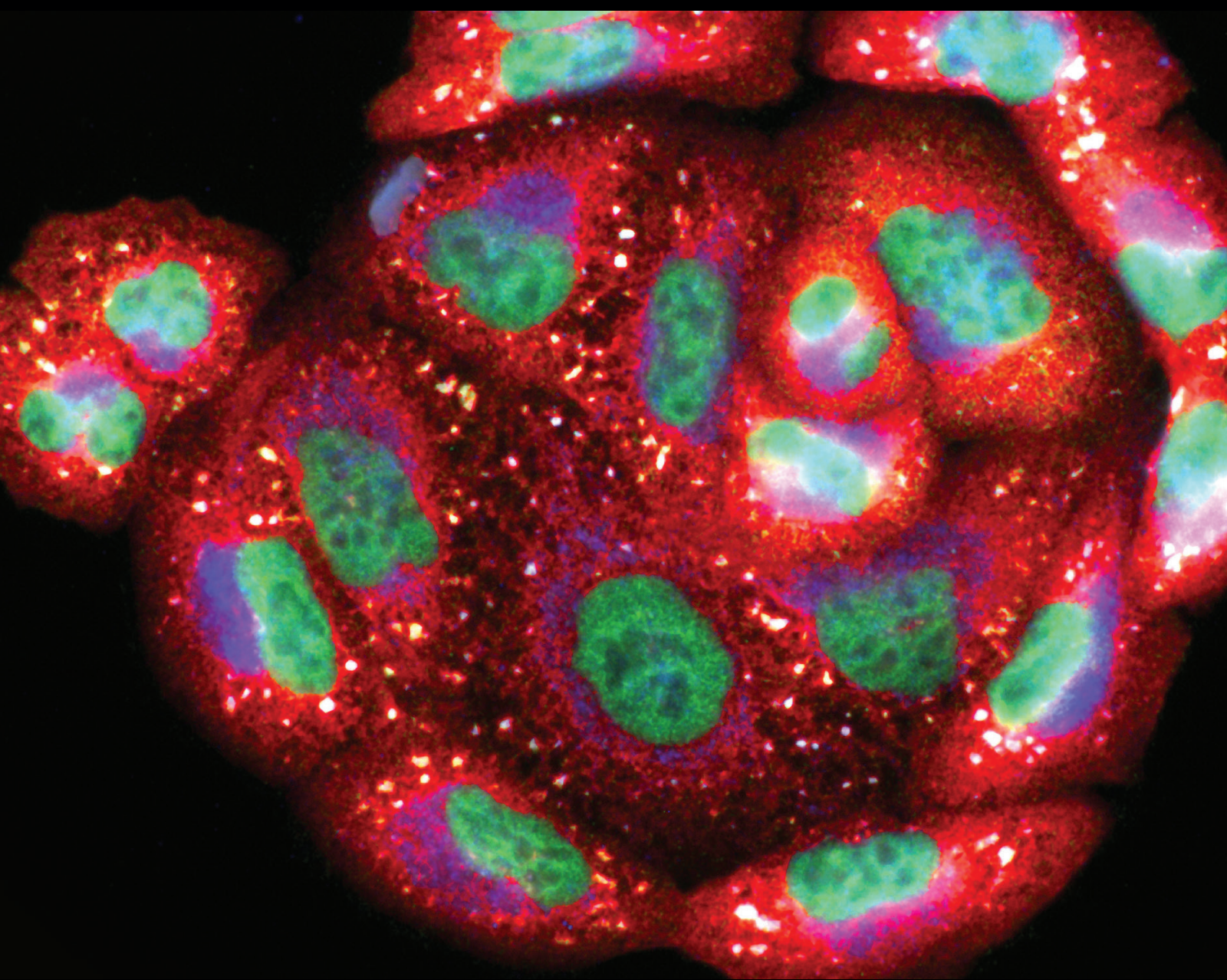
Oxidative Medicine and Cellular Longevity

New Trends in Antioxidant Compounds: A Precise Nutraceutical in Cardiometabolic Disorders

Lead Guest Editor: Cristiana Caliceti
Guest Editors: Norifumi Urao, Paola Rizzo, and Mariateresa Giuliano



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Editorial Board

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Editorial

New Trends in Antioxidant Compounds: A Precise Nutraceutical in Cardiometabolic Disorders

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Cardiometabolic disorders are among the leading causes of morbidity and mortality in the western world. The most cost-effective preventive approach still remains a personalized diet and physical activity, but novel therapeutic approaches are still needed to delay the progression of these conditions more efficiently compared to existing treatments. As shown with other pathologies, it is becoming increasing clear that a "precise and personalized" strategy holds more possibilities of success compared to traditional approaches. Most medical treatments are designed for the "average patient" as a "one-size-fits-all-approach," which may be successful for some patients but not for others. Precision medicine is an innovative approach to tailor disease prevention and treatment that takes into account differences in genetic background, environment, and lifestyles. The goal of precision medicine is to administer the right treatments to the right patients at the right time.

Research on food bioactive molecules represents an emerging strategy to evaluate the role of functional foods and supplements in health and disease prevention. There is well-established evidence of the pharmacological properties of micronutrients that render them therapeutically effective in chronic inflammatory diseases. Although caution should be exercised in using antioxidant supplementation, antioxidant foods as dietary components play an important role in the management of cardiometabolic disorders. There is documented evidence of disease-modifying effects of nutritional compounds with anti-inflammatory and antioxidant effects. These compounds have specific applications in ameliorating oxidative stress-induced inflammatory diseases such as diabetes mellitus and cardiovascular diseases. However, due to the limited number of studies, the role of many of these supplements in chronic disease prevention is still unclear. Observational studies have suggested that foods such as fruits and vegetables, nuts, chocolate, and fatty fish, as well as beverages such as tea, wine, and coffee, are associated with a wide range of health benefits. As a result, many have postulated that various bioactive molecules and/or nutrients in these foods may be responsible for the observed health-related effects. Yet annual sales of dietary supplements continue to rise in the US, Europe, and Asia. This may be explained in part by the perception that supplements containing bioactive molecules and nutrients help ensure an adequate intake not only to prevent deficiency of essential vitamins and minerals but also to potentially reduce the risk of major chronic diseases.

In this issue, G. Aquila et al. review the effects of widely used nutraceuticals on the Notch pathway, a major regulator of the functions of endothelial cells and macrophages, the predominant cell types involved in cardiometabolic disorders. The knowledge of the type of modulation, if any, of

the Notch pathway by specific compounds could help to identify the most effective nutraceutical, based on its effect on Notch, for a specific individual and/or patient. Similarly, the review article by J. Lietava et al. on the activities of Cornelian cherries at different steps of atherosclerosis provides useful information of the best time for intervention with this compound. Precise and personalized treatments are possible only by dissecting the molecular mechanisms modulated by a specific agent. In this issue, J. Tian et al., by showing that Ginkgo biloba restores autophagy through the mTOR pathway, provide new insights on the well-known protective action of this nutraceutical in the context atherosclerosis, whereas M. Ucci et al. report novel aspects of β-carotene and lycopene-mediated protection of vascular endothelial cells under diabetic conditions. Lastly, C. Caliceti et al. identified the specific peptides from cauliflower leaves able to protect vascular cells, thus providing a further example of "precision" approach to obtain increased yield of the specific, bioactive compounds from agriculture waste. Given the growing social and environmental interest for the efficient reuse of agriculture waste co- and by-products, often rich in bioactive compounds, the identification and recovery of such compounds represent an efficient strategy to improve the ecosustainability of cultivations by reducing waste disposal.

Conflicts of Interest

The authors have no conflict of interest to declare.

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Norifumi Urao
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Review Article

The Use of Nutraceuticals to Counteract Atherosclerosis: The Role of the Notch Pathway

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Despite the currently available pharmacotherapies, today, thirty percent of worldwide deaths are due to cardiovascular diseases (CVDs), whose primary cause is atherosclerosis, an inflammatory disorder characterized by the buildup of lipid deposits on the inside of arteries. Multiple cellular signaling pathways have been shown to be involved in the processes underlying atherosclerosis, and evidence has been accumulating for the crucial role of Notch receptors in regulating the functions of the diverse cell types involved in atherosclerosis onset and progression. Several classes of nutraceuticals have potential benefits for the prevention and treatment of atherosclerosis and CVDs, some of which could in part be due to their ability to modulate the Notch pathway. In this review, we summarize the current state of knowledge on the role of Notch in vascular health and its modulation by nutraceuticals for the prevention of atherosclerosis and/or treatment of related CVDs.

1. Introduction

"Prevention is better than cure" is the proverb that better describes the impact of eating habits in prophylaxis of many pathologies, including cardiovascular disease (CVD), one of the leading causes of death in industrialized countries [1]. The causes of CVDs are multifactorial and some of these, such as modifiable lifestyle (i.e., tobacco smoking and physical activity) and especially dietary habits, play a major role in this context. In fact, it has been widely demonstrated that a healthy diet, with balanced intake of vegetables, fruits, olive oil, and whole grains, reduces the risk of developing CVDs due to atherosclerosis. Therefore, in the last few years, novel nutritional strategies have been implemented, including dietary modifications and consumption of innovative functional foods and dietary supplements which fall in the category of nutraceutical products. Coined in the end of the '90s by Dr. Stephen De Felice, the neologism "nutraceutic" is a combination of the terms "nutrition" and "pharmaceutic" and indicates a discipline that studies the substances that are "a food or part of a food" which can provide "medical or health benefits, including the prevention and treatment of disease" [2]. More recently, nutraceuticals have been defined as "a substance that is cultivated/produced/extracted or synthesized under optimal and reproducible conditions and, when administered orally to normal subjects or patients, would provide the nutrient(s) required for bringing altered body structure and function back to normal, thus improving the health and well-being of the patients" [3]. Consistently, preclinical and clinical studies conducted in the last 20 years have

shown an effect of nutraceuticals on prevention and progression of CVDs, specifically on atherosclerosis. Nevertheless, there are studies that did not confirm the protective effect of nutraceuticals, and to shed further light on the action of these compounds, different doses and modality of administration, taking also under consideration the presence/absence of other medications, are being investigated, together with studies of pharmacodynamics and pharmacokinetics. These contrasting results could be also due to the limited number of pathways so far investigated. An example in this context is provided by probiotics, whose supplementation efficacy has given contrasting results. A recently published study shows that probiotic supplements promote endothelial functions in humans with coronary artery disease (CAD) without altering traditional cardiovascular risk factors or the microbiota population but only their transcriptional activity, as indicated by their plasma metabolites profile [4]. Thus, by only looking at the different bacteria population of the microbiota, these effects of probiotics would have been missed. Additionally, by increasing the number of biomarkers of vascular function modified by nutraceuticals, the identification of "super responders" could be achieved, paving the way for "personalized nutraceutical treatments."

The Notch pathway, originally studied for its role in promoting the survival of cancer cells, is emerging as a key player in the maintenance of the vascular wall health and in the regulation of inflammation [5, 6]. This pathway responds to agents, such as inflammatory cytokines and lipopolysaccharides, whose effects are contrasted by nutraceuticals. The aim of this review is to describe existing studies on the regulation of Notch by nutraceuticals, in order to determine whether the analysis of components of this pathway could represent novel clinical surrogates providing useful information when trying to assess the effect of traditional or emerging nutraceuticals.

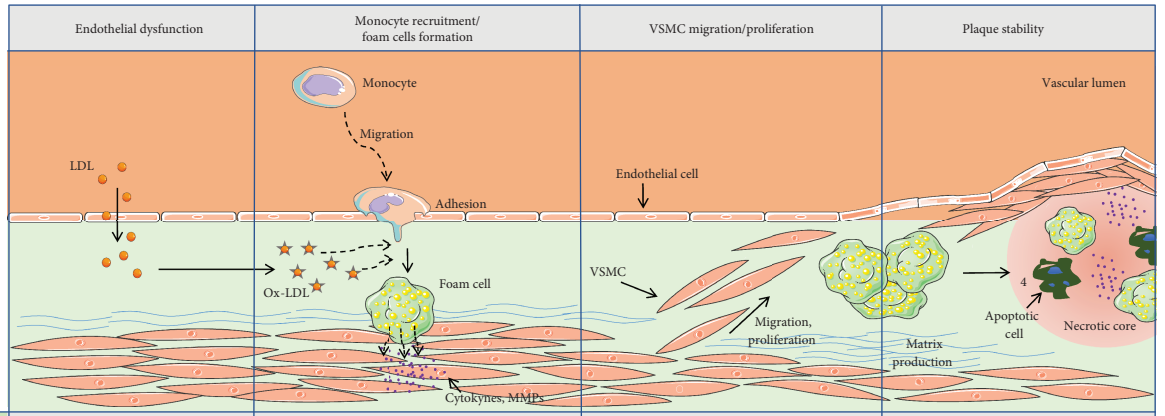
2. The Steps of Atherosclerosis

Atherosclerosis is a complex multifactorial and multistep chronic inflammatory condition characterized by the progressive accumulation of lipid-loaded fibrous plaques within the artery wall. This pathology, arising from a cumulative damage of the vessel wall, can culminate in atherosclerotic plaque rupture and subsequent thrombus formation [7], leading to its most common clinical manifestations, namely, myocardial infarction (MI) or stroke [8]. The first stage of atherosclerosis development takes place in the endothelium, the intima layer of artery walls. The vascular endothelium is a selective semipermeable continuous monolayer of endothelial cells (ECs) that plays a major role in controlling the vascular tone and vascular wall thickness by synthesizing a plethora of autocrine and paracrine substances, such as nitric oxide (NO), prostacyclin, histamine, prostacyclin, angiotensin II (Ang II), heparin, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-I), which affect vascular smooth muscle cell (VSMC) proliferation, leukocyte migration, platelet aggregation, and adhesion [9, 10]. Numerous systemic and hemodynamic risk factors contribute to the onset of endothelial dysfunction (defined as reduced levels of NO, increased endothelium permeability, caused by EC apoptosis and reduction of junctions between ECs, and increased expression of adhesion molecules) which is the first step toward plaque formation [11]. Endothelium discontinuity favors the entry of cholesterol-containing low-density lipoprotein (LDL) particles in the intima of large arteries [10], an event mediated by interaction between LDL apolipoprotein B100 (ApoB100) and matrix proteoglycans. LDL retention predisposes them to oxidative modification [12], and the resulting oxidized LDLs (oxLDL), by binding to a specific receptor, lectin-like oxLDL receptor-1 (LOX1), induce the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) by ECs [13, 14]. These adhesion molecules, along with chemotactic molecules, such as monocyte chemoattractant protein-1 (MCP-1) secreted by ECs and VSMCs, mediate the transmigration of monocytes in the subendothelium, where differentiate into macrophages. These later adopt a M1 proinflammatory phenotype and encode a wide range of scavenger receptors (SRs) (i.e., SR-A1 and CD36), useful for facilitating the endocytosis oxLDLs [15]. This oxLDL overload results in acquisition of the "foam cell" phenotype by macrophage and fatty streak formation.

Furthermore, mast cells, T cells, and other inflammation mediators penetrate lesions and, together with foam cells, contribute to the maladaptive proatherosclerotic inflammatory response. Foam cells secrete chemotactic growth factors and metallopeptidases which, through degradation of the extracellular matrix (ECM), support proliferation and migration of VSMCs and leukocytes into the intima [16]. In the intima, the VSMCs produce interstitial collagen and elastin and form a fibrous cap that covers the plaque. This cap overlies the macrophage-derived foam cells, some of which die by apoptosis and release lipids that accumulate extracellularly. This process leads to the formation of a lipid-rich pool called the "necrotic core" of the plaque [7]. The stability of the plaques is influenced by the balance between cap and necrotic core. Stable plaques are characterized by the presence of a small core rich in lipids covered by a thick fibrous cap rich in collagen. Instead, unstable plaques have a thin fibrous cap over a large fatty core [17] and are prone to rupture which leads to the exposure of tissue factors to blood flow and the activation of coagulation cascade with consequent thrombus formation [7, 18]. An intraluminal thrombus in the coronary arteries may decrease blood flow causing ischemic cardiomyopathy: a complete vascular occlusion will cause MI.

3. Nutraceutical Supplementation for Atherosclerosis Prevention

Nutraceuticals are classified into (i) dietary supplements: products intended to supplement the diet, which contain one or more dietary ingredients with well-known nutritional functions (i.e., vitamins, minerals, herbs, or herbal active compounds) and (ii) functional foods: consumed as part of a normal diet, they consist in whole foods, along with "fortified, enriched, or enhanced" foods supplemented with



Olive oil derivates	↓ expression of VCAM-1, ICAM-1 ↑ NO production	↓ PGE2, TNF-α and superoxide anion release		
n-3 PUFAs	↓ expression of VCAM-1, ICAM-1	↓ monocytesadhesion ↓ chemokine (MCP-1, CCL-20) production		↑ atherosclerotic plaque stability
Flavonoids	↓ oxidative stress and NF-κB activation ↑ eNOSlevel ↑ expression of SIRT-1	↓ expression of MMP-9, MCP-1, and TLR4 ↓ oxLDL uptake ↓ ROS formation ↓ foam cell formation	↓ VSMC migration, proliferation, and contraction ↓ oxLDL-induced calcification in VSMCs	
SCFAs	↓ expression of VCAM-1 ↓ activity of HDAC ↓ inflammasome activity	↓ proinflammatory cytokine (TNF-α, IL-2, IL-6 and IL-10) production	↓ neointima formation	
Vitamins	↓ apoptosis and permeability ↑ eNOSactivity, ET-1 secretion, and NO production	↓ LDL oxidation ↓ proinflammatory cytokine (TNF-α and IL IL-6) production	↑ synthesis of type IV collagen ↓ VSMC proliferation and migration	↓ atherosclerotic lesion formation

Figure 1: Beneficial effects of major nutraceuticals on atherogenesis key steps. Highlights of the main findings of *in vitro* and *in vivo* studies which have investigated the mechanisms underlying the benefits of the major nutraceuticals (olive oil derivates, n-3 PUFAs, flavonoids, SCFAs, and vitamins) at different stages of atherosclerosis development, including endothelial dysfunction, monocyte recruitment, foam cell formation, VSMC migration and proliferation, and plaque stability.

known biologically active compounds that, in addition to macro- and micronutrients, provide physiological benefits and are intended for reducing the risk of developing chronic diseases [19]. Some examples of functional foods are garlic, apples, or soybean as remedies for treatment or prevention of a number of diseases, but also vitamin D-fortified milk, useful for counteracting osteoporosis [20]. Lastly, functional food category includes yogurts for their content in probiotics [21], live microorganisms with positive impact on the host through a beneficial action on the intestinal tract, and prebiotics, organic substances, found in several vegetables and fruits, which selectively enhance the activity of some groups of bacteria [22].

This traditional definition of nutraceutical classes intended that both nutraceutical ingredients and functional foods derived preferentially from food products. However, the hodiern nutraceutical includes also non-food-derived active metabolites, which are safe and useful as novel sources for modern nutraceuticals and functional foods, such as medicinal plant-derived compounds [23], marine bioactive compounds [22], and amino acids derived from bacteria fermentation [24].

Several classes of nutraceuticals have been shown to have potential benefits in the treatment of atherosclerosis (reviewed in [25]) (Figure 1) and CVDs, and the ones for which strong evidence exists for atherosclerosis protection are described below and summarized in Table 1.

3.1. Olive Oil Derivates. Nutritional intake of olive oil, a key component of Mediterranean diet, has been associated with the prevention of CVDs, thanks to its content in monounsaturated fatty acids [26]. The 75% of fatty acids in olive oil is represented by monounsaturated oleic acid which counteracts endothelial dysfunction and reduces the tumor necrosis factor-alpha- (TNF-α-) induced apoptosis in VSMCs [27]. Olive oil is also a source of diverse phenolic compounds, such as hydroxytyrosol, oleocanthal, oleuropein, lignans, and pinoresinol, and numerous experimental, clinical, and epidemiological investigations support the beneficial properties of these olive derivates in CVDs [28, 29]. For instance, as for oleic acid, hydroxytyrosol reduces the expression of cell surface adhesion molecules (VCAM-1, ICAM-1, and E-selectin) in human umbilical vein endothelial cell line (HUVEC)[30]. Furthermore, hydroxytyrosol, as well as its derivates, inhibits the release of superoxide anions, prostaglandin E2 (PGE2), and TNF-α and the expression of cyclooxygenase 2 (COX2) in human monocytes [31]. Similarly, peracetylated hydroxytyrosol, a hydroxytyrosol derivate, attenuates lipopolysaccharide- (LPS-) induced proinflammatory cytokine production in murine peritoneal macrophages [32]. Interestingly, a diet rich in polyphenol powder, obtained from olive mill wastewater, enhanced antioxidant mechanisms and reduced oxidative stress-induced damage in chickens [33]. Moreover, *in vitro* treatment with hydroxytyrosol, or polyphenol extracts, from extra virgin olive oil,

Table 1: Summary of cardiovascular benefits of major nutraceuticals in human studies.

Nutraceutical	Study name	Study type	Number of participants/studies analyzed	Duration	Intervention	Summary of findings	References
Olive oil	NUTRAOLEUM	Clinical trial	58	5 months	30 mL/d of three virgin olive oils (VOOs): (1) a VOO (124 ppm of phenolic compounds and 86 ppm of triterpenes), (2) an optimized VOO (OVOO) (490 ppm of phenolic compounds and 86 ppm of triterpenes), and (3) a functional olive oil (FOO) high in phenolic compounds (487 ppm) and enriched with triterpenes (389 ppm)	Improved plasma HDL levels Decreased the level of systemic ET-1	[35]
	VOHF	Clinical trial	33	3 weeks	VOO (80 $mg \cdot kg^{-1}$), FVOO (500 $mg \cdot kg^{-1}$), and FVOO enriched with phenolic compounds from thyme FVOOT (500 $mg \cdot kg^{-1}$; 1 : 1)	Enhanced HDL content Increased endogenous antioxidant enzymes Reduced DNA oxidation level Increased fecal microbial metabolic activity Ameliorated endothelial function	[36, 37]
n-3 PUFAs	DART	Clinical trial	2.033	6 months	Advised to eat about 300 g/week of oily fish or fish oil supplements giving an equivalent amount of n-3 PUFAs	29% reduction in all-cause mortality	[311]
	GISSI-Prevenzione	Clinical trial	11.324	3.5 years	Supplements of n-3 PUFA (1 g/d), vitamin E (300 mg/d), both, or none	20% reduction for total deaths 30% reduction for cardiovascular death 45% reduction for sudden deaths	[52]
	JELIS	Clinical trial	18.645	5 years	EPA (1800 mg/d) with statin or statin	19% reduction in major coronary events	[51]
		Meta-analysis	7.951			Reduced overall mortality and sudden death Reduced mortality due to MI	[2]
		Meta-analysis	77.917			No significant associations with CHD events and death No significant associations with nonfatal MI	[56]
	Omega-FMD	Clinical trial	74	3 months	Supplements of n-3 PUFA (2 g/d) or placebo	No improvement of endothelial function indices	[57]
	ASCEND	Clinical trial	15,480	7.4 years	Supplements of n-3 PUFA (1 g/d) or placebo	No reduction in the rates of nonfatal serious adverse events	[58]

Table 1: Continued.

Nutraceutical	Study name	Study type	Number of participants/studies analyzed	Duration	Intervention	Summary of findings	References
	REDUCE-IT	Clinical trial	19.212	4.9 years	Supplements of icosapent ethyl (4 g/d) or placebo	25% reduction in primary composite cardiovascular endpoint 26% reduction in secondary composite cardiovascular endpoint	[60]
Flavonoids	Zutphen Elderly Study	Prospective cohort study	805	5 years	(-)-epicatechin (100 mg/d), quercetin-3-glucoside (160 mg/d), or placebo	Reduced risk of CHD mortality Reduced incidence of MI	[99]
	Rotterdam Study	Prospective cohort study	4807	5.6 years		reduced incidence of MI	[100]
	The Caerphilly study	Prospective cohort study	1900	14 years		No change in incidence of ischemic heart disease	[101]
	The Health Professionals Study	Prospective cohort study	45589	2 years		No association between tea consumption and CVD	[102]
	FLAVO	Clinical trial	37	4 weeks		Only (-)-epicatechin improved endothelial function and reduced inflammation	[103]
SCFAs		Umbrella meta-analysis	31 (meta-analysis)			7-24% reduction in CHD and stroke 17-28% reduction in overall morbidity and mortality	[108]
		Meta-analysis	752,848	12.4 years		23% reduction in CVD mortality	[109]
Vitamins	ASAP	Clinical trial	520	3 years	d-Alpha-tocopherol (182 mg/d), slow-release vitamin C (500 mg/d), both, or placebo	Delayed progression of atherosclerosis	[153, 154]
	Women's Health Study	Clinical trial	39.876	10.1 years	Natural-source vitamin E (600 IU) on alternate days	Reduced cardiovascular mortality in healthy women	[155]
	MRC/BHF	Clinical trial	20.536	5 years	Vitamin supplementation (vitamin E, 600 mg/d; vitamin C, 250 mg/d; β-carotene, 20 mg/d)	No significant reduction in the incidence of cardiovascular events and CVD-related mortality	[156]
	GISSI-Prevenzione	Clinical trial	11.324	3.5 years	Supplements of n-3 PUFA (1 g/d), vitamin E (300 mg/d), both, or none		[52]
	VEAPS	Clinical trial	353	3 years	DL alpha-tocopherol (400 IU/d) or placebo		[157]
	HOPE	Clinical trial	9.541	4.5 years	Natural-source vitamin E (400 IU/d) or placebo		[158]
	SU.VI.MAX	Clinical trial	1.162	7.2 ± 0.3 years	Combination of antioxidants (vitamin C, 120 mg/d; vitamin E, 30 mg/d; beta carotene, 6 mg/d; selenium, 100 μg/d; zinc 20 mg/d) or placebo		[159]

Table 1: Continued.

Nutraceutical	Study name	Study type	Number of participants/studies analyzed	Duration	Intervention	Summary of findings	References
		Meta-analysis	51 (trials)			No significant reduction in mortality and cardiovascular risk	[160]
		Meta-analysis	15.871			No significant reduction in mortality and cardiovascular risk	[161]
	CARET	Clinical trial	18.314	6 years	Beta-carotene (30 mg/d) and vitamin A (25000 IU/d) or placebo	26% increase of CVD-related mortality	[162]
	Meta-analysis	Meta-analysis	2,000,000			No prevention of heart attacks, strokes, or cardiovascular death Reduced risk of CHD incidence	[164]

protects against the endothelial dysfunction induced by hyperglycemia and free fatty acids through modulation of NO production and endothelin-1 (ET-1) expression [34]. These observations have been confirmed by the NUTRAOLEUM study, a randomized double-blind controlled trial, which supported the hypothesis of beneficial effects of virgin olive oils on biomarkers of endothelial dysfunction in healthy adults. This study demonstrated that administration of olive oil enriched in phenols, especially in hydroxytyrosol, other than improving plasma high-density lipoprotein (HDL) levels, ameliorates the systemic ET-1 levels [35]. These data mirror those obtained in two other clinical trials which tested the effect of polyphenol-enriched olive oils on HDL- and endothelial-related markers in hypercholesterolemic subjects. In these studies, these functional olive oils promoted cardioprotective effects, as indicated by increased levels of fat-soluble antioxidants and antioxidant enzymes, improved HDL subclass distribution, reduced the level of DNA oxidation, and ameliorated endothelial function [36, 37]. Very recently, it has been also demonstrated that hydroxytyrosol blunts endothelial dysfunction by ameliorating mitochondrial function and reducing mitochondrial oxidative stress [38], suggesting a potential mitochondria-targeting antioxidant activity of hydroxytyrosol in the inflamed endothelium.

3.2. N-3 Polyunsaturated Fatty Acids. Polyunsaturated fatty acids (PUFAs) are fatty acids that contain two or more bonds in their carbon chains. Depending on the position of the carbon-carbon double bond closest to the omega (methyl) end of the molecule, PUFAs can be classified in omega-6 and omega-3 series of fatty acids which, other than being vital constituents of cell membranes, constitute the substrates for synthesis of eicosanoids (i.e., prostaglandins, prostacyclins, thromboxanes, and leukotrienes), mediators of inflammatory response and regulators of blood pressure and coagulation [39]. In the end of the 80's, an examination of the composition of Eskimos' diet revealed an association between the low mortality rate due to cardiovascular events in this ethnic group and their diet rich in PUFAs derived from sea fish [40]. This observation prompted the researchers to investigate the biological functions of these compounds and their role in human health and pathology prevention. During the past 20 years, the research on the role of omega-3 PUFAs in CVD has flourished, with the majority of scientific research being focused on eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both displaying beneficial cardiovascular effects. In fact, behind the possible cardioprotective effect of these omega-3 fatty acids, other than the reduction of triglyceride (TG) levels [39], additional modes of actions have been proposed, which include hypotensive effect [41], thrombosis reduction [42], and also decrease in malignant arrhythmias [43]. Moreover, numerous *in vitro* and *in vivo* investigations have shown that omega-3 PUFAs are able to modulate diverse key steps involved in atherosclerotic plaque formation (reviewed in [44]). Specifically, omega-3 PUFAs exert potent anti-inflammatory properties in polarized macrophages [45, 46] and are able to inhibit the expression of adhesion molecules by ECs [39, 47], thus decreasing leukocyte infiltration into the vascular wall [48]. In $ApoE^{-/-}$ mice, omega-3 PUFA treatments significantly attenuated the development and destabilization of atherosclerotic plaques [49]. In diabetic mice, supplementation with ω-3 fatty acids extracted from microalgae decreased the percentage of T lymphocyte $CD4^{+}$-producing proinflammatory cytokines [45].

Several clinical trials have been conducted to assess the cardiovascular benefits of EPA and DHA treatments (i.e., diet and reinfarction trial (DART) [50], Japan EPA Liquid Intervention Study (JELIS) [51], and Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto (GISSI) [52]). In 2002, a meta-analysis of eleven randomized clinical trials revealed that ω-3 PUFA supplementation reduced overall mortality, mortality due to myocardial infarction, and sudden death in patients with coronary heart disease (CHD) [53]. In the same year, the American Heart Association (AHA) recommended doses of total EPA and DHA between 2 and 4 g/day, to achieve a TG level reduction by 25-30% in normal and hyperlipidemic individuals [54]. After the publication of these guidelines, a large number of observational studies on omega-3 PUFA intake and CHD risk were conducted. In 2017, in an updated scientific advisory of AHA, the authors confirmed omega-3 fish oil supplements, in consultation with a physician, as a secondary—and not primary—prevention of sudden cardiac death in patients with prevalent CHD and in patients with heart failure [55]. In contrast, a recent meta-analysis of 10 trials involving 77917 individuals did not provide support for the AHA recommendations in individuals with a history of CHD for the prevention of fatal CHD, nonfatal MI, or any other vascular events [56]. Consistently, in a recent clinical trial, three months of treatment with PUFAs at a dose of 2 g/die did not improve endothelial function in patients with type 2 diabetes and high cardiovascular risk [57]. Similarly, in the ASCEND study, after a follow-up of 7.4 years, patients with diabetes and no evidence of cardiovascular disease, who received a daily supplement of ω-3 fatty acids, did not show a significantly lower incidence of serious vascular events than those who received placebo [58]. Despite the evidence from these last randomized trials that omega-3 has little or no effect on cardiovascular outcomes, clinical guidelines still recommend the use of omega-3 fatty acid supplements for the secondary prevention of CHD [55] and consider the beneficial effect of consuming fish and seafood, for their content in omega-3 PUFAs [59]. To date omega-3 PUFA effects on cardiovascular endpoints remain still unclear and might vary based on different types/doses of dietary omega-3 intakes and the presence of other medications (i.e., statins) in the clinical trials performed so far. Consistently, the recent results of the REDUCE-IT trial, which involved 19212 patients with elevated triglyceride levels at risk for ischemic events, showed that treatment with 4 g/die of EPA, a dose twice and 4-times higher compared to the dose tested in the Omega-FMD study [57] and ASCEND study [58], respectively, resulted in decreased risk of primary and secondary composite cardiovascular end points (i.e., cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization, or unstable angina) of 25 and 26%, respectively [60]. Several ongoing large randomized clinical trials (i.e., EVAPORATE [61], VITamin D and OmegA-3 TriaL, VITAL [62], STatin Residual Risk

Reduction With EpaNova in HiGh CV Risk PatienTs With Hypertriglyceridemia, and STRENGTH (ClinicalTrials.gov Identifier NCT02104817)) will shed more light on the possible associations between omega-3 supplementation and reduction of risk of major cardiovascular events.

3.3. Flavonoids. Flavonoids, antioxidants present in fruit and vegetables, represent the most abundant polyphenolic constituents in foods of plant origin [63]. It has been estimated that the daily dietary intake of flavonoids is at least 1 gram per person and thus it is higher than that of all other known dietary antioxidants (e.g., vitamins C and E intake from food are estimated less than 100 mg/day) [64, 65]. Flavonoids present a 15-carbon (C6-C3-C6) skeleton, which generally consists of two phenyl rings and a heterocyclic ring. Approximately 8000 flavonoids have been identified based on C3 structure variations and degree of oxidation, and these include flavonols, flavones, isoflavones, flavanones, flavan-3-ols, and anthocyanidins [66]. In the context of atherosclerosis, flavonoids exert various beneficial effects, such as improvement of endothelial function and reduced oxidative stress and inflammation, and also antiplatelet, antihypertensive, and vasodilatory actions, inhibition of cholesterol synthesis, alteration of HDL function, and increased insulin sensitivity (reviewed in [67–69]). Flavanols are the subclass of flavonoids [70, 71] which have attracted most of the attention in the cardiovascular research community, since numerous epidemiological and mechanistic studies have supported the role of flavanols, particularly catechins, contained in cocoa and green tea, in counteracting endothelial dysfunction and atherosclerosis development [63, 72–76]. The mechanisms underlying the cardiovascular protective effects of catechins may include the inhibition of endothelial cell apoptosis by decreasing oxidative stress and ameliorating mitochondrial injury [77] and attenuation of proliferation of VSMCs by regulating anti-inflammatory and antioxidative enzyme heme oxygenase-1 (HO-1) [78]. In macrophage cell lines, catechins also mediate suppression of expression of metalloproteinase-9 (MMP-9), monocyte chemoattractant protein-1 MCP-1, and Toll-like receptor 4 (TLR4), which have a major role in atherosclerosis [79]. Moreover, in macrophages, epigallocatechin-3-gallate (EGCG) suppresses oxLDL uptake and foam cell formation [80]. In $ApoE^{-/-}$ mice, catechin consumption has been associated with reduced susceptibility of LDL to oxidation and aggregation and thus to the reduced atherosclerotic lesion area [81]. Recently, *in vivo* findings show that catechins reduce circulating LDL cholesterol and protect HUVECs against oxidative injury and decrease arterial vasoconstriction through reduction of H_2O_2 activity and eNOS level restoration [82, 83].

Similarly to flavanols, flavonols, and primarily quercetin, reduce atherosclerosis lesion area *in vivo* by affecting the oxidative stress status [84, 85]. The mechanism underlying this protection, which resembles those of flavanols, may involve attenuation of endothelial dysfunction [86], induction of HO-1 [87] and sirtuin-1 (SIRT-1) [88], and reduction of NF-κB activity [89]. It has been also suggested that quercetin may also inhibit VSMC migration, proliferation, and contraction [90, 91] and oxLDL-induced calcification in VSMCs, by targeting the ROS/TLR4 signaling pathway [92]. Recently, it has been also demonstrated that quercetin inhibits ox-LDL-induced ROS formation in mouse peritoneal macrophages by limiting the activation of NADPH-oxidase, which in turn may lead to the observed attenuation of high-fat diet-induced atherosclerosis in $ApoE^{-/-}$ mice [93, 94]. Additionally, quercetin reduces ROS levels in aortas of hypertensive rats by interfering with MMP-2 activity, thus limiting vascular remodeling [95]. Similarly, quercetin metabolites, like quercetin-3-glucuronide, generally found in onions, broccoli, and apples, have been shown to exhibit antioxidant, anti-inflammatory, and also antihyperglycemic properties both *in vitro* and *in vivo* [96–98].

The Zutphen Elderly Study was the first investigation in 805 men (aged 65-84 years, followed up for 5 years) which assessed the inverse correlation between flavonoid intake from tea, with a high content in quercetin, and the mortality from CHD, together with the incidence of MI [99]. Such correlation was confirmed by the Rotterdam Study, in which it was found that increased intake of tea and flavonoids may contribute to the primary prevention of ischemic heart disease [100]. Conversely, the protective effect of consumption of quercetin-rich tea, against ischemic heart disease, was not confirmed in other studies, such as the Caerphilly Study of Welsh men [101] and the Health Professionals Follow-Up Study [102]. Recently, the FLAVO study, a randomized, double-blind, placebo-controlled crossover trial performed in 37 (pre)hypertensive men and women (40-80 years), compared the cardioprotective effects of quercetin and epicatechin supplementation, both exerting similar *in vitro* and *in vivo* atheroprotective functions. The study has shown that, unlike quercetin-3-glucoside, supplementation of pure epicatechin improves endothelial function and reduces inflammation, thus reducing CVD risk factors [103].

Together, these data suggest that flavonoids, especially quercetin and catechins, might exert cardiovascular health benefits but larger trials are needed to draw conclusive evidence for the cardiovascular protective effects of these natural antioxidants, in the presence and absence of commonly used therapies for CVD.

