Cardiovascular Therapeutics

Natural Active Compounds as Cardiovascular Therapeutics: A Gender View

Lead Guest Editor: Annalisa Noce
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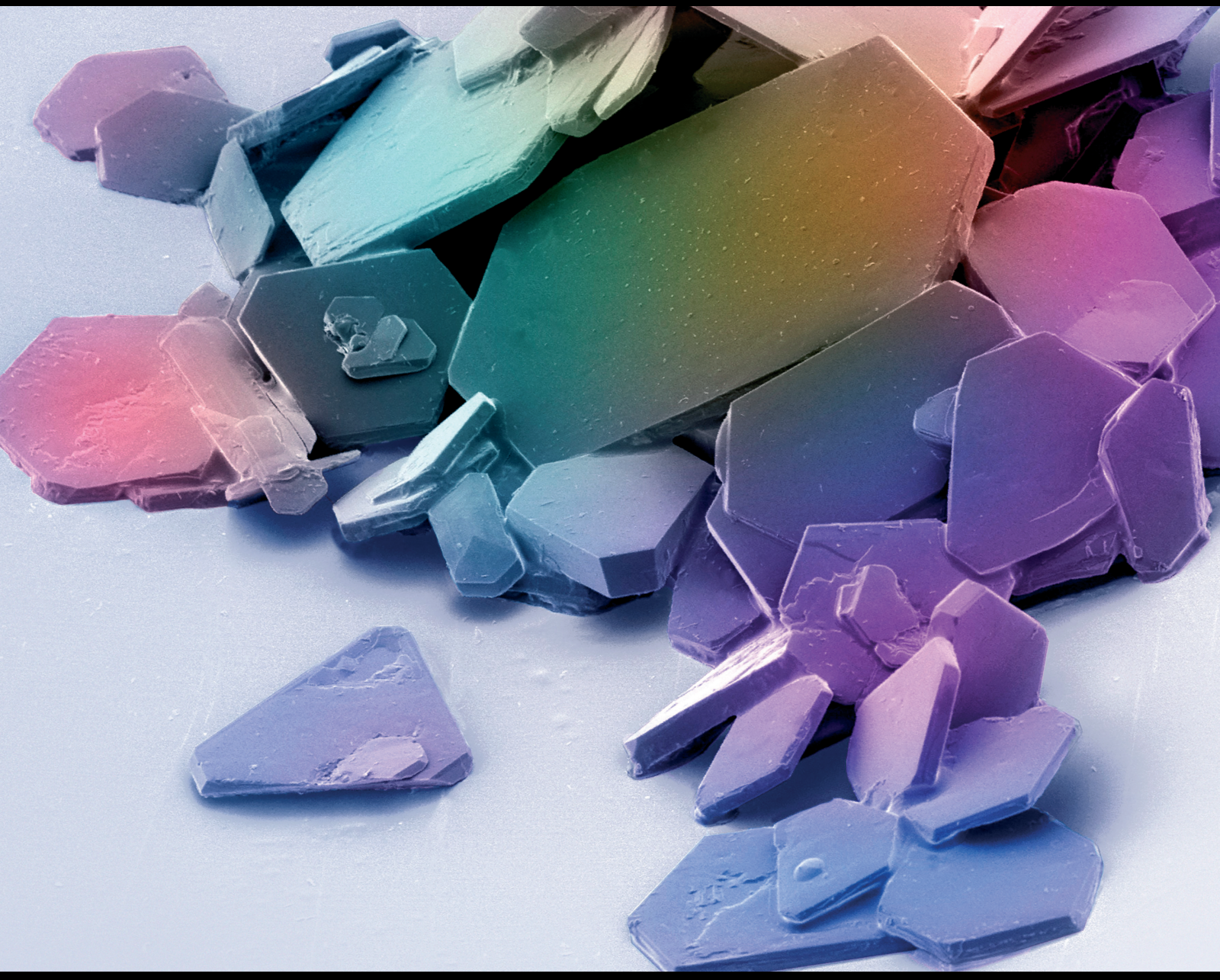

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Research Article

Evaluation of Cardiovascular Risk in Hidradenitis Suppurativa Patients Using Heart Rate Variability (HRV) Analysis

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Guest Editor: Annalisa Romani

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease associated with elevated prevalence of comorbidities, especially metabolic and cardiovascular diseases. We used a tool called Heart Rate Variability (HRV) in order to assess the correlation between HS and alterations of the sympathetic-vagal equilibrium in the autonomic cardiovascular regulation system. We found increased sympathetic activity, associated with a higher risk of cardiovascular disease. HS, according to our results, is an independent cardiovascular risk factor.

1. Introduction

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disease characterized by recurrent skin nodules, abscesses, and draining sinus tracts affecting primarily the apocrine gland-rich regions of the skin, especially the axillae and groin [1, 2].

HS has a chronic evolution with unpredictable periodic exacerbations. The lesions show a tendency to turn into fistulous tunnels, draining externally a serum-purulent material healing with the formation of retracting scars. Prevalence is estimated from <1% to 4% [3, 4], and the incidence is 11.4 cases per 100,000 patients, which is steadily increasing. Previous studies revealed a female predominance of 3 : 1 in the general population [5, 6]. Genetic factors play a fundamental role in the pathogenesis of HS, and one-third of patients report a positive family history of the disease. However, although the HS pathogenetic mechanism is still unknown, follicular hyperkeratinization probably characterizes the first step of the inflammatory process [7]. Histological examination revealed follicular hyperkeratosis and lymphocytic perifollicular infiltration followed by the disruption of the follicular unit.

Furthermore, the immunohistochemistry studies revealed increased production of proinflammatory molecules, like tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1b, IL-12, IL-17, and IL-23.

In addition, TNF-α is one of the main cytokines that can be associated with the development of insulin resistance and hyperlipidemia, characteristics associated with metabolic syndrome.

Recent works suggest that HS should be viewed as a systemic inflammatory disease linked most commonly to metabolic, gastrointestinal, rheumatologic, psychiatric, and cardiovascular (CV) comorbidities.

The prevalence of the sympathetic branch of the autonomous cardiovascular system in the population reflects an increased CV risk. In this study, we used Heart Rate Variability (HRV) as a noninvasive tool to assess the sympathetic-vagal equilibrium in the autonomous CV regulation system [8–10]. Our aim was to evaluate whether autonomic function

is affected in young HS patients and whether to consider HS as an independent cardiovascular risk factor [11].

2. Material and Methods

2.1. Subjects. Subjects are those affected by moderate to severe HS who attended our Outpatient Service of Dermatology from October 2018 to April 2019.

The heart rate (HR), RR trait (in ms), PQ interval (v.n.: 120-220 ms), QRS (v.n.: <100 ms), and QTc (v.n.: <430 ms) were measured.

Patients were informed not to assume any modifiers of autonomic nervous system functions such as psychoactive drugs and caffeine within 4 hours prior to the electrocardiogram (ECG). Informed consent was collected from each patient.

2.2. Assessments. We used the Cardiolab CE pocket PC ECG system in order to obtain an 8 min digital ECG at rest in supine position and during 8 minutes of orthostatic position obtained by the use of a passive tilt table.

With dedicated software (Cardiolab—Xai Medica), we analyzed the HRV indexes in the time and frequency domains. We will also analyze the nonlinear dynamics of HRV by Kubios software [12].

2.3. Linear Methods. We used linear methods in order to obtain a direct estimation of HRV through two types of analysis: time-domain analysis and frequency-domain analysis.

The time-domain method is based on NN intervals which are related to RR intervals in the ECG. They are analyzed to acquire variables such as SDNN (Standard Deviation of all NN intervals) and RMSSD (Root Mean Square of Successive Differences between adjacent NNs); both indexes represent parasympathetic tone.

Frequency-domain analysis, which is based on the power spectral density of the heart rate time series, highlights the issue of the underlying rhythms of the mechanisms controlling the heart rate (HR) and identified three major spectral peaks: high frequency (HF: 0.15-0.4 Hz), low frequency (LF: 0.04-0.15 Hz), and very low frequency (VLF: below 0.04 Hz) in the adult HR spectrum.

LF is an indicator of both sympathetic and parasympathetic activities. HF reflects parasympathetic activity. The LF/HF ratio is an index representing overall balance between sympathetic and parasympathetic systems. Higher values reflect dominance of the sympathetic branch, while lower ones reflect the predominance of the parasympathetic action.

2.4. Nonlinear Methods. The nonlinear methods (Poincarè plots and Detrended fluctuation analysis) are not modified by environmental features and measure the complex mechanisms regulating the signal [13].

Poincarè plots is a two-dimensional vector analysis that was used to quantify the shape of the plots (Figure 1). In this quantitative method, short-term (SD1) and long-term (SD2) RR interval variability and the ellipse area of the plot are quantified separately. SD1 is an indicator of vagal modulation of the sinus node. The HR correlations were defined separately for short-term (<11 beats, $\alpha 1$) and longer-term (>11 beats, $\alpha 2$) RR interval data. The $\alpha 1$ index was positively correlated with LF in normalized units. The other nonlinear method is the detrended fluctuation analysis (DFA) that consists of a procedure utilized to measure the fractal scaling assets of short-term and intermediate-term variability in the RR ECG intervals. The decrease of the $\alpha 1$ index is considered a mortality prognostic factor in patients affected by severe cardiovascular conditions such as ischemic events and cardiac insufficiency.

2.5. Data Analysis. Our study results were examined through two types of software: Cardiolab, Xai Medica (used to investigate HRV linear methods), and Kubios HRV 2.0 (for the HRV nonlinear analysis examination). We expressed our data as the mean ± standard error. We calculated the t-test in order to estimate the quantitative variables, and the chi-squared test was performed to estimate the qualitative ones.

We performed a statistical analysis using the software SigmaStat 3.5 (Systat Software Inc., Point Richmond, CA, USA). We considered a P value with a significance level < 0.05.

3. Results

Sixteen HS patients were recruited, including 9 females and 7 males aged between 16 and 48 years. Four of sixteen patients have normal weight, 6/16 were overweight, 3/16 patients presented with grade I obesity, 2/16 patients presented grade II obesity, and one patient presented with grade III obesity. In addition, eight of sixteen patients were smokers.

Information about gender, age, weight, height, BMI, and smoking habits is reported in Table 1.

We did not observe any significative difference when we catalogued patients according to BMI, though an increase of the heart rate (RR) was observed in subjects with a BMI > 25 (Table 2).

On the difficulty of having clean data, we analyzed 8/16 patients in detail, as preliminary data: 3 males and 5 females aged between 16 and 48 years. Of these subjects, only 2 patients presented with a normal weight while 6 patients had a BMI greater than 24.99 kg/m^2 (2 patients were overweight, 3 patients had a grade I obesity, and 1 patient had a grade III obesity). Five patients were smokers, and 3 patients were nonsmokers. In all patients, the age of onset was between 10 and 28 years while the age of diagnosis was between 15 and 45 years. The ECG recorded did not show any alteration, except in one patient, where it showed a right conduction disorder (Table 3).

As expected, from the supine to orthostatic position, RR interval showed a significant decrease (from 796.3 ± 110.8 to 674.68 ± 56.95 msec—$P < 0.001$); systolic and diastolic arterial pressure showed a slight increase as in normal subjects (Tables 4 and 5).

We also compared the HRV indexes of subjects with HS with those observed in a group of 14 normal subjects from our previous work. We observed a reduction of total HRV in the time and frequency domain: the STD showed a significant decrease in HS patients (40.32 ± 5.79 to 33.1 ± 19.4 msec). The sympathetic indexes in HS patients are higher than those

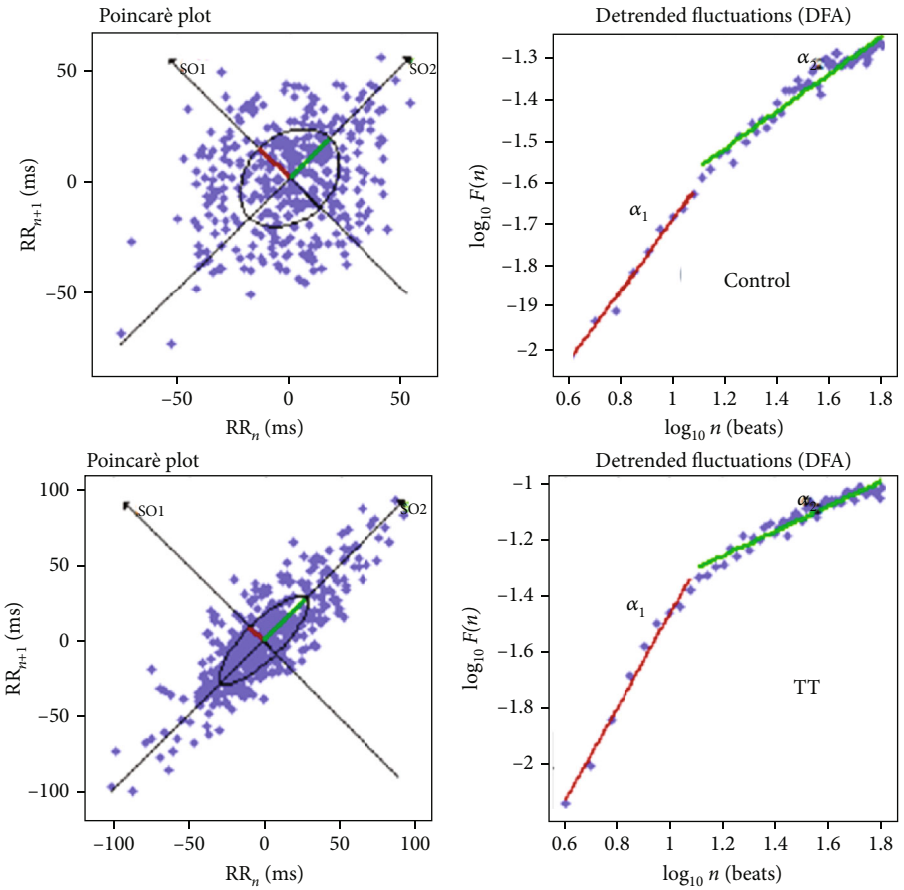

Figure 1: An example of Poincarè plot and DFA during a Tilt Test (TT) in a patient with HS.

