises the scientific community's interest in plant-derived nanovesicles, recognized for their distinctive characteristics, such as low immunological risk and, notably, enhanced bioavailability. Their lipidic biolayer can exert an essential role in

the preservation of the metabolites, RNA, and proteins packed into them, increasing the bioavailability of all these compounds [185, 186, 187].

Here, we aim to investigate the hepatoprotective effect of Lemon nanovesicles in THLE-2 cells and HFD-fed rat model. Findings correlated to plant-derived nanovesicles have indeed emerged as a crucial starting point for investigating and understanding cross-kingdom communication and numerous studies have been conducted to analyze the interaction between nanovesicles derived from plants and mammalian targets.

Our study focuses not only on the evaluation of the hepatoprotective effects of nanovesicles produced in the laboratory but also on those produced on an industrial scale. Industrial production may represent a useful and excellent way to have easier access to lemon nanovesicles making them better applicable in the therapeutic and nutraceutical fields for a large-scale use. We have previously conducted a qualitative and quantitative metabolomic characterization of lemon nanovesicles produced at laboratory scale [71] and at industrial scale [167], showing overlapping profiles in terms of flavonoid and organic acid content.

Nowadays, antioxidants derived from plants are directly employed to tackle diseases linked to oxidative stress [188]. In their research, Savcı *et al*. evaluated how EVs isolated from grapefruit promote wound healing [52] and reduce the level of intracellular ROS increased via H_2O_2-induced oxidative stress in HaCaT cells, a human epidermal keratinocyte cell line. In accordance with that, we recently found the capability of LNVs in reducing ROS levels and in activating the antioxidant pathway of Nrf2 in a fibroblast model stimulated with H_2O_2 and UVB, and in zebrafish embryos stimulated with LPS [49]. Here, we further expanded our data and underlined the ability of LNVs and iLNVs to reduce ROS levels in human healthy hepatocytes stimulated with menadione, a quinone and vitamin K analog. Menadione can transfer electrons from ETC complex I to oxygen, thereby producing superoxide [189], and has been widely used to induce oxidative stress and cell damage [190, 191, 192, 193].

Undoubtedly, overproduction of ROS is a primary mechanism contributing to mitochondrial dysfunction, leading to lipid peroxidation and that consequently can induce the development of several diseases, such as hepatic disorders [177, 194]. As mentioned above, to maintain the level of ROS at a physiological level, the liver is equipped with various antioxidant systems with enzymatic activity. Among these processes, some rely on the activation of ARE, in turn, regulated by Nrf2 [110] whose gene expression can be modulated by administering nanoparticles derived from plants [50].

For example, studies have shown that nanovesicles derived from *Aloe vera* contribute to wound healing by exerting antioxidant effects, by the activation of Nrf2 signaling [195]. In line with this, Ginger EVs, increased the nuclear translocation of Nrf2, which is implicated in the modulation of antioxidant-related genes, such as HO-1 [59]. In this study, we demonstrate that LNVs and iLNVs can induce the upregulation of the Nrf2/HO-1 signaling and a higher expression of nuclear Nrf2, which is consequently associated with its active transcriptional status. Furthermore, the different effect provided by the iLNVs and by the two doses of LNVs is potentially due to the number of particles/mL. Specifically, the 2.5µg/mL dose of iLNVs corresponds to $3x10^8$ number of particles/mL, the 10 µg/mL dose of LNVs to $8x10^8$ number of particles/mL, and the 25 µg/mL dose of LNVs to $2x10^9$ number of particles/mL.

Considering these powerful beneficial properties, plant-derived nanovesicles can be used to prevent or fight against multiple diseases. Recent studies indicate that exosome-like nanovesicles derived from yams may serve as effective oral treatments for osteoporosis [196], whereas nanovesicles from orange juice might be useful for managing obesity-related intestinal issues [197]. In line with these studies, plant-derived nanovesicles can also exert hepatoprotective effects *in vivo*. Ginger-derived nanoparticles possess a protective impact against alcohol-induced liver damage in mice [59] while the administration of mice with blueberry-exosome-like nanovesicles improves liver function, halts vacuole formation, and reduces the accumulation of lipid droplets in the livers of animals exposed to a high-fat diet [50].