3.4. Short-Chain Fatty Acids. In 1954, Walker and Ardvidsson [104] and Higginson and Pepler [105] were the first to suggest that low plasma lipid levels and the absence of atherosclerosis in primitive African populations were mostly due to their fiber-rich diet. In the 70's, this hypothesis was formalized by Burkitt et al. [106] and Trowell [107], proposing that the interaction among the dietary components can disturb the course of atherosclerosis onset, pointing out at the pivotal role played by fibers in modulating the serum lipid level. Since then, diverse epidemiological studies have reported an inverse relationship between fiber consumption and cardiovascular risk and that the beneficial effects of fibers would primarily reside in an improvement of the serum lipid profile (reduced total serum and LDL cholesterol levels) and reduction in blood pressure and systemic inflammation. In a recent umbrella review of all published meta-analyses (from January 1, 1980 to January 31, 2017), it emerged that dietary fibers can reduce

chances of developing CHD and stroke between 7% to 24% and the overall morbidity and mortality caused by cardiovascular disease from 17% to 28% [108]. These observations mirror those described in another systematic meta-analysis review, performed in 2014, which investigated the association of fiber consumption and all-cause and cause-specific mortality in 42 cohorts (from 25 studies). This analysis showed a 23% reduction of the mortality rate for CVDs and a concomitant 17% decrease for cancer-related death and a 23% reduction for all-cause mortalities [109]. Based on these scientific evidences, the Food and Drug Administration (FDA) strongly recommends consumption of fiber-rich food for health promotion and disease prevention, suggesting that an adequate intake of dietary fibers should be at least 25 grams/day for a 2000-calorie diet (https://www.accessdata.fda.gov/scripts/InteractiveNutritionFactsLabel/factsheets/Dietary_Fiber.pdf).

The beneficial effects on lowering total serum cholesterol are attributed to three major mechanisms: (i) prevention of bile salt reabsorption from the small intestine which leads to increased fecal excretion of bile acids, (ii) reduced glycemic index and insulin resistance, which can result in the inhibition of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase activity and hepatic cholesterol synthesis [110, 111], and (iii) production, from anaerobic bacterial fermentation of undigestible fibers, of short chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate [112]. SCFAs inhibit the production of proinflammatory cytokines and the recruitment of immune cells to ECs, mechanisms mediated by binding to the free fatty acid (FFA) receptor types 2 and 3 and G-protein-coupled receptor 109A (GPR109A) and by inhibiting intracellular activity of histone deacetylases (HDACs), enzymes which can regulate multiple molecular processes involved in atherogenesis [97, 113–115]. Recently, it has emerged that SCFAs inhibit LPS- and TNF-α-induced endothelial expression of VCAM-1, through activation of GPR41/43, which are SCFA receptors expressed in ECs, and inhibition of HDAC activity [116, 117]. The anti-inflammatory activity of SCFAs has been also evaluated *in vivo*: in a partially ligated carotid artery (PLCA) mouse model of atherosclerosis, it has been found that butyrate repressed endothelial Nlrp3 (Nlr family pyrin domain-containing 3) inflammasome activation in ECs, via redox signaling pathways, and prevented arterial neointima formation. On the contrary, acetate and propionate exerted minimal inhibitory effects on inflammasome activation and even seem to augment the arterial neointima formation in the PLCA model [117]. The antiatherosclerotic activity of SCFAs was also confirmed by a recent study by Chen and collaborators showing that SCFAs from pectin fermentation are able to inhibit intestinal cholesterol absorption, to decrease serum total and low-density lipoprotein cholesterol and to protect ApoE$^{-/-}$ against diet-induced atherosclerosis [118]. Also, in this case, the authors showed that the beneficial effects of SCFAs are mediated only by butyrate through a mechanism which involves increased expression, in the small intestinal mucosa, of ATP-binding cassette (ABC) transporters G5 (*Abcg5*) and G8 (*Abcg8*), which limit intestinal absorption and facilitate biliary secretion of cholesterol [118, 119].

3.5. Vitamins. An adequate intake of vitamins, from food or dietary supplements, is commonly considered as indispensable for maintenance of good health, and thus, since their discovery in the early 1900s, vitamins have been considered the most promising nutraceuticals for the prevention of diverse pathologies, including atherosclerosis-derived CVDs. In this context, vitamins C and E have received the most attention since, according to the "oxidation hypothesis" of atherosclerosis onset [120, 121], antioxidant organic compounds, like these two vitamins, could represent the first line of defense against LDL oxidation, the first step in the formation of atherosclerotic plaques. Moreover, during the last two decades, other antiatherogenic mechanisms of action have been ascribed to vitamins C and E (reviewed in [25, 122, 123]), such as stimulation of endothelial cell proliferation, thanks to their ability to increase the synthesis of type IV collagen, reduction of apoptosis induced by high glucose conditions, LPS or TNF-α [122, 123], enhancement of eNOS activity by stabilization of the eNOS cofactor tetrahydrobiopterin (BH4) [124], and tightening of the permeability barrier of ECs [124]. Similarly, for vitamin E, the antiatherosclerotic action has been found, which is independent from its antioxidant properties, such as reduced cholesterol synthesis by inhibiting HMG-CoA reductase, increased eNOS activity, reduced NF-κB-dependent synthesis of ICAM-1 and VCAM-1, reduced platelet aggregation, and inhibition of VSMC proliferation [123, 125].

These *in vitro* findings were confirmed by studies in animal models of atherosclerosis (as reviewed in [126]). For instance, vitamin E, in an age-dependent manner, is able to inhibit atherosclerosis in ApoE$^{-/-}$ mice by decreasing serum oxLDL and vasculature mRNA expression of genes involved in cholesterol transportation [127]. Similarly, α-tocopheryl phosphate (a natural form of vitamin E) limits atherosclerosis lesions in hypercholesterolemic rabbits [128] and in ApoE$^{-/-}$ mice by limiting aortic superoxide formation and by reducing circulating plasma levels of proinflammatory markers [129], Similarly, vitamin E deficiency, caused by disruption of the α-tocopherol transfer protein gene, increased the severity of atherosclerotic lesions [130].

Likewise, vitamin C limits endothelial dysfunction in animal models of atherosclerosis: long-term supplementation (26/28 weeks) of vitamin C restored eNOS activity in the aorta of ApoE$^{-/-}$ mice [131], whereas chronic hypoascorbemia has been associated to an elevated lipoprotein(a) (Lp(a)) accumulation in the vasculature and increased atherosclerotic lesion development in gulonolactone-oxidase-deficient (Gulo$^{-/-}$)/Lp(a)$^{+}$ mice, a model lacking of endogenous ascorbate synthesis and expressing human Lp(a) [132].

In vivo studies have investigated the antiatherosclerotic effect of a combined vitamin C and vitamin E supplementation in mouse models of this pathology: vitamin C/vitamin E cocktail inhibited the development of fatty streak lesions in the LDLr$^{-/-}$ mice [133] and limited aortic *Vegf* and *Vegfr-2* expression in ApoE$^{-/-}$ mice [134] compared to nontreated littermates. In a very recent study, in atherogenic diet-fed (scavenger receptor class B type 1) SR-B1 KO/ApoER61$^{h/h}$ mice, a murine model of dyslipidemia, progressive atherosclerosis, CHD and premature ischemic death,

combined with administration of vitamins C and E reduced serum total cholesterol and triglyceride levels, improved HDL antioxidant function, and lowered serum TNF-α levels [135].

Other than vitamins C and E, vitamins A and D show potential antiatherosclerotic properties (as reviewed by [136]). The wide range of vitamin D beneficial functions includes reduction of endothelial dysfunction and VSMC proliferation and migration, as well as downregulation of the atherosclerosis-related inflammatory and immune processes [136]. Less is known about vitamin A (retinol) in this context: retinoic acid (RA) metabolites, derivatives of vitamin A, limit VSMC growth, differentiation, and proliferation [137] and prevent high-fat diet- (HFD-) induced atherogenesis in ApoE$^{-/-}$ mice via the upregulation of aforementioned transporters ABC-A1 and ABC-G1 [138]. Peculiarly, in the same animal model, vitamin A deficiency stimulates atherogenesis, prevented by β-carotene supplementation [139]. All-trans retinoic acid (ATRA), a derivative of vitamin A, has been recently shown to reduce the plaque size in a rabbit model of HFD-induced atherosclerosis by inhibiting platelet aggregation, by decreasing caveolin-1 expression and ET-1 secretion and by enhancing eNOS activity and NO formation [140–143].

Despite the promising *in vitro* and *in vivo* findings supporting the antiatherosclerotic properties of vitamins and their metabolites, at the clinical level, the results obtained have been contradictory: albeit the vast majority of the literature have correlated a low level of the above described vitamins with early atherosclerosis onset, major risk of a CVD events, and heart failure in human [144–152], it is undeniable that the various clinical trials, performed so far, lack consistency. For instance, a few studies have evidenced that supplementation of vitamins C and E, alone or in combination, may delay the progression of atherosclerosis [153, 154] and reduce cardiovascular mortality in healthy women [155], whereas many other trials, like the MRC/BHF Heart Protection Study Heart Protection Study Collaborative Group [156], GISSI-Prevenzione [52], VEAPS [157], HOPE [158], and Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) [159], reported that these vitamins do not produce any significant difference in the incidence of cardiovascular events and CVD-related mortality. Contextually, in 2011, a systematic review of 51 trials showed no significant reduction in mortality and cardiovascular risk associated with vitamin D supplementation [160], having conclusions mirroring those obtained from vitamin C-centered meta-analysis performed in 2016 [161]. It is expected that new insights will arise from the ongoing randomized, double-blind, placebo-controlled VITAL trial, which will evaluate the long-term effects of high-dose vitamin D supplementation on CVD events in 25874 U.S. adults [62].

Only a few trials have been performed to assess the effects of vitamin A on CVDs. In 1996, the beta-carotene and retinol efficacy trial (CARET) tested the effect of beta carotene and vitamin A supplementation on the incidence of lung cancer and cardiovascular death in 18314 high-risk participants, specifically smokers and workers exposed to asbestos [162]. This trial was stopped because participants randomly assigned to vitamin supplementation, compared to the placebo group, exhibited a 28% increase in incidence of lung cancer and a 17% increase of overall mortality rate and a higher rate (26%) of cardiovascular disease mortality, which decreased during the 6-year follow-up after the vitamin integration was stopped [162, 163].

A recent meta-analysis of 18 studies, which involved a total of 2 million participants, concluded that taking multivitamins does not prevent heart attacks, strokes, or cardiovascular death, even though it seems to be associated with a lower risk of CHD incidence [164]. In the absence of studies showing their efficacy in primary prevention of CVDs, the AHA does not recommend vitamin supplementation for healthy subjects, and similarly, the U.S. Preventive Services Task Force (USPSTF) states that the current evidence is insufficient (I) to assess the balance of benefits and harms of the use of multivitamins for the prevention of cardiovascular disease or cancer (I statement). The USPSTF also discourages (D) the use of β-carotene or vitamin E supplements for the prevention of cardiovascular disease or cancer (D recommendation) [165].

3.6. Other Emerging Antiatherogenic Nutraceuticals

3.6.1. Berberine. Berberine (BBR), a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids (5,6-dihydrodibenzoquinolizinium derivative) found in *Berberis* species plants (Berberidaceae), exhibits many different types of biological activities, including its effectiveness in lipid disorders and hyperglycemia [166]. The poor intestinal absorption and bioavailability of BBR are the main drawback when orally administered even though its metabolites maintain higher concentration in plasma, behaving like the pharmacologically active forms of BBR; its main metabolite berberrubine (M1) tautomerizes to a highly conjugated, electroneutral quinoid structure [167] reaching a high plasma concentration as a consequence of a more efficient intestinal absorption [167].

Several preclinical studies as well as clinical trials suggest a beneficial role of BBR in endothelial dysfunction and dyslipidemia [166]; in ECs, BBR attenuates LDL oxidation induced by ROS and reduces apoptosis modulation, chromosome condensation, cytochrome c release, and caspase-3 activation. It has also been reported that BBR reversed NOX4-derived ROS production in HUVECs, at least in part due to the regulation of adenosine monophosphate-activated protein kinase (AMPK) activation. In both cultured endothelial cells and blood vessels isolated from rat aorta, BBR enhanced eNOS and promoted a glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) hyperactivation in the liver of mice, attenuating H_2O_2-induced ROS [168]. BBR elevates LDL receptor (LDLR) expression in human liver cells through inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) transcription, an enzyme that posttranscriptionally upregulates LDLR [169]. In rat liver, a combination of BBR with simvastatin increased the LDLR gene expression to a level significantly higher in comparison to monotherapies [166]. In human macrophage-derived foam cells treated with oxLDL, BBR inhibits the expression

of LOX-1 as well as the oxLDL uptake by macrophages and reduces foam cell formation in a dose-dependent manner by activating the AMPK-SIRT1-PPARγ pathway [170]. We recently reported that BBR prevents the oxLDL- and TNF-α-induced LOX1 expression and oxidative stress in HUVECs, key events leading to NOX, MAPK/Erk1/2, and NF-κB activation linked to endothelial dysfunction [171], and consistently, Abidi and colleagues had previously shown, both *in vitro* and *in vivo*, that BBR reduces VCAM-1 expression induced by LPS [172]. We recently observed that M1 inhibited intracellular xanthine oxidase activity, one of the major sources of ROS in vasculature, and reduced the expression ICAM-1 [173]. The lipid-lowering activity of BBR, alone or in association with other nutraceuticals, has been clearly confirmed in a relatively large number of randomized clinical trials which support the safety of a short-term use of this nutraceutical, especially when used at a lipid-lowering dose [166].

3.6.2. Carotenoids. Carotenoids represent a group of pigments widely distributed in nature. They contribute to the red, orange, and yellow colors found in many flowers, fruits, and vegetables, where they act as photoprotectors and as attractant for insects and animals for pollination and seed dispersal. Since animals and humans are unable to synthesize carotenoids de novo, carotenoids are essential nutrients and important health beneficial compounds [174, 175]. Carotenoid consumption improves the metabolic profile, decreasing the incidence of diabetes, lowers LDL levels, and improves blood pressure by ameliorating the bioavailability of NO [175]. Apart from the well-established function of carotenoids as provitamin A, some carotenoids, such as lycopene and astaxanthin, are strong antioxidants and have a protective function in reducing the risk of both cancer and cardiovascular diseases [175, 176]. Xanthophylls, such as lutein and zeaxanthin, are essential components of the macular pigments in the eyes and offer protection against macular degeneration, the leading cause of age-related blindness [175, 177, 178]. Lutein exhibits strong antioxidant properties *in vitro* and *in vivo* [178], and low serum levels of lutein have been associated both with increased values of common carotid intimal medial thickness (CCA-IMT) and myocardial infarction [179]. An 8-year follow-up study, performed on 43.738 men with no history of cardiovascular disease or diabetes, showed a significant inverse correlation between lutein intake and risk for ischemic stroke [179]. A case control study found that the risk for MI was inversely correlated with adipose tissue lutein content and inversely proportional to dietary lutein intake [178].

Due to their antioxidant activity, lycopene, lutein, zeaxanthin, and astaxanthin are able to attenuate the atherosclerotic process. Lycopene, a fat-soluble carotenoid without provitamin A activity, is the pigment responsible for the distinctive red color in tomatoes and watermelon, and it is a powerful antioxidant and free radical quencher [180]. High plasma lycopene levels have been associated with reduction in aortic stiffness in patients with metabolic syndrome [181]. Conversely, low plasma levels of lycopene were associated with increased risk of atherosclerotic lesions and with an increased risk of acute coronary events or stroke [179]. Similarly, in a case control study performed in patients suffering from heart failure (NYHA class II-III), the left ventricular ejection fraction was significantly and positively correlated with plasma lycopene levels: NYHA class II patients showed significantly higher levels of lycopene than class III patients [179]. High lycopene consumption has been associated with a decreased risk of CVD, including atherosclerosis, myocardial infarction, and stroke. In a study performed on healthy male volunteers, lycopene supplementation improved the endothelial function, together with a significant decrease in serum levels of CRP, ICAM-1, and VCAM-1 and an improvement in the atherosclerotic risk factors (lipid profile and systolic blood pressure level) [181]. A meta-analysis using a random effects model of all studies between 1955 and September 2010 investigating the effect of lycopene on blood lipids or blood pressure for a minimum duration of 2 weeks suggests that lycopene taken in doses ≥ 25 mg daily is effective in reducing LDL cholesterol by about 10% which is comparable to the effect of low doses of statins in patients with slightly elevated cholesterol levels [180].

Ketocarotenoid astaxanthin is the main carotenoid present in aquatic animals (salmon, trout, red seabream, shrimp, lobster, and fish eggs), contributing to the pinkish-red color of their flesh, and also in some birds (flamingoes and quails in particular) [179]. Astaxanthin exhibits a free radical-quenching potency that is, on an equimolar basis, double than the potency of β-carotene [178], about 100-fold greater than the antioxidant potency of α-tocopherol [178], and approximately 6000 times the potency of ascorbic acid [178]. Astaxanthin demonstrated to exert beneficial effects on the heart, both by reducing inflammation and by modifying blood levels of LDL-C and HDL-C; moreover, it reduces macrophage infiltration and apoptosis in vascular lesions, thus improving plaque stability by increasing adiponectin [179]. Astaxanthin inhibits also the production of oxLDL [178] and their uptake by activated intravascular macrophages [178] and inhibits the release of atherogenic ROS, NO, and proinflammatory cytokines [178]. 8 weeks of a dietary supplementation with 2 mg of astaxanthin daily by a group of healthy postmenopausal women produced a significantly greater increase in total plasma antioxidant machinery than what was elicited by placebo and a significantly greater decrease in the plasma concentration of thiobarbituric acid-reactive substances (the mixed reaction products of nonenzymatic oxidative lipid peroxidation) [178]. Dietary astaxanthin also contributes to healthy blood flow through the vasculature by promoting aortic and coronary artery vasodilation and increases the flexibility of red blood cell membranes (with an acceleration of red blood cell flow through the blood vessels) [178].

3.6.3. Red Yeast Rice. Red yeast rice (RYR) is a Chinese herbal supplement produced by fermenting white rice with the yeast, *Monascus purpureus*, used to flavour, color, and preserve foods and as a traditional medicine for many years. RYR contains a variety of monacolins, which inhibit HMG-CoA reductase, the rate-limiting step in cholesterol synthesis.

Approximately 90% of the total monacolin content of RYR consists of monacolin K, chemically identical to lovastatin, and its hydroxy acid form, monacolin KA [182]. Other active ingredients with the potential to lower cholesterol in commercially available RYR products include plant sterols (β-sitosterol, campesterol, and stigmasterol), isoflavones, and monounsaturated fatty acids [183]. The first prospective, double-blind, placebo-controlled study evaluating RYR in an American population was conducted by Heber et al. in 1999 in eighty-three healthy adults with untreated hyperlipidemia that followed the AHA cardioprotective diet (less than 10% of calories from saturated fat and less than 300 mg from cholesterol per day). They were randomly assigned to receive 2.4 g per day of RYR or placebo, for 12 weeks. Compared to baseline, LDL-C levels decreased by 22% in the RYR-treated group [184]. Other clinical trials have found that a relatively small dose of RYR (equivalent to a daily lovastatin dose of 5 to 7 mg) is as effective as 20 to 40 mg of pure lovastatin in lowering cholesterol [185]. Becker et al. compared the efficacy of an alternative treatment composed by RYR, fish oil, and therapeutic lifestyle changes with simvastatin 40 mg per day in 74 primary prevention patients with known or newly diagnosed hypercholesterolemia [186]. Depending on baseline LDL-C, patients took 1200 mg of RYR (10 mg lovastatin) or 1800 mg (15 mg lovastatin) twice a day, for 12 weeks. At the end of the study, both groups had a similar reduction in LDL-C and no significant differences were found between the two groups. Interestingly, participants in the RYR-fish oil and life style change group lost more weight during the study (-4.7 ± 2.4 kg vs -0.3 ± 2.2 kg) and had a significant reduction in triglycerides compared with the simvastatin group. Li et al. published a large meta-analysis, which examined the effectiveness and safety of RYR as an alternative approach for treating dyslipidemia. Thirteen randomized, placebo-controlled trials were included (from 1999 to 2013) with treatment duration of 4 weeks to 6 months and no serious side effects were reported. Overall, RYR significantly lowered total and LDL-C levels ($P < 0.001$) compared with placebo and this effect did not appear to be related to the dose, duration of therapy, or geographic location [187]. In another small observational study, 25 dyslipidemic patients with a history of intolerance to lipid-lowering medications were treated with RYR for more than 4 weeks. In accordance with other studies, RYR significantly lowered LDL-C by 21% in this clinical population during the period of treatment [188]. The China coronary secondary prevention study (CCSPS) so far is the only randomized, double-blinded, placebo-controlled, multicentered study demonstrating that monacolin K reduces cardiovascular risk [189]. This trial recruited 4870 Chinese patients with a history of MI and moderate hypercholesterolemia. Patients were randomized to receive twice-daily treatment of a capsule of Xuezhikang (XZK), containing 2.5 to 3.2 mg of monacolin K equivalent to a total daily lovastatin dose of 10 to 12.8 mg or placebo. After 4.5 years, XZK was associated with a highly significant reduction in frequency of coronary events (10.4% in the placebo vs 5.7% in the XZK group) and a relative risk reduction of 45% [189]. Treatment with XZK also significantly decreased total mortality by 33%, cardiovascular deaths by 30%, and the need for coronary revascularization by 33%. Total cholesterol and LDL-C levels decreased by 13 and 20%, respectively, compared to baseline. Adverse effects were similar in both groups, and the XZK appeared to be well tolerated. A substudy of elderly hypertensive patients in the same CCSPS cohort found that monacolin K was effective in lowering the rates of coronary events and death from CHD compared with placebo [190].

Several clinical trials have shown RYR to be safe, effective, and well tolerated both alone or in combination with other nutraceuticals; however, the studies are small and of short duration [191]. Even if RYR is perceived as a "natural" product providing fewer side effects, it should be taken into account that monacolin K is identical to lovastatin and therefore may present an increased risk of muscular and other side effects especially in patients with a history of SAM. Myopathy, hepatoxicity, and rhabdomyolysis have all been reported in patients taking RYR, as one would expect from any statin therapy [192]. For this reason, RYR should be taken under the guidance of a physician who will closely monitor its efficacy, safety, and tolerability [193]. In the USA, RYR has been used as an alternative to statin therapy in treating patients with mild to moderate hypercholesterolemia, especially among patients who might be intolerant to standard therapy due to statin-associated myalgia (SAM). For this reason, the FDA has prohibited the sale of all RYR products containing monacolin K, because it is considered an unapproved drug; however, many RYR supplements are still on the market.

3.6.4. Allicin. Garlic (*Allium sativum*) has been used as a spice, food, and medicine for over 5000 years and is one of the earliest documented herbs utilized for the maintenance of health (as a diuretic and for the immune system and gastrointestinal health) and for treatment of disease, including circulatory disorders and infections [194]. Functional sulfur-containing components described in garlic include alliin, allicin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, and S-allylcysteine. Allicin is a thiosulfinate and in nature is produced after damage of the plant tissue by an enzymatic reaction [195].

Aged garlic extract in cell culture prevented endothelial cell dysfunctions caused by oxidative stress by increasing cellular concentrations of thiol antioxidants, such as cysteine and glutathione (GSH) [196]. A correlation between low red blood cell glutathione (GSH), which plays important roles in cellular redox status and signaling, and increased plasma homocysteine (HCy) has been linked to an increased incidence of hypertension [197]; in an animal model of hyperhomocysteinemia, induced by a severely folate-depleted diet in rats, aged garlic extract decreased plasma HCy concentrations by 30% [198]. The potential effect of garlic on HCy levels has been reported in a small clinical trial of atherosclerosis patients randomized to aged garlic extract ($P = 0.08$) [199]. Garlic has been also shown to have blood pressure- (BP-) lowering properties in hypertensive patients [200]: allicin, decomposes rapidly to its degradation products which results in the release of hydrogen sulfide (H_2S) [201], a potent gaseous signaling molecule which lowers blood pressure (BP) by the relaxation of smooth muscle cells

surrounding the blood vessel [194]. The H_2S-dependent BP-reducing effect is thought to be primarily mediated through sulfhydration of ATP-sensitive potassium (K_{ATP}) channels, which in turn leads to voltage-sensitive channel opening and relaxation of vascular smooth muscle cells [202]. However, other potassium channels may also be affected by H_2S and additional mechanisms have been suggested in determining the opening/closing of K^+ channels, including a possible cooperation between H_2S and NO [202]. In CVD models, the administration of H_2S prevents myocardial injury and dysfunction [203] and aged garlic extract was shown to normalize NO output from endothelial cells by preventing the decline of BH4 levels [194]. In addition, garlic, due to the high content of polysulfides, may help in providing the nutrients needed for maintaining optimum redox balances for several eNOS-dependent signaling pathways important in vascular relaxation [194]. Additionally, allicin is able to suppress cholesterol biosynthesis [204–206] and platelet aggregation. So far, five trials have demonstrated a strong effect of garlic on inhibition of platelet aggregation, whereas one trial reported no effect [207].

3.6.5. Curcuminoids. Curcuminoids, extracted from the rhizomes of *Curcuma longa*, are naturally occurring polyphenols used for centuries in indigenous medicine to treat various diseases, such as common colds, arthritis, diarrhea, and upper respiratory disorders. The curcuminoids best characterized are curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC), all belonging to the diarylheptanoid family [208]. A lot of evidence suggests that curcuminoids have a diverse range of molecular targets; curcumin, the main component of curcuma extract, has several biological and pharmacological properties including anti-inflammatory, antioxidant, antithrombotic, antiatherosclerotic, anticonvulsant, and anticancer properties and cardio- and neuroprotective activities (reviewed in [209]).

Curcuminoid treatment improved glycemic factors, hepatic function, and serum cortisol levels in subjects with overweight and impaired fasting glucose in a randomized double-blind placebo-controlled trial involving 80 overweight subjects [210]. Among curcuminoids, curcumin is the best characterized and studied; its antioxidant and anti-inflammatory properties are therefore considered a multifunction phytochemical that can interact with multiple molecular targets, modulating cell growth, inflammation, and apoptosis signaling pathways (reviewed in [211]). Firstly studied for its beneficial properties in the gastrointestinal tract, it has been observed that curcumin has a beneficial role in chronic conditions such as intestinal dysmotility disorders and in the prevention and maintenance of remission of intestinal bowel disease (IBD), requiring long-time treatment [212]. Moreover, curcumin seems to show protective properties from metabolic syndrome, decreasing insulin resistance, obesity, hypertriglyceridemia, and hypertension and preventive properties from complications. It has been evidenced that curcumin possesses hypolipidemic effects, which together with its antioxidant and anti-inflammatory activities can contribute to reducing the incidence of atherosclerosis [213]. The remarkable antioxidant capacity of curcumin reduces lipid peroxidation and the generation of oxLDL and, consequently, reduces the inflammatory response and the progression of atherosclerosis [213].

Recently, it has been observed that curcumin can inhibit hypoxia-inducible factor 1α (HIF-1α) thus repressing the total cholesterol and lipid level in macrophage under hypoxic condition [214]. The benefit of curcumin in patients at risk of atherosclerosis has also been described; after 6 months of curcumin dietary supplementation, patients with type 2 diabetes had lower pulse wave velocity which improved the metabolic profile [215]. Furthermore, the use of curcumin for 8 weeks improved flow-mediated dilatation in 32 postmenopausal women [216]. A major limitation to using curcuminoids as a nutraceutical is its poor bioavailability, owing to inadequate absorption in the gut and as it is rapidly broken down and quickly excreted from the body. Several strategies are being pursued in an attempt to increase their bioavailability, including the use of liposomal curcumin, nanoparticles, and a curcumin phospholipid complex.

4. Notch Signaling Modulation by a Nutraceutical Approach

The Notch signaling is a highly conserved short-intercellular communication system deeply investigated for the possible role as a novel therapeutic target in cancer [217], which is becoming more and more recognized as a key player in the maintenance of vascular homeostasis. In the next paragraphs, we will discuss the basics of this pathway, the role played by its dysregulation in atherosclerosis and what is currently known about the effects of nutraceuticals on Notch.

4.1. The Basics of Notch Signaling. In mammals, there are four highly homologous receptors (Notch-1-4) and five ligands belonging to Delta-like (Dll-1, 3, and 4) or Jagged (Jagged-1 and 2) families. Notch receptors are synthesized as single-chain precursors and are cleaved by Furin (S1 cleavage) into an extracellular domain (NECD, rich in epidermal growth factor- (EGF-) like repeats) and a transmembrane subunit in the Golgi apparatus, generating the functional heterodimeric receptor, linked by Ca^{2+}-dependent noncovalent bonds. Here, the EGF-like domains can be modified by the adding of O-fucose glycans by the Glycosil transferase Fringe [218], thus determining which ligands can be bound by the Notch receptors. Similarly, the ligands are also single-pass type I transmembrane proteins and present an extracellular domain formed by EGF-like repeats [219]. The canonical activation of Notch signaling arises from the interaction between the Notch receptors and their ligands on adjacent cells (Figure 2).

Accordingly to the "pulling force" theory, the Notch signaling is activated when the E3 ubiquitin-protein ligase (MIB1) modifies the Notch ligands, when bound to NECD, allowing the ligand endocytosis and generating the mechanical force necessary for exposing the second cleavage site of Notch receptors, thus driving the successive proteolytic cleavages mediated by A disintegrin and metalloprotease (ADAM) surface protease (S2 cleavage) [220]. A second intramembranous cut by γ-secretase (S3 cleavage) mediates

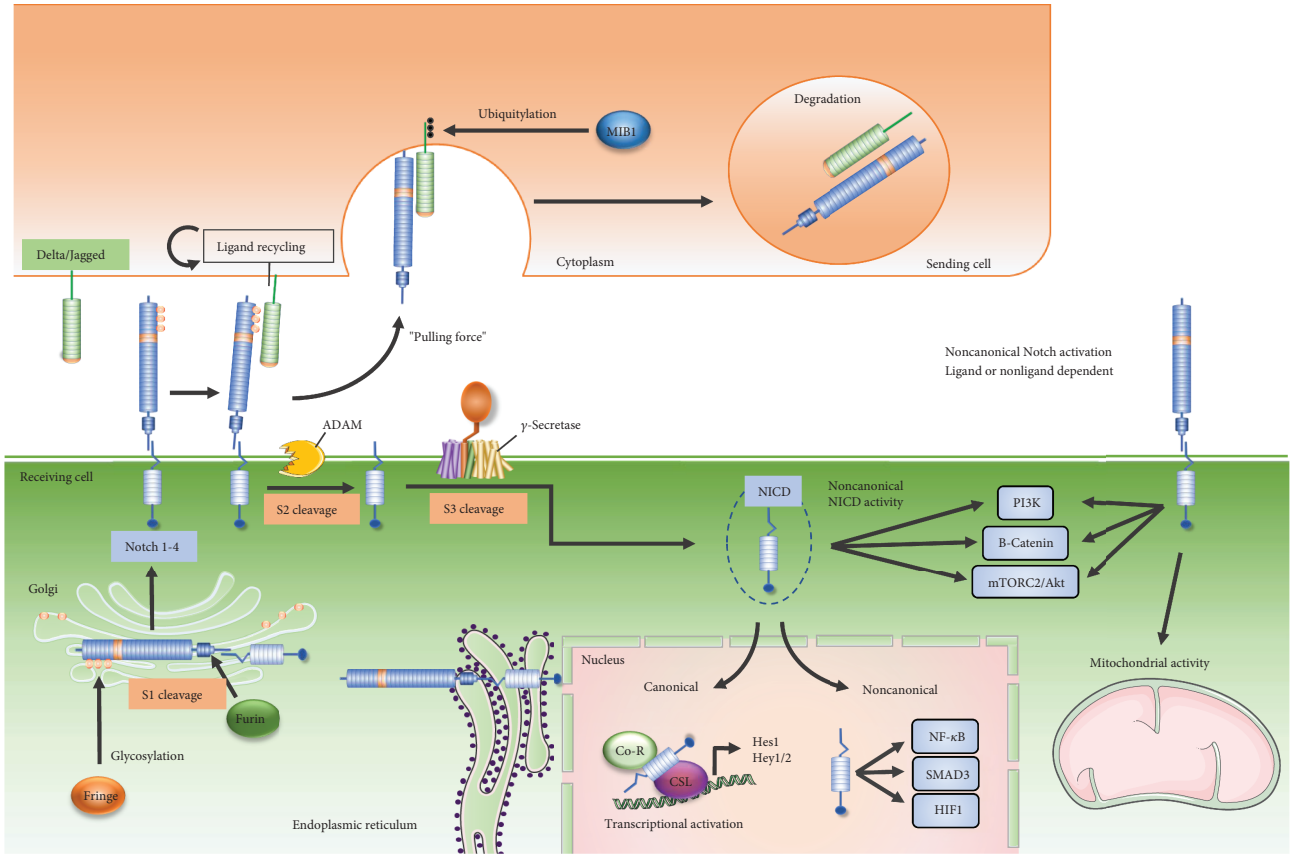


Figure 2: Canonical and noncanonical Notch signaling pathway. In the canonical Notch pathway, precursor of Notch receptors undergoes Furin-mediated cleavage (S1) in the Golgi apparatus, which is necessary to form the functional heterodimeric receptor. Upon Notch glycosylation by the Fringe family of glycosyltransferases, the Notch receptor translocates to the plasma membrane, where it interacts with a Delta/Jagged ligand, present on the surface of an adjacent cell. Notch signaling is activated when the ligand, bound to the receptor, is ubiquitylated by MIB1, an event that generates the mechanical force necessary for exposing the second cleavage site of Notch receptors. This event leads ADAM to perform the second cleavage (S2). The third cleavage (S3), by the γ-secretase complex, promotes the release of the intracellular domain of the receptor (NICD). NICD translocates into the nucleus where it promotes the transcription of canonical Notch target genes, such as Hey1 and 2 and HES1. The noncanonical Notch signaling pathway may be γ-secretase dependent or independent. This later may also occur either in the presence or in the absence of its ligand. Noncanonical Notch signaling is also independent of CSL, and it is mediated by the interaction with PI3K, mTORC2, AKT, Wnt, NF-κB, YY1, or HIF-1α pathways at either the cytoplasmic and/or nuclear levels.

the release of the Notch intracellular domain (NICD), the active form of the receptor. NICD translocates into the nucleus where it binds the transcription factor CSL (CBF-1, suppressor of Hairless and Lag-1) also known as RBP-Jκ (recombinant signal-binding protein 1 for Jκ) transcription factor, thus promoting the transcription of Notch target genes. The most characterized direct Notch target genes are the negative regulator of transcription and belong to the Hairy and Enhancer of Split (HES) and Hairy and Enhancer of Split with YRPW motif (HEY) gene families [221] although several other targets have been described [222]. Under pathological conditions, like cancer and activation of the immune system, Notch signaling can also act in a RPB-Jκ-independent manner ("noncanonical" fashion) signaling [223]. In the noncanonical pathway, NICD interacts with other transcription factors, such as SMAD3 (small mother against decapentaplegic 3), YY1 (Yin Yang 1), HIF-1α, and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), into the nucleus, whereas, in the cytoplasm, this signaling may occur via the uncleaved Notch receptor, still bound to membrane, or via the NICD, through interaction with PI3K (phosphoinositide 3-kinase)/Akt/Wnt/β-catenin pathways [224]. Recently, it has been also reported that Notch, by interacting with PTEN-induced kinase 1 (PINK1), can activate the mTORC2/Akt pathway, thus influencing the mitochondrial function and cell survival [225]. Posttranslational modifications (methylation, hydroxylation, acetylation, ubiquitylation, and phosphorylation) and the interplay with other signaling pathways, such as NF-κB, estrogen receptor- (ER-) alpha, G protein-coupled ER (GPER), ErbB2, and vascular endothelium growth factor receptors (VEGFRs) [226–231], increase the complexity of this signaling pathways which, in a cell type- and context-dependent manner, can influence a plethora of biological processes.

4.2. Notch in the Endothelium. A large number of *in vitro* and *in vivo* studies have convincingly established the critical

role of Notch signaling during development of the vascular system in which Notch is indispensable for a correct arteriovenous specification [232] and its malfunctioning has been correlated to vascular abnormalities, not compatible with life. Briefly, Notch-1 homozygous mutant and Notch-1/Notch-4 double homozygous mutant embryos displayed severe defects in angiogenic vascular remodeling [5, 233, 234]. Similarly, expression of active Notch-4 in vasculature leads to an anomalous vessel structure and embryonic lethality at embryonic day 10 (E10) [235]. Furthermore, the vascular defects of Notch transcriptional regulators (RBP-Jκ or Mib1) on homozygous mutant mice embryos are similar to defects developed by mutant embryos for Notch receptors and died prior to E11.5 [236, 237]. Likewise, Hey1 or Hey2 and Dll-4 or Jagged-1 mutant mice exhibit defects in their vasculature during embryogenesis and died from massive hemorrhage [238–241].

Given the pivotal role played by Notch signaling in vascular embryogenesis, it is not surprising that Notch is also essential in maintaining the homeostasis of the adult vasculature. In ECs, all Notch receptors, Dll-1 and 4 and Jagged-1 and 2 ligands, are expressed [242] and it is well-known that Notch, by intricate crosstalk with VEGF-A, controls arterial angiogenesis, also under proinflammatory conditions [243], by affecting the balance between tip cells and stalk cell [244].

During the last decade, Notch has been under the spotlight of the atherosclerosis research field since, except for a few studies [116, 245–247], numerous studies demonstrated that Notch may counteract endothelial dysfunction and atherosclerotic plaque development. Quillard et al. provided the first *in vitro* evidence that proinflammatory conditions (i.e., TNF-α) dysregulate Notch signaling leading to increased levels of ICAM-1 and VCAM-1 and to NF-κB-mediated apoptosis [248, 249]. These data were corroborated by Briot et al. showing that in human aortic ECs, siRNA-mediated reduction of Notch-1 is sufficient to increase the expression of inflammatory markers and adhesion molecules and that treatment of ECs with oxidized lipids and proinflammatory cytokines (TNF-α and interleukin-1-beta (IL1β)) decreased Notch-1 expression [250]. In agreement with this study, we have recently demonstrated that 17β-estradiol is able to limit TNF-α-induced apoptosis in ECs by activating Notch-1 [226]. In support of the protective role of Notch in the endothelium, Wang and collaborators reported that in bone marrow ECs, RBP-Jκ inhibits miR-155/NF-κB axis activation [251], data being reinforced by the Nus et al. study which showed that RBP-Jκ heterozygous inactivation results in aortic valve calcification under dyslipidemic condition [252]. Similarly, in human-induced pluripotent stem cell- (iPSC-) derived ECs, Notch-1 haploinsufficiency interferes with EC response to shear stress, causing the unlock of proosteogenic and inflammatory networks [253]. The interplay between Notch and shear stress is an emerging relevant topic in the context of vascular biology [6]. Diverse Notch signaling components in the endothelium respond to shear stress [254], and Notch-1 is essential for preserving EC tight junctions and their normal transcriptional/epigenetic response to shear stress [253, 255]. Polacheck et al. suggested that Notch-1 may be activated by shear stress through a Dll-4-dependent mechanism, triggering a noncanonical Notch pathway [256]. Schober et al. showed instead that in aorta regions exposed to turbulent flow, thus prone to plaque formation, disturbed shear stress may lead to expression of the Notch inhibitor Delta-like 1 homolog (Dlk1), through the downregulation of miR126-5p, leading to reduced expression of *HES5*, a Notch-1 target gene essential for restoring the dyslipidemia-injured endothelium [257]. Noteworthy, we have recently demonstrated that heart rate reduction by ivabradine treatment induces an atheroprotective gene profile and *HES5* expression in the aortic arch endothelium of apolipoprotein E-deficient (ApoE$^{-/-}$) mice, which was linked to maintenance of endothelial integrity and reduction in the plaque area in their aortic root [258].