Table 1: HS patients included in the study and assessed with ECG.

Age (years)	Sex	Height (cm)	Weight (kg)	BMI	Smoke	HR (bpm)	RR (ms)	PQ (ms)	QRS (ms)	QTc (ms)
41	F	1.68	83	29.4	Yes	78	768	114	88	324.00
16	F	1.65	72	26.4	No	93	642	148	58	296.00
31	M	1.7	95	32.9	Yes	81	740	140	92	344.00
15	M	1.62	93	35.4	No	87	690	140	78	332.00
48	M	1.8	90	27.8	Yes	60	1004	170	84	410.00
13	F	1.42	79	39.2	No	89	672	134	80	303.00
28	F	1.7	85	29.4	Yes	74	816	98	70	361.00
16	M	1.85	85	24.8	No	59	1020	152	88	404.00
36	M	1.66	77	27.9	No	70	856	148	88	370.00
19	F	1.76	72	23.2	No	78	768	122	76	324.00
32	F	1.65	83	30.5	Yes	79	758	148	86	322.00
28	F	1.57	76	30.8	Yes	67	892	144	84	349.00
24	F	1.7	130	45	Yes	93	644	156	88	297.00
16	F	1.67	60	21.5	No	83	720	172	108	314.00
22	M	1.85	95	27.8	Yes	74	806	104	110	359.00
21	M	70	1.85	20.5	No	69	866	120	88	372.00
25.38		6.23	79.80	29.53		77.125	791.375	138.125	85.375	342

Table 2: HS patients included in the study and assessed with ECG, divided by BMI.

(a) Normal weight

Physical condition	Smoke	HR	RR	PQ	QRS	QTc
Normal weight	No	59	1020	152	88	404.00
Normal weight	No	78	768	122	76	324.00
Normal weight	No	83	720	172	108	314.00
Normal weight	No	69	866	120	88	372.00
		72.25	843.5	141.5	90	353.50

(b) Overweight and obesity

Physical condition	Smoke	HR	RR	PQ	QRS	QTc
Obesity I	Yes	81	740	140	92	344.00
Obesity I	Yes	79	758	148	86	322.00
Obesity I	Yes	67	892	144.0	84	349.00
Obesity II	No	87	690	140	78	332.00
Obesity II	No	89	672	134	80	303.00
Obesity III	Yes	93	644	156	88	297.00
Overweight	Yes	78	768	114	88	324
Overweight	No	93.0	642	148	58	296.00
Overweight	Yes	60	1004	170	84	401.00
Overweight	Yes	74	816	98	70	361.00
Overweight	No	70	856	148	88	370.00
Overweight	Yes	74	806	104	110	359.00
		78.75	774	137	85.38	338.17

in normal subjects even if not all of them reached statistical significance (LF/HF ratio 1.13 ± 0.24 vs. 1.42 ± 1.1, DFA $\alpha 1$ 0.85 ± 006 vs. 1.04 ± 0.29).

4. Discussion

HS is an inflammatory skin disease of the terminal hair follicle. It manifests clinically with the onset of painful nodules, abscesses, and draining sinus tracts eventually resulting in scars, involving multiple regions of the body.

Some recent studies have suggested a connection between HS and a significantly increased risk of ischemic cardiovascular events, such as myocardial infarction and stroke. It was also suggested that the risk of ischemic events is higher in subjects affected by HS when compared to severe psoriasis patients [14, 15]. The association between inflammatory markers and sympathetic hyperactivation indexes remained significant after correcting important risk factors and confounding factors such as age, Body Mass Index, heart rate, and smoking. Sympathetic activation can also be triggered by reflex mechanisms (arterial baroreceptor impairment), psychological stress, oxidative stress, obstructive sleep apnea, inflammation, and metabolic factors as I.R. and dysregulated production and secretion of adipokines from visceral fat with a particular important role of leptin [16].

Interestingly, a Danish cross-sectional study showed that the mean heart rate in severe HS patients in resting conditions was suggestively higher when compared with that of controls [17]. Both HS and atherosclerosis have a chronic inflammatory subset, and cardiovascular and metabolic diseases are the most common comorbidities found in HS [18, 19]. Atherosclerosis, a chronic inflammatory disease affecting the blood vessels, is considered a major cause of cardiovascular diseases. Markers of inflammation are associated with both cardiovascular risk and the severity of HS inflammatory manifestations. It is known that inflammation represents the core in the bridge between HS and the pathogenesis of cardiovascular diseases, though many doubts remain regarding its mechanism of action. An important role is played by interleukin- (IL-) 17, which is found to be upregulated in lesional and perilesional skin of HS patients [20]. Overexpression of various other cytokines, such as IL-1, TNF-α, IL-10, and IL-11, as well as antimicrobial peptides (AMPs), beta-defensin 2, psoriasin, and cathelicidin, has been observed in the lesional setting.

Interleukin- (IL-) 32 expression is significantly enhanced in plaque of atherosclerosis, and its proinflammatory function could help increase the risk of CV events in patients affected by chronic inflammatory diseases [21, 22]. Moreover, HS patients generally present a higher incidence of other cardiovascular risk factors such as obesity, smoking history, diabetes, hypertriglyceridemia, and metabolic syndrome [23].

The present study was based on the analysis of HRV as a noninvasive tool to evaluate the association between HS and an increased CV risk. The mechanism leading to depressed HRV in heart failure is complex and not perfectly described, but it could partially be related to the alteration of neurohumoral activity [24].

The results indicate that the sympathetic activity increases during the Tilt Test more than in normal subjects as shown by the tachycardia and by the increase of the arterial pressure and LF/HF ratio and the decrease of the STD. The Tilt Test induces, also, an increase in the properties of short-term fractal correlations of heart rate dynamics (DFA) accompanied with a decrease in all the nonlinear indexes of HRV, confirming the thesis that these indexes are an expression of the cardiac sympathetic-vagal balance.

HS patients, if compared to the rest of the population, tend to present a higher incidence of carotid atherosclerosis. The role of smoking in the severity of HS is still not clear because it is not known whether smoking cessation improves the course of the disease.

A higher resting heart rate and a lower heart rate variability are linked to subclinical chronic inflammation in adult and elderly patients. The higher death rate that was described in this kind of situations could therefore manifest a shared etiology. A disproportion of the sympathetic branch of the autonomic nervous system could correlate with inflammatory events to play a major role in atherosclerosis.

It has been previously demonstrated that the use of HRV allows us to evidence an upregulated CV risk in other inflammatory dermatological disorders, such as psoriasis [25]. As reported in patients with psoriasis, in patients with HS, the sympathetic activation indexes (LF/HF, SD2/SD1, and $\alpha 1/\alpha 2$) are higher than those in normal subjects too.

Table 3: Characteristics of HS patients evaluated with HRV (Heart Rate Variability).

Age	Weight (kg)	Height (m)	BMI	Physical condition	Smoke	Age of onset	Age of diagnosis	ECG
16	72	1.65	26.4	Overweight	No	10	15	Normal
31	95	1.7	32.9	Obesity I	Yes	20	30	Normal
48	90	1.8	27.8	Overweight	Yes	20	45	dx conduction disorder
19	72	1.76	23.2	Normoweight	No	14	18	Normal
32	83	1.65	30.5	Obesity I	Yes	28	28	Normal
28	76	1.57	30.8	Obesity I	Yes	15	25	Normal
24	130	1.7	45	Obesity III	Yes	13	23	Normal
21	70	1.85	20.5	Normoweight	No	14	20	Normal

Table 4: HRV values obtained using both linear and nonlinear methods in clinostatic position.

Clino	RR (ms)	STD RR (ms)	LF (%)	HF (%)	LF/HF (-)	SD_1 (ms)	SD_2 (ms)	SD_2/SD_1	α_1	α_2	α_1/α_2
	675	24.5	71	23.2	3.06	12.6	32.3	0.39	1.411	0.302	4.67
	757.1	16.9	67.3	23.8	2.83	11.5	20.9	0.55	1.272	0.46	2.77
	965.5	34.3	33.3	62.1	0.54	33.3	35.4	0.94	0.627	0.372	1.69
	795.8	36.6	40.9	51.5	0.79	27.9	43.7	0.64	0.839	0.436	1.92
	766.2	26.1	38.8	49.7	0.78	19.1	31.7	0.6	1.015	0.443	2.29
	860.6	42.3	29.6	62.3	0.48	19.6	28.5	0.69	0.69	0.39	1.77
	642.8	10.4	55.6	31	1.79	7	12.9	0.54	1.024	0.525	1.95
	907.6	73.6	35.9	61.4	0.58	57.1	87.1	0.66	0.829	0.203	4.08
Mean	796.3	33.1	46.6	45.6	1.4	23.5	36.6	0.6	1	0.4	2.6
SD	110.8	19.4	15.9	17.1	1.1	16.1	22.4	0.2	0.3	0.1	1.1

Table 5: HRV values obtained using both linear and nonlinear methods in orthostatic position.

Ortho	RR (ms)	STD RR (ms)	LF (%)	HF (%)	LF/HF (-)	SD_1 (ms)	SD_2 (ms)	SD_1/SD_2	α_1	α_2	α_1/α_2
	599.5	27.9	79.1	7.5	10.55	9	38.4	0.23	1.714	0.486	3.53
	724.5	18.5	63.2	23.4	2.70	9.6	24.4	0.39	1.401	0.558	2.51
	746.7	30.3	57.6	34.4	1.67	25.8	34.2	0.75	0.975	0.373	2.61
	741.8	25.3	47.7	46.9	1.02	14.3	32.8	0.44	1.126	0.422	2.67
	669.8	20.2	63.9	20.7	3.09	11	26.3	0.42	1.299	0.63	2.06
	646.8	23.2	86	5.3	16.23	6.8	32.1	0.21	1.759	0.522	3.37
	614.3	13.7	54.6	36	1.52	7.6	17.8	0.43	1.080	0.576	1.88
	654	41.8	54.4	39.3	1.38	23.3	54.4	0.43	1.198	0.329	3.64
Mean	674.68	25.11	63.31	26.69	4.77	13.43	32.55	0.41	1.32	0.49	2.78
SD	56.95	8.58	13.07	15.05	5.57	7.26	10.94	0.16	0.29	0.10	0.66

Studies conducted on patients with known HS indicate that many of them are overweight. Obesity is one of the main risk factors associated with the development of HS, although its role is yet to be defined. We did not observe any significant difference when reorganizing patients according to the BMI, although an increase of heart rate (RR) was observed in subjects with a Body Mass Index (BMI) > 25. Furthermore, we observed that the sympathetic activation indexes during passive orthostatism had increased compared to clinostatism.

5. Conclusions

Our data show a balanced reduction of the parasympathetic influence on the sinus node in patients with moderate to severe HS. These results could correlate with an increased risk of cardiovascular disease; thus, HS should be considered as an independent CV risk factor.

Our data seems to indicate an increase in the sympathetic hyperactivity indices in HS patients, but since our study was based on a limited number of cases, we consider it to be

preliminary and more studies are necessary in order to obtain statistical significance. Identification of individuals at risk for subsequent morbidity and nonrisk groups requires future prospective studies to determine the sensitivity, specificity, and predictive values of HRV.

Moreover, our data demonstrates that biological therapy and other anti-inflammatory therapies could alter the autonomous cardiac function in patients with moderate to severe HS, as reported in a previous work on psoriasis [26]. The important connection between HS and cardiovascular diseases underlines the necessity of a cardiovascular screening in HS patients, particularly if other risk factors are present.

Although the exact role of smoking in the pathogenesis of HS remains to be determined, HS could give us another opportunity to encourage our patients to change their lifestyle.

Dermatologists must be conscious of the comorbidities related to HS. The aim is to create a condition in which patients are managed by the proper specialists. This may lead to an improvement in the disease course and in the quality of life of patients affected by this disease.

Data Availability

The authors are available to provide data supporting the research.

Conflicts of Interest

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

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Research Article

MFGE8, ALB, APOB, APOE, SAA1, A2M, and C3 as Novel Biomarkers for Stress Cardiomyopathy

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Background. Stress cardiomyopathy (SCM) is a transient reversible left ventricular dysfunction that more often occurs in women. Symptoms of SCM patients are similar to those of acute coronary syndrome (ACS), but little is known about biomarkers. The goals of this study were to identify the potentially crucial genes and pathways associated with SCM. *Methods*. We analyzed microarray datasets GSE95368 derived from the Gene Expression Omnibus (GEO) database. Firstly, identify the differentially expressed genes (DEGs) between SCM patients in normal patients. Then, the DEGs were used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Finally, the protein-protein interaction (PPI) network was constructed and Cytoscape was used to find the key genes. *Results*. In total, 25 DEGs were identified, including 10 upregulated genes and 15 downregulated genes. These DEGs were mainly enriched in ECM-receptor interaction, dilated cardiomyopathy (DCM), human papillomavirus infection, and focal adhesion, whereas in GO function classification, they were mainly enriched in the extracellular region, positive regulation of the multicellular organismal process, establishment of localization, and intracellular vesicle. *Conclusion*. Seven hub genes contained APOE, MFGE8, ALB, APOB, SAA1, A2M, and C3 identified as hub genes of SCM, which might be used as diagnostic biomarkers or molecular targets for the treatment of SCM.

