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Corso di Dottorato in Salute dell'Uomo

ANTI-INFLAMMATORY AND ANTI-ADIPOGENIC ACTIVITY OF OLIVE LEAF EXTRACT AND ITS BIOACTIVE COMPOUNDS

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CHAPTER 1: INTRODUCTION

The Interest in the plant world for bio-medical applications has increased exponentially in recent decades,(Shipkowski et al., 2018) however the benefits of many plant compounds have been known since ancient times. The current social and economic pressures to integrate natural and conventional medicines have been driven largely by the enormous prevalence of chronic, non-communicable degenerative diseases and the complex management of patients affected by them. Therefore, an increase in scientific research into the antiviral, antibacterial, antitumour, antioxidant, cardioprotective and neuroprotective the effects of many natural substances have been examined in *in vitro* and *ex vivo* models. In several cases those activities have also been confirmed in *in vivo* studies.

In several cases those activities have also been confirmed in *in vivo* studies (Gurău et al., 2018; Soldati et al., 2018; Voltes et al., 2020).

A very innovative aspect of the research concerns the anti-ageing properties of certain natural compounds; the great interest is due to the increase in life expectancy of the population in developed countries and the consequent increase in the incidence of age-related diseases (ARDs). Cellular senescence is now considered to be one of the causal mechanisms of the ageing process which in turn represents the risk factor for degenerative diseases; therefore the "senolytic" properties, i.e., the ability to selectively eliminate senescent cells, are currently a topic of great scientific interest. Moreover, cellular senescence is characterized by a senescence associated secretory phenotype (SASP) which contributes to chronic, sterile, low-grade inflammation, named *inflammaging.* The possibility of using certain natural compounds as adjuvants in the treatment of age-related diseases due to their anti-inflammatory properties may be a valuable strategy to contribute to healthy ageing. An aspect not to be neglected when talking about natural substances is the reduced environmental impact and the low cost for their extraction that often takes place from waste products and therefore make them ideal candidates of the circulatory economy Furthermore, natural compounds can often be obtained by waste-products of food industry and therefore, readily available at low cost.

1.1 NATURAL COMPOUNDS

1.1.1 Classification and main sources

As it is well known, the energy provided by diet is derives from the so-called macronutrients, i.e. carbohydrates, fats and proteins. In addition to these, there are the essential micronutrients, such as vitamins and minerals, which are provided by both animal and plant foods. The plant world, however, also provides a wealth of lesser-known chemical compounds that have attract increasing attention in recent years. These compounds are commonly referred to as phytochemicals and include thousands of small molecules that are classified according to their chemical structure and/or botanical origin (Biesalski et al., 2009). In living organisms, chemical compounds are synthesised and degraded through a series of reactions, each of which is carried out by an enzyme.

These processes are generally known as metabolism and include catabolism (degradation) and anabolism (synthesis).

All organisms have similar metabolic pathways, through which they synthesise and utilise essential chemical components: sugars, amino acids, fatty acids, nucleotides, and polymers derived from them (polysaccharides, proteins, lipids, RNA and DNA) (Wang et al., 2019).

This is the primary metabolism and the compounds listed above, which are essential for the survival of an organism, - are primary metabolites.

Many organisms also use other metabolic pathways, through which they produce compounds that are generally of no apparent use, the so-called secondary metabolites.

The biosynthetic pathways and the utilisation of these molecules therefore constitute the secondary metabolism.

Secondary metabolic pathways are as much a result of the genetic setting of the organism as the primary ones, but are probably only activated during particular conditions of growth and development, or during periods of 'stress' caused by nutritional deficiencies or microbial attack. In reality, the dividing line between primary and secondary metabolism is quite blurred: the primary

metabolism gives rise to the formation of a number of small molecules, which are then used as 'starting material' in many important secondary metabolic pathways.

The biosynthetic building blocks for secondary metabolites derive therefore from primary metabolites and from fundamental processes such as photosynthesis, glycolysis and the Krebbs cycle (Li et al. 2020).

Plants synthesise about 100000 secondary metabolites. Some substances are specific to that species or genus, while others are ubiquitous, making it difficult to establish a single classification criterion (Zaynab et al., 2018). The following groups of compounds are considered the most significant or emerging at the present time **(Figure 1)**.

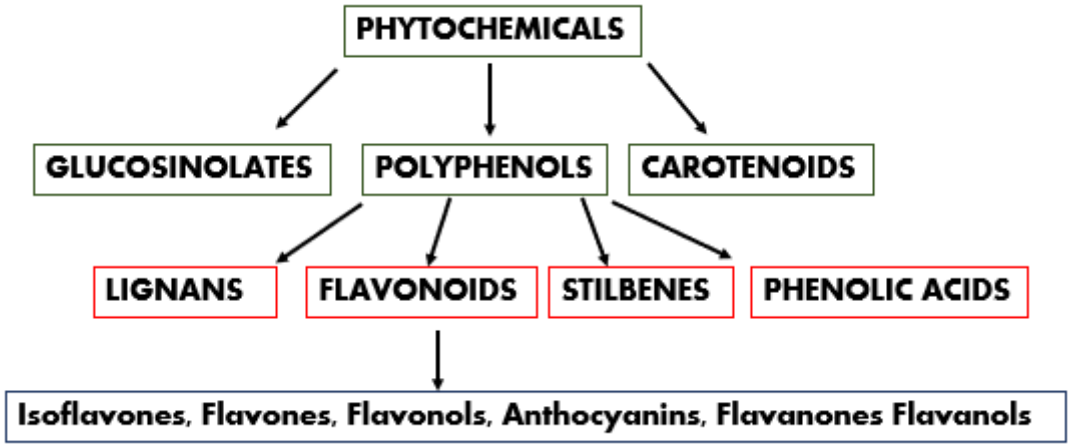


Figure 1: Classification of phytochemical compounds.

1.1.1.2 Polyphenols

In plants, polyphenols are ubiquitous compounds that are fundamental to plant physiology, contributing to their organoleptic characteristics and to the resistance against microorganisms and insects, pigmentation, and to preserve their integrity due to continuous exposure to environmental stresses, including UV and high temperatures. Among polyphenols-derived protective compounds anthocyanins play a pivotal role. For these reasons, mediterranean vegetables and fruit are particularly rich in them (Singla et al., 2019).

Although the thousands of molecules have different biological functions, they have a common chemical structure: they are benzene derivatives with hydroxyl groups bonded to aromatic rings **(Figure 2)** (Scalbert and Williamson, 2000). This structure allows these compounds to function actively as scavengers to stabilise free radicals, reducing agent chelators of pro-oxidant metals, and quencher of singlet oxygen formation (Ji et al., 2020).

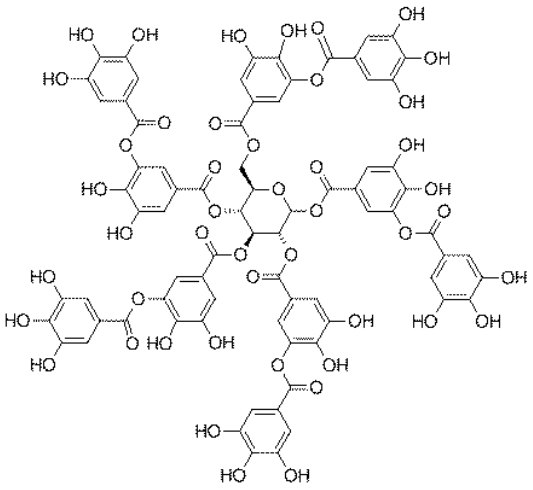


Figure 2: Chemical structure of a polyphenol.

One way of classifying polyphenols is to distinguish them into flavonoids and non-flavonoids: the former are structurally made up of three hexatomic rings, two of which are bonded together, the latter are characterised by one or two phenolic rings (Serafini et al., 2010).

1.1.1.2.1 Flavonoids

Flavonoids represent the most important and heterogeneous group of polyphenols. The basic structure consists of a "flavanic nucleus" containing 15 carbon atoms (C6-C3-C6) arranged in 3 rings, indicated by the letters A, B and C, where the bridge with 3 carbon atoms is commonly cyclized with oxygen **(Figure 3)** (Hanrahan et al., 2011).

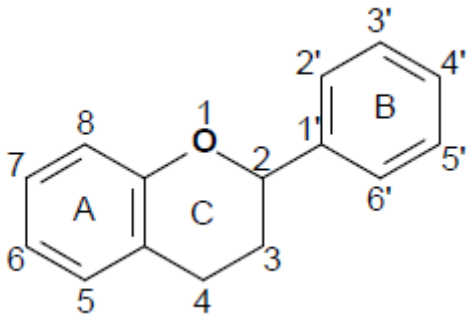


Figure 3: Chemical structure of flavonoid.

The different classes of flavonoids differ in their oxidation levels and in the substitution pattern of the C, while in each class the various compounds differ in the number and arrangement of phenolic groups on the A and B rings, as well as in the nature and extent of alkylation and/or glycosidation of these groups; the most interesting are the flavones, the flavanones, flavonols, flavanonols, flavan-3-ols, anthocyanidins and isoflavones.

Within this class of phenolic compounds, it is important to mention i) anthocyanins, which characterise the colouring of apples and citrus fruits, ii) quercetin, which belongs to the flavonol class and is one of the most studied molecules in natural pharmacology and is present in various vegetables and fruits, and iii) catechins, present in high concentrations in green tea (Wojdyło et al., 2007; Silva et al., 2017).

1.1.1.2.2 Non Flavonoids

Compared to flavonoids, non-flavonoid compounds are a less extensive but equally widespread class in nature. The common basic structure of all non-flavonoid compounds is an aromat (Singla et al., 2019). However, the main molecules known in nature for their biological properties are structurally made up of two aromatic rings **(Figure 4)**.

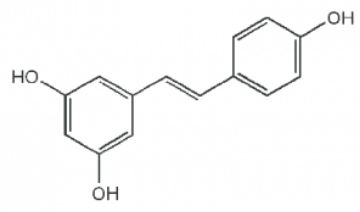


Figure 4: Chemical structure of Resveratrol, a non-flavonoid

Phenolic acids, stilbenes and lignans belong to this polyphenol group. Curcumin and caffeic acid, found in turmeric and green coffee respectively, are two of the main phenolic acids studied in recent decades for their antioxidant, anti-inflammatory, antibiotic, and anti-cancer properties (Baspinar et al., 2017; Nieber, 2017; Giordano and Tommonaro, 2019; Matacchione et al., 2021a). Similarly, resveratrol, a stilbene found in various plant foods and particularly in grape skins, has been extensively studied for its antioxidant, anti-inflammatory and anti-ageing properties (Quadros Gomes et al., 2018; Matacchione et al., 2021a).

1.1.1.3 Non phenolic compounds (Glucosinolates and Carotenoids)

Glucosinolates are a group of phytochemicals that include more than 130 different compounds that are widely distributed mainly in the Cruciferous family, particularly in Brassicaceae such as cauliflower, brussels sprouts and broccoli.

From a chemical point of view, they consist of a common glycone, characterised by β-D-thioglucosides N-hydroximinosulfates and an aglycone derived from amino acids, namely methionine, phenylalanine, tyrosine and tryptophan. Following tissue breakdown the glucosinolates, contained in the cell, are rapidly hydrolysed by myrosinases (thioglucosidases) to unstable intermediates that rearrange themselves spontaneously into isothiocyanates, thiocyanates or nitriles (Talalay and Fahey, 2001; Bell et al., 2018; Soundararajan and Kim, 2018).

Although this class of compounds has a low bioavailability, interest in these degradation products has increased in recent years. Several studies demonstrated a chemoprotective activity of the mentioned molecules, attributing an inversely proportional relationship between Brassicaceae consumption and

the development of different types of cancer (Cornblatt et al., 2007; Balasubramanian et al., 2011; Novío et al., 2016).

Carotenoids are plant pigments of a lipid nature. In plants act as photoprotectors and their main function is to capture excess light not absorbed by chlorophyll (Eggersdorfer and Wyss, 2018; Maoka, 2020).

Chemically, carotenoids are polyene terpenoids, formed by 40 carbon atoms, and consisting of a long central hydrocarbon chain of 22 atoms. Carotenoids include several conjugated double bonds, and two terminal units that may contain different functions (alcoholic, ketone, epoxy, etc.). The conjugated double bonds form the chromophore system responsible for the intense colour of these substances. Carotenoids can be divided into two broad categories: carotenes, which are simple hydrocarbons, and xanthophylls, which contain oxygen atoms in their structure (Haas et al., 2019). The carotenoids best known for their beneficial properties for human health are the carotenes in carrots, the lycopene in tomatoes, and the xanthophyll or lutein found in all green plants. The main properties attributed to this class of compounds are antioxidant, anticarcinogenic, antimicrobial and anti-inflammatory (Chung et al., 2017; Beynon et al., 2019; Pan et al., 2021).

1.1.2 Natural compounds and their activities on human health

Interest in the plant world for bio-medical applications has increased exponentially in recent decades (Shipkowski et al., 2018). The human benefits of many plant compounds have been known since ancient times. The current social and economic pressures to integrate natural and conventional medicines have been driven largely by the huge prevalence of chronic, non-communicable degenerative diseases and the complex management of patients with such diseases. Therefore, an increasing interest of scientific research into the effects of numerous natural substances using both *in vitro* and *in vivo* models has been observed. *In vitro* studies have made it possible to identify numerous activities of compounds of plant origin, such as antiviral, antibacterial, anti-tumour, antioxidant, cardioprotective and neuroprotective activities which, in several cases, have also been confirmed in in vivo studies (Gurău et al., 2018; Nardiello et al., 2018; Soldati et al., 2018; Voltes et al., 2020).

1.1.2.1 Antimicrobial activities

The antimicrobial activity of individual natural compounds or plant extracts is one of the areas of greatest interest for the scientific community. In the last few decades many scientific reports demonstrated in *in vitro* studies this important property. For example, Resveratrol (3,4′,5-trihydroxystilbene), a molecule found in various foods and plant matrices such as wine, grapes, peanuts or berries, has a strong bacteriostatic and bactericidal activity against various pathogenic bacteria for humans. Paulo et al. (2010) tested different doses of resveratrol on some of the main pathogenic microorganisms. The minimal inhibitory concentration (MIC) of the gram positives tested (*Bacillus cereus, Staphylococcus aureus* and *Enterococcus faecalis*) was very low (MIC: 50-100 µg/mL). For gram negatives, it was not possible to obtain the MIC. Due to its maximum solubility (the maximum testable concentration of resveratrol is 400 µg/mL) which is sufficient to exert an inhibitory activity of 81, 80 and 58% against Escherichia *coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* respectively (Paulo et al., 2010). These effects has been confirmed in many other studies that have identified resveratrol as one of the main natural compounds with antimicrobial activity (Dadi et al., 2009; Paulo et al., 2011; Taylor et al., 2014; Ferreira and Domingues, 2016).

In general, numerous *in vitro* studies have shown that gram-negative bacteria are more resistant to polyphenols than gram positive. A plausible explanation is the structure of the cell wall of these microorganisms: although gram negatives have a thinner layer of peptidoglycan than gram positives, they have a thick layer of lipopolysaccharide (LPS), which would slow down the entry of natural molecules into the bacterial cell (Cui et al., 2012; Biswas et al., 2013; Bobis et al., 2015).

However, *Porphyromonas gingivalis,* a gram negative bacterium involved in periodontal disease, is inhibited by theaflavins (Kong et al., 2014). Similarly, (-)-epigallocatechin-3-gallate (EGCG) (a catechine) appears to have strong inhibitory activity against *Escherichia coli* O157:H7 (gram negative) and also *Staphylococcus aureus (gram positive)* and*)*, two widely distributed pathogens (Cui et al., 2012): both compounds are contained in tea.

Tea is a drink that originated in Chinese culture. Today, it is one of the most popular beverages in the world. There are different types of tea, but they all derive from the same plant (*Camellia sinensis*) (Luo et al., 2020). A common feature of the different varieties is the high polyphenols amount that characterise the drinks made from this plant's leaves. In particular, catechins and theaflavins are the compounds most commonly found in aqueous extracts

The antimicrobial properties of these natural compounds are very important in light of the increasingly relevant problem of antibiotic resistance. This serious problem for human and animal health is due to several causes: the increased use of these drugs (including inappropriate use) in both human and veterinary medicine; the use of antibiotics in animal husbandry and agriculture; the spread of hospital infections caused by antibiotic-resistant micro-organisms (and the limited control of these infections); and the increased spread of resistant strains due to increased international travel and migration flows (Martinez, 2014).

In this respect, the appropriate use of natural compounds can be a solution in different areas, from the food industry to the hospital setting (Rahmani et al., 2015; Bouarab Chibane et al., 2019; Huang et al., 2019; Ez zoubi et al., 2020).

1.1.2.2 Antioxidant activities

Oxidative stress is defined as the mechanism of cellular damage caused by an excess of chemical substances called free radicals or reactive oxygen species (ROS). This mechanism is characterised by high reactivity and chemical instability, produced at cellular level mainly in the mitochondria (Ito et al., 2019). Oxidative stress is one of the causes of chronic inflammation which in turn can lead to the development of several diseases. The daily intake of the right dose of phytochemical compounds through food or supplements helps to maintain the oxidative balance and thus the proper physiological functioning of the body (Mileo and Miccadei, 2016; Gurău et al., 2018).

In fact, for over thirty years, one of the main feature attributed to phytochemical compounds, and in particular to polyphenols, is to neutralize ROS's action (Ruby et al., 1995). The mechanisms by which polyphenols exert their antioxidant activity are multiple. Their action can be direct, acting as a

scavenger or inhibiting enzymes involved in the formation of ROS, or indirect, stimulating the body's antioxidant defences (A. et al., 2013). Liew and al. (2018) characterised hydroalcoholic extracts of *Citrus sinesis* and found a high content of phenolic acids (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid and ferulic acid), flavan-3-ols (catechin and epigallocatechin), followed by flavanone (luteolin, apigenin and vitexin). Then, their antioxidant activity was assessed by the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, and oxygen radical absorbance capacity (ORAC) assay). The positive correlation between TPC and TFC of the extracts and antioxidant activity that was observed in all three assays suggests the functionality of the above-mentioned compounds (Liew et al., 2018). In another study, the antioxidant capacity of individual compounds and total extract of sweet potato (*Ipomoea batatas* L.) leaf was evaluated and compared with the total phenols from tea and grape seed: it shows that caffeic acid contained in these leafs has a higher scavenging and oxygen radical absorbance capacity than total potato leaf extract, tea and grape seed phenols (Sun et al., 2018). Oxidative stress is one of the causes of chronic inflammation which in turn can lead to the development of several diseases. The daily intake of the right dose of phytochemical compounds through food or supplements helps to maintain the oxidative balance and thus the proper physiological functioning of the body (Mileo and Miccadei, 2016; Gurău et al., 2018).

1.1.2.3 Anti-inflammatory activities

Non-steroidal anti-inflammatory drugs, commonly known as NSAIDs, are widely used for different types of diseases. They have the ability to reduce inflammation and pain, such as joint disorders, headaches, toothaches and neuralgia. They also have an antipyretic effect, because they can lower the body temperature in the event of a fever. Like all drugs, they are not without side effects, which is why it is important not to abuse them, as can happen in cases of chronic inflammation (Cornejo-garcia et al., 2009). In this respect, the scientific community has investigated extensively for natural molecules that can replace or at least support the action of NSAIDs.

A study carried out on two cellular models such as the primary endothelial cell line, HUVECs and the monocytic cell line, THP-1 showed the anti-inflammatory capacity of bioCurcumin, resveratrol and β-caryophyllene. These compounds were able to inhibit of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, TNF-α) production and to downregulate miR-146a and miR-21 expression whether they were produced as a result of an acute inflammatory stimulus (LPS, in THP-1 model) or as a result of chronic inflammation due to cell senescence (in HUVEC model). Furthermore, the best results were obtained by administering the three compounds simultaneously, assuming a synergistic effect (Matacchione et al., 2021a).

One of the major natural compound with anti-inflammatory effect is oleuropein, the main polyphenol in olive oil and leaves. A recent scientific report shows that Oleuropein Attenuates Lipopolysaccharide-Induced Acute Kidney Injury *in vitro* and *in vivo* using human macrophage and BALB/c mice treated with LPS as models. Oleuropein treatment inhibited NF-κB/p65 nuclear translocation and downregulated IL-1β, IL-6 and TNF-α in macrophages and alleviated LPS induced acute kidney injury, decreased serum creatinine and blood urea nitrogen levels and proinflammatory cytokines in mice (Cui et al., 2021).

Chlorogenic acid is another phenolic compound with anti-inflammatory properties widely used in the plant world. Green coffee beans in particular are an excellent source of it (Macheiner et al., 2019). In an *in vitro* model for osteoarthritis, which is a pathology characterised by chronic inflammation, chlorogenic acid was able to inhibit inflammation both by acting on the NF κB signalling pathway and by reducing the iNOS/NO, IL-6, MMP-13 and COX-2/PGE2 production (Liu et al., 2017).

1.1.3 Olea europaea L.

The olive tree (*Olea europaea L.*) is an 'evergreen tree' cultivated throughout the Mediterranean region. However, cultivation of this plant can also be found in other regions such as the Arabic peninsula, India and various other parts of Asia (Somova et al., 2003). Olives and oil are the main products of this cultivation and, to date, Italy, Spain and Greece are the largest producers (Oil et al., 2019; Montoya and Alarc, 2020).

The numerous testimonies of its beneficial use for human health have contributed to increase the consideration of this plant over time. Since ancient times, the symbolic values attributed to this plant are numerous: during the Roman and Greek eras, it was considered a symbol of peace, wisdom, glory and prosperity (Ferreira et al., 2007; Kalogeropoulos and Tsimidou, 2014).

In modern times, the scientific community has taken a growing interest in the *Olea europaea L.* and its derived products. Several studies have found a positive correlation between moderate consumption of olive oil, typical of the Mediterranean diet, and the maintenance of good health (Kopel et al., 2014; Rodríguez-Rejón et al., 2014). The beneficial effects of olive oil consumption have been attributed not only to the high content of unsaturated fatty acids (98-99% of the total weight of extra-virgin olive oil) but also to a class of small phenolic compounds which, although present in low concentrations (1-2%), may help to prevent different diseases over time (Oil et al., 2019).

Fatty acids	**Range**
Palmitic (C16:0)	6.99-11.05
Plamitoleic acid(C16:1)	0.49-1.11
Stearic acid (C18:0)	2.61-4.43
Oleic (C18:1)	76.52-82.49
Linoleic(C18:2)	3.07-6.62
Linolenic acid (C18:3)	0.48-0.95

Table 1: Total Fatty acids composition (%) of Olive oil.

Several beneficial effects (anti-inflammatory, -viral, -bacterial, antioxidant, cardioprotective and neuroprotective) of natural compounds in extra-virgin olive oil (EVOO) have been demonstrated *in vitro* and *in vivo* studies (Giamarellos-Bourboulis et al., 2006; Yu et al., 2016; Lockyer et al., 2017a). Importantly, in the list of EFSA (the European Food Safety Authority) health claims which has been made on foods, as referred to in Article 13 of Regulation (EC) No 1924/2006, a health claim has been

established for olive oil polyphenols. The claim, as registered, is that *"olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"*.

Interestingly, many natural compounds are present at high concentrations not only in oil but also in olive leaves. Olive leaves are a source of many phytochemicals like phenolics and flavonoids. Moreover, they represent a waste by-product for olive oil sector, therefore readily available at low cost (Medina et al., 2019; Tarchoune et al., 2019).

1.1.3.1 Phytochemical constituents of olive tree leaves

Olive leaves are a waste product of olive oil processing. They are commonly used as animal feed or burned together with excess branches from pruning. Interest in this waste product for possible biomedical applications has grown in recent times. This can be related to its high concentration of phenolic compounds and its low cost and availability. Moreover, olive leaves have a higher polyphenol content than olive oil (Japón-Luján et al., 2006).

The variety of the olive tree, its origin, climatic factors, seasonality included, and the presence or absence of pests are key factors determining the type of compounds and their total content in the leaves (Martín-García and Molina-Alcaide, 2008).

Scientific community generally classify the phenolic compounds present in olive leaves into 5 categories:

- secoiridoids (oleuropein, oleacin and verbascoside generically called oleuropeosides)
- flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin and diosmetin)
- flavonols (rutin)
- flavan-3-ols (catechin)
- derived phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid)

The main compound present in olive leaves is oleuropein, whose concentration varies between 17 and 23%. In addition, several The concentration of the other active compounds found in olive leaves (hydroxytyrosol, tyrosol, verbascoside, caffeic acid, rutin, luteolin 7-O-glucoside, luteolin 4-O-

glucoside, apigenin-7-O-rutinoside and apigenin 7-O-glucoside, Diosmetin, Vanillic acid, Vanillin, Catechin) is variable and depends on the parameters mentioned above (Singh et al., 2008; Şahin and Şamli, 2013; Şahin et al., 2017; Mmopele et al., 2018).

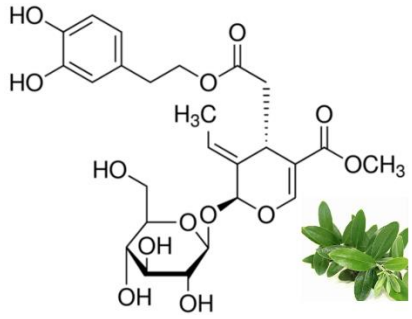


Figure 5: Chemical structure of oleuropein, the main compound of olive leaf

However, the concentration and proportion of the extracted compounds depends also by the extraction process (temperature, time, solvent). Yateem et al. (2014) demonstrated that by increasing the temperature (from 25 °C to 60 °C) and lowering the pH (from 9 to 3) using the same solvent results in a significant improvement in the extraction efficiency of oleuropein. Similarly, using hybrid organic solvents (e.g. water/ethanol, water/methanol, water/acetonitrile) is a much more effective extraction method than using pure solvents. In agreement with this, it has been shown that by using solvents consisting of water and ethanol/methanol (50% v/v), the final concentration of total phenol content (TPC), total flavonoid content (TFC) and oleuropein increases significantly (Goldsmith et al., 2015).

Obtaining extracts rich in bioactive compounds combined with a view of their possible use in the biomedical field is one of the main objectives of the research in this field, however, it is essential that the extraction process would be non polluting and safe and the products are non-toxic for human health. For these reasons, research is moving towards what are commonly referred to as 'green methods'. The use of the eco-friendly substance 2-hydroxypropyl-β-cyclodextrin (CD) as a co-solvent at low concentrations (7% w/v) has been shown to improve the extraction efficiency of the main polyphenols from olive leaves such as oleuropein, Luteolin diglucoside, Luteolin

glucoside, Rutin, Apigenin rutinoside, Apigenin rhamnoside, Luteolin glucoside, Luteolin glucoside and Luteolin derivative (Mourtzinos et al., 2016). Another extraction system is the solvent-free microwave-assisted extraction (SFMAE). This method offers numerous advantages: first of all, by avoiding the use of organic solvents, there is a considerable saving in money and a reduction in environmental impact and then reduction of time in the extracting process and the possibility of obtaining a healthier product for humans, are equally important factors that are directing the research towards this innovative technology (Li et al., 2011, 2013; Baghdikian et al., 2016). The use of SFMAE for extraction from olive leaves shows that excellent extraction performance can be achieved in terms of TPC and oleuropein content. In addition, the products obtained have a high antioxidant and antimicrobial activity, making them compatible for use in the food industry, for example as additives or nutraceuticals (Sahin et al., 2017).

1.1.3.2 Main activities of natural olive leaf compounds

The term 'Mediterranean Diet' (MD) was coined by the American scientists Ancel and Margaret Keys in the 1970s. They dedicated their lives to epidemiological studies focusing on the relationship between diet and the incidence of various diseases. In particular, the population longevity of southern Italy led them to formulate the hypothesis of a positive correlation of it with a lifestyle that included socio-cultural aspects with diet as a cornerstone. In 2010, UNESCO approved the inscription of the Mediterranean Diet in the Intangible Cultural Heritage List, recognising the maternity of Italy, Spain, Greece and Morocco, and then from 2013 also Cyprus, Croatia and Portugal (Russo et al., 2021).

EVOO is a staple of the MD. There are many beneficial properties attributed to the moderate consumption of this food. This is due not only to its high content of unsaturated fatty acids, but also to the presence of polyphenols that characterise this plant. As mentioned above, these same polyphenols can also be obtained from olive leaves, a waste material in food production that is readily available at low cost and which is abundant in the Mediterranean region. This has aroused great interest in the scientific community which, over the last 20 years, has devoted much attention studying the extraction processes, bioavailability and biological properties of these compounds (Boss

et al., 2016a). Oleuropein is the main polyphenol in EVOO and olive leaves. Its antioxidant and anti-inflammatory properties have been confirmed by several studies. One of the consequences of oxidative stress is the development of diabetes mellitus, which in turn can lead to serious complications, in some cases fatal. In alloxan-induced diabetic rabbits model, the administration of oleuropein (20 mg/kg body weight) for 4 months was able to restore the enzymatic and non-enzymatic antioxidant systems, as well as bringing blood glucose levels back to values similar to those of non-diabetic controls (Al-Azzawie and Alhamdani, 2006). Janahmadi et al. (2017) investigated the effect of oleuropein on an animal model of rats with heart failure. Oleuropein was able to attenuate heart failure through an antioxidant and anti-inflammatory action as demonstrated by, inter alia, restoration of the enzyme systems superoxide dismutase and glutathione reductase and reduction of serum values of, interleukin-1β, and tumour necrosis factor-α (Janahmadi et al., 2017).

Oleocanthal is another compound with very interesting properties. The importance and peculiarity of oleocanthal lies in its highly antioxidant and anti-inflammatory properties. Its anti-inflammatory action on the body is very similar to ibuprofen, one of the most widely used NSAIDs (Yi-Fen Chiang, Hui-Chih Hung, Hsin-Yuan Chen, Ko-Chieh Huang and Jen-Yun Chang, 2020). LeGendre et al. (2015) demonstrated that *in vitro* oleocanthal was able to kill cancer cells in 30 minutes without damaging healthy cells. Oleocanthal would act by destroying lysosomes, organelles with metabolic waste, from malignant cells (LeGendre et al., 2015). Moreover, the administration of oleocanthal to C57BL/6 wild-type mice seems to reduce the accumulation of beta-amyloid plaque, the cause of Alzheimer's disease (Abuznait et al., 2013).

Hydroxytyrosol, a molecule derived from the hydrolysis of oleuropein, is the most abundant phenolic compound in EVOO. However, good concentrations have also been found in its leaves(Breakspear and Guillaume, 2020). Hydroxytyrosol is considered one of the most biologically active compounds in the plant world. Scientific studies show that the antioxidant activity of hydroxytyrosol is higher than that of vitamins C and E, and is even around 10 times greater than that of green tea and twice that of coenzyme Q10, a molecule naturally present in our cells and acting as an antioxidant. Recently,

D'Andrea et al. (2020) demonstrated that hydroxytyrosol reverses the neural ageing process in adult and aged wild-type mice. The study shows that oral intake of hydroxytyrosol for one month keeps alive the new neurons produced during this period, both in adults and even more so in the elderly, in which it also stimulates the proliferation of stem cells from which neurons are generated. In addition, thanks to its antioxidant activity hydroxytyrosol also succeeds in 'cleaning up' nerve cells. This is because it also leads to a reduction in certain markers of ageing such as lipofuscins, which are accumulations of debris in neuronal cells (D'Andrea et al., 2020). Oleacein, a secoiridoid chemically very similar to oleuropein, appears to have cardiovascular protective effects. Its high antioxidant power counteracts the effects of oxidative metabolism induced by ROS and, consequently, delays the development of atherosclerosis (Singh et al., 2008). A recent study showed that oleacein would exert its anti-inflammatory activity by acting on the iron present in the catalytic site of the enzyme 5-lipoxygenase (5-LOX), an enzyme that catalyses the initial steps in the biosynthesis of pro-inflammatory leukotrienes (Vougogiannopoulou et al., 2014).

1.2 INFLAMMATION

Inflammation is the main response resulting from the body's innate immunity. This response is triggered by physical (trauma, high or low temperatures), chemical (endogenous or exogenous harmful substances) or biological (bacteria, viruses) damage. Inflammation plays a key role in neutralising the pathological agent, in tissue repair and in restoring normal functions of the body (Arulselvan et al., 2016; Germolec et al., 2018).

Inflammation can be acute or chronic: acute inflammation has a sudden onset, a rapid resolution andis characterised by phenomena mainly involving the dilation of blood vessels, increased blood flow followed by formation of oedema; chronic inflammation is long-lasting, and it can occur asymptomatically or as a consequence of acute inflammation that has not completely healed andoccurs when the cause of the inflammation cannot be completely eliminated or when the functioning of the processes involved in healing are defective.

In contrast to acute inflammation, chronic inflammation is characterised by infiltration of mononuclear cells including macrophages, lymphocytes and plasma cells, proliferation of blood vessels, fibrosis, and cell necrosis (Taniguchi and Karin, 2018; Varela et al., 2018).

1.2.1 Acute inflammation caused by biological agents: lipopolysaccharide (LPS)

LPS is one of the components of the cell wall of Gram-negative bacteria. These molecules perform fundamental functions for the survival of bacteria and are involved in the defence against antimicrobial agents. LPS is only released following replication or cell lysis and is therefore classified as a bacterial 'endotoxin'**.** The LPS consists of three main regions: i) The hydrophobic domain known as **lipid A**, ii)A non-repeating 'core' oligosaccharide and iii) The **antigen O** which represent the outer portion of the LPS.

While the toxicity of the compound is associated with the lipid A component, immunogenicity (the capacity to trigger an immune response) is due to the polysaccharide region.

The LPS core region is similar in all Gram, however, he O antigen is a polysaccharide chain that differs between species and, in some cases, between strains. This diversity determines the pathogenicity of the bacterium. *Pseudomonas aeruginosa, Helicobacter pylori, Salmonella enterica, Salmonella tiphimurium, Escherichia coli* are some examples of species that produce this endotoxin (Maldonado et al., 2016; Cochet and Peri, 2017; Zhou, 2017)**.**

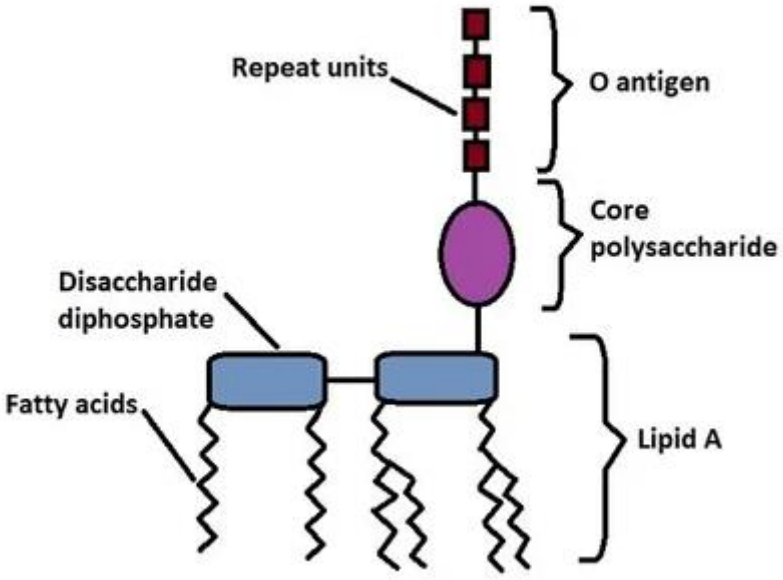


Figure 6: Diagrammatic representation of LPS

1.2.1.1 Mechanisms activated by LPS

Toll-like Receptors (TLRs) are receptor proteins that play a key role in humans and animals in the recognition of molecular structures associated with common pathogen-associated molecular patterns (PAMPs), including viruses, bacteria, protozoa and fungi. Among the 11 types of TLRs known in humans, TLR-4 is the specific sensor of bacterial endotoxins and, in particular, recognises LPS present on the outer membrane of Gram- bacteria. Therefore, TLR-4 has a protective role for the body by triggering immune and inflammatory responses to pathogens. However, if TLR-4 activation is too powerful or too prolonged, the excessive release of molecular mediators of inflammation, i.e. cytokines, can have serious pathologicalconsequences, including sepsis and septic shock (Jeong and Lee, 2011; Vidya et al., 2018).

The complex molecular mechanism of TLR-4 activation and subsequent signalling leading to the production of inflammatory cytokines has been almost completely elucidated in recent years in the case of TLR-4 being activated by bacterial LPS (Płóciennikowska et al., 2015).

From a molecular point of view, the activation of the innate immune response consists of a series of specific interactions between LPS and receptors that act sequentially. These include Lipid Binding Protein (LBP), CD14 (a monocyte differentiation antigen), and Myeloid Differentiation Factor 2 (MD-2) in complex with TLR4 . Thus, the molecular process of TLR4 activation on the cell surface begins with the extraction of a single LPS molecule from the aggregates in solution by LBP, which transfers the molecule to CD14, which in turn transfers it to the dimeric complex consisting of MD-2 and TLR4. Subsequently, from the cell surface, the homodimeric molecular complex (LPS with MD-2/TLR4) initiates intracellular signalling. Once the activated complex is formed, specific adaptor proteins bind to the intracellular domain of TLR4, triggering different signalling pathways (Płóciennikowska et al., 2015; Lai et al., 2017; Ryu et al., 2017; Tsukamoto et al., 2018). In particular, the complex intracellular signal transduction cascade results in the activation of interleukin-1 receptor associated kinase (IRAK) and the subsequent phosphorylation and degradation of IkBα, which is the inhibitor of the nuclear factor kappa light chain enhancer (NF-kB). Degradation of IkBα results in the activation of rapid NF-kB nuclear translocation. NF-kB controls directly or with the cooperation of other transcription factors, the activity of more than 100 genes that regulate numerous cellular processes of vital importance for inflammation and immune response. In particular, activation of this factor determines the transcription of genes encoding for pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, chemokines such as IL-8, adhesion molecule such as ICAM-1 and VCAM and antimicrobial molecules. Some of the products of gene activation by NF-kB (e.g. TNF-α, IL-1β) are themselves activators of NF-kB (Karaky et al., 2018; Su et al., 2018; Tang et al., 2021). Prolonged activation of NF-kB has been demonstrated in many chronic inflammatory diseases, including asthma, rheumatoid arthritis, chronic inflammatory bowel disease and psoriasis (García-Vásquez et al., 2018).

1.2.2 Cellular senescence and inflammation

The aging process is considered a universal and inevitable process of physiological decline associated with a greater vulnerability to disease and death. This vulnerability is linked to the complexity of the organism, which comes from the myriad of interactions and feedback controls that operate between its different structural units. These mechanisms allow cells, tissues, and entire organisms the ability to respond and adapt to stressful environmental conditions.