4.3. Notch in Vascular Smooth Muscle Cells. The correct morphology and functionality of VSMCs are also indispensable for guaranteeing the stability and function of adult vasculature, and in the first stages of atherosclerosis, VSMCs, switching from a contractile/quiescent to a secretory/inflammatory/migratory state, play a role in plaque formation. Many studies suggest that Notch is necessary for maintaining VSMCs in a contractile/quiescent phenotype. In rat VSMCs, the IL-1β-induced secretory/migrating phenotype is blunted by Notch-3 overexpression and enhanced by treatment with DAPT, a γ-secretase inhibitor [259] but, contrary to this finding, DAPT also seems to prevent SMC migration and proliferation induced by AngII [260].

We recently demonstrated that cholesterol-induced VSMC transdifferentiation is associated with reduced levels of Jag1 and Hey2 and increased levels of Dll-4 mRNAs [261]. In human aortic VSMCs, Notch-3 also promotes transcription of prosurvival genes, which resulted to be significantly decreased in the aortas of Notch-3$^{-/-}$ mice [262]. Similarly, Notch-3 knockdown by RNA interference caused VSMC to have higher proliferation, migration, and apoptosis rates, with a concomitant abnormal morphology configuration [263]. Consistently, Ragot et al. showed that the loss of the Notch-3-RBP-Jκ pathway in VSMCs led to cardiac vasculature alterations in response to AngII-induced hypertension, thus to the development of cardiac hypertrophy [264]. Reduced expression of Notch-1 and Dll-4 has been also found in the aortic wall of patients with abdominal aortic aneurysm, which was correlated to decreased VSMC content in the vessels [265]. Chen and collaborators found that Notch-1 repression is required for miR-34a-mediated VSMC proliferation and migration [266]. Redmond et al. also reported that Notch-1 activation may guide the neointimal formation and VSMC proliferation in the carotid artery ligation mouse model, phenomenon prevented by Notch-1 siRNA injected following carotid ligation [267]. In conclusion, based on the existing data, which seem to indicate an opposite effect of Notch-1 and Notch-3 in controlling VSMC activity and phenotype, the role played by Notch in this context still needs to be clarified.

4.4. Notch in Macrophages. During atherosclerotic plaque formation, intraintima macrophages respond to extracellular

and intracellular signals which regulate their phenotypes, resulting in high levels of heterogeneity and plasticity among macrophage subtypes [268]. The "classical" model of macrophage activation indicates that the predominant phenotypes are characterized by a proinflammatory M1 and alternative M2 profiles [268]. Many studies *in vitro* clearly indicate that the Dll-4/Notch-1 axis is involved in promoting a M1 phenotype in macrophages [269–272]. Outtz et al. demonstrated that macrophages from Notch-$1^{+/-}$ mice displayed decreased LPS/IFNγ-mediated induction of proinflammatory IL-6, IL-12, and TNF-α compared with wild-type mice [273]. These data have been confirmed by Xu and collaborators that showed in macrophages isolated from Notch-$1^{-/-}$ mice a decreased basal and LPS-induced NF-κB and HIF-1α activation, indicative that induction of the M1 pathway is dependent on Notch signaling [274]. Defects in NF-κB p50 nuclear localization were observed in DAPT-treated macrophages and in RBP-Jκ-deficent macrophages, indicative of crosstalk between Notch and NF-κB pathways [275]. Lastly, DAPT treatment during MI diminished the number of macrophages in the infarcted area and significantly increased the M2 macrophage polarization [276], data resembling those obtained by Singla et al. that confirmed reduction of the proinflammatory M1 phenotype following monocyte treatment with DAPT or Notch-1 siRNA [277].

Pabois et al., by using an EC/monocyte coculture system, demonstrated that endothelial Dll-4 induces M1 polarization [278], and consistently, Koga et al. showed that Dll-4 antibody administration resulted in reduced vein graft lesion development in $LDLr^{-/-}$ mice and concomitantly decreased macrophage accumulation and expression of proinflammatory M1 genes [279]. Recently, the Pagie et al. study showed that Dll-4, other than promoting LPS/IFNγ-mediated M1 polarization, interferes with IL-4-mediated M2 macrophage polarization [280]. In elucidating the mechanism underlying the Notch signaling-mediated M1/M2 polarization, Lin et al. suggested that activation of Notch signaling might downregulate, through HES family corepressors, the signal regulatory protein α (SIRPα), a M2 phenotype inducer, which conversely resulted to be upregulated in RBP-Jκ-deficient bone marrow-derived macrophages [281]. Consistently, bone marrow-derived macrophages from RBP-$J\kappa^{-/-}$ mice displayed, under LPS/IFNγ stimulation, a M2 phenotype (reduced expression of TNF-α, IL-6, and inducible-nitric oxide synthase (iNOS)), which can be reversed by transfection with miR-148a-3p mimic, a Notch-1-target miRNA which promotes M1 polarization [282]. Similarly, Miranda and collaborators, in an attempt to identify the link between the macrophage subtype and the resistance to insulin in HFD-induced obesity mouse models, found that miR-30, targeting Dll-4, is associated with obesity-induced inflammation and proinflammatory cytokine production in adipose tissue macrophages isolated from visceral fat of obese mice [283]. Specifically, they demonstrated that miR-30 inhibition is sufficient to promote the Dll-4/Notch-1 axis and proinflammatory cytokine (TNF-α and CCL2) production, this later is blunted by using anti-Dll-4 antibody. Conversely, lentiviral overexpression of miR-30 in RAW264.7 cells resulted in reduced M1 polarization and TNF-α/CCL2 production [283]. Taken together, with the previously published studies [269], these studies indicate that Dll-4/Notch-1 assume in macrophage a central function in determining the balance of M1/M2 subpopulations; thus, it could represent a valid anti-inflammatory target for limiting excessive activation of proinflammatory programs during atherosclerosis onset.

4.5. Nutraceuticals Acting through the Notch Pathway. Several authors reported that natural extracts can be used in cancer prevention; few of them focused the studies on Notch signaling, obtaining interesting results. EGCG is able to inhibit Notch signaling in BALB/c nude mice, previously injected with cancer stem cells (CSC), inhibiting tumor formation [284]. Honokiol, a phenolic compound isolated from the bark of *Magnolia officinalis* Rehder (Magnoliaceae), is able to counteract the growth of melanospheres formed by CSC isolated from two melanoma cell lines downregulating Notch-2 receptor, HES1, and cyclin D1 expression [285]. Withaferin-A (WA), a bioactive compound derived from Withania somnifera, inhibits Notch-1 signaling and cell proliferation in three colon cancer cell lines [286]. Two bioactive compounds (tricin and p-coumaric acid) present in leaf extract of *Sasa quelpaertensis* Nakai (Poaceae) are able to inhibit the growth of CSCs deriving from different colon cancer cell lines through Dll-1 and Notch-1 inhibition and downregulation of biomarkers related to tumor vascularization (VEGF and HIF-1α) [287]. Curcumin inhibits the activation of Notch-1 and the expression of Jagged-1 as well as HES1 in esophageal cancer cell lines [288]. It has also been shown that curcumin inhibits the expression of Notch-1-specific microRNAs including miR-21and miR-34a and upregulates the cancer suppressor let-7a miRNA. Moreover, Notch downstream genes are overexpressed in pancreatic cancer and curcumin induces apoptosis through reduction of the Notch-1 signaling pathway and downregulation of cyclin D1 and Bcl-xL. Curcumin downregulated the expression of Notch-1 leading to increased apoptosis and cell cycle arrest in hepatic and oral cancer cells, activating NF-κB and its target genes (Bcl-2, cyclin D1, VEGF, and MMP-9) [289]. Curcumin inhibits the DNA-binding ability of NICD in prostate cancer cells [290] and decreases also CSC markers in lymphoma/leukemia cells, at least in part through inhibiting their self-renewal [291]. Resveratrol, a natural phenol present in grape skin with known anticancer effects [292], is able to suppress proliferation and to induce a p53-mediated apoptosis in acute lymphoblastic leukemia cell via inhibition of the Notch signaling pathway [293]. These studies suggest that curcumin treatment is an attractive new strategy for several types of cancers at least in part thanks to its capability to downregulate Notch-1 signaling.

In human epidermal growth factor receptor 2- (HER-2-) positive breast cancer cells, characterized by HER2 gene amplification [294], all-trans retinoic acid (ATRA) inhibited γ-secretase and Notch-1 processing, involved in cell migration and proliferation [295]. Oroxylin A, a natural compound extracted from the root of *Scutellaria baicalensis*, inhibited the hypoxia-induced invasion and migration of ERα-positive breast cancer cells by suppressing the Notch

Table 2: List of nutraceuticals acting through Notch signaling modulation.

Nutraceutical	Disease	Major findings	Role of Notch	References
Epigallocatechin-3-gallate (EGCG)	Cardiovascular	EGCG inhibits macrophage accumulation and inflammation response in the skin wounds of STZ-induced diabetes mellitus	EGCG reduces expression of Notch-1 and 2 in wound tissues of diabetic mice	[299]
		In RAW 264.7, EGCG limits LPS-mediated release of proinflammatory IL-1β	EGCG reduces expression of Notch-1 and 2 and of Notch target gene HES1. EGCG binds Notch-1 and limits its activity	
		In HUVECs, EGCG induces expression of iNOS and eNOS and inhibits oxLDL-mediated apoptosis	EGCG restores the expression of Jagged-1 and of target proteins (MATH1, HES1, and HES5)	[276]
			Jagged-1 is the key effector of EGCG-protective effect against oxLDL-induced endothelial dysfunction	
		EGCG attenuates the HFD-induced accumulation of atherosclerotic plaque in ApoE-deficient mice	EGCG protects ApoE-KO mice from atherosclerosis through the Jagged-1/Notch-1 pathway	
	Cancer	EGCG inhibits the self-renewal capacity of head and neck squamous carcinoma (HNSC) cancer stem cells (CSCs) by suppressing their sphere forming capacity and attenuates the expression of stem cell markers. EGCG augments cisplatin-mediated chemosensitivity	EGCG decreases HNSC CSC traits by inhibiting the Notch-1 pathway	[225]
Norisoboldine	Cardiovascular	Norisoboldine suppresses VEGF-induced HUVEC migration	Norisoboldine induces VEGF-mediated migration through activation of Notch-1	[301]
Docosahexaenoic acid (DHA)	Cardiovascular	DHA significantly decreases VSMC migration/proliferation induced by IL-1β as well as fibrinolytic/MMP activity	DHA increases Notch-3 expression and HES1 transcription and enhances γ-secretase complex activity	[300]
Diosgenin	Cardiovascular	Diosgenin reduces the HFD-induced atherogenesis in rat aorta	Diosgenin prevents nuclear translocation of NICD in aorta and in differentiated macrophage cells	[302]
Berberine (BBR)	Cardiovascular	BBR significantly improves cardiac function recovery and decreases myocardial apoptosis, infarct size, serum creatine kinase, and lactate dehydrogenase levels in rats following myocardial IRI	Both *in vitro* and *in vivo*, BBR upregulates NICD translocation and HES1 expression	[304]
		In H9C2, BBR attenuates simulated IRI-induced myocardial apoptosis	*In vitro*, antiapoptotic effect of BBR is blocked by Notch-1 or HES1 siRNA	
Polydatin	Cardiovascular	Following myocardial IRI, polydatin preserves cardiac function, ameliorates myocardial oxidative/nitrative stress damage, and reduces myocardial infarct size in STZ-induced diabetic rats	Polydatin exerts cardioprotection against diabetic myocardial IRI by activating myocardial Notch-1/HES1 signaling. DAPT blunts the beneficial effects of polydatin	[305]

Table 2: Continued.

Nutraceutical	Disease	Major findings	Role of Notch	References
2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG)	Cardiovascular	TSG significantly improves cardiac function and suppresses IRI-induced myocardial apoptosis	Both *in vitro* and *in vivo*, TSG upregulates NICD and HES1 expression	[306]
		In H9C2, TSG pretreatment dose-dependently decreases simulated IRI-induced apoptosis	*In vitro*, antiapoptotic effect of TSG is blocked by DAPT	
Honokiol	Cancer	Honokiol inhibits the growth of melanospheres formed by CSC	*In vitro*, Honokiol downregulates Notch-2, HES1, and cyclin D1 expression	[285]
Withaferin-A	Cancer	In three colon cancer cell lines, Withaferin-A mediates c-Jun-NH(2)-kinase-mediated apoptosis	Withaferin-A inhibits Notch-1 signaling	[286]
Tricin and p-coumaric acid	Cancer	Tricin and p-coumaric acid inhibits the growth of CSCs and VEGF and HIF1α expression	Tricin and p-coumaric acid inhibits Dll-1 and Notch-1 expression	[287]
Curcumin	Cancer	Curcumin inhibits hepatocellular cancer cell (HCC) proliferation	Curcumin decreases NICD expression in HCC	[289]
		Curcumin treatment results in a 40% decrease in tumor growth in a nude mouse xenograft model		
		Curcumin inhibits proliferation and colony formation in esophageal cancer cell lines and upregulates expression of let-7a miRNA	Curcumin reduces Notch-1 activation and expression of Jagged-1 and HES1	[288]
			Curcumin reduces expression of Notch-1-specific microRNAs (miR-21 and miR-34a) and upregulates tumor suppressor let-7a miRNA	
		Curcumin decreases markers associated with CSCs in Burkitt lymphoma and acute myeloid leukemia cells	Curcumin reduces expression of Notch-1 and cyclin D1	[93]
Resveratrol	Cancer	Resveratrol increases apoptosis and suppresses proliferation in MOLT-4 acute lymphoblastic leukemia cells	Resveratrol reduces NICD levels in a dose-dependent manner and inhibits the expression of HES1	[293]
	Cardiovascular	Resveratrol inhibits phenotypic switching of neointimal VSMCs after balloon injury in rats	Resveratrol decreases Notch-1, Jagged-1, Hey1, and Hey2 mRNA in balloon-injured arteries at 7 days	[303]
All-trans retinoic acid (ATRA)	Cancer	ATRA exerts a strong antimigratory action in the HER2-positive SKBR3 cell line	ATRA inhibits Notch-1 pathway	[295]
Oroxylin A	Cancer	Oroxylin A inhibits the hypoxia-induced invasion and migration of ERα-positive breast cancer cells	Oroxylin A inhibits NICD translocation into the nucleus	[296]
Alpinetin	Cancer	Alpinetin suppresses the proliferation and invasiveness of glioma stem cells (GSCs) and induces their apoptosis	Alpinetin reduces Notch-1 activity. Notch reactivation, by using recombinant Jagged-1, rescues the effect of alpinetin on GSCs	[297]
Cowanin	Cancer	Cowanin shows potent cytotoxicity against human leukemic HPB-ALL cells	Cowanin degrades nicastrin, a component of γ-secretase, thus hampering Notch-1 activation	[298]

processing [296]. Alpinetin can suppress the proliferation and invasiveness of gastric stem cells (GSCs) and induce apoptosis through Notch inhibition in glioma stem cells [297]. Cowanin has a strong inhibitory activity of the Notch signaling target proteins, HES1 and HES5, affecting the activity of the γ-secretase complex on several cancer cells [298].

Still, little is known about the modulation of Notch signaling by natural bioactive compounds and the consequent control of many features that characterize endothelial dysfunction. EGCG prevents the oxLDL decrease of Jagged-1 and Notch pathway-related proteins (MATH1, HES1, and HES5) and inhibits apoptosis *in vitro* in human vascular

ECs and in ApoE$^{-/-}$ mice [276]. Furthermore, EGCG inhibits macrophage accumulation and inflammation response in the skin wounds of streptozotocin- (STZ-) induced DM mice at least in part through Notch signaling modulation [299]. DHA, an omega-3 fatty acid, increases Notch-3 and HES1 transcription and enhances γ-secretase complex activity, thus reducing fibrinolytic/MMP activity in transdifferentiated VSMCs toward a migrative/proliferative phenotype [300]. Norisoboldine, an alkaloid compound isolated from *Radix Linderae*, suppresses synovial angiogenesis, thanks to its inhibition of VEGF-induced endothelial cell migration via a cAMP-PKA-NF-κB/Notch-1 signaling pathway [301]. Both in rat aortic subintimal macrophages and in *in vitro*-differentiated macrophage cell diosgenin, a phytosteroid sapogenin extracted from the tubers of *Dioscorea* wild yam suppresses the nuclear translocation of NICD [302]. Resveratrol can attenuate neointimal VSMC hyperplasia in rats, following balloon injury, through a mechanism that involves inhibition of the Notch signaling [303]. Activation of Notch signaling reduces myocardial ischemia reperfusion injury (MI/RI), by activating key components of survival pathways, namely, PI3K-Akt, NOS, and mitochondrial K^+-ATP (mitoKATP) channels [231]; BBR, polydatin, and 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside upregulate Notch-1/HES1 signaling attenuating myocardial apoptosis both in cultured cardiomyocytes and in rats subjected to MI/RI [304–306].

Nutraceuticals have been studied also in the context of age-related diseases [307] and few studies reported an involvement of Notch in the effect of natural extract treatment on Alzheimer disease. Dihydroergocristine (DHEC), a component of ergoloid mesylates approved by the FDA for the treatment of hypertension and dementia, suppresses the production of Aβ peptides by inhibiting the γ-secretase complex in neurons [308]. Also, (20S)-Rg3, a triterpene natural compound known as ginsenoside and one compound (SPI-014) isolated from *Actaea racemosa* reduced Aβ peptide levels in neurons *in vitro* and in a mouse model of Alzheimer disease, at least in part decreasing the association of presenilin 1 (PS1) fragments with catalytic components of the γ-secretase complex localized in lipid rafts [309].

Indeed, the modulation of Notch signaling in several age-related diseases is an emerging approach in clinical practice and the correct use of plants could help to decrease the incidence as well as the healthcare costs of these pathologies.

5. Conclusions

The increased understanding of how diet affects disease, together with high healthcare costs and the aging population, has generated interest in food as a tool for disease prevention and health enhancement. There is growing evidence that components of food may play an integral role in the link between food and health; thus, a diet, mainly based on plant and plant-derived nutraceuticals, can contribute to reduce health care costs, while supporting economic development in rural communities.

Preclinical investigations have consistently shown that bioactive compounds, present in plants and certain foods, inhibit those biological processes linked to atherosclerosis onset. In the clinical settings, while a large number of studies have shown a close association between an imbalanced diet, with low consumption of fruits, vegetables, fibers, vitamins, and fish, and the increase risk of incidence of CVDs, other studies have failed to show results in primary, or secondary, prevention of atherosclerosis-related coronary artery disease, stroke, and heart failure trough consumption of nutraceuticals. This suggests that there is still much left to be understood about the stability of these compounds *in vivo*, the best route and dose of administration, and differences in response related to gender and age. Moreover, more investigations are needed to fully understand how nutraceuticals' effect varies in the presence of those medications commonly used by the subjects involved in this type of studies. This gap could be filled by a wider characterization of the molecular pathways regulated by these compounds. The Notch signaling is a well-known master regulator of embryogenesis and postnatal maintenance of self-renewing tissues, and its detrimental role when trying to achieve cancer cell apoptosis is unquestioned. To date, γ-secretase inhibitors (GSIs) are the most studied Notch-inhibiting agents which increase response to chemotherapy [310]. Furthermore, the modulation of Notch signaling by nutraceuticals to interfere with cancer progression (Table 2) is a research field that has been steadily growing in the last ten years.

As discussed in this review, the implications of Notch in atherogenesis are wide ranging, from protection against endothelial dysfunction to the modulation of VSMCs and macrophage phenotypes, but a detailed analysis of the signaling upstream and downstream of Notch, in each cell context, has not been performed yet. Additionally, due to the distinct roles played in the above cited cell type (anti-inflammatory in endothelial cells vs proinflammatory in macrophages), targeting Notch in the context of atherosclerosis could result to be particularly challenging. There is a limited number of studies showing that some classes of nutraceutical compounds exert antiatherosclerotic activity, at least partially, through modulation of Notch signaling (Table 2). Given the important role of Notch in atherosclerosis, it could be of interest to investigate the effect on vascular Notch of widely investigated and used nutraceutical compounds, in order to gain a better understanding of their biological effects. Additionally, high-throughput analyses could be applied to investigate the effects on Notch of novel, still uncharacterized plant compounds. Based on the established role of Notch as anticancer therapy and on the emerging role of this pathway in atherosclerosis, it is not implausible to imagine a combination of specific tissue-targeted nutraceutical Notch modulators as a novel therapeutic strategy in this context.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Cristiana Caliceti and Paola Rizzo contributed equally to this work.

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Research Article

Ginkgo Biloba Leaf Extract Attenuates Atherosclerosis in Streptozotocin-Induced Diabetic ApoE-/- Mice by Inhibiting Endoplasmic Reticulum Stress via Restoration of Autophagy through the mTOR Signaling Pathway

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Background. There is a crosstalk between endoplasmic reticulum stress (ERS) and autophagy, and autophagy could attenuate endoplasmic reticulum stress-mediated apoptosis. Ginkgo biloba leaf extract (GBE) exerts vascular protection functions. The purpose of the present study is to investigate the role of autophagy in diabetic atherosclerosis (AS) and the effect of GBE on autophagy and ERS. *Methods*. Network pharmacology was utilized to predict the targets and pathways of the active chemical compounds of Gingko biloba leaf to attenuate AS. ApoE$^{-/-}$ mice were rendered diabetic by intraperitoneal ingestion with streptozotocin combined with a high-fat diet. The diabetic mice were divided into five groups: model group, atorvastatin group, rapamycin group, and low- and high-dose GBE groups. Serum and tissue markers of autophagy or ERS markers, including the protein expression, were examined. *Results*. The mammalian target of rapamycin (mTOR) and NF-κB signaling pathways were targeted by the active chemical compounds of GBE to attenuate AS predicted by network pharmacology. GBE reduced the plaque area/lumen area and the plaque lipid deposition area/intimal area and inhibited the expressions of CD68, MMP2, and MMP9. Rapamycin and GBE inhibited the expression of mTOR and SQSTM1/p62 which increased in the aorta of diabetic mice. In addition, GBE reduced the expression of ERS markers in diabetic mice. GBE reduced the serum lipid metabolism levels, blood glucose, and inflammatory cytokines. *Conclusion*. Impaired autophagy and overactive endoplasmic reticulum stress contributed to diabetic atherosclerosis. mTOR inhibitor rapamycin and GBE attenuated diabetic atherosclerosis by inhibiting ERS via restoration of autophagy through inhibition of mTOR.

1. Introduction

Atherosclerosis as one of the vascular complications of diabetes mellitus is accelerated by oxidative stress and endoplasmic reticulum stress. Therefore, amelioration of endoplasmic reticulum stress-mediated apoptosis is significant for the attenuation of diabetic atherosclerosis. Three pathways of endoplasmic reticulum stress, including IRE1-XBP1, PERK-eIF2α, and IRE1-JNK, contribute to activation of autophagy [1]. Autophagy is a nonapoptotic cell death manner, without increasing the inflammation of plaques. Furthermore, autophagy attenuates apoptosis by degrading unfolded proteins and impaired cell organelles. The protective function of macrophage autophagy involves the regulation of cholesterol metabolism [2]. Free cholesterol was hydrolyzed from cholesteryl esters by autophagy lysosomes

in foam cells, and its efflux was mediated by ATP-binding cassette transport protein 1. The impaired macrophage autophagy results in decreased free cholesterol efflux and accumulation of apoptotic foam cells attributable to lipid overload, leading to the increased lipid content in the plaques, increased necrotic core, and secondary inflammatory reaction, which consequently promote the vulnerability of atherosclerotic plaques [3]. In the models of atherosclerotic ApoE-/- mice, high-fat diet inhibits Beclin1-mediated protective effects of macrophage autophagy, thus accelerating the progression of atherosclerosis [4]. Rapamycin has the ability to restore impaired autophagy by inhibiting the mammalian target of rapamycin (mTOR), leading to selective clearing of macrophages, increased cholesterol efflux, reduction of apoptotic cells in the plaques, and stabilization of atherosclerosis in ApoE-/- mice [5–7].

There is a crosstalk between endoplasmic reticulum stress and autophagy, and autophagy protectively inhibits endoplasmic reticulum stress-mediated apoptosis in a negative feedback manner. According to Liao et al. [2], autophagy inhibits oxidative stress and endoplasmic reticulum stress-mediated macrophage apoptosis. Glomerular autophagy has the ability to attenuate endoplasmic reticulum stress and cell injury in mesangial cells induced by advanced glycation end products (AGE) [8]. Autophagy exerts a protective role in AGE-induced early injury of human vascular endothelia cells, featured by restoration of LC3II [9]. 7-Ketone-induced autophagy attenuates atherosclerosis by inhibition of endoplasmic reticulum stress [10]. On the other hand, the excess of autophagy also contributes to atherosclerosis as a form of cell death, indicating moderate autophagy protecting against atherosclerosis. Phosphatidylinositol 3 kinase- (PI3K-) Akt-mTOR and AMP-activated protein kinase- (AMPK-) mTOR are the two major pathways regulating autophagy. Although it has been well established that rapamycin is protective in atherosclerosis by inducing autophagy via inhibition of mTOR, the role of mTOR in diabetes mellitus is complex. Activation of mTOR promotes the secretion of insulin and increases insulin sensitivity; on the contrary, mTOR may lead to glucose intolerance through blocking of the insulin receptor substrate 1 (IRS1) by phosphorylation of p70S6K in a negative feedback loop manner [11, 12]. Hyperleptinemia can coexist with diabetes mellitus and has the ability to stimulate the activity of mTOR and promote the vascular smooth muscle cell proliferation [12]. The mTOR pathway was activated in the liver and skeletal muscle of obese rats involved in obesity-induced insulin resistance [13]. Autophagy is essential to islet function and survival; on the contrary, inadequate autophagy can lead to islet degeneration and reduced insulin secretion [14, 15]. According to den Hartigh et al., low-grade chronic mTORC1 inhibition improved insulin sensitivity attributable to signaling through reduced phosphorylated p70S6K, which might be beneficial in antiobesity and anticardiovascular disease therapies [16]. AMPK, upstream of mTOR, exerts cardiovascular protective function by inhibiting mTOR [17]. However, this protective effect attenuates in the context of high glucose due to inactivation of AMPK [18]. In the condition of diabetes, the cardio SIRT1 is a molecule directly or indirectly linked to the mTOR signaling pathway and autophagy. SIRT1 prevents endothelial senescence induced by hyperglycemia and ox-LDL through upregulation of autophagy via activation of AMPK [19, 20]. SIRT1 inhibition promotes atherosclerosis through impaired autophagy [21]. SIRT1 overexpression in neurons promotes neurite outgrowth and cell survival [22] and blocks the senescence of mesangial cells induced by high glucose through inhibition of mTOR signaling [23]. According to Hou et al. [24], inhibition of mTOR pathways prevents high glucose-induced inhibition of autophagy and cardiomyocyte injury. Consequently, it is hypothesized that the overactivated mTOR increases insulin resistance and the risk of cardiovascular diseases. However, there is lack of study regarding the activity of mTOR signaling pathways in diabetic atherosclerosis.

The impairment of the initiation stage of autophagy is characterized by decreased LC3II and increased SQSTM1/p62. Failure of the terminal phases of autophagy is characterized by increased SQSTM1/p62 in the cell, indicating an inability to clear autophagosomes and degrade p62 [25]. The increased expression and bioactivity of SQSTM1/p62 are closely associated with atherosclerosis [26]. The high concentration of ox-LDL blocked autophagic flux, leading to impairment function of autophagic degradation and SQSTM1/p62 aggregation. The accumulation of SQSTM1/p62 is involved in the increased MMP9 expression mediated by the NF-κB pathway, resulting in instability of the atherosclerotic plaques [27]. According to Fetterman et al. [25], inadequate autophagy featured by increased p62 promotes endothelial dysfunction in diabetic patients. Zhang et al. [28] revealed that high glucose inhibits autophagy in cardiac microvascular endothelial cells (CMECs), featured by activation of the mTOR pathway and increased SQSTM1/p62 levels, enhancing the apoptosis of CMECs. On the contrary, rapamycin inhibits high glucose-induced CMEC apoptosis by inhibiting mTOR signaling and through degrading SQSTM1/p62. The expressions of these hallmarks of autophagy in the context of diabetic atherosclerosis are still unknown.

Recently, accumulated studies reported that herbals could protect against ischemic cardiomyopathy by regulating autophagy [29]. Gingko biloba leaf extract (GBE), which contains terpenoids, flavonoids, alkylphenols, polyprenols, and organic acids, has long been used for the treatment of cardiovascular disease [30]. The mechanism by which GBE exerts vascular protection effects involves anti-inflammation, antioxidant, and antiplatelet. Ginkgo biloba K has been shown to attenuate endoplasmic reticulum stress and cell apoptosis in the infarct myocardium through upregulation of autophagy by inducing XBP1 [1]. GBE protects against myocardial ischemic/reperfusion injury in the context of high glucose by the mechanism of antioxidants [31]. According to our previous study, GBE attenuated streptozotocin- (STZ-) induced diabetic ApoE-/- mouse injury by attenuation of endoplasmic reticulum stress [32]. In vitro, GBE inhibited the adhesion of macrophages to endothelial cells induced by high glucose through reduction of the expression of IL-6 [33]. Currently, there are few studies regarding the effect of GBE on diabetic atherosclerosis in vivo. Furthermore, it is

still unclear if GBE has the ability to attenuate diabetic atherosclerosis through regulating the interaction between autophagy and endoplasmic reticulum stress. The present study is aimed at investigating autophagy and endoplasmic reticulum stress activity in diabetic atherosclerosis and whether GBE ameliorates diabetic atherosclerosis by inhibiting endoplasmic reticulum via upregulation of autophagy.

2. Materials and Methods

2.1. Prediction Analysis of Pharmacological Mechanism Based on Network Pharmacology. Gingko biloba leaf contains terpenoids (including ginkgolides and bilobalide), flavonoids (primarily quercetin, kaempferol, isorhamnetin, luteolin, rutin, apigenin, and myricetin), alkylphenols, polyprenols, and organic acids. 223 chemical compounds were obtained from the TCMSP database (http://lsp.nwsuaf.edu.cn/tcmsp.php). A bioavailability (OB) >30%, drug-like >0.18, was set as the threshold for further extraction and optimization of the ingredients in ADEM, and 25 active chemical compounds were used for further analysis [34]. 227 targets for the 25 active chemical compounds were obtained in the TCMSP database. 348 targets for atherosclerosis were achieved in the DrugBank database (http://www.drugbank.ca/), TTD database (http://bidd.nus.edu.sg/BIDD-Databases/TTD/TTD.asp), GAD database, and available literature. The gene symbols were retrieved in the UniProtKB database (http://www.uniprot.org) using protein names. 81 targets were subsequently achieved by intersection of targets for active chemical compounds and targets for atherosclerosis. Then, 6 active chemical compounds with degrees from 58 to 19 (quercetin, luteolin, kaempferol, beta-sitosterol, isorhamnetin, and stigmasterol) and 79 corresponding targets were used for further analysis. Inflammation cytokines IL-1β, IL-6, TNF, and MMP were included in these targets. The compound-target network was constructed by Cytoscape software (Figure 1). DAVID database (https://david.ncifcrf.gov/) was applied for KEGG pathway analysis, and 25 pathways for these 6 active compounds were achieved. The target-pathway network and compound-pathway network were constructed by utilizing Cytoscape software (Figures 2 and 3). These 6 active chemical compound target pathways include mTOR signaling pathways and NF-κB-mediated inflammation signaling.

2.2. Reagents. GBE powder was purchased from Beijing Handian Pharmaceutical Co. Ltd., atorvastatin and rapamycin were purchased from Pfizer Pharmaceuticals Co. Ltd., and streptozotocin (STZ) was purchased from Sigma Co. Ltd. Ginkgo flavonoid and terpenoid contents in GBE in the present are 44.9% and 6.3%, respectively, whereas the amount of ginkgo acid is limited to <1 ppm.

2.3. Experimental Protocol. Male ApoE-/- mice, aged 6-7 weeks (weighing 19-21 g (C57BL/6J background, introduced from Jackson Laboratory of USA by Peking University Health Science Center Laboratory Animal Science Department; quality certification number SCXK (Beijing) 2016-0012)), were rendered diabetic by intraperitoneal injection of 50 mg/kg/day STZ diluted with citrate buffer (pH 4.5; final concentration, 1%) for 5 consecutive days [35], after rearing with high-fat diet for 4 weeks. The mice with plasma glucose level > 12 mmol/L served as models [35, 36]. The mice that received normal saline water served as model controls ($n = 10$). 20 C57BL/6J mice fed with chow diet served as normal controls. Diabetic mice were divided into five groups, namely, model group (normal saline water i.g., $n = 16$), atorvastatin group (10 mg/kg/day i.g., $n = 16$) [37], rapamycin group (1 mg/kg/day i.g., $n = 16$) [38], low-dose GBE group (200 mg/kg/day i.g., $n = 18$), and high-dose GBE group (400 mg/kg/day i.g., $n = 18$). The dose of GBE in the present study was based on a previous study [32, 39, 40]. The intragastric administration was performed one week after STZ injection. Atorvastatin, rapamycin, and GBE were dissolved and delivered by normal saline water. *Considering the less toxicity of normal saline compared to organic solvent when administered to mice, normal saline is a feasible choice for intragastric administration. To avoid precipitation, the drugs were blended before medication.*

All the mice were sacrificed after another 12-week gavage plus high-fat diet. At the end of the study course, there were 11 mice that survived in the model group; 8 mice in the model control group survived. In the atorvastatin group and rapamycin group, there were 14 and 15 survivors, respectively; the survivor mice in the low-dose GBE and high-dose GBE groups were 16 and 17, respectively. All the 20 mice in the normal group survived (see Figure 4). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences, in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.4. Body Weight and Plasma Glucose Changes. The body weight and fasting plasma glucose level were recorded before the initial treatment of GBE and every four weeks thereafter. A glucometer (Roche) was used to determine the plasma glucose levels by the cutting tail method.

2.5. Tissue Preparation and Histological and Immunohistochemical Measurement. The chests were opened after the mice were anesthetized. The heart was perfused with heparin saline, and the hearts containing aortic root, together with the aortic arch to the iliac artery bifurcation, were harvested under aseptic conditions and fixed in 4% polyformaldehyde. The full length of the aorta is used for en face analysis by red "O" staining. The cross sections of the aortic sinus fixed in 4% paraformaldehyde and embedded in paraffin were used for H&E staining, MASSON staining, and immunohistochemical staining. Immunohistochemical staining was performed as the following: for α-SMA (rabbit polyclonal to α-SMA antibody; ab5694; 1 : 50), CD68 (rat monoclonal to CD68 antibody; ab53444; 1 : 50), MMP2 (rabbit polyclonal to MMP2 antibody; ab97779; 1 : 400), MMP9 (rabbit polyclonal to MMP9 antibody; ab38898; 1 : 100), mTOR (rabbit polyclonal to mTOR antibody; ab2732; 1 : 2000), Beclin1 (rabbit polyclonal to Beclin1; ab62557; 1 : 300), LC3B (rabbit polyclonal to LC3B; ab48394; 1 : 1000),

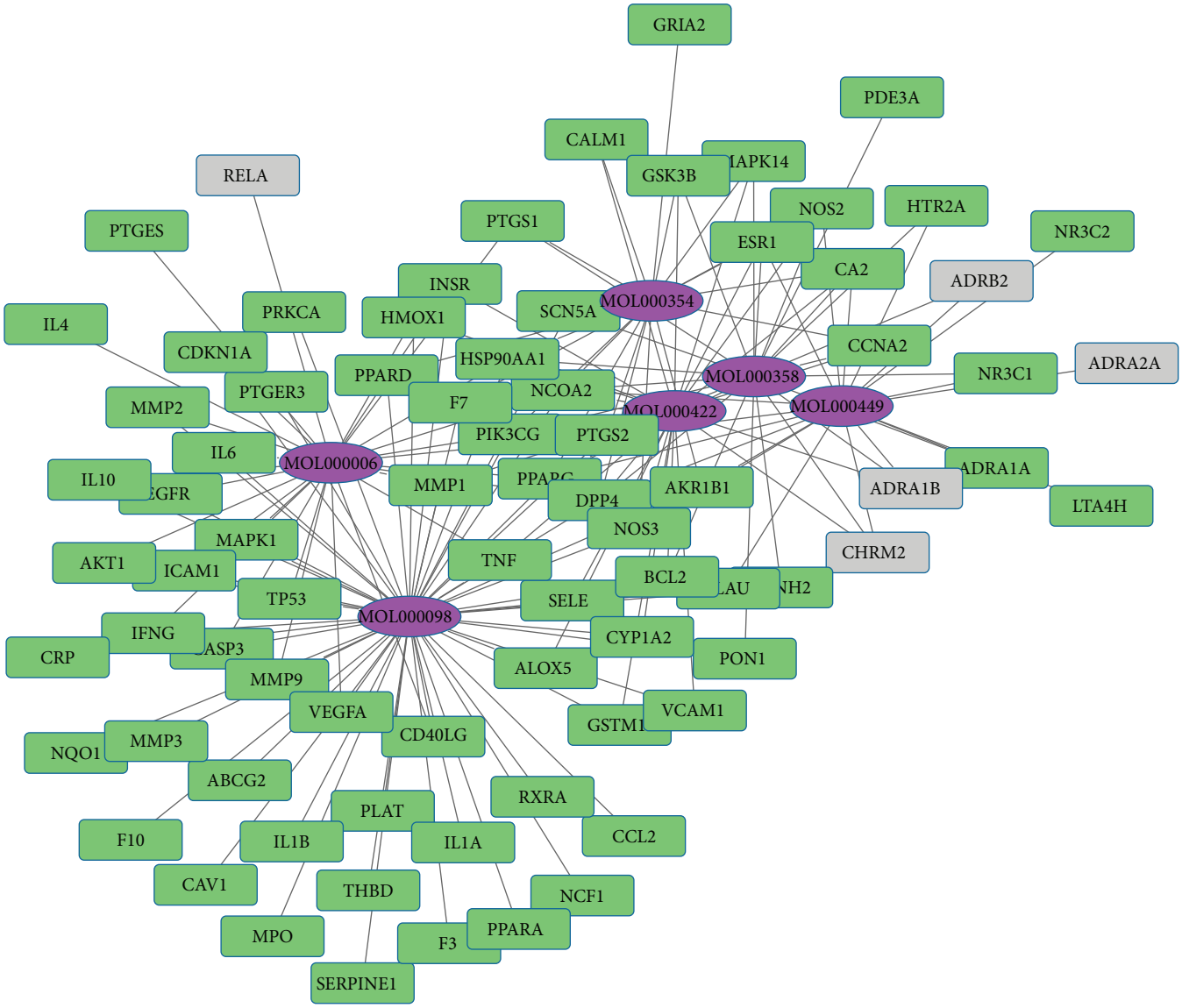


Figure 1: Compound-target network. The purple nodes indicate the 6 active chemical compounds with degrees from 58 to 19. The other nodes indicate corresponding targets for atherosclerosis. The lines indicate the interaction between these chemical active compounds and targets.

and SQSTM1/p62 (rabbit polyclonal to SQSTM1/p62; ab91526; 1 : 100). Immunohistochemical semiquantitative analysis was conducted by an automated image analysis system (Image-Pro Plus 6.0; Media Cybernetics, MD Rockville, USA). The positive expressions of these indexes were represented by integral optical density (IOD).