1. Introduction

Stress cardiomyopathy, also known as takotsubo cardiomyopathy (TTC), was first reported in Japan in 1990 [1], characterized by transient systolic and diastolic left ventricular (LV) dysfunction with multiple wall dyskinesia, most commonly occurring in postmenopausal women, especially in populations with a recent history of mental or physical stress [2]. The reversibility of cardiac insufficiency is one of the most prominent features of the disease. It is sometimes considered fairly benign, but it has a 4.1% in-hospital mortality rate, especially in the early stages analogous to acute coronary syndrome (ACS), and is also likely to have cardiogenic shock and fatal arrhythmias [3]. It estimates that 1 to 2% of suspected ACS patients were eventually diagnosed with stress cardiomyopathy.

The exact pathophysiology of stress cardiomyopathy is unknown and seems to be associated with excess plasma catecholamine, which is caused by stress conditions. Myocardial ischemia appears to play a crucial role in takotsubo syndrome, both in human heart muscle specimens and in experimental models of SCMP. Most cases occur in patients with risk factors for endothelial dysfunction [4].

Most patients with stress-induced cardiomyopathy (>95%) have electrocardiogram abnormalities that typically show ischemic ST-segment and T-wave changes, but his appearance is most likely to associate the presence of ACS than SCM [5, 6]. Previous studies have shown that cardiac biomarkers such as cardiac troponin T or I, creatine-kinase myocardial band (CK-MB), and b-type natriuretic peptide (BNP) may play a role in the early clinical recognition of SCM, but the accuracy is low [7, 8]. In patients with suspected stress

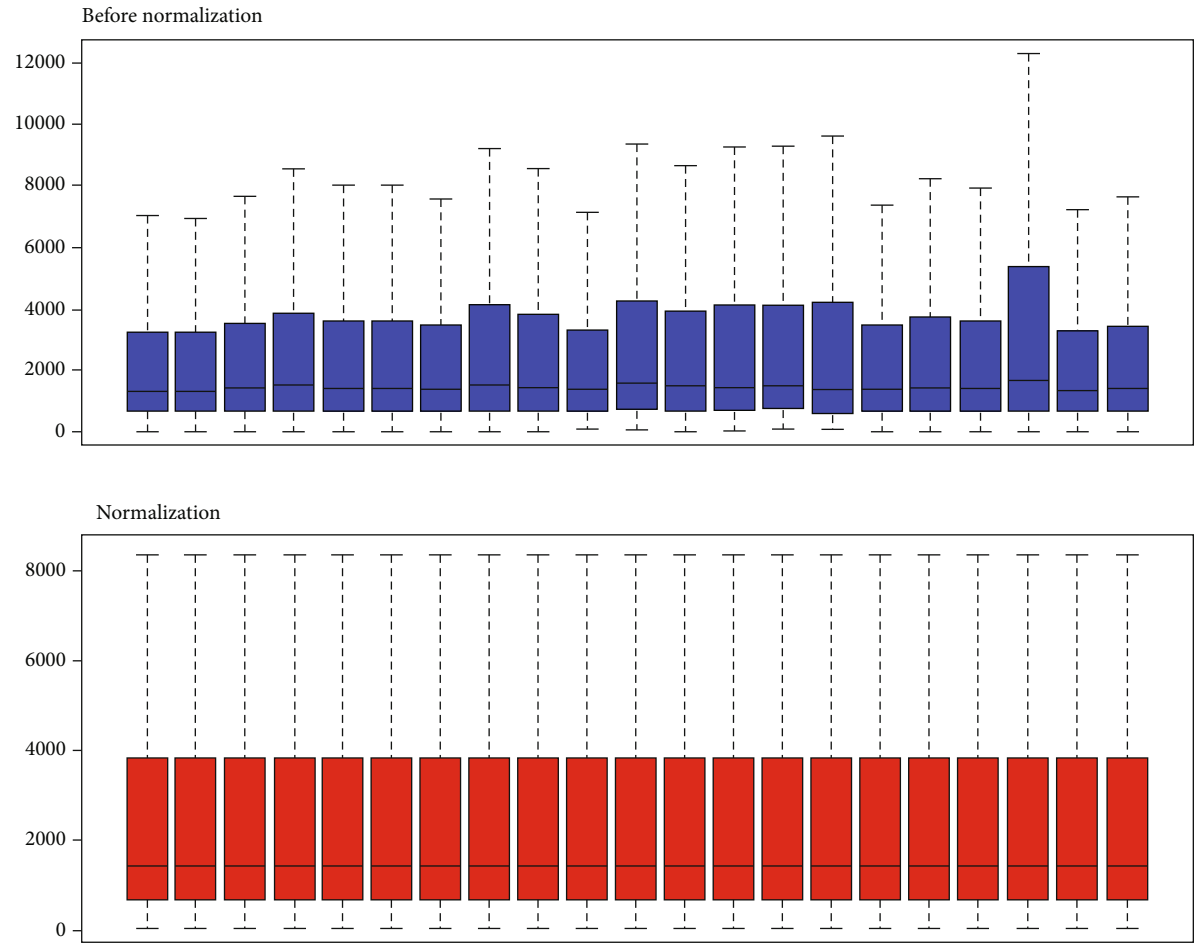

Figure 1: Standardization of gene expression. The blue bar represents the data before normalization, and the red bar represents the normalized data.

cardiomyopathy, transthoracic echocardiography with color and tissue doppler is the preferred noninvasive imaging trial [5]. A recent study reports that STE echocardiographic index may be more accurate and unique than traditional echocardiography for the early detection of subtle abnormalities [9]. Because of the reversibility of SCM's condition, it is not possible to determine the diagnosis at the time of presentation, although some clinical features are highly predictive of stressful myocardial disease [10].

In this study, we further revealed biomarkers related to SCM by analyzing gene expression profile (GSE95368) deposited by Yvonne Edwards et al. (2017). Identifying key genes and pathways contributes to a better understanding of the pathophysiological mechanism of disease development, which provides innovative ideas for the diagnosis and treatment of SCM.

2. Materials and Methods

2.1. Data Sources. Gene expression data of GSE95368 is available for download from the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). The expression profiling arrays were generated using the GPL23119 platform (SOMAscan human proteomic 1.3k assay). A total of 21 serum samples in this database, including 15 from SCM patients and 6 from normal serum samples, were analyzed.

2.2. Data Preprocessing and Identification of DEGs. Genetic matrix files and platform files were downloaded to eliminate errors and make the experimental group comparable between control groups, so the obtained data were standardized. The estimation package is based on the KNN (k-nearest neighbor) algorithm is used to fill the missing values. After this, the probes were converted into gene symbols on the basis of the annotation platform file. If there are multiple probes corresponding to a gene, take the average as the final value. If the probe without gene symbol was removed, the normalized between array function in the limma package is applied to standardize the data. Then, the expression data were log2 transformed and the limma functional package in R software was used to compare gene expression in SCM and control samples to identify DEGs. The screening criteria for DEGs were p value < 0.05 and $|\log 2\text{foldchange (FC)}| \geq 1$.

2.3. GO and KEGG Pathway Enrichment Analysis of DEGs. Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov) and KOBAS

Table 1: Screening upregulated and downregulated DEGs.

DEGs	Gene symbol
Upregulated (15)	LTA4H APOB ALB IL36A PRKACA NAMPT XPNPEP1 ITGA2B ITGB3 CA13 C3 EHMT2 PTPN6 AZU1 A2M MFGE8
Downregulated (10)	GAPDH APOE CRP NPPB PKM2 GDF15 PLAT ITGA1 ITGB1 THBS2 SAA1

Abbreviation: DEGs: differentially expressed genes.

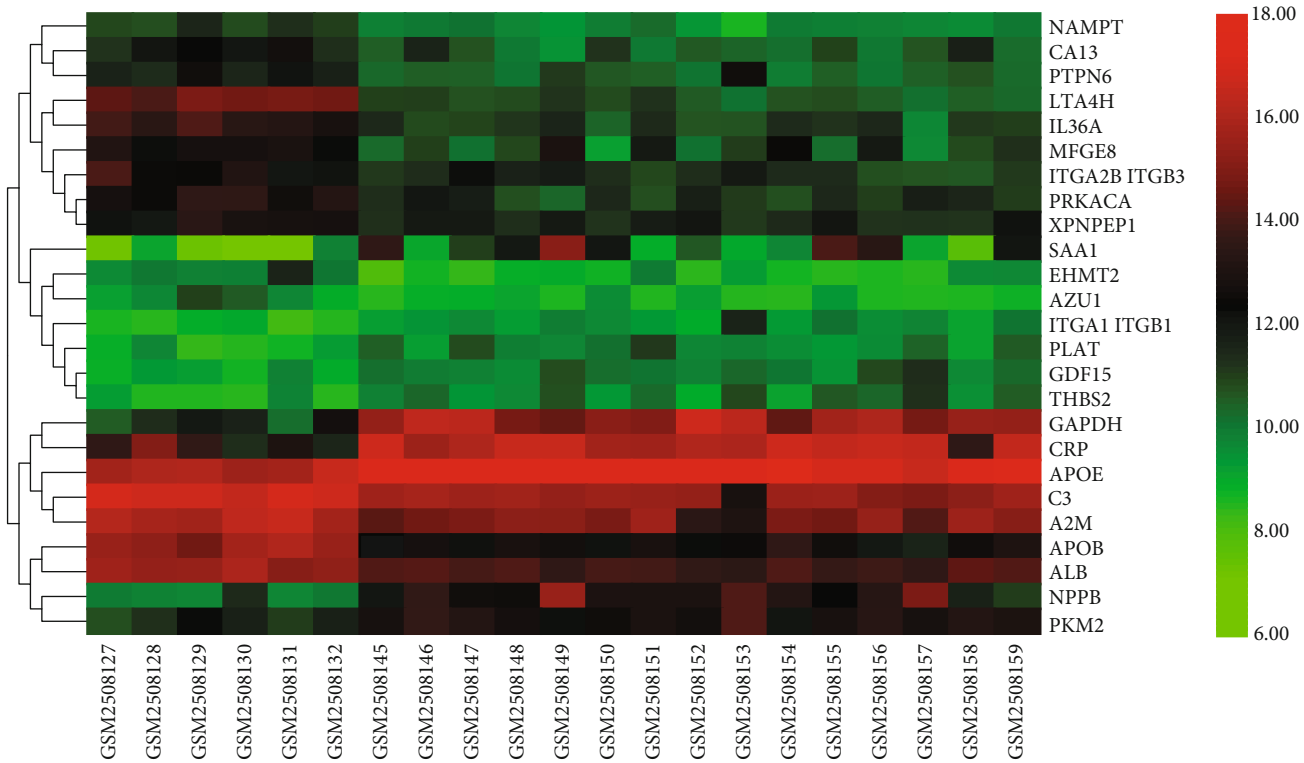

Figure 2: Heatmap results of DEGs. Abbreviation: DEGs, differentially expressed genes.

(http://kobas.cbi.pku.edu.cn) were applied for GO annotation and KEGG pathway analysis. Because the KOBAS online tool uses gene ID for data analysis, so we first use DAVID to convert the gene symbol of DEGs into gene ID and then use KOBAS for GO and KEGG enrichment analysis of DEGs. Finally, we use R software to visualize the results, to further understand the potential functions of the identified DEGs.

2.4. PPI Network Analyses. PPI network analyses can show the functional link between proteins and proteins, using string software (http://www.string-db.org) for PPI network analysis of differential genes. When building a protein interaction network, the settings are all set by default. Subsequently, Cytoscape software was applied to visualize and analyze the PPI network.

2.5. Module Analysis and Selection of Hub Genes. Molecular Complex Detection (MCODE) can find the interacting dense region in the PPI network, and the dense regions of interest can also be extracted and visualized. So we use MCODE to discover modules across the network. The hub genes were identified by using the plug-in cytoHubba of the Cytoscape software, including Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC), and Maximum Neighborhood Component (MNC).

3. Results

3.1. Identification of DEGs. The results of standardizing the matrix file are shown in Figure 1. We identified a total of 25 DEGs in SCM samples compared with normal samples, including 10 upregulated genes and 15 downregulated genes, which were statistically significant (adjusted $p < 0.05$, |log fold change (FC)| >1) (Table 1). The cluster heatmap plot and volcano plot of the DEGs are shown in Figures 2 and 3.