The fact that age represents the common risk factor for almost all age-related diseases (ARDs) supports the idea that there are pathogenetic mechanisms common both to aging and to the development of the same ARDs.

Two of these mechanisms have been identified to date and extensively studied in cellular models, animals and in humans: cellular senescence and the pro-inflammatory, chronic-systemic state of low-grade defined 'inflammaging'. These two phenomena are not independent but closely interconnected, since cellular senescence feeds inflammaging and inflammaging promotes the increase of senescent cells in the body. A cell is defined as senescent when it irreversibly loses its replicative capacity.

In fact, the main feature of cellular senescence (CS) is the arrest of the cell cycle which results in permanent blockage of cells in G1 phase (Prattichizzo et al., 2016).

In addition to this, senescent cells are characterized by morphological and functional changes. The most important functional change is that the senescent cell remains metabolically active and acquires a pro-inflammatory secretory phenotype which is called senescence-associated secretory phenotype (SASP). Numerous molecules are part of the senescent cell secretome, such as cytokines, chemokines, lipid mediators, non-coding RNAs (microRNAs) and DNA fragments. The number of senescent cells increases with age, and their SASP phenotype fuels the inflammaging that characterizes human aging and that increases the risk of developing ARDs (Coppé et al., 2010).

1.2.2.1 Senescence-associated secretory phenotype (SASP)

The components of the SASP include proteins that are generally conserved between cell types, although some differences can be observed also between tissues. SASP, which is fuelled by the DNA damage response (DDR), is characterized by NF-kB and NLRP3 inflammasome pathways activation, and by the consequent synthesis and release of a plethora of proinflammatory factors such as interleukins, chemokines, growth factors, matrix- degrading enzymes, reactive oxygen species, and non-coding RNA, i.e. miRNAs (Coppé et al., 2010; Malaquin et al., 2016). The SASP involves secretion of hundreds of molecules, of which interleukin (IL)-1 α/β, IL-6, IL-8, transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α are the best characterized (Coppé et al., 2010).

Most of these cytokines are transcriptionally regulated by NF-kB induces a variety of inflammatory

SASP components (e.g. interleukin [IL]-6, IL-1 and tumor necrosis factor [TNF-α]), which are essential cell-autonomous regulators of senescence . Upstream, Janus kinase (JAK), p38, and other MAP kinases have all been implicated in SASP control (Freund et al., 2011). Importantly, the SASP components, under the control of the inflammasome, are also able to propagate paracrine senescence to neighboring cells, which in turn become capable of acquiring the SASP phenotype.

Epigenetic modifiers, i.e. non-coding RNAs, have also been reported to play a role in the SASP and

its systemic spread. Increasing evidence suggests that specific miRNAs, such as miR-21, miR-126, and miR-146a can modulate cellular senescence being involved in the modulation of inflammatory

responses and inflammaging (Matacchione et al., 2021b); moreover, other complex epigenetic mechanisms seem to modulate the pathways involved in SASP acquisition. There is strong evidence that SIRTs, can modulate SASP and senescence, extending lifespan/health-span, in different animal models (Hayakawa et al., 2015).

However, it should be noted that the SASP is a double-edged sword. In fact, localised and time-limited SASP can be helpful in resolving tissue damage, at least in young people. In fact, the release

of SASP factors, including proteins and nucleic acids, at a paracrine and systemic level fuels inflammation thus recruiting immune cells om order to eliminate damaged cells from tissues.

However, chronic SASP is associated with the widespread senescence, an elevated proinflammatory state, and a faster rate of ageing. As part of the ageing process, the excessive accumulation of SCs and the systemic spread of SASPs have thus transformed a protective response into a pathogenic mechanism (Coppé et al., 2008, 2010; Franceschi and Campisi, 2014).

1.3 BONE MARROW ADIPOSE TISSUE (MAT)

In the late 1970s and 1980s the term of marrow adipose tissue (MAT) began to appear in selected manuscripts in recognition that the marrow fat exists as a distinct adipose tissue organ. After a period of discovery which focused on elucidating the basic nature of MAT, in the 1980s the bone biology community became very concerned about the potential relationship between MAT accumulation and bone loss. This led to many clinical and animal studies on this topic. Today MAT is considered a negative factor in diseases such as osteoporosis. Research on the relationship between MAT and skeletal metabolism became a main topic for many groups on a worldwide scale. In addition to understanding the impact of MAT on bone, several groups are working to define the physiological role of MAT, the biology of its progenitor and to understand the fundamental genetic, epigenetic, and metabolic differences between MAT and white adipose tissue (WAT).

Like WAT, MAT has the ability to respond to nutritional status. The most puzzling example

in recent years is that in states of calories restriction and anorexia, the amount of MAT

increases while peripheral WAT is lost (Bredella et al., 2009). The earliest fatty change of the human marrow in the phalanges of the toe begins at or slightly before birth, regardless of prematurity, and rapidly accelerates between 4 and 8 weeks of age The marrow in the phalanges gradually matures and reaches full fatty conversion after 1 year (EMERY and FOLLETT, 1964). MAT continues to

accumulate in the appendicular skeleton from distal to proximal until age 20–25, with gradual MAT conversion continuing in the axial skeleton throughout life.

1.3.1 Adipocyte differentiation of Bone Marrow-derived-Mesenchymal Stem Cells

Bone is a dynamic connective tissue that is continuously formed, resorbed, and reformed throughout life. Maintenance of healthy bone quality and quantity depends on the coordinated development and activity of bone-forming osteoblasts and bone-resorbing osteoclasts within local sites of bone remodelling called basic multicellular units. During skeletal remodelling, cells of the osteoblast lineage synthesize and secrete proteins that can initiate or control osteoclast differentiation. Similarly, cells of the osteoclast lineage secrete chemical signals that impact osteoblast differentiation. Intercellular communication between these two lineages therefore plays a key role in coordinating the development and function of key effectors and processes required for healthy bone remodelling. Disorders characterized by bone loss such as osteoporosis are often associated with changes in these chemical signals and a consequent loss of the homeostatic control over balanced bone remodelling. Within bone, osteoblasts are derived from mesenchymal stem cells (MSCs), while osteoclasts originate from hematopoietic stem cells (HSCs). Importantly, MSCs are multipotent, and in addition to osteoblasts can give rise to several other distinct cell types including adipocytes, myocytes, chondrocytes, endothelial cells, and vascular smooth muscle cells (Muruganandan et al., 2009). An imbalance in the regulation of osteoblast and adipocyte differentiation is commonly seen with conditions such as aging and diabetes mellitus, and can result in fatty bone marrow, impaired osteoblast renewal, and chronic bone loss.

Among MSC potential fates, differentiation to the osteoblast and adipocyte lineages has a particular relevance to the maintenance of normal bone homeostasis. For example, considerable evidence exists to support that a shift in MSC differentiation to favour the adipocyte lineage over the osteoblast lineage can directly contribute to imbalances in bone formation/resorption and ultimately lead to bone loss. In support of this reciprocal relationship, many studies on bone marrow-derived MSC have

shown that factors inducing adipogenic differentiation inhibit osteoblast formation and, likewise, factors that promote osteoblastogenesis inhibit adipocyte formation.

In bone marrow, the fate of MSCs is largely determined by the expression of specific groups of transcription factors that act as molecular switches to drive the differentiation of uncommitted precursors down a specific lineage (Muruganandan et al., 2009).

Adipogenesis is a multistep process characterized by two major phases: the determination phase and the terminal differentiation phase. During the first one, multipotent MSCs become committed to the adipocyte line. In the second one, fibroblast like pre-adipocytes are converted to spherical, mature adipocytes that can synthesize and transport lipid, secrete adipocyte specific proteins.

The main regulator of adipogenesis is the transcription factor PPARγ (Wan et al., 2007).

Bone homeostasis can be affected by different mechanisms and imbalance between bone-forming osteoblasts and bone-resorbing osteoclasts, stimulated by pro-adipogenic factors, can occur. Furthermore, adipocytes release pro-inflammatory cytokines fuelling inflammation and exherting detrimental effects. However, experimental models have shown that different adipocyte-derived signalling molecules, including leptin and adiponectin may be involved in bone homeostasis and useful for systemic health. Leptin acts directly on precursor cells to promote osteoblast differentiation and proliferation. It is also able to inhibit adipogenesis and to suppress osteoclastogenesis (Holloway et al., 2002). Adiponectin, has been reported to have a direct negative effect on MSC osteoblastogenesis (Liu et al., 2010).

1.3.2 Diseases related to excessive accumulation of bone marrow fat

Since the 1980s, MAT action has been correlated with a negative regulation of bone formation. Accordingly, an increase in MAT accumulation has been associated to a decrease in cortical bone and low bone mineral density (Shen et al., 2007).

However, controversies remain about a full definition of the role of increased levels of MAT that could be directly implicated to regulate bone formation and strength or simply represent a passive response to bone loss or changes in the marrow microenvironment. In any case, it seems indisputable that both MAT accumulation and bone loss occur with aging and are temporally linked in many conditions. For this reason, MAT accumulation may be able to serve as a biomarker to diagnose patients at high risk for future loss of BMD.

1.3.2.1 Aging bone and osteoporosis

Aging is a risk factor for osteoporosis and osteoporotic fractures (Berry et al., 2007). Osteoporosis is significantly associated with morbidity and mortality (Hasserius et al., 2005) and, for this reason should be early diagnosed and carefully managed.

The pathophysiology of age-related bone loss is complex and involve the fat and bone connection. Marrow adipocytes and osteoblasts derive from multipotent common precursors, the BM-MSCs and bone differentiation programs are mutually exclusive. The imbalance in bone turnover seen in age-related bone loss may be due to predominant MSC differentiation into adipocytes and osteoclast oversupply with progressive lack of active osteoblasts. As a main consequence, high levels of bone resorption and insufficient bone formation occur.

Kirkland JL et al. (2002) proposed that MSC are by default programmed to differentiate into adipocytes. In young bone, optimal conditions for osteoblastogenesis include a normal MSC number and confluence, appropriate bone marrow blood supply and appropriate levels of secreted growth factors which are able to prevent MSC to differentiate into adipocytes. In contrast, most of these conditions are lost due to the aging processes and therefore adipogenesis would be the predominant fate of MSC within the bone marrow (Kirkland et al., 2002).

The consequences of age-related fat infiltration in several organs and tissues, including bone marrow, are not completely understood. Different studies have suggested that the bone marrow adipocytes

secretion of fatty acids could have a lipotoxic effect on osteoblasts (Maurin et al., 2000). Accordingly, increased levels of oxidized fatty acids in the aging bone marrow have been reported (Moerman et al., 2004). In summary, several evidence suggests that osteoporosis has the characteristics of a lipotoxic disease in which marrow fat affects both cellular components of bone remodelling, formation and resorption. The paracrine secretion of toxic signals affects osteoblast function and survival thus decreasing bone formation, whereas expression of high levels of PPARγ is associated with increased bone resorption.

1.3.2.2 Lipodystrophy

Lipodystrophy has been linked to mutations involving different genes and it is characterized by a complete or partial loss of body fat. Its characteristics underline the main role of MAT in skeletal maintenance, homeostasis and systemic metabolism. Surprisingly, loss of body fat can result in insulin resistance and diabetes and can be associated to pathologic accumulation of lipid thus suggesting that a normal amount of endocrine adipose tissue is necessary for proper glucose homeostasis. Patients with lipodystrophy have low circulating levels of the adipokines leptin and adiponectin (Parker et al., 2011). Depending on the genetic variant, lipodystrophy occurs as either a failure of adipocyte formation from birth or as a failure of adipocyte maintenance and gradual loss of WAT mass over time.

Patients with congenital generalized lipodystrophy (CGL), in the first decade of life, present with prominent muscularity, accelerated bone growth, advanced skeletal age, cortical thickening, and skeletal sclerosis (Bandeira et al., 2007). After the first 10 years of life (Senior, 1964) growth slows, and adults with CGL are of average stature. Further, aging is often associated with an increase in radiodensity in the appendicular skeleton, accompanied by metaphyseal sclerosis with similar, though less prevalent, changes noted in the axial skeleton. The mechanism is unknown, but this may represent a shift in osteoblast activity or number in response to the absence of MAT that would normally be forming in the appendicular skeleton at this age. In adolescence, a subset of patients with CGL

develop osteolytic cyst-like lesions in the long bones and occasionally the phalanges that are progressive and can result in pathologic fractures (Fleckenstein et al., 1992). Biopsy results have been reported in several cases and generally contain bone fragments, normal hematopoietic marrow, and a proliferation of vascular structures that has been likened to cystic angiomatosis. Given the lack of marrow fat in these patients, the expected development of MAT and conversion of red to yellow marrow during childhood and adolescence fails to occur. Thus, cyst formation at this time may represent a local reaction to the failure of MAT conversion. There are several important observations that can be derived from the human condition. First, neither MAT nor WAT is necessary for basic skeletal patterning and formation. However, loss of WAT in CGL is sufficient to induce skeletal changes ranging from osteosclerosis, advanced bone age, and cortical thickening. These findings suggest a bell-shaped curve in which both too little MAT or too much MAT are not ideal. In addition to effects on the skeleton, the lipodystrophies raise the possibility that MAT may be able to functionally contribute to systemic metabolism, especially in times when WAT is decreased or absent. It is clear that the presence of MAT is not sufficient to completely compensate for the loss of endocrine WAT, however, the prevalence of hyperinsulinemia, high serum triglycerides, acanthosis nigricans (a sign of insulin resistance), and diabetes is lower in patients with CGL4 (MAT present) when compared to CGL1 or CGL2 (MAT absent). This could imply that preservation of MAT decreases the severity of diabetes and insulin resistance. Therefore, MAT preservation may improve insulin sensitivity through secretion of adipokines, including adiponectin.

1.3.2.3 MAT and systemic metabolism: effect of caloric restriction, anorexia nervosa and cancer therapy

The mechanisms explaining the involvement of MAT in the regulation of systemic metabolism are largely unknown. MRI studies have demonstrated that in adult normal weight subjects, MAT ranges from 0.51 to 3.28 L, making up 1.1–43.2% (mean _7%) of the total adipose tissue volume. In anorexic patients, the proportion of MAT is further amplified because MAT, unlike WAT, increases during calories restriction (Devlin et al., 2010). These findings suggest that MAT in humans is largely and

more represented with respect to peripheral WAT and able to exert a significant contribution to metabolism, especially in states of low body fat. Several hypotheses regarding the function of MAT have been suggested. It is possible that MAT promote appetite by a secretion of adipokines, such as adiponectin, that can increase insulin sensitivity and facilitate the metabolic homeostasis during the early stages of starvation. Further, accumulation of MAT may serve as an energy reserve that can be leveraged during the final stages of starvation to provide several additional days of life (Devlin, 2011). There is also the possibility that MAT accumulates in response to loss of bone mineral, perhaps regulated by signals from the osteocyte. To understand this metabolic role, the composition and function of the bone should be taken in consideration. In adults the skeleton, in addition to the role of structural support, is involved in blood cells production. Both functions require significant amounts of energy whereas marrow fat is relatively inert in its energy requirements. In the case of a lowering in energy reserves, a physiologic favourable response could consist in a decrease of the energy allocation to bone turnover and hematopoiesis in favour of MAT formation. As a result, energy reserves increase for critical life-sustaining processes. On the other hand, it could be argued that marrow is a life-sustaining organ and that MAT increases the potential energy reserves to maintain basal hematopoiesis and skeletal function.

Cawthorn et al. (2014) observed that MAT increases during caloric restriction and in different clinical conditions. Further, they showed that adiponectin secretion is greater from MAT than WAT, expecially in caloric restriction conditions or in other adverse states, such as cancer therapy in humans (Cawthorn et al., 2014). To fully explain these observations, the systemic effects of the hormone adiponectin should be considered. Adiponectin promotes insulin sensitivity, fat oxidation, anti-atherogenic, and anticancer effects. Serum adiponectin is also a well-established biomarker for insulin resistance and cardiovascular disease. Accordingly, circulating adiponectin levels are reduced in subjects with metabolic syndrome, probably due to a decreased adiponectin expression and secretion, which may result from mitochondrial dysfunction or aberrantly increased inflammation, hypoxia, or endoplasmic reticulum stress (Ye and Scherer, 2013). Conversely, serum adiponectin increases in lean,

insulin-sensitive states such as with calorie restriction in animals and anorexia nervosa in humans (Combs et al., 2003; Dolezalova et al., 2007).

It has also been demonstrated that both MAT and circulating adiponectin increase during cancer therapy in humans, despite no change in total body fat (Cawthorn et al., 2014). This suggests that MAT might contribute to circulating adiponectin in contexts beyond caloric restriction or anorexia nervosa.

CHAPTER 2: AIM OF THE STUDY

The main objective of this doctoral thesis was to verify the possibility of using olive leaves, a by-product of the processing of EVOO, as a raw material to obtain plant phenolic extracts and to evaluate its possible activities applicable in the biomedical field to be proposed to the food and/or pharmaceutical industry.

According to the starting proposal of the present doctoral thesis, the research activity carried out during the three years has been concentrated in the achievement of the following objectives:

1) Obtain an aqueous extract with a high content of polyphenols and that at the same time is not cytotoxic.

2) Evaluate the potential use of the total extract and its isolated compounds as adjuvants for treatments of diseases by using human cellular models.

To achieve these objectives, the study was developed in several phases: i) production and characterization of olive leaf extract ii) analysis of the anti-inflammatory activity of OLE and its purified active compounds in cellular model of acute (LPS-treated HUVECs and THP-1) and chronic (replicative senescence HUVECs) inflammation; iii) analysis of the anti-adipogenic activity of OLE and its active compounds in human Bone Marrow- derived -MSC, in relation to the fact that the accumulation of bone marrow fat is also closely related to the development of different ARDs.

CHAPTER 3: MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Olive leaves were picked from trees of the Marche region (Italy) cultivated without any kind of chemical treatment , washed with distilled water dried and stored at – 20 °C before use.

3.2 SAMPLE PREPARATION

Olive leaves were grinded with a home blender in order to obtain a fine powder which was immediately used for the extraction.

3.3 EXTRACTION PROCEDURE

5 grams of olive leaf powder were macerated in 50 ml of PBS for 24 hours at 4 °C. The extract obtained was centrifuged for 10 minutes at 1500 RPM to separate coarse particles and then filtered through a 0,22 µm membrane filter to ensure sterility. The extract was stored at – 20 °C before use.

3.4 OLE COMPOSITION

3.4.1 Total Phenolic content

Folin–Ciocalteu assay, with minor modifications, was used to determine the total phenolic content (TFC) of OLE.

Gallic acid at different concentrations was used as standard.

1 ml of Folin-Ciocalteu reagent diluted 1:10 in distilled water was mixed with 0,1 ml of OLE or standard. 0,9 ml of Na_2CO_3 solution (7,5 % w/v) was added precisely after 3 minutes and the reaction has been left in the dark for 90 minutes at room temperature.

The TFC amount was determined by measuring the optical density (OD) at 760 nm using a microplate reader (MPT Reader, Invitrogen, Milano, Italy).

3.4.2 Chemical characterization of OLE

The chemical characterization by HPLC method of the main compounds present in OLE has been carried out thanks to the collaboration with the Department of Health Sciences of the University Magna Graecia of Catanzaro, directed by Professor Antonio Procopio. The single molecules present in OLE, used in this study, were then isolated and purified by the same laboratory.

3.5 MTT ASSAY

Cell viability was assessed through the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine dose-dependent cytotoxic effects of OLE. BM-MSC were seeded in 12-well plates at a density of 5000/cm^2. Cells were treated with different doses in terms of TFC or with the single compounds for 24 hours. Untreated cells were used as control group.

MTT solution (1 mg/ml) was added in each well (10 µl/ 100µl Medium) and Cells were incubated for 2,5 hours. After removing the medium, 400 µl of dimethyl sulfoxide (DMSO) were added to solubilize formazan salt. The measurement of absorbance at OD 590 nm (proportional to the number of viable cells) was performed using a microplate reader (MPT Reader, Invitrogen, Milano, Italy).

Cell viability was calculated as ratio between OD of treated group and control group.

3.6 CELL CULTURE AND TREATMENTS

HUVECs were obtained from a pool of donors and purchased from Clonetics (Lonza, Switzerland).Cells were maintained in humidified atmosphere at 37°C and 5% CO_2 and medium was changed every 48 hours, seeded at a density of 5000/cm^2 in T 75 flasks (Corning Costar, Sigma Aldrich, St. Louis MO, USA) with endothelial basal medium EBM, supplemented with EGM-2 endothelial growth medium (EGM-2) Single Quots (CC-4176, Lonza) (Lonza, Switzerland) containing 0.1% human recombinant epidermal growth factor (rh-EGF), 0.04% hydrocortisone, 0.1% vascular endothelial growth factor (VEGF), 0.4% human recombinant fibroblast growth factor (rh-FGF-B), 0.1% insulin-like growth factor-1 with the substitution of arginine for glutamic acid at position 3 (R3- IGF-1), 0.1% ascorbic acid, 0.1% heparin, 0.1% gentamicin and amphotericin-B (GA-1000), and 2% fetal bovine serum (FBS).

After culture reached the confluence of 80-90%, cells were trypsinized and seeded at lower density.

Characterization of senescent cells

Replicative senescence (RS) was achieved after a number of replicative passages (measured as population doubling-PD).Population doublings (PDs) were calculated using the following formula: $(\log_{10}F - \log_{10}I) \times 3{,}32$ where F represents the number of cells at the end of the passage and I is the number of seeded cells. Cumulative population doublings (cPD) were calculated as the sum of PD changes.

SA-β-Gal activity was evaluated by using a Senescence Detection Kit (BioVision Inc., Milpitas, CA, USA). Briefly, HUVECs were seeded at a density of 5000/cm^2 in 12-well plats and incubated overnight. Later on, Cells were fixed for 15 minutes at room temperature, and then washed twice in phosphate-buffered saline (PBS). Finally, β- Gal staining solution containing 1 mg/ml 5-bromo-4-chloro-3indolyl- β-D-galactopyranoside (X-gal) was added and Cells were incubated overnight at 37°C. the percentage of SA- β-gal positive cells was determined by counting at least 200 cells in each well using light microscopy.

HUVECs were classified as young or senescent based on cPD and on senescence-associated (SA)-β-galactosidase activity. Young cells were characterized by cPD < 15 and SA-β-Gal positive cells < 5%. Senescent cells were characterized by >30 and SA-β-Gal positive cells > 60%.

To confirm these results, we also evaluated $p16^{INK4}$ expression by RT-qPCR and western blot analysis.

Human monocytic THP-1 cells were purchased from ATCC (Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with 10 % heat-inactivated fetal bovine serum, 1 % penicillin/streptomycin, and 1 % L-glutamine (all from Euroclone, Milano, Italy).

Cells treatments

Young HUVECs were seeded at a density of 5000/cm^2 in 6-well plates. After an overnight incubation, medium was changed and cells were pre-treated with OLE (2 hours) or with the single

compounds and then stimulated with lipopolysaccharide (LPS) (500 ng/ml, 3 hours) in order to induce an inflammatory response.

Cells cultured in the same condition but without OLE were used as control to evaluate the anti-inflammatory effect of the extract; unstimulated-LPS Cells were used as control to ensure the inflammatory effect of LPS.

THP-1 were seeded at a concentration of $200x10^3$/mL, left in suspension and treated with the same condition after 24 hours

Senescence HUVECs were also treated with OLE or with the single compounds for 24 hours to evaluate its effect on the basal inflammatory status.

The optimum concentration of OLE and the compounds in the medium was selected according to the results of preliminary experiments performed to establish the cytotoxicity effect of the natural extract.

At the end of the experiments pellets and medium were stored respectively at - 80 and - 20 °C.

Human BM-MSCs were obtained from Lonza (Basel, Switzerland).BM-MSC were seeded at a density of 5000/cm^2 in T 75 flasks (Corning Costar, Sigma Aldrich, St. Louis MO, USA) with Alpha Minimum Essential Medium Eagle (α-MEM) supplemented with 10% FBS (Gibco), 1% di 2mM L-glutamine, 1% 100 U/mL penicillin/streptomycin and maintained in humidified atmosphere at 37°C and 5% CO_2.

Adipogenic differentiation

For adipogenic differentiation experiments, BM-MSC in early passages (3-6) were seeded at a density of $10x10^3/cm^2$ in 6-well plates.

After 24 hours (Day 0), medium was supplemented with 3-isobutyl-1-methylxanthine (IBMX, 0.45 mM), indomethacin (0.2 mM Sigma-Aldrich), dexamethasone (0.5 µM) and human insulin (5 ug/mL) with or without the presence of OLE. BM-MSC cultured in the same condition but without adipogenic induction were considered as controls. At day 14 of differentiation the

experiments were stopped. Pellets and medium were stored respectively at - 80 and - 20 °C.Medium was changed every 3 days for all conditions. Each condition has been replicated 3 times.

3.7 ISOLATION OF RNA AND cDNA SYNTHESIS

Total RNA was isolated using Total RNA Purification kit (Norgen Biotek corp.,#37500, Thorold, ON, Canada), according to datasheet's instructions. RNA was immediately used for cDNA Synthesis or stored at −80 °C until use.

Total RNA quantification was determined by spectrophotometric quantification with Nanodrop ONE (NanoDrop Technologies, Wilmington, DE, USA).

For each sample, cDNA Synthesis was obtained according to PrimeScript™ RT Reagent Kit (Perfect Real Time)' s datasheet (#RR037B). cDNA was stored at -20 °C until use.

3.8 mRNA EXPRESSION BY RT-qPCR

mRNA expression was evaluated by RT-qPCR using TB Green™ Premix Ex Taq™(Cat:RR420A) in a Rotor-Gene Q (Qiagen). mRNA quantification was assessed using the $2^{-\Delta CT}$ method. ACTINE and IPO8 were chosen as housekeeping gene.

3.9 WESTERN BLOT

Cell pellets were lysed by adding RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1 % SDS, 1.0 % Triton X-100, 5 mM EDTA, pH 8.0) with a protease inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA).

Protein quantification was obtained using the Bradford assay.

Each Sample and pre-stained molecular weight marker were separated into 12% SDS-PAGE gel and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). 5% skim milk was used to block the membrane that was then incubated overnight with the primary antibody.

3.10 ELISA ASSAY

At the end of each experiment, medium was collected and stored at −20°C until use. Interleukin-6 (human) ELISA Kit (Invitrogen) and Adiponectin ELISA kit (Cayman chemical) was used to

detect the concentration of human IL-6 an adiponectin released in the medium, according with the manufacturer's instructions.

3.11 OIL-RED-O STAINING

Oil-Red-O staining was used to evaluate lipid droplet accumulation after 14 days.

Cells were washed with PBS and fixed with 4% paraformaldehyde (w/v) for 10 minutes.

Oil-Red-O stock solution was diluted 3:2 with distilled water and then filtered through a 0,22 µm membrane filter.

Cells were covered with the solution for 20 minutes and left in the dark. Later, cells were washed two times with PBS to remove the excess dye and images were obtained using light microscopy. Moreover, in order to obtain a quantification of intracellular dye, Cells were lysed by adding 400 µl of DMSO. The amount of intracellular dye was determined by measuring the OD at 540 nm using a microplate reader (MPT Reader, Invitrogen, Milano, Italy).

3.12 STATISTICAL ANALYSIS

Summarized data are shown as mean of at least three independent experiments ± SD, SEM or frequency (%). Paired sample T test was used for the analysis of real-time PCR, ELISA and densitometric data. Data analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp, Armonk, NY, USA). Statistical significance was defined as a two-tailed p-value < 0.05.

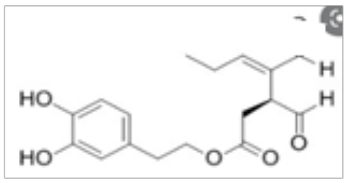
CHAPTER 4: RESULTS

4.1 CHEMICAL CHARACTERIZATION OF OLE

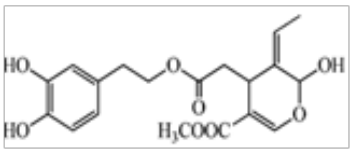
OLE was initially characterised in TPC terms. We obtained the average concentration of 569,5±22,142 µg/mL. Thanks to the collaboration with the Department of Health Sciences, University "Magna Græcia" of Catanzaro, the main compounds and their concentrations in OLE were identified using the HPLC method **(Table 2)**.

OLE T.P.C (Total Phenolic Content): 569,5±22,142 µg/mL

MOLECULES	CONCENTRATION (gr/L)
Oleuropein	140,3±12,2
hydroxytyrosol	67,9±4,8
Oleacein	42,9±6,1
Oleuropein-Aglycone	27,8±4,4



Oleacein



Oleuropein Aglycone

Table 2: Chemical characterization of OLE.

4.2 CELL VIABILITY ASSESSMENT OF HUVEC, THP-1 AND BM-MSC TREATED WITH OLE, OC OR OA

Initially the effect of OLE, OC and OA on viability was studied in all the cellular models used in the study, HUVEC, THP1 and BM-MSC, by MTT assay.

For this purpose cells were treated with different doses of each compound and OLE and the concentrations that would ensure al least 85% were chosen for the study. **Figure 7** shows the results obtained. Concentrations of 5 Mm for OC and OA and 4 µg/Ml for OLE were selected because of their efficacy and compatibility with all three cell models used in this study.

In this preliminary phase inflammation marker expression under LPS stimulation was also tested in HUVEC and THP-1.

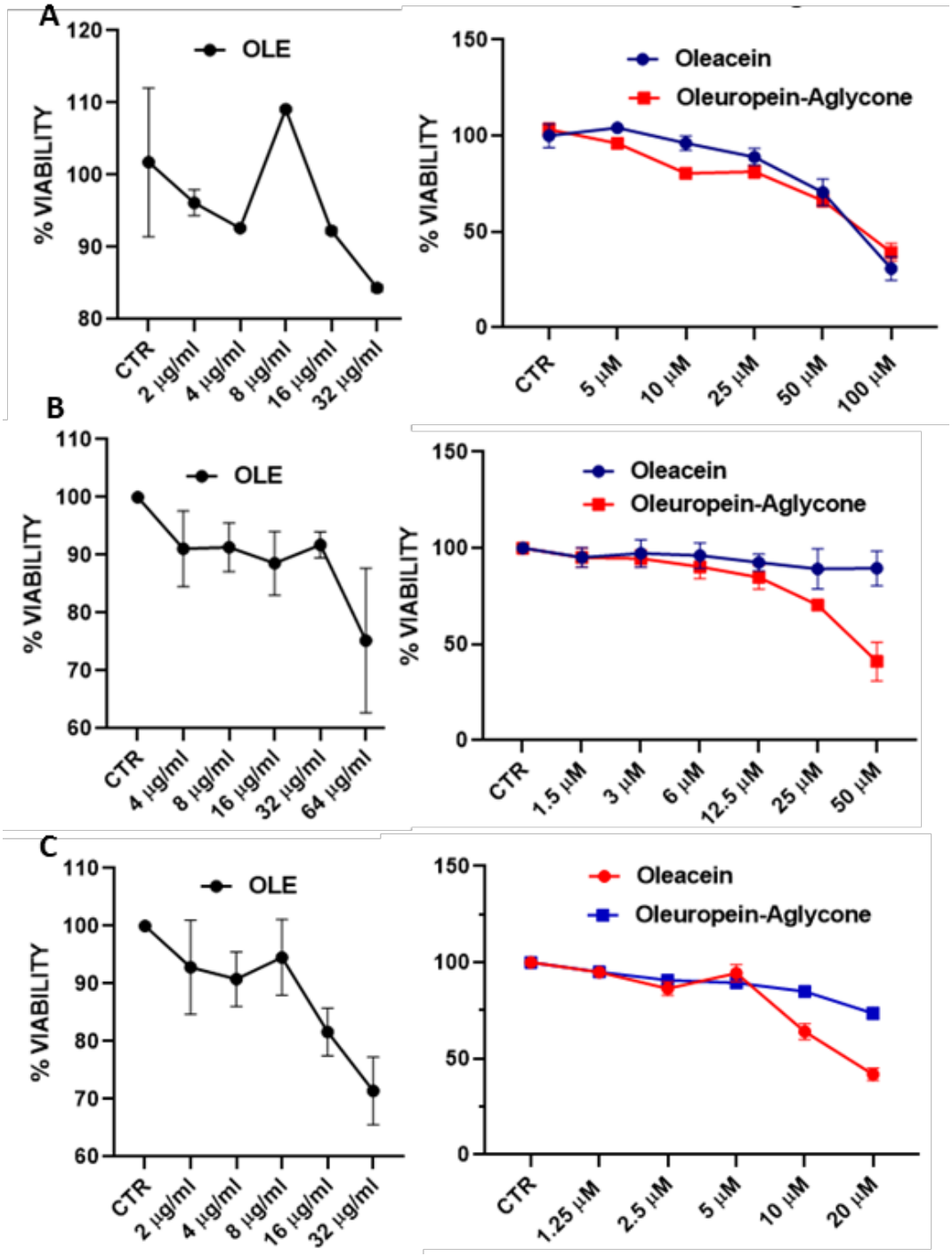


Figure 7: *Effect of OLE, OC and OA on yHUVECs (**A**),THP-1 cells (**B**) and MSCs (**C**)*. Cells were treated with different concentrations of OLE, OC and OA for 24 h. The viability of cells was determined by MTT assay. The results are expressed as a percentage of cell viability normalized to the viability of DMSO treated cells (CTR) and presented as mean value ± SEM from three independent biological experiments.

4.3 STUDY OF THE ANTI-INFLAMMATORY EFFECT OF OLE, OC AND OA

4.3.1 Effect of OLE, OC and OA in LPS-induced inflammation

Endotoxins are among the most potent bacterial immune cell activators. LPSs activate monocytes/macrophages after binding to the cell's CD14, which transfers the molecule to the Toll-like 4 (TLR4)/myeloid differentiation protein 2 (MD2) complex (Schildberger et al., 2013). This mechanism initiates a series of reactions that have been described in previous chapters. For these reasons, we decided to evaluate the potential anti-inflammatory activity of OLE, OC and OA on LPS-stimulated HUVEC and THP-1 cells .. HUVECs were pre-treated with OLE or OC or OA and the effect was studied analysing the expression modulation of IL-1β, IL-6, TNF-α, IL-8 cytokines of ICAM-1 and VCAM adhesion molecules and the release of IL-6. OLE and OC significantly decreased the expression levels of all cytokines tested , of the adhesion molecules Fig. x) and inhibited of the concentration of IL-6 released into the conditioned medium **(Figure 8)**. OA was effective on IL-8 modulation and and IL-6 release, but there was no significant modulation of the other cytokines and adhesion molecules tested was observed.

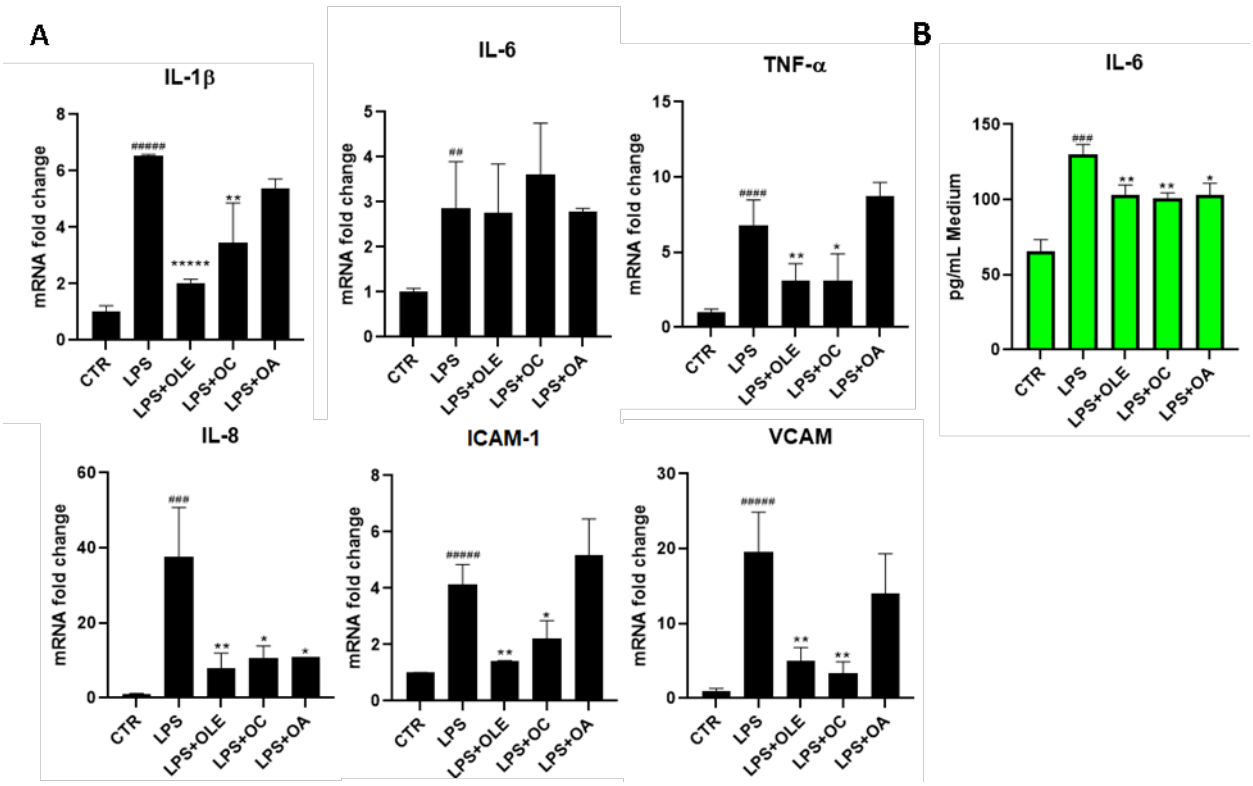


Figure 8: *Anti-inflammatory activity of OLE, OC and OA in LPS-stimulated HUVEC cells*. **A** IL-1β, IL-6,TNFα, IL-8, ICAM-1 and VCAM mRNA expression. Data were reported as fold change vs untreated LPS-stimulated HUVEC according to 2-ΔCt method and using actine as housekeeping. **B** Concentration (pg/ml) of IL-6 in the culture medium of untreated and treated HUVEC cells; histograms represent the mean of three different experiments ± SD. Paired *t* test, *p < 0.05, **< 0.01 versus HUVEC+ LPS; ## p < 0.01, ###< 0.001, ###<0.0001 versus CTR (LPS-untreated HUVEC)

In THP-1 cells in addition to cytokines was analyzed the mRNA expression of NF-kB and the chemokine MCP-1 Pre-treatment with OLE and OC before LPS stimulation, seems to modulate the activation of this pathway. OLE seems to be more effective than OC and OA: with OLE mRNA expression of all markers analysed was significantly reduced. OC and OA did not inhibited significantly the expression of IL-1 nor of IL-6 and MCP-1 but was effective on NF-kB expression both at mRNA and protein level and strongly on TNF-a which is modulated to values similar to those of unstimulated controls **(Figure 9)**.