2.6. Western Blot Analysis. The aortic tissues were snap-frozen in liquid nitrogen, weighed, homogenized, and resuspended in ice-cold lysis buffer. Protein concentration was determined with the bicinchoninic acid method. Equal amounts of protein from each sample were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Nonspecific sites were blocked by incubating the membranes with 5% nonfat milk and 0.2% Tween 20 in Tris-buffered saline for 2 h at room temperature. Primary antibodies incubated overnight at 4°C are as follows: mTOR (ab2732; 1 : 1000), Beclin1 (ab62557; 1 : 1000), LC3B (ab48394; 1 : 2000), SQSTM1/p62 (ab91526; 1 : 2000), CHOP (CST 2895S; 1 : 2000), p-JNK (CST 9251S; 1 : 1000), JNK (CST 9252S; 1 : 1000), and Caspase-12 (CST 2202S; 1 : 1000). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. The expressions of amTOR, Beclin1, LC3B, SQSTM1/p62, CHOP, and Caspase-12 were adjusted for GAPDH. The expression levels of p-JNK were adjusted for total JNK.

2.7. Blood Biomarker Detection. At the end of the 12-week period, fasting serum glucose and lipid profiles, including high-density lipoprotein cholesterol (HDL-c), total cholesterol (TC), triglycerides (TG), and low-density lipoprotein

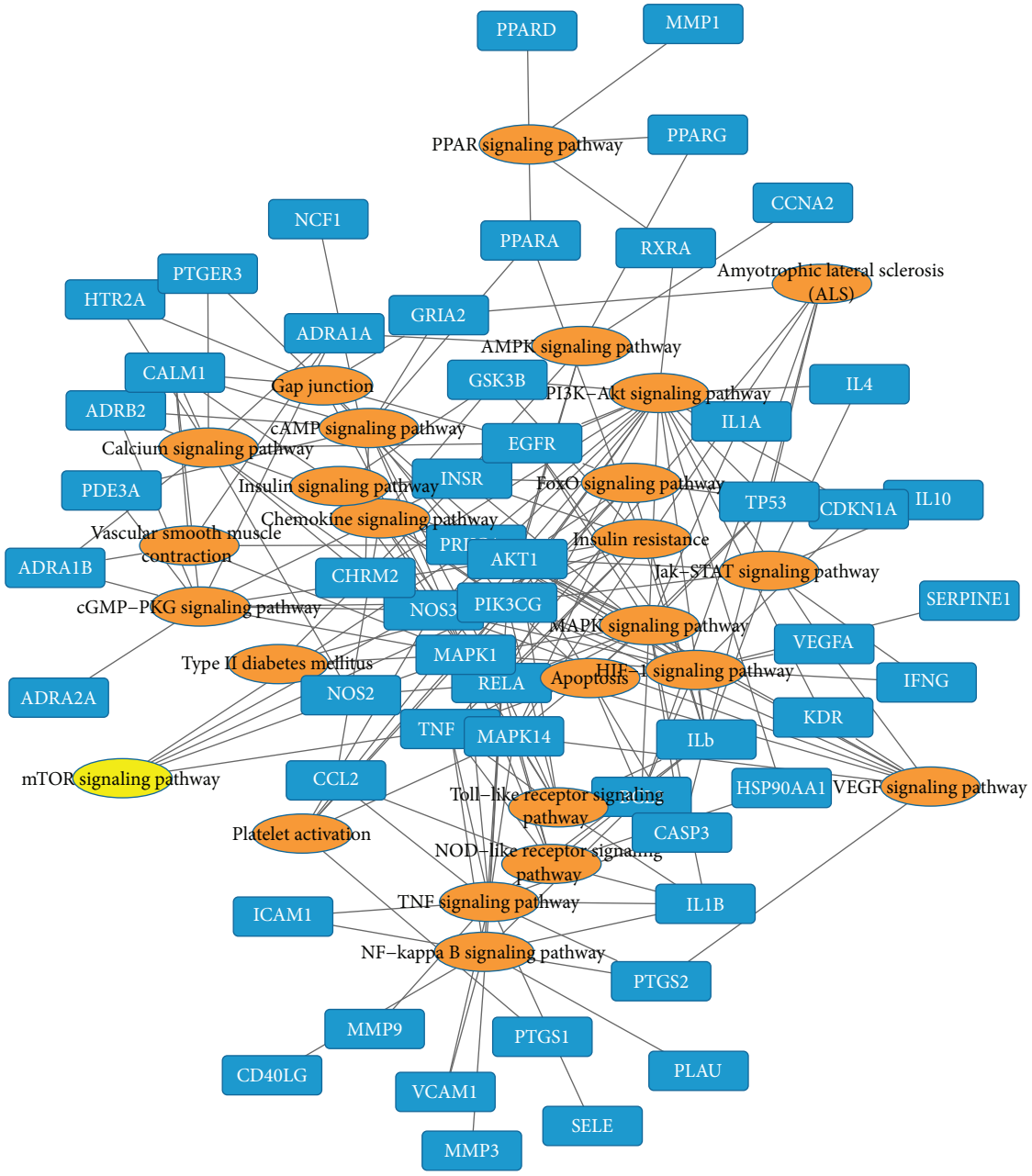


Figure 2: Target-pathway network. The blue nodes indicate 79 targets for atherosclerosis. The orange nodes indicate the corresponding pathways obtained in the DAVID database, and the yellow node indicates mTOR signaling. The lines indicate the interaction between these targets and signaling pathways.

cholesterol (LDL-c) levels, were determined by an automated system before the mice were sacrificed. Blood samples were collected and centrifuged at 3000 rpm for 10 min.

Serum levels of inflammatory cytokines (IL-1, IL-6, IL-1β, TNF-α, and iNOS) were detected using commercially available ELISA kits, purchased from Beijing Fang Cheng Jia Hong Technology Co. Ltd. Five serial dilutions of the standard were prepared according to the manufacturer's instructions. Blank and sample wells were set, respectively. Sample diluent (40 μL) was added to the sample wells in the precoated ELISA plates, followed by addition of the samples (10 μL). After sealing the plates with a closure plate membrane, they were incubated for 30 min at 37°C. HRP-conjugated reagent (50 μL) was added to all wells, except for the blank well. After incubation at 37°C, the liquid in the wells was removed, and the plate was washed with a wash liquid. Chromogen solution A (50 μL) and chromogen solution B (50 μL) were added to each well. The plates were incubated in the dark at 37°C for 15 min. The blank well was considered zero, and the absorbance of each well was measured at 450 nm within 15 min after adding the stop solution.

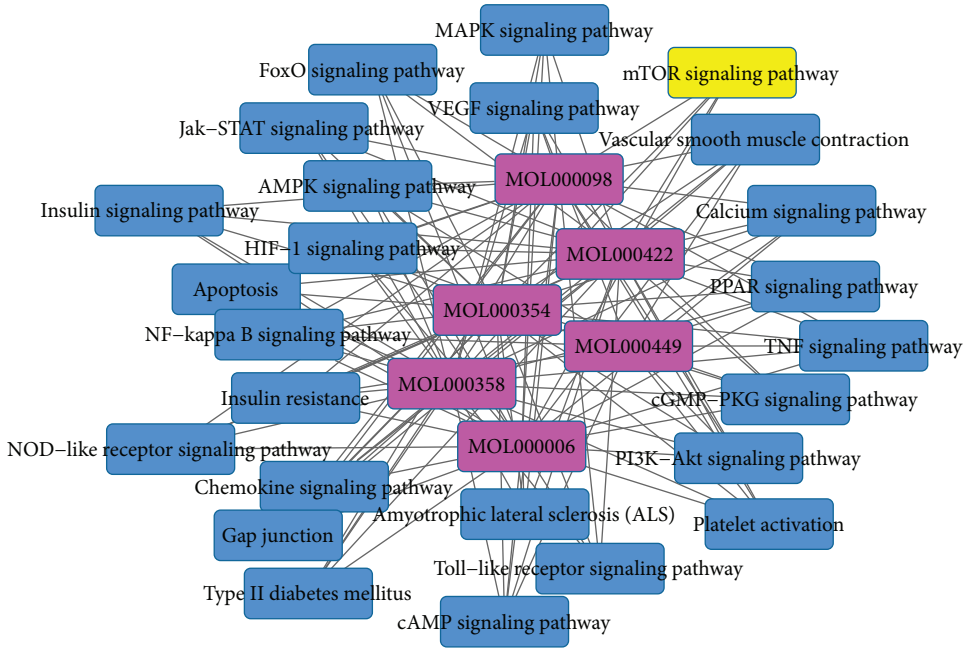


Figure 3: Compound-pathway network. The purple nodes indicate the 6 active chemical compounds. Blue and yellow nodes indicate signaling pathways corresponding to these active chemical compounds. The lines indicate the interaction between compound and signaling pathways.

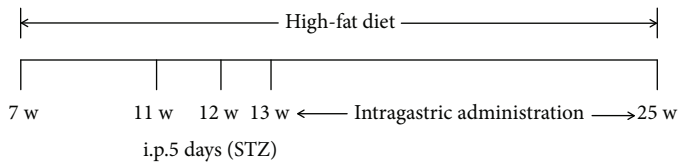


Figure 4: Experimental protocol.

2.8. Statistical Analysis. SPSS 14.0 was used for data analysis. The measurement data were presented using means ± standard deviation (x ± s). One-way analysis of variance (ANOVA) was applied for comparing means among the groups. The least significant difference (LSD) test was used to multiple comparisons between the model group and the other groups. $P < 0.05$ was considered statistically significant. GraphPad Prism 5.0 software was used for graphical presentation.

3. Results

3.1. The Effects of GBE on Body Weight and Plasma Glucose Levels. The body weight was significantly reduced in diabetic mice compared to the model control group after the intraperitoneal injection of STZ for 1 week (22.3 ± 1.8 g vs. 28.7 ± 3.9 g, $P < 0.01$). The body weight in the model group was lower compared to that in the model control group at the end of the study course (26.4 ± 2.7 g vs. 32.0 ± 6.1 g, $P < 0.01$). Atorvastatin, rapamycin, and GBE (200 mg/kg/day) did not affect the body weight of diabetic mice.

There was a significant increase in the plasma glucose level induced by STZ in the model group compared to the model control group (model group vs. model control group: 14.7 ± 2.1 mmolmmol/L vs. 7.4 ± 1.0 mmol/L, $P < 0.01$) and normal group (model group vs. normal group: 14.7 ± 2.1 mmol/L vs. 5.3 ± 0.8 mmol/L, $P < 0.01$) detected by a rapid blood glucose meter using the tail blood sample. After 12-week gastric administration, compared to the model group, GBE at both doses of 200 mg/kg/day and 400 mg/kg/day decreased the plasma glucose level [(200 mg/kg/day GBE group vs. model group: 18.8 ± 6.5 mmol/L vs. 23.4 ± 6.4 mmol/L, $P < 0.01$); (400 mg/kg/day GBE group vs. model group: 15.3 ± 7.1 mmol/l vs. 23.4 ± 6.4 mmol/l, $P < 0.05$)].

3.2. The Effects of GBE on Serum Lipid and Glucose Profiles. The serum LDL-c, TC, TG, and glucose levels were significantly elevated in the model group compared to the normal group and model control group ($P < 0.05$, Figures 5(a)–5(c)), whereas the HDL-c level in the model group was lower in the model group compared to the normal group ($P < 0.05$, Figure 5(d)). Compared to the model group, atorvastatin and GBE (200 mg/kg/day and 400 mg/kg/day) significantly reduced the levels of LDL-c, TC, and TG ($P < 0.05$, Figures 5(a)–5(c)), without affecting the HDL-c level.

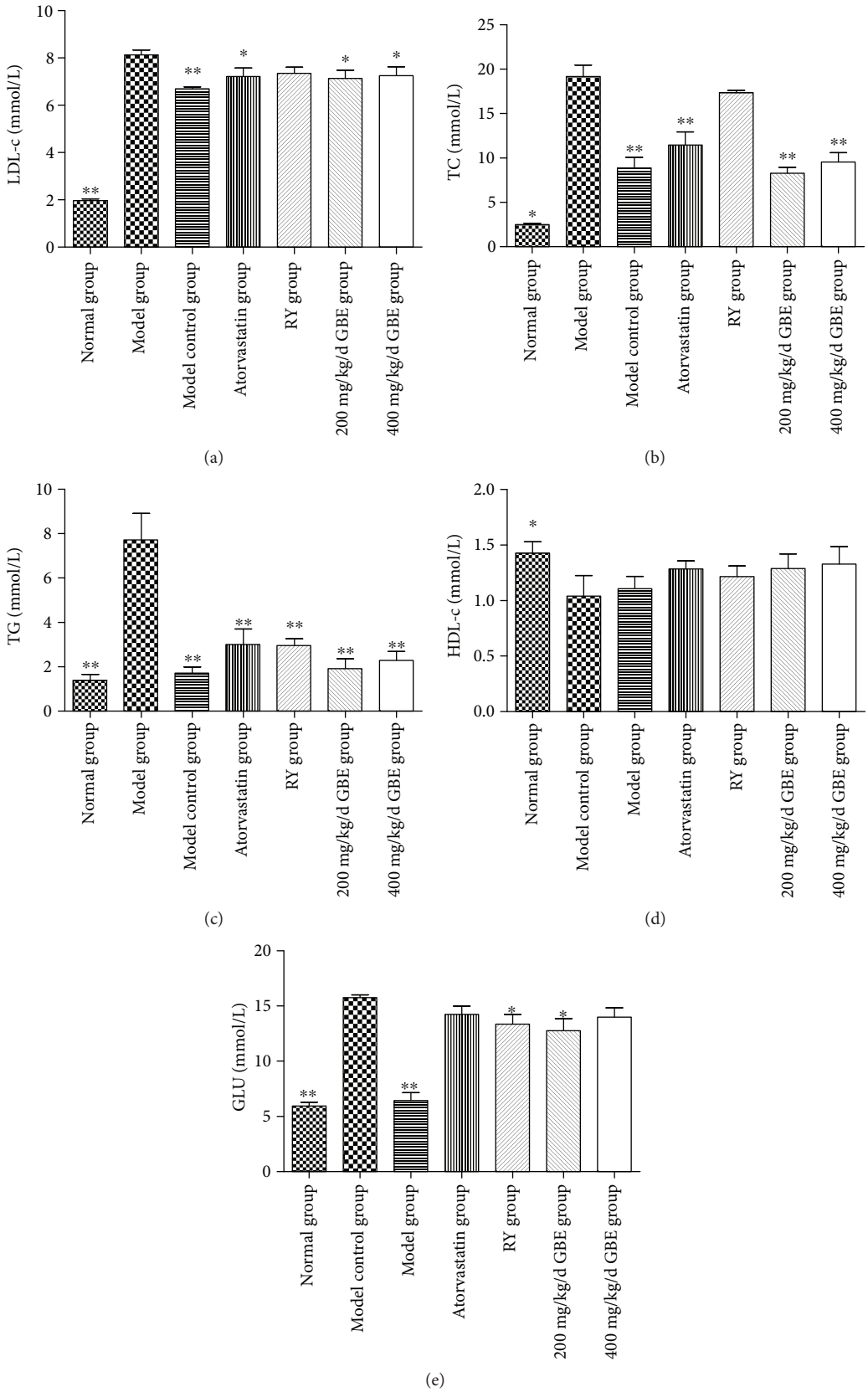


Figure 5: Effects of GBE on serum lipid and glucose profiles. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the model group. GBE: ginkgo biloba leaf extract (normal group $n = 18$, model group $n = 7$, model control group $n = 7$, atorvastatin group $n = 11$, rapamycin group $n = 15$, 200 mg/kg/day GBE group $n = 10$, and 400 mg/kg/day GBE group $n = 12$).

Compared to the model group, rapamycin did not affect the serum levels of LDL-c, TC, and HDL-c ($P > 0.05$, Figures 5(a), 5(b), and 5(d)), whereas rapamycin decreased the serum TG level (rapamycin group vs. model group: 7.7 $\pm$ 3.2 mmol/L vs. 3.0 $\pm$ 1.2 mmol/L, $P < 0.05$) (Figure 5(c)). The serum TG level in the rapamycin group was higher than that in the model control group, but the difference was not statistically significant ($P > 0.05$). The serum glucose level was higher in the model group compared to the normal group and model control group; rapamycin and 200 mg/kg/day GBE decreased the serum glucose level of diabetic mice ($P < 0.05$, Figure 5(e)), but the levels were still higher than those in the model control group ($P < 0.05$).

3.3. The Effects of GBE on Serum Inflammatory Cytokines. The serum inflammatory cytokines including IL-1β, TNF-α, and iNOS were significantly elevated in the model group compared to the normal group and model control group ($P < 0.05$, Figures 5(c)–5(e)). The levels of IL-1 and IL-6 in the model group and model control group were significantly higher than that in the normal group ($P < 0.05$); the difference between the model group and the model control group was not statistically significant ($P > 0.05$, Figures 6(a) and 6(b)). GBE at both doses of 200 mg/kg/day and 400 mg/kg/day reduced the serum levels of IL-6, IL-1, IL-1β, TNF-α, and iNOS of diabetic mice ($P < 0.01$, Figures 6(a)–6(e)). Atorvastatin and rapamycin significantly decreased the serum levels of IL-1β, TNF-α, and iNOS ($P < 0.01$, Figures 6(c)–6(e)). Furthermore, rapamycin has been revealed to decrease the serum levels of IL-1 ($P < 0.05$, Figure 5(b)).

3.4. The Effects of GBE on Plaque Lipid Disposition. Compared to the model control group, plaque lipid disposition area/intima area in the model group was significantly increased; atorvastatin, rapamycin, and GBE (200 mg/kg/day and 400 mg/kg/day) decreased the plaque lipid disposition area/intima area, and the difference was statistically different ($P < 0.05$) (Figures 7(a) and 7(b)). The effect of 200 mg/kg/day and 400 mg/kg/day GBE on the plaque lipid disposition area/intima area was not significantly different.

3.5. The Effect of GBE on Plaque Area and Collagen Area. Compared to the model control group, the plaque area/lumen area in the model group was significantly increased in the model group, whereas the collagen area/plaque area in the model group was significantly reduced. Atorvastatin, rapamycin, and GBE (200 mg/kg/day and 400 mg/kg/day) significantly (200 mg/kg/day and 400 mg/kg/day) decreased the plaque area/lumen area ($P < 0.05$, Figures 8(a) and 8(c)). On the contrary, they increased the collagen area/plaque area ($P < 0.05$, Figures 8(b) and 8(d)). Compared to the model control group, the effects of GBE at 200 mg/kg/day or 400 mg/kg/day on the plaque area/lumen area and collagen area/plaque area were not statistically different.

3.6. The Effect of GBE on Inflammatory Markers and α-SMA in the Atherosclerotic Plaques. Compared to the normal group and model control group, the expressions of CD68, MMP2, and MMP9 were significantly increased in the model group; atorvastatin, rapamycin, and GBE (200 mg/kg/day and 400 mg/kg/day) significantly decreased the expression of CD68, MMP2, and MMP9 in the plaques ($P < 0.05$, Figures 9(a)–9(d)). There was no statistical difference among the groups according to the expression of α-SMA. There was an increasing trend in the expression of α-SMA when the diabetic mice were treated with atorvastatin ($P = 0.074$, Figure 9(e)).

3.7. GBE Attenuates Endoplasmic Reticulum Stress by Upregulating Autophagy. Immunohistochemical staining and Western blotting revealed that the expression of mTOR was significantly increased in the model group and model control group compared to the normal group ($P < 0.05$, Figures 10(a), 10(b), 11(a), and 11(b)). Rapamycin, and GBE (200 mg/kg/day and 400 mg/kg/day) significantly inhibited the increased expression of mTOR in diabetic mice ($P < 0.05$, Figures 10(a), 10(b), 11(a), and 11(b)). There was an increasing trend in the expressions of mTOR in atherosclerotic plaques and aorta of the model group compared to the model control group, but the difference was not statistically significant ($P > 0.05$, Figures 10(a), 10(b), 11(a), and 11(b)).

There were no significant differences among the groups according to the expressions of LC3B and Beclin1 in the atherosclerotic plaques analyzed by immunohistochemical staining (Figures 10(a), 10(c), and 10(d)). There was an increasing trend in Beclin1 expression in the atherosclerotic plaques of the GBE group compared to the model group ($P = 0.081$, Figures 10(a) and 10(c)). Western blotting showed that there was no statistically significant difference between the model group and model control group according to the expressions of Beclin1 and LC3II/LC3I in the aortas ($P > 0.05$, Figures 11(c)–11(f)). Western blotting showed that GBE at a dose of 400 mg/kg/day significantly increased the expression of Beclin1 in aortas compared to the model group ($P < 0.05$, Figures 11(c) and 11(d)).

Immunohistochemical staining showed that the expression of SQSTM1/p62 in the atherosclerotic plaques of the model group significantly increased compared to the model control group ($P < 0.05$, Figures 10(a) and 10(e)). Western blotting displayed that there was a high level of SQSTM1/p62 in the aorta of the model group compared to the normal group and model control group ($P < 0.05$, Figures 11(g) and 11(h)). There was an increasing trend in the expression of SQSTM1/p62 in the aorta of the model group when compared to the model control group, but the difference was not statistically different. Rapamycin and GBE (400 mg/kg/day) inhibited the SQSTM1/p62 expression in atherosclerotic plaques and aortas of diabetic mice ($P < 0.05$, Figures 10(a), 10(e), 11(g), and 11(h)). Atorvastatin has a trend to decrease the expression of SQSTM1/p62 in the aorta, but the difference was not statistically different (Figures 11(g) and 11(h), $P = 0.061$).

Western blot revealed that the expressions of endoplasmic reticulum stress markers including p-JNK, CHOP, and Caspase-12 in the aortas of the model group were higher than those in the model control group ($P < 0.05$, Figures 12(a)–12(f)); atorvastatin and GBE at a dose of 400 mg/kg/day decreased the expressions of p-JNK, CHOP,

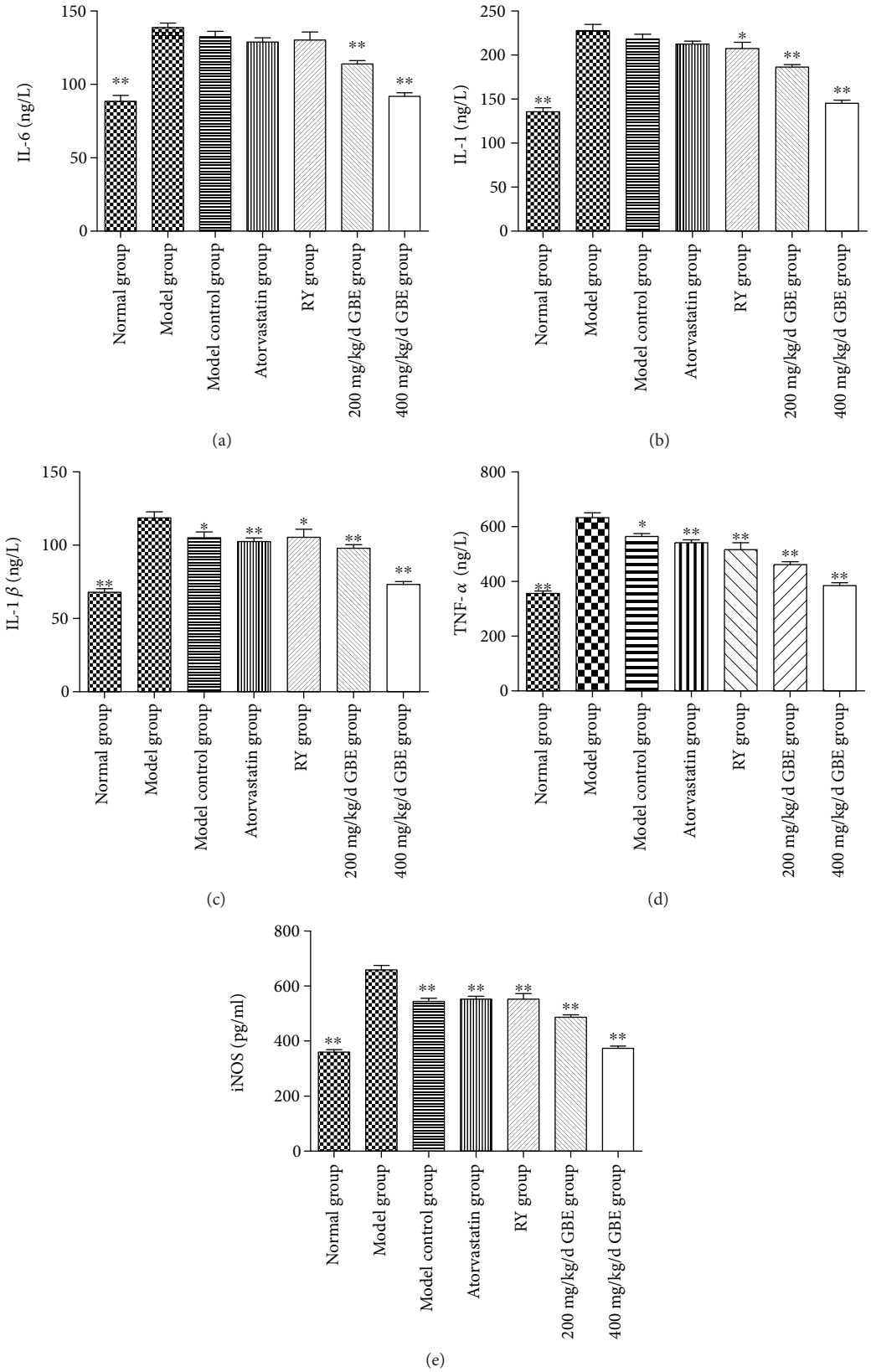
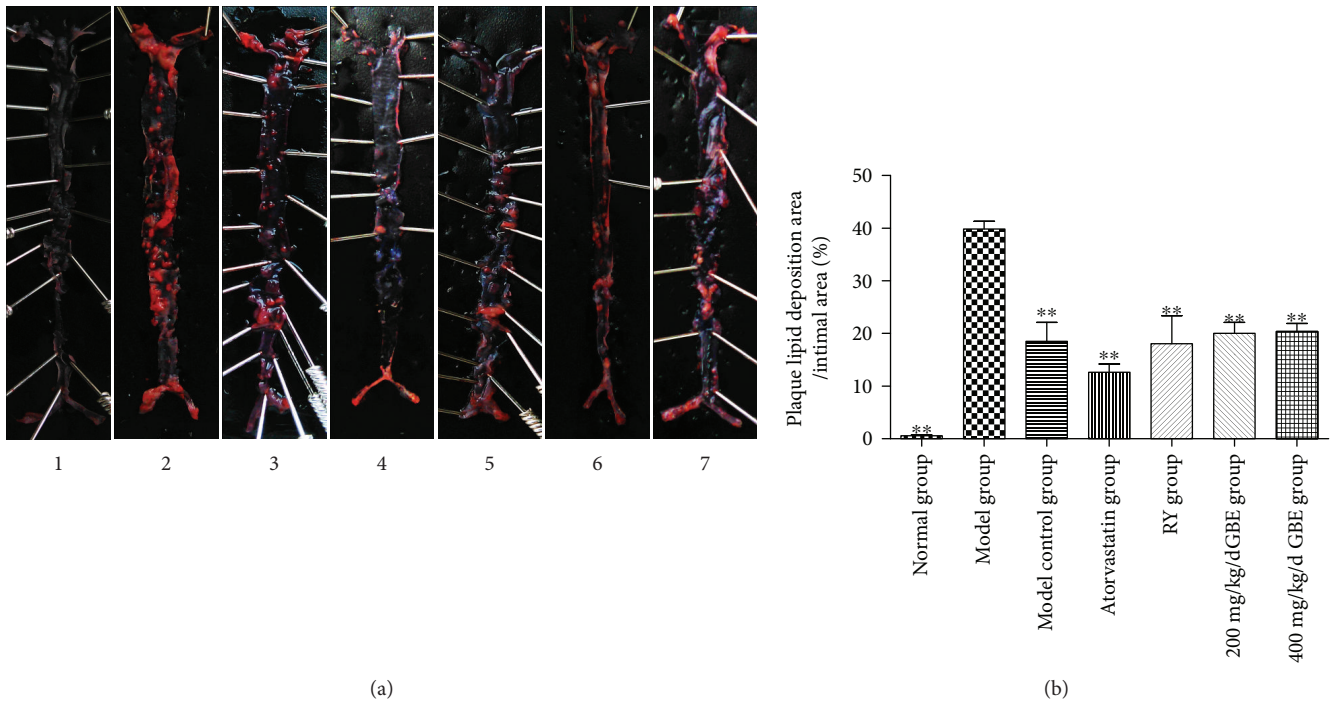


Figure 6: Effects of GBE on serum inflammatory cytokines. The level of IL-6, IL-1, IL-1β, TNF-α, and iNOS were determined by ELISA. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the model group (normal group $n = 18$; model group $n = 7$, model control group $n = 7$, atorvastatin group $n = 12$, rapamycin group $n = 15$, 200 mg/kg/day GBE group $n = 10$, and 400 mg/kg/day GBE group $n = 12$).



(a) (b)

Figure 7: Effects of GBE on plaque lipid disposition. (a) Red "O" staining for en face analysis. (b) Plaque lipid disposition area/intima area. $^{**}P < 0.01$, compared to the model group. (1) Normal group ($n = 3$), (2) model group ($n = 3$), (3) model control group ($n = 3$), (4) atorvastatin group ($n = 3$), (5) rapamycin group ($n = 3$), (6) 200 mg/kg/day GBE group ($n = 3$), and (7) 400 mg/kg/day GBE group ($n = 3$).

and Caspase-12 ($P < 0.05$, Figures 12(a)–12(f)). Rapamycin decreased the expression of CHOP and Caspase-12 ($P < 0.05$, Figures 12(c)–12(f)); the difference of the p-JNK level was not statistically different ($P > 0.05$, Figures 12(a) and 12(b)).

4. Discussion

Endoplasmic reticulum stress is a common pathological mechanism for diabetes mellitus and atherosclerosis; thus, attenuating endoplasmic reticulum stress is one of the important strategies for inhibition of diabetes mellitus and diabetic vascular complications. Endoplasmic reticulum stress has been exposed to upregulated autophagy, which in turn protectively inhibits unfolded protein response in a feedback manner [41]. Atherosclerosis is one of the main complications of diabetes mellitus. STZ-induced ApoE-/- mice were commonly used to establish diabetic atherosclerosis models [42]. Network pharmacology provides a new approach for exploring drug targets. The network pharmacology utilized in the present study predicted that ginkgo biloba leaf attenuates atherosclerosis through mTOR and NF-κB-mediated inflammation signaling pathways. Animal experiments showed that high glucose accelerated atherosclerotic plaques, characterized by an increase in both plaque area/lumen area and plaque lipid disposition area/intima area and a decrease in the collagen area/plaque area. Furthermore, the increased expressions of CD68, MMP2, and MMP9 in the atherosclerotic plaques were induced by high glucose. High glucose promotes the expression of endoplasmic reticulum stress markers including p-JNK, CHOP, and Caspase-12. The expressions of mTOR and SQSTM1/p62 were upregulated in the aorta of the model group compared to the normal group. In addition, the expression of SQSTM1/p62 in the atherosclerotic plaques of the model group was higher than that in the model control group. mTOR inhibitor rapamycin and GBE stabilized atherosclerotic plaques, featured by a decreased plaque area/lumen area and plaque lipid disposition area/intima area. The expressions of CD68, MMP2, and MMP9 in the atherosclerotic plaques were decreased by rapamycin and GBE. Rapamycin and GBE inhibited the SQSTM1/p62 expression of diabetic ApoE-/- mice and, consequently, reduced the expression of endoplasmic reticulum stress markers, including p-JNK, CHOP, and Caspase-12, via inhibition of the mTOR-dependent signaling pathway. GBE reduced serum glucose and inflammatory cytokines including IL-1, IL-6, IL-1β, TNF-α, and iNOS. Consistent with the previous studies, the TC, LD, and TG levels were increased in STZ-induced ApoE-/mice compared to ApoE-/- mice and C57BL/6J mice [42]. GBE has a positive regulating effect on the lipid profiles.

Moderate autophagy has the ability to prevent the onset and progression of diabetes mellitus and atherosclerosis [43, 44]. Overactivation of mTOR is linked to impaired autophagy. It has been demonstrated that metformin, an agent used to control hyperglycemia in diabetes mellitus, inhibits mTOR activity and promotes autophagy and protects against endothelial cell senescence [45, 46]. The present study showed that mTOR is activated in the aorta of diabetic ApoE-/- mice compared to C57BL/6J mice; furthermore, there was an increasing trend in the expression of mTOR in the atherosclerotic plaques and aortas of the model group

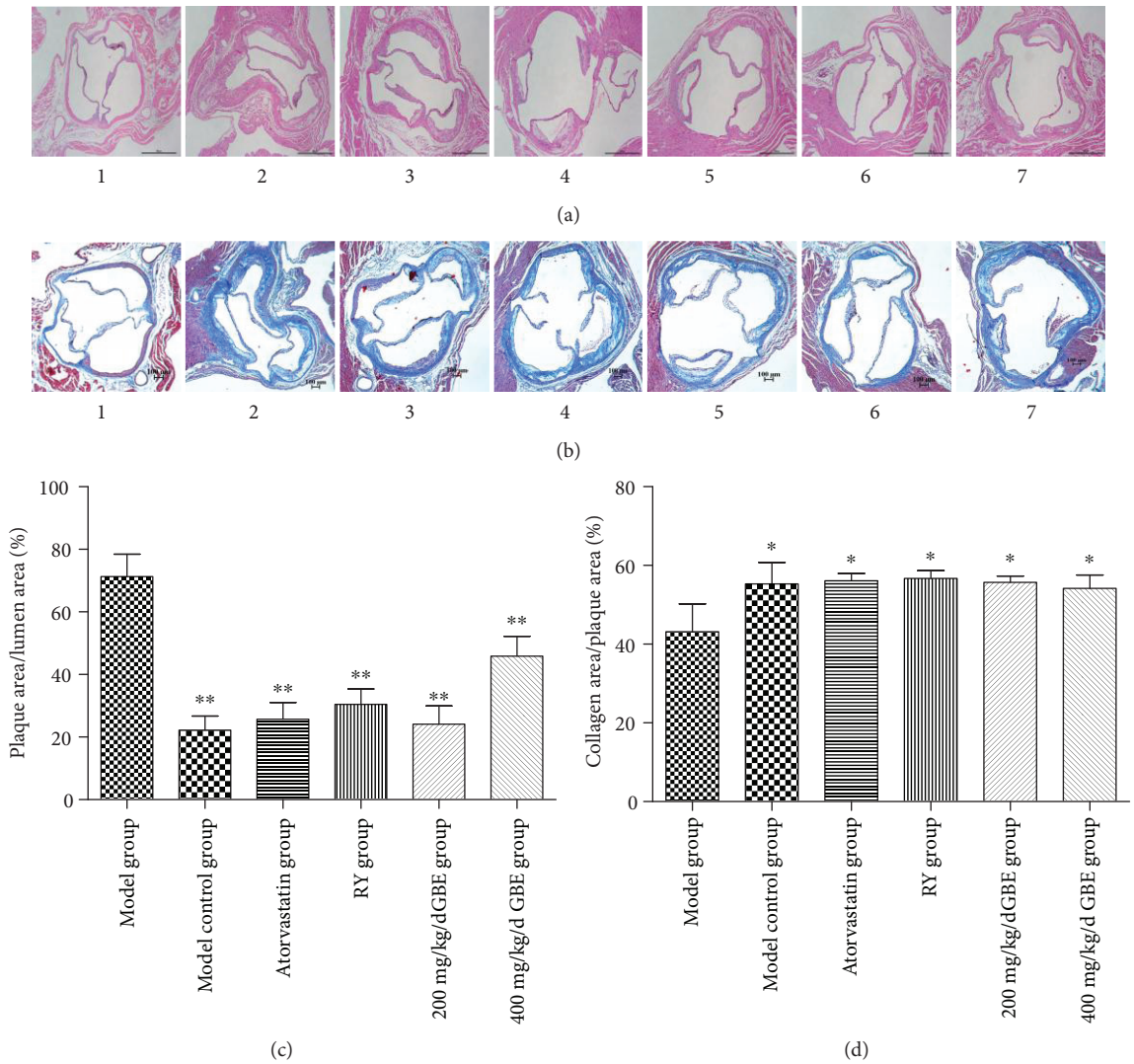


Figure 8: Effects of GBE on plaque area/lumen area and collagen area/plaque area. (a, c) HE staining for determination of plaque area/lumen area; (b, d) MASSON staining for determination of collagen area/plaque area. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the model group. (1) Normal group ($n = 5$), (2) model group ($n = 3$), (3) model control group ($n = 3$), (4) atorvastatin group ($n = 4$), (5) rapamycin group ($n = 5$), (6) 200 mg/kg/day GBE group ($n = 4$), and (7) 400 mg/kg/day GBE group ($n = 4$). (a, b) 40x.

compared to the model control group. As mentioned above, SQSTM1/p62 accumulation is closely associated with impaired autophagy. The present study revealed that the expression of SQSTM1/p62 in the aortas of diabetic ApoE-/- mice and nondiabetic ApoE-/- mice were both increased compared to C57BL/6J mice; additionally, SQSTM1/p62 was upregulated in atherosclerotic plaques of diabetic ApoE-/- mice compared to nondiabetic ApoE-/- mice. These findings were supported by the study of Fetterman et al. [25] that inadequate autophagy contributes to endothelial dysfunction in patients with diabetes. According to them, SQSTM1/p62 aggregated in endothelia indicating an impaired autophagy; LC3II has shown an increasing trend in a feedback manner. We also showed that rapamycin and GBE inhibited the expression of SQSTM1/p62 which increased in diabetic mice via blocking of mTOR signaling. Collectively, overactive mTOR and SQSTM1/p62 contribute to diabetic atherosclerosis [12, 25, 27, 28]. The downregulating effect of rapamycin on SQSTM1/p62 was coincident with the previous study [28] that high glucose promotes the activation of mTOR signaling, while rapamycin inhibited the accumulation of SQSTM1/p62 and microvascular endothelial cell apoptosis induced by high glucose by blockage of the upregulation of mTOR. Furthermore, the effect of GBE on autophagy in the present study supported by Zhang et al. [47] that luteolin, one of the active compounds of GBE which attenuate foam cell formation and macrophage apoptosis by promoting autophagy.