3.2. GO Enrichment Analysis of DEGs. The GO analysis consists of biological processes (BP), cellular component (CC), and molecular function (MF) terms. The different genes with adjusted p value < 0.05 were obtained from GO functional enrichment. The GO enrichment analysis results reveal that the DEGs were mainly involved in the extracellular region, positive regulation of the multicellular organismal process,

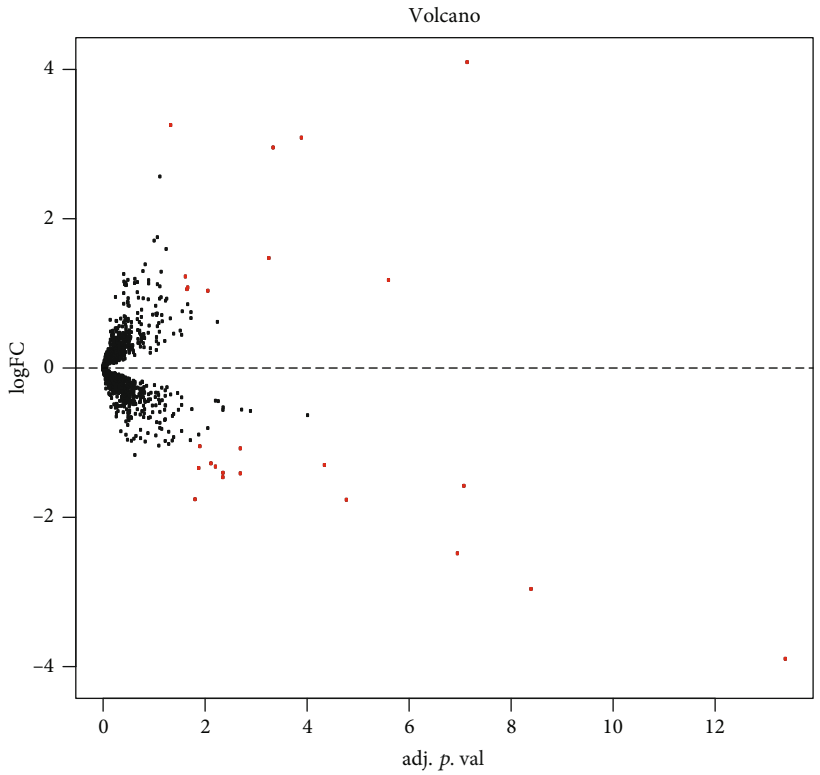

Figure 3: Differential expression of data between two sets of samples.

Table 2: GO enrichment analysis of differentially expressed genes.

Term	Description	Count	*p* value
GO:0005576	Extracellular region	7	4.42*E*-07
GO:0051240	Positive regulation of the multicellular organismal process	5	5.94*E*-07
GO:0051234	Establishment of localization	7	6.12*E*-07
GO:0097708	Intracellular vesicle	5	8.56*E*-07
GO:0098802	Plasma membrane signaling receptor complex	3	1.31*E*-06
GO:1901576	Organic substance biosynthetic process	7	3.12*E*-06
GO:0030198	Extracellular matrix organization	3	6.13*E*-06
GO:0050794	Regulation of cellular process	8	6.88*E*-06
GO:0032102	Negative regulation of response to external stimulus	3	9.39*E*-06
GO:0008152	Metabolic process	8	1.10*E*-05
GO:0032270	Positive regulation of cellular protein metabolic process	4	1.32*E*-05
GO:0140096	Catalytic activity, acting on a protein	5	1.78*E*-05
GO:0031399	Regulation of protein modification process	4	1.89*E*-05
GO:0007229	Integrin-mediated signaling pathway	2	2.37*E*-05
GO:0050810	Regulation of steroid biosynthetic process	2	2.37*E*-05
GO:0120039	Plasma membrane-bounded cell projection morphogenesis	3	4.40*E*-05
GO:0001568	Blood vessel development	3	4.80*E*-05
GO:0030667	Secretory granule membrane	2	5.16*E*-05
GO:1990266	Neutrophil migration	2	5.84*E*-05
GO:0072562	Blood microparticle	2	7.34*E*-05

Abbreviation: GO: Gene Ontology.

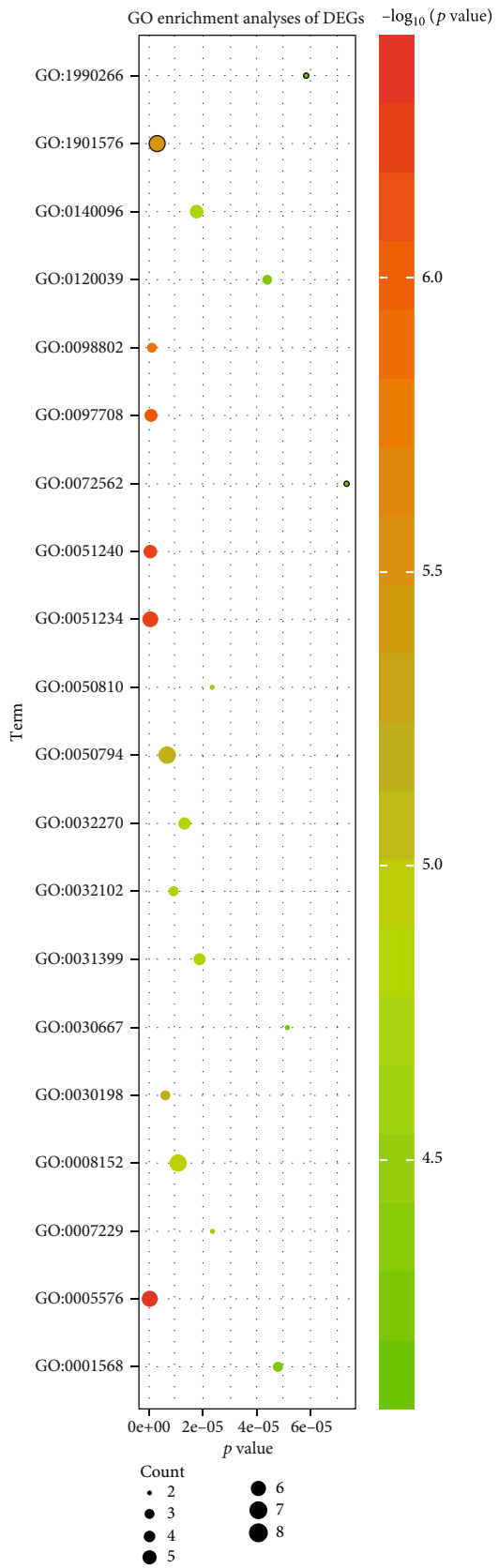

Figure 4: The DEGs significantly enriched GO (top 20). Abbreviation: DEGs, differentially expressed genes; GO, Gene Ontology.

Table 3: KEGG pathway analysis of DEGs.

Pathway	ID	Count	p value	Genes
ECM-receptor interaction	hsa04512	5	4.03E-09	ITGA1|ITGB3|THBS2|ITGB1|ITGA2B
Dilated cardiomyopathy (DCM)	hsa05414	5	6.84E-09	ITGA1|ITGB3|PRKACA|ITGB1|ITGA2B
Human papillomavirus infection	hsa05165	6	8.06E-08	THBS2|ITGB1|ITGB3|PRKACA|ITGA1|ITGA2B
Focal adhesion	hsa04510	5	2.31E-07	ITGA1|ITGB3|THBS2|ITGB1|ITGA2B
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	hsa05412	4	2.55E-07	ITGA1|ITGB3|ITGB1|ITGA2B
Hypertrophic cardiomyopathy (HCM)	hsa05410	4	4.65E-07	ITGA1|ITGB3|ITGB1|ITGA2B
Platelet activation	hsa04611	4	1.60E-06	ITGB3|PRKACA|ITGB1|ITGA2B
Phagosome	hsa04145	4	3.52E-06	ITGB3|THBS2|ITGB1|C3
PI3K-Akt signaling pathway	hsa04151	5	3.71E-06	ITGA1|ITGB3|THBS2|ITGB1|ITGA2B
Proteoglycans in cancer	hsa05205	4	1.08E-05	ITGB3|PRKACA|ITGB1|PTPN6
Regulation of actin cytoskeleton	hsa04810	4	1.32E-05	ITGA1|ITGB3|ITGB1|ITGA2B
Leishmaniasis	hsa05140	3	1.89E-05	ITGB1|PTPN6|C3
Complement and coagulation cascades	hsa04610	3	2.29E-05	PLAT|A2M |C3
Hematopoietic cell lineage	hsa04640	3	4.15E-05	ITGA1|ITGB3|ITGA2B
Fluid shear stress and atherosclerosis	hsa05418	3	0.000117572	ITGB3|PLAT|ITGA2B
Cholesterol metabolism	hsa04979	2	0.00056172	APOE|APOB
Thyroid hormone synthesis	hsa04918	2	0.001195438	PRKACA|ALB
Pertussis	hsa05133	2	0.001258577	ITGB1|C3
Rap1 signaling pathway	hsa04015	3	0.00038722786	ITGB3|ITGB1|ITGA2B
ECM-receptor interaction	hsa04512	5	4.03E-09	ITGA1|ITGB3|THBS2|ITGB1|ITGA2B

Abbreviation: KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: differentially expressed genes.

establishment of localization, and intracellular vesicle (Table 2, Figure 4).

3.3. KEGG Pathway Analysis of DEGs. The KEGG pathways of the DEGs were analyzed using DAVID and KOBAS. The top 20 of the KEGG pathways is shown in Table 3 and Figure 5, the DEGs chiefly enriched in ECM-receptor interaction, dilated cardiomyopathy (DCM), human papillomavirus infection, and focal adhesion.

3.4. Establishing the PPI Network, Conducting Module Analysis, and Selection of Hub Genes. Use the string online tool to create a PPI network to gain a better understanding of the biological properties of DEGs. There were 24 nodes and 51 edges in this network, as shown in Figure 6. Subsequently, Cytoscape is applied to confirm a vital module throughout the network; the most significant modules were selected, as shown in Figure 7. Nine key genes were identified, including SAA1, C3, CRP, ALB, APOE, APOB, MFGE8, GAPDH, and PLAT, finally utilizing the cytoHubba plug-in to determine APOE, MFGE8, ALB, APOB, SAA1, A2M, and C3 as the hub gene, as shown in Figure 8 and Table 4.

4. Discussion

In this study, we performed an integrated analysis of gene expression profiles from serum samples without/with SCM aiming to identify the DEGs, related key signaling pathways, and hub genes for the disease. A total of 25 DEGs, including 10 upregulated and 15 downregulated genes, were identified from the GSE95368 database. The GO enrichment analysis showed that these differential genes associated with SCM were mainly enriched in the extracellular region, positive regulation of the multicellular organismal process, establishment of localization, and intracellular vesicle. From the KEGG pathway enrichment analysis, we identified that these DEGs were mainly enriched in the pathway of the ECM-receptor interaction and dilated cardiomyopathy (DCM). Through the construction and module analysis of the PPI network, we identified 9 key genes, including SAA1, C3, CRP, ALB, APOE, APOB, MFGE8, GAPDH, and PLAT. Finally, APOE, MFGE8, ALB, APOB, SAA1, A2M, and C3 are regarded as hub genes for the development of SCM.

MFGE8, a secreted glycoprotein, is associated with a variety of pathophysiological processes, including anti-inflammatory [11], antifibrosis [12], antiatherosclerosis [13], and inhibition of cardiac hypertrophy [14]. Recent studies have shown that MFGE8 is a part of the arterial inflammatory signaling network that promotes endothelial cell apoptosis [15]. MFGE8 is an inflammatory mediator that coordinates multiple cellular interactions and is involved in the pathogenesis of various diseases. In a study investigating postischemic injury, MFGE8 may alleviate postischemic injury by integrin beta3-dependent inhibition of inflammatory bodies [15]. Cardiomyocyte ischemia plays a critical role in the development of SCM, so we propose that MFGE8 may play an important role in SCM by participating in the process of myocardial ischemia.

C3 is the most abundant complement component in serum, mainly macrophage and liver synthesis, and plays

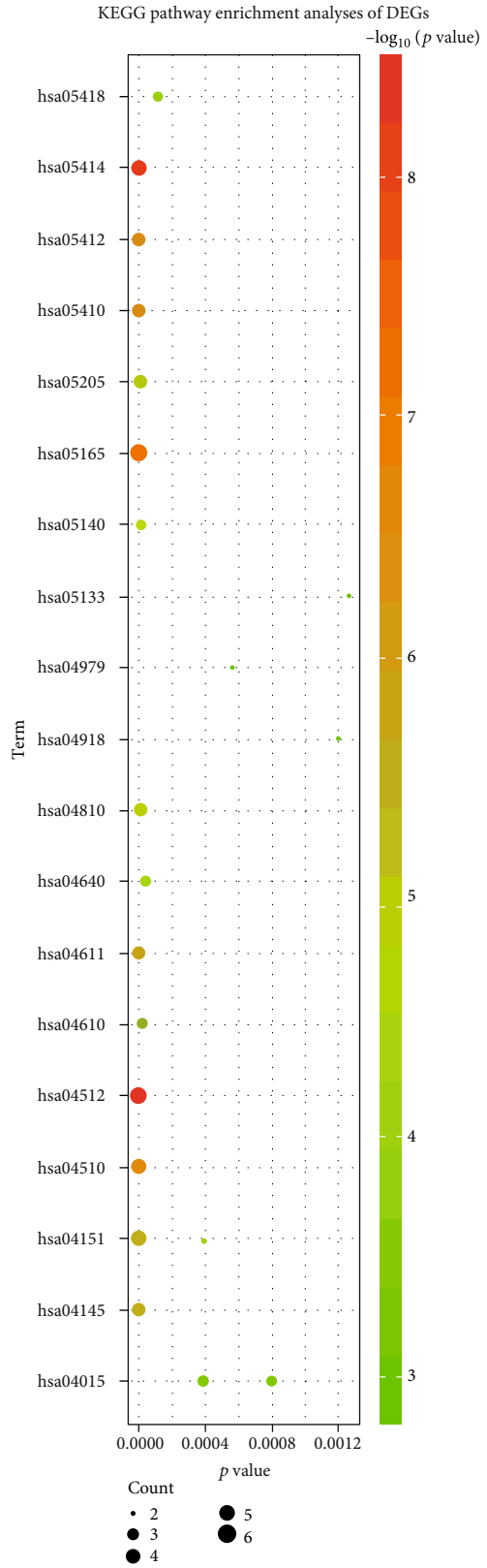

Figure 5: KEGG pathway analysis of DEGs. Abbreviation: KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: differentially expressed genes.