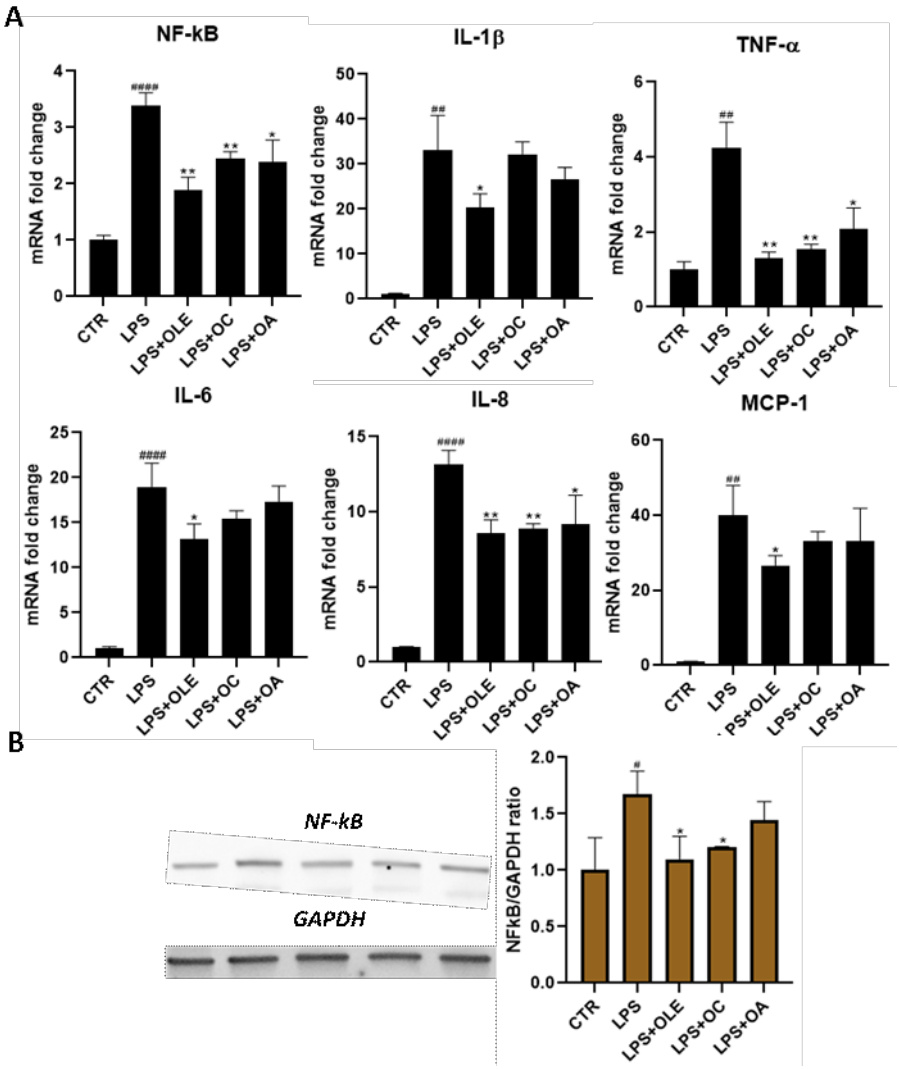


Figure 9: *Anti-inflammatory activity of OLE, OC and OA in LPS-stimulated THP-1 cells*. A NF-kB, IL-1β, IL-6 ,TNFα, IL-8, MCP-1 mRNA expression. Data were reported as fold change vs untreated LPS-stimulated THP-1 according to 2-ΔCt method and using actine as housekeeping. B NF-kB protein expression was analysed by western blotting and densitometric analysis. Data were normalized using GAPDH as internal control and reported as fold change of LPS-stimulated THP-1 vs untreated cells with OLE, OC AND OA; histograms represent the mean of the mRNA or concentration detected in three different experiments ± SD. Paired *t* test, *p < 0.05, **< 0.01 versus HUVEC+ LPS; ## p < 0.01, ###< 0.001, ###<0.0001 versus CTR (LPS-untreated HUVEC)

4.3.2 Effect of OLE, OC and OA on senescent cell pro-inflammatory state

Senescent cells are characterised by the so called SASP, a secretory phenotype associated to senescence. Secreted factors, i.e. pro-inflammatory cytokines, determines the onset of a chronic, low-level state of systemic and sterile (in the absence of overt infection) inflammation, called

inflammaging which represents a pervasive feature of human aging and probably one of its major causes. In this study HUVECs were used as a model of replicative senescence in order to study the role of OLE, OC and OA in modulateing this pro-inflammatory condition. Senescence was evaluated by SA- β-Gal activity and $p16^{ink4a}$ protein expression. HUVECs were defined as young (yHUVECs) with Population Doublings (Cpd) < 9 and SA-β-Gal positive cells < 5% whereas cells were considered senescent (RS-HUVECs) at . Cpd > 18 with a percentage of SA-β-Gal positive cells > 60% **(Figure 10)**.

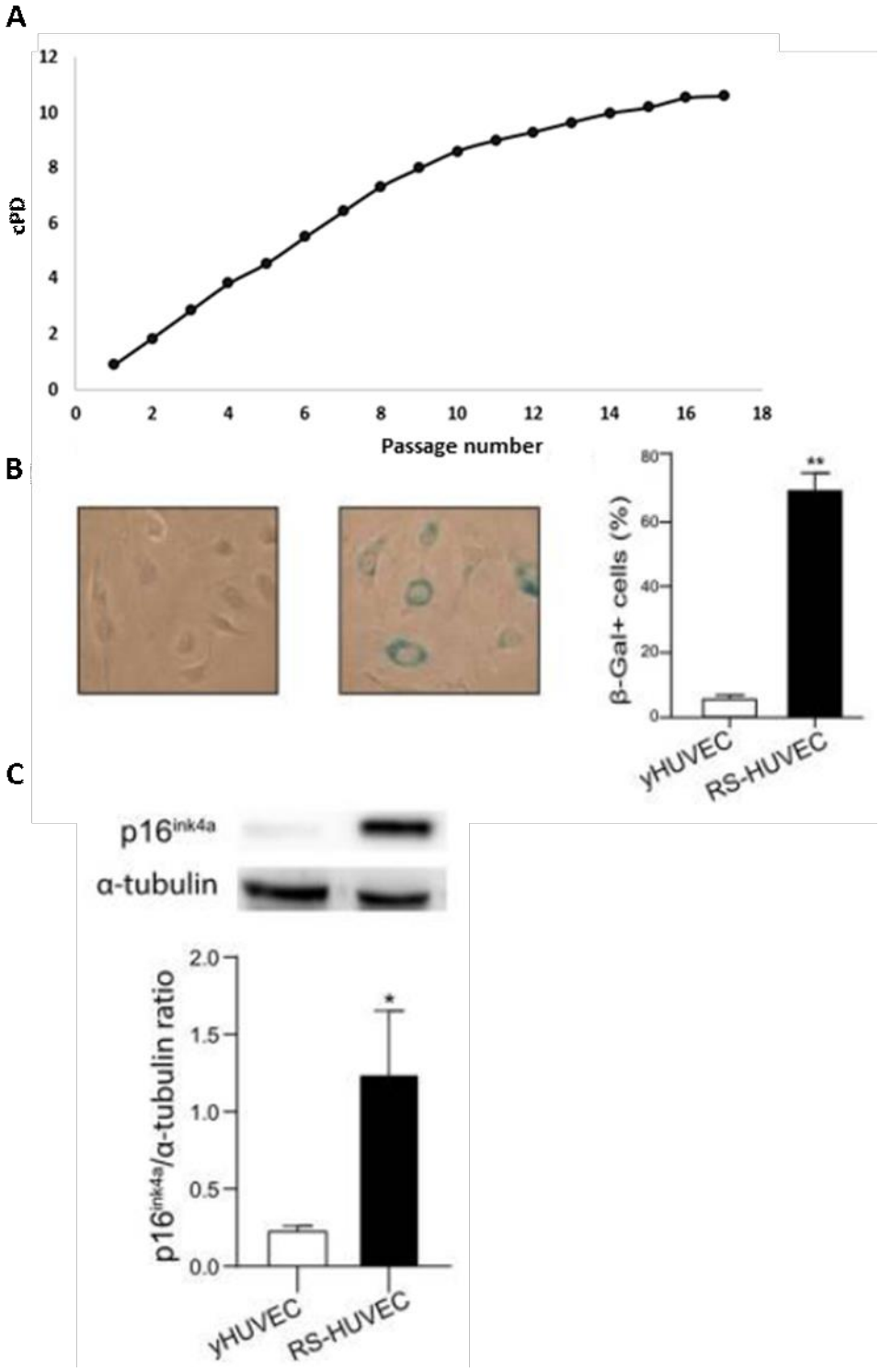


Figure 10: *Characterization of endothelial cell senescence* . **A** Growth curve of a pool of HUVECs—Y-axis: Cumulative Population Doubling (Cpd); X-axis: cell passages from P1 to P18; **B** representative pictures of SA-β-Gal staining of young and senescent HUVECs and % of SA- β-Gal positive cells. Cell cultures with SA-β-Gal < 10% were considered young (Yhuvec), while those with SA- β-Gal > 60% were identified as senescent cells (RS-HUVECs); **C** western blot and densitometric analysis of $p16^{ink4a}$ in RS-HUVEC. Histograms represent the mean of the protein level and the relative expression measured in three different experiments ± SD. Paired *t* test, *p < 0.05 versus Yhuvec. *RS* replicative senescence

As shown in **Figure 11** RS-HUVECs express higher level of IL-1β, IL-6 and TNF-α, IL-8, ICAM and VCAM compared to younger cells. As observed in both LPS-stimulated yHUVECs and THP-1, treatment of RS-HUVECs with OLE or OC or OA for 24 hours significantly reduces the expression of these inflammatory markers, however, the two active compounds, the one that worked the most is OC : in particular OC was able to reduce strongly all the markers tested. And the release of IL-6 except for IL-8. In the case of ICAM-1 and VCAM, the values return almost to the level of young cells. Similar results were obtained with OLE and OA.

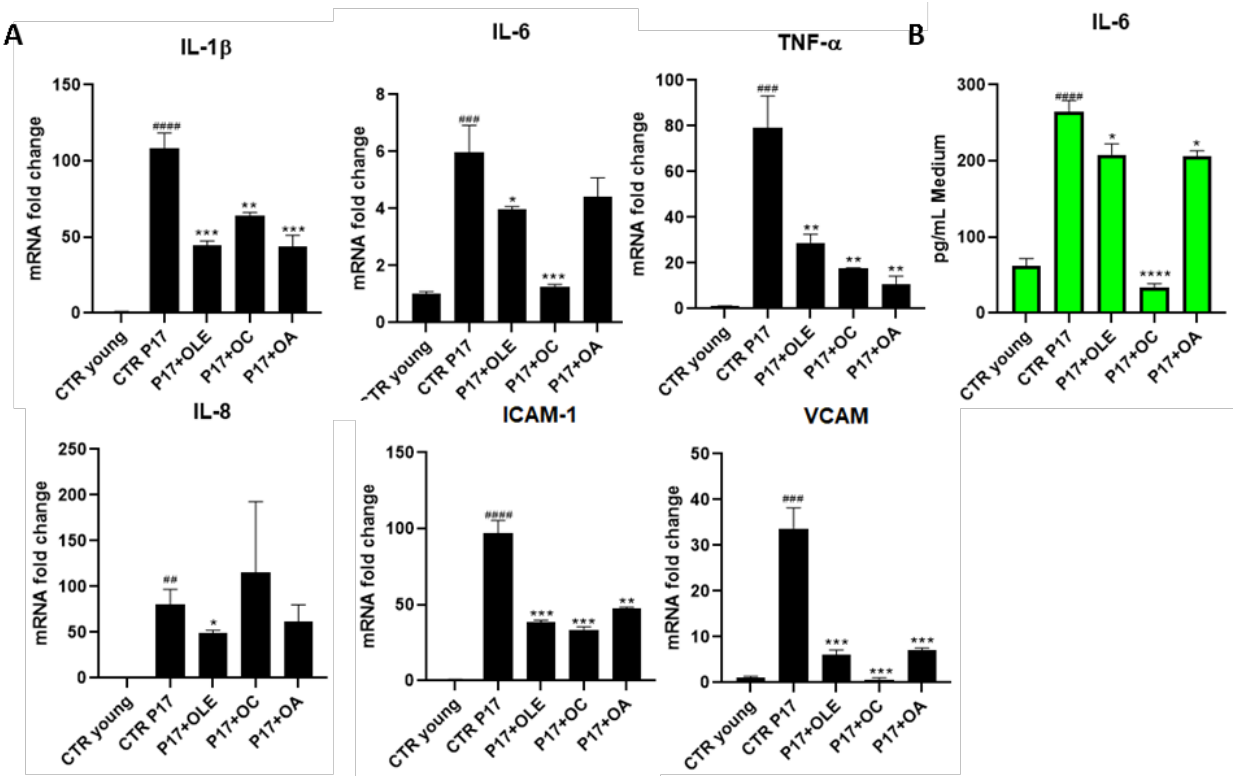


Figure 11: *Anti-inflammatory activity of OLE, OC and OA in RS-HUVECs* **A** IL-1β, IL-6 ,TNFα, IL-8, ICAM-1 and VCAM Mrna expression in RS-HUVECs treated for 24 hours with *OLE, OC and OA*. Data were reported as fold change vs CTR (Yhuvec) according to 2-ΔCt method and using actine as housekeeping. **B** Concentration (pg/ml) of IL-6 in the culture medium of untreated yHUVECs and RS-HUVECs and treated RS-HUVECs; histograms represent the mean of three different experiments ± SD. Paired *t* test, *p < 0.05, **< 0.01, ***< 0.001 versus untreated Shuvec; ## p < 0.01, ###< 0.001, ###<0.0001 versus CTR (Yhuvec).

4.4 STUDY OF THE EFFECT OF OLE, OC AND OA ON ADIPOGENIC DIFFERENTIATION

During the last decade several reports have suggested that olive leaf extract can be effective in treating obesity by Modulating the Expression of Molecules Involved in Adipogenesis and inducing Thermogenesis pathways (Shen et al., 2014; Palmeri et al., 2016). Adipose tissue generates a chronic low-grade inflammation which is, in turn, mechanistically linked to metabolic disease and organ tissue complications in the overweight and obese organism (Kawai et al., 2021). Since this chronic inflammation is initiated and sustained over time by dysfunctional adipocytes that secrete inflammatory adipokines, we decided to study the role of olive leaf active compounds on reducing adipogenesis by using human Bone Marrow derived Mesenchymal Stromal Cells (MSC), a cellular model largely used in our laboratory. For our purpose MSC were induced to differentiate in adipogenic medium alone or integrated with OLE for 14 days.

We initially analysed OLE effect both in undifferentiated cells and in the differentiated ones by quantifying lipid droplets accumulation by Oil Red O-stained staining Quantification showed that constant administration of OLE during differentiation strongly reduces the production of lipid droplets and therefore the acquisition of the adipocyte phenotype **(Figure 12)**.

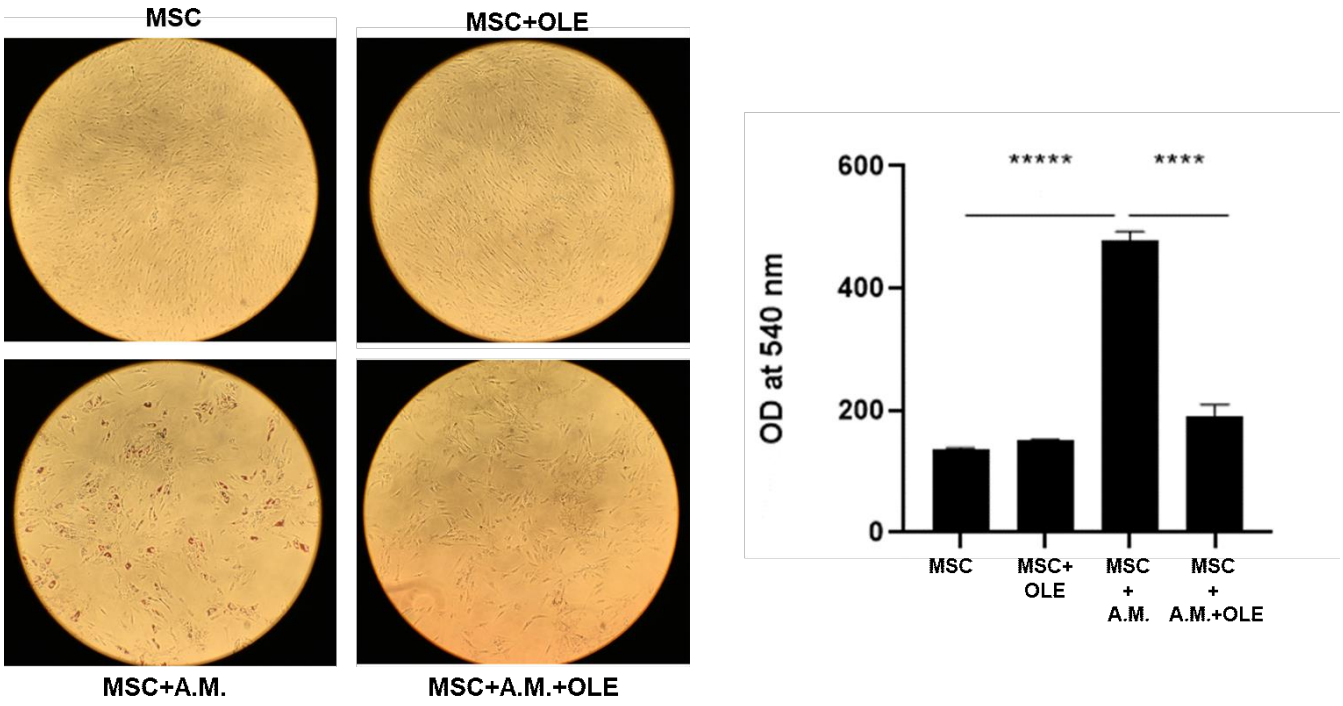


Figure 12: *Lipid droplets accumulation measured in undifferentiated MSC or induced to differentiate in the presence or absence of OLE.* Lipid droplet accumulation was analysed by Oil Red Staining and adipogenesis measured as the relative absorbance (OD 490 nm) of Oil Red at day 14 after inducing adipogenesis as described in Section "Materials and Methods" . All values are expressed as mean ± SEM of detected in three different experiments ± SD (****$P < 0.0001$). A.M. Adipogenic Medium.

Subsequently, in order to investigate which genes involved in MSC adipogenesis can be modulated by the extract, we analysed the expression of those who play a leading role in the process and also of those who are active in differentiated adipocytes. . In particular we observed that OLE was able to reduce with high efficiency the mRNA levels of all genes upregulated by adipogenic medium alone: the transcription master of adipogenesis PPAR-γ, those responsible for the formation of mature adipocytes FABP4 and Adiponectin and finally Perilipin (PLIN-1), associated with lipid droplet formation . The only exception is leptin, whose reduction is not significant but for which a trend in line with the other markers is observed **(Figure 13)**.

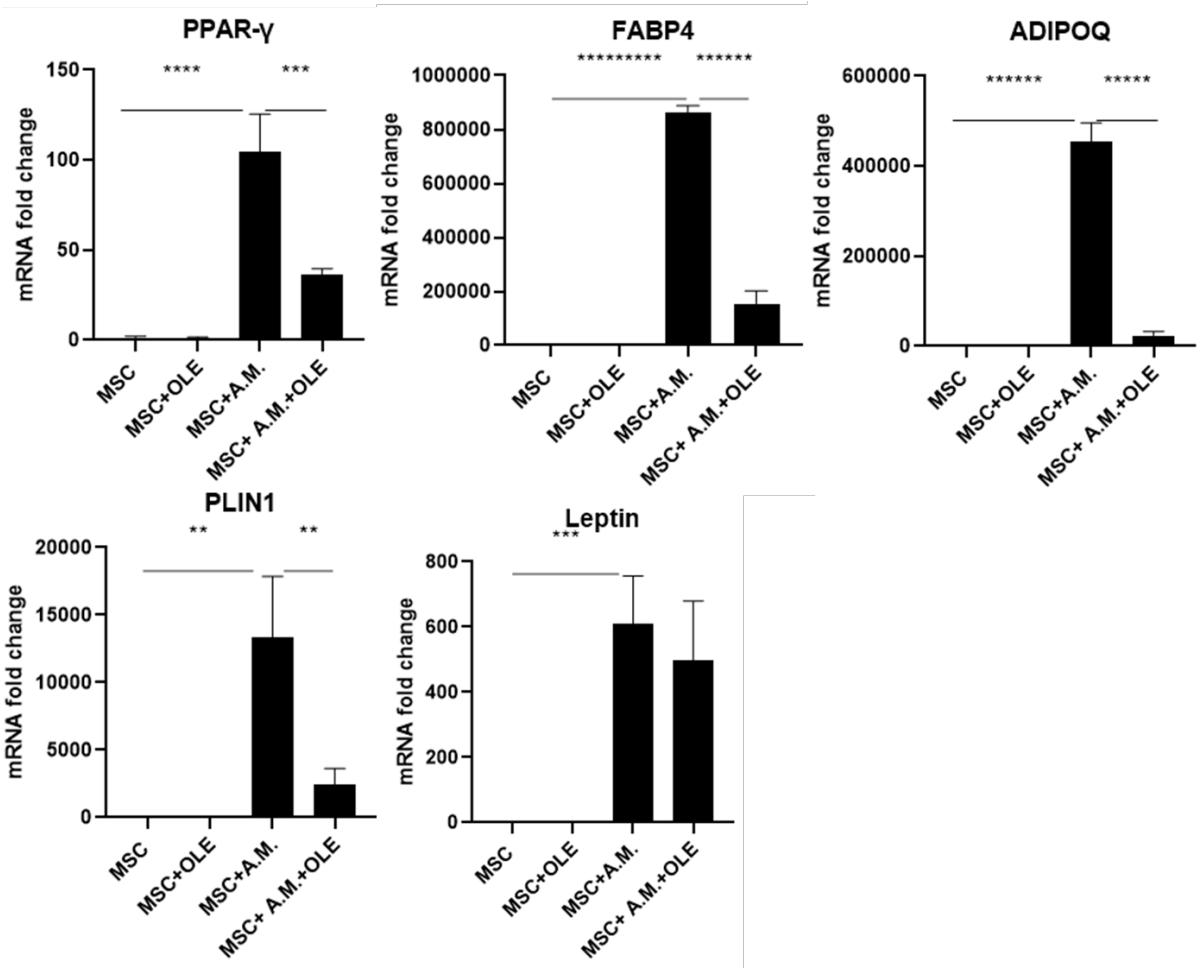


Figure 13: *Anti adipogenic activity of OLE on MSC induced to adipogenic differentiation.* PPARγ, FABP4, ADIPOQ, PLIN1 and Leptin mRNA expression. Data were reported as fold change vs undifferentiated MSC according to 2-ΔCt method and using IPO8 as housekeeping. histograms represent the mean of the mRNA expression analysed in three different experiments ± SD. Paired t test, *$p < 0.05$, **< 0.01, ***< 0.001, ****< 0.0001 etc versus undifferentiated MSC or versus differentiated MSC.

Results on genes expression were confirmed by protein expression analyses of PPAR-γ by western blotting and and by quantification of adiponectin release into the medium by ELISA **(Figure 14)**.

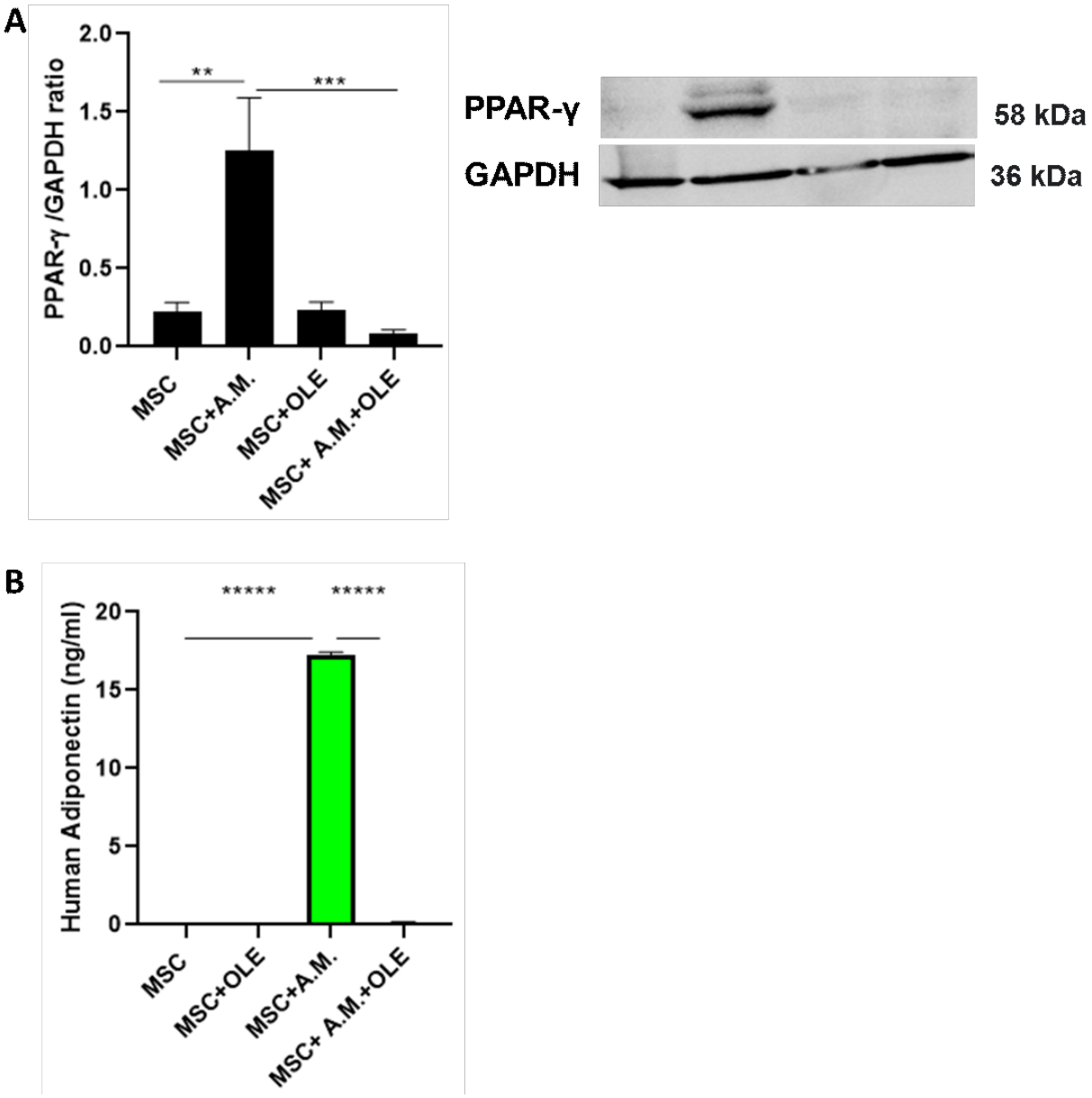


Figure 14: *Effect of OLE on PPARγ and adiponectin production.* **A** wstern blotting and densitometric analysis of PPARγ in undifferentiated and differentiated MSC in the absence or presence of OLE. **B** Adiponectin concentration (ng/ml) in the culture medium of differentiated or undifferentiated MSC with or without of OLE. Histograms represent the mean of the protein level and the relative expression measured in three different experiments ± SD. Paired *t* test, **p < 0.01; ***p< 0.001.

At the moment, research is continuing with the aim of identifying the main OLE molecules that exert the anti-adipogenic action. Due to the good results obtained with OC and OA in i the experiments performed to study their anti-inflammatory effect, we are testing these molecules in adipogenesis.

Results obtained so far are showing that OA is more effective than OC in reducing the mRNA expression of adipogenesis-related genes be in fact, OA reduces significantly all the genes tested, whereas OC seems not to be significantly effective in modulating FABP4 and PLIN1; nevertheless, their modulation shows a trend in line with the other markers analysed **(Figure 15)**. More analyses are needed to definitely confirm these encouraging results, in particular those concerning protein expression analyses.

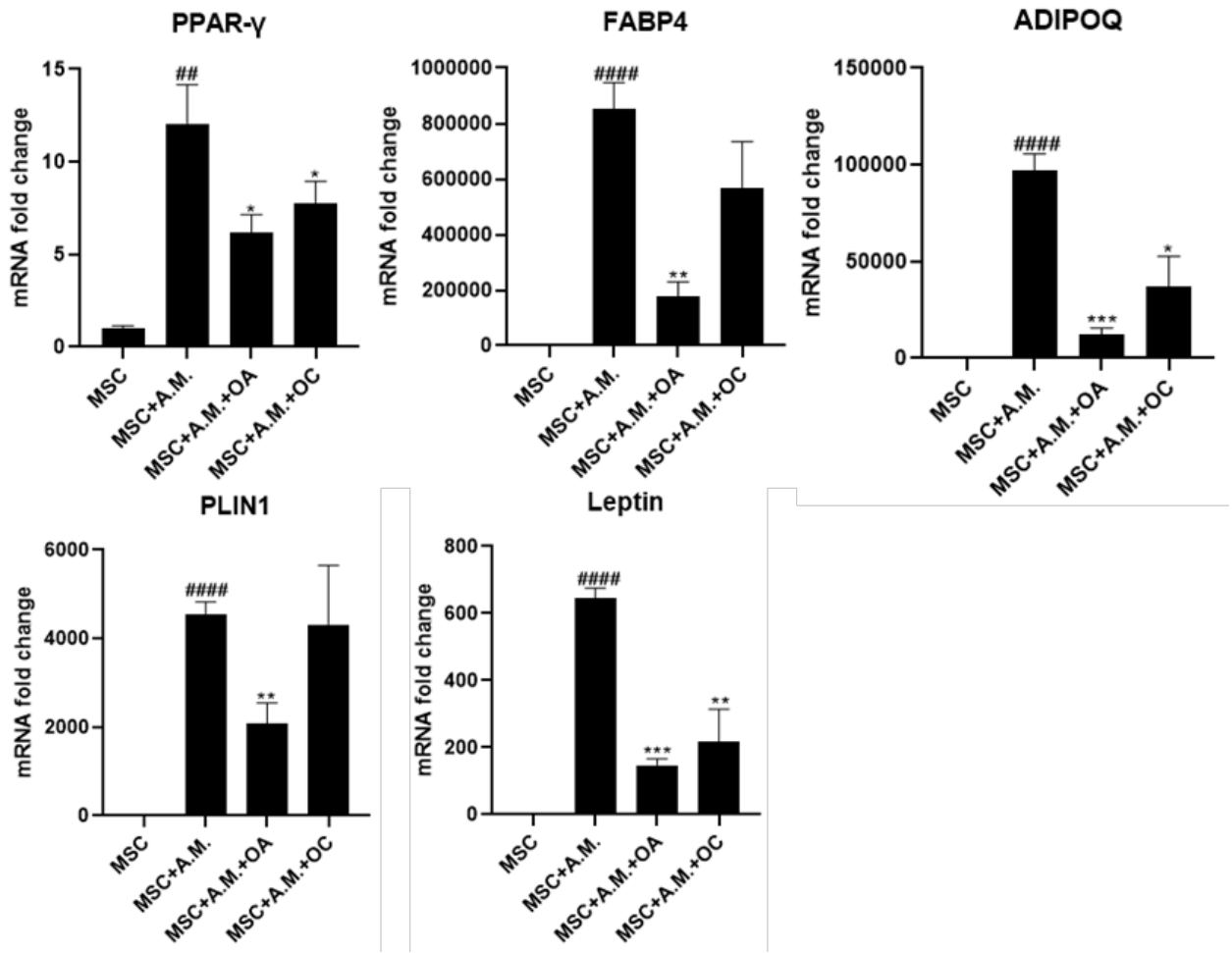


Figure 15: *Anti adipogenic activity of OC and OA*. PPARγ, FABP4, ADIPOQ, PLIN1 and Leptin mRNA expression were analysed in MSC induced to differentiate with adipogenic medium alone or supplemented with OC or OA. Data were reported as fold change vs undifferentiated MSC according to 2-ΔCt method and using IPO8 as housekeeping. Histograms represent the mean of the mRNA expression detected in three different experiments ± SD. Paired t test, *p < 0.05, **< 0.01, ***< 0.001, ****< 0.0001 etc versus undifferentiated MSC or versus differentiated MSC.

CHAPTER 5: DISCUSSION

The objectives of this study was to demonstrate that olive leaf extracts and its active compounds are useful as anti-inflammatory agents and that they could be used in acute inflammatory conditions, i.e. sepsis or viral infections characterized by a cytokine storm, and also in chronic ones such as inflammaging or inflammation due to adipocity. The idea to study the properties of olive leaf extract and of several compounds was born in collaboration with Prof. Antonio Procopio of Magna Graecia University, one of the greatest experts in the world of olive properties. He developed a low environmental impact methodology for the extraction of secoiridoid phenols and in particular of biocompatible oleuropein (Ole) from olive leaves, and its hydrolysis to obtain Ole-aglicone (OA) without the use of solvents and reagents which are dangerous to human health. Furthermore, he developed a simple and eco-sustainable semi-synthetic process to produce oleacein (OC) from OLE, by decarboxylation of Krapcho promoted by microwaves in an aqueous environment (Costanzo et al., 2018).

The anti-inflammatory properties of a group of secoiridoid phenols from the olive tree have aroused enormous interest in the scientific community. An initial study published in 2005 in NATURE recognised that oleocanthal, responsible for the spicy flavour of EVOO, had COX inhibition activity similar to that of ibuprofen (Beauchamp et al., 2005). A year later, the PREDIMED clinical trial demonstrated the anti-inflammatory effect of the Mediterranean diet supplemented with EVOO (Nediani et al., 2019), and in particular the modulation of biomarkers associated with systemic inflammation and those involved in the recruitment of inflammatory cells (chemokines and adhesion molecules of endothelial and monocytic cells) (Estruch, 2010). Similar effects were also attributed to olive leaf extract (Boss et al., 2016b; Lockyer et al., 2017b; Burja et al., 2019), which is effective in sepsis, tissue repair (Gong et al.; Koca et al.; de Paula Ramos et al., 2019)and prevention of respiratory diseases (Somerville et al., 2019). Attention was then focused on the possibility of using individual phenolic compounds from the olive tree as promising anti-inflammatory agents, demonstrating both *in vitro* and *in vivo* the efficacy of oleocanthal, oleuropein and hydroxytyrosol in modulating

inflammatory parameters and reducing cytokine synthesis (Impellizzeri et al., 2011; Takeda et al., 2013).

In addition, it is very important to take into account that olive leaves are a waste material in the EVOO industry and, for this reason, available at low cost. The possibility of using waste products is part of the concept of sustainable development which has among its principles the need to move from a linear production model, based on extraction, transformation, production, consumption and waste to an economic model based on circularity, i.e. on the possibility not only to limit the use of materials and energy as much as possible, but also and above all to pay extreme attention to the reduction of waste, minimizing losses at every stage of the production process. As an evidence of the importance attributed today to the development of the circular model, it should be noted that it represents one of the main themes of the 2030 Agenda for Sustainable Development launched by the United Nations. According to what is indicated in the platform of measures on the circular economy presented by the European Commission, by 2030 procedures will have to be in place to recycle high percentages of waste products in various sectors, including agriculture and food industry

Therefore, in view of these considerations, our study focused on assessing the properties of OLE and its most active compounds within it. Specifically, the anti-inflammatory properties were first evaluated on two cellular models of acute inflammatory response, LPS-treated HUVECs and THP-1. OLE and OC and OA, were able to reduce the expression of the main cytokines, chemokines and adhesion molecules that are overexpressed following endothelial and monocytic activation by one of the most widespread bacterial antigens, the LPS.

In addition, OLE, OC and OA were also evaluated on replicative senescence HUVECs, characterised by a SASP phenotype. The SASP, one of the main features of Cellular Senescence, is a crucial contributor to inflammaging, and the ability of OLE and individual compounds to inhibit the synthesis and release of pro-inflammatory cytokines, and furthermore the expression of adhesion molecules and thus the recruitment of leucocytes, may represent a strategy to preserve the health of the

endothelium and of tissues from dysfunction. In fact, endothelial dysfunction is considered one of the main causes of the development of the most common ARDs, and controlling the spread of this stage in the endothelium can help to prevent and delay this from happening, ensuring what is known as 'healthy aging'.

These results suggested to us to submit the project "OLEA-ACT, Olive Leaf Extract Active Ingredients-Against Covid-19 Activity" to the call FISR 2020 (MUR) aimed at studying the potential use of active OLE compounds to reduce the cytokine storm observed during the SARS-Cov2 virus infection. The project was approved and financed by the MUR in June 2021 (OLEA-ACT project (IP_ 02077). In Humans, the virus infects the airways and causes pneumonia which, in a limited number of people, can evolve towards a worsening, potentially lethal clinical condition. In fact, this clinical picture is not only caused by the viral infection itself, which leads to the death of the cells expressing ACE2 (used as a receptor by the viral protein SPIKE) (Hoffmann et al., 2020), but also by the intense inflammatory response of the host, characterised by the hyper activation of epithelial, endothelial cells and alveolar macrophages (Hoffmann et al., 2020; Tay et al., 2020). In the final stage of the disease, this response becomes uncontrolled and is characterised by the so called cytokines storm, with serious armful local (acute respiratory distress syndrome and bacterial and fungal superinfections) and systemic effects and multi organ failure. Therefore, the cytokines storm represents a negative prognostic factor, and is believed to be the direct cause of death from COVID-19 (Tay et al., 2020). Timely targeted therapeutic strategies, based on drugs capable of blocking the cytokine cascade and controlling the activation of the cells involved is therefore necessary. In this context, the proposal of natural molecules active against cytokines storm, which have already been used on humans, may be a potential therapeutic option.

Another major aspect concerning the development of ARDs is the excessive accumulation of i) adipose tissue, which is responsible of a low but chronic and systemic inflammatory condition and ii) Marrow Adipose Tissue. For example, the shift in the differentiation of bone marrow MSCs to favour

adipocyte lineage over osteoblast lineage can directly contribute to imbalances in bone formation/reabsorption and ultimately lead to bone loss. In addition to this, as described at length in this thesis, excessive accumulation of MAT is closely related to several other diseases such as lipodystrophy, anorexia nervosa and also is a consequence of cancer therapy. In this study we tried to understand if OLE could somehow address the differentiation potential of MSCs, and in particular inhibit adipogenic differentiation. OLE administration strongly inhibited the expression of several pro-adipogenic markers, first of all PPARγ, the master transcription factor of adipogenesis. Accordigly OLE inhibited the accumulation of lipid droplets accumulation within the cell. The promising preliminary results obtained with OC and OA also prompted us to continue the study and focus on possible formulations using these two molecules alone or in combination. Furthermore, by virtue of what has been described in the literature, it will also be interesting to evaluate the ability of OLE and its compounds to direct the differentiation potential of MSCs towards the formation of osteoblasts and thus to investigate the possibility of using a supplement in the prevention of bone loss.