Endoplasmic reticulum stress-mediated apoptosis accelerates diabetic atherosclerosis. Autophagy is required to remove misfolded proteins and eliminate nonfunctioning mitochondria. Endoplasmic reticulum stress markers including p-JNK, CHOP, and Caspase-12 were increased in diabetic ApoE-/- mice in the present study, supported by previous studies [48, 49]. Rapamycin and GBE inhibited the expressions of p-JNK, CHOP, and Caspase-12 by reducing

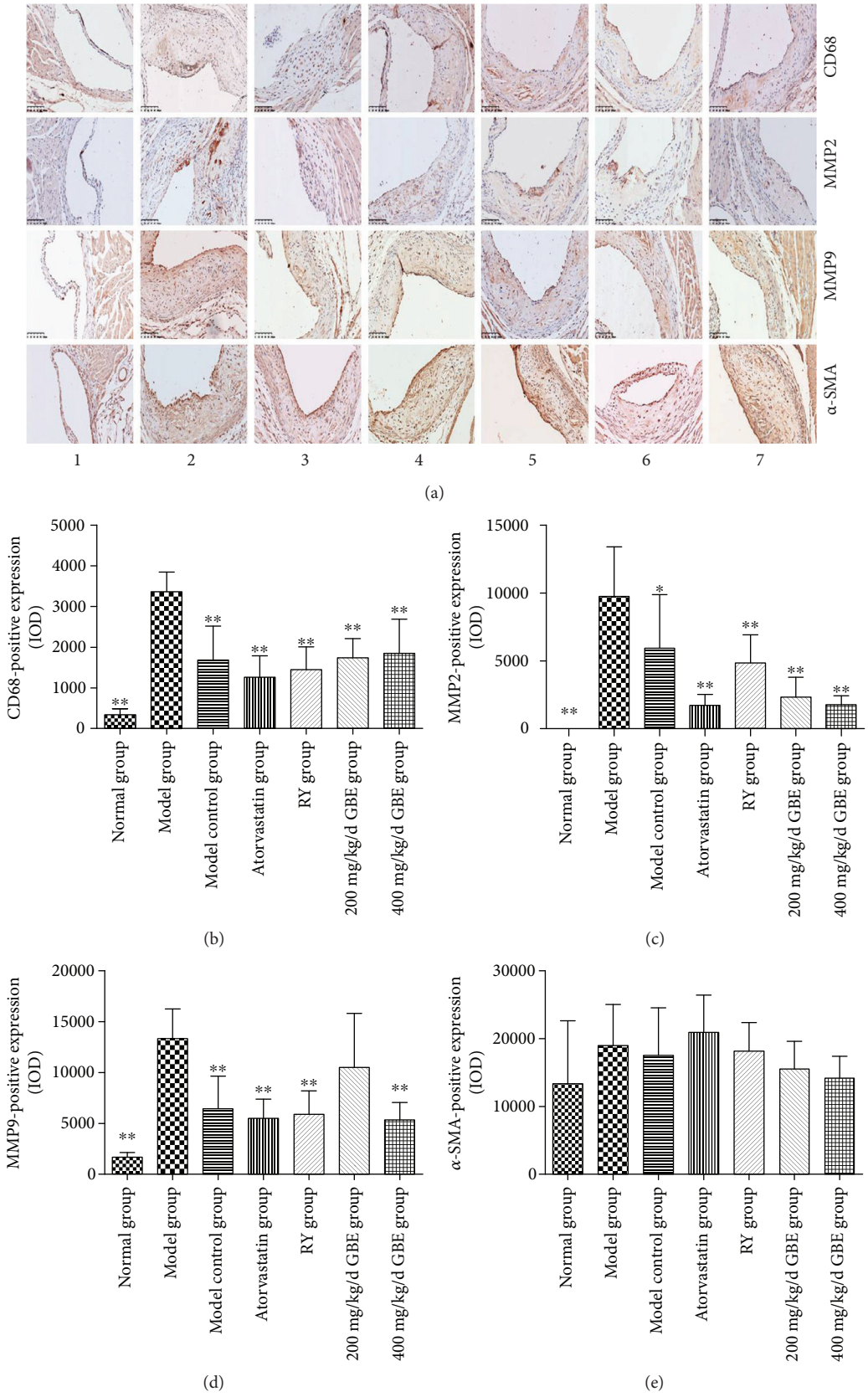


Figure 9: Immunohistochemical staining for the expression of CD68, MMP2, MMP9 and α-SMA in the atherosclerotic plaques. $^{**}P < 0.05$, $^{**}P < 0.01$, compared to model group. (1) Normal group ($n = 3$), (2) model group ($n = 5$), (3) model control group ($n = 5$), (4) atorvastatin group ($n = 5$), (5) rapamycin group ($n = 5$), (6) 200 mg/kg/day GBE group ($n = 5$), and (7) 400 mg/kg/day GBE group ($n = 5$) (immunohistochemical staining 200x).

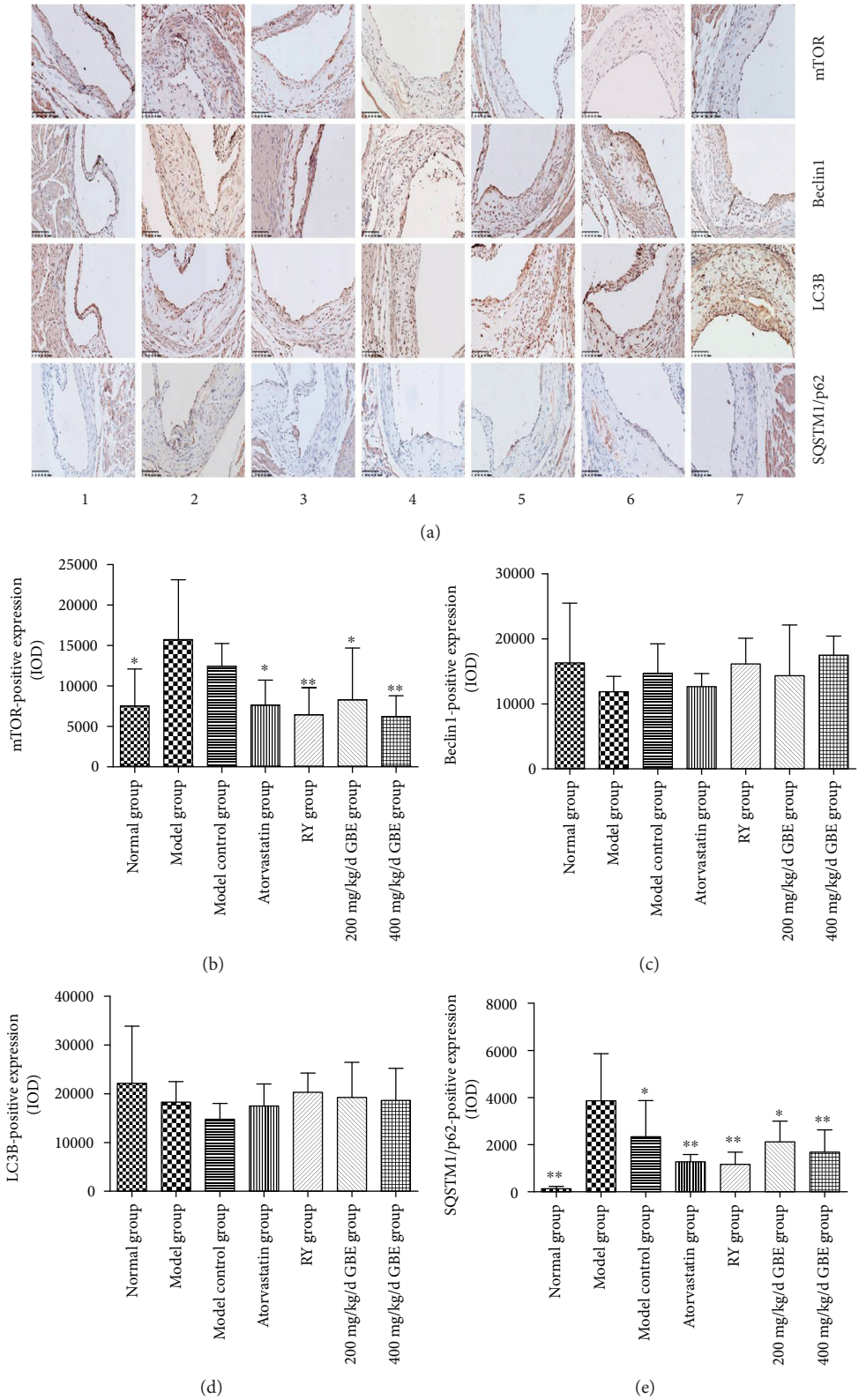


Figure 10: Effects of GBE on autophagy hallmark expressions in the atherosclerotic plaques. Immunohistochemical staining for the expression of mTOR, Beclin1, LC3B, and SQSTM1/p62 in the plaques. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the model group. (1) Normal group ($n = 3$), (2) Model group ($n = 5$), (3) model control group ($n = 5$), (4) atorvastatin group ($n = 5$), (5) rapamycin group ($n = 5$), (6) 200 mg/kg/day GBE group ($n = 5$), and (7) 400 mg/kg/day GBE group ($n = 5$) (immunohistochemical staining 200x).

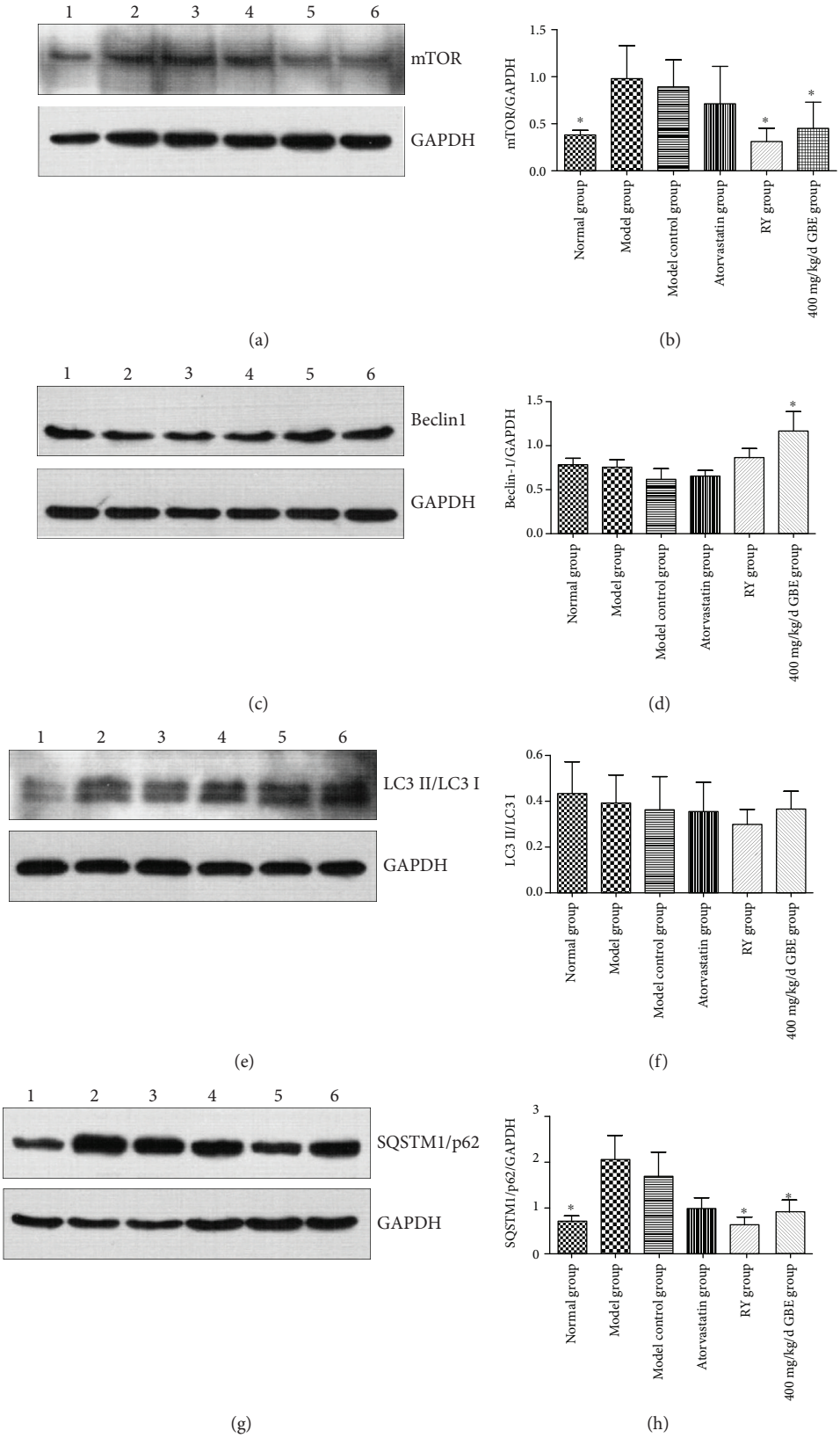


Figure 11: Effects of GBE on autophagy hallmark expressions in the aorta of mice. The expression of mTOR, Beclin1, LC3II/LC3I, and SQSTM1/p62 in the aorta was determined by Western blotting. (a, b) The expression of mTOR ($n = 3$ in each group); (c, d) the expression of Beclin1 ($n = 6$ in each group); (e, f) the expression of LC3II/LC3I ($n = 5$ in each group); (g, h) the expression of SQSTM1/p62 ($n = 3$ in each group). $^*P < 0.05$, compared to the model group. (1) Normal group, (2) model group, (3) model control group, (4) atorvastatin group, (5) rapamycin group, (6) 200 mg/kg/day GBE group, and (7) 400 mg/kg/day GBE group.

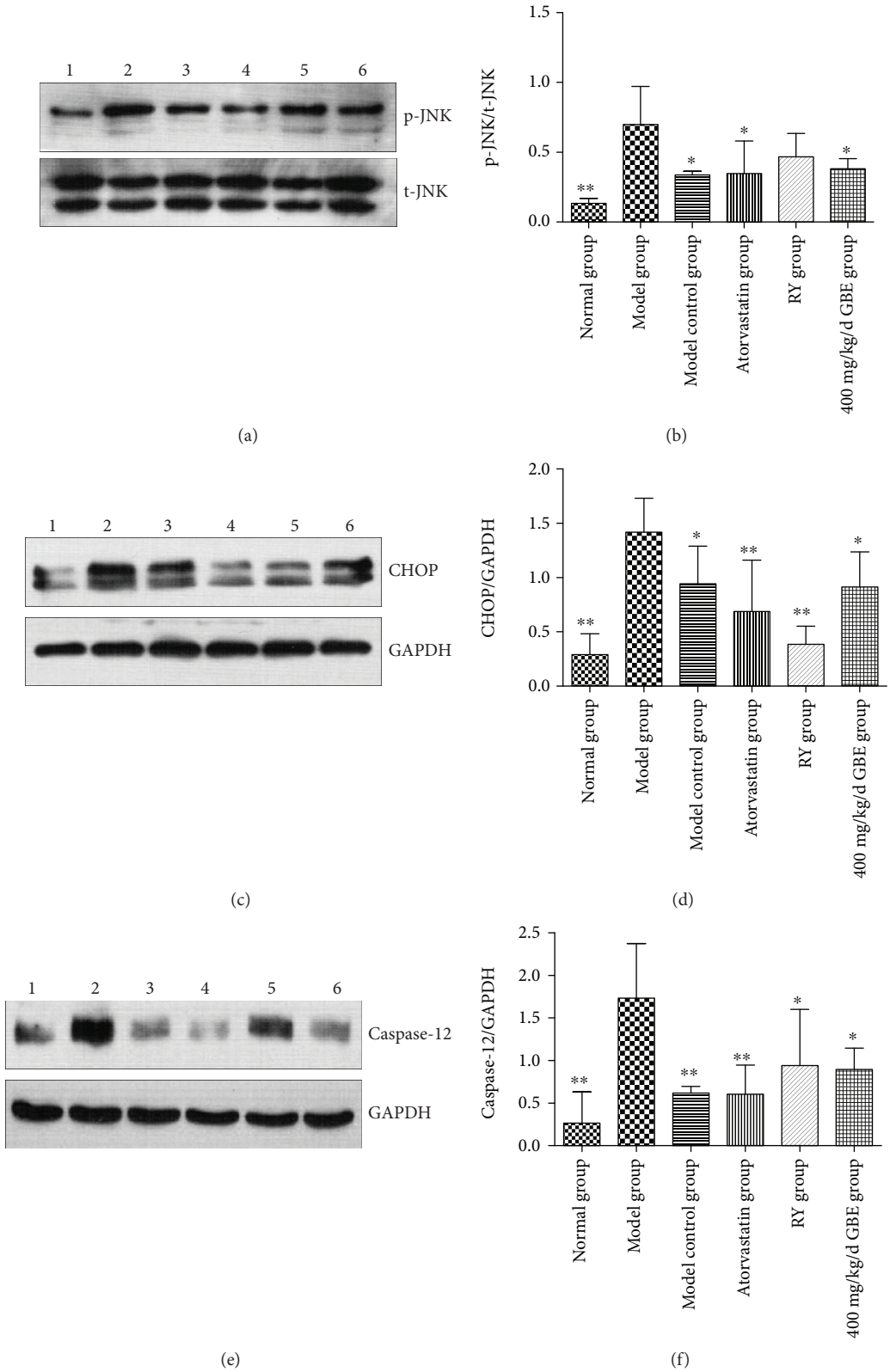


Figure 12: Effects of GBE on endoplasmic reticulum stress hallmark expressions in the aortas of mice. The expressions of p-JNK, CHOP, and Caspase-12 were determined by Western blotting. (a, b) The expression of p-JNK ($n = 3$ in each group); (c, d) the expression of CHOP ($n = 4$ in each group); (e, f) the expression of Caspase-12 ($n = 3$ in each group). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the model group. (1) Normal group, (2) model group, (3) model control group, (4) atorvastatin group, (5) rapamycin group, (6) 200 mg/kg/day GBE group, and (7) 400 mg/kg/day GBE group.

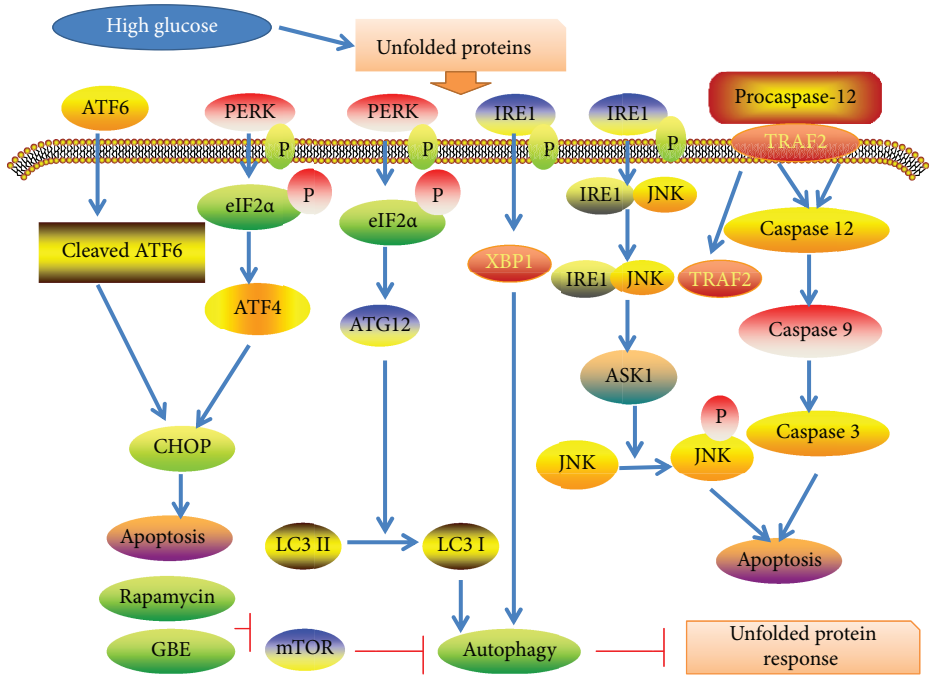


Figure 13: The mechanism by which GBE attenuates diabetic atherosclerosis. GBE attenuates unfolded protein response by upregulation of autophagy via inhibition of mTOR.

SQSTM1/p62 via inhibition of mTOR signaling, indicating a protective role of autophagy in diabetic atherosclerosis (Figure 13).

High glucose, representing a disordered metabolism, is also a chronic inflammatory process. Endoplasmic reticulum stress which was upregulated in the context of high glucose activates NF-κB by its IRE1α and PERK signaling, attributable to macrophage adhesion to the vascular wall and development of atherosclerosis [50]. These inflammatory cytokines increased in the diabetic condition, promote the biogenesis of ROS, and activate NF-κB in a positive feedback manner, leading to further generation of inflammatory cytokines [49, 50]. Atorvastatin, rapamycin, and GBE attenuated diabetes-induced endoplasmic reticulum stress in diabetic ApoE-/- mice, blocking the positive feedback loop of endoplasmic reticulum stress and inflammation, which consequently reduced the inflammation in vivo, characterized by decreased inflammation cytokines, and decreased infiltration of macrophages and inhibited MMP expression. The reduced expression of MMP by GBE is probably attributable for attenuation of endoplasmic reticulum stress or inhibition of SQSTM1/p62 [27].

The present study revealed that GBE downregulated the blood glucose level. According to previous studies, the mechanisms by which GBE reduced blood glucose involved antioxidant, pancreas protection, and insulin sensitivity enhancement [51, 52]. In the present study, 400 mg/kg/day GBE reduced the plasma glucose level, whereas it did not affect the serum glucose level, which might be attributable to different detecting methods and the predisposition to stress response in the model group mice when tail blood samples were obtained. The mechanisms that cause different effects of 400 mg/kg/day GBE on plasma glucose and serum glucose level need further investigation. Furthermore, the attenuation of endoplasmic reticulum stress in the present study was probably another mechanism for GBE against hyperglycemia. The findings about the effect of rapamycin on blood glucose are controversial. It has been shown that activation of mTOR contributes to the secretion of insulin and increase in insulin sensitivity. On the contrary, other studies revealed that enhancing macrophage autophagy by the mTOR inhibitor may be a viable therapeutic or preventative approach to inflammatory disease, obesity insulin resistance, and diabetes [44, 53]. Interestingly, the present study showed that rapamycin at a dose of 1 mg/kg/day exerts a positive effect on serum glucose in STZ plus high-fat diet-induced diabetic ApoE-/- mice, and the finding was supported by den Hartigh et al. [16]. Further studies needed to investigate the effect of rapamycin on blood glucose in STZ plus high-fat diet-induced ApoE-/- mice.

According to Elloso et al. [54], ApoE-/- mice dosed q.o.d. with 1, 2, 4, or 8 mg/kg of sirolimus for 13 weeks associated with approximately 30% increase in LDL-c, regardless of the dosage implemented. Sirolimus treatment of 8 mg/kg was associated with an increase in HDL-c of more than 40 mg/dL. Zhao et al. [38] showed that low-dose oral sirolimus (0.1 mg/kg, 0.3 mg/kg, and 1 mg/kg for 8 or 16 weeks) effectively delayed the progression of atherosclerosis and modulated the plaque phenotype in LDL-rKO mice, without influencing total lipid levels, indicating that low-dose oral sirolimus is well tolerated and exerts a potent antiatherosclerotic effect. The present study showed that low dose of rapamycin (1 mg/kg/day) did not affect LDL-c and TC levels of diabetic ApoE-/- mice; low dose of rapamycin reduced the

TG level compared to the model group, but the level was still higher than that in the model control group ($P > 0.05$). Therefore, the downregulating effect of GBE on TG was probably attributable to glucose metabolism improvement. The lipid regulation effect of GBE against hyperlipidemia was consistent with the study of Wei et al. [55] that GBE inhibited high-fat diet-induced increased of serum TG, TC, and LDL-c levels. According to Zhang et al. [56], GBE exerts multidirectional lipid-lowering effects by limitation of the absorption of cholesterol, inactivation of HMGCoA, and favorable regulation of profiles of essential polyunsaturated fatty acid.

5. Study Limitations

Firstly, the present animal experiment was not capable of verifying each active compound that protects against atherosclerosis included in network pharmacology. However, network pharmacology indeed provides a feasible approach for predicting the potential active compound and target. Due to the limited available information of ginkgolide, the antiatherosclerotic effect needs further investigation. The expressions of mTOR and SQSTM1/p62 were elevated in the aorta of both the model group and the model control group when compared to the normal group. Secondly, in the present study, beside a significantly increased expression of SQSTM1/p62 in the atherosclerotic plaques in the model group compared to the model group, there was an increasing trend of mTOR and SQSTM1/p62 expressions in the aorta of the model group compared to the model group, maybe due to a relatively small sample. However, we concluded that overactive mTOR and SQSTM1/p62 were contributors to atherosclerotic plaques. Thirdly, the dose-dependent manner of GBE against diabetic atherosclerosis was not found, and it was not clear whether there exists a dose-dependent manner in attenuation of diabetic atherosclerosis with the dose of GBE less than 200 mg/kg/day. Fourth, GBE at doses of 200-400 mg/kg/day may reach a platform in attenuation of diabetic atherosclerosis. The optimal dose of GBE for attenuation of diabetic atherosclerosis needs further investigation, because there was lack of study on the protective effect of diabetic atherosclerosis in vivo. Furthermore, GBE may reduce SQSTM1/p62 accumulation by activating the high glucose-inhibited AMPK upstream of mTOR. However, it did not affect the findings of the present study. The protective effect of mTOR inhibitor rapamycin against diabetic atherosclerosis indicated that GBE attenuated diabetic atherosclerosis and endoplasmic reticulum stress-mediated apoptosis by blockage of SQSTM1/p62 via direct inhibition of mTOR. Lastly, given the critical role of macrophage autophagy in atherosclerotic progression, further study should focus on the effect of GBE on autophagy and endoplasmic reticulum stress in macrophages.

6. Conclusions

Our study demonstrates that impaired autophagy was associated with diabetic atherosclerosis, characterized by upregulated mTOR expression and SQSTM1/p62 accumulation. Both mTOR inhibitor rapamycin and GBE protected against diabetic atherosclerosis by attenuation of endoplasmic reticulum through downregulation of SQSTM1/p62 by inhibiting mTOR signaling.

Abbreviations

GBE: Ginkgo biloba leaf extract
mTOR: Mammalian target of rapamycin
AGE: Advanced glycation end products
PI3K: Phosphatidylinositol 3 kinase
AMPK: AMP-activated protein kinase
IRS1: Insulin receptor substrate 1
CMECs: Cardiac microvascular endothelial cells
STZ: Streptozotocin.

Data Availability

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

Ethical Approval

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Xiyuan Hospital, China Academy of Chinese Medical Sciences, in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Yue Liu contributed to the topic conception, manuscript revision, and decision to submit for publication. Jinfan Tian, Mohammad Sharif Popal, and Yanfei Liu participated in the animal study. Mohammad Sharif Popal and Rui Gao helped to revise the manuscript. Shuzheng Lyu and Keji Chen participated in the conception of the study design.

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Review Article

Effects of Cornelian Cherry on Atherosclerosis and Its Risk Factors

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Functional food represents an important alternative management of atherosclerosis, its risk factors, and clinical complications. Atherosclerosis is characterized by microinflammation, formation of atheromatous lipoprotein-rich plaques, and protrombogenic status. Cornelian cherry (*Cornus mas L.*, CC) contains polyphenols influencing all three components of atherosclerosis. Its high antioxidant potential, verified in experimental studies, exhibited a pronounced decrease of inflammatory markers. CC treatment demonstrated a favourable effect on lipid spectrum (comparable with statins), decrease of glycemia, and increase of insulin (comparable with glibenclamide). Polyphenols identified in CC exhibited both direct antiplatelet effects and reduction of platelet hyper-reactivity mediated via attenuation of oxidative stress. The first clinical trials confirmed a clinically relevant decrease of total and low-density lipoprotein cholesterol, triacylglycerols, lipoproteins, amelioration of inflammatory activity, and insulin secretion improvement after the treatment with CC polyphenolic compounds. However, the limitation of published studies is the use of undefined cultivars of CC, their experimental nature, small scale, and missing longitudinal trials. Nevertheless, biochemical properties of CC, hitherto described, predispose its products for the adjuvant management of atherosclerosis.

1. Introduction

Cornelian cherry (*Cornus mas L.*, CC) is used as a food item as well as a traditional herbal drug in wide belt from middle and south Europe, through the Asia Minor and Caucasus to the Sub-Himalayan region. The archeological evidence of its use is dated to the La Tène period (range ca 450 B.D. to 100 A.D.) [1]. A Traditional medicine practice utilizes its anti-inflammatory and hypoglycemic properties in therapy of fever, dyspepsia and diabetes mellitus, rheumatic pain, nonspecific skin and urinary tract infections, and liver diseases in either a form of over-the-counter (OTC) herbal medical products or natural fruits and leaves [2–4]. The fruit of *C. mas*, together with the fruit of *C. officinalis*, has also a long history of use in traditional Chinese medicine. Known as *shānzhūyú*, it is used to retain the jing, essence, to tonify the kidneys, and in cases of spermatorrhea [5].

Beneficial effects of CC result from its favourable properties, mainly (i) fresh cornelian cherry fruits contains twice as much ascorbic acid as oranges; (ii) compared to other juices obtained from plum, pear and apple, cornelian cherry juice contains high levels of calcium, reaching 10 folds higher than other juices; (iii) CC has high contents of potassium and magnesium but is low in sodium and other essential minerals like Cu, Mn, Fe, and Zn; (iv) CC levels of toxic elements are also negligible; and (v) the fruits are excessively rich in organic acids, tannins, anthocyanins, phenols, and other antioxidants [6–9].

The first scientific analysis reported the CC content of anthocyanins in 1939 [10] confirmed long-term research on active compounds of CC. The crude extracts of fruits

and other parts of the plant as well as their pure isolates reach in polyphenolic compounds exhibit a broad spectrum of pharmacological activities such as antimicrobial, antidiabetic, antiatherosclerotic, antiparasitic, anticancer, cytoprotective, hepatoprotective, neuroprotective, and renal-protective, antiplatelet, and antiglaucomic activities [11, 12].

Interest in new sources of anti-inflammatory and antioxidant compounds has recently become a major research issue. Cornelian cherry receiving particular attention for its significant amounts of phenolic compounds and vitamins, which exhibit a wide range of biological and pharmacological properties, may represent a promising source capable of fighting against atherosclerosis. This study was aimed at increasing knowledge regarding the effect of Cornelian cherry on an atherosclerotic process.

2. Atherosclerotic Process

Atherosclerosis, its clinical complications, and associated diseases such as myocardial infarction, stroke, diabetes mellitus, peripheral arterial occlusive disease, and hypertension are the leading causes of total mortality in industrial countries [13]. The detailed recognition of the etiopathology provides a background for the targeted therapy.

Life lasting microinflammation has a crucial role in the pathogenesis of atherosclerosis and subsequent vascular damage [14, 15]. The complex atherosclerotic process is generalized in humans independently of age [16], and it is accelerated by risk factors such as dyslipidemia, hyperglycemia and/or diabetes mellitus, obesity and/or unbalanced nutrition, hypertension, sedentarism, smoking, permanent stress, and environmental factors [17]. Atherosclerosis is in short characterized by excessive fibrosis of the intima, fatty plaque formation due to accumulation of predominantly low-density lipoprotein (LDL) cholesterol, proliferation of smooth muscle cells due to increased oxidative stress and/or cytokines, and infiltration and/or migration of a group of cells such as monocytes, T cells, and platelets, which are formed in response to progressing microinflammation [18]. Penetration of the cells into the vascular wall is conditioned by the expression of leukocyte and chemokine adhesion molecules in which the transcription is performed by nuclear factor-κB (NF-κB). Proinflammatory molecules, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 18 (IL-18) cytokines, C-reactive protein (CRP), adhesion molecules, matrix metalloproteinases (MMP-9 or gelatinase B) produced by monocytes, macrophages and/or adipose tissue further potentiate microinflammation, and oxidative stress [19, 20].

Inhibitory monocyte chemokine protein (MCP-1) increased level is early and persistent markers of progression of the process. Another important consequence of endothelial damage is the dysfunction of endothelial nitric oxide synthase (eNOS)/NO pathway with the decrease of eNOS or NO levels and impairment of a natural antiproliferating and anti-inflammatory effect of NO [21–24].

Atherosclerosis accelerates by an increased oxidation of LDL cholesterol to oxLDL cholesterol and/or by hyperglycemia [25, 26]. Severe damage to the arterial wall is associated with increased secretion of cytokines and growth factors as interleukin 1 (IL-1) and tumor necrotising factors by smooth muscles and endothelial cells, which causes penetration of various cells into a plaque. The core of a plaque consists of debris from cell lesions, foam cells, calcium, cholesterol esters, and a mass of fatty substances [27]. Their exposition to the blood initiates coagulation cascade in a vulnerable plaque [28]. An atherosclerotic process itself is associated with an increased level of fibrinogen, acute-phase reactant responding to inflammations or infections, and with increased level of factor VII, coagulate protein involved in thrombogenesis [29, 30].

3. Active Antiatherosclerotic Compounds in Cornelian Cherry

The CC fruit content varied dependently on the region and genotype in the range of 0.1–0.3% fat, 0.4% protein, 21.7% carbohydrate, 0.8% ash, 0.5% dietary fibre, 6.6–25.2% total sugar, (33.1–43.1% fructose and 53.6–63.1% glucose), 4.22–23.01% reducing sugars, and 14.96–38.87 mg/100 g of vitamin C, as well as 15 amino acids [31–34].

CC juice contains high level of calcium (323 mg/L) exceeding 10-fold content in plum, pear, or apple juices and a comparable amount of potassium, sodium, zinc, and magnesium, while copper level is significantly lower [9].

CC fruit has been found to contain a wide range of phytochemicals with a biological effect, including tannins (131.51–601.2 mg/L), organic acids (4.6–7.4%), anthocyanin, fatty acids, flavonoids [35], and at least 16 phenolic compounds which generally vary from 29.76 to 74.83 mg/g dry matter [36]. The content of CC anthocyanins varied between 36.35 and 116.38 mg/100 g and consists of delphinidin 3-O-beta-galactopyranoside, cyanidin 3-O-beta-galactopyranoside, and pelargonidin 3-O-beta-galactopyranoside, myricetin-3-arabinose, quercetin-3-galactoside, and gallic acid [32, 36, 37]. Of special interest is the high level of polyphenolic compound gallic acid (45.5 mg/g) with highly expressed antioxidant activity evaluated as a ferric-reducing antioxidant power (FRAP), which was 10-fold higher in CC in comparison with apple, pear, or plum [6, 9]. Sochor et al. [38] identified a chlorogenic acid in all assayed samples of Eastern and Middle Europe CC cultivars (Nero, Titus, and Vygotsky) as the major polyphenolic compounds. In contrast, Pawlowska et al. [39] found a quercetin 3-O-D-glucuronide as the major flavonoid constituent of CC fruits from the Italian region. In South-East Serbia CC genotypes, high level of (+) catechin and (–) epicatechin (41.23% and 41.96%, respectively) together with lower content of procyanidin B2 (16.80%) has been found [40].