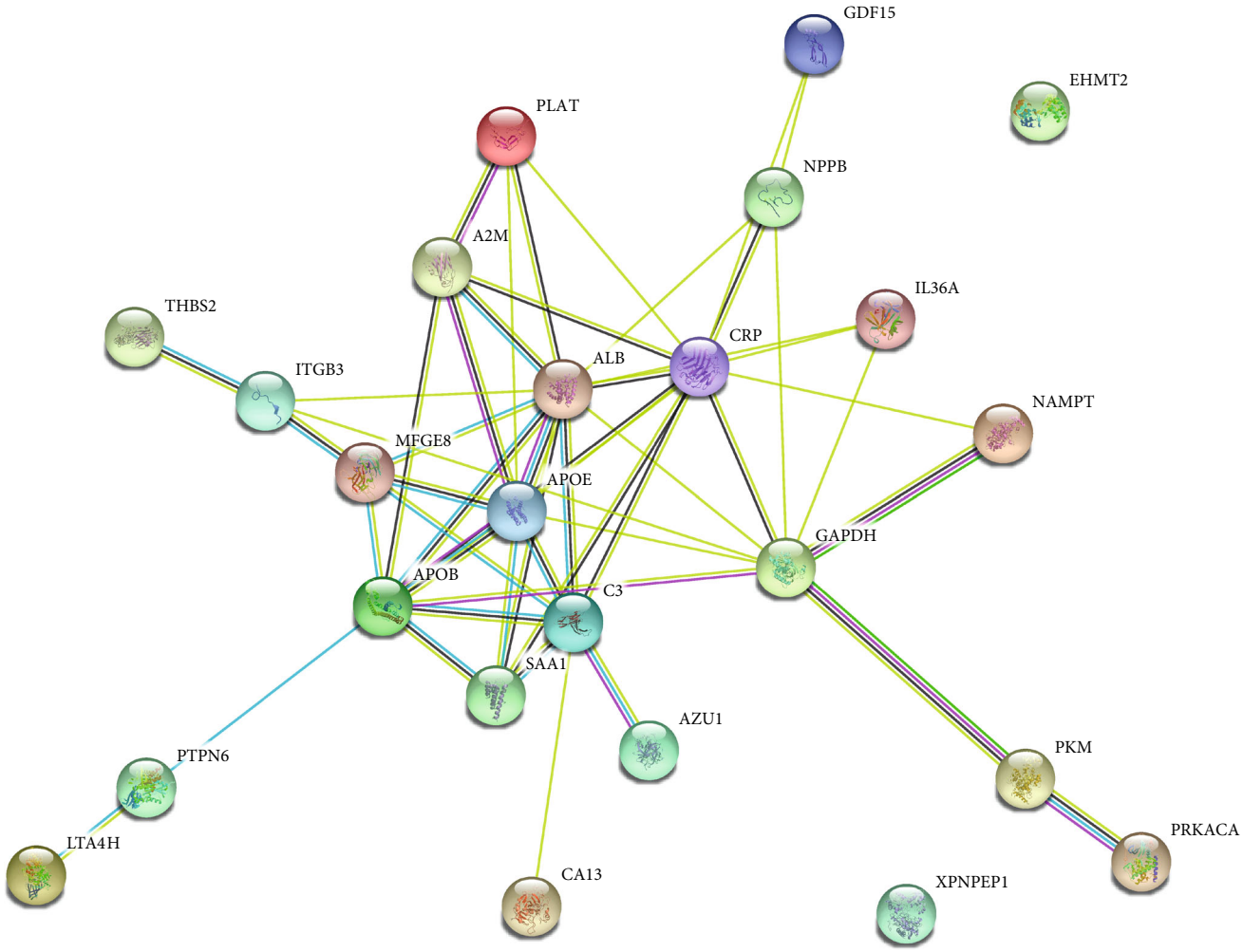

Figure 6: Results of PPI network analysis of DEGs. Abbreviation: PPI: protein-protein interaction; DEGs: differentially expressed genes.

an important role in complementing the classical activation pathway and bypass activation pathway [16]. C3 increases are common in the early stages of some acute inflammation or infectious diseases [17]. Although the specific mechanism of SCM is unclear, most patients are subjected to stress status. C3 is one of many neurohumoral factors released as a result of a series of adaptive responses in the body under stress [18]. So C3 may be involved in the pathological process of SCM through this pathway. SAA promotes chemotaxis of monocytes and neutrophils and plays a key role in various functions such as lipoprotein metabolism, cholesterol transport, and host defense [19]. Previous studies have shown that SAA1 plasma levels have increased dramatically in response to tissue damage, infection, and various emergencies [20, 21]. So SAA1 is also involved in the pathogenesis of SCM, and its mechanism is similar to that of C3.

Alpha-2-macroglobulin (A2M) is a broad-spectrum protease-binding protein of the vertebrate innate immune system that prevents pathogen invasion [22, 23]. In our study, both A2M and C3 were enriched in the complement and coagulation cascade pathway, suggesting an important role in coagulation. A recent study suggests that SCM is a prethrombotic state [24], which is consistent with our findings, so we speculate that A2M and C3 may be involved in the SCM pathological process through this pathway.

Serum albumin (ALB) is synthesized in the liver and is the most abundant protein in vertebrate plasma. Its main function is to maintain plasma colloid osmotic pressure and participate in the transport of various substances [25, 26]. We found that ALB is mainly associated with the synthesis of thyroid hormones, which increase in response to human mood swings and emergencies [27]. So thyroid hormone is involved in the process of SCM, and therefore, ALB is also likely to be involved in the pathogenesis of SCM. In addition, several studies have shown that serum ALB has a predictive value for a variety of thrombotic diseases [28, 29]. As mentioned above, SCM is also a prethrombotic state, so we guess that serum ALB also seems to have some predictive effect on SCM.

Apolipoprotein is a protein that can bind and transport blood lipids to tissues for metabolism and utilization [30]. A large number of studies have found that apolipoprotein gene mutation can affect blood lipid metabolism and

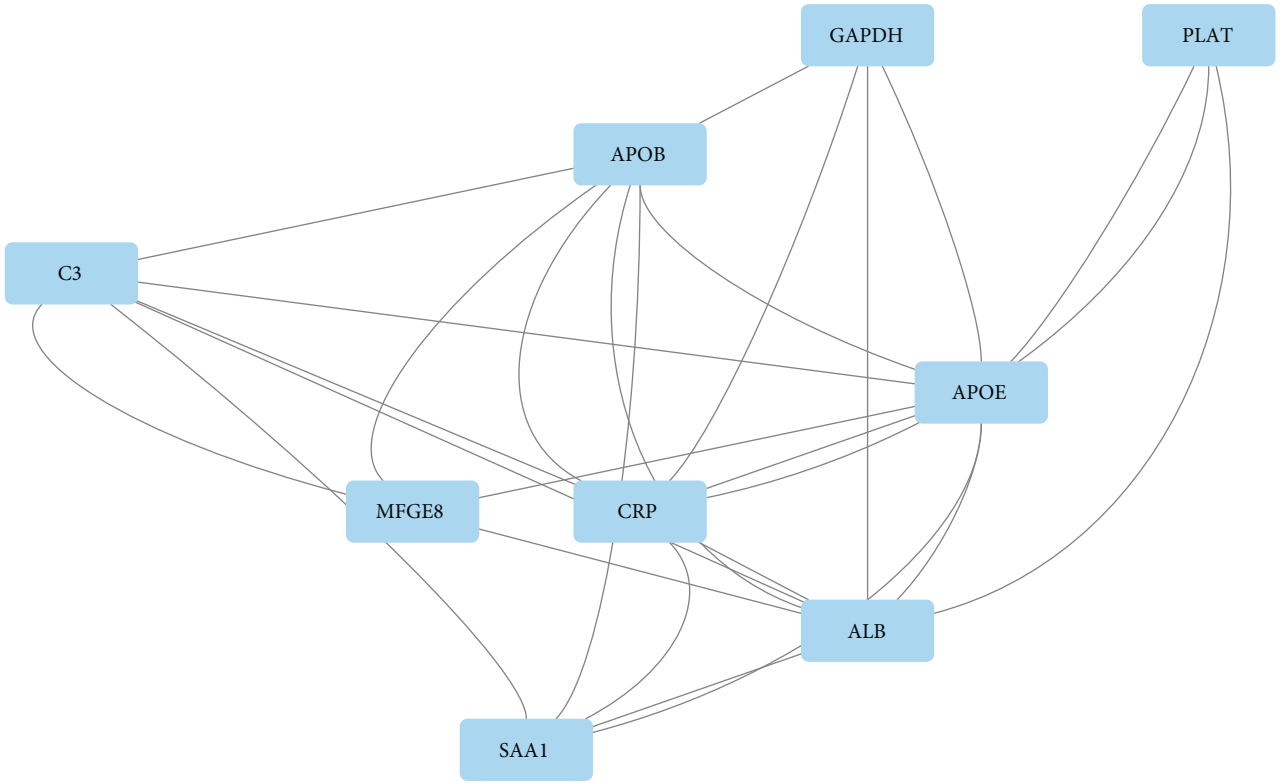

Figure 7: PPI network of module. Abbreviation: PPI: protein-protein interaction.

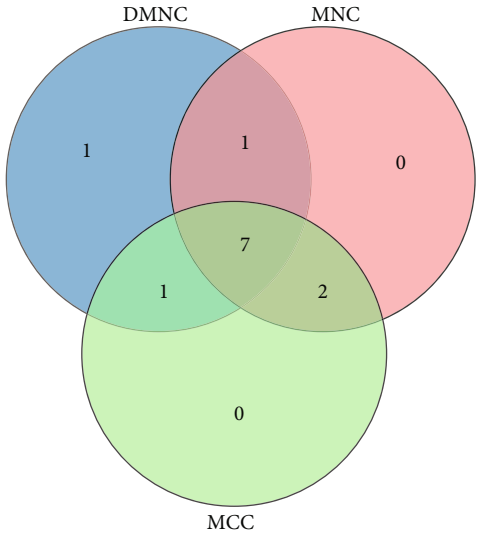

Figure 8: Venn diagram of common hub genes based on three methods. Abbreviation: MCC: Maximal Clique Centrality; DMNC: Density of Maximum Neighborhood Component; MNC: Maximum Neighborhood Component.

Table 4: Hub genes based on cytoHubba.

Projects	Methods in cytoHubba		
	MCC	MNC	DMNC
Gene symbol top 10	**ALB**	**ALB**	**SAA1**
	APOE	CRP	**C3**
	CRP	**APOE**	**A2M**
	APOB	**APOB**	PLAT
	C3	GAPDH	**APOB**
	SAA1	**C3**	**APOE**
	A2M	**A2M**	IL36A
	GAPDH	**MFGE8**	**MFGE8**
	MFGE8	**SAA1**	**ALB**
	PLAT	NPPB	NPPB

Bold gene symbols were the overlap hub gene. Abbreviation: MCC: Maximal Clique Centrality; DMNC: Density of Maximum Neighborhood Component; MNC: Maximum Neighborhood Component.

utilization, thus growing up to be a risk factor for cardiovascular and cerebrovascular diseases, diabetes, and other diseases [31, 32]. APOE and APOB are members of the apolipoprotein family, and so far, no studies have shown a direct relationship between apolipoprotein and SCM. Previous studies have shown that diabetes is a risk factor for SCM, and hyperlipidemia is a risk factor for diabetes [33, 34], so it can be speculated that hyperlipidemia is a potential risk factor for SCM, so compared with the normal population, APOE and APOB genes are upregulated in SCM patients.

In this experiment, we analyzed gene chips to obtain SCM possible key genes and related pathway information. However, due to the defects of the study itself, the conclusion needs basic and clinical experimental verification. Most regrettably, due to the limited experimental conditions, the conclusions drawn in this paper cannot be further

investigated. But we hope to be able to provide new ideas for SCM diagnosis based on this study and expect other scientific researchers to further explore this.

5. Conclusions

In summary, our study provides an integrated bioinformatics analysis of DEGs of SCM. In the present study, we identified some key genes and pathways. However, the key genes and signaling pathways related to SCM derived from this study still need further experimental verification due to the defects of analytical methods and sample size.

Data Availability

The data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

GSE95368 datasets were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/). *(Supplementary Materials)*

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Review Article

Female Sex as a Thromboembolic Risk Factor in the Era of Nonvitamin K Antagonist Oral Anticoagulants

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Sex-specific differences have been definitively demonstrated in cardiovascular (CV) diseases. These differences can also impact on the effects of CV therapies. Female sex is recognized as an independent predictor of thromboembolic risk, particularly in older patients. Most of strokes are due to atrial fibrillation (AF). Women affected by AF have higher stroke risk compared to men. The introduction of novel oral anticoagulants (NOACs) for long-term anticoagulation completely changed the anticoagulant therapeutic approach and follow-up of patients affected by nonvalvular atrial fibrillation (NVAF). CHA2DS2-VASc stroke risk scoring in use in the current international guidelines attributes 1 point to "female sex". Besides, no anticoagulation is indicated for AF female patients without other risk factors. Interestingly, NOACs seem to normalize the differences between males and females both in terms of safety and efficacy, whereas residual higher stroke risk and systemic embolism persist in AF women treated with vitamin K antagonist anticoagulants VKA with optimal time in therapeutic range. Based on the CHA2DS2-VASc score, NOACs represent the preferred choice in NVAF patients. Moreover, complete evaluation of apparently lower risk factor along with concomitant clinical conditions in AF patients appears mandatory, particularly for female patients, in order to achieve the most appropriate anticoagulant treatment, either in male or in female patients. The present review was performed to review sex differences in AF-related thromboembolic risk reported in the literature and possibly highlight current knowledge gaps in prevention and management that need further research.