In conclusion, the possibility of finding adjuvant treatments to traditional ones seems to be an extremely promising field of research for several reasons. In the field of diseases in which the inflammatory component plays an important pathogenetic role, the profile of side effects of non-steroidal and steroidal anti-inflammatory drugs is well known, especially in conditions requiring prolonged treatment. The use of natural substances, i.e. active OLE compounds, should obviously not be seen as a replacement proposal: their validity should be carefully assessed with targeted studies in order to test the hypothesis of an adjuvant role that could lead to a more favourable modulation of traditional therapies, for example by reducing the doses or the time of administration of traditional drugs while maintaining their efficacy and making traditional therapies more favourable.

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CHAPTER 7: FUNDED PROJECTS AND PUBLICATIONS

Domanda

Codice: **FISR2020IP_02077**

Proponenti (Art. 2 Comma 1) e Costi (Art. 3)

Proponenti	A) Personale	B) Strumenti	C) Consulenze	D) Generali	E) Esercizio	Totale
Università degli Studi "Magna Graecia" di CATANZARO	8.000,00	0,00	0,00	4.800,00	27.200,00	**40.000,00**
Università Politecnica delle MARCHE	8.000,00	0,00	0,00	4.800,00	27.200,00	**40.000,00**
Totale	**16.000,00**	**0,00**	**0,00**	**9.600,00**	**54.400,00**	**80.000,00**

Ambito e Area (Art. 1 Commi 2 e 4)

Area Life Sciences

Ambito Risposta all’emergenza, sviluppando soluzioni relative alla fase di espansione della pandemia

Idea progettuale (Art. 2 Comma 5)

Acronimo OLEA-ACT

Durata in mesi 6

Titolo IT Principi Attivi dell'Olivo Contro il COVID-19

Titolo EN Olive Leaf Extract Active Ingredients-Against Covid-19 Activity

Descrizione della proposta (IT) Il virus SARS-CoV2 ha suscitato un improvviso allarme globale a causa della potenziale mortalità del COVID-19, patologia ad esso associata. Negli esseri umani, il virus infetta le basse vie respiratorie e causa una polmonite che, in un numero limitato di persone, può evolvere verso un quadro clinico ingravescente, potenzialmente letale. Tale quadro non è dovuto soltanto all’infezione virale stessa, che causa la morte delle cellule esprimenti ACE2, usato come recettore dalla proteina virale Spike (1), ma anche all’intensa risposta infiammatoria dell’ospite, caratterizzata da iper-attivazione delle cellule epiteliali, endoteliali e macrofagi alveolari (2-3). Nell’ultimo stadio della malattia, tale risposta diventa incontrollata ed è caratterizzata dalla cosiddetta tempesta citochinica, con gravi effetti dannosi non solo locali (Sindrome da Distress Respiratorio Acuto e superinfezioni batteriche e fungine) ma anche sistemici con insufficienza multiorgano. La tempesta citochinica pertanto rappresenta un fattore prognostico negativo ed è ritenuta la causa diretta della morte per COVID-19 (4). Strategie terapeutiche tempestive mirate, fondate su farmaci capaci di bloccare la cascata citochinica e controllare l’attivazione delle cellule coinvolte nella loro produzione, migliorerebbero sicuramente l’esito clinico della malattia. Poiché l’emergenza sanitaria e il contenimento della diffusione del coronavirus esigono tempi di risposta molto più brevi di quelli spesso richiesti dall’approvazione di nuovi farmaci, la proposta di molecole naturali attive contro la tempesta citochinica, e già utilizzate sull’uomo, può rappresentare una potenziale opzione terapeutica. Negli ultimi anni, le proprietà antiinfiammatorie di un gruppo di fenoli secoiridoidi dell’olivo ha suscitato un enorme interesse nella comunità scientifica. Un primo studio, apparso nel 2005 su Nature, ha riconosciuto all’oleocantale, responsabile del sapore piccante dell’olio extra vergine di oliva (EVOO), un’attività di inibizione della COX simile a quella dell’ibuprofene (5). L’anno dopo, lo studio clinico PREDIMED dimostrava l'effetto anti-infiammatorio della dieta mediterranea (MD) integrata con EVOO (6) ed in particolare la modulazione dei biomarcatori associati all’infiammazione sistemica (PCR, IL-6) e di quelli coinvolti nel reclutamento di cellule infiammatorie (chemochine e molecole di adesione delle cellule endoteliali e monocitiche) (7). Simili effetti sono stati attribuiti anche all’estratto di foglie d’olivo (8-10), efficace nella sepsi, nella riparazione tissutale (11-13) e nella prevenzione delle patologie delle vie respiratorie (14). L’attenzione si è quindi focalizzata sulla possibilità di utilizzare i singoli composti fenolici

dell'olivo come promettenti agenti antinfiammatori, dimostrando sia in vitro che in vivo, l'efficacia dell'oleocantale, dell'oleuropeina e dell'idrossitirosolo nel modulare parametri infiammatori e ridurre la sintesi di citochine (15-17). L'unità di ricerca dell'Università Magna Græcia, UMG (UO1) diretta dal Prof Procopio è impegnata da più di un decennio nell'analisi, separazione, manipolazione semisintetica e indagine delle potenzialità farmaco-biologiche dei composti fenolici dell'olio d'oliva ai quali l'EFSA ha riconosciuto di contribuire alla protezione dei lipidi del sangue dallo stress ossidativo (18) (Figura). Presso il Green Chemistry Laboratory (G.C.L.) di UMG è stata sviluppata una metodologia a basso impatto ambientale per l'estrazione dell'oleuropeina (Ole) dalle foglie dell'olivo (prodotto di scarto della lavorazione dell'olio) e la sua successiva idrolisi per ottenere Ole-aglicone (Ole-A). I processi utilizzati, oggetto del brevetto internazionale WO2008136037A2, consentono di ottenere i prodotti Ole e Ole-A in maniera totalmente biocompatibile evitando l'uso di solventi e reagenti pericolosi per la salute umana. Più recentemente, G.C.L. ha sviluppato un processo semi-sintetico semplice ed ecosostenibile per produrre oleaceina (Oln) a partire dall'Ole mediante la decarbossilazione di Krapcho promossa dalle microonde in ambiente acquoso (19). Le proprietà antiinfiammatorie dei secoiridoidi dell'EVOO ottenuti mediante l'applicazione di queste metodologie sono oggetto di studi sia in vitro che in vivo (20-24). La Prof Rippo, responsabile dell'Unità di ricerca di Patologia Sperimentale dell'Univ Politecnica delle Marche (UO2) è impegnata da diversi anni nello studio dell'infiammazione cronica sistemica caratteristica dell'invecchiamento (inflamm-aging), delle patologie età-associate, nella ricerca di biomarcatori di infiammazione sistemica, ponendo particolare attenzione al ruolo delle cellule endoteliali e dei monociti/macrofagi (25-28). Con il presente progetto, nella sua fase 1, l'UO1 metterà a disposizione dell'UO2 Ole, Ole-A, Oln, HTyr e Tyr per testare in vitro la capacità di modulare l'attivazione di cellule umane, principalmente coinvolte nella risposta infiammatoria che si scatena a seguito dell'infezione da Sars-Cov2: cellule epiteliali alveolari (polmonari), monociti/macrofagi e cellule endoteliali giovani e senescenti. In particolare, l'UO2 testerà l'efficacia nel contrastare i) la produzione di citochine infiammatorie e chemochine, ii) di inibire l'espressione delle molecole di adesione (che favoriscono l'ulteriore reclutamento di cellule infiammatorie dal circolo) e iii) l'espressione delle proteine ACE2 e TMPRSS2, utilizzate dal virus per infettare la cellula ospite e iv) di modulare il fenotipo dei macrofagi da M1 (pro-infiammatorio) a M2 (anti-infiammatorio e promuovente il processo di riparazione). Nel corso del progetto, la UO1 si occuperà di progettare la preparazione dei derivati mancanti secoiridoidi ligstroside (Lig), suo aglicone (Lig-A) e oleocantale (p-HPEA-EDA), e di studiare una formulazione dei principi attivi per la somministrazione via aerosol e orale, come la microincapsulazione (29, 30). Sarà obiettivo del progetto lo sviluppo di microparticelle innovative costituite da materiali biocompatibili (chitosano, ac. ialuronico, derivati del polossamero, PLGA/PLA) contenenti i composti attivi al fine di potenziare le loro caratteristiche bio/mucoadesive e promuoverne un rilascio organo-specifico (somministrazione di aerosol per i polmoni). I microsistemi saranno caratterizzati in termini di dimensioni medie, carica superficiale, carico utile del principio attivo e profilo di rilascio. L'efficacia farmacologica verrà valutata in funzione della concentrazione del principio attivo e del tempo di incubazione sui modelli in vitro già descritti e poi, nella fase 2, in vivo su modelli animali di infiammazione e sepsi di cui può disporre l'UO2, al fine di valutarne le proprietà anti-infiammatorie locali e sistemiche e di promozione della riparazione. Schema attività: Q1 UO1-Preparazione Ole, Ole-A, Htyr, Tyr e 3,4-DHPEA-EDA Q2 UO2-Allestimento modelli cellulari per test in vitro di valutazione efficacia Ole, Ole-A, Htyr, Tyr, 3,4-DHPEA-EDA. Q3 UO1-Preparazione Lig. Lig-A Q4 UO2-Valutazione in vitro efficacia di Lig. Lig-A e p-HPEA-EDA Q5 UO1-Sviluppo microparticelle materiali biocompatibili per somministrazione aerosol e orale Q6 UO2-Valutazione efficacia microparticelle in vitro. Personale (oltre ai responsabili di UO) e costi: UO1- prof. Donato Cosco (associato) dott.ssa Monica Nardi (Ricercatore a t.d. - t.pieno (art. 24 c.3-b L. 240/10)), dott.ssa Manuela Oliverio (ricercatore) - 27.200 euro (reagenti e solventi chimici, materiale cromatografico). [redacted], assegnista Dott.ssa Angelica Giuliani - 27.200 euro (materiale monouso sterile, cellule e terreni per colture, anticorpi e kit ELISA per analisi proteine, kit di estrazione, primers per RT-PCR). 1. M. Hoffmann et al. Cell. 2020. doi:10.1016/j.cell.2020.02.052) 2. Y. 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Descrizione della proposta (EN)

The SARS-CoV2 virus has caused a sudden global alarm due to the potential mortality provoked by its associated disease, COVID-19. In humans, the virus infects the airways and causes pneumonia which, in a limited number of people, can evolve towards a worsening, potentially lethal clinical condition. This clinical picture is not only caused by the viral infection itself, which leads to the death of the cells expressing ACE2 (used as a receptor by the viral protein Spike) (1), but also by the intense inflammatory response of the host, characterized by the hyper-activation of epithelial, endothelial cells and alveolar macrophages (2-3). In the final stage of the disease, this response becomes uncontrolled and is characterized by the so-called cytokine storm, with serious harmful local (Acute Respiratory Distress Syndrome and bacterial and fungal superinfections) and systemic effects and multi-organ failure. Therefore, the cytokine storm represents a negative prognostic factor, and is believed to be the direct cause of death from COVID-19 (4). Timely targeted therapeutic strategies, based on drugs capable of blocking the cytokine cascade and controlling the activation of the cells involved in their production, would certainly improve the clinical outcome of the disease. With the health emergency and containment of the coronavirus diffusion requiring much shorter response times than those often needed for the approval of new drugs, the proposal of natural molecules active against cytokine storm, which have already been used on humans, may be a potential therapeutic option. In recent years, the anti-inflammatory properties of a group of secoiridoid phenols from olive trees have aroused enormous interest among the scientific community. A first study, published in 2005 in Nature, recognized in oleocanthal, responsible for the spicy taste of extra virgin olive oil (EVOO), a COX inhibition activity similar to that of ibuprofen (5). The following year the PREDIMED clinical study demonstrated the anti-inflammatory effect of the Mediterranean diet (MD), integrated with EVOO (6), and in particular the modulation of biomarkers associated with systemic inflammation (PCR, IL-6), as well as those involved in the recruitment of inflammatory cells (chemokines and endothelial and monocytic cell adhesion molecules) (7). Similar effects have also been attributed to olive leaves extract (8-10), which is effective in sepsis, tissue repair (11-13) and in the prevention of respiratory diseases (14). Researchers therefore focused on the possibility to use the individual phenolic compounds of olive trees as potential anti-inflammatory agents, demonstrating, both in vitro and in vivo, the effectiveness of oleocantale, oleuropein and hydroxysuccinate in modulating inflammatory parameters and reducing cytokine synthesis (15-17). The research unit of the Magna Græcia University, UMG (UO1) directed by Prof Procopio has been active for more than a decade in the analysis, separation, semi-synthetic manipulation and investigation of the pharmaco-biological potential of olive oil phenolic compounds, which have been recognized by the EFSA for their protective anti-oxidant role towards circulating lipoproteins (18). UMG's Green Chemistry Laboratory (G.C.L.) has developed a low environmental impact methodology for the extraction of oleuropein (Ole) from olive leaves (waste product of oil processing) and its hydrolysis to obtain Ole-aglicone (Ole-A). The processes used, which are the subject of the international patent WO2008136037A2, make it possible to obtain Ole and Ole-A products in a wholly biocompatible way, avoiding the use of solvents and reagents which are dangerous to human health. More recently, G.C.L. has developed a simple and eco-sustainable semi-synthetic process to produce oleacein (Oln) from OLE, by decarboxylation of Krapcho promoted by microwaves in an aqueous environment (19). The anti-inflammatory properties of EVOO secoiridoids obtained by the application of these methodologies are the subject of both in vitro and in vivo studies (20-24). Prof Rippo, head of the Experimental Pathology Research Unit of the Università Politecnica delle Marche (UO2) has been engaged for several years in the study of chronic systemic inflammation characteristics of aging (inflamm-aging), age-related diseases and molecular biomarkers of systemic inflammation, paying particular attention to the role of endothelial cells and monocytes/macrophages (25-28). With this project, in its Phase 1, UO1 aims to provide UO2 with enough quantities of Ole, Ole-A, Oln, Htyr and Tyr to test in vitro the ability to modulate the activation of the human cells which are mainly involved in the inflammatory response resulting from the Sars-Cov2infection: alveolar epithelial cells (pulmonary), monocytes/macrophages and young and senescent endothelial cells that the UO2 already possesses, or that are commercially available. In particular, UO2 will test the effectiveness in

modulating i) the production of inflammatory cytokines and chemokines, ii) the expression of adhesion molecules (which promote further recruitment of inflammatory cells from the circle) and iii) the expression of the proteins ACE2 and TMPRSS2, used by the virus to infect the host cell and iv) the phenotype of macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory and promoting the repair process). During the project, UO1 will design the preparation of the missing secoiridoid derivatives ligstroside (Lig), its aglicone (Lig-A) and oleocantale (p-HPEA-EDA), and study a formulation of the active principles for aerosol and oral administration, such as microencapsulation (29, 30). Therefore, one of the project's objectives will be the development of innovative microparticles consisting of biocompatible materials (chitosan, hyaluronic acid, poloxamer derivatives, PLGA/PLA) containing the active compounds, in order to enhance their bio/mucoadesive characteristics and promote an organ-specific release (administration of aerosols for the lungs). Microsystems will be characterized in terms of average size, surface charge, active substance loading and release profile. Pharmacological efficacy will be assessed on the basis of the active substance concentration and incubation time on the in vitro models described above and then, in Stage 2, in vivo on animal models of inflammation and sepsis available to UO2, in order to assess local and systemic anti-inflammatory properties and promote tissue repair. Below, a schematization of the tasks, dedicated staff (in addition to UO managers) and research costs of per Unit. Q1 UO1- Ole, Ole-A, Htyr, Tyr e 3,4-DHPEA-EDA synthesis Q2 UO2- Development of cellular models to test in vitro efficacy of Ole, Ole-A, Htyr, Tyr , 3,4-DHPEA-EDA. Q3 UO1- Lig. Lig-A synthesis Q4 UO2-Evaluation of efficacy Lig. Lig-A and p-HPEA-EDA in in vitro models Q5 UO1- Development of biocompatible microparticles for aerosol and oral administration Q6 UO2- Evaluation of microparticles efficacy in in vitro models Personnel (in addition to UO managers) and costs: UO1-UO1- prof. Donato Cosco (associate professor), Doctor Monica Nardi (Researcher, under fixed – full time (art. 24 c.3-b L. 240/10), Doctor Manuela Oliverio (Researcher). 27.200 euros (solvents and chemicals, chromatographic materials) UO2-1 PhD student, 1 Research Fellow. 27.200 euros (sterile disposable material, cells and culture media, antibodies and ELISA kits for protein analysis, extraction kits, Primers for RT-PCR). 1. M. Hoffmann et al. Cell. 2020. doi:10.1016/j.cell.2020.02.052) 2. Y. Shi Y, et al. 2020. doi:10.1038/s41418-020-0530-3) 3. M. Z. Tay et al. Nat Rev Immunol. 2020. doi:10.1038/s41577-020-0311-8) 4. G. Jia et al. Cytokine and Growth Factor Reviews, 2020. doi: 10.1016/j.cytogfr.2020.05.002]. 5. G. K. Beauchamp et al. Nature 2005, 437, 45-46 6. C. Nediani et al., Antioxidants 2019 and references therein reported. doi:10.3390/antiox8120578] 7. R. Estruch. Proc Nutr Soc. 2010. doi:10.1017/S0029665110001539. 8. S. Lockyer et al. Eur J Nutr. 2017. doi:10.1007/s00394-016-1188-y) 9. A. Boss et al. Int J Mol Sci. 2016. doi:10.3390/ijms17122019) 10. B. Burja et al. Front Cardiovasc Med. 2019. doi:10.3389/fcvm.2019.00056) 11. M. F. P. Ramos et al. PeerJ. 2019. doi:10.7717/peerj.7219) 12. U. Koca et al. J Med Food. 2011. doi:10.1089/jmf.2010.0039) 13. D. Gong et al. J Med Food. 2011. doi:10.1089/jmf.2010.1153) 14. V. Somerville et al. Nutrients. 2019. doi:10.3390/nu11020358) 15. A. Procopio et al. Clin. Nutr. 2011. doi.org/10.1016/j.clnu.2011.02.004] 16. A. Procopio et al., Biochem Pharmacol 2012.doi.org/10.1016/j.bcp.2012.02.001 17. Takeda R, et al. Phytomedicine. 2013. doi:10.1016/j.phymed.2013.03.021 18. EFSA Journal 9 (4), 2033, http://dx.doi.org/10.2903/j.efsa.2011.2033, 25 pp. 19. A. Procopio et al. Food Chem. 2018 ,doi.org/10.1016/j.foodchem.2017.10.097 20. A. Procopio et al. J. Agric. Food Chem. 2009. doi.org/10.1021/jf9033305 21. A. Procopio et al. Front Endocrinol. 2018. doi.org/10.3389/fendo.2018.00116 22. A. Procopio et al. Nutrients 2017.doi: 10.3390/nu9040391 23. A. Procopio et al. Curr Med Chem 2012. doi.org: 10.2174/092986712802002536 24. Reg Prot n. 147, 15 /6/17: Somministrazione topica di un preparato a base di oleuropeina in pazienti da rettocolite ulcerosa distale 25. Sabbatinelli J et al. Front Physiol. 2019. doi:10.3389/fphys.2019.01523 26. A. Giuliani et al. Oncotarget. 2016. doi:10.18632/oncotarget.7858 27. A. Costantini et al. Aging (Albany NY). 2018. doi:10.18632/aging.101465 28. F. Olivieri et al. Free Radic Biol Med. 2013. doi:10.1016/j.freeradbiomed. 29. Cosco D. et al. Carbohydr Polym. 2016; 152:583-591 30. Cosco D. M. Carbohydr Polym. 2019; 212:430-438

Parole chiave

	IT	EN
Parola 1	olio extra-vergine d'oliva	extra-vergin olive oil
Parola 2	fenoli secoiridoidi dell'olio d'oliva	olive oil secoiridoid phenols
Parola 3	oleuropeina	oleuropein
Parola 4	oleacina	oleacein
Parola 5	idrossitirosolo	hydroxytyrosol

Parola 6	tirosolo	tyrosol
Parola 7	oleocantale	oleocanthal
Parola 8	tempesta citochimica	cytokine storm
Parola 9	foglie d'olivo	olive leafs

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RESEARCH ARTICLE

Anti-SASP and anti-inflammatory activity of resveratrol, curcumin and β-caryophyllene association on human endothelial and monocytic cells

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Abstract A challenging and promising new branch of aging-related research fields is the identification of natural compounds able to modulate the senescence-associated secretory phenotype (SASP), which characterizes senescent cells and can contribute to fuel the inflammaging. We investigated both the anti-SASP and anti-inflammatory activities of a nutritional supplement, namely Fenoxidol^{TM}, composed of turmeric extract bioCurcumin (bCUR), Polydatin (the natural glycosylated precursor of Resveratrol-RSV), and liposomal β-caryophyllene (BCP), in two human cellular models, such as the primary endothelial cell line, HUVECs and the monocytic cell line, THP-1. Replicative and Doxorubicin-induced senescent HUVECs, both chosen as cellular models of SASP, and lipopolysaccharides (LPS)-stimulated THP-1, selected as a model of the inflammatory response, were treated with the three single natural compounds or with a combination of them (MIX). In both senescent HUVEC models, MIX treatment significantly reduced IL-1β and IL-6 expression levels and $p16^{ink4a}$ protein, and also increased SIRT1 protein level, as well as downregulated miR-146a and miR-21 expression, two of the so-called inflamma-miRNAs, more effectively than the single compounds. In THP-1 cells stimulated with LPS, the MIX showed a significant effect in decreasing IL-1β, IL-6, TNF-α, and miR-146a expression levels and Caspase-1 activation, in association with an up-regulation of SIRT1 protein, compared to the single compounds. Overall, our results suggest that the three analysed compounds can have a combined effect in restraining SASP in senescent HUVECs as well as the inflammatory response in LPS-stimulated THP-1 cells.

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Keywords Nutraceuticals · Polyphenols · Senescence · Inflammaging · SASP

Introduction

An exciting cutting-edge topic of research concerns the identification of natural compounds that can improve human health. As the population is progressively aging and this phenomenon is accompanied by an increased incidence of age-related diseases (ARDs), the discovery of natural substances able to decelerate the aging process and postpone the development of the most common ARDs has become of widespread interest (Forni et al. 2019). A number of natural compounds capable of interacting with biological processes are contained in food and were therefore named "nutraceuticals" (Biesalski et al. 2009). In recent years, the role of nutraceuticals was extensively investigated in cellular and animal models, as well as in humans, with the aim to prevent and/or contribute to treat ARDs. One of the most interesting hypotheses on the mechanism by which bioactive compounds contained in food could improve health status is their ability to restrain inflammaging (Gurau et al. 2018; Pazoki-Toroudi et al. 2016). Inflammaging is the systemic, low-grade, inflammatory status associated with aging, which is considered a shared risk factor for the most common ARDs (Franceschi 2017; Fulop et al. 2017). The increased burden of senescent cells (SCs) is recognized as key player in promoting inflammaging since SCs are characterized by the acquisition of a senescence-associated secretory phenotype (SASP) with proinflammatory activity (Coppe et al. 2010). SASP, which is fueled by the DNA damage response (DDR), is characterized by NF-kB and NLRP3 inflammasome pathways activation, and by the consequent synthesis and release of a plethora of proinflammatory factors such as interleukins, chemokines, growth factors, matrix-degrading enzymes, reactive oxygen species, and non-coding RNA, i.e. miRNAs (Ishida et al. 2019; Meyer et al. 2017; Munk et al. 2017). Increasing evidence suggests that specific miRNAs, such as miR-21, miR-126, and miR-146a can modulate cellular senescence being involved in the modulation of inflammatory responses and inflammaging (Dimmeler and Nicotera 2013; Harris et al. 2008; O'Connell et al. 2008; Olivieri et al. 2013c; Tili et al. 2007).

Notably, increasing evidence suggests that another main culprit of inflammaging is the repeated stimulation of innate immune responses over time (Prattichizzo et al. 2016). In this framework, both an increased burden of senescent cells during aging and a hyper-stimulation of macrophages over time can be considered key pillars of inflammaging.

Cellular senescence was firstly obtained by long-term culturing of human primary cells (replicative senescence) but can be prematurely triggered in response to several stressors, including oxidative, genotoxic, oncogenic and, therapeutic (induced senescence) (Venturini et al. 2020). In this context, human umbilical endothelial cells (HUVECs) in replicative senescence were used as a well-established in vitro model of SASP and, more recently, Doxorubicin-treated HUVECs were shown to acquire a premature senescent phenotype (Hwang et al. 2020; Venturini et al. 2020). Taking into account that almost all the most common age-related diseases are characterized by endothelial dysfunction, a number of studies on inflammaging were focused on senescent HUVEC models (Hwang et al. 2020; Olivieri et al. 2013a; Wong et al. 2019).

In vivo, the cross-talk between endothelial cells and macrophages is the first step of innate immune activation. Further, endothelial cells can modulate macrophage polarization and function. Monocytic cell line (THP-1) stimulated with LPS can be considered as one of the best-characterized in vitro models of the innate immune pro-inflammatory response (Prattichizzo et al. 2016).

Thus, restraining or delaying SASP acquisition and reducing the increased pro-inflammatory burden of a plethora of antigens over time are emerging as new challenges in aging research (Sabbatinelli et al. 2019; Song et al. 2020).

Several nutraceuticals, including Resveratrol (RSV) and *Curcuma longa,* have been investigated for their potential ability to modulate a number of pathways relevant in the aging process (Howitz et al. 2003; Olivieri et al. 2013c; Schilder et al. 2009; Shishodia 2013). A multitude of data affirmed that the healthful effect on aging is promoted by RSV through the stimulation of sirtuins activities and the inhibition of the inflammatory and stress-related responses (Argyropoulou et al. 2013; Latorre et al. 2017). The

biological properties of *Curcuma longa* depend mainly on a yellow-orange lipophilic polyphenolic substance called curcumin family of curcuminoids (McCubrey et al. 2017), which is acquired from the rhizomes of the plant (Shishodia 2013), whereas RSV is a natural phytoalexin found in grapes and red wine (Liu et al. 2017). The age-modulating proprieties of Curcumin has been demonstrated in different animal models, including C.elegans, Drosophila and mice. This compound was found to extent both healthspan and lifespan, mainly suppressing the most relevant proinflammatory pathway NF-kB (Argyropoulou et al. 2013).

BCP and β-caryophyllene oxide (BCPO) are two other naturally occurring phytocannabinoids with bicyclic sesquiterpene structure, present in a large number of plants, that are currently evaluated for their effects on inflammation and pain (Fidyt et al. 2016). BCP can act as an agonist of the peripherally expressed cannabinoid receptor type 2 (CB2), and a number of studies have shown that it is involved in the modulation of inflammatory responses and neuropathic pain, as well as in the modulation of glycaemic and lipidic metabolism (Baldissera et al. 2017; Basha and Sankaranarayanan 2016; Sharma and Kanneganti 2016).

Increasing evidences support the hypothesis of potential additive or synergistic effects of the mixtures compared to single compounds (Iwuchukwu et al. 2011; Masuelli et al. 2014). For example, curcumin synergizes with BCP, exerting an anti-inflammatory activity in human articular chondrocytes indicating the efficacy of the natural compound combination (D'Ascola et al. 2019).

Therefore, we focused our interest on a novel nutraceutical, namely Fenoxidol^{TM} (Mivell S.r.l., Jesi, AN, Italy), composed of bioCurcumin (bCUR), Polydatin and, liposomial β-caryophyllene (BCP). Polydatin is the natural glycosylated precursor of Resveratrol (RSV), characterized by increased bioavailability in humans compared to RSV (Henry-Vitrac et al. 2006).

In this study, we first characterized physicochemically the active ingredients of Fenoxidol^{TM} and subsequently, we evaluated the anti-SASP and anti-inflammatory activity of bCUR, RSV and, BCP, alone or in combination (MIX) as in the Fenoxidol^{TM}, on two different human cellular models involved in the fuelling of inflammaging, *i.e.,* endothelial cells and monocytes.

Materials and methods

Natural extracts composition

The natural extracts utilized in this study and in the nutritional supplement Fenoxidol^{TM} were analysed in order to characterize their compositions. For the bioCurcumin (bCUR), dissolved in methanol, a high-performance liquid chromatography (HPLC Agilent 1260 Infinity II, Agilent, USA) method was developed to detect at the same time three curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and the main terpene ar-turmerone. The separation was carried with a Zorbax Eclipse XDB-C18, 4.6 × 250 mm, 5 µm column (Agilent, USA) maintained at room temperature. The mobile phase was composed of water containing 0.4% (v/v) acetic acid (A) and acetonitrile (B) with a gradient as follows: 55% A at 0–13 min, 55–44% A at 13–16 min, 44% A at 16–50 min, and 44–0% A at 50–55 min with 10 min of 55% A re-equilibration between individual runs for a total time of 70 min. The injection volume was 20 µL with a constant flow rate of 1 mL/min. The HPLC system was equipped with a diode array detector (DAD) that registered two wavelengths at the same time: 240 nm for the detection of ar-turmerone and 430 nm for the detection of curcuminoids (Chao et al. 2018). Four different calibration curves, one for each evaluated component, were constructed by plotting the mean peak area vs. the concentration of the reference obtaining optimal coefficient of determination (R^2: 0.9996 for curcumin, 0.9976 for demethoxycurcumin, 0.9997 for bisdemethoxycurcumin and 0.9993 for ar-turmerone) in the concentration range of 0.01–0.1 mg/mL. As for the bioCurcumin, for the evaluation of RSV and polydatin, an HPLC method was utilized. The quantification was carried out in an Agilent Poroshell 120 EC-C18, 100 × 4.6 mm, 2.7 µm column (Agilent, USA) with an isocratic elution composed by 77% of water and 23% of methanol. The injection volume was 20 µL and the flow rate was 1 mL/min. The detection signal was recorded at 306 nm keeping the analysis system at room temperature (Xu et al. 2020). The calibration curve was constructed by dissolving the standard in

methanol in a range from 0.01 to 0.1 mg/mL with an R^2 of 0.9998. The black pepper extract titrated as BCP was provided incapsulated in lyophilized liposomes so, after rehydration in water, the suspension was characterized in terms of average particle size (Z-average) and polydispersity index (PDI) by dynamic light scattering (DLS) using a Malvern Zetasizer Nano S instrument (Malvern Instruments Ltd, UK). The amount of BCP present in the extract was evaluated using a gas chromatograph mass spectrometer (GCMS-QP2010S, Shimadzu, Japan) after a dissolution in acetone. The active molecule was analysed on a Agilent DB-5MS (Agilent Technologies Inc, USA) capillary column (30 m length, 250 μm internal diameter, 0.25 μm film thickness) with the following temperature program: 55 °C for 10 min followed by a temperature rise at a 5 °C/min rate to 220 °C (held for 20 min). Carrier gas was He with a constant flow of 1 mL/min and an injection of 0.2 μL. A calibration curve was constructed from 0.5 to 500 nL/mL with a R^2 of 0.9995 using BCP standard dissolved in acetone.

HUVEC and THP-1 culture

HUVECs, primary human umbilical vein endothelial cells, obtained from a pool of donors, were purchased from Clonetics (Lonza, Switzerland) and cultured in endothelial basal medium (EBM-2, CC-3156, Lonza) supplemented with SingleQuot Bullet Kit (CC-4176, Lonza) containing 0.1% human recombinant epidermal growth factor (rh-EGF), 0.04% hydrocortisone, 0.1% vascular endothelial growth factor (VEGF), 0.4% human recombinant fibroblast growth factor (rh-FGF-B), 0.1% insulin-like growth factor-1 with the substitution of arginine for glutamic acid at position 3 (R3- IGF-1), 0.1% ascorbic acid, 0.1% heparin, 0.1% gentamicin and amphotericin-B (GA-1000), and 2% fetal bovine serum (FBS). The cells were seeded at a density of 5000/cm^2 in T75 flasks (Corning Costar, Sigma Aldrich, St. Louis MO, USA).

Human monocytic THP-1 cells were purchased from ATCC (Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (all from Euroclone, Milano, Italy). The cells were seeded at a density of 2×10^5 cells/ml in T75 flasks.

Induction and characterization of senescent cells

Replicative senescence (RS) was achieved after a number of replicative passages (measured as population doubling-PD). Population doublings (PDs) were calculated by the formula: $(\log_{10}(F) - \log_{10}(I))/\log_{10}(2)$, where F is the number of cells at the end of the passage and I is the number of seeded cells. Cumulative population doublings (cPD) were calculated as the sum of PD changes. Drug-induced senescence (IS) was obtained by treating HUVECs with Doxorubicin hydrochloride (50 nM) (Sigma Aldrich, Italy) for 24 h. Treated cells were harvested following a 96 h period with fresh medium to acquire the SASP. HUVECs were classified as young or senescent based on cPD as well as on senescence-associated (SA)-β-galactosidase activity and $p16^{ink4a}$ expression. SA-β-Gal activity was detected by using Senescence Detection Kit (BioVision Inc., Milpitas, CA, USA). Briefly, non-confluent, 12-wells plates cultured HUVECs were fixed for 15 min at room temperature, and then washed twice in phosphate-buffered saline (PBS) and incubated overnight at 37 °C with Staining Solution Mix (containing X-Gal). The percentage of β-Galactosidase-positive cells was determined by counting at least 200 cells per well using light microscopy. $p16^{ink4a}$ expression was evaluated by RT-qPCR (Fw: CATAGATGCCGCGGAAGGT; Rv: CTAAGTTTCCCGAGGTTTCTCAGA) and western blot analysis.

Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to test cell viability. Cells were grown for 24 h in 24-well plates at a density of 5000 cells/cm^2 before treatments with different doses of the natural compound for 3 h. Briefly, MTT (1 mg/ml) solution was added and incubated for 4 h; the insoluble formazan salt product was solubilized by adding 200 μl of dimethyl sulfoxide (DMSO) and its amount was determined by measuring the optical density at 540 nm using a microplate reader (MPT Reader, Invitrogen, Milano, Italy).

Cell viability was calculated according to the equation (T/C) × 100%, where T and C represent respectively the mean optical density of the treated group and the control group.

Natural compound treatments

All substances were dissolved in DMSO at a 0.1% final concentration of DMSO in all the solutions. Based on the results of the viability assay, cells were treated with bCUR- 2 μg/ml, RSV- 2 μg/ml and, BCP 20 μg/ml. Considering the total polyphenolic content was 2 μg/ml for all the three tested compounds, we maintained the same amount of polyphenols for the combination treatments, thus the MIX was composed of a total of 2 μg/ml of polyphenols. In addition, a weight ratio equal to 1:1:0.2 (1.6 μg/ml bCUR, 1.6 μg/ml BCP and 0.32 μg/ml RSV) was established to preserve the ratio applied in Fenoxidol™. The treatments with the single compounds and the MIX were carried out for 3 h.

THP-1 cells were treated with 500 ng/ml of LPS (lipopolysaccharide) without or with the single compounds and the MIX for 3 h.

RNA isolation, mRNA and mature miRNAs expression by RT-qPCR

Total RNA was isolated using the Norgen Biotek Kit (#37500, Thorold, ON, Canada), according to the manufacturer's instructions. RNA was stored at− 80 °C until use. RNA amount was determined by spectrophotometric quantification with Nanodrop ONE (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (1 μg) was reverse-transcribed using TAKARA Kit (PrimeScript™ RT reagent Kit with gDNA Eraser, Cat: RR047A) based on the manufacturer's instructions. qRT-PCR was performed in a Rotor-Gene Q (Qiagen) using TB Green™ Premix Ex Taq™ (Cat: RR420A) in a 10 μl reaction volume. mRNA quantification was assessed using the $2^{-\Delta CT}$ method. GAPDH and β-actin were used as an endogenous control.

MiRNAs expression was quantified by quantitative real-time PCR (RT-qPCR) using TaqMan miRNA assay (Catalog #4427012—TermoFisher Scientific), according to the manufacturer's protocol. Data were analysed with Rotor Gene Q (Qiagen, Hilden, Germany) with the automatic comparative threshold (Ct) setting for adapting baseline. RT-qPCR data were standardized to RNU44. The $2^{-\Delta CT}$ method was used to determine miRNA expression.

Western blot analysis

Cell lysates were obtained using RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 5 mM EDTA, pH 8.0) with protease inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein concentration for each sample was evaluated by Bradford assay. Proteins (30 μg) were analysed by SDS-PAGE, and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). 5% skim milk was used to block the membrane that was then incubated overnight with the primary antibody.

Mouse anti-SIRT1 (Abcam), rabbit anti-Caspase-1 p10 (Santa Cruz Biotechnology), mouse anti-$p16^{ink4a}$ (Santa Cruz) and, rabbit anti-α-tubulin (Cell Signaling), were used as primary antibodies.

Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from horse and goat respectively (Vector laboratories, CA, USA). Protein bands were visualized using Clarity ECL chemiluminescence substrate (Bio-Rad) with Uvitec Imager (UVItec,Cambridge,UK) and then quantified using ImageJ software.

Caspase 1 activity was measured as the ratio between band intensity of the pro-Caspasi-1 and of the cleaved Caspasi-1. Each measure was normalized with α-tubulin.

ELISA assay

Cell culture supernatants were collected at the end of each incubation, centrifuged, and stored at− 20 °C until use. IL-6 ELISA Kit (Cayman chemical, Ann Arbor, USA), IL-1β ELISA Kit (Booster biological technology, Pleasanton, CA) and TNF-α ELISA Kit (Booster biological technology, Pleasanton, CA) were used to measure the concentration of IL-6, IL-1β and TNF-α released in the medium respectively, according to the manufacturer's instructions.

Statistical analysis

Summarized data are shown as mean of at least three independent replicates ± SD, SEM or frequency (%). Paired sample T test was used for the analysis of real-time PCR, ELISA and densitometric data. Data analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp, Armonk, NY,

USA). Statistical significance was defined as a two-tailed p-value < 0.05.

Results

Natural extracts composition

With the optimized HPLC–DAD technique, the four main molecules of bioCurcumin (bCUR) (Fig. 1a) were detected in a single injection (Fig. 1b) and quantified in 66.3 ± 1.8% of curcumin, 16.4 ± 0.9% of demethoxycurcumin, 8.1 ± 0.3% of bis-demethoxycurcumin and 5.9 ± 0.5% of ar-Turmerone.