In addition, terpene and other secondary metabolites [41] may importantly contribute to high antioxidant capacity and beneficial properties of different CC cultivars. Although the terpenes with proven antioxidant properties like α-phellandrene and β-myrcene were found in different investigated fruits, the highest content of these compounds was measured in cherry silver berry fruits [42]. Since secondary metabolites represent an integrated defence mechanisms of the plants, such properties may reflect beneficial effects on

the health [43]. Due to strong antioxidant and anti-inflammatory properties, all polyphenolic compounds mentioned above have a big chance to fight against all phases of the atherosclerotic process.

Similarly, different polyphenolic compounds may significantly contribute to the nutraceutical effect of plants such as grapevine [44, 45], *Aronia melanocarpa* [46] and many others.

4. Effects of Cornelian Cherry on Atherosclerosis

4.1. "In Vitro" and Animal Studies. CC is capable to positively influence the classical risk factors of atherosclerosis. There is important evidence for the mutual antioxidant and hypolipidemic effect of CC, which could be mediated mainly by an antioxidant-related effect shown both *in vitro* and *in vivo* experimental conditions.

Sozański et al. [47] studied effects of CC fruit lyophilisate on peroxisome proliferator-activated receptor α (PPAR α) protein expression and atheromatous plaque changes in hypercholesterolemic rabbits. CC in a dose of 100 mg/kg BW caused a 44% decrease in triacylglycerols (TG) and prevented the development of atheromatous changes in the aorta. Amelioration of atherosclerosis was associated with a significant elevation of hepatic PPAR α protein expression which was considered a central mechanism protecting against fibrinogenesis and neoformation of collagen [48]. An antiatherogenic effect of CC on hepatic function was supported also by Celep et al. [49] who observed increased total antioxidant capacity of the liver, however, without changes in the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and hepatic lipid peroxidation.

Favourable hypolipidemic effects of CC were found by Asgary et al. [50] in rats with alloxan-induced diabetes. The rats exhibited also an antihyperglycemic effect comparable with glibenclamide therapy in the control group. Rasoulian et al. [51] confirmed the hypoglycemic effect of CC in hamsters fed with CC fruits with subsequent elevation of insulin level partially independent on the dose. Preventive use of CC fruits two or three times daily did not provide any additional benefit in comparison with one dose. According to the experimental study using an immortalized proximal tubule epithelial cell line, anthocyanins, to which CC are rich, decrease a high-glucose-induced enhance cholesterol efflux and ATP-binding cassette transporter (ABCA1) expression [52]. Furthermore, anthocyanins cause an increase of PPAR α and liver X receptor α expression and a decrease of the high-glucose-induced expression of the proinflammatory cytokines, intercellular adhesion molecule-1 (ICAM-1), MCP1, and transforming growth factor-β1 (TGF-β1), as well as NF-κB activation [52], which may explain the favourable effect of anthocyanins on blocking cholesterol deposition and their beneficial anti-inflammatory action.

Asgary et al. [53] compared the effect of 1 g/kg BW of CC powder with 100 mg/kg BW of lovastatin on fibrinogen levels in hypercholesterolemic rabbits. The study proved significant reduction of fibrinogen levels in both arms; in lovastatin one demonstrated a decrease of about 30 mg/dL, and in CC arm a decrease of about 50 mg/dL revealing more profound anti-inflammatory property of CC. Another study of Asgary et al. [50] in alloxan-induced diabetic rats demonstrated a beneficial effect on lipid spectrum, protecting the worsening of the spectrum in the CC-treated group. Generally, the hypolipidemic effect in animal studies corresponds to that induced by less potential statins. In hypercholesterolemic rabbits, Sozański et al. [54] found a decrease of total and LDL cholesterol, oxidised cholesterol, and TG as well as an increase of HDL cholesterol and hepatic PPARS α and γ after 60 days of therapy with anthocyanins derived from CC.

Protective effects of CC on endothelial function are much less studied despite predisposing favourable properties of the plant. The only relevant histological observation was made by Sozański et al. [54] in the group of cholesterol-fed rabbits, in which CC-derived anthocyanins significantly improved the composition of the arterial vessel, wall increasing intima thickness, and intima/media ratio in the thoracic aorta.

Leskovac et al. [55] demonstrated reduced incidence of radiation-induced micronuclei (19.23%) and reduced lipid peroxidation (50.04%) and two-fold enhancement of cell apoptosis in human peripheral blood lymphocyte cell cultures treated by an extract from the CC leaves. An analogous protective effect was found in Wistar rats treated with freeze-drying lyophilisate CC powder and exposed to high-fat or fructose diets. CC caused the decrease of the plasmatic while the increase in brain catalase activity suggesting increased cerebral protection. In turn, with regard to paraoxonase activity in both brain tissue and plasma, it had a stimulating effect. [56]. In the kidney injury induced by an injection of carbon tetrachloride, Banihabib et al. [8] observed the decrease of antioxidant enzyme activities (SOD, catalase, and GPx) and impairment of renal functions (increased serum creatinine, urea, and uric acid and decreased albumin). Treatment of rats with different doses of CC fruit extract (300 and 700 mg/kg) significantly ameliorated the alterations induced with carbon tetrachloride in lipid peroxidation, antioxidant defences, and biochemical and renal lesions.

Study of Asgary et al. [53] demonstrated strong attenuation of fibrinogen production after short-term therapy by CC extracts; however, studies of CC direct effect on atherothrombogenesis are missing. Another study of Williams et al. [57] using purified anthocyanins revealed reduction of expression of P-selectin suggesting an ability of anthocyanins to reduce thrombocyte activation. Authors supposed a potential contra-reaction of anthocyanins to oxidative stress-induced dysfunction of platelets. Yang et al. [58] confirmed a significant inhibition of platelet aggregation in the reduction of thrombus growth in human and murine blood using delphinidin-3-glucoside, which is also an important component of CC anthocyanins.

4.2. Human Studies. In the only two PubMed published controlled clinical studies, Asgary et al. [59] studied the hypolipidemic and anti-inflammatory effect of 100 grams of CC fruits added to a diet for 6 weeks in 40 dyslipidemic children

Table 1: Review of studies on effects of Cornelian cherry on atherogenetic, inflammatory, and antioxidant markers.

Authors	Study group(s)	Cornelian cherry	Duration	Dose	Parameters and results
Sozański et al. [47]	Hyper CH rabbits	Fruit lyophysate	60 d	100 mg/kg BW	↑ PPAR expression ↓ TG 44% ↓ Ao plaque ↓ ROS
Francik et al. [56]	22-week-old Wistar rats	Freeze-dried wild fruits	5 w	10% of daily intake	In brain tissue ↑ catalase ↑ PON1 ↑ FRAP ↓ protein carbonyl ↓ thiols
Banihabib et al. [8]	Wistar male rats, injected	Wild fruits, dried, methanol extracted powder	16 d	300 and 700 mg/kg BW	Kidney ↓ MDA ↑ CAT ↑ SOD ↑ GPx ↓ lipid peroxidation
Abdollahi et al. [7]	Wistar male rats	Hydromethanolic extract	21 d	50, 200, 400 mg/kg BW	Blood count improved in 400 mg group
Alavian et al. [63]	Wistar male rats, injected with CCl_4	Wild fruits, dried, methanol extracted powder	16 d	300 res. 700 mg/kg BW	↓ hepatic markers AST, ALT, ALP ↓ hepatic lipid peroxidation
Asgary et al. [50]	DM rats (nonspecified)	Wild fruits, dried powdered	4 w	2 g/d	↓ G, TCH, LDL-CH, AST, ALT, ALP comparable to glibenclamide
Celep et al. [49]	Sprague–Dawley male rats	Leaves—80% methanolic extract	21 d	500 mg/kg BW	*In vitro*—free radical scavenging +metal reducing activity *In vivo*—↑ total antioxidative capacity of liver ↔ SOD, CAT, GPx, lipid peroxidation
Asgary et al. [59]	Dyslipidemic children 9-16 yrs	Wild fruits, fresh	6 w	100 gr fruits/d	↓ TCH, LDL-CH, TG, apoB, I-CAM1, VCAM-1 ↑ HDL-CH, apoA
Celep et al. [64]	Control rats vs. rats treated with CCl_4	80% methanolic extract of CC leaves	21 d	100, 200, 500 mg/kg BW	Partial return of SOD, CAT, GPx, MDA, TEAC (depending on the dose)
Rasoulian et al. 2012 [51]	Hamsters	Fruits	20 d	5, 10, 15 g/d	↑ insulin ↓ body weight, ↓ G (only in 5 g/d)
Forman et al. [65]	Human breast cancer cells (MCF-7)	Aqueous leaf extracts	24, 48, 72 h	50-750 μg/mL	Antiproliferative effects
Savikin et al. [66]	*In vitro* HeLa cells, LS174 cells of human cancer	Fruits and leaves	NA	Not specified	Direct correlation with antioxidant capacity
Yousefi et al. [67]	*In vitro* tumor cells	Hydroalcoholic CC extract	NA	5, 20, 100, 250, 500, 1000 μg/mL	Inhibition of proliferation of different tumor cells in a dose-independent manner
Leskovac et al. [55]	*In vitro* human lymphocytes irradiated by gamma-rays	Wild leaves air-dried, powdered	NA	Extracts 0.1-0.4 mg/mL	Lowest dose—best results ↓ radiation-induced micronuclei ↓ lipid peroxidation
Jayaprakasam et al. [68]	C57BL mice	Fruits derived and purified anthocyanin vs. ursolic acid	8 w	1 g/kg of anthocyani-ns and 500 mg/kg of ursolic acid	Anthocyanine mice ↓ 27% weight gain ↓ TG ↓ lipid liver accumulation Both ↑↑ insulin

Ao, aortic; apoA, apolipoprotein A; apoB, apolipoprotein B; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BW, body weight; CAT, catalase; CC, cornelian cherry; DM, diabetes mellitus; FRAP, ferric-reducing antioxidant power; G, glycemia; GPx, glutathione peroxidase; HDL-CH, high-density lipoprotein cholesterol; ICAM-1, intercellular adhesion molecule 1; MDA, malondialdehyde; LDL-CH, low-density lipoprotein cholesterol; PON, paraoxonase 1; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOD, superoxide dismutase; TCH, total cholesterol; TEAC, trolox equivalent antioxidant capacity; TG, triacylglycerols; VCAM-1, vascular cell adhesion protein 1.

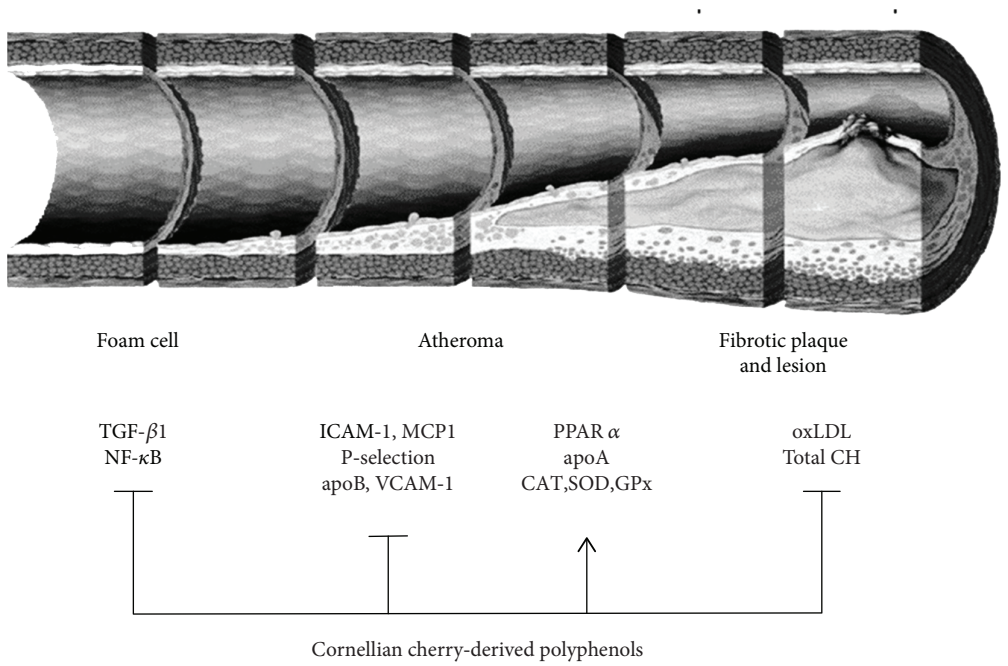

Figure 1: Effects of Cornelian cherry- (CC-) derived polyphenols on the atherosclerotic process. CC-derived polyphenols lead to the decrease of transforming growth factor-β1 (TGF-β1), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), monocyte chemokine protein 1 (MCP1), P-selectin, apolipoprotein B (apoB), vascular cell adhesion protein 1 (VCAM-1), oxidized low-density lipoprotein (oxLDL), and total cholesterol (CH) and the increase of peroxisome proliferator-activated receptor α (PPAR α), apolipoprotein A, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx).

and adolescent aged 9-16 years. The intervention group demonstrated a significant decrease of total cholesterol, TG, LDL cholesterol, apoB, ICAM-1, and VCAM-1 levels after six weeks. However, only apoA1 and ICAM-1 were significantly decreased compared to control groups. Clear improvement of lipid spectrum and inflammation markers after mild intake of CC added to usual diet predisposes the fruits as a supportive therapy of the main risk factors of atherosclerosis.

A randomized double-blind placebo-controlled clinical trial of Soltani et al. [60] on 60 patients with type 2 diabetes randomly was divided into two groups, treated either with 150 mg of anthocyanins or with placebo measured fasting plasma levels of glucose, insulin, $HgbA_{1C}$, and TG as well as 2-hour postprandial glucose. After 6 weeks of intervention, an increase in insulin level ($1.13 \pm 1.90\ \mu$U/mL versus $-0.643 \pm 1.82\ \mu$U/mL) and a decrease in $HgbA_{1C}$ ($-0.24 \pm 0.429\%$ versus $0.023 \pm 0.225\%$) and TG (-23.66 ± 55.40 mg/dL versus 2.83 ± 15.71 mg/dL) were observed. The authors concluded that daily consumption of the fruit extract of CC improves glycemic control by increasing insulin level and reduces TG serum level in type 2 diabetic adult patients.

The effects of CC on atherogenetic, inflammatory, and antioxidant markers in experimental as well as clinical studies are summarized in Table 1. It seems that CC similar to other nutraceuticals reach in polyphenolic compounds [61, 62] may have beneficial effects on cardiovascular, diabetes, and obesity-related diseases including atherosclerosis (Figure 1). However, more studies on mechanisms, actions, pharmacokinetics, as well as adverse effects of CC extracts, their bioactive constituents, and their effective doses in humans are needed for the applications of CC extracts in clinical practice and treatment of atherosclerosis especially.

5. Conclusions

Cornelian cherry similar to other nutraceuticals is capable of favourable interaction with risk factors of atherosclerosis and can contribute to the prevention of atherosclerosis through a positive effect on lipid spectrum and glycemia, reduction of free radicals and inflammation, amelioration of endothelial dysfunction, and prothrombogenic status. Until now, an identification of the main responsible compound, dosing, and duration of therapy as well as clinical experience in prospective studies are missing.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Peptides from Cauliflower By-Products, Obtained by an Efficient, Ecosustainable, and Semi-Industrial Method, Exert Protective Effects on Endothelial Function

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The large amount of cauliflower industry waste represents an unexplored source of bioactive compounds. In this work, peptide hydrolysates from cauliflower leaves were characterized by combined bioanalytical approaches. Twelve peptide fractions were studied to evaluate unexplored biological activities by effect-based cellular bioassays. A potent inhibition of intracellular xanthine oxidase activity was observed in human vascular endothelial cells treated with one fraction, with an IC50 = 8.3 ± 0.6 μg/ml. A different fraction significantly induced the antioxidant enzyme superoxide dismutase 1 and decreased the tumor necrosis factor α-induced VCAM-1 expression, thus leading to a significant improvement in the viability of human vascular endothelial cells. Shotgun peptidomics and bioinformatics were used to retrieve the most probable bioactive peptide sequences. Our study shows that peptides from cauliflower waste should be recycled for producing valuable products useful for the prevention of endothelial dysfunction linked to atherogenesis progression.

1. Introduction

Agricultural and food waste management is a great challenge for global security and environmental governance, directly linked with global competitiveness, increasing population and other economic related factors. Under the European 2020 growth strategy launched in 2010, Europe has set itself the goal of shifting from linear to circular models of production and consumption. This important issue needs advanced efficient alternatives other that landfilling or composting, in order to maximize the value derived from such an important waste source. The food waste, including both edible food and inedible parts, has been estimated in Europe of 88 million tons (9 million tons comes from primary production) directly associated with around 143 billion euros of costs [1].

In the last decade, an increasing attention has been devoted to the recycling of protein or other functional ingredients from fruit and vegetable by-products. In the perspective of biosustainable development and renewable resource technologies, by-products and waste represent a relatively cheap source of material suitable for bioactive molecules production [2], which would reduce both the amount of waste and the related costs of disposal, while producing value-added nutritional products [3]. Indeed, leaf protein has been considered as a supplementary protein source since the 1960s [4, 5]. In particular, food processing wastes and by-products have been considered for the production of antioxidant and ACE inhibitor peptides [3]. These peptides are often functionally inactive within the native proteins and must be released by proteolysis (*in vivo* digestion,

in vitro enzymatic hydrolysis, or bacterial fermentation) to achieve their potential "bioactive" roles.

As a representative example, the cultivation and consumption of cauliflower *(Brassica oleracea L. ssp botrytis)* have increased rapidly over the last few years with a large waste production, except for cauliflower curd (the sole edible part of cauliflower). Tons of cauliflower by-products (stems and leaves) are also generated during the harvest every year. Cauliflower is well known to contain various beneficial molecules, such as vitamin C, glucosinolates, carotenoid, and leaf protein [6, 7]. Numerous extraction techniques have been developed for bioactive compound extraction, such as supercritical fluid extraction [8], microwave-assisted extraction [9], and ultrasonic-assisted extraction [7], in order to treat larger quantities at the industrial scale still controlling the cost of the entire process.

Protein hydrolysates from cauliflower by-products have shown antioxidant [10] and angiotensin I-converting enzyme (ACE) inhibitory [11] activities in cell-free systems; therefore, they may be potential complementary to antihypertensive drugs [12]. It has also been reported that they regulate the glucose consumption and glycogen content in HepG2 cells, indicating an important role also in glucose metabolism [7]. In addition, several authors studied numerous antimicrobial peptides from plants, such as thionins, defensins, proline-rich peptides, lipid transfer proteins, cyclotides, and snakins [13, 14] that are also found in *Brassicaceae* species [15].

However, few researchers have focused on the study of protein fractions and preparation of their hydrolysates from cauliflower by-products and its biological activities [7, 11, 16, 17] in order to exploit them as preventive biomolecules for people genetically predisposed to diseases or within the framework of a healthy lifestyle.

Therefore, the aim of this paper was the development of a combined "ad hoc" bioanalytical approach based on an efficient recovery of peptides from cauliflower leaves, a characterization of their functional properties as potential nutraceuticals with highly predictive effect-based bioassays in cells and an in silico identification of the most active peptides.

The study of peptide bioactivity, with highly predictive cell models, is an efficient and reliable tool to reproduce *in vivo* physiological conditions avoiding the use of animal experiments to observe their effects on a wide range of biological activities, from endothelial dysfunction to antimicrobial properties.

2. Materials and Methods

2.1. Materials/Chemicals. Xanthine oxidase from bovine milk, luminol sodium salt, xanthine, oxypurinol, PBS tabs, Na-EDTA salt, gelatin from bovine skin, penicillin/streptomycin, trypsin-EDTA, Trolox, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium perborate, boric acid, NaOH, and $FeCl_2$ were from Carlo Erba (Milan, Italy). M200 medium, low serum growth supplements, and fetal bovine serum, RNaseOUT, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RNeasy Mini Kit was from QIAGEN (Hilden, Germany). Primers for RT-PCR were purchased from IDT (Coralville, IA, USA). Cell counting kit-8 (CCK8) and LDH assay kit were purchased from Dojindo Molecular Technologies (Rockville, MD, USA). SuperScript® III First-Strand Synthesis SuperMix and EXPRESS SYBR® GreenER™ qPCR SuperMix were purchased from Life Technologies (Carlsbad, CA, USA). All the other chemicals and solvents were of the highest analytical grade.

2.2. Peptidomic Workflow. The entire peptidomic workflow was performed as previously reported [17] with some modifications. The procedure is reported in Supplementary Material S1. Briefly, 1 kg of lyophilized cauliflower by-products was extracted using an ecofriendly saline buffer consisting of 50 mmol L-1 Tris-HCl (pH 8.8) and 15 mmol L-1 KCl. The extracted proteins were digested by Alcalase® enzyme and the whole obtained hydrolysate was purified by a semipreparative reverse phase high-performance liquid chromatography (SP-RP-HPLC) in order to simplify the complex mixture. Twelve fractions were collected and subsequently tested for specific and less unexplored bioactivities. The fractions with positive bioactivity were further analyzed by nano-HPLC coupled to high-resolution mass spectrometry. The peptides in the most active fractions were identified by peptidomic technologies and screened for bioactivity by the use of bioinformatics, to retrieve most probable bioactive peptide candidates.

2.3. Sample Preparation for Analysis. Stock solutions were prepared solubilizing cauliflower lyophilized fractions derived from HPLC separation in 1 ml of PBS buffer 0.1 M pH 7.4 by sonication. The protein content for each stock solutions was determined by absorbance spectroscopy, at 280 nm by NanoDrop 2000c (Thermo Fisher Scientific Inc., Massachusetts, USA). Stock solutions were diluted in PBS buffer 0.1 M pH 7.4 to obtain a final concentration of 10 mg/ml in protein content. After filtration, fractions were sampled and stored at -20°C for further analysis.

2.4. Cell Culture. In order to study the protective effect of peptide fractions on endothelial dysfunction, experiments were performed in human umbilical vein endothelial cells (HUVECs), a robust *in vitro* model for the study of endothelial cell physiology and function [18].

HUVECs pools, purchased from Life Technologies, were plated on gelatin-coated tissue culture dishes and maintained in phenol red-free basal medium M200 (Life Technologies) containing 10% FBS and growth factors (LSGS, Life Technologies) at 37°C with 5% CO_2. Cells from passages 3 to 7 were actively proliferating (70–90% confluent) when samples were harvested and analyzed [19].

2.5. Cell Viability Bioassay. The cell viability was assessed by WST8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo Molecular Technologies, Japan) that, in the presence of an electron mediator, is reduced by dehydrogenases in cells (as a vitality biomarker) to formazan dye which is soluble in the tissue culture medium. The amount of the

formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells [20]. The decrease in absorbance between the treatment after 24 h (representing t_1) and the control (representing t_0) was monitored at 37°C at 450 nm using a Varioskan™ flash multimode reader.

HUVEC cells were seeded in a transparent 96-well plate at a density of 5×10 cells/well [3]. The next day, cells were treated with stock dilutions (1×10^0-1×10^{-2} mg/ml in protein content) in complete culture medium for 24 h.

2.6. Cell Cytotoxicity: Lactate Dehydrogenase Release. Lactate dehydrogenase (LDH) release from HUVECs was monitored by collecting aliquots of medium at different times, using a standard spectrophotometric method [16]. The method is based on a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD^+ reduction to NADH. Diaphorase reduces tetrazolium salt, oxidizing NADH, to a red formazan product that can be measured at 490 nm. Medium derived from HUVECs treated with stock dilutions of protein fractions (1×10^0-1×10^{-2} mg/ml in protein content) for 24 h was collected and the increase in absorbance between the treatment after 24 h (representing t_1) and the control (representing t_0) was monitored at 37°C using a Varioskan™ flash multimode reader.

2.7. Intracellular Total Oxidant Fluorescent Detection. Intracellular oxidant levels were evaluated by using the oxidant-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA).

Briefly, the probe is not fluorescent until the acetate groups are removed by intracellular esterases; in the presence of oxidants, the probe is oxidized within the cells producing a fluorescent signal related to intracellular oxidant levels that was measured using a microtiter plate reader (Varioskan™ flash multimode reader, Thermo Fisher Scientific). Excitation wavelength was 485 nm and emission wavelength was 535 nm. HUVECs were treated with cauliflower peptide fractions (1×10^0-1×10^{-2} mg/ml) for 24 h and Trolox (100 μM) was used as reference. After treatment, cells were incubated with 5 μM H_2DCFDA for 20 min at 37°C and then subjected or not to oxidative stress generated by 50 μM H_2O_2 for 30 min. The decrease of fluorescence signal between cells treated with cauliflower peptide fractions and control was reported as the percentage of intracellular reactive oxygen species (ROS) normalized with H_2O_2 treatment alone [21].

H_2DCFDA can be used as a redox indicator probe for detecting intracellular oxidant formation caused by changes in iron or heme signaling or peroxynitrite ($ONOO^-$) formation. The fluorescent response based on the oxidation of DCFH provides an index for the total oxidants present in biological systems, not for cell-derived H_2O_2. This limitation determines a low selectivity toward H_2O_2 [22–24].

2.8. RNA Extraction. HUVECs were preincubated with cauliflower peptide fractions (1 mg/ml) for 8 hours at 37°C before 24 h of exposure to TNF-α (10 ng/ml). Total RNA was extracted using a commercial RNA extraction kit (QIAGEN) [20].

2.9. Real-Time PCR. RNA concentration and purity were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). 25 ng of total RNA was reverse transcribed using the SuperScript® III First-Strand Synthesis SuperMix (Life Technologies, Carlsbad, CA, USA) and amplified using the EXPRESS SYBR® GreenER™ qPCR SuperMix (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol in a final volume of 20 μl. Real-time PCR reactions were conducted on a Rotor-Gene Q QIAGEN Real-Time PCR System (QIAGEN GmbH, QIAGEN Strasse 1, D-40724, Hilden), with an initial 5 min incubation at 60°C, then 2 min at 95°C, followed by 40 cycles of amplification: 95°C for 15 s and 60°C for 1 min and examined on by Rotor-Gene Real-Time Analysis Software 6.0 (QIAGEN GmbH, QIAGEN Strasse 1, D-40724, Hilden). Primer concentration was 500 nM. The following primers were used: LOX1: forward 5′-TCGGGCTCATTTAACTGGGAA-3′, reverse 5′-TTGCTGGATGAAGTCCAGATCA-3′; NOX2: forward 5′-GTCTCAGGCCAATCACTTTGC-3′, reverse 5′-CATTATCCCAGTTGGGCCGT-3′; NOX-4: forward 5′-TCTGGCTCTCCATGAATGTCC-3′, reverse 5′-GACACAATCCTAGCCCCAACA-3′; VCAM: forward 5′-GGTATCTGCATCGGGCCTC-3′, reverse 5′-TAAAAGCTTGAGAAGCTGCAAACA-3′; ICAM: forward 5′-AGCTTCGTGTCCTGTATGGC-3′, reverse 5′-TTTTCTGGCCACGTCCAGTT-3′; eNOS: forward 5′-ATCTTCAGCCCCAAACGGAG-3′, reverse 5′-GATCAGACCTGGCAGCAACT-3′; SOD: forward 5′-AGGCATGTTGGAGACTTGGG-3′, reverse 5′-TGCTTTTTCATGGACCACCAG-3′; HO-1: forward 5′-CAACAAAGTGCAAGATTCTG-3′, reverse 5′-TGATTCACATGGCATAAAG-3′; XOD: forward 5′-CTACAGCTTTGAGACTAACTC-3′, reverse 5′-TCTTATGATCTCCTGTTAGGC-3′; p65: forward 5′-TGGGGACTACGACCTGAATG-3′, reverse 5′-GGGGGCACGATTGTCAAAGA-3′; p52: forward 5′-CCGTTGTACAAAGATACGCGG-3′, reverse 5′-CATCCAGACCTGGGTTGTAGC-3′; p50: forward 5′-AATGGGCTACACCGAAGCAA3′, reverse 5′-AGCTCGTCTATTTGCTGCCT-3′; SOD-2: forward 5′-GCTCCCCGCGCTTTCTTA-3′, reverse 5′-GCTGGTGCCGCACACT-3′; GPx1: forward 5′-TATCGAGAATGTGGCGTCCC-3′, reverse 5′-TCTTGGCGTTCTCCTGATGC-3′; catalase: forward 5′-CTCCGGAACAACAGCCTTCT-3′, reverse 5′-ATAGAATGCCCGCACCTGAG-3′; and RPL13A: forward 5′-CACCCTGGAGGAGAAGAGGA-3′, reverse 5′-CCGTAGCCTCATGAGCTGTT-3′. Changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ formula using RPL13A as reference gene.

2.10. Chemiluminescent Intracellular Xanthine Oxidase Assay. To monitor xanthine oxidase activity, 5×10^3 cells/well were plated in a 96-black well microtiter plate; the day after, cells were incubated at 37°C with CL reaction cocktail solution containing different amounts of cauliflower peptide fractions ranging from 1×10^0-1×10^{-2} mg/ml and the CL emission produced after the addition of xanthine (2.0 mM) was monitored for 20 min using the Luminoskan™ Ascent luminometer automatic plate reader (Thermo Fisher Scientific,

Roskilde, Denmark). The detailed procedure is reported in a previous paper [25].

2.11. Antioxidant Capacity Using a Chemiluminescent (CL) Method. The chemiluminescence method for measurement of antioxidant effect is based on the competition between the reaction of peroxyl radicals with luminol, giving rise to light emission, and the scavenging of peroxyl radicals by antioxidants. Indeed, the addition of a solution of known antioxidants to a glowing steady-state chemiluminescent reaction temporarily quenches light output. The extent of light emission quenching is related to the amount and the strength of antioxidant added. The procedure is reported in [26].

2.12. Antimicrobial Activity. The *in vitro* antimicrobial activity of the cauliflower peptide fractions was evaluated towards a panel of reference bacterial strains from the American Type Culture Collection (ATCC) including *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 9591 and the yeast *Candida albicans* ATCC 10231.

The peptide fractions were assayed by means of a broth microdilution method as previously described, with minor modifications [27, 28]. Briefly, for antibacterial determinations, a suspension at 0.5 McFarland of each reference strain was diluted 1 : 200 in Mueller-Hinton broth (Sigma-Aldrich) or in Brain heart infusion broth (Biolife) for *E. faecalis* and incubated with tenfold dilutions of cauliflower peptide fractions starting from 1 mg/ml and with gentamicin as reference drug. For antifungal determinations, yeast suspension was diluted 1 : 20 in RPMI-1640 medium (Gibco®, Thermo Fisher Scientific Inc., Waltham, USA), containing glucose 2%, 0.3% levoglutamine, and 0.165 M 3-(N-morpholino)--propanesulfonic acid (MOPS), pH 7.0, and then incubated with tenfold dilutions of peptide fractions starting from 1 mg/ml and with fluconazole, as reference drug. As additional control, cells were incubated in regular medium in the absence of fractions to check both background turbidity and the sterility of the procedure. Following 24 h of incubation at 37°C, microbial growth was determined by adding in each well the WST-8 dye (Microbial Viability Assay kit-WST, Dojindo Laboratories) and measuring the absorbance at 450 nm using the Multiskan Ascent microplate reader (Thermo Fisher Scientific Inc., Waltham, USA). Percentage values of samples at the different experimental conditions were determined as relative to the positive growth controls. Determinations were performed in triplicate and in two independent experiments.

2.13. Computational Methods. Molecular docking simulations were performed using the open-source program AutoDock Vina [29] along with AutoDockTools (ADT) [30], a graphical user interface compliment to the AutoDock software suite. In order to run the Vina docking program, both peptidic ligands and protein structure must be first refined and then prepared in a specific file format (.pdbqt). The peptide models of sequences (FKDENGGKLIGF, GNIFDGIQRPL, GYNPSYGARPL, and KWAGGKPEKPILR from fraction 8) were generated using PEP-FOLD3 server that provides a general framework for the structural characterization of peptides and returns in a few minutes the five best models. The models with the lowest energy conformations were selected for docking runs [31–33]. The xanthine oxidase model from bovine milk source (1FIQ) was the most used for docking simulation as reported in literature [34, 35] because of its suitable crystallographic resolution (~2 Å) assuring best docking results. The xanthine oxidase model from bovine milk source (1FIQ) was downloaded from the RCSB protein data bank (http://www.rcsb.org/) and refined by molecular graphic PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). Then, ADT was used to create the necessary .pdbqt files of both peptides and xanthine oxidase (XOD) structure that are read by Vina. The identification of candidate regions of the protein surface likely to be involved in the interaction with a peptide sequence required to assist in silico experiments was obtained using PEP-SiteFinder server [36].

2.14. Statistical Analysis. Results are expressed as mean ± SD of at least three independent experiments. Differences between the means were determined by one-way ANOVA followed by the Bonferroni multiple comparison test using the GraphPad Prism software, version 6.0 (GraphPad Software Inc., La Jolla, CA), and a P value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Peptide Hydrolysate Safety. Peptide hydrolysates from cauliflower by-products were obtained by Alcalase®, a low-cost enzyme compatible with large-scale applications [37]. Alcalase® displayed a greater degree of hydrolysis over the other common used enzymes, but it was employed in most cases just to obtain antioxidant and ACE inhibitory peptides [3]. It is certainly interesting to test hydrolysates for less-studied bioactivity since it was suggested that this kind of sample could be a promising source of understudied bioactive peptides [3]; therefore, hydrolysates were subjected to dose-effect safety experiments in HUVECs. Fractions 1, 2, and 3 showed a reduction of cell viability after a 24 h treatment with 1 mg/ml while the others did not affect cell viability. Then, lactate dehydrogenase (LDH), a marker for cell death both *in vitro* and *in vivo*, was quantified in cell culture medium and we did not observe any toxic effect (Figures 1(a) and (b)).

Fractions 4-12 were subsequently investigated to evaluate several unexplored biological activities, such as the protection against endothelial dysfunction and antimicrobial effects.

3.2. Peptides from Fractions 8 and 12 Reduce Oxidative Stress through Intracellular Endogenous Antioxidant Enzyme Modulation in HUVEC Cells. Fractions 8 and 12 decreased intracellular ROS levels after acute exposure to H_2O_2 ($P < 0.05$ and $P < 0.01$, respectively) (Figure 2(a)), suggesting they exert a protective effect against oxidative stress in the vasculature, process involved in endothelial dysfunction.

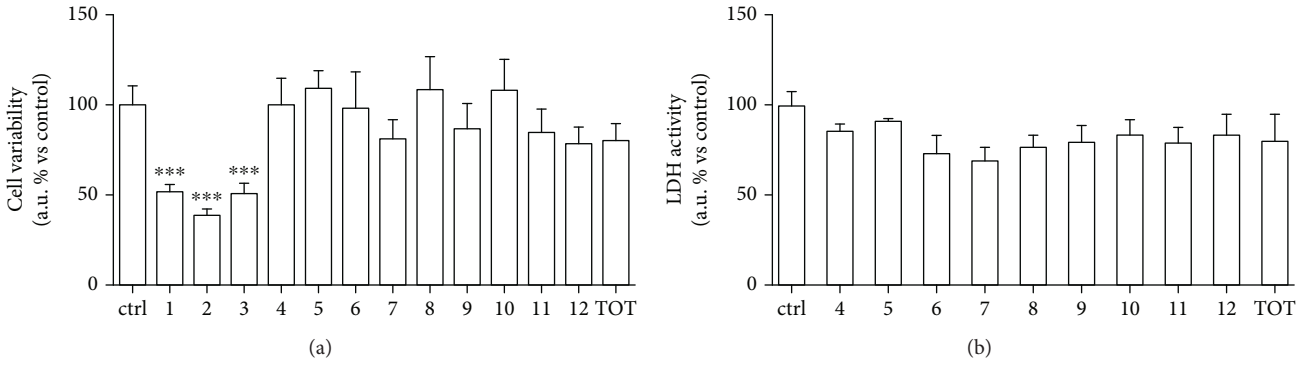

Figure 1: HUVECS were treated with peptide fractions (1-12) and the whole hydrolysate [13], the total (1 mg/ml) for 24 hours. (a) Cell viability was spectrophotometrically detected through formazan production in the presence of dehydrogenases in cells. (b) LDH activity was spectrophotometrically quantified in cellular medium as index of cytotoxicity.

Even if H_2DCFDA is still used to detect intracellular oxidant species from the scientific community, this probe suffers of some limitations as artefactual amplification of the fluorescence intensity via a redox cycling mechanism involving an intermediate radical, $DCF^{\cdot-}$, and responds to changes in intracellular iron signaling or enhanced peroxidase activity [24]. In fact, DCFH does not directly react with superoxide, H_2O_2, or nitric oxide. Instead, DCF fluorescence results from oxidation by potent oxidants, such those produced from metal ion- and peroxidase-catalyzed reactions and from decomposition of $ONOO^-$. Moreover, DCF-dependent fluorescence can be self-amplified by redox cycling of the one-electron oxidized dye [23]. Indeed, we observed that DCFH probe showed a dose-dependent increase in fluorescence intensity proportional to increase amount of Fe^{2+} (0.3-10 μM) in the presence of 50 μM H_2O_2 (Figure S1) in a cell-free system, with a limit of detection (LOD) = 0.6 ± 0.3 μM and a limit of quantification (LOQ) = 2.4 ± 0.3 μM, while we did not observe a direct correlation of DCFH-related fluorescence as a function of increase amount of H_2O_2 (0.5-100 μM) (data not shown).

Most peptides derived from hydrolysis of food proteins such as those from milk, egg, meat, wheat, and soy were characterized with chemical assays in cell-free *in vitro* conditions, generally for radical-scavenging activity or for metal chelating activity [38]. However, these methods do not allow evaluating the bioactivity of antioxidant peptides under physiological conditions, in order to establish their real protective roles in diseases. We demonstrated that peptide form cauliflower fractions 8 and 12 reduced intracellular oxidant species, acting as good antioxidants in HUVECs.

To clarify the possible mechanisms of action, we investigated the expression of several prooxidant (NADPH oxidases 2 and 4, lectin-type-oxidized LDL receptor 1, endothelial nitric oxide synthetase, and xanthine oxidase) and antioxidant biomarkers (superoxide dismutases 1 and 2, heme oxygenase 1, catalase, and glutathione peroxidase 1). Peptide fraction 12 at the higher concentration (1 mg/ml) significantly increased superoxide dismutase- (SOD-) 1 and glutathione peroxidase- (GPx-) 1 expression (Figure 2(b)) ($P < 0.01$ and $P < 0.05$, respectively), important intracellular antiatherogenic enzymes that counteract oxidative damage in the vascular endothelium [39, 40].