1. Introduction

There is no doubt that men and women are biologically different in terms of body weight, body surface area, total body water, the distribution of extracellular, and intracellular water, as well as differences in the response to drug treatments. Several possible potential reasons are represented by biological differences between men and women [1] such as coagulation mechanisms, in e.g., during different female hormonal status at various ages (menstrual cycle, pregnancy, postmenopause), endothelial function, oral contraceptive therapy, and hormone replacement therapy. The risk for ischemic stroke in women doubles between the ages of 55 and 65, coinciding with the menopausal period, when severe estradiol levels and estrogen receptor reduction occurs. This condition could also favour a hypercoagulable state also through an increased production of inflammatory cytokines [2]. Several prothrombotic biomarkers, such as D-dimer, von Willebrand factor, and beta-thromboglobulin are present in higher concentrations in patients affected by atrial fibrillation (AF) [3, 4].

Von Willebrand factor and soluble E-selectin are also known markers of endothelial dysfunction or damage, as well. Sex differences have been found in von Willebrand

factor concentration, which is higher in women compared to males [5].

The hemorrhagic burden includes various factors involved in endothelial function, platelet aggregation, and vascular changes during different biological phases and coagulation factor activation.

Differences in the volume of distribution, larger "free fraction" of drugs, as well as differences in drug clearance, may lead to drug overexposure in women. Differences in receptor numbers, in receptor binding, and in the signal transduction pathway following receptor binding may all make women more sensitive to drug effects [6].

Differing gene expression directly reflects on vascular function, myocardial response to a stress condition, and sex-specific drug metabolism. These are all referred to as "sex differences" and may be reproduced in animal models.

Conversely, "gender characteristics" arise from sociocultural environments, different behaviours, nutrition, dietary habits, environmental exposure, attitudes towards lifestyle, and compliance to therapy. They are unique to the human. Both sex and gender are equally important for cardiovascular (CV) diseases. Some authors use sex/gender definition for medical differences between men and women [1, 7].

Women take a greater amount of medications compared to men, leading to a higher potential for drug interactions. In the setting of oral anticoagulation, the impact of sex on the effectiveness and safety of warfarin has been previously analysed, but not fully elucidated.

The ATRIA study [8], a large cohort prospective study, reported a similar rate of major bleeding between sexes, during warfarin treatment, even if women showed a lower risk of intracranial hemorrhage (ICH). Besides, women had higher rates of ischemic stroke and peripheral embolism while not taking warfarin, than men did.

In the last decade, much interest has been developing in sex and gender-specific aspects of many areas of medicine, in terms of pathophysiology, clinical manifestation, and management, as well. The European Union (EU) current research framework programme "Horizon 2020", included "gender" into biomedical research as one of the most relevant requirements for a more accurate improvement of scientific quality and knowledge. As far as CV disease is concerned, biological differences between men and women reflect also on several CV clinical patterns and incidence, pathophysiology, response to treatment, and clinical outcomes. Such differences can affect prognosis, with important implications in terms of management and public health.

The present review was performed to analyse the influence of the female sex on AF-related thromboembolic risk in either non-anticoagulated AF patients or AF patients on vitamin K antagonist anticoagulants (VKAs) compared to Novel Oral Anticoagulants (NOACs). Female patients undergoing VKAs therapy exhibit a higher risk of stroke and systemic embolism (SE), despite an optimal time in therapeutic range (TTR), NOACs seem to resolve sex differences both in terms of safety and efficacy. Concern arises about the revised CHA2DS2-VASc risk stratification score, according to which, female sex is recognized as an independent predictor of thromboembolic risk. A higher incidence of stroke in female patients was reported by several authors [9–11]. Female sex was significantly associated with an increased incidence of stroke particularly among patients aged ≥75 years. Indeed, the 2012 edition of the European Society of Cardiology guidelines [12] suggested to apply one point only to females aged ≥65 years. Some authors [5] found a higher stroke incidence in women of all ages. Data on stroke risk of females aged 65-75 years are conflicting. Women with AF can be more symptomatic and present with more comorbidities because of the older age. The aim of the present review is to focus on the difference between male and female sex in terms of thromboembolic risk and in terms of therapeutic choice in order to highlight the need to offer proper diagnostic tools and management to both men and women [13].

2. Methods-Search Strategy

A literature search was conducted on PubMed, electronic database using the keywords "atrial fibrillation" [Title/Abstract] and "Novel Oral Anticoagulants" [Title/Abstract] or "NOACs" [Title/Abstract] or "sex"/"gender" [Title/Abstract] or "thromboembolic risk" [Title/Abstract].

Reference lists and related records were manually reviewed. The search was limited to English language papers published until January 20th, 2020.

3. Stroke and Atrial Fibrillation

Every year, about 17 million people all over the world die from CV diseases, heart attack, and stroke, about 5 million die from this disease [14]. The majority of strokes are ischemic-induced [15]. Patients undergoing AF are up to five-fold likely to suffer from ischemic stroke, compared to patients without AF, and a high percentage suffers for permanent disabilities. AF is the most common supraventricular arrhythmia in the world [16]. Although not directly life-threatening, it can precipitate acute heart failure, pulmonary edema, stroke, and sudden death. AF negatively influences the quality of life, has heavy implications on work activity, and increases the risk of hospitalization. Several differences in epidemiological patterns, clinical manifestations, and incidence of stroke have been reported between women and men, affected by AF, particularly in the elderly population. Aged women have higher blood pressure than men and a higher prevalence of heart failure with preserved ejection fraction [17]. As widely reported in the literature from epidemiological studies [16], AF incidence is progressively increasing and represents an independent risk factor for ischaemic stroke. A number of 2.7 million new cases *per* year for men and 2.0 million for women have been estimated [18, 19]. In the EU, 4-17 million AF patients are anticipated by 2030 with 120000-215000 newly diagnosed patients *per* year [13]. A 2.5-fold increase in the prevalence of AF is projected by the year of 2050 [17, 19].

A five-fold increase in CV accidents and systemic embolization has been demonstrated in patients with AF. Epidemiological studies highlighted a progressive global burden of AF

incidence, with a significant impact on public health and subsequent increase of mortality related to it [13, 18, 20].

Several studies described sex differences in epidemiology and prognosis of AF, as widely demonstrated [15, 17], there is a significant association between stroke and women aged ≥75 years. Of note, patients aged <65 years, without any other risk factor, had low stroke rates, either males or females [21].

AF incidence increases with age (3.7%–4.2% of those aged 60–70 years, and 10%–17% of those aged >80 years) [17]. Moreover, women make up the greater proportion (60%) of AF patients who are over the age of 75 years, because of their longer lifespan. Women with AF have a higher mortality rate, even after adjustment for baseline comorbid conditions and treatment with anticoagulants [15].

Despite substantial advances in rhythm control therapy, anticoagulation plays a major role in AF treatment for the prevention of thromboembolic stroke [15].

4. Anticoagulation Benefit

Based on European and North American epidemiological studies, different prevalence and prognosis between men and women affected by AF have been largely demonstrated. While men have a higher risk of AF, female AF patients harbour a greater risk of having a stroke particularly in aged (≥75 years) patients and irrespectively of warfarin therapy [8, 15, 19, 21]. The risk of death in women with AF is similar or higher than that in men with AF. The Atrial Fibrillation Follow-up Investigation of Rhythm Management (AFFIRM) [22] found that women were more likely outside the TTR with consequent sub therapeutic international normalized ratios (INRs). Besides, those women with a comparably high TTR (>66%) still had significantly more ischemic strokes.

The largest prospective, observational, multicentre Global Anticoagulant Registry in the Field of AF (GARFIELD-AF) [15] explored the impact of sex on 1-year outcomes in patients with nonvalvular atrial fibrillation (NVAF). Outcomes of AF female patients markedly differ from men, with a higher incidence of stroke and higher mortality, even after adjusting for comorbidities. Notably, women were aged 75 or older at the time of diagnosis of NVAF, as reported from other studies [23]. Women population was more burdened by hypertension than men, whereas other risk factors, such as diabetes and prior stroke were similar in both sexes. Notably, there were small differences in stroke risk factors in men and women with newly diagnosed NVAF, beyond the impact of sex. The pattern of treatment was almost identical in the two groups (male and female).

Of a total of 63.8% women and 62.9% men receiving anticoagulant therapy, respectively, 46.8% and 46% received VKAs. The response to anticoagulant therapy was significantly more evident in men compared to women in terms of reduction of stroke/SE, similar for major bleeding and all-cause mortality.

The authors conclude that of the 28.624 patients enrolled, women showed a higher risk of stroke/SE and the reduction on event rates with AC therapy was less than in men.

The lower benefit on stroke rates in women has been partially explained by the lower weight and lower adherence to therapy, leading to wider variations in anticoagulation control and poorer anticoagulation in women compared to men [24].

Several reports actually describe differences in epidemiology, clinical patterns, and both thromboembolic and bleeding risk between males and females [17].

The possible existence of differences between males and females in thrombotic and haemorrhagic risk and in the outcomes during anticoagulant therapies may pose the question of whether any sex-specific management is required.

5. Female Sex as a Risk Factor

In the large observational ATRIA study on "gender differences in the risk of ischemic stroke and peripheral embolism in AF", female patients while off-warfarin showed a higher risk of stroke, than did men, even when adjustment for clinical risk factors was performed. Both younger and aged female patients showed higher rates of thromboembolism. Similar rates of major bleeding were reported between sexes, whereas female patients presented a lower risk of ICH. The authors conclude that female sex is an independent risk factor for thromboembolism and should be taken into account for a correct anticoagulant therapeutic choice in patients affected by AF. These findings indicate that women with AF face a higher absolute risk for thromboembolism independently of other risk factors and should gain more from anticoagulant therapy [8]. Besides, no homogeneous evidence was previously reported in the literature and, if some studies found an increased risk in women, others were not able to confirm this finding and female sex as one of the independent risk factors for stroke [9] was not included in 2007 stroke risk score.

The systematic review and large meta-analysis carried out by Wagstaff et al. [10] included 17 studies, five randomized-controlled trials, and 12 observational studies on anticoagulated and non-anticoagulated AF patients. The analysis found that women carry a risk ratio of 1.31 (95% CI 1.18-1.46) for stroke, and the risk appears greater for women ≥75 years. The authors conclude that women affected by AF have increased risk of stroke, regardless of oral anticoagulation therapy. A correct and comprehensive evaluation of stroke risk should include female sex, as a risk factor in all AF patients.

In 2010, the stroke risk stratification score used in the international guidelines has been revised to the CHA2DS2-VASc score, for a more accurate evaluation of risk [12].

The revised CHA2DS2-VASc (congestive heart failure, hypertension, age ≥ 75 years "doubled", diabetes, stroke "doubled"-vascular disease, age "65-74", and sex "female") score attributes 1 point to female sex. Female sex was finally accepted as an independent stroke risk factor [25], in that it increases the risk of stroke in older women, when other risk factors coexist. Moreover, it is considered as a "risk modifier" as it does not seem to increase stroke risk when no other risk factor is present [10].

According to the 2016 ESC Guidelines for the management of AF developed in collaboration with EACTS [13], anticoagulation is recommended with a 1 point or more

"CHA2DS2-VASc" score for men and 2 or more for women. Moreover, anticoagulation is not indicated in women without other risk factors and receives only a Class IIa-LoEB recommendation when only one additional risk factor is present. The increased risk in women compared to men, is not yet completely understood.

6. From VKA to NOACs

After decades of VKAs, NOACs changed both the approach and the follow up of patients on anticoagulant therapy. Dabigatran, rivaroxaban, apixaban, and edoxaban have been studied in large randomized trials, in which they emerge as the preferred choice, particularly in naïve patients, as mentioned in the European Heart Rhythm Association (EHRA 2018) [26]. Physicians are getting more and more familiar with the use of these drugs in clinical practice, but many unresolved questions on how to optimally use these agents remain, especially in specific clinical situations. Very well-designed multicentre clinical trials (Table 1) on the effectiveness of the newer direct oral anticoagulants and VKAs were performed, but sex-associated risk, during NOACs treatment, is not completely understood.

Panchloy et al. conducted a large meta-analysis (sixty-four randomized studies), which addressed the issue of sex-related outcome differences in either warfarin or NOACs therapy in NVAF. The results indicated that AF female patients have a significantly higher residual risk of cerebrovascular accidents (CVA)/SE, when warfarin is prescribed, as compared to men. The authors conclude that sex difference disappears when a pooled population treated with NOACs is analysed. Moreover, they suggest an increased net clinical benefit of NOAC agents compared with warfarin in treating women with AF. Less major bleeding risk was observed in NOACs female patients compared to male patients [19].

The large meta-analysis by Poli and Antonucci [17] clearly established that women with AF carry a persistently higher stroke risk compared to men, even when adequate anticoagulation is prescribed. The higher stroke risk among AF women seems to be confirmed, although none of the phase III trials has been powered to determine a sex difference in the efficacy of NOACs [27–30]. However, the benefit of anticoagulation was similar between males and females (Table 2).

Reports from a large cohort study performed by Law et al., comparing NOACs vs warfarin efficacy and safety in men and women according to anticoagulation control (TTR), demonstrated comparable results of NOACs versus warfarin in male patients. NOACs treatment led to a lower risk of ICH and all-cause mortality, only in women. The association of lower ICH risk remained when compared to warfarin patients with good anticoagulation control [31].