In addition to the evaluation of the effects of the nanovesicles on *in vitro* models, in our study, we reported the hepatoprotective effect of iLNVs in HFD-fed rats with MetS. NAFLD, the hepatic manifestation of MetS, is indeed one of the main causes of loss of hepatic function in the Western world and is often correlated with type 2 diabetes and obesity [198, 199]. In obesity conditions, the gathering of fat in the abdominal area influences both lipid and glucose metabolism, resulting in a fat-laden and insulin-resistant liver [200]. Our results reveal that the oral administration of iLNVs for 4 weeks to HFD rats is linked to an improvement in biometric parameters such as the final body weight and the body weight gained by rats throughout the study. These favorable outcomes are in line with the amelioration of glucose and lipid homeostasis encountered in HFD rats supplemented with iLNVs. Indeed, we demonstrated that iLNVs ameliorated glucose tolerance by reducing AUC. This is in line with the idea that administering natural compounds to HFD-fed animals can influence the deleterious effect of MetS on glucose metabolism by decreasing blood

glucose levels, inducing a hypoglycemic effect, and improving the histopathological characteristics of hepatic tissue [168, 201]. Moreover, plant derivates can improve the homeostasis of lipid parameters in the HFD-fed state. Accordingly, here we found that iLNVs can decrease TG and LDL levels and can increase the amount of HDL cholesterol. The effects of iLNVs on lipid homeostasis, support their functional modulatory role, which is indicative of improved hepatic lipogenesis.

Crucially, our assessment of plasma redox balance unveiled that systemic oxidants are markedly reduced, alongside an enhancement of antioxidant defenses such as SHp and endogenous anti-ROMs.

In particular, the improved markers of oxidative state suggest that after iLNVs the hepatic production of primary lipoperoxide and hydroperoxide radicals, respectively assessed by LP-Cholox and dROMs, is reduced. Hydroperoxides, along with lipoperoxidation, have been identified as significant factors contributing to severe alterations that initiate oxidative damage in the liver of both humans and animals [202].

In accordance with this, we found improved levels of SHp and of the endogenous antiROMs also including the antioxidant defenses exerted by uric acid, bilirubin, and polyphenols. However, the non-significant effect of iLNVs on exogenous antiROMs, in terms of vitamins, agrees with the low levels of liposoluble vitamins present in the iLNVs [167]. In this context, SHp level is an established marker of endogenous antioxidant status since it denotes the homeostasis between thiols and disulfides, essential for cellular signaling pathways including, cell signaling cascades, apoptosis, detoxification processes, enzymatic efficacy, and transcriptional regulation [203]. Excessive production of reactive oxygen species (ROS) can lead to the oxidation of thiol groups, forming disulfide bridges and shifting the balance toward a more oxidized state. However, this process is reversible. Treatments that enhance endogenous antioxidant defenses can indeed shift the balance back in favor of thiols, as observed following iLNVs supplementation in our research and in previous studies [168, 204]. Intriguingly, Nfr2 has been indicated as an upstream activator of the thiol-dependent redox signaling [205], hence supporting the putative influence of iLNVs on Nrf2/HO-1 antioxidant signaling which we found up-regulated in HFD rat liver. Nrf2 indeed regulates the expression of various thiol-based antioxidant enzymes, such as thioredoxin 1 and its corresponding reductase, sulfiredoxin, and peroxiredoxins 1 and 6 [206, 207, 208]. Consequently, the treatment with iLNVs could determine the activation of endogenous antioxidant mechanisms and have an effect in reducing Nrf2-mediated oxidation of thiol groups. In connection with this, we presumed that the antioxidant and hepatoprotective effect

of lemon nanovesicles is not mediated by the action of a single compound, but by the combination of multiple contents within the nanovesicles and protected by the lipidic bilayer.

Based on this assumption, vesicles and their contents can perform multiple functions not only in a complex system such as the liver but also in the regulation of processes fundamental to the defense of human health. The liver, as previously described, is constantly connected to the gut which, in case of alteration of the barrier's permeability, can determine the translocation of the microbiota bacteria through the portal vein in the liver. On the other hand, the scientific literature indeed reports the ability of various natural compounds to activate immune cells and potentially recognize the pathogen and damage-associated molecular patterns (PAMPs, DAMPs) of bacteria translocated from the gut. A recent study evaluated the modulation mediated by citral in S. aureus infection through the expression of surface molecules, cytokine dosage, and expression of innate and adaptive immune response genes in monocytes [209]. Furthermore, it was demonstrated that botanical polysaccharides enhance macrophage immune responses, increasing reactive oxygen species (ROS) production, and enhancing secretion of cytokines and chemokines. In particular, studies have shown that natural polysaccharide activates macrophages via the TLR4 signaling pathway. Furthermore, Yeo Dae Yoon and colleagues found that a polysaccharide extracted from *Platycodon grandiflorus* increases the DNA binding activity of AP-1 and activates three MAPK subgroups (ERK1/2, SAPK/JNK, and p38 MAPK) in macrophage RAW264.7 cells. Inhibition of these kinases prevented PG's activation effect, indicating that MAPK and AP-1 are crucial in this process. Additionally, Wang's research demonstrated that a homogalacturonan from *Hippophae rhamnoides* L. Berries enhances macrophage activity in immunosuppressed mice, likely through TLR4. The activation involves increased MyD88 and p-IκB-α expression, which is blocked by TLR4 inhibition, confirming TLR4's role in this natural compound-mediated macrophage activation [210]. Here, for the first time, we demonstrated that LNVs significantly enhance macrophage activity, leading to a more effective reduction in bacterial growth. Therefore, lemon-derived nanovesicles can be considered efficient vehicles, able to release their content inside mammalian cells. This is the main step to understanding the recent intriguing aspect of the cross-kingdom interaction and its revolutionary and functional role in regulating mammalian pathological processes, such as hepatic disease.