Among the antioxidant enzymes, SOD-1 is the most abundant and ubiquitous isoform, with a great physiological significance and therapeutic potential in CV diseases because the endothelium is particularly sensitive to oxidant injury [39], so we investigated its expression in HUVECs upon treatment with peptide fractions in the presence of TNF-α. Numerous studies suggest that SOD-2 is perhaps one of the most famous NF-κB targets with antioxidant activity in the vascular endothelium [41–44], at least in part via nuclear transcription factor p65 [45]. We investigated SOD-2 gene expression, but we did not observe any significant changes cells upon treatment with peptide fraction 12 in the presence of TNF-α.

GPx-1 is the most abundant selenoperoxidase form in mammalian tissues and a key antioxidant enzyme in many cell types including endothelial cells. GPx-1 consumes reduced glutathione to convert H_2O_2 to water and lipid peroxides to their respective alcohols [46]; it also acts as an $ONOO^-$ reductase [47]. Mice with a disrupted GPx1 gene exhibit increased susceptibility to oxidative stress-inducing agents [48], while induction of this isozyme has been shown to provide protection against oxidative damage in endothelial cells [49] GPx1 deficiency causes endothelial dysfunction [50, 51] and endothelial progenitor cell dysfunction in mice [52]. Furthermore, transgenic GPx1 expression was observed to impair endothelial dysfunction [51].

As some authors observed [53], food-derived peptides can display protective effects by induction of gene expression of proteins that protect cellular components from oxidative stress-induced deterioration; however, to the best of our knowledge, this is the first time that was reported an induction of SOD-1 and GPx-1 expression caused by peptides from cauliflower leaves.

In the endothelium, ROS predominantly arise from the isoforms of NAPDH oxidases 2 and 4 [54]; however, XOD and endothelial nitric oxide synthase (eNOS) play a physiologic role in inflammatory signaling regulation of NO production and vascular function [55]. The oxidative stress generated by these enzymes induces endothelial dysfunction, leading to atherosclerosis, cardiovascular diseases, and

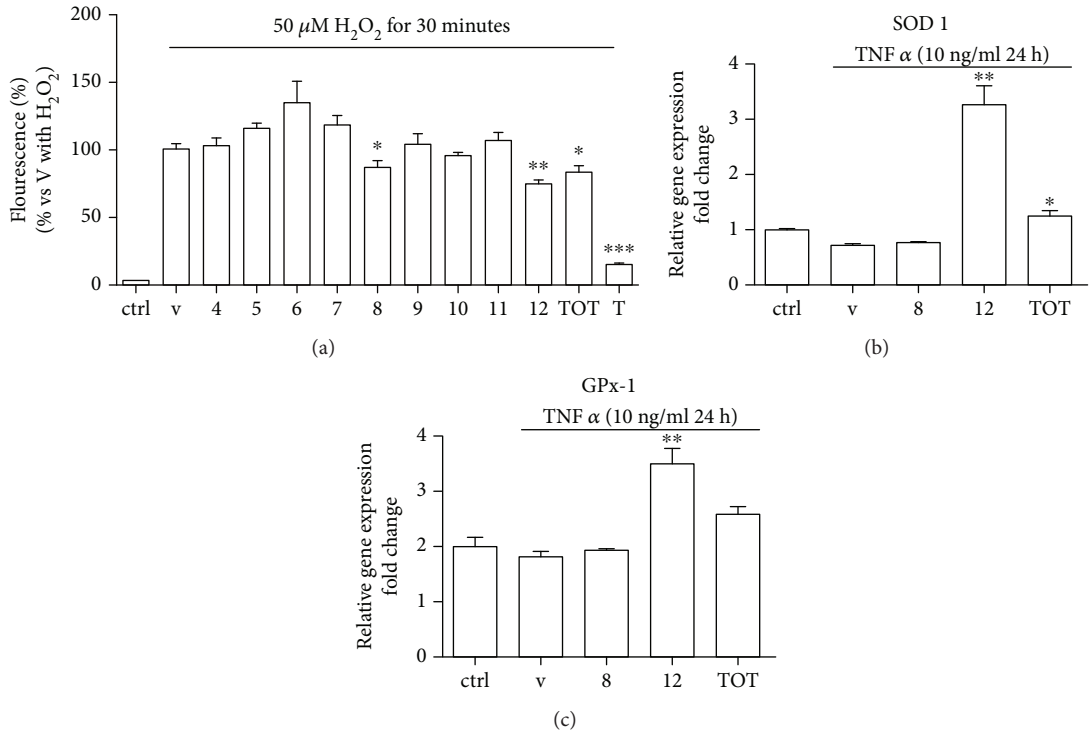

Figure 2: (a) HUVECs were treated with peptide fractions (4-12) and the whole hydrolysate at a concentration of 1 mg/ml for 24 hours and then exposed to oxidative stress generated by 50 μM H_2O_2 for 30 min. Treatment with 100 μM Trolox (T) for 24 hours was used as reference. Intracellular ROS levels were measured by means of H_2DCFDA assay as described in Materials and Methods. HUVECs were pretreated with cauliflower fractions (1 mg/ml) for 8 h before 24 h of exposure to TNF-α (10 ng/ml). Total RNA was extracted, and qRT-PCR analysis was performed to determine (b) SOD-1 gene expression and (c) GPx-1 expression. Relative changes in mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using RPL13A as reference gene. Results are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ significantly different from the vehicle (V, DMSO).

metabolic syndrome. Indeed, XOD activity is inversely related to endothelium-dependent vasodilation, since it was located primarily in cells derived from the vasculature and especially in endothelial cells [56]; elevation of XOD activity is associated to poor clinical outcomes [57]. Therefore, to date, XOD is recognized as an important biomarker, incentivizing extensive exploration of inhibition strategies to address disease processes where oxidative stress is contributory, such as cardiovascular disorders [58].

Peptide fraction treatment did not modulate XOD expression in HUVECs (data not shown) while fraction 8 inhibited intracellular XOD activity (Figures 3(a) and (b)). To quantify intracellular XOD activity, we previously developed an ultrasensitive cell-based biosensor reporting that the xanthine oxidase activity in living endothelial cells (HUVECs) was $(6 \pm 1) \times 10^{-7}$ mU/ml/cell and the IC50 of oxypurinol, the active metabolite of allopurinol, was 152 ± 76 ng/ml [25]. After 20 minutes of incubation, the intracellular IC50 of fractions 4-12 was evaluated by a dose-response curve, obtaining that fraction 8 has an IC50 = 8.3 ± 0.6 μg/ml (Figures 3(a) and (b)). This cell-based biosensor utilizing whole cells takes into consideration also the bioavailability of the compound, especially the ability to cross cell plasma membranes, so it is more representative and predictive to human situation. Moreover, we previously excluded the possible interferences of all the peptide fractions in the intracellular CL reaction, exploiting an assay based on enhanced chemiluminescent (ECL) detection method, able to reveal different species of ROS [20], demonstrating that fractions 8 and 12 cannot be considered direct ROS scavenger (data not shown). Moreover, to confirm that peptide fractions 8 and 12 act as intracellular antioxidant, we detect intracellular H_2O_2 level exploiting cell-based assays with a bioluminescent detection using a boronic probe selective for H_2O_2. We treated cells with menadione, one of the simplest quinones, widely used for evaluating the cellular effects of oxidative stress in endothelial cells [59, 60]. The major mechanism caused by menadione is the intracellular production of ROS by redox cycling, where one-electron reduction of O_2 by the semiquinone form of menadione generates superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is an extremely unstable ROS that rapidly dismutates in the cells to H_2O_2 either spontaneously or enzymatically catalyzed by SOD [61]. As it is shown in Figure S2, peptide fractions 8 and 12 reduced menadione-derived intracellular H_2O_2. These results confirmed the intracellular antioxidant activities of fractions 8 and 12.

3.3. Peptides from Fraction 12 Ameliorate TNF-α-Triggered Endothelial Dysfunction in HUVEC Cells. To better clarify the protective effect of fractions 8 and 12 in respect with TNF-α-induced endothelial dysfunction, we evaluated the cell viability in inflammatory conditions. As shown in

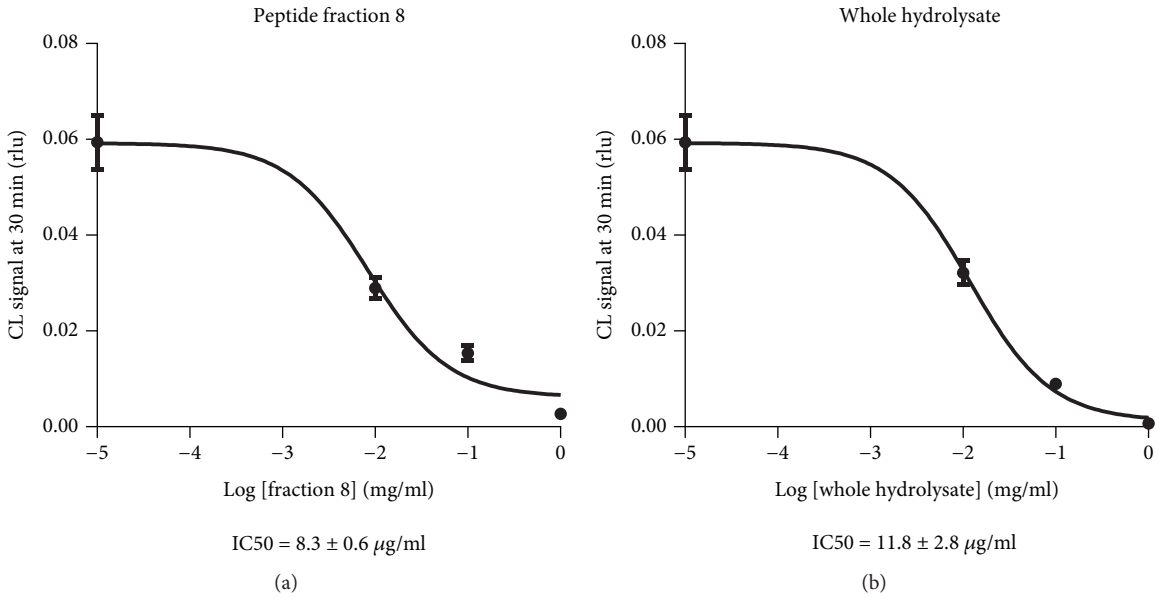

Figure 3: Concentration-response plot of intracellular XOD inhibition obtained by analyzing CL signals after 20 min of incubation with Fe^{2+}-EDTA-luminol reaction cocktail in HUVECs treated with peptide (a) fraction 8 and (b) whole hydrolysate (range 1–0.0001 mg/ml).

Figures 4(a) and (b), we observed that TNF-α treatment decreased cell viability in HUVECs; the addition of fractions 8 and 12 (1 mg/ml for 24 hours) significantly counteracted the effects induced by TNF-α ($P < 0.05$), while lower doses had slight, not significant effect (data not shown). NF-κB signaling is an attractive target for the development of novel anti-inflammatory drugs and the ability of certain small cell-penetrating peptides to enter cells inhibiting NF-κB signaling offer exciting potential also in the clinical setting. Classical NF-κB activity regulates the expression of many genes involved in inflammatory and survival responses, including those encoding cytokines (e.g., IL-1, IL-2, and IL-6), leukocyte adhesion molecules (e.g., E-selectin, ICAM-1, and VCAM-1), and antiapoptotic proteins (e.g., Bcl2, Bcl-XL, and XIAP) [62]. Therefore, the expression of the cellular adhesion proteins VCAM-1 and ICAM-1 was investigated; as it is represented in Figure 4(b), fractions 8 and 12 significantly decreased TNF-α-induced VCAM-1 expression ($P < 0.001$) in HUVECs while had no effect in ICAM-1 expression (not shown). To clarify the mechanism of action, the expression of NF-κB-related nuclear transcription factors p65, p50, and p52 was also investigated. As shown in Figures 4(c) and (d), fraction 12 treatment significantly decreased p65 expression while peptide fraction 8 decreased p50 expression ($P < 0.001$ and $P < 0.01$, respectively). p65 protein binds to the promoter of SOD-2 gene [45], so a decrease in p65 expression induced by fraction 12 explains results obtained in SOD-2 expression. NF-κB was the first transcription factor shown to be redox-regulated [63] and it has been demonstrated that overexpression of SOD-1 suppressed ischemia-induced activation of NF-κB through a decrease in nuclear translocation protein levels [64]. It has been also shown that transfection of endothelial cells with SOD-1, but not catalase, inhibited NF-κB signaling and expression of VCAM-1 induced by TNF-α [65].

NF-κB activation is regulated by reactive species [63], and ROS derived from intracellular XOD activity is implicated in heart failure [66] possibly through NF-κB-related p50 modulation.

Therefore, a decrease in intracellular oxidative stress by fraction 8 and 12 treatment can inhibit NF-κB signaling in endothelial cells, which can suppress its downstream effects. This notion was supported by the effects of fractions 8 and 12 on VCAM-1 mRNA level measured by qPCR.

Notably, the anti-inflammatory activity of a peptide derived from ovotransferrin, which is present in the albumen of eggs, was recently explained through the NF-κB-related p50 and p65 inhibitions [67].

Increased reactive oxygen species (ROS) production together with increased adhesion molecules and thrombogenic tissue factor expression on endothelial cells has a key role in proatherogenic mechanisms [68]. Therefore, peptides able to decrease the expression of inflammatory biomarkers could be useful for reducing the severity of atherosclerosis progression.

3.4. Peptides from All of the Fractions Do Not Show Any Antimicrobial Activity. The characterization of several plant protein hydrolysates demonstrated that they have antimicrobial activity, thus qualifying as functional foods [69], but we did not observe inhibitory activity of the cauliflower peptide fractions towards the human pathogenic bacteria and yeast strains selected in the present study.

3.5. Identification of Bioactive Peptides from Fractions 8 and 12. Fractions 8 and 12, the most active ones, were analyzed by nano-HPLC-MS/MS method. The obtained MS/MS raw files were searched by the Proteome Discoverer software to obtain peptide sequences. The identified peptides were manually validated taking into consideration only the most abundant peptides in both fractions; peptide abundance is

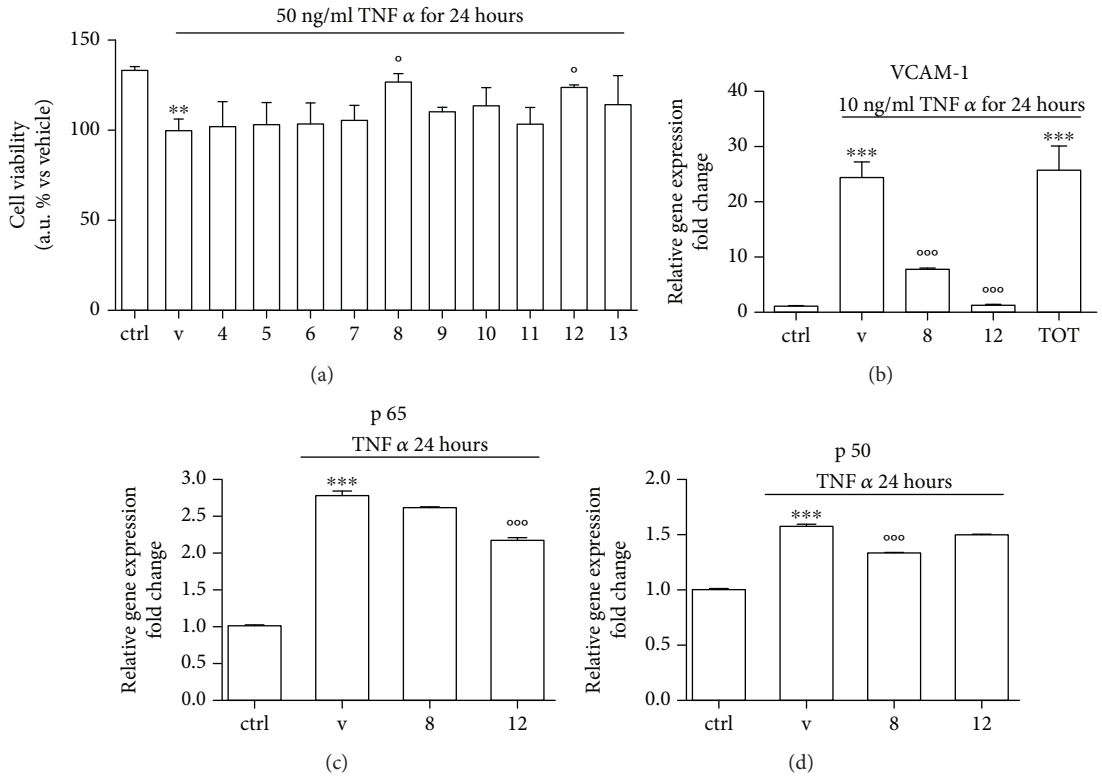

Figure 4: HUVECs were pretreated with peptide fractions (1 mg/ml) for 8 h before 24 h of exposure to TNF-α (50 ng/ml). (a) Cell viability was spectrophotometrically detected through formazan production in the presence of dehydrogenases in cells. Total RNA was extracted and qRT-PCR analysis was performed to determine (b) VCAM-1 gene expression, (c) p65 gene expression, and (d) p50 gene expression. Relative changes in mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using RPL13A as reference gene. Results are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from the control; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, and $^{\circ\circ\circ}P < 0.001$ significantly different from the vehicle (V, DMSO).

related to their area; thus, peptides were filtered according to it and only the ones with an area larger than 10^7 and higher score were accepted. A total of 181 peptides were identified after this manual validation. The complete list of identified peptides coming from the two most active fractions from the first chromatographic dimension, with sequence and related data, is reported in Supplementary Material S2.

After that, an in silico analysis using PeptideRanker [70] was carried out to further mine data. In this way, each peptide was assigned a score based on the probability of being bioactive, probability that the built-in N-to-1 neural network computed on the basis of the peptide primary sequence (the complete list of probability scores assigned to each identified peptide is reported in Supplementary Material S2). Such algorithm is capable of predicting the bioactivity of peptides because of the general features that different bioactive peptide functional classes have in common; therefore, PeptideRanker represented a useful tool to select the most probable bioactive peptides. Since the software labelled as "bioactive" any peptide possessing a score above the 0.5 threshold, we applied a higher 0.7 threshold in order to reduce the number of false positive hits. After such filtering of peptide scores, most peptide sequences were rejected. Twenty-three peptides have shown a score higher than 0.7 in fraction 12, and among these, only one has shown a probability higher than 0.9, namely, IDNIFRF. As it is possible to see in Supplementary Material S2, only four potentially bioactive peptides come from fraction 8, and all the others belong to fraction 12. The treatment with fraction 12 increased SOD-1 and reduced VCAM-1 expression in the presence of TNF-α. Both genes are modulated by NF-κB signaling [43, 71], suggesting that one or more peptides in fraction 12 could act as inhibitor of NF-κB pathway machinery. Several bioactive peptides have been described in literature as inhibitors of classical NF-κB signaling by either disrupting the IKK complex or by inhibiting critical events downstream of IKKβ [72]. In addition, several peptides that target upstream intermediates in the NF-κB pathway or other signaling mechanisms, such as the ERK and JNK pathways, have also been developed. IDNIFRF seems the most promising candidate due to the presence of both the cationic (arginine) and hydrophobic side chains (isoleucine) that can facilitate its uptake across the plasma membrane. An in silico determination of the hydrophobic character of this peptide was performed exploiting Peptide 2.0 ProtParam tool of ExPASy bioinformatics resource portal and peptide synthesis and proteotypic peptide analyzing tool of Thermo Scientific confirmed a hydrophobicity of 30.90, with an aliphatic index of 111.43 and a grand average of hydrophobicity (GRAVY) of 0.443 [73]. These results suggested that IDNIFR is an adequate

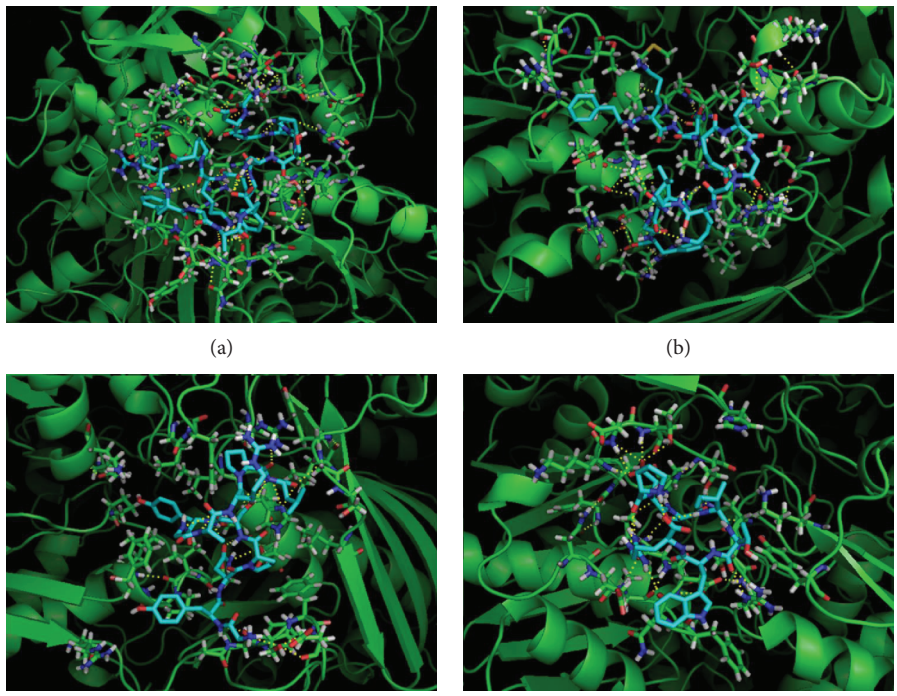

Figure 5: Picture of peptides docked in XOD enzyme (1FIQ), showing polar interactions within active sites for (a) GDSNPSNPKPRFGAY, (b) FKDENGGKLIGF, (c) GYNPSYGARPL, and (d) PDSITWR sequences, calculated by AutoDock Vina and elaborated by PyMOL software.

Table 1: Comparison between inhibition constant values (K_i) derived from cell-based assay and in silico molecular docking simulations of bioactive peptides in cauliflower fraction 8.

K_i calculated from cell-based assay	Peptide sequence	Energy affinity	K_i calculated from in silico simulation results
8.6 $\mu g\,ml^{-1}$	GDSNPSNPKPRFGAY	-28.03 kJ/mol	20 $\mu g\,ml^{-1}$
	PDSITWR	-25.10 kJ/mol	34 $\mu g\,ml^{-1}$
	GYNPSYGARPL	-25.52 kJ/mol	40 $\mu g\,ml^{-1}$
	FKDENGGKLIGF	-25.10 kJ/mol	52 $\mu g\,ml^{-1}$

hydrophobic peptide able to cross plasma membranes. However, to the best of our knowledge, more studies are needed to determine its efficacy as NF-κB signaling modulator.

3.6. Molecular Docking Simulations for Xanthine Oxidase. To validate the experimental work conducted to determine the intracellular XOD inhibition, molecular docking simulations have been used as a tool to augment the molecular level interpretation of the data. In particular, to seek the molecular explanation of the inhibition behavior of fraction 8 against XOD activity in the cell-based assay, we undertook a series of molecular docking studies using AutoDock Vina. The in silico calculations provided nine best output results for each docking run. From the analysis of docking study, the peptide sequence GDSNPSNPKPRFGAY showed the best docked conformation (Figure 5) with the lowest energy affinity of -28.03 kJ/mol than other peptides (Table 1). The docking results indicated that medium-weak interactions between protein surface and peptides exist. Then, theoretical dissociation constants K_i (ranged from 20 to 52 $\mu g\,ml^{-1}$) for peptide sequences were calculated from docking outputs and compared with experimental K_i, derived from cell-based assay results. The *in silico* simulations referred only to a single isolated peptide and did not consider cell permeability of inhibitors. Moreover, the discrepancy between experimental and computational K_i values can be explained considering a positive synergic effect of the four inhibitors [74]. Indeed, Figure 5(b) shows four different regions of the protein surface involved in the interaction with each peptide, respectively. The synergistically interaction of the four sequences to different sites of XOD can justify the increased inhibitory effect, as observed in *in vitro* assays. Molecular docking simulations demonstrated that the trend of in silico prediction of peptide effects is in agreement with experimental *in vitro* results and confirmed inhibitory effect of cauliflower fraction 8 on XOD activity as obtained from cell-based assay, providing a theoretical explanation of molecular inhibition mechanism by the identification of protein-peptide interactions (Figures 5 and 6).

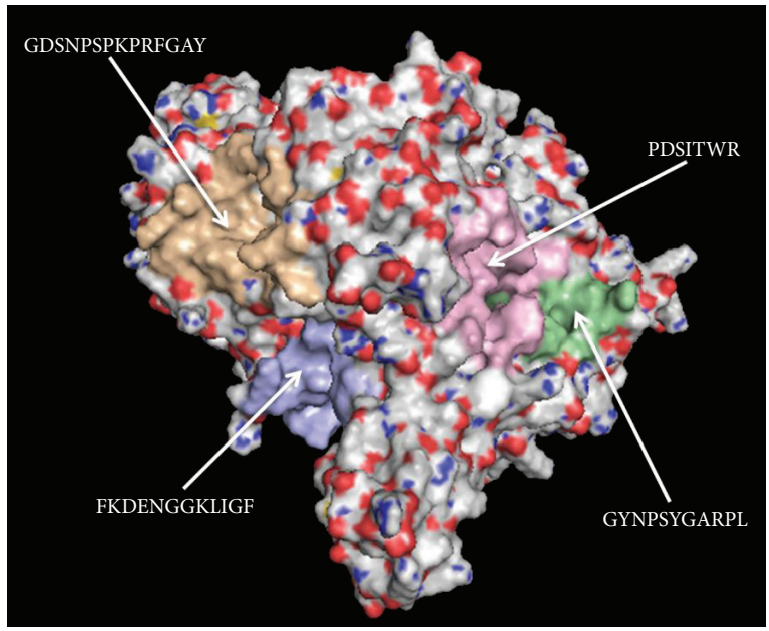

Figure 6: Picture of XOD enzyme polar surface, showing that each sequence (GDSNPSNPKPRFGAY, PDSITWR, FKDENGGKLIGF, and GYNPSYGARPL) interacts with a different site of XOD, calculated by PEP-SiteFinder server and elaborated by PyMOL software.

4. Conclusions

We developed an innovative combined (bio)analytical approach, based on a rationale recovery of bioactive peptides from cauliflower waste through an advance peptidomic-based strategy integrated with an *in vitro* activity characterization utilizing highly predictive cell-based bioassays. Our study suggests that cauliflower peptides from two fractions possess antioxidant and anti-inflammatory effects in the vasculature, at least in part through inhibition of intracellular XOD activity and modulation of SOD-1 and VCAM-1 expression. *In silico* analysis showed that four peptides from fraction 8 and one from fraction 12 could be the most probable bioactive candidates exerting protective effects against endothelial dysfunction. Moreover, one peptide from fraction 8 able to synergistically inhibit XOD was detected through a detail *in silico* docking analysis.

Advancements in the biopharmaceutical industry have resulted in the development of several new peptide-based therapeutics; to the best of our knowledge, this is the first attempt to determine biological effects of peptides from cauliflower waste, in order to evaluate their possible application into valuable functional components in nutraceutics and pharmaceutics as well as in animal feed.

Oral administration is most preferred because of patient compliance and acceptability; however, to exercise their effects in the target organ, peptides need to remain intact during the digestive process. To solve this crucial issue, several approaches including chemical modifications (lipidation), physical methods (microencapsulation), use of mucoadhesive polymers, formulation design, and use of enzyme inhibitors have been developed to improve their bioavailability after oral ingestion [58].

This translational (bio)analytical approach represents a smart and powerful tool that allows to open new perspectives on the possibility to find new uses of waste, even outside the agricultural field, contributing to the creation of sustainable value chains in the farming and processing sectors.

Data Availability

The in vitro data obtained in human umbilical vein endothelial cells used to support the findings of this study are included within the article. The description of the in vitro method to determine antimicrobial activity is included within the article; however, no data are available since peptide fractions did not show any significant antimicrobial activity. Previously reported data regarding the multidimensional liquid chromatography characterization of peptide fractions were used to support this study and are available at DOI: 10.1016/j.jff.2018.02.022. These prior studies are cited at relevant places within the text as references [17]. The data obtained by peptidomic analysis and bioinformatics used to support the findings of this study are included within the article and in supplementary information files 1 and 2.

Conflicts of Interest

The authors have declared no conflict of interest.

Acknowledgments

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Authors' Contributions

CC had substantial contributions to the conception and design of the work; the acquisition, analysis, and interpretation

of data for the work; and drafting the work. ALC did mass spectrometry analysis and revised for important intellectual content. DC did the acquisition, analysis, and interpretation of data for the work and molecular docking simulations using AutoDock software. FB did the acquisition, analysis, and interpretation of data for the work and revised the paper critically for important intellectual content. RCC did the mass spectrometry analysis and computational analysis and revised the paper critically for important intellectual content. CMM did the acquisition and analysis of data for the work. SP did the acquisition and analysis of data for the work. MZ revised the paper critically for important intellectual content. MM had an agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. PS did the acquisition and analysis of data for the work. AL revised the paper critically for important intellectual content and final approval of the version to be published. AR revised the paper critically for important intellectual content and final approval of the version to be published.

Supplementary Materials

Supplementary 1. Supplementary Material S1: a detail description of the entire peptidomic workflow, procedures, and results regarding the intracellular oxidants measurement were reported.

Supplementary 2. Supplementary Material S2: the complete list of identified peptides coming from the two most active fractions from the first chromatographic dimension, with sequence and related data, was reported.

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Research Article

Anti-inflammatory Role of Carotenoids in Endothelial Cells Derived from Umbilical Cord of Women Affected by Gestational Diabetes Mellitus

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Diabetes is associated with vascular inflammation, endothelial dysfunction, and oxidative stress, promoting the development of cardiovascular diseases (CVD). Several studies showed that a carotenoid-rich diet is associated to a reduced cardiovascular risk in healthy and diabetic subjects, although the mechanisms of action are still unknown. Here, the potential role of β-carotene (BC) and lycopene (Lyc) in human endothelial cells isolated from human umbilical cord vein (HUVECs) of women with gestational diabetes (GD) and respective controls (C) has been investigated. Results showed that BC and Lyc reduced the tumor necrosis factor alpha- (TNF-α-) stimulated monocyte-endothelium interaction (adhesion assay), membrane exposure (flow cytometry), and total expression levels (Western blot) of VCAM-1 and ICAM-1 in both cell types. Moreover, the treatment with BC and Lyc reduced the TNF-α-induced nuclear translocation of NF-κB (image flow cytometry) by preserving bioavailability of nitric oxide (NO, flow cytometry, and cGMP EIA kit assay), a key vasoactive molecule. Notably, BC and Lyc pretreatment significantly reduced peroxynitrite levels (flow cytometry), contributing to the redox balance protection. These results suggest a new mechanism of action of carotenoids which exert vascular protective action in diabetic condition, thus reinforcing the importance of a carotenoid-rich diet in the prevention of diabetes cardiovascular complications.

1. Introduction

Cardiovascular diseases represent the major complications and the main cause of reduced life expectancy in type 2 diabetic patients [1].

Diabetes is a chronic low-grade inflammatory condition featured by the increased plasma levels of TNF-α, a primary mediator of inflammation and insulin resistance [2], and reactive oxygen species (ROS), both playing an important role in the promotion of endothelial dysfunction and cardiovascular complications [3].

Nitric oxide is an important molecule playing a pleiotropic role in preserving vascular wall homeostasis. It is produced by endothelial nitric oxide synthase (eNOS) via the conversion of the amino acid L-arginine into L-citrulline. Once released, it diffuses to the vascular smooth muscle cells (vSMC), where it activates the enzyme guanylate cyclase (GC), inducing the production of cyclic guanosine monophosphate (cGMP), a molecule involved in vascular relaxation. Moreover, nitric oxide (NO) modulates platelet aggregation and monocyte adhesion and infiltration into the vascular wall and inhibits vSMC proliferation and migration [4, 5]. Thus,

the maintenance of nitric oxide availability is mandatory to avoid the activation of the inflammatory process and the endothelial dysfunction.

However, several studies found that under hyperglycemic conditions NO rapidly reacts with superoxide anion ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), a highly potent oxidant molecule that diffuses across phospholipid membranes, resulting in substrate nitrosylation and nitric oxide bioavailability decline [6–8].

Several natural molecules seem to have a beneficial effect on oxidative stress and vascular dysfunction [9–11]; among them, carotenoids are the most characterized. Carotenoids include a large family of fat-soluble molecules noted for their antioxidant action [12]. However, besides their antioxidant effect, they also exert an anti-inflammatory effect, playing an important role in the prevention of cardiovascular complications [13, 14].

Among more than 700 carotenoids discovered, the main and better characterized are β-carotene and lycopene with free radical scavenger activity and nutritional relevance [15]. Interestingly, several studies showed that carotenoids are able not only to prevent but also to ameliorate diabetes and its subsequent complications by reducing oxidative stress [16, 17].

In preclinical reports using streptozotocin- (STZ-) induced hyperglycemic rats as a model to evaluate the effect of chronic lycopene treatment [18–20], it was found that this molecule acts as an antidiabetic agent, attenuating endothelial dysfunction by its antioxidant action.

Moreover, other studies also indicated that dietary lycopene administration markedly reduced serum lipid levels and the formation of atherosclerotic plaques in New Zealand White (NZW) rabbits fed a high-fat diet [21–23], further indicating that lycopene could play a significant role in the prevention of cardiovascular consequences.

As regard human studies, although the evidence on the beneficial effects of carotenoids in the reduction of diabetes incidence is controversial [24–26], it was demonstrated that serum concentration of carotenoids was inversely associated with future oxidative stress, inflammation, and endothelial dysfunction [27]. In addition, prospective investigations highlighted that adequate dietary intakes of carotenoids were associated with a reduced risk for type 2 diabetes mellitus (T2DM) [28–30], inducing to establish a "carotenoid health index" to better evaluate the cardiovascular risk according to established plasmatic carotenoid concentrations [31].

Although several *in vitro* cellular models have been used to evaluate the role of carotenoids in reducing the development and the progression of the atherosclerotic plaque [17, 32, 33], it is however mandatory to better delineate the molecular events involved in the beneficial effect of carotenoids in slowing down the endothelial dysfunction and the atherosclerotic process in diabetes mellitus.

In this study, we used an endothelial cell model of chronic hyperglycemia derived from the human umbilical cord vein of women affected by gestational diabetes (GD-HUVECs). Recently, we found that these cells exhibit durable proatherogenic modifications of cellular homeostasis potentially predisposing to endothelial dysfunction and atherosclerosis development [34, 35], making them a useful model for studying endothelial dysfunction related to diabetes. Thus, we aim to investigate the molecular mechanisms of new natural molecules such as β-carotene and lycopene on the prevention or the delay of the vascular damage induced by hyperglycemia.

In particular, to better outline the way of action of carotenoids in endothelial dysfunction prevention in diabetes, we pretreated TNF-α-stimulated GD-HUVECs with β-carotene and lycopene. Interestingly, we found that the exposure of diabetic HUVECs to β-carotene and lycopene resulted in an increased nitric oxide bioavailability, probably induced by the scavenging action of carotenoids, and in the reduction of the oxidative and inflammatory stress damage.

2. Materials and Methods

2.1. Materials. Phosphate-buffered saline (PBS, CAT. D8662), Dulbecco's modified Eagle medium (DMEM, CAT. D6046), M199 endothelial growth medium (CAT. M4530), 0.5% trypsin/0.2%, ethylenediaminetetraacetic acid (EDTA) solution (CAT. 59418C), bovine serum albumin (BSA), L-glutamine (CAT. G7513), penicillin-streptomycin (CAT. P4333), phorbol myristate acetate (PMA, CAT. P1585), ionomycin (Iono, CAT. I0634), anti-β-actin mouse monoclonal antibody (CAT. A5441), 7-aminoactinomycin D (7-AAD, CAT. A9400), and TNF-α (CAT. T0157) were purchased from Sigma-Aldrich (Saint Louis, USA). Fetal bovine serum (FBS, CAT. 41A0045K) was from Life Technologies (Monza, Italy), and L-nitro-arginine-methyl ester (L-NAME, CAT. ALX-105-003) was purchased from Alexis Biochemicals (San Diego, CA, USA). Anti-vascular cell adhesion molecule-1 (VCAM-1, CAT. sc-13160) and anti-intercellular adhesion molecule-1 (ICAM-1, CAT. sc-107) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PE-labeled anti-VCAM-1 (phycoerythrin-labeled, CAT. 305806) and FITC-labeled anti-ICAM-1 (fluorescein isothiocyanate--labeled, CAT. 313104) antibodies were from BioLegend (San Diego, CA, USA). Anti-NF-κB p65 (CAT. 4764) primary antibody was from Cell Signaling (Danvers, MA, USA). Alexa Fluor 488-conjugated antibody was from Invitrogen (CAT. 11034). DAF-2DA probe was from Calbiochem (CAT. 251505). HKGreen-4A was synthesized by Prof. Dan Yang's lab [36]. An enzyme immunoassay (EIA) kit was taken from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Lycopene (Lyc, CAT. L9879) was purchased from Sigma-Aldrich, and β-carotene (BC, CAT. 22040) from Fluka (Hamburg, Germany): both were dissolved in tetrahydrofuran (THF, CAT. 401757, Sigma-Aldrich) and used as described in our previous work [37].

2.2. Cell Cultures and Experimental Protocol. Primary human umbilical vein endothelial cells (HUVECs) were explanted by umbilical cords obtained from randomly selected mothers affected by gestational diabetes (GD) and healthy Caucasian mothers (Control, C), according to the previously reported methods [38]. The characteristics of C-mothers ($n = 10$) and GD-mothers ($n = 12$) selected for this work are described in Table 1. All procedures were in agreement with the

Table 1: Clinical characteristics of control (C, $n = 10$) and gestational diabetic (GD, $n = 12$) women.