Both RSV utilized during the in vitro studies and polydatin present in the nutraceutical formulation was characterized by high purity (> 98%) meanwhile the amount of BCP included in the pro-liposomal formulation contained in Fenoxidol^{TM} was 8.9 ± 0.2%. After resuspension of pro-liposomal powder in water, the main particle size was 204 ± 15 nm with a narrow distribution.

Resveratrol, β-caryophyllene and bioCurcumin effect on HUVECs and THP-1 cell viability

RSV, BCP, bCUR and, the MIX cytotoxic effect was evaluated on young HUVECs (yHUVECs) and THP-1 cells (Fig. 2) and on replicative and Doxorubicin-induced senescent HUVEC cells (RS-HUVECs and IS-HUVECs) (Suppl. Figure 1) after 3 h of treatments by MTT assay. Further experiments were performed considering the concentration of compounds that gave approximately 95–75% of viability of treated yHUVECs and THP-1 cells (2 µg/ml bCUR and RSV, 20 µg/ml BCP whereas 1.6 µg/ml bCUR, 1.6 µg/ml BCP, and 0.32 µg/ml RSV were used for the MIX), accordingly to previous reports (Castejon et al. 2017; Faragher et al. 2011; Moghaddam et al. 2019; Zhang et al. 2018).

Replicative and doxorubicin-induced senescence in HUVECs

The effects of natural compounds were analysed in yHUVECs and RS-HUVECs or IS-HUVECs. Senescent status was evaluated by replicative ability, SA- β-Gal activity and p16^{ink4a} expression. yHUVECs were characterized by cPD < 9 and SA-β-Gal positive cells $< 5\%$. RS-HUVECs were defined as cPD > 18 and SA-β-Gal positive cells $> 60\%$ (Fig. 3a). To achieve IS-HUVECs model, Doxorubicin cytotoxicity was tested by MTT assay and the concentration of 50 nM corresponded to 80% of cell viability and SA-β-Gal positive cells $> 60\%$, was selected (Fig. 3b). In addition, a significant increase of both p16^{ink4a} mRNA (Fig. 3c) and protein (Fig. 3d) levels, compared to yHUVECs, were selected as cut-off values to establish the senescent status (both RS- and IS-HUVECs).

Combined natural compounds exert anti-SASP activity on RS- and IS- HUVECs

RS- and IS-HUVECs are both characterized by the acquisition of the senescence-associated secretory phenotype (SASP). Therefore, the effect of natural compounds to restrain the SASP was investigated

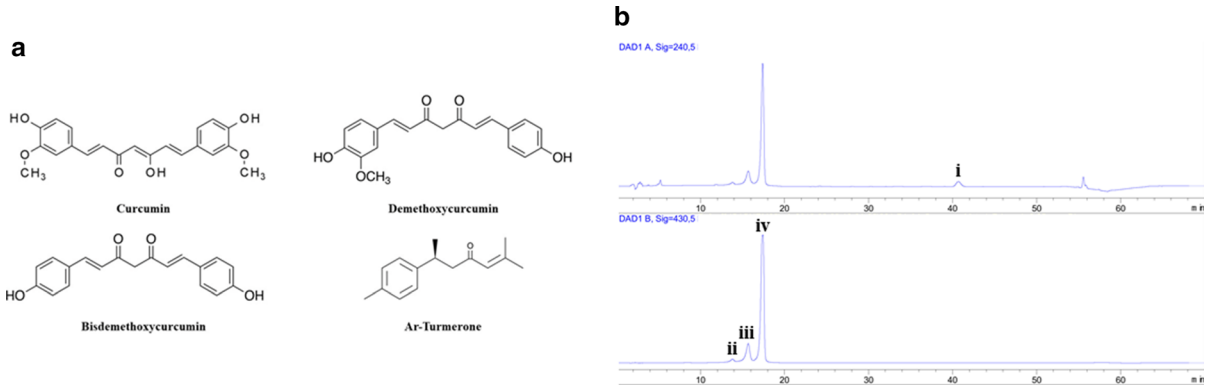


Fig. 1 **a** The main molecules of bioCurcumin **b** HPLC–DAD chromatogram with the peaks of ar-turmerone (i) at 240 nm, bisdemethoxycurcumin (ii), demethoxycurcumin (iii) and curcumin (iv) at 430 nm

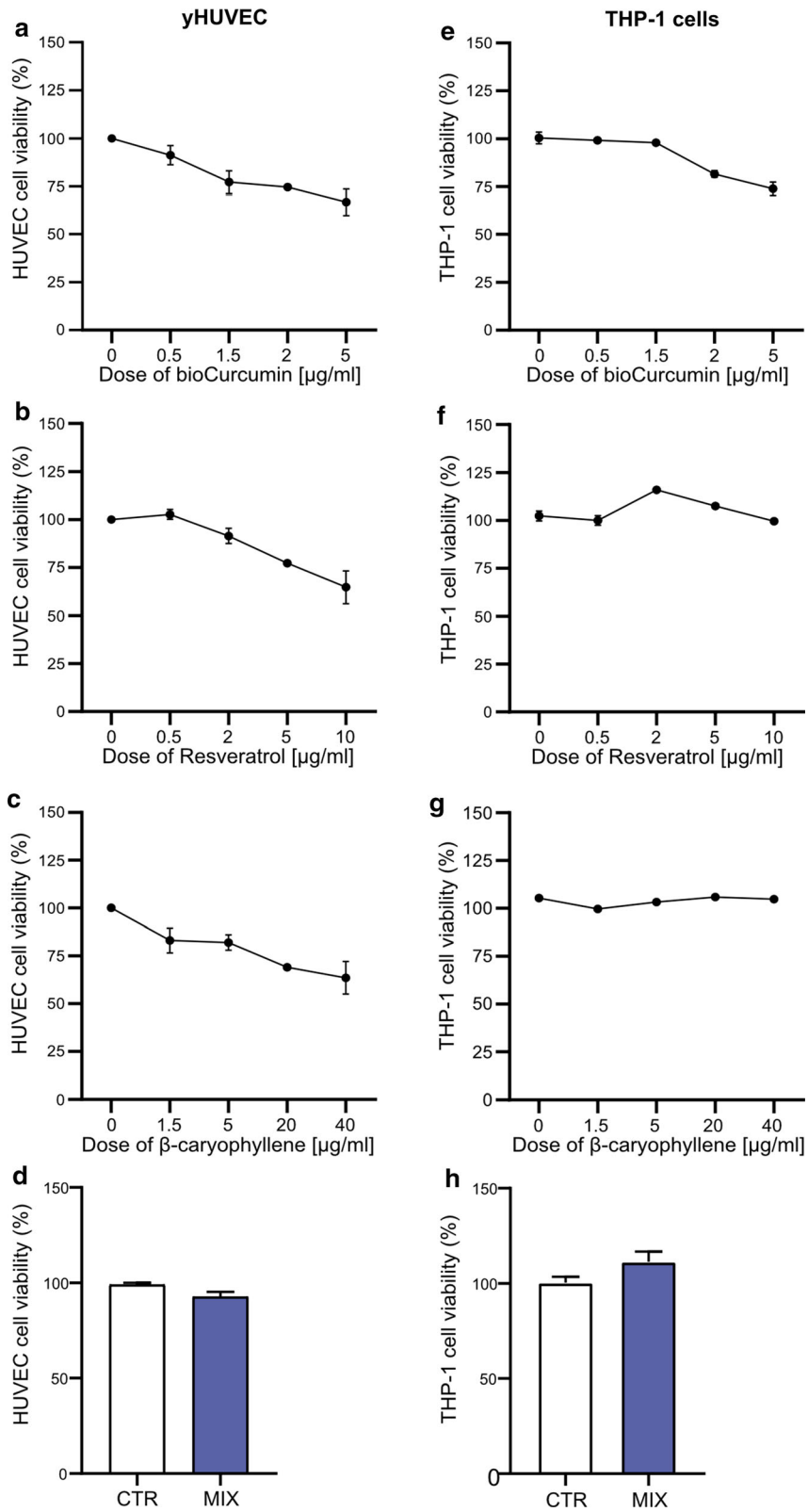


Fig. 2 Effect of bCUR, RSV, BCP and MIX on yHUVECs (**a–d**) and THP-1 cells (**e–h**). Cells were treated with different concentrations of bioCurcumin (from 0.5 to 5 µg/ml), Resveratrol (from 0.5 to 10 µg/ml), β-caryophyllene (from 1.5 to 40 µg/ml) and MIX for 3 h. The viability of cells was determined by MTT assay. The results are expressed as a percentage of cell viability normalized to the viability of DMSO treated cells (CTR) and presented as mean value ± SEM from three independent biological experiments. *bCUR* bioCurcumin, *RSV* resveratrol, *BCP* β-caryophyllene

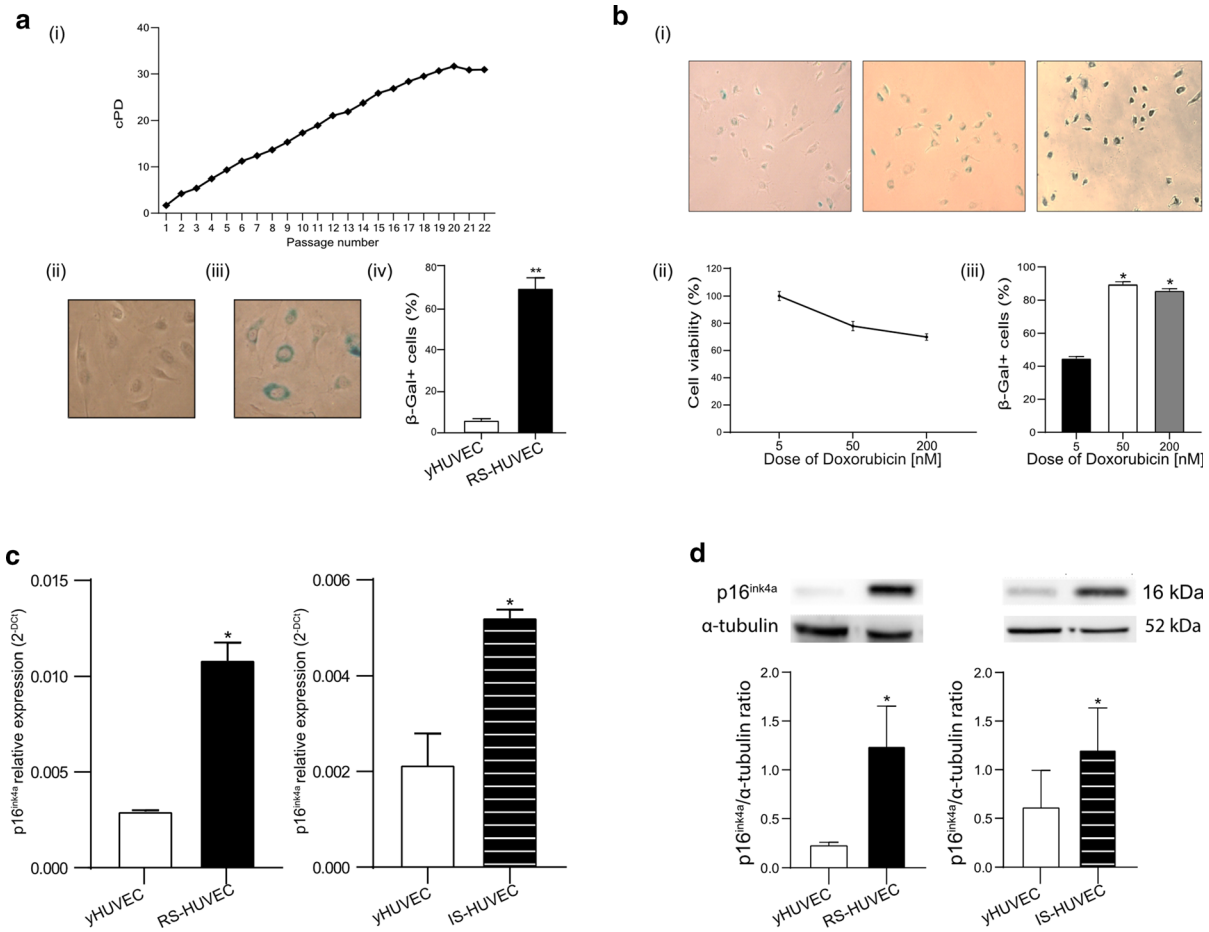


Fig. 3 Characterization of endothelial cells in RS- and IS-HUVEC models. **a** Growth curve of a pool of HUVECs—Y-axis: Cumulative Population Doubling (cPD); X-axis: cell passages from P1 to P22 (i); two representative pictures of SA-β-Gal staining of young (ii) and senescent (iii) HUVECs; % of SA- β-Gal (iv). Cells with SA-β-Gal < 10% were considered young cells (yHUVEC), while those with SA- β-Gal > 60% were identified as senescent cells; **b** representative picture of SA- β-Gal-positive cells (i), dose–response curve (ii) and % of SA- β-Gal (iii) after treatment with 5 nM (left image, 50 nM (central image) and 100 nM (right image) of Doxorubicin; **c** relative expression of $p16^{ink4a}$ mRNA in yHUVEC (P4), RS-HUVECs (P21) and IS-HUVECs (Doxorubicin 50 nM); **d** densitometric analysis of $p16^{ink4a}$ protein level in RS-HUVEC and IS-HUVEC. Histograms represent the mean of the protein level and the relative expression measured in three different experiments ± SD. Paired *t* test, *p < 0.05 versus yHUVEC. *RS* replicative senescence, *IS* induced senescence

analysing the expression levels of proinflammatory cytokines, such as IL-1β and IL-6, and some *inflamma-miRs*, like miR-21, -146a and, -126. In addition, three different proteins (i.e., $p16^{ink4a}$, SIRT1 and Caspase-1) involved in the acquisition of SASP were analysed. RS- and IS-HUVECs were treated with the single natural compounds or the MIX. The expression and secretion of IL-6 and IL-1β as well as the expression of miR-146a, miR-126 and miR-21 were significantly increased in RS- and IS-HUVECs compared to yHUVECs.

The most relevant result was that the treatment of RS- and IS-HUVECs with the MIX was able to significantly decrease IL-1β and IL-6 expression levels in both models of senescence. On the contrary, no single compounds were able to induce the same effects in both RS- and IS- HUVECs. (Fig. 4a and d).

Analysing the levels of IL-1β and IL-6 released in the culture medium, we observed that the MIX was able to induce an important reduction of both cytokines in RS-HUVECs but not in IS-HUVECs (Fig. 4b and e).

Moreover, the treatment with the MIX caused a strong down-regulation of miR-21 and miR-146a expression in RS-HUVECs (Fig. 4c) and IS-HUVECs (Fig. 4f) respectively as well as a significant up-regulation of miR-126 level in both senescent HUVEC models (Fig. 4c and f). As regards to protein analysis, as expected, both RS- and –IS-HUVECs showed a significant increase of $p16^{ink4a}$ (Fig. 5a and c) and a reduction of SIRT1 levels (Fig. 5b and d) compared to yHUVECs. Notably the treatment of senescent cells with the MIX downregulated $p16^{ink4a}$ expression in both RS- and IS- HUVECs compared to the non-treated senescent cells (Fig. 5a and c) whilst significantly restored the level of SIRT1 as in yHUVECs (Fig. 5b and d). Furthermore, the pro-Caspase-1 level showed a decreasing trend, which is still not significant, in both RS-and IS-HUVECs treated with the MIX compared to the not-treated senescent HUVECs (Suppl. Figure 2).

Anti-inflammatory effect of combined natural compounds on THP-1 cells

The potential anti-inflammatory activity of the natural compounds alone or in combination was also evaluated analysing the expression of IL-1β, IL-6, TNF-α, miR-146a and, miR-21 in LPS-stimulated THP-1 cells. The MIX significantly decreased the expression levels of all tested cytokines (IL-1β, IL-6, TNF -α) (Fig. 6a) and the release of IL-1β in the medium (Fig. 6b) in LPS-stimulated THP-1 cells more efficiently than the single compounds. MiR-146a was found significantly down-regulated by the MIX compared to the single treatments whereas miR-21 was not significantly modulated (Fig. 6c). Regarding the effects on pro-Caspase-1 activation and SIRT1 levels, we observed that the treatment with the MIX was associated with a significant increase of SIRT1 protein level (Fig. 6d) and a significant reduction of pro-Caspase-1 activation (Fig. 6e).

Discussion

The potential anti-aging function of a number of natural compounds has been extensively investigated in vitro, as well as in pre-clinical tests and clinical trials in humans, suggesting a role either in preventing age-related diseases (ARDs) and in "slowing down" aging itself (Correa et al. 2018; Furst and Zundorf 2014). Cellular senescence is recognized as one of the main triggers of the aging process, since the senescent cells acquired the senescence-associated secretory phenotype (SASP), thus fuelling inflammaging. Several natural compounds have been investigated for their anti-senescence and anti-aging potential effects through the modulation of SASP (Orjalo et al. 2009).

Among the components of SASP, in addition to the well-characterized pro-inflammatory cytokines, there are also some miRNAs, named *inflamma-miRs,* as well as epigenetic-related enzymes, i.e. SIRT1 (Hekmatimoghaddam et al. 2017; Yamakuchi 2012).

Therefore, with the aim to analyse the anti-SASP and the anti-inflammatory effects of some nutraceuticals, we characterized the modulation of a number of SASP related molecules induced by bioCurcumin (bCUR), Polydatin and, β-caryophyllene (BCP), which are the main components of the food supplement Fenoxidol™. The biological properties of the natural compounds were analysed when used individually or combined (MIX) (with the same proportions described in Fenoxidol™) on two different human cellular models, such as HUVECs, in replicative and Doxorubicin-induced senescence, and THP-1 cells stimulated with LPS.

Curcumin and Resveratrol (RSV) have been deeply described for their anti-inflammatory property both in monocytic and endothelial cells (Rana et al. 2016; Schwager et al. 2017; Sun et al. 2015), whereas BCP was only recently identified as an anti-inflammatory compound (Yamaguchi and Levy 2019). However, to the best of our knowledge, this is the first study evaluating the effect in vitro of bCUR, RSV and, BCP combined together (data are summarized in Table 1), as described in the novel dietary supplement Fenoxidol™.

The main result of our study is that the treatment with the MIX promotes significant anti-SASP effects on HUVECs and anti-inflammatory effects on LPS-stimulated THP-1 cells.

In both models of HUVEC senescence, as well as in THP-1, a downregulation of IL-1β and IL-6 mRNA expression was observed. This is a key finding if we consider the primary role of IL-1β in fuelling the inflammatory burden: such cytokine is able to stimulate the expression of several target genes (such as IL-6, MCP-1, IL-8) in different cell types, activating the nuclear factor-kB (Nf-kB) signalling (Weber et al.

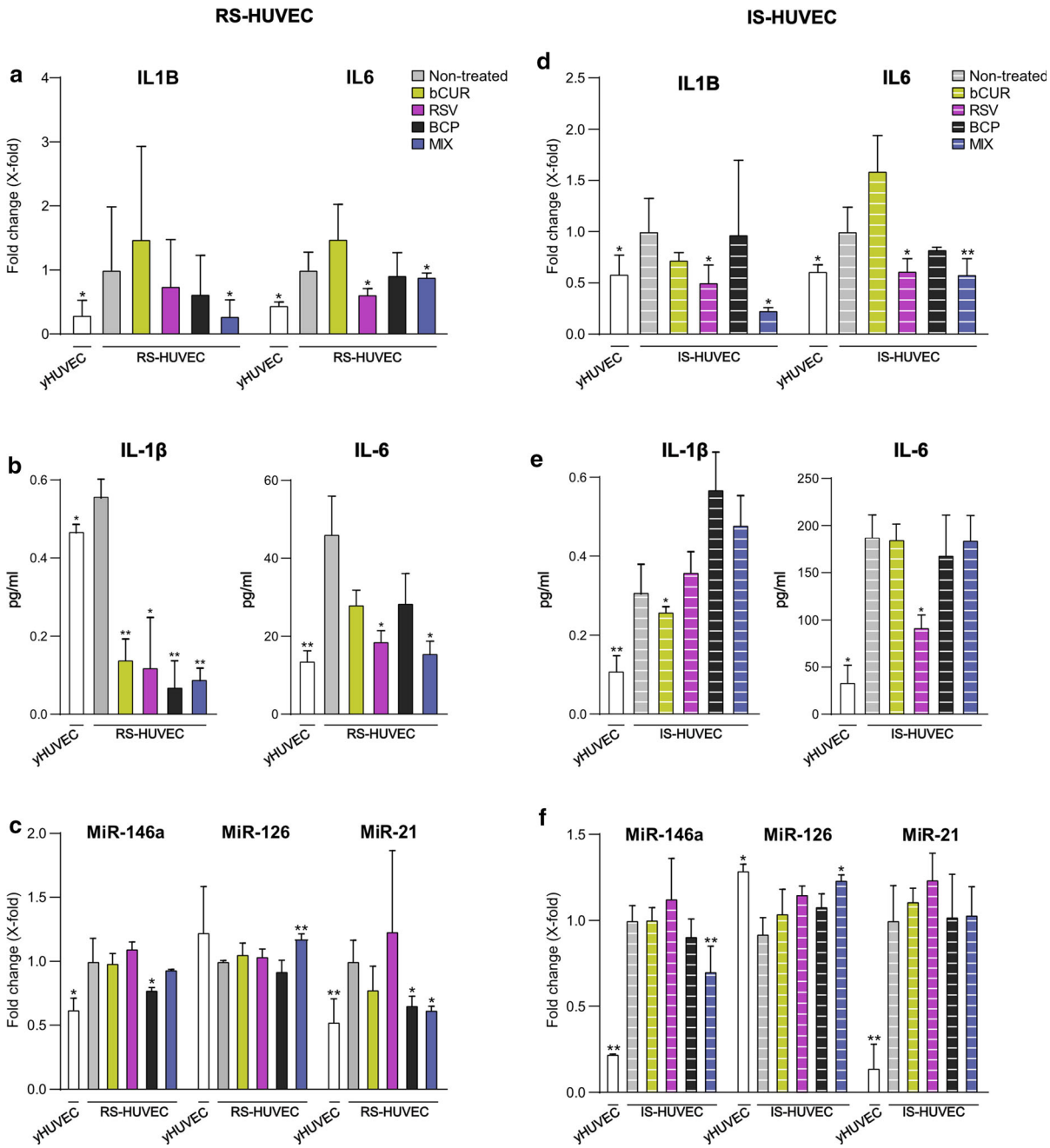


Fig. 4 mRNA and miRNA expression, IL-6 and IL-1β concentration after 3 h of treatment with bCUR, RSV and BCP as single compound and in combination in RS- and IS-HUVECs. **a** and **d** mRNA expression of IL-1β and IL-6 in RS- and IS-HUVECs; **b** and **e** concentration (pg/ml) of IL-1β and IL-6 in the culture medium of RS- and IS-HUVECs; **c** and **f** miRNA expression of miR-146a, miR-126 and miR-21 in RS- and IS-HUVEC. Data are reported as fold change *vs* untreated senescence HUVECs according to 2-ΔCt method and using β-actin and RNU48 (respectively for mRNA and miRNA) as housekeeping; histograms represent the mean of the fold change detected in three different experiments ± SD. Paired *t* test, *p < 0.05 versus RS- and IS-HUVECs; **p < 0.01 versus RS- and IS-HUVECs. *bCUR* bioCurcumin, *RSV* resveratrol, *BCP* β-caryophyllene, *RS* replicative senescence, *IS* induced senescence. RS-HUVECs data are depicted in the left panel (solid-coloured histograms); IS-HUVECs data are reported in the right panel (striped histograms)

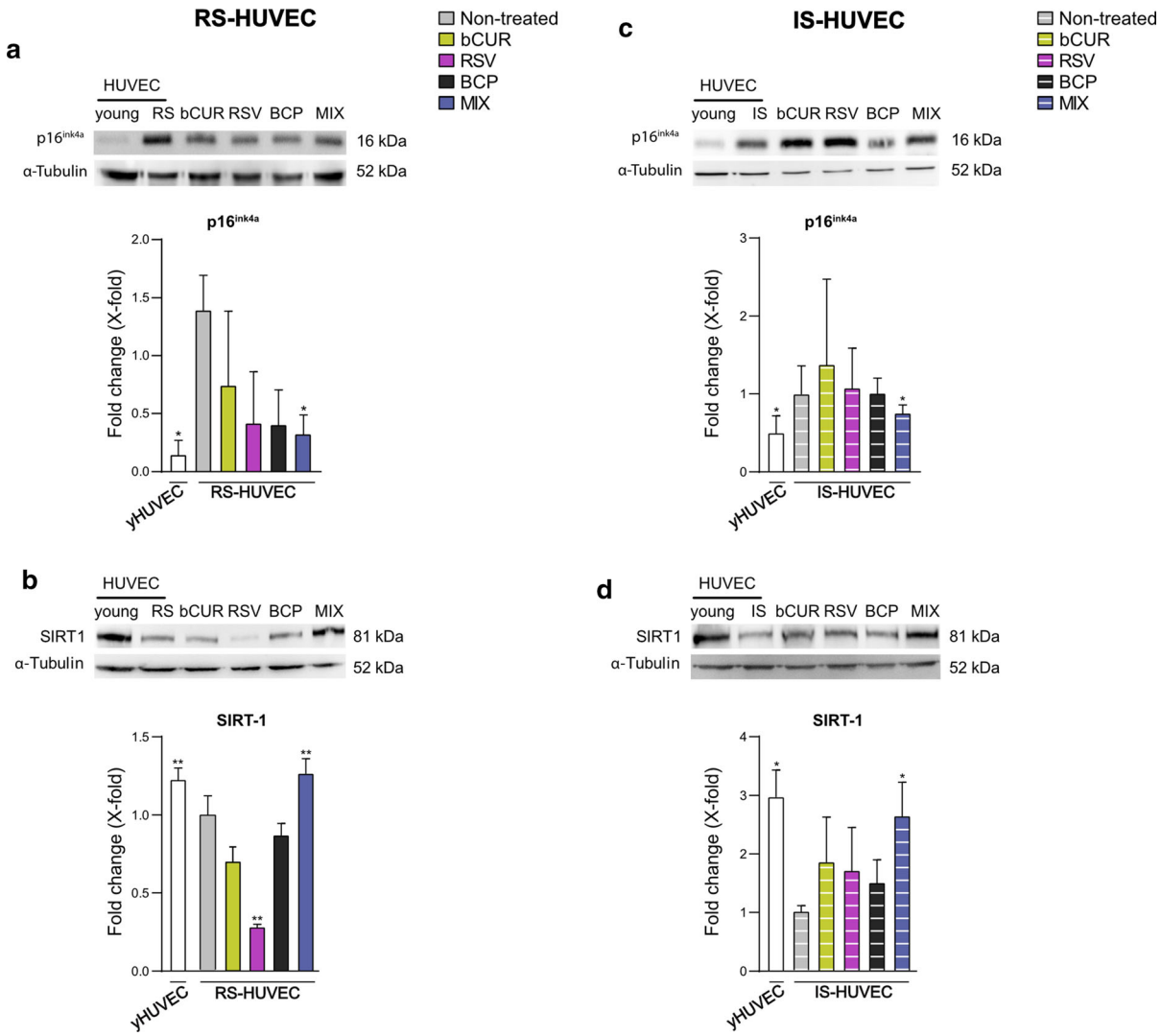
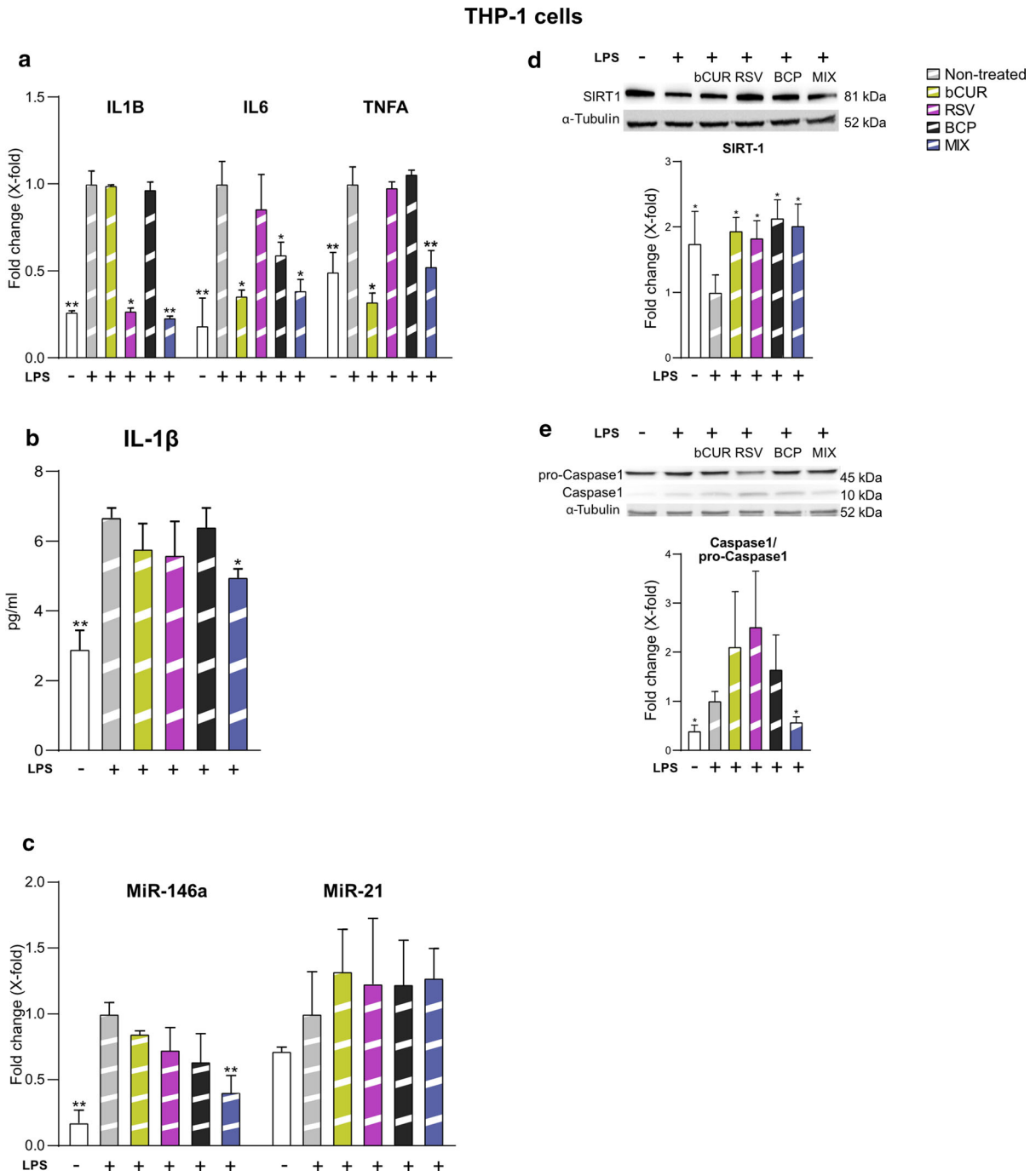


Fig. 5 $p16^{ink4a}$ and SIRT1 protein level after treatment with bCUR, RSV and BCP and their combination in RS- and IS-HUVECs. **a** and **c** $p16^{ink4a}$ protein level and densitometric analysis; **b** and **d** SIRT1 protein level and densitometric analysis. Data are reported as fold change vs untreated senescent HUVECs. All data were normalized using α-tubulin as internal control. Bands were quantified by ImageJ; histograms represent the mean of the protein expression detected in three different experiments ± SD. Paired *t* test *$p < 0.05$ versus RS- and IS-HUVECs; **$p < 0.01$ versus RS- and IS-HUVECs. bCUR, bioCurcumin; RSV, resveratrol; BCP, β-caryophyllene; RS, replicative senescence; IS, induced senescence. RS-HUVECs data are depicted in the left panel (solid-coloured histograms); IS-HUVECs data are reported in the right panel (striped histograms)

2010). Evidence on the anti-inflammatory activity of polyphenols are continuously expanding: Curcumin can decrease several markers of inflammation such as cytokines TNFα and IL-1, the adhesion molecules ICAM-1 and VCAM-1, and some prostaglandins and leukotriens (Shimizu et al. 2019). While it is known that Resveratrol plays anti-inflammatory proprieties which are mainly responsible for its cardioprotective effects (Yahfoufi et al. 2018), it was recently demonstrated that BCP exerts anti-inflammatory activity in high glucose-treated glomerular mesangial cells via the suppression of the NF-κB pathway and the Nrf2 activation (Li et al. 2020).



We observed a significant decreased release of both IL-1β and IL-6 cytokines in the medium of HUVECs in replicative senescence (RS) but not in HUVECs in Doxorubicin-induced senescence (IS). The higher percentage of SA-β-galactosidase (Gal)- positive cells in IS-HUVECs in comparison to RS-HUVECs and the consequent increased amount of cytokines synthesized and released by IS–HUVECs could explain, almost in part, why the three-hour treatment with the MIX is not sufficient to counterbalance the massive release of

◀**Fig. 6** Anti-inflammatory activity of bCUR, RSV and BCP as single compound and in combination in LPS-stimulated THP-1 cells **a** IL-1β, IL-6 and TNFα mRNA expression. Data were reported as fold change vs untreated LPS-stimulated THP1 cells according to 2-ΔCt method and using GAPDH as housekeeping **b** Concentration (pg/ml) of IL-1β in the culture medium of untreated and treated THP-1 cells; **c** miR-146a and miR-21 expression. Data were reported as fold change vs untreated LPS-stimulated THP-1 cells according to $2^{-\Delta Ct}$ method and using RNU48 as housekeeping. **d** and **e** SIRT1 and Caspase-1/pro-Caspase-1 protein level and densitometric analysis. Data were normalized using α-tubulin as internal control and reported as fold change versus LPS-stimulated THP-1 cells untreated with natural compounds. Bands were quantified by ImageJ; histograms represent the mean of the protein expression detected in three different experiments ± SD. Paired *t* test, *p < 0.05 versus THP-1 + LPS; **p < 0.01 versus THP-1 + LPS. *LPS* lipopolysaccharide, *bCUR* bioCurcumin, *RSV* resveratrol, *BCP* β-caryophyllene

SASP factors induced by Doxorubicin treatment (Bielak-Zmijewska et al. 2014).

Notably, our study provided evidence that natural compounds exert their biological effects also modulating microRNAs expression (Lin et al. 2017). It has been previously reported that RSV down-regulates some miRNAs involved in the development of cancer (miR-21, miR-30a-5p, miR-19) (McCubrey et al. 2017; Wang et al. 2015). Here we show that in senescent HUVECs the treatment with the MIX was able to reduce the expression of some *inflamma-miRs* (Olivieri et al. 2013b), such as miR-21 and miR-146a and to increase the expression levels of miRNAs involved in the maintenance of endothelial cells functions, *i.e.* miR-126. A significant downregulation of miR-146a was observed in THP-1 cells stimulated with LPS and treated with the MIX. Remarkably, miR-146a and miR-21 can target some proteins belonging to NF-kB pathway, like the tumour necrosis factor receptor-associated factor 6 (TRAF6), IL-1 receptor-associated kinase 1 (IRAK-1) (Taganov et al. 2006), MyD88 (Chen et al. 2013) and programmed cell death protein 4 (PDCD4), respectively (Sheedy et al. 2010). We recently reported that miR-21 loaded on exosomes released by senescent HUVECs can promote features of senescence in younger cells (Mensa et al. 2020). Further, miR-126 plays a pivotal role in modulating vascular development and homeostasis, targeting specific mRNAs such as CXCL12, VCAM-1, SPRED-1 and PIK3R2 (Olivieri et al. 2014). Thus, the significant effect of MIX treatment in increasing miR-126 observed in both senescent HUVEC models

Table 1 Summarized data of the anti-SASP and anti-inflammatory effect of bCUR, RSV and BCP alone and in combination (MIX) on RS- HUVECs, IS-HUVECs and THP-1 cells

	RS-HUVEC				IS-HUVEC				THP-1 + LPS			
	bCUR	RSV	BCP	MIX	bCUR	RSV	BCP	MIX	bCUR	RSV	BCP	MIX
IL-1β	ns	ns	ns	*↓	ns	*↓	ns	*↓	ns	*↓	ns	**↓
IL-6	ns	*↓	ns↓	*	ns	*↓	ns	**↓	*↓	ns	*↓	*↓
TNF-α	–	–	–	–	–	–	–	–	*↓	ns	ns	**↓
miR-146a	ns	ns	*↓	ns	ns	ns	ns	**↓	ns	ns	ns	**↓
miR-126	ns	ns	ns	**↑	ns	ns	ns	*↑	–	–	–	–
miR-21	ns	ns	*↓	*↓	ns	ns	ns	ns	ns	ns	ns	ns
IL-1β (pg/ml)	**↓	*↓	**↓	**↓	*↓	ns	ns	ns	ns	ns	ns	*↓
IL-6 (pg/ml)	ns	*↓	ns	*↓	ns	*↓	ns	ns	–	–	–	–
SIRT1	ns	**↓	ns	**↑	ns	ns	ns	*↑	*↑	*↑	*↑	*↑
$p16^{ink4a}$	ns	ns	ns	*↓	ns	ns	ns	*↓	–	–	–	–
Caspase-1	ns	*↓	ns	ns	ns	*↓	ns	ns	ns	ns	ns	*↓

LPS lipopolysaccharide, *bCUR* bCurcumin, *RSV* resveratrol, *BCP* β- caryophyllene, *RS* replicative senescence, *IS* induced senescence, *Ns* not significantly modulated, *down arrow* significantly decreased, *up arrow* significantly increased,—data not present

Student's *t* test *p < 0.05 versus RS- and IS-HUVECs

**p < 0.01 versus RS- and IS-HUVECs; p < 0.05 versus THP-1 + LPS

**p < 0.01 versus THP-1 + LPS

strongly suggest its effectiveness in promoting endothelial cell function. In addition the treatment with the MIX was associated with increased SIRT1 levels in senescent HUVECs and with decreased pro-Caspase-1 activation in inflamed THP-1, which are molecules involved in inflammation, aging and ARDs (Cordero et al. 2018). SIRT1 is the most studied sirtuin in mammals, with many roles in several tissues and organs, including the ability to restrain SASP at the transcriptional level (Hayakawa et al. 2015); it also regulates the cellular response to stressors and delays vascular senescence by activating eNOS and suppressing the p53 and NF-kB pathways (Kida and Goligorsky 2016). Notably, SIRT1 blocks the monocyte transmigration into the arterial wall, playing anti-inflammatory effects in endothelial cells (Kida and Goligorsky 2016). Interestingly, increasing evidence suggests that a number of dietary intake of RSV, catechins, EGCG, propolis extracts, creosol, and luteoloside may promote health and extend the lifespan via multiple mechanisms, including also the suppression of NLRP3 activation (Chuang et al. 2014). The NLRP3 inflammasome is essential to mediate the immune responses through the activation of caspase-1 and IL-1β. Indeed, the inflammasome association prompts a proteolytic cleavage of dormant pro-Caspase-1 into active Caspase-1, which converts the cytokine precursors pro-IL-1β into mature and biologically active IL-1β (He et al. 2016).