Despite the promising results obtained in demonstrating the hepatoprotective effects of lemon nanovesicles, further investigations will be necessary to determine the pathways

responsible for nanovesicle effects. Further studies will be also aimed at evaluating whether the effects of nanovesicles could be gender-related in the HFD rat model. In addition to this the small sample sizes presented in this project may limit the generalizability of the findings and can raise questions about the reproducibility of these results in larger populations. Consequently, expanding the sample sizes in future studies to strengthen the validity and robustness of the conclusions drawn will be a key pillar for further evaluations. Moreover, implementing larger sample groups would not only increase statistical power but also enhance the study's ability to reflect potential variations across a broader population.

Furthermore, investigating long-term efficacy and safety is crucial for a comprehensive understanding of Cfr-LEO and LNVs. While this thesis provides foundational data on immediate responses, longitudinal studies would indeed allow us to evaluate sustained effects and the overall safety profile of these interventions over time. Therefore, future research will focus on long-term studies to address these critical aspects, as they are fundamental to translating these findings into practical, long-lasting applications. This direction would significantly enhance the clinical relevance and applicability of the research.

6.2 Conclusion

In conclusion, this work lays the foundation for the development of specific foods/drinks, made from scientifically tested citrus essential oils and LNVs, aimed at preventing or alleviating chronic conditions associated with liver dysfunction.

First, identifying the specific compounds in Cfr-LEO responsible for the observed health effects could pave the way for more targeted formulations of foods and beverages with beneficial properties. Our future studies will be focused on evaluating the beneficial effects of selected Cfr-LEO components to understand which of them could act as a “TLR4 antagonist,” thus determining the observed protective effects of the fraction. Therefore, having evaluated its nontoxicity and beneficial properties in a healthy human hepatocyte cellular model, we confirm that Cfr-LEO can certainly be applied not only in the agri-food industry for its organoleptic properties, but can also represent a preventive tool for improving human health, exerting a protective effect against hepatotoxic stimuli.

Secondly, lemon-derived nanovesicles can be also applied to human health, particularly for their role in the prevention and treatment of diseases. Here for the first time, we demonstrated the antioxidant and hepatoprotective properties of nanovesicles isolated from lemon both *in*

vitro, using human healthy hepatocytes, and *in vivo*, in HFD-fed rats. Although further research is required to gain a deeper understanding of the mechanisms involved in the LNVs' actions, this study supports their application for the management of metabolic syndrome disorders and hepatic oxidative stress. Furthermore, LNVs isolated using size-exclusion chromatography (SEC) have shown potential in modulating immune responses, particularly in activating macrophages against bacterial pathogens. These nanovesicles can interact with macrophages, potentially enhancing their ability to recognize and respond to bacterial infections. Even though further research is essential to better understand the mechanisms at play with LNVs in the activation of macrophages, this study can pave the way to endorse their use in the management of gram-positive and gram-negative infection, assessing the capability of these Citrus nanovesicles in improving the effect of THP-1 M0 against bacteria colonies formation. Our next studies will also focus on the molecular mechanisms by which LNVs exert these effects, including their interaction with immune cell receptors and the subsequent signaling pathways that lead to immune activation.

Furthermore, our next step will be considering the transition from preclinical research to clinical trials which will be essential to making lemon derivates applicable in clinical settings for nutraceutical and therapeutic use. Consequently, this process will transform our laboratory discoveries into practical health applications. While *in vitro* and *in vivo* models provide foundational insights into safety and efficacy, clinical trials are needed to evaluate these findings in human contexts. For lemon derivates, this transition is particularly significant as it involves validating their preventive and therapeutic potential, optimizing dosing strategies, and identifying any potential side effects when administered to humans. In particular, the necessity of designing clinical trials with long-term follow-up to assess sustained efficacy and safety, and how insights from preclinical studies will be the next step. Although our studies have evaluated the preventive in vitro and reversal in vivo effects of lemon derivates, our goal is to translate these findings into an application for human health, following a preventive and protective approach. Cfr-LEO and LNVs, which show promising hepatoprotective effects, will undergo clinical testing to establish their effectiveness in managing liver conditions. This transition will also allow us to assess variability in human responses and monitor any adverse reactions that may not have emerged in animal studies. In addition to this, recruiting a diverse patient population will improve the generalizability of results and expedite the path to market approval.