Characteristic	C-women	GD-women
Age (years)	35 ± 7.1	34 ± 5.67
Height (cm)	163.75 ± 5.66	162.4 ± 7.93
Pregestational weight (kg)	68.14 ± 13	67.1 ± 10.73
BMI (kg/m^2)	27.49 ± 5.18	27.81 ± 2.97
OGTT values (*mmol/L*)		
Basal glycaemia	4.5 ± 0.24	5.1 ± 0.24**
1 h glycaemia	8.1 ± 0.99	10.2 ± 1.16**
2 h glycaemia	6.54 ± 1.14	8.04 ± 1.71*
OGTT gestational week	27.9 ± 2.4	24.4 ± 4.7
SBP (mm/Hg)	107.6 ± 8.87	105.5 ± 10.7
DBP (mm/Hg)	71.4 ± 9.1	68.4 ± 10.57

Data are expressed as the mean ± SD. BMI: body mass index, OGTT: oral glucose tolerance test, SBP: systolic blood pressure, DBP: diastolic blood pressure. $^{**}p < 0.05$; $^{*}p < 0.0001$.

ethical standards of the Institutional Committee on Human Experimentation (reference number 1879/09COET) and with the Declaration of Helsinki principles. For experiments, C- and GD-HUVECs were grown to subconfluence in a DMEM/M199 medium (ratio 1 : 1) supplemented with 20% FBS, 10 μg/mL heparin and 50 μg/mL endothelial cell growth factor. Serum-starved cells (in medium with 0.5% FBS) were incubated with TNF-α at concentration 1 ng/mL for 16 hours, following 24-hour preincubation with β-carotene or lycopene (2.5 μmol/L).

All experiments were performed in technical duplicate or triplicate using at least 3 different cellular strains ($n = 3$) obtained from umbilical cords of C- or GD-women.

2.3. Monocyte-HUVEC Adhesion Assays. The adhesion assay was performed in C- and GD-HUVECs in the basal state and after incubation for 24 hours with BC or Lyc (2.5 μmol/L) before stimulation with or without 1 ng/mL TNF-α for 16 hours. The cells were grown to confluence in six-well tissue culture plates and U937 cell lines (European Collection of Authenticated Cell Cultures (ECACC)) were used to evaluate the adhesion to HUVEC monolayers as previously described [39]. One hour before the assay, HUVECs were treated with antibodies against VCAM-1 or ICAM-1 at saturating concentrations (1 μg/1 × 10^6 cells) as negative technical controls. Photos were randomly chosen high-power fields taken at a half-radius distance from the centre of the well in one of three comparative experiments of a similar design, showing U937 monocytoid cell adhesion to endothelial cells.

2.4. Western Blot Analysis. C- and GD-HUVECs were stimulated as described in the experimental protocol, and Western blot analysis was performed as previously described [10]. For the specific experiment, cells were lysed and 30 μg total protein was resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using mouse monoclonal anti-VCAM-1 and ICAM-1 (1 : 1000 and 1 : 500, respectively) and mouse monoclonal anti-β-actin (1 : 10.000). The membranes were then incubated with peroxidase-conjugated secondary antibodies (1 : 10.000). Band densities of proteins were detected and quantified by using the Alliance Chemiluminescence Imaging System (UVItec Limited, Cambridge, United Kingdom). Densities of VCAM-1 and ICAM-1 proteins were divided by those of β-actin content, and the ratio was indicated as arbitrary units.

2.5. cGMP EIA Kit Assay. Control and GD-HUVECs were grown to confluence in six-well tissue culture plates and were stimulated as described in the experimental protocol. To stimulate endogenous NO production, C- and GD-HUVECs were incubated with ionomycin (2 μmol/L for 24 h) with or without L-NAME preincubation (1 mmol/L for 45 minutes). Intracellular cGMP levels were evaluated by using a commercial enzyme immunoassay (EIA) kit, following the instruction provided by the supplier.

2.6. Flow Cytometry Analysis. At the basal state and after stimulations, nonpermeabilized cells were detached by 5 mM EDTA, washed, and resuspended in 0.5% BSA solution. Cells were treated and incubated with anti-VCAM-1 PE conjugate (1 : 100) and with anti-ICAM-1 FITC conjugate (1 : 100) as previously described [38]. To determine cytoplasm-nucleus translocation of NF-κB p65, cells were permeabilized by an Intrasure kit (CAT. 641778, BD Biosciences), processed, and incubated with anti-NF-κB p65 (1 : 100) primary antibody and then secondary antibody Alexa 488 conjugate (1 : 100). Nuclear staining was performed by incubating the cells with 7-AAD (1 : 100) for 10 minutes at room temperature, and all samples were analysed by imaging flow cytometry (ImageStream AMNIS by using IDEAS software, BD).

To evaluate NO levels, 5 × 10^5 C- and GD-HUVECs were incubated with the cell-permeable fluorescent nitric oxide probe DAF-2DA (2 μmol/L for 30 minutes at 37°C). For the evaluation of intracellular levels of peroxynitrite ($ONOO^-$), about 5 × 10^5 cells were incubated with the HKGreen-4A probe as previously described [38]. 10.000 events for each sample were analysed using a FACS Calibur or FACS Canto II flow cytometer (BD Biosciences, California, USA). All data were analysed using FACS Diva (BD Biosciences), FlowJo v.8.8.6 (TreeStar, Ashland, OR), and CELL Quest 3.2.1 software (BD Biosciences).

All results are expressed as the percentage (%) of positive cells or the MFI (mean fluorescence intensity) ratio, calculated by dividing the MFI of positive events by the MFI of negative events (MFI of secondary antibody).

2.7. Statistical Analysis. Results are presented as the means ± standard deviation (SD) of at least 3 different experiments using at least 3 different cellular strains ($n = 3$) both of C-HUVECs and of GD-HUVECs. Student's t-test and ANOVA test followed by the Bonferroni multiple comparison test for post hoc comparisons were used to analyse the differences between the two cell strains and between the different treatments. Significance was defined as a p value less than 0.05.

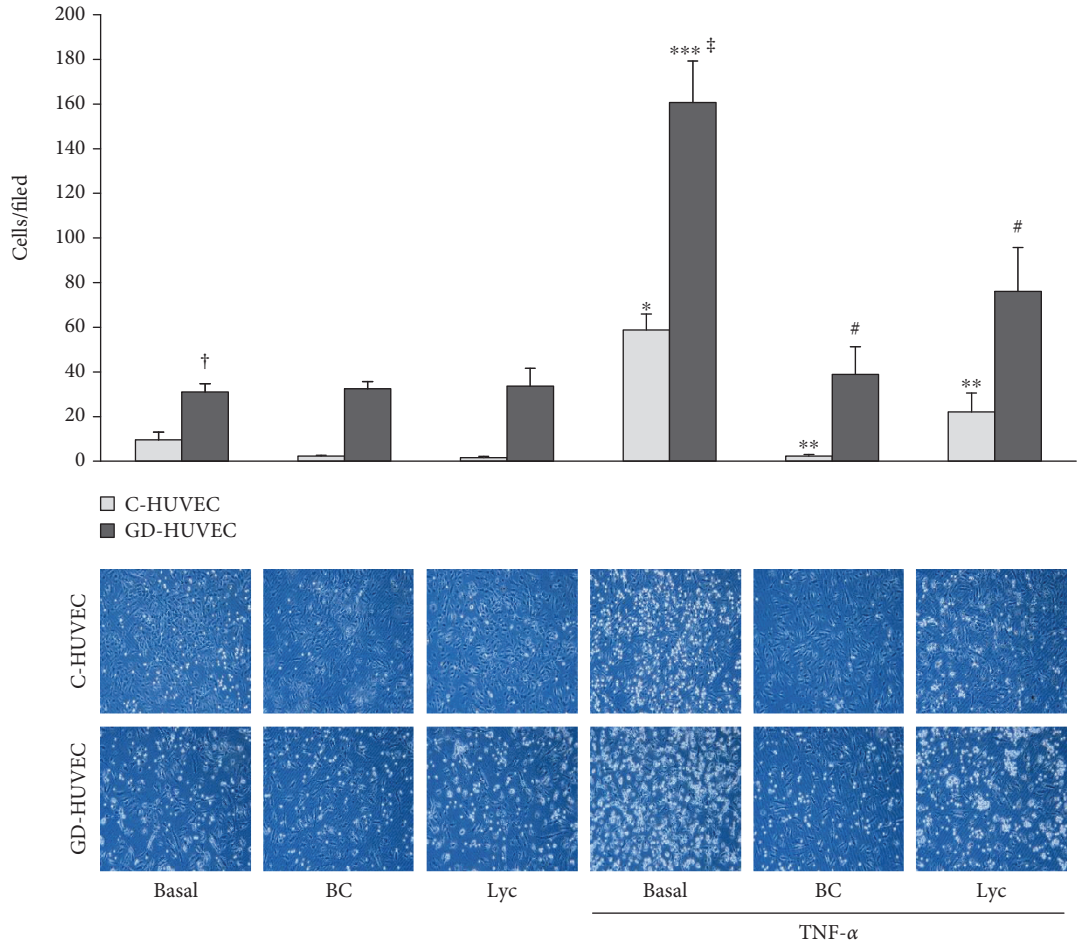

Figure 1: Effect of carotenoids on TNF-α-induced monocyte interaction in C- and GD-HUVECs. Monocyte-HUVEC adhesion in C- and GD-HUVECs untreated (Basal) and incubated for 24 h with BC or Lyc (2.5 μmol/L) and then stimulated for 16 h with or without TNF-α (1 ng/mL). In the histogram (upper side), quantitative data express the number of U937 cells adhering within a high-power field (3.5mm^2). Each measurement is expressed as the mean ± SD of adhering cells from 3 experiments ($n = 3$), each consisting of 8 counts per condition. In the lower side, representative photos of C- and GD-HUVECs for each experimental condition. *ANOVA and Bonferroni multiple comparison test:* $^*p < 0.05$ vs. basal C-HUVECs, $^{**}p < 0.05$ vs. TNF-α C-HUVECs, $^{***}p < 0.05$ vs. Basal GD-HUVECs, $^{\#}p < 0.05$ vs. TNF-α GD-HUVECs. *Student's t-test:* $^{\dagger}p < 0.0002$ basal GD-HUVECs vs. basal C-HUVECs, $^{\ddagger}p < 0.0001$ TNF-α GD-HUVECs vs. TNF-α C-HUVECs.

3. Results

3.1. Effect of Carotenoids on Monocyte-HUVEC Interaction. The effects of carotenoids on human monocyte line U937 adhesion rate to control and GD-HUVECs, in basal or TNF-α-stimulated conditions, were investigated.

Figure 1 shows that, in the basal state, the monocyte-GD-HUVEC interaction was significantly higher compared to C-HUVECs ($p < 0.0002$). The exposure to 1 ng/mL TNF-α further increased this difference ($p < 0.05$). Interestingly, pretreatment with 2.5 μmol/L of BC or Lyc for 24 hours significantly resulted in the reduction of monocyte adhesion induced by TNF-α to both cell types ($p < 0.05$).

3.2. Effect of Carotenoids on Adhesion Molecule Membrane Exposure and Expression. The exposure of the adhesion molecules on the endothelial cell membrane is the major mechanism responsible for the monocyte-endothelial cell interaction. We thus evaluated VCAM-1 and ICAM-1 membrane exposure and total protein expression in C- and GD-HUVECs with or without the pretreatment with BC or Lyc (2.5 μmol/L for 24 h) and in the presence or absence of the inflammatory stimulus TNF-α.

Figure 2 shows that both basal VCAM-1 (Figure 2(a)) and ICAM-1 (Figure 2(b)) exposure is greater on GD-HUVEC membrane compared to control cells ($p < 0.001$ and $p = 0.05$, respectively). TNF-α increased the exposure of VCAM-1 and ICAM-1 in both cell types ($p < 0.05$). The increased exposure induced by TNF-α was significantly reduced in the presence of 2.5 μmol/L for 24 h BC or Lyc ($p < 0.05$). Interestingly, in GD-HUVECs, Lyc is able to reduce ICAM-1 exposure on the endothelial membrane also in the basal state ($p < 0.05$).

After 16 h of 1 ng/mL TNF-α stimulation, a significant increase in VCAM-1 (Figure 2(c)) and ICAM-1 (Figure 2(d)) total protein levels was observed. The increase was more pronounced in GD-HUVECs as compared to C-HUVECs ($p < 0.05$). Remarkably, in TNFα-stimulated

C- and GD-HUVECs, the pretreatment with 2.5 μmol/L for 24 h of BC (left) or Lyc (right) significantly decreased adhesion molecule protein levels ($p < 0.05$), supporting the idea of the potential role played by these carotenoids in the reduction of the monocyte adhesion.

3.3. Effect of Carotenoids on NF-κB p65 Nuclear Translocation. Figure 3 shows the nucleus-NF-κB p65 colocalization rate expressed as the histogram (Figure 3(a)) and single-cell images (Figure 3(b)) in the presence or absence of BC and Lyc (2.5 μmol/L for 24 h) in C- and GD-HUVECs with or without the inflammatory stimulus TNF-α.

As expected, compared to C-HUVECs, GD-HUVECs at basal condition showed an enhanced NF-κB p65 nuclear translocation level ($p < 0.01$), which was further significantly increased following TNF-α stimulation in both cell types ($p < 0.05$), and it resulted to be more evident in GD-HUVECs ($p < 0.005$ vs. C-HUVECs). Interestingly, 24 h pretreatment with BC or Lyc was associated to a significant reduction of TNF-α-induced NF-κB nuclear translocation in both GD- and C-HUVECs ($p < 0.05$). It is noteworthy how in BC and Lyc pre-treated control cells a reduction of NF-κB nuclear translocation was also evident in basal condition.

3.4. Effect of Carotenoids on NO Bioavailability. As shown in Figure 4, as compared to basal condition, TNF-α stimulation significantly decreased nitric oxide levels both in C- and in GD-HUVECs ($p < 0.05$). Pretreatment with 2.5 μmol/L β-carotene and lycopene restored NO bioavailability in both the cell types. Moreover, after TNF-α exposure, both the cell types displayed decreased levels of cGMP, a biological target of NO activity, compared to their basal condition, and this was more evident in GD-HUVECs ($p < 0.05$). Notably, cGMP content significantly increased after pretreatment with BC and Lyc in both TNF-α-stimulated C-HUVECs and TNF-α-stimulated GD-HUVECs ($p < 0.05$). As a positive control, eNOS activator ionomycin (Iono) stimulation was used and a significant increase in nitric oxide bioavailability and cGMP levels ($p < 0.05$) was observed in C-HUVECs, the effect that was abolished by the preincubation with the eNOS inhibitor L-NAME ($p < 0.05$).

3.5. Effect of Carotenoids on Peroxynitrite Production. To better understand if the increased NO levels after BC and Lyc stimulation were associated with a reduced peroxynitrite formation, we evaluated the intracellular $ONOO^-$ production in C- and GD-HUVECs in the presence or absence of an inflammatory stimulus.

Figure 5 shows that, in the basal state, GD-HUVECs display greater peroxynitrite levels compared to C-HUVECs ($p < 0.05$). The pretreatment with 2.5 μmol/L of BC or Lyc for 24 h decreased the peroxynitrite levels in both TNFα-stimulated cell types. This result is more evident in GD-HUVECs ($p < 0.05$). The potent oxidant molecule PMA (200 ng/mL) and Ionomycin (50 nmol/L) pretreatment for 30 minutes highly increased the percentage of positive cells for peroxynitrite probe ($p < 0.05$), mostly in control with respect to GD-HUVECs, confirming the efficiency of the assay.

4. Discussion

Cardiovascular complications are the major consequences of chronic hyperglycemia and represent the main reason for impaired life expectancy in diabetic patients [40]. In fact, cardiovascular events represent the most prevalent cause of morbidity and mortality in diabetic patients [40]; thus, an optimal control of both hyperglycemia and cardiovascular risk factors is necessary to prevent adverse outcomes in type 2 diabetic patients [41]. More in depth knowledge of the complex of mechanisms controlling vascular damage in diabetes suggested that some natural molecules could be able to address multiple aspects of diabetes and its complications and, most importantly, to reduce disease-related morbidity and mortality [14].

Although several studies have been performed regarding the potential protective role of some natural antioxidant molecules, disparate results have been obtained regarding the beneficial effect of antioxidant therapy in the reduction of diabetes incidence and the prevention of its cardiovascular complications [42].

Carotenoids are among the main characterized natural antioxidants studied in order to find new potential protective molecules for chronic inflammation and oxidative stress [12]. However, controversial data have been found on their effects. Indeed, it is likely that the structure of carotenoids makes these molecules highly susceptible to oxidation under certain conditions, such as the oxygen partial pressure (PO_2) and their high amount [43–46], inducing a reduction in their nutritional value and their beneficial action [47, 48].

In addition, several studies also showed that the mechanisms of action and the antioxidant capacities of carotenoids could be totally different and strongly dependent upon their interaction with other antioxidant compounds [49]. Indeed, an increase in the concentration of one might reduce the absorption of another with a great antioxidant capacity reducing the overall effectiveness [50]. In fact, the effect of antioxidant molecules has to be considered the result of a complex network involving several reactive species and other biological targets, and the redox regulation has to consider not only the ROS imbalance in a quantitative manner but also their chemical structure, cellular location, formation and degradation rate, and physiological functions [51]. Furthermore, the presence of chemical interactions between reactive oxygen species, which influence the response of organism to environmental challenges and stressors ensuring its homeostasis, must be considered. Then, it is necessary not to discard the idea that inappropriate removal of ROS could be also be self-defeating [52].

Hence, there is still not a definite scenery regarding the potential benefic effect of carotenoid diet administration in diabetes cardiovascular complication prevention and their mechanism of action, so further analyses are needed.

In the present study, we investigated the mechanisms potentially involved in carotenoid prevention of vascular inflammation and atherogenesis under chronic hyperglycemic condition.

In particular, we evaluated the effect of β-carotene and lycopene on the modulation of the inflammatory and nitro-oxidative state of GD-HUVECs, which represent a

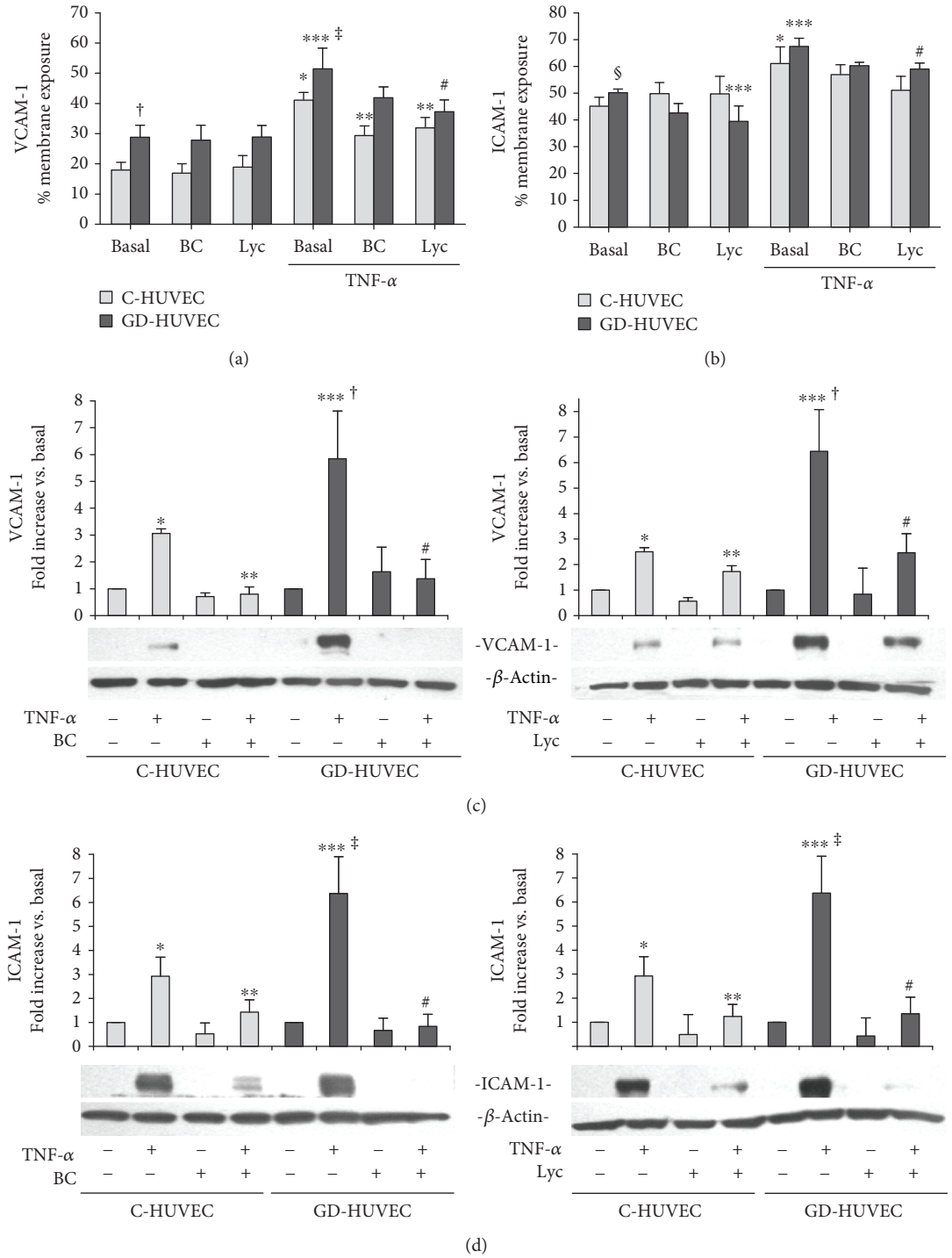

Figure 2: The effect of carotenoids on adhesion molecule membrane exposure and total expression after TNF-α-stimulation in C- and GD-HUVECs. VCAM-1 (a) and ICAM-1 (b) membrane exposure in C- and GD-HUVECs untreated (basal) and incubated for 24 h with BC or Lyc (2.5 μmol/L) and then stimulated for 16 h with or without TNF-α (1 ng/mL). Quantitative data in histograms result from 4 different experiments ($n = 4$). The results are expressed as the percentage of positive cells for surface exposure on the plasma membrane of VCAM-1 and ICAM-1 in not permeabilized cells. Representative Western blot and its histogram for VCAM-1 (c) and ICAM-1 (d) total protein expression in C- and GD-HUVECs untreated (Basal) and incubated for 24 h with 2.5 μmol/L of BC (left panels) or Lyc (right panels) and then stimulated for 16 h with or without TNF-α (1 ng/mL). Quantitative data in histograms result from 3 different experiments ($n = 3$). The results for the VCAM-1 or ICAM-1 and β-actin ratio are expressed as arbitrary units, and data are shown as fold increase vs. basal condition of the mean ± SD from three independent experiments. *ANOVA and Bonferroni multiple comparison test:* $^{*}p < 0.05$ vs. basal C-HUVECs, $^{**}p < 0.05$ vs. TNF-α C-HUVECs, $^{***}p < 0.05$ vs. basal GD-HUVECs, $^{\#}p < 0.05$ vs. TNF-α GD-HUVECs. *Student's t-test:* in (a) and (b), $^{\dagger}p < 0.001$ and $^{\S}p = 0.05$ basal GD-HUVECs vs. basal C-HUVECs, $^{\ddagger}p < 0.05$ TNF-α GD-HUVECs vs. TNF-α C-HUVECs; in (c) and (d), $^{\dagger}p < 0.03$ and $^{\ddagger}p < 0.05$ TNF-α GD-HUVECs vs. TNF-α C-HUVECs.

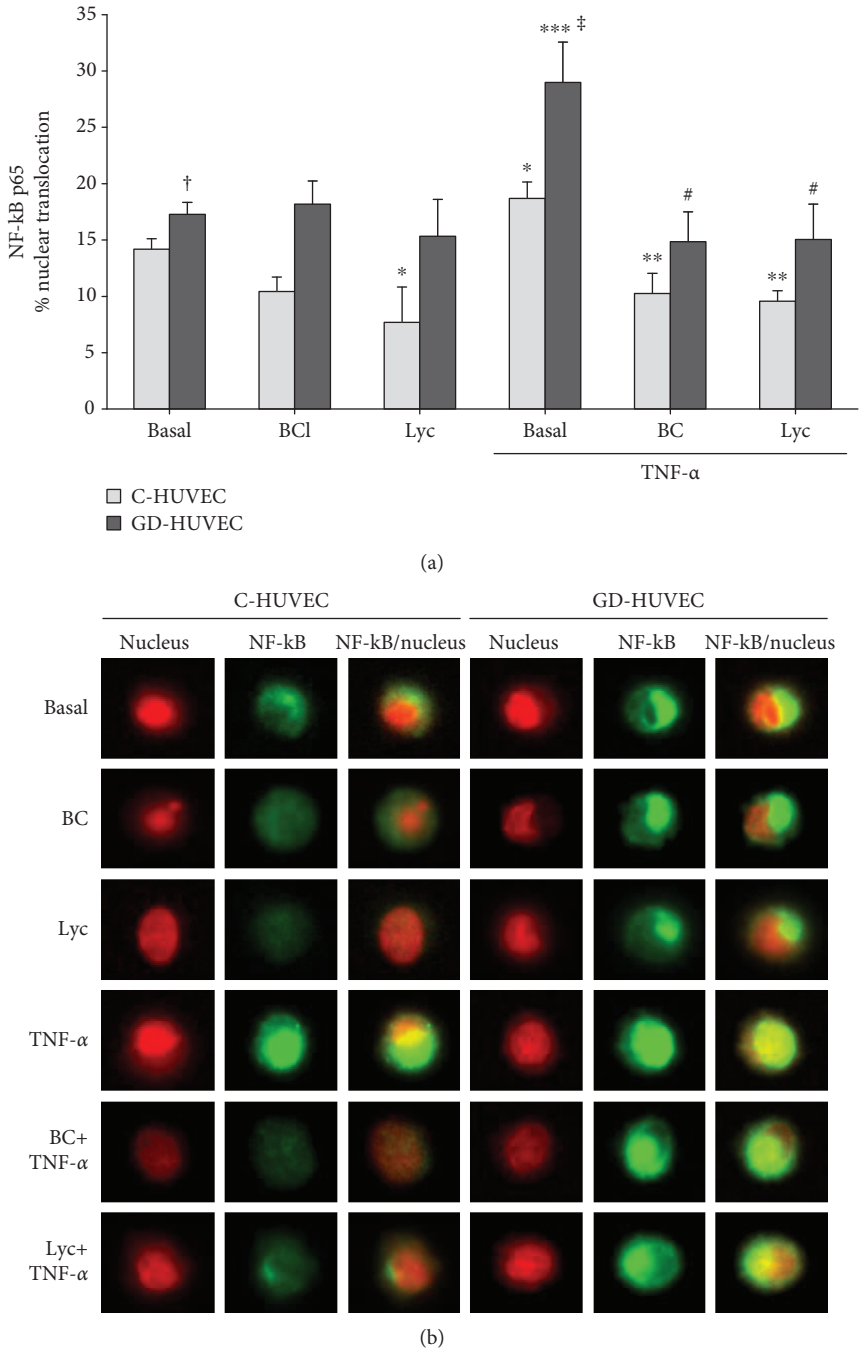

Figure 3: The effect of carotenoids on TNF-α-increased NF-κB p65 nuclear translocation levels in C- and GD-HUVECs. The histogram (a) and representative single-cell images (b) of NF-κB p65 cytoplasm-nucleus translocation in untreated (basal) or TNF-α stimulated C- and GD-HUVECs after preincubation for 24 h with of BC or Lyc (2.5 μmol/L). In (a), data in the histogram result from 3 independent experiments ($n = 3$) and are expressed as the percentage of positive cells for nucleus-NF-κB p65 colocalization. In (b), nuclei are stained in red and NF-κB p65 in green for each experimental condition. *ANOVA and Bonferroni multiple comparison test:* $^{*}p < 0.05$ vs. basal C-HUVECs, $^{**}p < 0.05$ vs. TNF-α C-HUVECs, $^{***}p < 0.05$ vs. basal GD-HUVECs, $^{\#}p < 0.05$ vs. TNF-α GD-HUVECs. *Student's t-test*: $^{\dagger}p < 0.01$ basal GD-HUVECs vs. basal C-HUVECs, $^{\ddagger}p < 0.005$ TNF-α GD-HUVECs vs. TNF-α C-HUVECs.

useful cellular model of endothelial dysfunction occurring during hyperglycemic conditions [34]. Cells were exposed *in vitro* to β-carotene and lycopene concentration comparable to circulating levels of those molecules reached after oral administration, *in vivo*.

The results obtained suggest a new hypothesis regarding the mechanism of action of carotenoids in the prevention of diabetes-related cardiovascular complications.

At first, in order to evaluate carotenoids' anti-inflammatory action, we determined the monocyte-

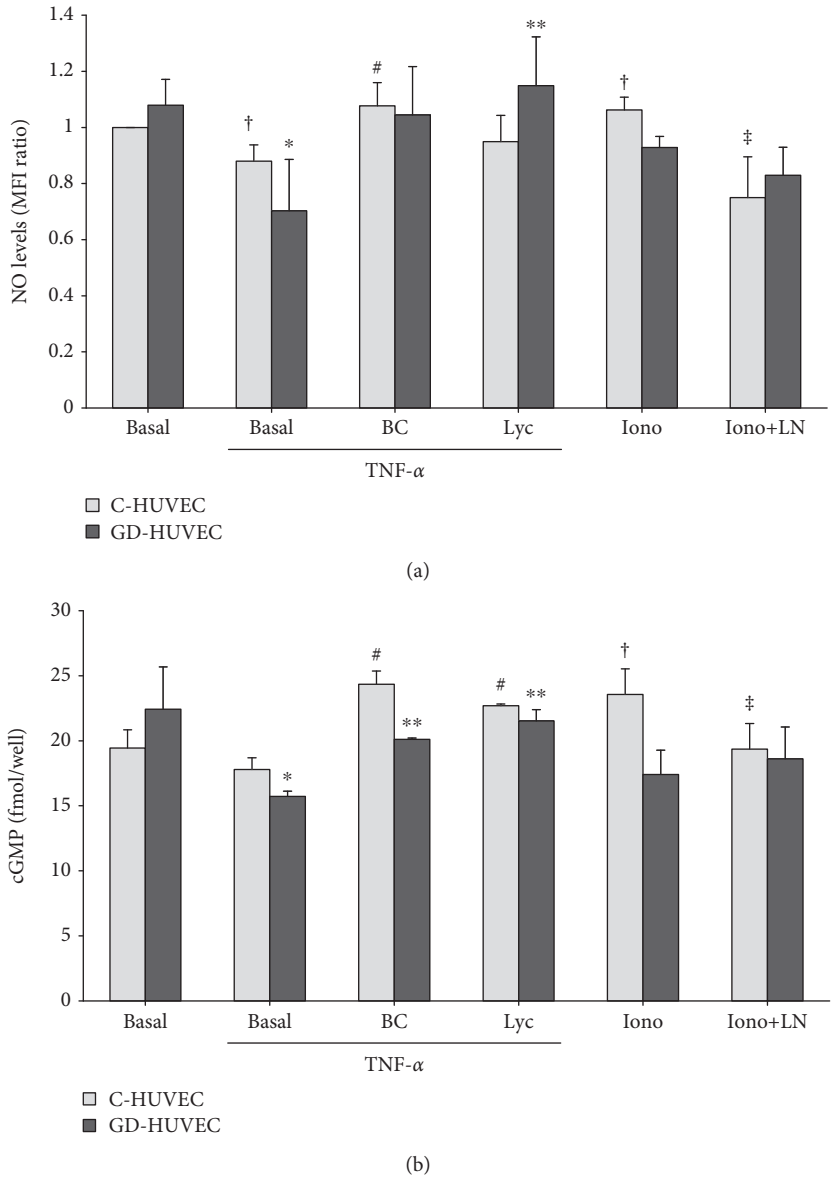

Figure 4: The effects of carotenoids on NO bioavailability in C- and GD-HUVECs. (a) Nitric oxide generation measured by DAF-2DA cytometric analysis and (b) cGMP levels measured by an EIA kit in HUVECs pretreated with BC or Lyc (2.5 mmol/L) in the presence or absence of 16 h stimulation with TNF-α (1 ng/mL). The stimulation with ionomycin (Iono, 2 μmol/L) for 24 h with or without L-NAME (LN, 1 mmol/L) preincubation (45 minutes) is used as a positive control. In (a), data are expressed as the mean fluorescence intensity (MFI) ratio (signal to noise ratio) from 4 independent experiments ($n = 4$). In (b), data result from 3 different experiments ($n = 3$) and are expressed as fmol/well. *ANOVA and Bonferroni multiple comparison test:* $^{*}p < 0.05$ vs. basal and $^{**}p < 0.05$ vs. TNF-α in GD-HUVECs, $^{\#}p < 0.05$ vs. TNF-α C-HUVECs. *Student's t-test*: $^{\dagger}p < 0.05$ vs. basal and $^{\ddagger}p < 0.05$ vs. Iono C-HUVECs.

endothelial cell interaction rate, finding that β-carotene and lycopene significantly reduced monocyte-HUVEC adhesion in TNF-α-stimulated C- and GD-HUVECs (Figure 1). Coherently, we also observed that BC and Lyc significantly decreased TNF-α-induced VCAM-1 and ICAM-1 membrane exposure and the total protein expression in both control and GD cells (Figure 2).

Moreover, it is noted that NF-κB plays a fundamental role in the expression of proinflammatory molecules such as cytokines, chemokine, and adhesion molecules [53] and several studies demonstrated that it is highly involved in metabolic disorders and atherosclerosis [54, 55], thus evaluating the effect of carotenoids treatment on NF-κB nuclear translocation.

Interestingly, we found that, under a TNF-α-stimulated state, carotenoid treatment significantly reduced NF-κB nuclear translocation in control cells as well as in GD-endothelial cells (Figure 3). Notably, these data highlight the ability of carotenoids to inhibit the inflammatory pathway not only in healthy conditions, as previously found [37], but also in a hyperglycemic state, suggesting their anti-inflammatory role in diabetes.

In this regard, we consider the effect of BC and Lyc pretreatment in the modulation of nitric oxide bioavailability,

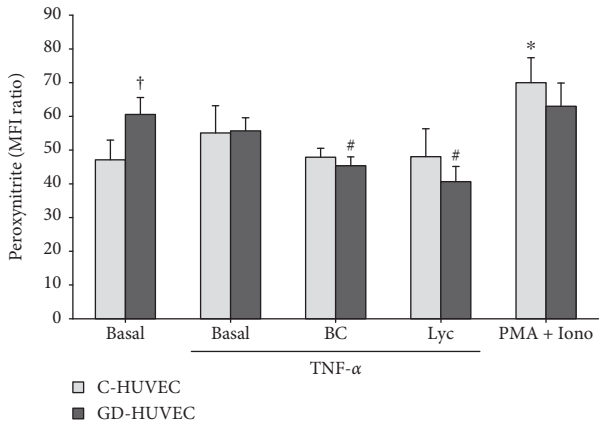

Figure 5: The effect of carotenoids on peroxynitrite levels in C- and GD-HUVECs. Intracellular peroxynitrite production in C- and GD-HUVECs incubated for 24 h with BC or Lyc (2.5 μmol/L) with or without TNF-α-stimulation (1 ng/mL) for 16 h. Data in the histogram are expressed as the mean fluorescence intensity (MFI) ratio (signal to noise ratio) of 4 independent experiments ($n = 4$). Phorbol myristate acetate (PMA, 200 ng/mL) and ionomycin (Iono, 50 nM) for 30 min before the assay are used as positive controls for endogenous peroxynitrite production. *ANOVA and Bonferroni multiple comparison test:* $^{*}p < 0.05$ vs. basal C-HUVECs, $^{\#}p < 0.05$ vs. TNF-α GD-HUVECs. *Student's t-test*: $^{\dagger}p < 0.05$ basal GD-HUVECs vs. basal C-HUVECs.

which is involved in the modulation of the NF-κB pathway [56] and thus in the vascular homeostasis balance.

Of note, in endothelial cells chronically exposed to high glucose and inflammation, despite an increase in NO production [57], the bioavailability of nitric oxide is decreased, as we previously demonstrated [34], probably as results of the "quenching" of NO, which rapidly reacts with the high levels of superoxide to produce peroxynitrite [58].

Here, we further confirm that the exposition to the proinflammatory stimulus TNF-α promotes a decline in NO levels and a decrease of the production of cGMP, its biological target, both in C- and in GD-HUVECs (Figure 4). However, remarkably, we also found that the exposure to β-carotene and lycopene induced an increase in NO bioavailability, particularly in GD-HUVECs.

In this regard, not surprisingly, the pretreatment with BC and Lyc resulted in the decreased peroxynitrite production in both TNF-α-exposed control and GD-HUVECs (Figure 5), confirming their antioxidant action and their role in the promotion of nitric oxide level maintenance.

All together, these results indicate that carotenoids contribute to restore endothelial homeostasis in an endothelial cell model chronically exposed to high-glucose levels by promoting nitric oxide bioavailability, exerting both antioxidant and anti-inflammatory actions.

5. Conclusion

In conclusion, data obtained in the present study elucidate the mechanisms of action of carotenoids in the modulation of the inflammatory and oxidative state induced *in vitro* by the proinflammatory molecule TNF-α on an endothelial cellular model of chronic hyperglycemia.

Then, while care must be taken regarding the safety of chronic and high-dose carotenoid supplementation, our results show that a diet amount administration of these natural food components could be important for the management of the vascular homeostasis in hyperglycemic conditions, speculating that a carotenoid-rich diet could prevent cardiovascular complications in diabetic patients.

Data Availability

The experimental data and materials used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

MU, PDT, and AP conceived and designed the experiments. MU, PDT, FT, VGPC, GB, and SDS performed the experiments. MU, PDT, PL, NDP, CP, and DM analysed the data. MU, PDT, GF, and AP drafted the paper. All authors read and approved the final manuscript. Mariangela Ucci and Pamela Di Tomo contributed equally to this work.

Acknowledgments

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