We observed an increased IL-1β synthesis and release by senescent HUVECs and by LPS-stimulated THP-1 cells. Importantly, the treatment with the MIX was associated with a significant reduction of IL-1β transcription levels in all analysed cellular models, suggesting the ability of MIX to restrain the pro-inflammatory responses and SASP.

Interestingly, we also observed a slight but significant reduction of $p16^{ink4a}$ protein level, a well-known regulator of cellular senescence (Campisi 2011), in both RS-and IS-HUVEC treated with the MIX for 3 h, suggesting a possible anti-aging effect of the three natural compound combination. Notably, our results on HUVECs are in accordance with data suggesting that quercetagetin 3,4′-dimethyl ether exerts anti-senescence effects, confirmed by the modulation of p53 and p21 protein levels, in RS-HUVECs (Yang et al. 2015).

Overall, our results suggest that the MIX of bCUR, RSV and, BCP exerts a combined anti-inflammatory/anti-SASP activity in THP-1 cells and RS- and IS-HUVECs. However, additional studies are needed to confirm the effect of the MIX analysing a larger panel of cell lines (e.g., HAEC, HCAEC). Moreover, further studies on miR-126 target genes (e.g., CXCL12, VCAM-1, SPRED-1 and PIK3R2), could clarify the effects of the MIX on endothelial homeostasis.

Considering that endothelial and innate immune cells are key players in activating and perpetuating the inflammatory responses that fuel inflammaging, which is a common risk factor for the development of the most common ARDs, our data reinforce the hypothesis that specific combinations of nutraceuticals could be more effective than single compounds in promoting healthy aging.

Author contributions Conceptualization, FO, GM and FG; methodology, AS, MT and LM; data curation, GM and AS; writing—original draft preparation, FG and GM; writing—review and editing, OC, LC, DV and MRR; project administration, RR and FM; funding acquisition, FO, and ADP. All authors have read and agreed to the published version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare no conflict of interest.

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Brief Report

Plasma levels of interleukin-38 in healthy aging and in type 2 diabetes

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ABSTRACT

Plasma levels of interleukin (IL)-38 were evaluated in patients with type 2 diabetes (T2DM) and healthy controls. Plasma IL-38 was higher in T2DM patients and positively related to waist/hip ratio, HbA1c, uric acid, liver function tests, triglycerides and total proteins. Patients suffering from diabetic nephropathy had the highest IL-38 levels.

1. Introduction

Type 2 diabetes (T2DM) is one of the most common age-related diseases (ARDs), a group of diseases characterized by a chronic low-grade inflammatory status termed 'inflammaging' [1]. Investigations on pro-inflammatory cytokines, e.g. interleukin (IL)-1β, IL-6, C-reactive protein (CRP) and tumor necrosis factor (TNF)-α, lend valuable insight into the pathophysiology of T2DM and provided new biomarkers of disease progression [2,3]. However, emerging biomarkers capable of tracking inflammaging and predicting the risk of ARD development and progression are still not routinely evaluated in the clinical setting [4].

IL-38, the latest member identified in the IL-1 cytokine family, has been reported to act as an immune modulator mainly by blocking the release of pro-inflammatory cytokines [5]. Recent findings supported the involvement of IL-38 in a variety of conditions [6], including gestational diabetes melli-

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tus (GDM) [7] and pediatric T2DM [8]. However, no evidence is available to our knowledge on the role of this cytokine in healthy aging, in adult patients with T2DM, and in T2DM complications. In the present study we evaluated the circulating levels of IL-38 in patients with T2DM and healthy control subjects and assessed the correlations between IL-38 and specific features of the disease.

2. Materials and methods

2.1. Study population and laboratory assays

Blood samples from 100 patients with T2DM (50 patients without complications and 50 patients with at least one T2DM complication) and 65 age- and gender-matched healthy control subjects (CTR) were selected from a previously collected cohort [9]. All the subjects gave their informed written consent to be enrolled in the study. The study protocol was approved by the Institutional Review Board of IRCCS INRCA (Ancona, Italy). T2DM was diagnosed according to the American Diabetes Association Criteria [10]. The presence/absence of diabetic complications was established as previously described [11]. Collected data included information on vital signs, anthropometric data, medical history, behaviors, and exercise. Blood cell count and biochemical variables were assessed by standard procedures in all subjects. Estimated glomerular filtration rate (eGFR) was calculated according to CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation based on serum creatinine, age, sex, and ethnicity. Human IL1F10 (IL-38) ELISA Kit was purchased from Wuhan Fine Biotech Co. Plasma IL-38 concentration was determined in triplicate according to the instructions from the manufacturers. The mean intra-assay coefficient of variation (CV) for IL-38 measurements was 2.4% ± 1.3%.

2.2. Statistical analysis

Continuous data were tested for normality by Shapiro Wilk's test and are reported as mean ± standard deviation (SD) or median [IQR] for normal and non-normal data, respectively. Receiver operating characteristics (ROC) curve analysis using Youden index and the area under curve (AUC) was used to evaluate the diagnostic accuracy and determine the optimal cutoff of IL-38 in T2DM. Pearson's correlation was used to test for correlations between IL-38 and other biochemical variables. Multiple linear regression analysis using the Enter method was used to evaluate factors predicting plasma IL-38 concentration. Analysis of covariance (ANCOVA) followed by Bonferroni post-hoc tests for multiple comparisons was used to compare the mean differences in biochemical, clinical, and anthropometric variables after adjustment for age and sex. Data analysis was performed using SPSS Statistics for MacOS, version 26.0 (IBM Corp, Armonk, NY, USA) and the R programming language, version 3.6.1. Non-linear correlations were estimated using the nlcor package (https://rdrr.io/github/ProcessMiner/nlcor/man/nlcor.html). Statistical significance was defined as two-tailed p-value < 0.05.

3. Results

3.1. Circulating IL-38 in healthy subjects and patients with T2DM

The clinical, anthropometric, and biochemical variables of 100 patients with T2DM and 65 age- and gender-matched healthy subjects (CTR) are reported in Table 1. A significant increase in the median plasma concentration of IL-38 was observed in T2DM patients compared with CTR (85.7 [93.9] vs. 43.6 [117.0] pg/mL, respectively; $p < 0.0001$) (Fig. 1**A**). When T2DM patients were grouped into two subgroups according to the absence (T2DM-NC) or presence (T2DM-C) of complications, IL-38 levels were significantly higher in both groups compared to CTR (T2DM-NC, 128.4 [93.9] pg/mL, $p < 0.001$; T2DM-C, 131.1 [97.0] pg/mL, $p = 0.002$), whereas no significant difference was observed between T2DM-NC and T2DM-C (Fig. 1**B**). The ROC curve generated to assess the discriminatory ability of IL-38 between CTR and T2DM revealed an optimal IL-38 cutoff of 44.0 pg/mL (sensitivity, 52.3%; specificity, 91.0%; positive predictive value, 79.1%; negative predictive value, 74.6%; AUC = 0.70, $p < 0.0001$; Fig. 1**C**). Multiple linear regression showed that IL-38 levels are associated with T2DM independently of gender, body mass index (BMI), hs-CRP, eGFR and total cholesterol ($p = 0.033$, Table 2). The regression model significantly predicted IL-38 concentration, $F[7,157] = 2.140$, $p = 0.042$, adj. $r^2 = 0.046$. Interestingly, age added significantly to the prediction model, $p = 0.015$. When considering T2DM complications separately and after adjusting for the presence of other complications, we found higher circulating IL-38 levels in patients with diabetic nephropathy compared to the other patients with T2DM (one-way ANCOVA, $F[1, 94] = 5.131$, $p = 0.026$; Fig. 1**D, Table S1**).

3.2. Correlations with clinical and biochemical variables

Correlation analysis showed that plasma levels of IL-38 in patients with T2DM (NC and C) and healthy subjects were positively related to age ($p = 0.026$), HbA1c ($p = 0.002$), uric acid ($p < 0.001$), gamma-GT ($p < 0.001$), triglycerides ($p = 0.001$), total protein ($p = 0.019$) and PAI-1 ($p = 0.014$), and negatively related to HDL-C ($p = 0.041$) and plasma IL-6 ($p = 0.008$). Interestingly, the direction of the association between IL-8 levels and HbA1c was positive ($p = 0.026$) in T2DM patients and negative ($p = 0.008$) in CTR subjects. Fig. 2**A** shows distinct correlation plots for the entire cohort, and for CTR and T2DM subjects separately. The complete correlation matrix is available as **Table S2**. Analysis of the whole dataset using a nonlinear correlation estimation tool confirmed the biphasic association between the two variables and revealed a change in the direction of the correlation at an HbA1c value of 6.2% (44 mmol/mol) (Fig. 2**B**). The coefficient of the correlation estimate is 0.256 ($p = 0.023$). Notably, circulating IL-38 levels displayed a significant age-dependent increase only in healthy subjects ($p < 0.001$), while no significant correlation with age was observed in patients with T2DM (Fig. 2**C**). To further substantiate this finding, subjects were split into subgroups according to a 65-year age cutoff and observed that a significant age-related increase in circulating IL-38 exists only in CTR subjects ($p = 0.002$). Young T2DM patients had higher IL-38 plasma levels compared to age-matched CTR

Table 1 – Comparison of biochemical and anthropometric characteristics between healthy control subjects (CTR) and patients with type 2 diabetes mellitus (T2DM).

Variables	CTR N = 65	T2DM N = 100	p-value
Age (years)	65.2 (9.5)	67.6 (7.1)	0.085
Gender (Males, %)	31 (48%)	48 (48%)	0.969
BMI (Kg/m^2)	27.0 (5.4)	28.5 (3.9)	0.032
Weight (Kg)	70.6 (11.0)	76.9 (11.1)	0.001
Waist-hip ratio	0.89 (0.08)	0.93 (0.07)	0.002
Total cholesterol (mg/dL)	221.5 (40.5)	214.1 (39.9)	0.253
HDL-C (mg/dL)	61.7 (15.8)	55.6 (17.1)	0.025
LDL-C (mg/dL)	132.9 (37.5)	124.4 (32.8)	0.128
Triglycerides (mg/dL)	98.4 (65.6)	145.2 (114.0)	0.001
ApoA1 (mg/dL)	184.2 (35.2)	169.8 (38.4)	0.016
ApoB (mg/dL)	107.0 (32.7)	106.6 (27.0)	0.942
Glucose (mg/dL)	93.7 (9.4)	163.8 (48.7)	<0.001
HbA1C (%)	5.7 (0.4)	7.5 (1.2)	<0.001
HbA1C (mmol/mol)	38 (4)	59 (13)	<0.001
Insulin (UI/mL)	5.4 (3.7)	6.6 (4.0)	0.058
HOMA index	1.25 (0.86)	2.71 (1.95)	<0.001
WBC (n/mm^3)	6.0 (1.5)	6.6 (1.5)	0.011
Platelets (n/mm^3)	226.4 (54.2)	222.7 (100.0)	0.736
hs-CRP (mg/L)	2.36 (2.69)	3.73 (3.61)	0.005
PAI-1 (ng/mL)	21.5 (12.2)	20.1 (9.6)	0.436
Fibrinogen (mg/dL)	301.2 (68.4)	292.5 (85.7)	0.552
Iron (μg/dL)	82.6 (21.9)	77.3 (23.2)	0.152
Ferritin (ng/mL)	126.7 (101.3)	129.4 (133.3)	0.891
Creatinine (mg/dL)	0.82 (0.22)	0.95 (0.39)	0.015
eGFR (mL/min)	89.8 (43.2)	76.4 (21.3)	0.005
Azotemia (mg/dL)	37.9 (7.4)	41.2 (11.9)	0.006
Uric acid (mg/dL)	4.8 (0.9)	5.0 (1.4)	0.196
Disease duration (years)	–	18.2 (1.4)	–
T2DM medications (n)			
Any medication	–	84	–
Metformin	–	40	–
Sulphonylureas	–	56	–
Glinides	–	1	–
Insulin	–	17	–
T2DM complications (n)			
Retinopathy	–	41	–
Nephropathy	–	15	–
Neuropathy	–	22	–
Peripheral artery disease	–	16	–
MACE	–	9	–

Variables are expressed as mean (standard deviation). P value from *t*-test for continuous variables and from chi-squared tests of association for categorical variables.

($p < 0.0001$), while no significant difference was reported between CTR and T2DM at advanced ages ($p = 0.915$) (Fig. 2D).

4. Discussion

In the present study, we found that the plasma concentration of IL-38, a natural suppressor of the inflammatory response, was significantly increased in T2DM, in line with the few available reports showing the upregulation of IL-38 in placentas from GDM patients [7] and in sera from pediatric T2DM patients [8]. The higher circulating IL-38 levels observed in patients with diabetic nephropathy compared to the other T2DM patients represent the first evidence of IL-38 modulation in a T2DM complication and are in line with a report showing a strong association between IL-38 upregulation and renal impairment in systemic lupus erythematosus [12].

While a previous study showed that circulating IL-38 is negatively related to insulin resistance [8], we found two distinct significant trends of IL-38 concentration in relation to HbA1c levels. Of note, the point of trend reversal (HbA1c = 6.2%) falls into the interval of 6.0–6.4% (42–47 mmol/mol), which is predictive of high T2DM risk [13]. This result points out the inability of IL-38 to counteract the excessive inflammatory response and metabolic damage in the presence of alterations in glycemic control. Furthermore, positive correlations were identified with PAI-1 in CTR and uric acid in T2DM patients, both involved in the pathogenesis of the age-related endothelial dysfunction [14]. In matched CTR subjects, we showed significant negative correlations between IL-38

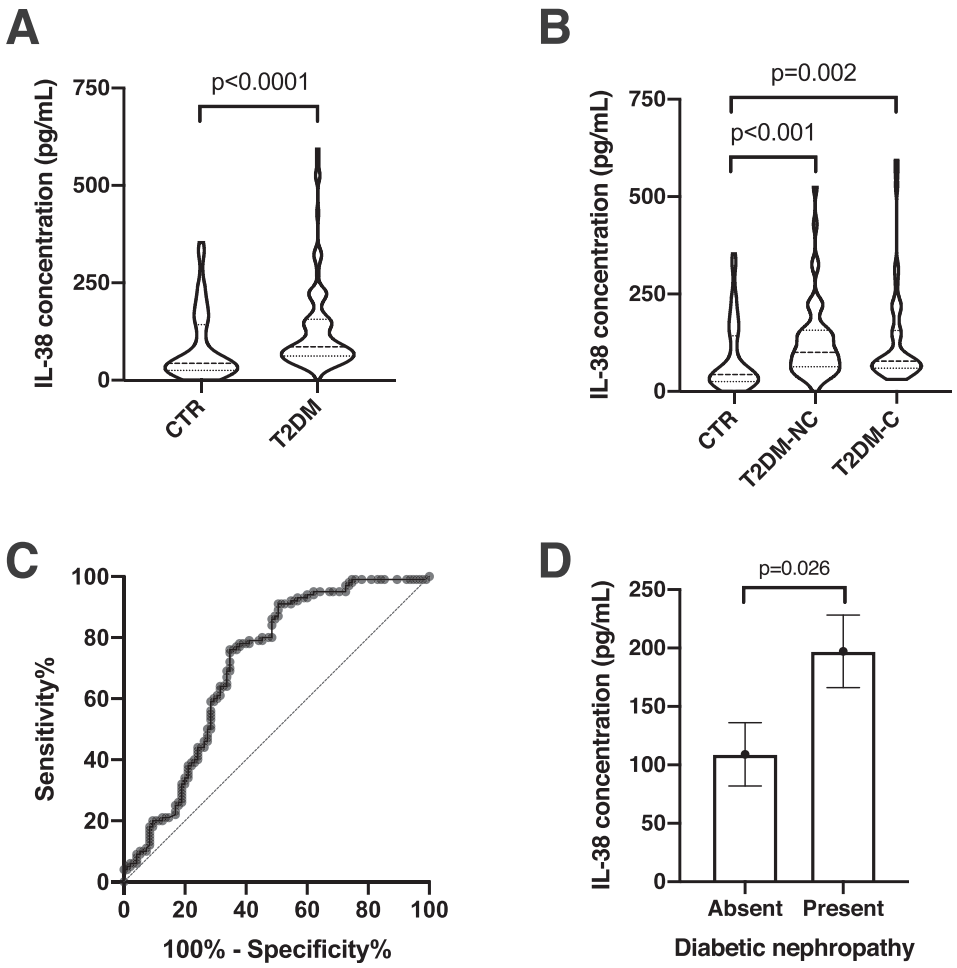

Fig. 1 – IL-38 plasma concentration (pg/ml) in T2DM patients and CTR. A. Plasma IL-38 levels in T2DM patients (NC and C) (n = 100) and healthy controls (CTR) (n = 65). B. IL-38 plasma concentration in T2DM patients with complications (T2DM-C) (n = 50) and T2DM patients without complications (T2DM-NC) (n = 50) compared with healthy controls (CTR) (n = 65). Data were determined by IL-38 ELISA kit. P values from Mann Whitney U and Kruskal Wallis tests followed by Dunn's multiple comparisons. C. Receiver-operating characteristic (ROC) curve analysis of plasma IL-38 for the diagnosis of T2DM. D. IL-38 plasma concentration (pg/ml) in patients with or without diabetic nephropathy. Data are shown as estimated marginal mean ± SEM. P value following analysis of co-variance (ANCOVA) with Bonferroni correction.

Table 2 – Multiple linear regression of IL-38 plasma levels as dependent variable adjusting for type 2 diabetes, gender, age, BMI, hs-CRP, eGFR, and total cholesterol.

Variable	B	S.E.	Beta	P value
Type 2 diabetes (absent/present)	38.465	17.896	0.174	0.033
Gender	9.864	17.139	0.046	0.566
Age (years)	2.525	1.029	0.191	0.015
BMI (Kg/m^2)	−1.601	1.904	−0.068	0.402
hs-CRP (mg/L)	1.380	2.642	0.042	0.602
eGFR (mL/min)	−0.129	0.283	−0.036	0.650
Total cholesterol (mg/dL)	0.037	0.219	0.014	0.864

B, unstandardized regression coefficient; S.E., standard error of the coefficient, Beta, standardized regression coefficient; BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate.

levels and both HbA1c and HDL-C, in agreement with the observation that IL-38 up-regulation attenuated obesity-related insulin resistance [15].

Epidemiological evidence and experimental data corroborate the consideration that aging is the major risk factor for developing ARDs, including T2DM [16]. Accordingly, we showed that age added significantly to a multivariate model

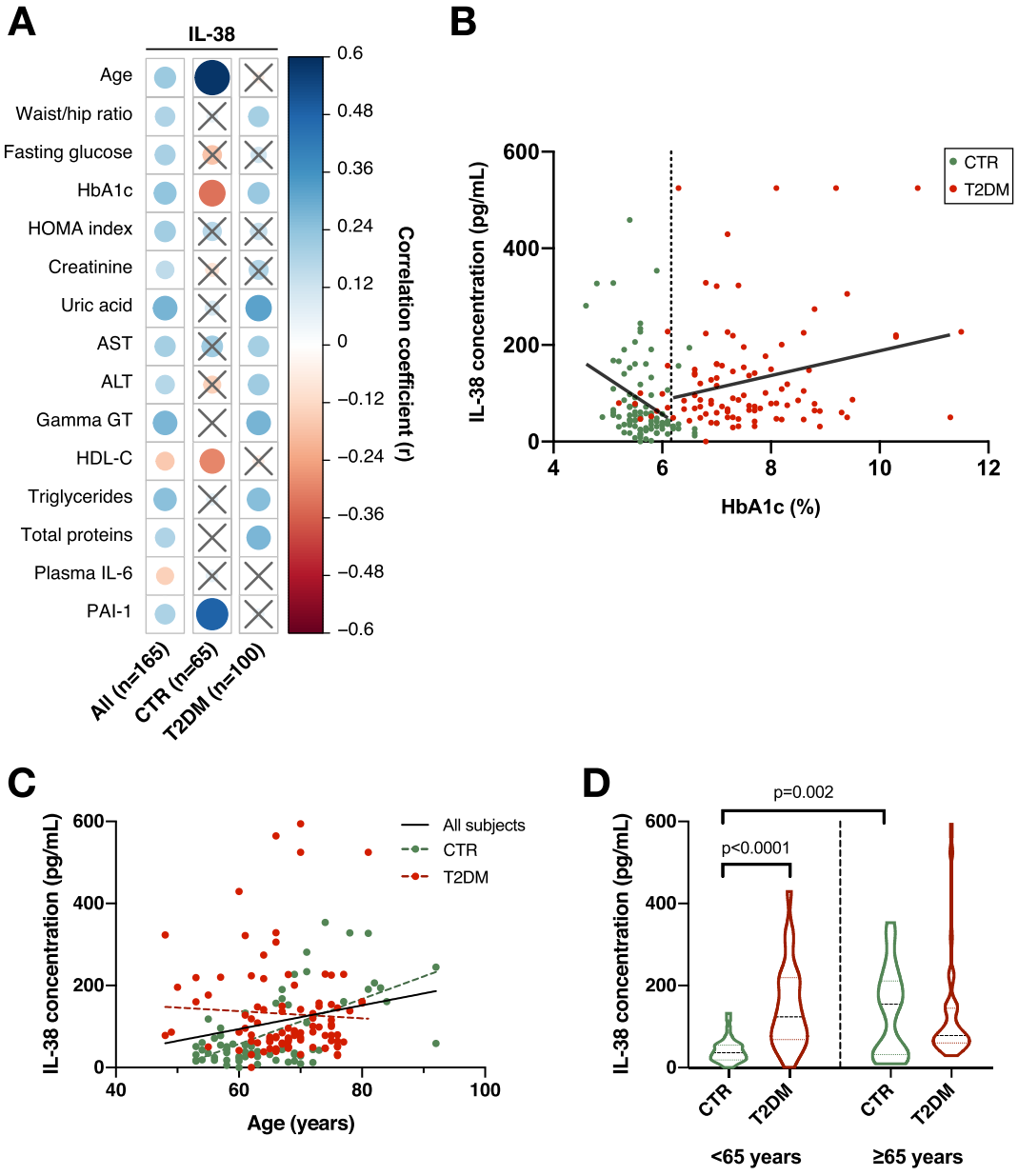

Fig. 2 – Correlations between circulating IL-38 levels and selected biochemical variables in T2DM and CTR subjects. A. **Correlation plot showing Pearson's correlations between different variables and IL-38 in the whole population (left), in healthy subjects (middle), and in T2DM patients (right). The intensity of the color and the size of the circle depend on the magnitude of the correlation. Crosses indicate non-significant ($p \geq 0.05$) correlations.** B. **Scatter plot showing the correlation between HbA1c and plasma IL-38. The regression line retrieved by the nonlinear correlation analysis is displayed (see text).** C. **Scatter plot showing the correlation between age and plasma IL-38. Regression lines are displayed for the whole population (black), CTR subjects (green), and T2DM patients (red).** D. **Plasma IL-38 levels in T2DM and CTR subjects according to age. P values from Mann Whitney U. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**

predicting IL-38 levels and including the presence of T2DM and other biochemical variables. Notably, we observed an increasing age-related trend of circulating IL-38 in CTR subjects and a significant inverse correlation between IL-38 and plasma levels of the pro-inflammatory cytokine IL-6 in the entire cohort. In agreement with the rising consideration of T2DM as a manifestation of accelerated aging [17], no age-related trend in IL-38 plasma levels was found in T2DM, with younger patients showing higher levels of the cytokine compared to their healthy counterparts. Similar differences in the age-related trends according to the presence or absence of T2DM were observed in previous works from our group [11] and reflect the typical profile of many established markers of biological aging [18].

Our findings are compatible with the evidence that T2DM is characterized by chronic low-grade inflammation recently

referred to as 'metaflammation', which originates from visceral adipose tissue and is driven by overnutrition and nutrient excess [17,19]. Consistently with the role of the liver as the main target of metaflammation and a major contributor to the inflammatory state that has been linked with the development of T2DM vascular complications [20], we observed positive correlations in T2DM patients between IL-38 levels and liver function tests.

In conclusion, we demonstrated that the circulating levels of IL-38 follow an age-related trend in healthy aging, while increased IL-38 levels were reached faster in patients with T2DM. Our results indicate that IL-38 upregulation is carried out to exert an anti-inflammatory response, as demonstrated in other *in vitro* and *in vivo* models of inflammatory joint diseases [21], and to mitigate the metabolic disorders of T2DM [22]. Despite the need to validate these results in further longitudinal studies on larger cohorts, which poses a main limitation to our study, IL-38 could be an innovative biomarker to detect deviations from a healthy aging trajectory and could be useful in the multidimensional evaluation of the disease progression.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2020.108585.

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Review

Pleiotropic effects of polyphenols on glucose and lipid metabolism: Focus on clinical trials

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ABSTRACT

Epidemiological evidence from observational studies suggests that dietary polyphenols (PPs) – phytochemicals found in a variety of plant-based foods – can reduce the risk of developing type 2 diabetes mellitus (T2DM). Clinical trials have also indicated that PPs may help manage the two key features of T2DM, hyperglycemia and dyslipidemia. Since the incidence of T2DM is dramatically increasing worldwide, identifying food-based approaches that can reduce the risk of developing it and help manage its main risk factors in early-stage disease has clinical and socioeconomic relevance.

After a brief overview of current epidemiological data on the incidence of T2DM in individuals consuming PP-rich diets, we review the evidence from clinical trials investigating PP-enriched foods and/or PP-based nutraceutical compounds, report their main results, and highlight the knowledge gaps that should be bridged to enhance our understanding of the role of PPs in T2DM development and management.

1. Introduction

Polyphenols (PPs) are phytochemicals found in a wide range of plant-based foods (Gurău et al., 2018; Kim et al., 2016b). The approximately 10,000 PPs identified to date have been classified according to their chemical structure into two main groups: flavonoids, including flavonols, flavones, flavonones, flavanols, isoflavones and anthocyanins, and non-flavonoids, comprising phenolic acids, lignans, stilbenes, and tannins (Gurău et al., 2018).

Whereas the antioxidant activity of PPs has extensively been investigated (Shibata et al., 2009), recent evidence suggests that they instigate a variety of mechanisms, including complex epigenetic modulations, thus affecting several major pathways (Nasir et al., 2019). Whereas their pleiotropic effects, including antioxidant, antibacterial and antiviral actions, have been described in a wide range of cell types (Bunte et al., 2019; Momtazi-Borojeni et al., 2019), other effects, such as coagulation system inhibition (Bijak et al., 2019) and the promotion of misfolded protein aggregate disassembly (resulting in inhibition of amyloid aggregation) (Dhouafli et al., 2018), are not widely known.

Human aging and age-related diseases are characterized by a chronic, systemic, low-grade, subclinical inflammatory process that has been designated as inflammaging (Fulop et al., 2018; Olivieri et al., 2018). PPs have been reported to exert a variety of beneficial health effects, including restraining inflammaging (Gurău et al., 2018). Given the well-known protective effect of the Mediterranean diet (MD) against a variety of age-related diseases (Estruch et al., 2018) and its high PP content (Guasch-Ferre et al., 2017), it has recently been hypothesized that PPs play a key role in mediating its benefits (Martucci et al., 2017). In particular, PPs have been suggested to exert a hormetic effect, *i.e.* one where low doses promote pro-longevity responses but higher amounts may be detrimental (Martucci et al., 2017; Silva-Palacios et al., 2018). These data suggest that PP-enriched foods may

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have the potential to attenuate inflammaging by promoting hormetic effects, such as a reduction in senescent cell accumulation and the activation of antioxidant and anti-inflammatory pathways, thus preventing or delaying the onset of the most common age-related conditions.

Mounting evidence supports the hypothesis that the pleiotropic effects of PPs are exerted either through their metabolites or *via* modulation of the human microbiota (Edwards et al., 2017; Gurău et al., 2018). Indeed, colonic bacteria can produce bioactive molecules from PP-enriched foods and most of the molecules contained in such foods reach the large intestine and are digested to smaller phenolic acids, which are probably the actual bioactive effectors (Edwards et al., 2017). Moreover, PP consumption exerts prebiotic-like activities by modulating the gut microbiota and its gene expression (Lyu et al., 2017). However, the mechanisms underpinning the effects of PPs on host gut microbiota are still largely unknown (Anhe et al., 2015; Etxeberria et al., 2015; Most et al., 2017; Van Hul et al., 2018), not least because plant-derived foods also contain other compounds that can act as prebiotics, including dietary fiber (Carvalho-Wells et al., 2010). As a result, establishing whether the prebiotic effects of enriched foods are indeed due to PPs is fraught with difficulties and hampers their evaluation.

Despite the intense work carried out to document the beneficial health and pro-longevity effects of PPs using *in vitro* and *in vivo* animal models, our knowledge of the molecular mechanisms that underpin their pharmacological activities is still too sketchy. Outstanding issues include the clinical relevance of the pleiotropic effects of PPs, which should be explored by clarifying whether the benefits of PP-enriched foods can indeed be ascribed to their PP content or are mediated by other components. Without information on the dose/concentration of the purified compound used, it is impossible to determine dose-related efficacy. Moreover, although cellular models can help identify a specific mechanism of action, they lack physiological relevance, since they largely use non-physiological PP doses, do not investigate interactions with the microbiota, and dose non-intestinal cells with whole extracts.

Another outstanding issue is the actual efficacy of PP-enriched foods and PP supplementation, since dietary PPs usually reach target organs at low concentrations (Brglez Mojzer et al., 2016). The molecular and metabolic pathways affecting PP bioavailability in the gut depend on the chemical structure and composition of the microbiota of each individual (D'Archivio et al., 2010), on PP chemical structure, *e.g.* different glycoside moieties (Actis-Goretta et al., 2015), on the food matrix (Bohn, 2014), and on host-related factors (Mecha et al., 2020).

The demonstration of significant effects of PPs in cellular or animal models is insufficient to predict their effects in humans. Several *in vitro* studies have used PP doses that cannot be achieved *in vivo*, even with dietary modifications that enhance PP bioavailability (*e.g.* quercetin) (Bunte et al., 2019). For instance, PP-depleted fermented green tea extract is still able to mediate the benefits of green tea (Seo et al., 2017). However, human studies investigating whether green tea alters gut microbiota composition have not provided unequivocal results (Janssens et al., 2016).

The main data obtained from the investigation of cellular and animal models and mechanisms to establish the glucose- and lipid-lowering potential of PPs are summarized in Tables 1 and 2. For the details, the reader is referred to the original works listed in the essential bibliography.

The following analysis and discussion examine studies evaluating the effects of PPs on the onset of type 2 diabetes mellitus (T2DM) and on glucose and lipid metabolism.

2. Effects of polyphenols on glucose and lipid metabolism

Nutritional interventions are essential to manage diabetes. Indeed, balancing macronutrient intake, reducing the glycemic index and the carbohydrate load, and adopting a healthy dietary regimen are emerging as treatment pillars. PPs can affect glycemic homeostasis through a variety of mechanisms that include: 1) inhibition of α-amylase and α-glucosidase, 2) inhibition of glucose transporters, 3) stimulation of insulin secretion, 4) effects on hepatic glucose homeostasis, 5) protection against oxidative stress and inflammation associated to hyperglycemia, and 6) reshaping of the microbiota.

The potential action of PPs on lipid metabolism can be summarized as follows: 1) inhibition/reduction of intestinal transport of cholesterol and triglycerides (TGs), 2) reduction of serum cholesterol, TGs and lipoproteins, and 3) interaction with cholesterol and TG synthesis and elimination.

A PubMed search using the terms "polyphenols", "type 2 diabetes", "glucose metabolism", "dyslipidemia", "lipid metabolism", and their MESH terms was performed for studies in English published from 2005 to 2019. Where duplicate information was available, the more recent hits were selected. The *in vitro* and animal studies of glucose metabolisms are reported in Table 1; the main anti-hyperlipidemic activities of the major PPs are reported in the papers listed in Table 2; finally, a schematic summary of the sites of action of PPs relevant for glucose and lipid metabolism is reported in Fig. 1. Since several effects of PPs on glucose/lipid absorption and metabolism may be mediated by other, non-PP, compounds in food supplements, the formulations used in animal studies are also reported.

3. Observational studies of the association between polyphenols and type 2 diabetes onset

Several papers have described a protective effect of PP consumption against T2DM development. A recent meta-analysis has found 18 prospective epidemiological studies showing an association between exposure to PPs and T2DM incidence (hazard ratio-HR: 0.56; 95 % confidence interval-CI: 0.34 – 0.93) (Rienks et al., 2018). Similar results have been obtained by subdividing the results in relation to the different PP subclasses, *i.e.* flavonoids, phenolic acids, stilbenes, and lignans. The fact that several of these associations were not linear (Rienks et al., 2018) suggests: i) that the "hormesis hypothesis" is supported by epidemiological evidence; and ii) that PP intake can reduce the risk of developing T2DM. However, controlled, randomized clinical trials assessing the effect of PPs on T2DM development are not yet available.

4. Clinical trials exploring the effects of polyphenol supplementation on glucose and lipid metabolism

Designing clinical trials that explore the health benefits of PPs is complicated by the absence of regulatory recommendations regarding the reporting of their content in supplemented foods. Moreover, careful selection is required to avoid the inclusion in trials of foods containing unnecessarily high PP doses and of products that can discourage the consumption of a naturally PP-rich diet. Despite the intrinsic difficulty of translating *in vitro* results to *in vivo* settings, the large number of clinical trials that have been conducted in the past decade demonstrates the strong interest attracted by the use of PPs in patients with a variety of conditions.

A search of the ClinicalTrials.gov website using the keyword 'polyphenols' found 467 trials, of which 316 are completed and 97 are active or recruiting. Only those involving PPs and T2DM or dyslipidemia were selected for the purpose of this review. The search identified 17 trials relating to PPs and dyslipidemia (13 completed, 3 active, 1 an unknown status) and 27 trials involving PPs and T2DM (17 completed, 5 recruiting or active, 4 in an unknown status). Data on the influence of PPs on glucose and lipids were sought in the website by identifying the papers associated to the clinical trials. The results are reported in Table 3.

As regards T2DM, we considered seven trials with published results. The sample size ranged from 8 to 86 subjects, who consumed the study products for periods ranging from a single time to 9 weeks. The trials

Table 1
Summary of the evidence for the glucose-lowering effects of polyphenols grouped according to their activity against the different features of diabetes.

Mechanism	Polyphenols	Models	References
Interaction with glucose absorption			
Inhibition of SGLT1 and GLUT2	Epigallocatechin gallate, epigallocatechin and epicatechin gallate	Caco-2 intestinal cells	(Johnston et al., 2005)
	Quercetin-3-O-rhamnoside, phlorizin, pelargonidin-3-O-glucoside and 5-caffeoylquinic acid	Caco-2 intestinal cells	(Manzano and Williamson, 2010)
	Apigenin and apigenin 7-O-glucoside	Caco-2/TC7 cells	(Villa-Rodriguez et al., 2018)
Inhibition of α-amylase, α-glucosidase and maltase	Quercetagetin, scutellarein and fisetin	Human salivary α-amylase	(Lo Piparo et al., 2008)
	Chlorogenic acid and 5-caffeoylquinic acid	Porcine pancreatic α-amylase	(Narita and Inouye, 2009)
	Proanthocyanidins	Porcine pancreatic α-amylase	(Grussu et al., 2011)
	Rosmarinic acid, olivetol, curcumin, resveratrol, p-coumaric acid, 1,1,2,2-tetrakis(p-hydroxyphenyl) ethane, isoliquiritigenin and caffeic acid phenethyl ester	α-glucosidase (from *Saccharomyces cerevisiae*) and α-amylase (porcine)	(Taslimi and Gulcin, 2017)
	Phlorizin, chlorogenic acid and tannin	C57BL/6 J mice (polyphenols in powder, in combination with maize starch and alone)	(Li et al., 2019)
Stimulation of insulin secretion and interaction with insulin pathways			
Increase in GLP-1 output	Caffeoylquinic acid and feruloylquinic acid	NCI-H716 cells, C57BL/6 J mice (d-(+)-glucose or starch plus glyceryl trioleate with or without solid extract of coffee polyphenol)	(Fujii et al., 2015)
	Anthocyanin	Caco-2 cells, HUVEC cells, Wistar rats (grape seed extract by oral gavage)	(Gonzalez-Abuin et al., 2014)
	Genistein	Albino rats (polyphenols alone or in combination with metformin)	(Rehman et al., 2019)
	Procyanidins	ICR mice (procyanidins from cocoa liquor, in fractions with different degree of polymerization)	(Yamashita et al., 2019)
Inhibition of DPP4	Catechin, epicatechin and their oligomer and polymer procyanidins	Human recombinant DPP4	(Ryan et al., 2017)
Inhibition of PDE	Resveratrol and curcumin	Mouse MIN6 cells, human pancreatic islet HP62 β-cells	(Rouse et al., 2014)
Activation of PKA	Genistein	Rat INS-1 and mouse MIN6 cells	(Liu et al., 2006)
	Vanillic acid	INS-1 cells, Wistar rats (polyphenol in pure form, orally administered)	(Mahendra, 2019)
Upregulation of GLUT4	Anthocyanins and procyanidins	KK-Ay mice (black soybean seed extract)	(Kurimoto et al., 2013)
		ICR mice (procyanidins from cocoa liquor, in fractions with different degree of polymerization)	(Yamashita et al., 2019)
	Epigallocatechin-3-O-gallate	Rat L6 cells	(Zhang et al., 2010)
Activity on liver glucose homeostasis			
Suppression of PEPCK and G6Pase genes	Procyanidins, cinnamic acid and catechin	H4IIE rat hepatoma cells C57BL/6 J mice (cinnamon extract cinnamic acid-enriched defatted soy flour, oral administration)	(Cheng et al., 2012)
	Chlorogenic acid	Human liver microsomes	(Henry-Vitrac et al., 2010)
	Epigallocatechin gallate	Primary hepatocytes from C57BL/6 J mice	(Collins et al., 2007)
		ICR mice (EGCG alone and in combination with starch, glucose, sucrose and maltose, oral administration)	(Li et al., 2018)
	Kaempferol	C57BL/6 J mice (kaempferol isolated from *Ginkgo biloba*, orally administered)	(Alkhalidy et al., 2018)
	Pterostilbene	H4IIE cells	(Ren et al., 2018)
Upregulation of glucokinase	Ferulic acid and p-coumaric acid	C57BL/KsJ *db/db* mice (pure form purchased, oral administration)	(Jung et al., 2007)
	Hesperidin and naringin	C57BL/KsJ *db/db* mice (pure form purchased, oral administration)	(Jung et al., 2006)
Protection against oxidative stress and inflammation			
Restoration of antioxidant enzymes and prevention of β-cell apoptosis	Epigallocatechin gallate and quercetin	INS-1 rat insulinoma cells	(Kim et al., 2010)
	Epicatechin, catechin, and procyanidins	ZDF rats (cocoa powder-rich diet)	(Fernandez-Millan et al., 2015)
	Anthocyanins	INS-1 cells	(Zhang et al., 2011)
Reduction of inflammation markers	Cyanidin 3-glucoside, procyanidins, anthocyanin and quercetin	C57BL/6 J mice (blueberry, blackberry, and blackcurrant powders in diet)	(Kim et al., 2016a)
		H4IIE rat hepatoma and HDFa cells	(Hoskin et al., 2019)

DPP4, dipeptidyl peptidase-4; G6Pase, glucose 6-phosphatase; GLP-1, glucagon-like peptide-1; GLUT2/GLUT4, glucose transporter 2/4; PDE, phosphodiesterase; PEPCK, phosphoenolpyruvate carboxykinase; PKA, protein kinase A; SGLT1, sodium-glucose transporter 1.

evaluated various outcome measures related to insulin sensitivity and oxidative stress. Although the protocols included a wide range of PP compositions, sources, and food matrices, the products were all designed to provide PP amounts consistent with a diet rich in fruit and vegetables and to avoid interference due to the presence of non-PP components, *e.g.* fiber or trace elements. A randomized crossover study of male T2DM patients found that meat meals seasoned with a PP-rich spice mixture improved endothelial function (Li et al., 2013). A trial involving 38 healthy obese or overweight first-degree relatives of T2DM patients demonstrated that supplementation of a high-fructose diet with

Table 2
Summary of the evidence for the lipid-lowering effects of polyphenols grouped according to their mechanism of action.