CHAPTER 7:

SCIENTIFIC PRODUCTS

7.1 Scientific publications in journals relevant to the topic

1. **Gasparro R**, Gambino G, Duca G, Majo DD, Di Liberto V, Tinnirello V, Urone G, Ricciardi N, Frinchi M, Rabienezhad Ganji N, Vergilio G, Zummo FP, Rappa F, Fontana S, Conigliaro A, Sardo P, Ferraro G, Alessandro R, Raimondo S. Protective effects of lemon nanovesicles: evidence of the Nrf2/HO-1 pathway contribution from in vitro hepatocytes and in vivo high-fat diet-fed rats. Biomed Pharmacother. 2024 Oct 8;180:117532. doi: 10.1016/j.biopha.2024.117532. Epub ahead of print. PMID: 39383731.
2. Tinnirello V, Zizzo MG, Conigliaro A, Tabone M, Ganji NR, Cicio A, Bressa C, Larrosa M, Rappa F, Vergilio G, **Gasparro R**, Gallo A, Serio RM, Alessandro R, Raimondo S. Industrial-produced lemon nanovesicles ameliorate experimental colitis-associated damages in rats via the activation of anti-inflammatory and antioxidant responses and microbiota modification. Biomed Pharmacother. 2024 May;174:116514. doi: 10.1016/j.biopha.2024.116514. Epub 2024 Apr 3. PMID: 38574618.
3. **Gasparro R**, Pucci M, Costanzo E, Urzì O, Tinnirello V, Moschetti M, Conigliaro A, Raimondo S, Corleone V, Fontana S, Alessandro R. Citral-Enriched Fraction of Lemon Essential Oil Mitigates LPS-Induced Hepatocyte Injuries. Biology (Basel). 2023 Dec 17;12(12):1535. doi: 10.3390/biology12121535. PMID: 38132361; PMCID: PMC10740427.
4. Urzì O, Cafora M, Ganji NR, Tinnirello V, **Gasparro R**, Raccosta S, Manno M, Corsale AM, Conigliaro A, Pistocchi A, Raimondo S, Alessandro R. Lemon-derived nanovesicles achieve antioxidant and anti-inflammatory effects activating the AhR/Nrf2 signaling pathway. iScience. 2023 Jun 7;26(7):107041. doi: 10.1016/j.isci.2023.107041. PMID: 37426343; PMCID: PMC10329147.
5. Urzì O*, **Gasparro R***, Ganji NR, Alessandro R, Raimondo S. Plant-RNA in Extracellular Vesicles: The Secret of Cross-Kingdom Communication. Membranes (Basel). 2022 Mar 23;12(4):352. doi: 10.3390/membranes12040352. PMID: 35448322; PMCID: PMC9028404.

7.2 List of publications or products not related to the project

1. Urzì O, **Gasparro R**, Costanzo E, De Luca A, Giavaresi G, Fontana S, Alessandro R. Three-Dimensional Cell Cultures: The Bridge between In Vitro and In Vivo

Models. Int J Mol Sci. 2023 Jul 27;24(15):12046. doi: 10.3390/ijms241512046. PMID: 37569426; PMCID: PMC10419178.

7.3 Abstracts, posters and oral presentation presented at scientific congresses

1. 2nd MOVE SYMPOSIUM – BELGRADE, SERBIA, abstract submission and **poster** presentation: **Gasparro R**, Duca G, Gambino G, Tinnirello V, Di Maio D, Ganji NR, Conigliaro A, Fontana S, Ferraro G, Sardo P, Alessandro R, Raimondo S, Hepatoprotective effects of nanovesicles derived from lemon: an in vitro and in vivo investigation (8-11 October 2024)
2. 3rd EVIta SYMPOSIUM - URBINO, ITALY, abstract submission and **poster** presentation: **Gasparro R**, Duca G, Tinnirello V, Ganji NR, Fontana S, Alessandro R, Raimondo S. Antioxidant effect of nanovesicles derived from lemon juice on hepatocytes. (September 13-15 2023)
3. 95° Congresso SIBS - TRIESTE, ITALY, abstract submission and **oral** presentation: **Gasparro R**, Pucci M, Moschetti M, Urzì O, Costanzo E, Tinnirello V, Conigliaro A, Raimondo S, Corleone V, Alessandro R, Fontana S, Citral-enriched fraction of lemon essential oil mitigates the lps-induced effects in human hepatocytes (April 12-15, 2023)

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