A large systematic review of the available evidences from randomized trials on NOACs along with a systematic meta-analysis of the 4 phase III clinical trials was conducted by Proietti et al. [32]. The aim was to investigate sex differences in stroke/SE events and major bleedings for a better assessment of major adverse outcomes according to sex, during the treatment. Data was collected directly from the original papers with the results on dabigatran, rivaroxaban, apixaban, and edoxaban in NVAF. Interestingly, only minor differences were found either in efficacy or in safety between males and females when NOACs were prescribed. A higher risk was found for the occurrence of stroke/SE in males. No difference was found for major bleeding between male and female patients. In the paper by Avgil Tsadok et al. [33], dabigatran users (both dabigatran 110 mg. and dabigatran 150 mg) were matched with warfarin users among AF patients, stratified according to sex (50,4% females, 49,6% males). The mean CHA2DS2-VASc score was higher in females compared to males. Female patients were older and found more burdened with hypertension and a previous history of stroke/transient ischemic attack (TIA). Females were less likely prescribed dabigatran compared with warfarin and when dabigatran was chosen, they received dabigatran 110 mg. After a median follow up of 1.3 years, incidence rates of stroke did not differ between male and female patients, both for dabigatran 110 mg and dabigatran 150 mg.

Moreover, Vinereanu et al. conducted a subgroup analysis of the Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) trial [34]. While women had more risk factors (age, hypertension, and previous thromboembolic events) and higher "CHA2DS2-VASc score" (as female sex scores 1 point), apixaban normalized the difference between males and females, when compared to warfarin. Notably, no differences in bleeding risk were described. Indeed, analysed data from the AVERROES (apixaban vs aspirin) trial [35] demonstrated that the use of Apixaban reduced the ischemic induced stroke and normalized the difference between males and females, who were even more burdened by other risk factors. Moreover, there was no difference in the reduction of stroke risk in comparison with aspirin, in both sexes. Data from "real life" about dabigatran use, even if female patients had, again, more risk factors, did not show any difference in the prevention of stroke/TIA occurrence. Additionally, a better safety profile was observed in women treated with apixaban [32]. Nonetheless, dabigatran showed better protection against bleeding events in male than in female patients, either with low or high dose dabigatran regimens [32]. A small advantage in terms of risk reduction was observed in women and data on bleeding demonstrated a better safety profile for female patients taking apixaban.

Preliminary data from the "Outcomes Registry for Better Informed Treatment of Atrial Fibrillation (ORBIT-AF) registry" [36], a multicentre, prospective, ambulatory-based registry of incident and prevalent AF, demonstrated that TTR in women is more often lower than in men, when warfarin is prescribed. Therefore, the elevated residual risk of CVA/SE observed in the female cohort has been possibly ascribed to this mechanism. This correlates to pharmacodynamics and pharmacokinetic advantages provided by NOACs, leading to a more stable anticoagulant effect, "superior" to warfarin [19].

Therefore, data from literature seem to actually highlight some differences basing on VKAs and NOACs treatment. On the other hand, as NOACs differ in pharmacologic

Table 1: Phase III clinical trial on non-vitamin K antagonist oral anticoagulants.

Trial	Year	Drug	Type of the study	Sample (*n*)	Mean TTR (%)	Mean CHA2DS2-VASc score	Follow-up (years)
RE-LY [27]	2009	Dabigatran	Open-label	Total patients 18,113 Men 11,514 Women 6,599	64	2.1	2.0
ROCKET-AF [28]	2011	Rivaroxaban	Double-blind	Total patients 14,264 Men 8,601 Women 5,663	55	3.5	1.9
ARISTOTLE [29]	2011	Apixaban	Double-blind	Total patients: 18,201 Men 11,785, Women 6,416	62.2	2.1	1.8
ENGAGE AF-TIMI 48 [30]	2013	Edoxaban	Double-blind	Total patients: 21,105 Men 13,065 Women 8,040	64.9	2.8	2.8

properties, Moseley et al. performed an indirect comparison of the 4 NOACs for efficacy and safety, using warfarin as a comparator, and no apparent difference for any single agent between males and females [37] was demonstrated. Besides, the authors underline that a very large patient population is required to find statistically significant differences.

Another recent observational study conducted by Avgil Tsadok et al. [21], demonstrated that the reduced risk of ischemic stroke in patients on rivaroxaban compared to dabigatran and warfarin was observed in men, while bleeding risk was higher in women.

Nonetheless, the real impact of sex-related differences is still unclear. More recently, from another large review by Kostopoulou et al. [38] on sex differences in risk assessment and prevention of AF- related stroke mechanism, all-cause mortality was found higher in women. Notably, a 2-fold increase in the risk of death in AF female patients was found, compared to a 1.5-fold increase in AF male patients.

The authors underline that, in terms of predictors of thromboembolism, differences in disease severity and type of treatment, which may influence stroke risk, are not incorporated in the CHA2DS2-VASc score, since both vascular disease and hypertension are binary variables. Renal failure is also a strong predictor of stroke, but is not included in CHA2DS2-VASc. Similarly, hyperthyroidism as a cause of increased risk of stroke is more common in women but whether the thrombotic risk is higher in females compared to males is unknown. In addition, the risk for systemic embolization in thyrotoxicosis is not precisely known, and anticoagulant therapy in hyperthyroidism AF patients remains unclear [39].

Interestingly, the European Society of Cardiology guidelines [13] and a recent review on sex differences in arrhythmias by the European Heart Rhythm Association (EHRA) [40] underline these sex-specific issues and recommend offering therapeutics options to women and men, equally.

Of note, the CHA2DS2-VASc score has been evaluated as a predictor of new onset of AF, CV morbidity, and mortality in non atrial fibrillation population [41]. The authors analysed a population-based cohort of 22.179 middle-aged individuals, either with or without a history of AF (n.18.367), over a median follow-up of 15 years. The authors conclude that the CHA2DS2-VASc score is a sensitive tool for predicting new-onset AF and adverse outcomes in subjects either with or without AF.

A better net clinical benefit of NOACs compared to VKAs was demonstrated by Patti et al. [42], who evaluated the 1-year clinical outcomes in elderly (≥ 75 years) patients with AF in a prospective registry setting. This "real world" data demonstrated the better net benefit in NOACs patients, primarily due to lower rates of major bleeding, also in high-risk patients with low body mass index (BMI) and age > 85 years.

In terms of clinical presentation, as reported in the review of Poli et al. [17], the risk of AF-related complications is not different between short AF episodes and sustained forms of the arrhythmia, because of the high frequency of silent episodes which are often self-terminating. Moreover, no difference between sexes has been found for the progression of AF from paroxysmal to permanent chronic form.

As far as cardioversion and ablation is concerned, a recent clinical trial by Kuck et al. [43] assessed the association of baseline covariates with clinical outcomes in 750 patients with drug-refractory paroxysmal AF enrolled in the FIRE and ICE Trial. The authors found that female sex was associated with an almost 40% increase in the risks of primary efficacy failure and CV rehospitalization. A history of direct current cardioversion and of hypertension had a negative impact on primary efficacy failure and rehospitalization, respectively [44].

7. Conclusions

Female sex has been included as an independent predictor of thromboembolic risk, particularly in the elderly female population. It appears that females are less likely to receive anticoagulation therapy, possibly due to the higher age of diagnosis of AF compared to men. Female sex appears to act as a *risk modifier*, in that it seems to intensify other risk factors, particularly in VKAs patients.

Table 2: Sex-based analysis of rate of stroke/SE and major bleeding studies.

Authors, year	Sample (*n*)	Type of the study	Mean CHA2DS2-VASc score	Rate of strokes/SE and major bleeding in female vs male patients	Anticoagulant therapy
Poli et al., (2013) [9]	Total patients 3,015 (aged > 80 years): Men 1,361, Women 1,654	Observational study	Females showed a higher risk score for all the classes of risk when CHA2DS2VASc was applied	Stroke: Men nr. 45 (1.3 per 100 person year) Women nr. 67 (1.6 per 100 person year) RR 1.2 (95% CI 0.8-1.8) Major bleeding: Men nr. 75 (2.2 per 100 person year) Women nr. 97 (1.4 per 100 person year) RR 1.6 (95% CI 1.1-2.3)	VKA treatment
Avgil Tsadok et al., (2015) [33]	Total patients: 15,918 Men 8,346 Women 7,572	Observational study	Men 2.6 Women 3.9	Stroke: Men HR (95% CI) 0.98 (0.78-1.23) Women HR (95% CI) 0.79 (0.56- 1.04) Major bleeding: Men HR (95% CI) 0.73 (0.64-0.84) Women HR (95% CI) 0.85 (0.71-1.01)	Dabigatran
Vinereanu et al., (2015) [34]	Total patients: 9,120 Men 5886 Women 3234	Randomized controlled trial	Men 3 (median) Women 4 (median)	Stroke/SE: Men HR (95% CI) 0.84 (0.66-1.05) Women HR (95% CI) 0.73 (0.54- 0.97) Major bleeding: Men HR (95% CI) 0.76 (0.64- 0.90) Women HR (95% CI) 0.56 (0.44- 0.72)	Apixaban vs warfarin
Camm et al., (2017) [15]	Total patients: 28, 624 Men 15 915, Women 12,709	Prospective study	Men 2.6 ± 1.5 Women 4.0 ± 1.4	Stroke/SE: Men 1.17 (CI 1.01-1.37) Women 1.62 (CI 1.41-1.87) Major bleeding: Men 0.79 (CI 1.01 - 0.95) Women 0.93 (CI 0.78 - 1.1.3)	Men: 33.8% VKA, 12.2% VKA+AP, 13.1% NOAC, 3.8% NOAC+AP, 24.9% AP, 12.2% none. Women: 37.1% VKA, 9.7% VKA+ AP, 13.9% NOAC, 3.2% NOAC+AP, 23.9% AP, 12.3% none

Table 2: Continued.

Authors, year	Sample (*n*)	Type of the study	Mean CHA2DS2-VASc score	Rate of strokes/SE and major bleeding in female vs male patients	Anticoagulant therapy
Law et al., (2018) [31]	Total patients: 15,292 Men 7,952 Women 7,340	Cohort study	Men 2.96 (SD 1.68) Women 4.34 (SD 1.79)	Stroke: Men HR (95% CI) 0.85 (0.65-1.12) Women HR (95% CI) 0.81 (0.63- 1.03) Major bleeding: Men HR (95% CI) 1.13 (0.73- 1.4) Women HR (95% CI) 0.89 (0.63- 1.27)	Warfarin vs DOAC

AP: Antiplatelet; CI: Confidence interval; DOAC: Direct oral anticoagulant; HR: Hazard ratio; NOAC: Nonvitamin K antagonist oral anticoagulants; RR: Risk ratio; SE: Systemic embolism; VKA: Vitamin K antagonists.

Women have a lower risk to experience AF, but when they do, the stroke risk is persistently higher, even when VKAs are prescribed with anticoagulation good control.

NOACs seem to normalize sex differences, both in efficacy and in safety. As recommended by the current guidelines, NOACs are the preferred choice in NVAF patients, according to the CHA2DS2-VASc stroke risk score, which attributes 1 point to "female sex". Anticoagulation is indicated with a 1 point or more for men and 2 or more for women. Notably, anticoagulation is not indicated for AF female patients without other risk factors. Moreover, they receive only a Class IIa-LoEB recommendation when only one additional risk factor is present. Women are frequently affected by concomitant modifiable risk factors, such as hypertension, obesity, metabolic syndrome [45, 46], thyroid dysfunction, which require accurate evaluation for an appropriate risk stratification, particularly in patients with low CHA2DS2-VASc score. When appropriate risk stratification indicates the need for anticoagulation, women should receive treatment.

A better knowledge of the different efficacy and safety profiles of NOACs compared with one another, in AF patients according to sex, could help the clinician in making the most appropriate and individualized anticoagulant therapy, either in male or in female patients.

Abbreviations

AF: Atrial fibrillation
BMI: Body mass index
CV: Cardiovascular
CVA: Cerebrovascular accidents
EU: European Union
ICH: Intracranial hemorrhage
INR: International normalized ratios
NOACs: Novel oral anticoagulants
NVAF: Nonvalvular atrial fibrillation
SE: Systemic embolism
TIA: Transient ischemic attack
TTR: Time in therapeutic range
VKAs: Vitamin K antagonist anticoagulants.

Conflicts of Interest

The authors declare that there is no conflict of interests in the publication.

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Research Article

Identification of Target Genes of Antiarrhythmic Traditional Chinese Medicine Wenxin Keli

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Wenxin Keli (WXKL) is a traditional Chinese medicine drug approved for the treatment of cardiovascular diseases. This study aimed to identify WXKL-targeting genes involved in antiarrhythmic efficacy of WXKL. The Traditional Chinese Medicine Systems Pharmacology (TCMSP) technology platform was used to screen active compounds of WXKL and WXKL-targeting arrhythmia-related genes. A pig model of myocardial ischemia (MI) was established by balloon-expanding the endothelium of the left coronary artery. Pigs were divided into the model group and WXKL group ($n = 6$). MI, QT interval, heart rate, and arrhythmia were recorded, and the mRNA expression of target genes in myocardial tissues was detected by PCR. Eleven active ingredients of WXKL and eight WXKL-targeting arrhythmia-related genes were screened. Five pathways were enriched, and an "ingredient-gene-path" network was constructed. WXKL markedly decreased the incidence of arrhythmia in the MI pig model ($P < 0.05$). The QT interval was significantly shortened, and the heart rate was slowed down in the WXKL group compared with the model group ($P < 0.05$). In addition, the expression of sodium channel protein type 5 subunit alpha (*SCN5A*) and beta-2 adrenergic receptor (*ADRB2*) was downregulated, while muscarinic acetylcholine receptor M2 (*CHRM2*) was upregulated in the WXKL group ($P < 0.05$). In conclusion, WXKL may shorten the QT interval and slow down the heart rate by downregulating *SCN5A* and *ADRB2* and upregulating *CHRM2* during MI. These findings provide novel insight into molecular mechanisms of WXKL in reducing the incidence of ventricular arrhythmia.