Mechanism	Polyphenols	Models	Reference
Reduction of the intestinal absorption of cholesterol and triglycerides			
Inhibition of intestinal cholesterol absorption mediated by NPC1L1	Luteolin and quercetin	Wistar rats (pure form purchased and dissolved in DMSO, oral administration)	(Nekohashi et al., 2014)
	Curcumin	Caco-2 cells	(Feng et al., 2010)
Reduction of cholesterol absorption	Silymarin and polyphenol fraction	Wistar rats (extracted from *Silybum marianum*, administered as a dietary supplement)	(Sobolova et al., 2006)
Inhibition of NPC1L1, SR-BI and ABCA1 expression and increase in ABCG5, ABCG8, and LDL-R expression	Proanthocyanidins and anthocyanins present in black chokeberry	Caco-2 cells	(Kim et al., 2013)
Interaction with blood cholesterol, triglyceride and lipoprotein levels			
Upregulation SR-BI, ATP-binding cassette transporters ABCA1 and ABCG1 or ApoA1	Caffeic acid and ferulic acid	C57BL/6 J mice (caffeic acid as cooled coffee liquid, ad libitum; ferulic acid dissolved in water, oral gavage)	(Uto-Kondo et al., 2010)
	Resveratrol	THP-1 cells	(Voloshyna et al., 2013)
	Catechins	Wistar rats (isolation of green tea GMB-4 clones in powder form)	(Erna Susanti et al., 2019)
	Quercetin	RAW264.7 cells	(Chang et al., 2012)
		Sprague Dawley rats (nanoemulsion, with diet)	(Son et al., 2019)
Reduction of plasma TC, LDL-C, TGs, and increase of HDL-C	Curcumin	*LDL-R*$^{-/-}$ mice (pure form purchased, in diet)	(Shin et al., 2011)
	Theaflavin and thearubigins	Wistar rats (pure form purchased and dissolved in experimental drinks)	(Imran et al., 2018)
	Proanthocyanidins, chlorogenic acid, catechins, and xanthones	Sprague Dawley rats (commercial extract consisting of *Theobroma cacao* and *Coffea robusta* beans, leaves of *Camellia sinensis* and fruits of *Garcinia mangostana*, supplemented in diet)	(Huang et al., 2016)
Inhibition of MTP activity; reduction of ApoB100 secretion; reduction of plasma TGs and TC; increase of HDL-C.	Naringenin	Wistar rats (supplements to western diet)	(Mulvihill et al., 2010)
	Nobiletin	*LDLR*$^{-/-}$ mice	(Mulvihill et al., 2011)
	Naringenin, hesperidin, nobiletin, and tangeretin	J774A.1 cells	(Whitman et al., 2005)
Influence on lipid metabolism			
Downregulation of HMGCR and sterol regulatory element-binding proteins (SREBPs)	Catechin and gallic acid from *Solanum nigrum*	C57BL/6 J mice (coffee polyphenols powder without caffeine, dietary supplement) and HepG2 cells	(Chang et al., 2017)
	Caffeoyl and feruloyl quinic acids	C57BL/6 J mice (roasted coffee bean extract, dietary supplement) and Hepa 1−6 cells	(Murase et al., 2011)
	Oligomeric proanthocyanidins	Sprague Dawley rats (Oligomeric proanthocyanidin isolated from *Crataegus oxyacantha,* oral gavage administration)	(Sankar et al., 2018)
Increase in cholesterol and bile acids fecal elimination	Epigallocatechin gallate	Bile salt micelles	(Kobayashi et al., 2014)
	Punicalgin and Inulin	C57BL/6 J mice (pomegranate extract and inulin alone or in combination, supplementation in high-fat/high-sucrose diets)	(Yang et al., 2018)
Interaction with the PPARγ-ABCA1/CYP7A1 pathway	Punicalgin and pomegranate ellagic acid	L-02 cells	(Lv et al., 2016)
	Quercetin	Sprague Dawley rats (quercetin and quercetin nanoemulsion)	(Son et al., 2019)
Regulation of lipid metabolism, reduction of visceral obesity, enhanced regulation of fatty acid β-oxidation by PPARα	Rutin, quercetin and gallic acid	Zebrafish (dry matter of wine lees extract)	(Caro et al., 2017)
	Theaflavins, thearubigin, and theabrownins	Sprague Dawley rats (oxidized tea polyphenols, oral administration)	(Wang et al., 2017)

ABCA1; ATP-binding cassette transporter 1; ABCG, ATP-binding cassette transporter subfamily G; ApoA1, apolipoprotein A1; ApoB100, apolipoprotein B100; CYP7A1, cytochrome P450 7A1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL-R, LDL receptor; NPC1L1, Niemann-Pick C1-Like 1; PPAR, peroxisome proliferator-activated receptor; SR-B1, scavenger receptor class B type 1; SREBP, sterol regulatory element-binding proteins.

2 g/day grape PPs for 9 weeks reduced the fasting hepatic insulin sensitivity index by 20 % compared to placebo (Hokayem et al., 2013). Recently, a study assessing the effect of strawberry and cranberry PPs on 41 subjects with a body mass index ≥ 25 kg/m^2 and plasma fasting insulin > 60 pmol/L found a 14 % increase in insulin sensitivity after consumption of a beverage containing 333 mg of strawberry and cranberry PPs for 6 weeks (Paquette et al., 2017). In contrast, a cocoa beverage containing 960 mg of PPs exerted no effect on insulin resistance in obese adult T2DM patients fed a high-fat fast-food style meal (Basu et al., 2015). Bilberry lowered the glycemic spikes and improved insulin release or impaired glucose tolerance in male patients with T2DM (Hoggard et al., 2013). In the most recent randomized controlled trial, 86 obese or overweight subjects with metabolic syndrome received 4 different diets: a control diet, a diet high in long-chain n-3 polyunsaturated fatty acids (LCn3), a high-PP diet, or a diet high in LCn3 (mainly from dentex, salmon and anchovy) and PPs (from vegetables, extra-virgin olive oil, decaffeinated green tea and coffee, dark chocolate, and blueberry jam). After 8 weeks, an oral glucose tolerance test demonstrated that the PP-rich (approx. 3 g/day) diet improved glucose metabolism by reducing plasma glucose and increasing insulin secretion (Bozzetto et al., 2015).

However, the wide heterogeneity of the trials hampers the isolation of the net effect of PPs on glucose metabolism. Notably, the addition of a PP-rich food to an individual's current diet often results in a reduced intake of other nutrient sources and in a "healthier", sometimes hypocaloric, dietary pattern. Moreover, PP-rich foods also contain molecules with putative glucose-lowering effects. Finally, these trials are characterized by marked heterogeneity in the primary endpoint, the study design, and the PP supplementation approach; as a result, three meta-analyses of the effects of green tea on glucose parameters have described negative (Wang et al., 2017), mixed (Liu et al., 2013), and positive (Zheng et al., 2013) results, respectively. A similar situation has been reported for chocolate, cocoa, coffee, cinnamon, grape, wine, and berries (Kim et al., 2016b). However, more solid data indicate that

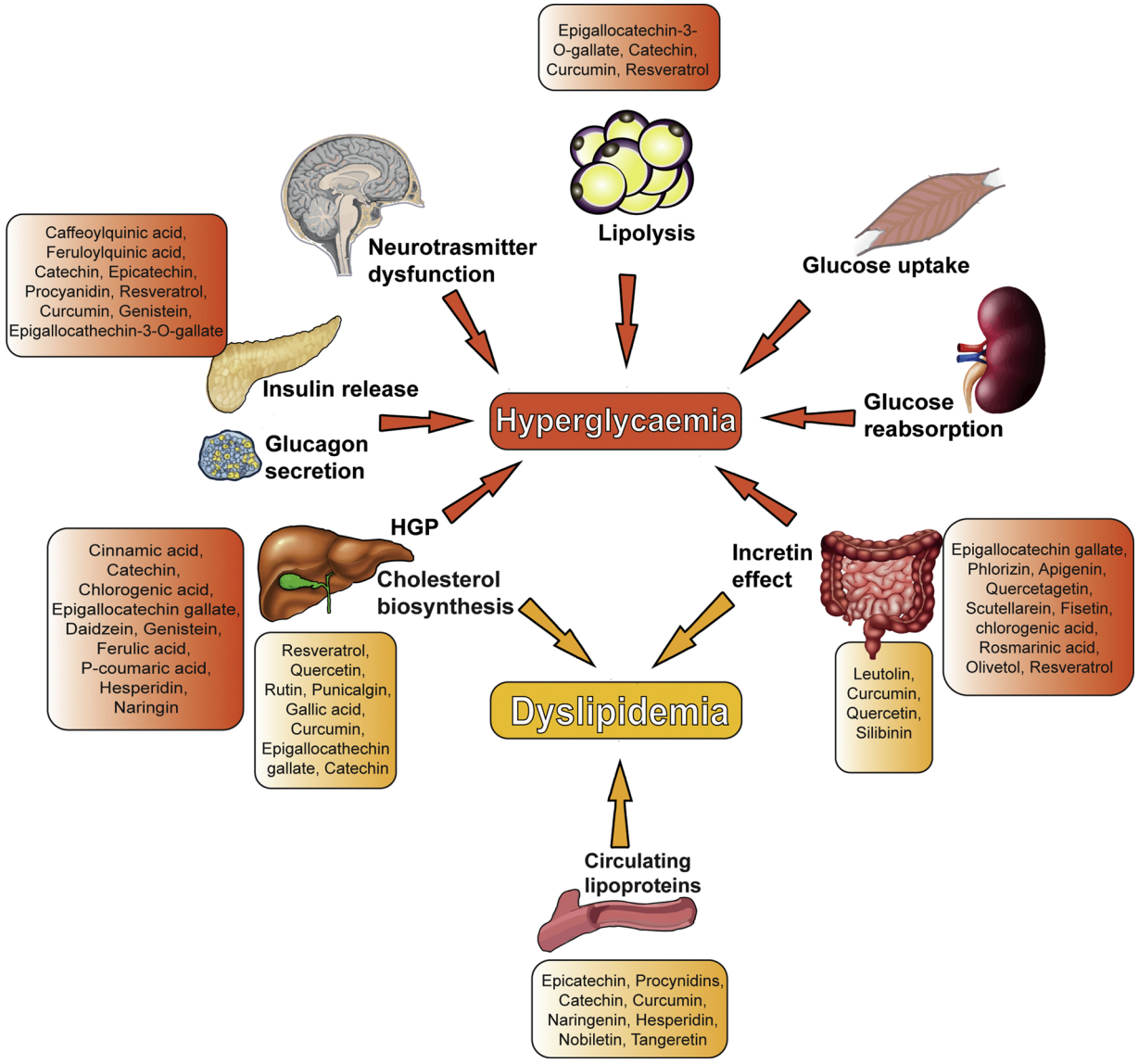

Fig. 1. Overview of the polyphenols acting on the organs involved in glucose and lipid metabolism dysfunctions. Adapted from (DeFronzo et al., 2015).

complex but specific dietary patterns such as PP-rich diets, the MD, and other dietary regimens high in fruit, vegetables, wholegrain, and unsaturated fats and involving a low calorie intake have a glucose-lowering effect (Chiu et al., 2018; Esposito et al., 2015; Kim et al., 2016b; Steven et al., 2016). A synergistic action of the compounds used in these diets very likely contributes to their beneficial effects. Studies of a single molecule would provide more accurate results. However, results for resveratrol, the only PP widely tested for T2DM-relevant endpoints in clinical trials, are also mixed (Ozturk et al., 2017).

Studies of untreated patients with mild hypercholesterolemia have demonstrated that PP supplements or extracts induced a significant reduction in total cholesterol (TC) and LDL-C but did not affect HDL-C. A diet rich in ellagic acid exerted significant effects on phosphatidylcholine, triacylglycerols, phosphatidylethanolamine, and cholesteryl esters (Puupponen-Pimia et al., 2013). Moreover, administration of a preparation containing sugar cane extract (90 % policosanols and 60 % octacosanol), red yeast rice (0.4 % monacolin K), and artichoke leaf dry extract (5–6% chlorogenic acid) three times a day for 16 weeks reduced TC, LDL-C and TGs respectively by 14.1 %, 22.4 %, and 12 % compared to the placebo group (Ogier et al., 2013). In a parallel study, a double dose did not provide additional benefits in reducing TC and LDL-C (Barrat et al., 2013). Red grape seed extract (200 mg) taken daily for 8 weeks significantly reduced oxidized LDL (-5.47 ± 12.12 mg/dL), TC (-10.68 ± 26.7 mg/dL) and LDL-C (-9.66 ± 23.92 mg/dL), highlighting the antioxidant effect of PPs (Razavi et al., 2013). Two studies assessing the effects of resveratrol on the lipid profile failed to yield significant results. However, their duration (1 month) and PP doses were insufficient to induce marked biological effects (Apostolidou et al., 2015; van der Made et al., 2015). The substantial heterogeneity of the results of the six trials evaluating the effects of PP administration on the lipid profile may depend on differences in the products and/or populations (*e.g.* mean age, degree of dyslipidemia, presence of other features of metabolic syndrome) considered.

A systematic review of clinical trials exploring the effects of resveratrol supplementation on circulating lipids has recently excluded a significant impact of this PP (Haghighatdoost and Hariri, 2018). However, since the sample consisted of patients on lipid-lowering drugs, the effect of resveratrol may have been overwhelmed; patients with early-stage mild dyslipidemia might have provided a more suitable population.

Finally, a recent systematic review of 21 studies (observational and trials) found that PPs can significantly increase HDL-C and lower LDL-C irrespective of study design, enrollment criteria, duration, and PP formulation used (Poti et al., 2019). Again, these studies were highly

Table 3
Summary of clinical trials investigating the effects of polyphenols on type 2 diabetes and dyslipidemia.

Intervention: polyphenol source and follow-up	Design and trial duration	Study population	Results	Clinical trial NCT# and Reference
Trials involving polyphenol (PP) administration to patients with type 2 diabetes mellitus (T2DM)				
Ground beef seasoned with a PP-rich spice mixture (cloves 4%, cinnamon 4%, oregano 26 %, rosemary 4%, ginger 11 %, black pepper 7%, paprika 30 %, garlic powder 13 %) or ground beef seasoned with salt	Randomized crossover, **one-time consumption.**	18 T2DM subjects treated with oral agents or subjects with impaired glucose tolerance taking no medications	Reduced urinary malondialdehyde, increased urinary nitrate/nitrite and improved postprandial endothelial function in men with T2DM.	NCT01076829 (Li et al., 2013)
Grape PP supplement or placebo plus 3 g fructose/kg daily (6 capsules) (333.33 mg grape extract per capsule)	Randomized, double-blind and placebo-controlled trial, **for 9 weeks.**	38 healthy overweight/obese first-degree relatives of T2DM patients (18 men and 20 women)	NA natural mixture of grape PPs at nutritional doses efficiently prevents fructose-induced oxidative stress and insulin resistance.	NCT01478841 (Hokayem et al., 2013)
Strawberry and cranberry PP beverage (333 mg PPs: polymers, phenolic acid, gallic acid, hydroxybenzoic acid, p-coumaric acid, m-coumaric acid, ferulic acid, vanillic acid, caffeoyl glucoside, coumaroyl glucoside, chlorogenic acid) daily or placebo beverage.	Parallel, double-blind, controlled randomized clinical trial, **for 6 weeks.**	41 overweight/obese and insulin resistant subjects • PP group (n = 20) • Control group (n = 21)	The PP group showed significantly improved insulin sensitivity compared to controls.	NCT01478841 (Paquette et al., 2017)
Cocoa beverage (960 mg total PPs; 480 mg flavanols: proanthocyanidins, epicatechins, catechins, theobromine, caffeine) or flavanol-free placebo (110 mg total PPs; <0.1 mg flavanols), with a high-fat fast food-style breakfast.	Randomized, double-blind, crossover study, **two-day consumption.**	Obese adults with T2DM (n = 18) (14 women; 4 men)	Cocoa increased HDL-C compared to placebo, but acute cocoa supplementation yielded no clear overall benefit in T2DM patients after a high-fat fast food-style meal	NCT01886989 (Basu et al., 2015)
Single capsule of either 0.47 g standardized bilberry extract (36 % (w/w) anthocyanins: elphinidin-3-galactoside, delphinidin-3-glucoside, delphinidin-3 arabinoside, cyanidin-3-galactoside and cyanidin-3-glucoside) or placebo.	Double-blinded crossover interventional, **2 weeks.**	Male volunteers with T2DM or with impaired glucose tolerance (n = 8)	The bilberry extract reduced venous plasma insulin AUC by 18 % compared to placebo	NCT01245270 (Hoggard et al., 2013)
Four different isoenergetic diets: (1) control, low in long chain *n*-3 polyunsaturated fatty acids (LCn3) and PPs; (2) rich in LCn3; (3) rich in PPs; or (4) rich in LCn3 and PPs (flavonoids (57%), mainly represented by flavanols (41%) and phenolic acids (43%)).	Randomized controlled trial, **8 weeks**.	86 overweight/obese subjects with metabolic syndrome (MetS) • Control diet (n = 20) • High LCn3 diet (n = 19) • High PP diet (n = 20) • High LCn3 and PP diet (n = 19)	PPs significantly reduced plasma glucose total AUC and increased early insulin secretion. Naturally PP-rich diets reduce blood glucose response, likely by increasing early insulin secretion and insulin sensitivity.	NCT01154478 (Bozzetto et al., 2015)
A bar containing 7.5 g of 70 % isolated soy protein; 16 mg isoflavones; 400 mg of cocoa polyphenols, twice a day.	Randomized, parallel, double-blind, placebo-controlled clinical trial, **8 weeks.**	60 patients with type 2 diabetes (24 on controlled diet and 36 on stable metformin therapy): • casein protein as placebo (n = 11) • soy protein alone (isoflavone free) (n = 13) • soy protein + isoflavones (n = 13) • soy protein + cocoa (n = 12) • soy protein + isoflavones + cocoa (n = 11)	Glycemic control achieved with soy but not with casein protein, whereas insulin resistance and LDL were improved by isoflavones. The cocoa supplement did not improve these indices.	NCT01754662 (Konya et al., 2019)
Trials involving dyslipidemic subjects				
789 mg of ellagitannins from 300 g fresh berries (100 g strawberry, 100 g raspberries, and 100 g cloudberries) a day	Randomized, controlled dietary intervention, **8 weeks.**	32 subjects with MetS (including dyslipidemia): • berry group (n = 20) • control group (n = 12)	Ellagic acid-rich diets have effects on phosphatidylcholine, triacylglycerols, phosphatidylethanolamines and cholesteryl esters.	NCT01414647 (Puupponen-Pimia et al., 2013)
Dietary supplement (DS, 3 tablets) of red yeast rice, sugar cane-derived policosanols and artichoke leaf extracts.	Double-blind, randomized, parallel controlled study, **16 weeks.**	39 untreated subjects aged 21–55 years with moderate hypercholesterolemia: • DS group (n = 19) • Placebo group (n = 20)	Reduction of LDL-C and TC on week 16 in the DS group compared with placebo group.	NCT01354327 (Ogier et al., 2013)
Dietary supplement of red yeast rice extract, policosanol and artichoke leaf extract in tablets (3 tablets, usual dose or 6 tablets a day.	Randomized, double-blind, placebo-controlled study, **4 weeks**.	45 subjects with untreated hypercholesterolemia in 3 arms: • 3 tablets (n = 15) • 6 tablets (n = 15) • placebo (n = 15) 52 mildly hyperlipidemic individuals	Twice the recommended dose appears to be safe, conferring no additional benefit	NCT01354340 (Barrat et al., 2013)

(*continued on next page*)

Table 3 (*continued*)

Intervention: polyphenol source and follow-up	Design and trial duration	Study population	Results	Clinical trial NCT# and Reference
200 mg/day of red grape seed extract in capsule (95% proanthocyanidins, 80% polyphenolic compounds)	Randomized double-blind placebo-controlled crossover study, **8 + 8 weeks.**		Reduced oxidized LDL, TC, LDL-C and beneficial effects on the lipid profile	NCT00713167 (Razavi et al., 2013)
Resveratrol capsules (150 mg a day).	Randomized, placebo-controlled crossover study, **4 weeks.**	45 overweight and slightly obese subjects, mean age 61 ± 7 years: • men (n = 25) • women (n = 20)	No change in metabolic risk markers in relation to cardiovascular health (ApoA-I, ApoB100, HDL-C, LDL-C and triacylglycerol).	NCT01364961 (van der Made et al., 2015)
Red wine from Northern Greece variety "tannat" (total anthocyanins, 566 mg/*L*; tannins, 6 g/L; resveratrol, 2.7 mg/L)	Placebo-controlled, cross-over study, **1 month.**	ealthy subjects: • asymptomatic hypercholesterolemic (n = 20) • normocholesterolemic (n = 17)	Total antioxidant capacity and Vitamin E increased after wine intervention in NC and AHC groups; no significant change in lipid profile.	NCT02409537 (Apostolidou et al., 2015)

heterogeneous under several respects, and the reported HDL-C increase and LDL-C reduction were not clinically significant (mean difference: HDL-C, +2.68 mg/dL; LDL-C, -4.39 mg/dL).

In this work we assessed the risk of bias of the 13 completed trials, for which a paper reporting the results is available, using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) (Sterne et al., 2019). RoB 2 explores five domains: the randomization process, deviations from the intended interventions, missing outcome data, outcome measurement, and the reporting of results. Its results – and whether information on the PP composition of products was available – are reported in Table 4. The analysis highlights a considerable risk of bias for most trials. One potential bias arises from participants' awareness of the assigned intervention, mainly due to the intrinsic nature of the study products, *i.e.* PP-enriched foods. A number of studies raise some concerns with regard to the reporting of results, due to the *per*-protocol analysis of the outcomes and to insufficient information on missing outcome data. Moreover, even though the analysis of data from the trial registry disclosed considerable heterogeneity in the choice of the outcomes related to glycemia or lipid control, substantial amendments to the primary and secondary outcomes following the study start date were reported for none of the trials.

The precise PP composition of the study products, either predetermined or determined *ex post* by the investigators, was reported in six of the 13 trials; in the other cases it was either not reported or reported in terms of total PP content.

5. Conclusions and future perspectives

The next decade is expected to be characterized by several health challenges, chiefly a further increase in the number of T2DM patients and the need to improve prevention strategies. It is essential to establish whether dietary PPs and/or PP supplements can help reduce the risk of developing T2DM and its complications (Coe and Ryan, 2016; Rienks et al., 2018). Since PPs exert pleiotropic effects on glucose and lipid metabolism, and provided that their contribution to maintaining a healthy lipid/glucose profile is demonstrated, PP-rich foods may provide a sound preventive approach – especially in individuals at low absolute risk of T2DM and cardiovascular disease according to classic algorithms (Landi et al., 2019; Pirro et al., 2017; Poli et al., 2018).

Follow-up duration appears to be the most severe limitation hampering human trials that study the effect of PPs in delaying the onset of age-related diseases. Since high-risk subjects should be treated when the disease is not clinically manifest, they should be followed up for decades to demonstrate without any doubt that PPs delay T2DM development and its complications. However, given that the duration of clinical trials evaluating PPs has never exceeded 2 months, their results allow drawing no conclusions on the long-term effects.

With regard to the effect of PPs in patients with overt T2DM, the evidence of their glucose- and lipid-lowering activity is potentially biased by a number of factors. Few trials have involved a well-characterized PP mixture (or food combination), a proper placebo control, a prespecified primary endpoint, and a sufficiently powered sample size. Preliminary data suggest that PPs do exert an effect on glycemia and insulin resistance in overweight patients. In contrast, their effect on dyslipidemia is highly variable, with the notable exception of studies using red yeast rice, whose content in lovastatin (monacolin K) exerts a marked effect on LDL in different settings (Gerards et al., 2015). Crossover clinical trials involving PP compound administration (in capsules, extracts or foods and beverages) to T2DM patients with dyslipidemia provide conflicting evidence on their protective effects on lipid and glucose metabolism due to a variety of reasons, such as small sample size (mainly due to narrow inclusion and exclusion criteria), inconsistent T2DM and dyslipidemia diagnostic criteria, and self-reporting of compliance and food intake rather than determination of blood/urine PP metabolites. Poor PP bioavailability and their fast metabolism also prevent drawing firm conclusions (Brglez Mojzer et al.,

Table 4
Results of the assessment of the clinical trials included in the Discussion using the Cochrane risk-of-bias tool (RoB 2). For each assessment domain, the icons indicate whether the risk of bias is low (green tick), high (red cross), or raises some concerns (exclamation mark). For additional information on the tool, please refer to (Sterne et al., 2019) and to the RoB 2 website (https://www.riskofbias.info). The last column reports whether the study provided information on the availability of the PPs content in the study products.

Trial	Cochrane risk-of-bias tool (RoB 2) assessment						Information on the PP composition of the study products
	Randomization process	Deviations from intended interventions	Missing outcome data	Measurement of the outcome	Selection of the reported results	Overall	
Trials involving type 2 diabetes patients							
Li et al. (2013)	!	✗	!	✗	!	✗	✗
Hokayem et al. (2013)	✓	!	✓	✓	✓	!	✓
Paquette et al. (2017)	✓	!	✓	✓	✓	!	✓
Basu et al. (2015)	✓	✓	✓	✓	✓	✓	✓
Hoggard et al. (2013)	✓	✓	✓	✓	!	!	✓
Bozzetto et al. (2015)	✗	✗	✓	✓	!	✗	✗
Konya et al. (2019)	✓	✗	✓	✓	!	✗	✗
Trials involving dyslipidemic subjects							
Puupponen-Pimia et al. (2013)	✗	✗	✓	✓	✓	✗	✓
Ogier et al. (2013)	✓	✓	✓	✓	✓	✓	✗
Barrat et al. (2013)	✓	✓	✓	✓	✓	✓	✗
Razavi et al. (2013)	✓	✗	!	✓	✓	✗	✗
van der Made et al. (2015)	✓	✓	✓	✓	✓	✓	✓
Apostolidou et al. (2015)	!	!	✓	!	✓	!	✗

2016).

Combinations of PPs and other natural compounds associated with current T2DM medications are also worth exploring. PP dosage and their interactions with the microbiome have a profound but scarcely controllable influence on their effects. Research into ways to unravel and improve the benefits of PPs and/or produce PP-rich preparations whose composition is capable of promoting specific metabolic mechanisms is clearly warranted (McDougall, 2017), as are clinical trials with a well-defined design, strict enrollment criteria, prespecified endpoints, longer follow-ups and, especially, well-characterized PP formulations.

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Declaration of interest

The authors have no conflict of interest to disclose.

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REVIEW
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MicroRNAs as Factors in Bidirectional Crosstalk Between Mitochondria and the Nucleus During Cellular Senescence

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Mitochondria are essential organelles that generate most of the chemical energy to power the cell through ATP production, thus regulating cell homeostasis. Although mitochondria have their own independent genome, most of the mitochondrial proteins are encoded by nuclear genes. An extensive bidirectional communication network between mitochondria and the nucleus has been discovered, thus making them semi-autonomous organelles. The nucleus-to-mitochondria signaling pathway, called Anterograde Signaling Pathway can be deduced, since the majority of mitochondrial proteins are encoded in the nucleus, less is known about the opposite pathway, the so-called mitochondria-to-nucleus retrograde signaling pathway. Several studies have demonstrated that non-coding RNAs are essential "messengers" of this communication between the nucleus and the mitochondria and that they might have a central role in the coordination of important mitochondrial biological processes. In particular, the finding of numerous miRNAs in mitochondria, also known as mitomiRs, enabled insights into their role in mitochondrial gene transcription. MitomiRs could act as important mediators of this complex crosstalk between the nucleus and the mitochondria. Mitochondrial homeostasis is critical for the physiological processes of the cell. Disruption at any stage in their metabolism, dynamics and bioenergetics could lead to the production of considerable amounts of reactive oxygen species and increased mitochondrial permeability, which are among the hallmarks of cellular senescence. Extensive changes in mitomiR expression and distribution have been demonstrated in senescent cells, those could possibly lead to an alteration in mitochondrial homeostasis. Here, we discuss the emerging putative roles of mitomiRs in the bidirectional communication pathways between mitochondria and the nucleus, with a focus on the senescence-associated mitomiRs.

Keywords: microRNA, senescence, mitochondria, mitonuclear communication, mitomiRs

INTRODUCTION

The aging process is considered a universal and inevitable process of physiological decline associated with a greater vulnerability to disease and death. This vulnerability is linked to the complexity of the organism, which comes from the myriad of interactions and feedback controls that operate between its different structural units. These mechanisms allow cells, tissues, and entire organisms the ability to respond and adapt to stressful environmental conditions. However, several studies suggest that this complexity diminishes with age due to the progressive loss of functions of cells, tissues, and organs and importantly of their ability to communicate, determining an increase of structural disorder; therefore, the complexity decrease is closely related to the increase in entropy, both determining the reduction of the functional reserve of older people (López-Otín et al., 2013). In this context, the Lorenz's Butterfly metaphor makes the concept of "instability of the aging system" easier: even the slightest change can cause consequences that are not proportionate to the initial event; a small accident can induce fatal effects in the elderly or biologically old individual just as "a flapping of the wings of a butterfly in Brazil can trigger a hurricane in Texas" (Lorenz, 1972). During organismal aging, senescent cells accumulate in tissues, where they alter microenvironment homeostasis. Many theories of the origin of cellular senescence have started from the observation of microscopic changes in aging cells. López-Otín et al. (2013) tried to identify and categorize common cellular and molecular hallmarks of aging: stem cell exhaustion, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing pathways and mitochondrial dysfunction. Senescent cells exhibit several functional, phenotypic, and molecular changes including a stable arrest of proliferation. The metabolic alterations and changes in gene expression allow these cells to remain viable and resist to apoptosis for a long time but are also the cause of the acquisition of a common secretory phenotype. Cell cycle arrest in senescence is largely mediated *via* the activation of either one or both p16/pRB and p53/p21 tumor suppressor pathways. Prolonged overexpression of these four components is sufficient to induce senescence: pRB and p53 are key transcriptional regulators whereas p16 and p21 are cyclin-dependent kinase inhibitors which negatively regulate cell cycle progression. p53 and its downstream effector p21, are activated by DNA damage caused by oncogenic or oxidative stress and telomere attrition; however, epigenetically induced senescence mostly acts by inducing p16 expression that prevents phosphorylation of RB and thus the transcription of genes required for cell cycle progression (Kumari and Jat, 2021).

The secretory phenotype, called Senescence Associated Secretory Phenotype (SASP), and the altered intercellular communication are both important aspects of aging cells because they cause a low grade systemic, chronic inflammation called inflammaging (Franceschi et al., 2000). This condition plays a key role in the pathophysiology of inflammatory age-related diseases (ARDs), i.e., cancer, diabetes, cardiovascular and neurodegenerative diseases. Judith Campisi and her group first coined the term SASP and demonstrated that genotoxic stress-induced senescent cells secrete a myriad of factors associated with inflammation and oncogenesis (Coppé et al., 2008). Since then, scientific data on the characterization and the pathogenetic role of the SASP has increased enormously, but from our understanding, SASP is represented by the release of different soluble factors, regardless of the type of senescence, i.e., induced or replicative, pro-inflammatory cytokines, chemokines, and non-coding RNAs (ncRNAs), including small (microRNAs), long (lncRNA) and circular RNA (Terlecki-Zaniewicz et al., 2018; Mensà et al., 2020). The SASP can propagate signals (proteins, lipoproteins, DNA and RNA) at systemic levels, which contributes to the communication between different types of cells and tissues (Fafián-Labora and O'Loghlen, 2020). Consolidated data have revealed that NF-κB signaling is the major signaling pathway which stimulates the appearance of the SASP and the production of pro-inflammatory mediators (Salminen et al., 2012).

MicroRNAs (miRNAs or miRs) are small non-coding RNAs (sncRNAs), about 18–25 nucleotides long, which can modulate various physiological and pathophysiological processes at a post-transcriptional level by binding the 3'-untranslated region of the target mRNA in the cytoplasm, inhibiting its expression. Their biogenesis, which has been elegantly described by other authors (Bartel, 2004; Ha and Kim, 2014; Wang et al., 2017a; Treiber et al., 2019) occurs in multiple steps, both in the nucleus and the cytoplasm. After pri-miRNAs are transcribed, they are subsequently cleaved to the more stable form pre-miRNAs by Drosha. Then they translocate in the cytoplasm where they associate with Ago2, after Dicer processing. It is only at this stage that the RNA-inducing silencing complex (RISC) takes shape, thus the binding with the target mRNA. MicroRNAs are potentially involved in all cellular functions, including development, proliferation, differentiation, apoptosis, and aging. A multitude of genome wide expression profile experiments have shown a differential modulation of non-coding RNA, including miRNAs, between proliferating and senescent cells (Faraonio et al., 2012; Giuliani et al., 2020). Most of ncRNAs and miRNAs play a pivotal role in inducing cellular senescence and related organismal dysfunction. MiRNAs can be actively released by living cells, shuttled by proteins and/or extracellular vesicles (EVs) and internalized by target cells, which spreads specific signals at paracrine and systemic levels. Senescent cells *via* the release of EVs containing a number of senescence-associated (SA)-miRNAs can spread the senescent

Abbreviations: ARDs, Age-related diseases; AMPK, AMP-activated protein kinase; DDR, DNA damage response; EVs, extracellular vesicles; Drp1, dynamin-related protein 1; ETC, electron transport chain; FOXO, forkhead box O; GPS2, G-Protein Pathway Suppressor 2; lncRNA, long non-coding RNA; miRNA, miR, microRNA; MDPs, mitochondrial-derived peptides; mtDNA, mitochondrial DNA; mitosRNA, mitochondrial genome-encoded small RNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ncRNA, non-coding RNA; NRF-1, nuclear-respiratory factor 1; ORF, open reading frame; OXPHOS, oxidative phosphorylation; PARK2, Parkin; piRNA, Piwi-interacting RNA; PPAR, peroxisome proliferator-activated receptor; PGC1α, PPARγ co-activator 1α; PINK1, PTEN induced kinase 1; pRB, retinoblastoma protein; RTG, retrograde; RISC, RNA-inducing silencing complex; ROS, reactive oxygen species; SA, senescent-associated; SASP, Senescence Associated Secretory Phenotype; SncmtRNA, sense mitochondrial ncRNA; ASncmtRNA, antisense mitochondrial ncRNA; SIRT1, sirtuin 1; sncRNA, small non-coding RNA; Tfam, mitochondrial transcription factor A; p53, tumor protein P53; UTR, untranslated region.

phenotype (Mariani et al., 2020; Mensà et al., 2020; Olivieri et al., 2021; Prattichizzo et al., 2021).

Several SA-miRNAs were shown to affect mitochondrial dynamics and bioenergetics. All known SA-miRNAs are coded by the nuclear genome and some of these have been found overexpressed within mitochondria of replicative senescent cells compared to younger, impacting on resident proteins and functions. The implications of nuclear-coded miRNAs targeting mitochondrial functions on the acquisition of the senescent phenotype (anterograde signals) and the onset of ARDs will be explored in this review. Furthermore, recent studies regarding retrograde signals (RTG) from mitochondria to the nucleus in the senescent cells will be discussed. The study of these relationships could be useful to figure out effective interventions in slowing aging and preventing ARDs.

MITOCHONDRIA AND SENESCENCE

Mitochondria are involved in many processes besides energy metabolism such as cell cycle regulation, apoptosis and inflammation (Nunnari and Suomalainen, 2012). This suggests a close relationship between the proper performance of these organelles and cellular senescence. Mitochondrial dysfunction is in fact associated with the aging process and with the pathogenesis of ARDs. Some features of aged mitochondria, such as accumulation of mutation in mitochondrial DNA (mtDNA), altered mitochondria dynamics and increased reactive oxygen species (ROS) generation accompanied by progressive decrease in energy production, will be discussed below.

The mtDNA is circular and extremely small (16,569 nucleotides, 37 genes): it contains 13 mRNAs coding for some of the protein subunits of the oxidative phosphorylation (OXPHOS) machinery, two ribosomal RNAs (12S and 16S rRNAs) and 22 transfer RNAs (tRNAs; Carelli and Chan, 2014). However, about 3,000 genes are needed to make a mitochondrion and therefore their resulting proteins must be transported from the nucleus to the developing organelle. Only about 3% of the genes needed to make a mitochondrion are for ATP production. The remaining genes are involved in other functions related to the specialised tasks of the differentiated cells in which they reside. The integrity of mtDNA plays a key role in maintaining cellular homeostasis. Several studies carried out both on *in vitro* and *ex vivo* cellular models have demonstrated a close positive correlation between mtDNA mutations and the activation of mechanisms that lead to cellular dysfunction and, more generally, to the aging process (Trifunovic, 2006). We understand that mtDNA undergoes a higher mutation rate compared to nuclear DNA. In a neuronal stem cells model, for example, the accumulation of mtDNA damage led to a greater predisposition to differentiation towards an astrocytic lineage to a detrimental neurogenesis (Wang et al., 2011).

Mitochondrial dynamics, which vary through the life cycle of the cell depending on energy demands and cell division state, are critical to maintain mitochondrial integrity. During their life, which is about 10 days, mitochondria are faced with fission and fusion (Hales, 2010). The former is required to remove depolarized, damaged, and dysfunctional mitochondria *via* autophagy (mitophagy). The second mechanism allows viable mitochondria maintenance since repolarized organelles can be recovered and restored by fusion with healthy elements of the mitochondrial network. During fusion events some functional components can be irregularly redistributed between mitochondria; as a consequence, dissimilar mitochondria can be generated by the next fission event (Westermann, 2010).

Several mitochondrial alterations, i.e., mtDNA mutation, ROS overproduction, depolarization, and misfolded protein lead to the accumulation of PTEN induced kinase 1 (PINK1) on outer mitochondrial membrane, which phosphorylates Parkin (PARK2). Parkin 2 in turn promotes the ubiquitination of proteins that are then recognised by the autophagic machinery. The autophagosome containing the dysfunctional mitochondria is then transported and fused to a lysosome where it is then degraded (Kazlauskaite and Muqit, 2015). However, during aging dysfunctional mitochondria accumulate, mainly for two reasons: autophagy declines (Wang and Klionsky, 2011), and fission overcomes fusion (Amartuvshin et al., 2020; Spurlock et al., 2020). Indeed, in senescent cells, the transcription factor p53 interacts with Parkin by inhibiting its accumulation on the outer membrane and consequently blocking the process of mitophagy (Hoshino et al., 2013; Badr et al., 2014; Correia-Melo et al., 2017; Manzella et al., 2018). Therefore, the number of mitochondria is greater in senescent cells than in young cells, but their functionality is severely compromised (Kim et al., 2018; Chapman et al., 2019).

We thoroughly explored the phenotype of mitochondria in human endothelial cells undergoing replicative senescence and observed elongated/branched morphology concomitantly with autophagic vacuole accumulation (Giuliani et al., 2018). Accordingly, Mai and co-workers suggest an hyper-fused state of the mitochondria due to downregulation of the fission regulating proteins, i.e., fission1 and dynamin-related protein 1 (Drp1; Mai et al., 2010). Similar mitochondrial morphology was observed in primary dermal fibroblasts induced to senescence with doxorubicin or hydrogen peroxide. These data show that mitochondrial hyperfusion can be associated with aging; this may appear contradictory with respect to the concept that mitochondrial fusion is an adaptive and protective response during stress. In this respect it has been suggested that in certain cell types an apparently compensatory mitochondria hyperfusion may have long-term negative consequences and accelerate aging.

This severe impairment of mitochondria function in senescent cells is also characterized by a higher basal oxygen consumption rate, which leads both to an increase in energy production and to a greater release of ROS (Hutter et al., 2004). Excessive ROS production provokes telomere shortening which culminates in the DNA-damage response, thus speeding up the senescence process (Passos et al., 2007; Rai et al., 2009; Hewitt et al., 2012).

Therefore, it has been postulated that mitochondrial morphology transitions might regulate mitochondrial function by RTG signaling (Picard et al., 2013; Walczak et al., 2017).

MITONUCLEAR COMMUNICATION

Mitochondria biogenesis is controlled by two physically separated genomes: the mtDNA and the nuclear genome. Therefore, an intense communication with the nucleus is required in order to provide mitochondria with nuclear-encoded proteins, which are necessary for mitochondrial homeostasis and function.

The coordination between the nucleus and the mitochondrion is mediated by a sophisticated communication system, named mitonuclear communication, which occurs in a bi-directional way, known as anterograde signaling (nucleus-to-mitochondrion) and retrograde signaling (mitochondrion-to-nucleus). The former mechanism reflects the accepted outlook of the nucleus as a regulatory factor, which coordinates the function of subcellular organelles, allowing mitochondria to adapt to the cellular milieu in response to endogenous alterations or extracellular stimuli. The retrograde (RTG) signaling is meant to be a feedback system of the mitochondrial functional state to the nucleus to initiate adaptive responses (Quirós et al., 2016; Bhatti et al., 2017). It might be considered as a quality control mechanism, which compensates for the loss of mitochondrial quality that naturally occurs with age.

Anterograde Signaling

The anterograde signals are induced by extracellular stimuli, like physical exercise, cold exposure and dietary restrictions, through the activation of several genes including transcription factors, such as nuclear-respiratory factor 1 (NRF-1) and GA-binding protein-α (known as "NRF2"), peroxisome proliferator-activated receptors (PPARs); mitochondrial transcription factor A, uncoupling proteins, oestrogen-related receptors and PPARγ co-activator 1α (PGC1α; Hock and Kralli, 2009; Whelan and Zuckerbraun, 2013; Quirós et al., 2016). For example, during exercise or caloric restriction, there is an overall increase of AMP/ATP ratio, which in turn triggers AMP-activated protein kinase, along with an increment of NAD^+ levels. This event leads to the activation of sirtuin 1 (SIRT1), a positive regulator of PGC1α, which is known to stimulate mitochondria metabolism and proliferation.

In the last few decades, there is growing evidence to underpin the involvement of ncRNAs in the regulation of mitochondrial homeostasis. Several research groups have examined their possible localization within mitochondria. In 2006, from an RNA sequencing experiment on rat's liver mitochondria, a few miRNAs were identified inside the organelle, the so-called mitomiRs. Although initially thought as cytosolic contamination, just a few years later, several independent studies involving microarray profiling confirmed the initial findings (Lung et al., 2006; Kren et al., 2009; Bandiera et al., 2013).

Extensive miRNA mapping analysis revealed that most of them are nuclear encoded, strengthening the assumption of a nuclear involvement in mitochondria homeostasis, which ultimately implies a miRNA import mechanism inside mitochondria. Only recently, we have started to discover mechanisms of RNA export and import into mitochondria, but none of which are miRNAs' specific (**Figure 1**; Zeng et al., 2008; Wang et al., 2010; Jannot et al., 2016).

Since Ago2, an essential protein for RISC functioning, and some of its targets, such as some tRNA genes, have been localized into mitochondria (Bandiera et al., 2011), Bandiera and colleagues proposed an Ago2 involvement in the transport of miRNAs into mitochondria, as a protein import system similar to those involved in RNA trafficking. It has been demonstrated that phosphorylation of Ago2 induces miRNA:Ago2 complex intake into cytoplasmic processing bodies (P-bodies), which are known to interact with mitochondria (Zeng et al., 2008; Huang et al., 2011).

An important role is also played by GW-bodies in the formation of structures where the protein GW182 holds the association miRNA:Ago2 in a cap-like structure to make a stable RISC. A subsequent deletion of the cap and of the poly(A) tail, or the detachment of GW182 alone, presumably induced by cellular stresses, leaves miRNA:Ago2 complex free to head towards mitochondria (Gibbings et al., 2009). The proposed mechanism of miRNA:Ago2 entry involves gates such as SAM50, TOM20 and TIM (Jusic and Devaux, 2020).

Another import mechanism might involve the polynucleotide phosphorylase PNPASE, a 3'→5' exoribonuclease and poly-A polymerase located in the mitochondrial intermembrane space, that regulates mitochondrial homeostasis and adenine nucleotide levels (Wang et al., 2010). Wang and co-worker showed how PNPASE imports RNA from the cytosol into the mitochondrial matrix by binding to specific stem-loop motifs in the RNA sequence, making assumption on its potential involvement in mitochondrial miRNA trafficking (Wang et al., 2012).

Finally, porins, highly conserved proteins located in the outer membrane of mitochondria, could participate in miRNA translocation from cytosol to mitochondria, as proposed by Bandiera et al. (2013).

Notably, there is increasing body of evidence to suggest that mitochondria or mtDNA are exposed to intracellular or intercellular transfer *via* exosome and tunnelling nanotubes (Spees et al., 2006; Borralho et al., 2014).

It is widely accepted that housekeeping mitochondrial nuclear-encoded ncRNAs regulate mitochondrial function and homeostasis (Gusic and Prokisch, 2020), but less is known about their deregulation and possible correlation with mitochondrial-related diseases. Several nuclear-encoded lncRNAs have been localized in mitochondria and it has been hypothesized that they could regulate mitochondrial functions (Vendramin et al., 2017). It has been demonstrated that SAMMSON, a nuclear-encoded lncRNA acts as an oncogene in several malignancies (Leucci et al., 2016; Zheng et al., 2020), as well as MALAT1, which is known to be associated with cancer and metastasis (Sun and Ma, 2019). However, it is still an uncovered field, since not much data are available and mitochondria import mechanisms have still to be elucidated; therefore, the presence and the function of these transcripts in mitochondria, especially during cellular senescence, need further investigation. As mentioned above, different nuclear-encoded ncRNAs, such as miRNAs, are involved in mitochondrial

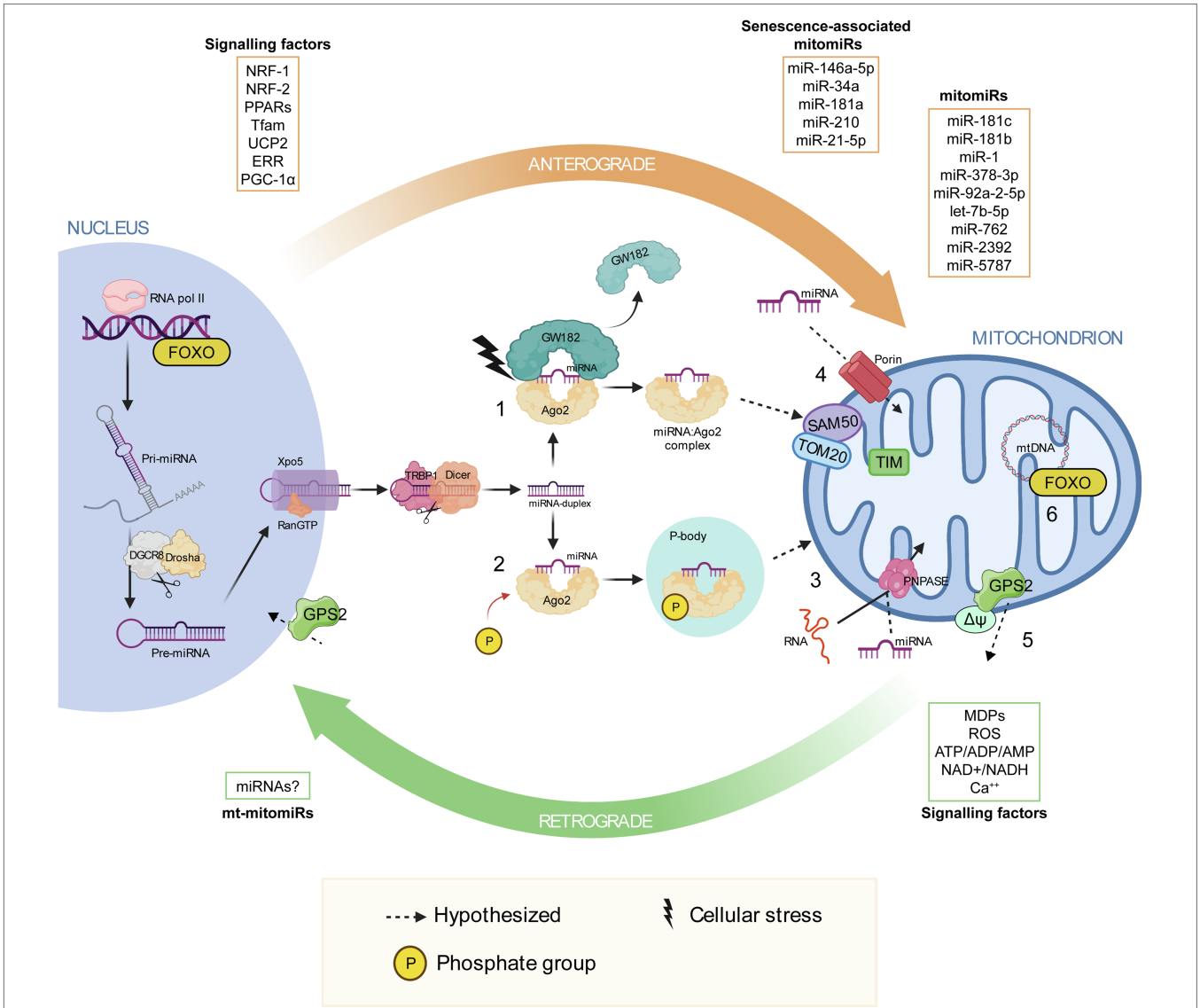

FIGURE 1 | Mitonuclear communication with a focus on the proposed mechanisms of miRNA import into mitochondria. Mitonuclear communication plays a key role in mitochondrial homeostasis. The nucleus coordinates a number of mitochondrial functions through the so-called anterograde signaling, which involve various signaling factors, including nuclear-respiratory factor 1, nuclear-respiratory factor 2, peroxisome proliferator-activated receptors, mitochondrial transcription factor A, uncoupling proteins, ERR and PPARγ co-activator 1α. MicroRNAs have been observed in this communication network, some are transported into the mitochondria (mitomiRs) and a few of which are associated with cellular senescence associated-mitomiRs. At the same time, mitochondria communicate their functional and oxidative state to the nucleus *via* the retrograde response, which involve mitochondria-derived peptides and metabolites, such as ROS, ATP/ADP/AMP, NAD^+/NADH and Ca^{2+}. After their biogenesis in the nucleus and their translocation in the cytosol, miRNA duplexes are unwound, they bind to the Argonaute protein 2 (Ago2) and are loaded into the RNA-inducing silencing complex. (1) Due to cellular stress GW182 dislocates from RISC, leaving the miRNA:Ago2 complex free in the cytosol. It has been proposed that miRNA:Ago2 might be imported into mitochondria through the SAM50 and TOM20 pores in the outer membrane and TIM in the inner mitochondrial membrane (Jusic and Devaux, 2020). (2) Phosphorylation of Ago2 induces miRNA:Ago2 intake into a P-body, which is known to interact with mitochondria. (3) Translocation of miRNA:Ago2 complex into mitochondria may be supported by polynucleotide phosphorylase. Polynucleotide phosphorylase facilitates mRNA import into mitochondria, by binding to stem loop motifs in the RNA sequence. (4) Porins participate in miRNA translocation into mitochondria, as proposed by Bandiera et al. (2013). (5) After mitochondrial depolarization, GSP2 translocates into the nucleus, triggering the expression of genes involved in mitochondrial homeostasis. (6) Upon sensing stress stimuli in the mitochondrion, Forkhead box O can induce specific sets of nuclear genes, including autophagy effectors and antioxidant activators. Parts of the figure were realized using BioRender (http://biorender.com).

homeostasis and are deregulated during senescence. The section "Anterograde signaling *via* nuclear-encoded mitomiRs in ARDs" will deeply describe nuclear-encoded miRNAs and their impact in mitochondria function and dysregulation.

Retrograde Signaling

The retrograde signaling pathway is activated when dysfunctional mitochondria try to communicate their oxidative, metabolic and respiratory stressful conditions to the nuclear compartment,

thus inducing a wide range of cellular adaptations (Passos et al., 2007; Quirós et al., 2016).

The most well studied retrograde signals are pleiotropic and include ROS, pro-apoptotic molecules such as ATP/ADP/AMP and metabolites such as NAD^+/NADH and Ca^{2+} (Picard et al., 2013), which, however, lack specificity as signaling molecules. Thus, the exact mechanism underlying the retrograde signaling by which mitochondria regulate cellular processes has not been completely unravelled yet.

Recently, mitochondrial-derived peptides (MDPs) encoded by a short open reading frame into mtDNA have been identified. Three types of MDPs have been discovered so far, including Humanin, MOTS-c and SHLP1-6. Their role is associated with cell survival, metabolism, inflammation and response to stressors with the final aim of maintaining mitochondrial function under stress conditions (Yang et al., 2019; Conte et al., 2021).

Recent reports highlight that the mammalian mitochondrial genome, in addition to the 37 known mitochondrial genes, encodes several classes of sncRNAs, "mitochondrial genome-encoded small RNAs" (mitosRNA), whose deregulation have been correlated with dysfunctional mitochondria and ultimately with a variety of diseases (Ro et al., 2013). To examine mitosRNA biogenesis, the presence of a mitochondrial RNAi machinery was investigated by Ro and colleagues, but neither Dicer nor AGO2 expression was detected. Therefore, mitosRNAs do not derive from RNA turnover, but they must be products of unknown mitochondrial ribonucleases (Ro et al., 2013).

NGS analyses performed by Larriba et al. (2018), who classified mitosRNAs into several groups of sncRNAs, emphasized the predominance of Piwi-interacting RNAs compared to other sncRNA categories, with regulatory functions in mitochondria and important for gametes and zygotic cells development, showing cell-type specific expression.

Despite mitosRNAs exact biogenesis and cellular trafficking are still uncertain (Vendramin et al., 2017), many studies about their possible function were carried out. Of note, mito-ncR-805 has been shown to have a protective effect to cigarette smoke in alveolar epithelial cells, as well as an increased expression of nuclear-encoded genes important for mitochondrial function, supporting the idea that mitosRNAs regulation and function might be cell-type specific (Blumental-Perry et al., 2020).

How mitosRNAs are exported from mitochondria and imported into the nucleus is still not known (Gammage et al., 2018), however studies about the expression of sense mitochondrial ncRNAs (SncmtRNAs) and antisense mitochondrial ncRNAs (ASncmtRNAs) between normal and cancer cells, demonstrated a cytoplasmic and nuclear localization of these mitochondrial transcripts, reinforcing the concept that they are exported from mitochondria (Villegas et al., 2007; Landerer et al., 2011; Borgna et al., 2020).

A direct mitonuclear communication strategy for mammals, similar to those found in yeasts and worms (Jazwinski and Kriete, 2012; Nargund et al., 2012, 2015), has been proposed by Cardamone et al., who characterized the G-Protein Pathway Suppressor 2 (GPS2; Cardamone et al., 2017), which also regulates insulin signaling, lipid metabolism and inflammation (Jakobsson et al., 2009). Cardamone and colleagues demonstrated that upon mitochondrial depolarization, GPS2 translocates into the nucleus, triggering the expression of genes involved in mitochondrial homeostasis. Other transcription factors involved in retrograde signaling have been identified, such as Forkhead box O (FOXO), which is known to help in the transcription of mitochondrial antioxidant enzymes, inducing mitophagy (Kim and Koh, 2017).

However, to our knowledge, since the details of this complex pathway are still unexplored, not much is known about other RNA export mechanisms from mitochondria, and if these also involve mitosRNAs.

New data support the idea of a mitonuclear communication involvement in aging. Since Ca^{2+} is the most important signaling molecule in the retrograde signaling pathway and studies in senescent human MRC5 fibroblasts correlated with increased mitochondrial biogenesis and Ca^{2+} alterations, it has been proposed that dysfunctional mitochondria might communicate with the nucleus *via* calcium signaling (Passos et al., 2007). Moreover, several studies reported that during induced or replicative senescence, mitochondria produce a higher amount of MDPs (humanin and MOTS-c), which can regulate mitochondrial energy metabolism, playing a cytoprotective role in ARDs. For example, humanin has a crucial role in reducing oxidative stress, while MOTS-c in glycolipids metabolism protects endothelial cells from atherosclerosis (Kim et al., 2018). Additional researchers have outlined that the increased production of ROS by defective mitochondria induces cytoplasmic chromatin fragments formation, which is JNK kinase-mediated, which is a trigger of SASP (Vizioli et al., 2020).

As far as we know, only a few studies have outlined the possibility of a relationship between mitosRNAs and ARDs. For example, SncmtRNA-1, SncmtRNA-2, ASncmtRNA-1 and ASncmtRNA-2 have been associated with cancer (Burzio et al., 2009; Vidaurre et al., 2014; Villota et al., 2019), whereas mt-lncRNA has been correlated with cardiovascular diseases (Yang et al., 2014). An interesting work by Shinde and colleagues (Shinde and Bhadra, 2015), demonstrates the expression of six novel mitochondrial genome-encoded miRNAs (mt-mitomiRs; Giuliani et al., 2019) in mitochondria and that the MT-RNR2 gene could be a potential target of two of them, hsa-miR-mit-3 and hsa-miR-mit-4. Curiously, MT-RNR2 gene also encodes for the humanin peptide, which has been correlated with Alzheimer's disease (Tajima et al., 2002), indicating a potential involvement of them in the development of the disease. MitosRNA association with ARDs makes them promising biomarkers of the diseases, therefore future studies will be required for a potential application in clinical practice.

ANTEROGRADE SIGNALING *VIA* NUCLEAR-ENCODED mitomiRs IN ARDs

One of the first piece of evidence that nuclear-encoded miRNA can regulate the expression of mitochondrial genome was provided by Das and colleagues. The authors showed that after its maturation in the cytoplasm, miR-181c translocated in the

mitochondrial compartment, where its principal target is the mt-COX-1 gene. Since mitochondrial DNA is sequentially transcribed as a polycistronic unit, miR-181c affects multiple proteins, including mt-COX2 and mt-COX3, resulting in complex IV remodelling (Das et al., 2012). Being the last complex of the respiratory chain, complex IV plays an important role in the transfer of electrons from cytochrome c oxidase to oxygen. Mutations or deregulation of complex IV lead to higher levels of ROS and mitochondrial dysfunction and are associated with a negative impact on lifespan and tissue integrity (Reichart et al., 2019). Cardiac myocytes are the cells with the highest volume density of mitochondria in the body and rely their extraordinary demand for continuous energy production on oxidative metabolism. As a result, these cells have been used to unravel mitomiR role in mitochondrial homeostasis. Das and co-workers confirmed their results *in vivo* using a lipid-based cationic nanoparticles miR-181c delivery system, which demonstrated that chronic overexpression of miR-181c is involved in heart failure (Das et al., 2014). Besides miR-181c, other miR-181 family members – i.e. miR-181a -181b, -181d – are found in mitochondria and have implications in heart mitochondrial health (Das et al., 2017). Interestingly, miR-181a and miR-181b have been shown to exert divergent roles in myocardial function. At the early stages of heart failure, miR-181a and -181b are consistently upregulated in cardiomyocyte mitochondria whereas, at the later stages, only miR-181b levels tend to remain stable, in association with a downregulation of miR-181a (Wang et al., 2017b). In this framework, mitomiRs acquire a critical role in cardiac function. Another miRNA widely studied in cardiac and skeletal muscle tissues is miR-1, which is actively involved in myogenesis and muscle proliferation (Chen et al., 2006). Increased expression of miR-1 was found in aging hearts, suggesting that this miRNA participates in additional cellular or pathophysiological functions other than myogenesis (Yang et al., 2007). MiR-1 has several cytosolic targets; however, during muscle differentiation it translocates to the mitochondria where it, surprisingly, enhances translation of mt-COX-1 and mt-ND1, resulting in boosted ATP generation. Zhang and colleagues showed that overexpression of miR-1 could have a negative impact on mitochondrial morphology and physiology in cancer stem cells, by targeting nuclear-encoded proteins required for mitochondria organization (Zhang et al., 2019). The non-canonical role as a translational activator of miR-1 seems to be linked to the lack of miRNA-mediated gene silencing GW182 inside the mitochondria, suggesting that the miRNA machinery is rearranged in this organelle (Zhang et al., 2014).

MitomiRs are also emerging players in the pathogenetic processes of diabetic heart diseases. Diabetes is associated with cardiac functional deficits, which may result from a decreased mitochondrial ATP output. Jagannathan et al. analyzed mitomiR distribution in the two spatially distinct mitochondrial subpopulations, i.e., subsarcolemmal and interfibrillar mitochondria, following diabetic insult. Of particular interest is mitomiR-378-3p, which originates from the first intron of peroxisome proliferator-activated receptor gamma, coactivator 1 beta gene that encodes PGC1β. This miRNA has been implicated in lipid metabolism, mitochondrial function, and shift towards the glycolytic pathway (Carrer et al., 2012; Krist et al., 2015). MitomiR-378 binds the ATP synthase F0 subunit 6 (ATP6), leading to a drop of ATP production following diabetes insult in interfibrillar mitochondria of mice (Jagannathan et al., 2015). In a condition of diabetic cardiomyopathy, mitomiR-92a-2-5p and let-7b-5p enter into the mitochondria to counteract cytochrome-b downregulation. Overexpression of miR-92a-2-5p enhances mitochondrial translation and reduces ROS production and lipid deposition, rescuing cardiac diastolic dysfunction in the db/db mouse model (Li et al., 2019). Also, miR-762 translocates to mitochondria upon ischemia/reperfusion model and downregulates ND2 leading to inhibition of ATP production and the enzyme activity of complex I, induction of ROS generation and apoptotic cell death in cardiomyocytes (Yan et al., 2019).

Metabolic reprogramming is a feature of cancer cells. Two interesting papers showed how mitomiRs can promote chemotherapy resistance by inducing glycolysis in cancer cells. Nuclear-encoded mitomiR-2,392 and -5,787 are involved in reprogramming metabolism *via* increase of glycolysis and inhibition of OXPHOS, resulting in enhanced chemoresistance in tongue squamous cell carcinoma cells (Chen et al., 2019; Fan et al., 2019).

The mitomiR-mediated switch of energy sources during cellular differentiation suggests a pivotal role of mitomiRs in all processes requiring metabolic reprogramming, including cellular senescence (Sabbatinelli et al., 2019). Indeed, during senescence mitochondrial function declines, which creates an energy deficit. Retrograde signaling tries to overcome this energy deficit by increasing mitochondrial biogenesis as well as glycolysis, as a compensatory measure. However, this compensation is partial and accompanied by an increase in ROS production, thus creating a cycle of further damage to the mitochondria itself and to the cell.

Overall, we can conclude that mitomiRs can interact with mitochondrial genome in multiple ways: (i) as prompt compensators after a negative insult — e.g., miR-92a-2-5p and let-7b-5p, (ii) as effectors of mitochondrial dysfunction — e.g., miR-738-3p, (iii) as mediators of physiological cellular processes — e.g., miR-1.

SENESCENCE-ASSOCIATED miRNAs IMPACT ON MITOCHONDRIAL FUNCTION

Senescence associated-mitomiRs have been demonstrated to affect all aspects of mitochondrial homeostasis. While the role of SA-mitomiRs on the expression of mitochondrial genes has yet to be elucidated, multiple pieces of evidence support their ability in modulating several processes linked to mitochondrial function.

Through an *in silico* analysis we have suggested that SA-mitomiRs may affect endothelial cell sensitivity to apoptosis through Bcl2 family member regulation (Rippo et al., 2014).

Furthermore, we have recently demonstrated that miR-146a-5p, miR-34a and miR-181a levels are increased during replicative senescence of endothelial cells and enriched in senescent mitochondria. Their overexpression induces permeability transition pore opening, ROS production, caspase-1 and -3 activation and autophagic vacuole accumulation at least due to Bcl-2 downregulation (Giuliani et al., 2018).

MiR-146a is one of the most extensively studied miRNAs in the field of senescence and inflammation (Olivieri et al., 2013a,b) The synthesis of miR-146a is intimately linked to inflammatory processes and its effects on cellular processes are highly stimulus- and context-dependent. Indeed, miR-146a, which is particularly enriched in the mitochondrial fraction of cardiomyocytes, exerts a cardioprotective role by inhibiting the mitochondria-dependent apoptotic pathway and attenuating the loss of mitochondrial membrane potential. Evidence from cardiomyocyte-specific knockout and overexpression experiments supported the hypothesis that adequate miR-146a levels are required to reduce the extent of myocardial infarction and cardiac dysfunction following ischemia/reperfusion damage (Su et al., 2021). Notably, acute cellular damage can affect the trafficking of miRNAs between the mitochondrial and cytosolic compartments. Following a severe traumatic brain injury (TBI), miR-146a-5p levels decrease in the hippocampal mitochondrial fraction, in association with an increase of its cytosolic expression. This compartmental shift was shown to be triggered by decreased mitochondrial bioenergetics following TBI.

MiR-146a cytosolic enrichment avoids uncontrolled activation of the NF-kB pathway by targeting its upstream members TRAF6 and IRAK1. Mitochondria can act as first-line responders to cellular stressors by triggering pathways leading to altered nuclear gene expression, also by affecting miRNA intracellular localization (Wang et al., 2015, 2021). Interestingly, miR-146a-5p also impacts mitochondrial dynamics by targeting PARK2, one of proteins involved in mitophagy. Decreased amounts of PARK2 lead to the accumulation of damaged and dysfunctional mitochondria, which exacerbates the ROS-induced neuronal damage (Jauhari et al., 2020). Parkin 2 is a component of a multiprotein E3 ubiquitin ligase complex that mediates the targeting of substrates, including mitochondrial proteins, for proteasomal degradation and mitophagy. Interestingly, PARK2 mutations have been associated with early onset Parkinson's disease (Kitada et al., 1998). Besides its neuroprotective and cardioprotective function, a role in liver homeostasis has been demonstrated for miR-146a. Its hepatocyte expression is needed to promote the mitochondrial oxidation of fatty acids and improve insulin sensitivity, which prevents the detrimental lipid accumulation in the liver. These effects are achieved by promoting both mitochondrial biogenesis and synthesis of electron transport chain complex subunits through targeting of MED1, a component of the mammalian mediator complex involved in adipogenesis and mitochondrial gene expression (Li et al., 2020). Moreover, bioinformatic analysis identified multiple miR-146a potential targets in mitochondrial genome, suggesting there is still much to uncover about the crosstalk between miR-146a and bioenergetic pathways (Dasgupta et al., 2015; Giuliani et al., 2017).

MiR-34a-5p has been implicated in the pathogenesis of ARDs accompanied by mitochondrial dysfunction and impairment of the autophagic flux, such as neurodegenerative disorders. MiR-34a-5p targets PINK1, a stress sensor that localizes to the outer mitochondrial membrane following the loss of mitochondrial potential. This triggers mitochondrial clearance. Similarly to PARK2, also PINK1 mutations have been involved in the pathogenesis of early onset Parkinson's disease. In this framework, overexpression of miR-34a-5p attenuates mitochondrial protein ubiquitination and prevents the recruitment of PARK2, thus delaying mitophagy (Tai et al., 2021). One of the main targets of miR-34a with a well-established role in aging is SIRT1. The rescue of SIRT1 levels following miR-34a inhibition reduced age-related hearing loss in C57BL/6 mice. Although the full signaling pathway responsible for this effect has still to be elucidated, the miR-34a/SIRT1 axis was hypothesized to affect the balance between mitophagy and mitochondrial biogenesis and to protect cochlear cells against oxidative stress-mediated apoptosis (Xiong et al., 2019). MiR-34a was also shown to accelerate renal aging by affecting the mitochondrial function of mesangial cells. MiR-34a targets the mRNA of thioredoxin reductase 2, a protein involved in the scavenging and detoxification of mitochondrial ROS. The levels of miR-34a were particularly high in senescent mesangial cells and, at the same time, miR-34a overexpression induced premature senescence in these cells due to the accumulation of dysfunctional mitochondria (Bai et al., 2011).

The link between mitochondrial function and miR-181a has been elucidated in multiple cellular models (Mancini et al., 2012; Indrieri et al., 2020). Interestingly, miR-181a is among the most characterized miRNAs in lymphoid tissue, with a well-documented role in T cell aging and immune senescence (Ye et al., 2018; Kim et al., 2021). The age-related decline in miR-181a expression in naive and memory T cells may account for some of the age-associated defects in T cell function. To this regard, restoration of miR-181a intracellular levels provided a feasible strategy to boost T cell response in the elderly (Li et al., 2012; Ye et al., 2018, 2021). Recently, it also has been shown a decline of miR-181a-5p in NK cells from the aged mice, impairing the production of IFN-γ (Lu et al., 2021). Moreover, miR-181a shows an age-dependent decline in peripheral blood mononuclear cells from donors of different ages (Xu et al., 2020). Similarly to miR-34a, miR-181a is a regulator of mitochondrial dynamics through the action on several proteins implied in the mitophagy process. Restoration of the expression of miR-181a in the skeletal muscle of old mice improved mitochondrial quality (Goljanek-Whysall et al., 2020). The prominent role of miR-181a-5p in age muscle homeostasis was elegantly reviewed by Borja-Gonzalez and colleagues (Borja-Gonzalez et al., 2020). MiR-181a is closely associated to inflammation and apoptosis in neuronal cells, where it targets mitochondria-related proteins, i.e., heat shock protein 70, glucose regulated protein 78, anti-apoptotic Bcl-2, and myeloid cell leukemia-1 (Ouyang et al., 2012; Hutchison et al., 2013).

MiR-210 is considered the master hypoxia-related miR, because of its prompt upregulation under hypoxia in most

TABLE 1 | Shows senescence-associated mitomiR targets.

SA-mitomiRs	Cytosolic target	Effect on senescence-related pathways	Mt-DNA target	Effect on mitochondrial functions
miR-21-5p	-NFIB and CDC25A (Dellago et al., 2013) -TLR8 (ligand; Zhang et al., 2018) -A20 (Xue et al., 2019) -PTEN (Buscaglia and Li, 2011; Ma et al., 2013) -PDCD4 (Matsuhashi et al., 2019)	-Cell proliferation arrest-Pro-inflammatory cytokine production-Activation of NF- κB pathway and NLRP3 inflammasome	-mt-Cyb (Li et al., 2016)	-enhanced mitochondrial translation
miR-146a-5p	-TLR4 (Xiao et al., 2019) -TRAF6 (Taganov et al., 2006) -IRAK1 (Taganov et al., 2006) -BCL2 (Giuliani et al., 2018)	-Pro-inflammatory cytokine production-Apoptosis sensitivity alteration-Activation of NF-κB pathway	-mt-ND1, mt-ND2, mt-ND4, mt-ND5, mt-ND6; -mt-ATP8 (PREDICTED; Dasgupta et al., 2015)	
miR-181a-5p	-SIRT1 (Di Val Cervo et al., 2012) -BCL2 (Giuliani et al., 2018) -PARK2 and p62/SQSTM1 (Goljanek-Whysall et al., 2020)	-Cell proliferation arrest-Apoptosis sensitivity alteration-Impaired autophagy		
miR-210	-NDUFA4 and SDHD (Puisségur et al., 2011) -ISCU1/2 (Chan et al., 2009)-RAD52 (Crosby et al., 2009) -E2F3 (Biswas et al., 2010)	-Mitochondrial dysfunction-DNA repair loss - Cell proliferation arrest		
miR-34a	-SIRT1/P53 axis-BCL2 (Giuliani et al., 2018) -Txnrd2 (Bai et al., 2011)	-Apoptosis sensitivity alteration-Cell proliferation arrest-Pro-inflammatory cytokine production-Increased oxidative stress		

Tumor necrosis factor, alpha-induced protein 3(A20); B-cell lymphoma 2 (Bcl-2); Cell division cycle 25 A(CDC25A); E2F transcription factor 3 (E2F3); Interleukin 1 Receptor Associated Kinase 1 (IRAK1); Iron–sulfur cluster assembly enzyme (ISCU); mtDNA-encoded cytochrome b (mt-Cytb); Nuclear factor 1 B-type (NFIB); Phosphatase and tensin homolog (PTEN); succinate dehydrogenase complex subunit D (SDHD); Sirtuin 1 (SIRT1); Sequestosome-1 (SQSTM1); Toll like receptor (TLR); TNF Receptor Associated Factor 6 (TRAF6); Thioredoxin Reductase 2 (Txnrd2).

cell types. MiR-210 is upregulated in senescent cells where it is involved in double-strand DNA breaks and ROS accumulation (Faraonio et al., 2012) *via* the inhibition of the electron transport chain (ETC) protein translation (Karshovska et al., 2019). MiR-210 directly targets NDUFA4 and SDHD — subunits of the ETC complex I and II, respectively — and induces mitochondrial dysfunction (Puisségur et al., 2011). Future studies are warranted to unravel its specific activity in mitochondrial function.

MiR-21, initially classified as an 'onco-miR' due to its modulation in different types of cancer, has an extensively established role in inflammatory and senescence processes (Olivieri et al., 2021). Increased expression of miR-21-5p was found in replicative and stress-induced models of senescence (Dellago et al., 2013; Mensà et al., 2020).

A particular myocardial enrichment of miR-21-5p has been observed in several models of cardiovascular disease, cardiac dysfunction, and heart failure, where it has been reported to prevent cardiomyocyte apoptosis by targeting the PDCD4 mRNA (Qin et al., 2012). *In vivo* silencing of miR-21, using a specific antagomir, has been found to attenuate cardiac fibrosis and cardiac dysfunction in pressure-overloaded hearts (Thum et al., 2008). Overexpression of miR-21 decreases mitochondrial fatty acid oxidation and concomitant mitochondrial respiration in rat cardiomyocytes, suggesting that miR-21 coordinates the shifting of cellular metabolism towards the glycolytic pathway (Nasci et al., 2019). This hypothesis was later confirmed by the evidence that miR-21 is able to translocate into the mitochondria and target mt-Cyb to enhance its translation in a spontaneous hypertensive rat model (Li et al., 2016). Moreover, miR-21-5p affects mitochondrial dynamics in a model of oxidized LDL-induced endothelial cell senescence by targeting Drp1 protein (Zhang et al., 2017).

Although miR-21 is also involved in several mitochondrial functions, the precise role of miR-21 in senescent mitochondria is far from being elucidated. **Table 1** summarizes the mitomiR targets with a well-recognized role on senescence. The involved pathways are functional to the acquisition of the senescent phenotype, including cell cycle arrest, mitochondrial dysfunction, and production of pro-inflammatory cytokines, that is, SASP.

CONCLUSION

The central role of mitochondria in cellular senescence is now a dogma recognized by all gerontologists in the world. Many biochemical and morphological changes to which these organelles meet are common to both stress-induced or replicative senescence: branching and elongation, ROS production, mtDNA mutations and membrane depolarization. Several of these phenomena are due to the sophisticated communication system (anterograde and retrograde signaling) between the nucleus and the mitochondrion. While an increasing number of research reports are shedding light on the anterograde signaling routes, retrograde signaling mechanisms are almost completely unknown although there is the certainty that they play important roles. Nuclear-encoded miRNAs shuttle within mitochondria, and at the epigenetic level, regulate both mt-DNA encoded proteins and those encoded by nuclear genes that are functional in mitochondria.

Since the mitochondrial alterations observed in the senescent cells represent etiopathogenetic factors in age-associated diseases, the deepening of the communication routes between nucleus

and mitochondria may lead to devise new preventive and therapeutic strategies.

AUTHOR CONTRIBUTIONS

CG, AS, and AG performed literature search, drafted the manuscript and prepared the figure. MR conceived the idea and participated in manuscript drafting. FO reviewed the manuscript. All authors approved the final version of the manuscript.

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