1. Introduction

Acute occlusion of the epicardial coronary artery leads to myocardial ischemia (MI) with a rapid onset of unstable electrocardiograph (ECG) activity, which usually induces fatal ventricular arrhythmias [1]. Wenxin Keli (WXKL) is the first traditional Chinese medicine (TCM) approved as an antiarrhythmic drug by China Food and Drug Administration. A meta-analysis showed that WXKL was effective in the treatment of cardiovascular diseases (angina, heart failure, and arrhythmia), although more high-quality evidence was needed to support its use in clinical settings [2]. Compared with Western medicine treatment alone, combined use with WXKL could lower the heart rate, reduce the occurrence of arrhythmia (ventricular premature beats, ventricular tachycardia, and ventricular fibrillation), and improve heart function [3, 4]. Moreover, WXKL significantly reduced ventricular arrhythmia after MI [5]. Additionally, a recent study reported that WXKL could relieve recent-onset atrial fibrillation, without significant difference in the efficacy on male or female patients [6].

TCMs are oral preparations and need to reach target organs and tissues through the absorption, distribution, metabolism, and excretion (ADME) process. The ADME process plays a role in oral bioavailability (OB) and drug-likeness (DL), two pharmacokinetic characteristics of TCMs [7]. OB refers to the relative amount and rate at which the drug is absorbed into blood circulation after oral administration. DL refers to the similarity between the compound and the known listed drug. Compounds with OB ≥30% and

DL ≥0.18 are considered potential active compounds for further analysis [8]. The TCM Systems Pharmacology (TCMSP) technology platform, which contains 499 herbs and their 29,000 chemical constituents, provides data on ADME properties of each compound, such as blood-brain barrier permeability, OB, and Caco-2 cell permeability, as well as targets for potentially active molecules (including 6,511 drug molecules in the DrugBank database and 3,987 proteins that interact with known compounds) and related disease information [9, 10]. The active components of WXKL and the targets related to arrhythmia can be retrieved from the platform. However, the effect of WXKL on cardiac electrical activity after MI and the mechanism of action of WXKL remain unclear.

The aim of this study was to investigate the effects of WXKL on arrhythmia and ECG activities after MI and identify WXKL-targeting genes involved in arrhythmia using TCMSP. We further confirmed WXKL-targeting genes in the animal model of MI.

2. Methods

2.1. Screening of Active Ingredients and Targets of WXKL. TCMSP was screened with "herb name" as the search item including the five ingredients of WXKL: *Nardostachys chinensis* Batal, *Codonopsis*, notoginseng,*Ambrum*, and rhizoma polygonati; OB was set to "Is greater than or equal to 30%"; DL was set to "Is greater than or equal to 0.18."

2.2. KEGG Enrichment Analysis and Construction of the "Ingredient-Gene-Path" Network. Target genes were analyzed for KEGG pathway enrichment using DAVID (the Database for Annotation, Visualization, and Integrated Discovery; https://david.ncifcrf.gov/) v6.8. The target genes were directly mapped to the pathway, the number of genes was proportional to the significance of pathway enrichment, and the pathway of drug target enrichment was considered the pathway of drug regulation. The corresponding ingredients, genes, and pathways were constructed into a network diagram through Cytoscape 3.0 software.

2.3. Experimental Animals. All animal procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee of Guangdong Pharmaceutical University (Guangzhou, China). Male miniature pigs (20–25 kg) were supplied by Guangdong Medical Laboratory Animal Center (Guangzhou, China) and randomized into two groups ($n = 6$): model group and WXKL group. In the WXKL group, WXKL purchased from Shandong Buchang Pharmaceuticals (8 g/kg, qd, mixed in the feed) was administered for 3 weeks prior to surgery. In the MI model, the pigs were anesthetized by injection with pentobarbital sodium (30 mg/kg) into the right common carotid artery, and the anterior descending branch of the left coronary artery (LAD) was expanded with interventional techniques. Then, 6*F* (1*F* = 0.33 mm) artery sheath tubes (Terumo Corporation, Japan) were introduced by guide wires through the iliac artery and placed in the left coronary artery under the C-arm X-ray machine (Artis zee III ceiling, Siemens, Germany). The balloon (Cordis; balloon : tube diameter = 1.3 : 1) entered the middle of the LAD via the guide wire and was inflated for 303.975 kPa for 30 s, repeated 3 times. The ECG monitor (Ruike Biotech, China) was used to continuously monitor the intraoperative and the postoperative electrocardiogram. The duration of QT, ST-T segment change, T-wave voltage, heart rate, and incidence of arrhythmia were recorded.

2.4. Quantitative PCR. The pigs were sacrificed, and myocardial tissue was removed and quickly frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted from the tissue using the TRIzol reagent (Invitrogen). cDNA was synthesized from RNA using the RT kit (DBI, USA), and PCR was performed using the PCR kit (Genecopoeia, USA) and the following primers: SCN5A GGATTGTAGCTCCTCTCACTTC and GGAAGGCATCACTCTCTTCTAC; KCNH2 GAGATC GCATTCTACCGGAAAG and CTTCTCCATCACCACCT-CAAAG; CHRM2 GCCTGCTATGCACTTTGTAATG and TCCTCTTGACTACCTTCCTTCT; ADRB2 CTCTTCCAT-CGTGTCCTTCTAC and CCTCAGACTTGTCGATCT TCTG; ADRB1 TCCGTCGTCTCCTTCTATGT and CGCAGCTGTCGATCTTCTT; ADRA1D GCAGACGGTC ACCAACTATT and ACCTCCATAGTGGCAGAGAA; and GAPDH CAGGTTGTGTCCTGTGACTT and TTGAC-GAAGTGGTCGTTGAG. The expression levels of target genes were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Statistical Analysis. Statistical analysis was conducted using the SPSS 21.0 software. The Mann–Whitney *U* test was used for all data analysis. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Arrhythmia-Related Target Genes of Active Ingredients of WXKL. Eleven active ingredients of WXKL were retrieved based on OB and DL. A total of eight WXKL-target genes related to arrhythmia were retrieved from TCMSP, including sodium channel protein type 5 subunit alpha (*SCN5A*), potassium voltage-gated channel subfamily H member 2 (*KCNH2*), beta-1 adrenergic receptor (*ADRB1*), beta-2 adrenergic receptor (*ADRB2*), alpha-1D adrenergic receptor (*ADRA1D*), muscarinic acetylcholine receptor M2 (*CHRM2*), alpha-2A adrenergic receptor (*ADRA2A*), and gap junction alpha-1 protein (*GJA1*) (Table 1).

3.2. I-G-P Network Construction and Analysis. Based on I-G-P network construction, we established the interactions among eleven active compounds of WXKL, eight targets, and six enriched KEGG pathways. *ADRB2* and *SCN5A* were the targets of the most ingredients, and calcium signaling pathway, neuroactive ligand-receptor interaction, adrenergic signaling in cardiomyocytes, and cGMP-PKG signaling

Table 1: Arrhythmia-related targets of active ingredients of WXKL.

Compound	OB	DL	Target gene
Acacetin	34.97	0.24	*ADRB2*
Cryptotanshinone	52.34	0.4	*ADRB2/SCN5A/ADRA1D*
Stigmasterol	43.83	0.76	*ADRB2/ADRB1/SCN5A/CHRM2/ADRA2A*
Quercetin	46.43	0.28	*ADRB2/GJA1*
Sitogluside	20.63	0.62	*ADRB2/ADRB1/KCNH2/SCN5A/ADRA1D*
β-Sitosterol	36.91	0.75	*ADRB2/KCNH2/SCN5A/CHRM2*
Diop	43.59	0.39	*ADRB2/SCN5A*
Frutinone A	65.9	0.34	*ADRB2/SCN5A*
Compound 1	32.16	0.41	*ADRB2*
DFV	32.76	0.18	*ADRB2*
Compound 2	71.12	0.18	*KCNH2*

Compound 1: 3-beta-hydroxymethylenetanshiquinone. Compound 2: (2R)-7-hydroxy-2-(4-hydroxyphenyl)chroman-4-one.

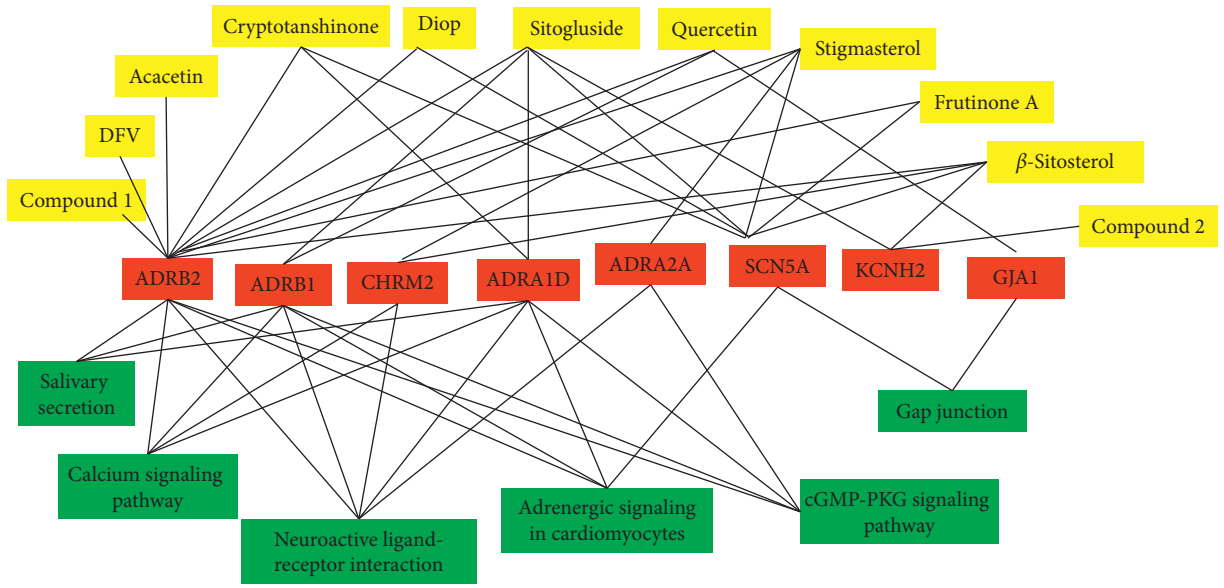

Figure 1: Ingredient-gene-path (I-G-P) network for the ingredients of WXKL, drug-target genes, and KEGG pathways. Yellow nodes represent eleven active compounds, red nodes represent eight targets, and green nodes represent enriched signal pathways.

pathway were the most important signaling pathways that may mediate antiarrhythmic effects of WXKL (Figure 1).

3.3. Electrophysiological and Antiarrhythmic Effects of WXKL. ST-segment abnormalities (different levels of T-wave low-level, inverted, ST-segment elevation) occurred after the balloon dilated the coronary artery, and all pigs developed ventricular tachycardia 3–6 minutes after the balloon began to expand, and all pigs had ventricular fibrillation about 5–7 minutes after expansion. Postoperative heart rates of both groups were significantly increased (all $P < 0.05$), but heart rates were significantly slower in the WXKL group compared to the model group ($P < 0.05$). There was no statistical difference in the QT interval before and after surgery, but the QT interval in the model group was significantly longer than that in the WXKL group ($P < 0.05$) (Table 2). In addition, the incidence of heart dysfunction was significantly lower in the WXKL group compared to the model group ($P < 0.05$) (Table 3). These data indicated the antiarrhythmic effects of WXKL.

3.4. WXKL Regulated the Expression of Targets in Myocardial Tissue. Finally, we selected 6 targets and examined the effects of WXKL on their expression in myocardial tissues of the animal models. PCR analysis showed that *SCN5A* and *ADRB2* mRNA levels were significantly lower and the *CHRM2* mRNA level was significantly higher in the WXKL group than in the model group, *KCNH2* and *ADRA1D* expressions showed no significant difference between two groups, while *ADRB1* was not expressed in two groups (Figure 2).

4. Discussion

ADRB1, *ADRB2*, *ADRA1D*, and *ADRA2A* are G-protein-coupled transmembrane receptors that mediate sympathetic nervous system activity by binding neurotransmitters such as catecholamines, epinephrine, and norepinephrine [11, 12]. CHRM2 binds to acetylcholine to mediate the activity of the parasympathetic nervous system. Cardiac autonomic nerves can induce or promote arrhythmias directly or indirectly by altering electrophysiological features

Table 2: QT interval and heart rate effects of WXKL.

Group	n	Preoperative		Postoperative	
		QT (ms)	HR (bpm)	QT (ms)	HR (bpm)
Model group	6	433.33 (419.10–439.90)	88.5 (78.00–99.00)	436.50 (408.30–453.36)	120.50 (112.00–134.00)
WXKL group	6	422.20 (337.90–438.60)	89.00 (73.00–98.00)	406.15* (318.90–425.30)	103.00* (85.00–120.00)

All data are expressed as the median, maximum, and minimum; $^*P < 0.05$ compared with the model group and preoperation.

Table 3: Comparison of the incidence of ventricular arrhythmias.

Group	n	VT	VF	Incidence (%)
Model group	6	2	2	4 (67)
WXKL group	6	2*	0*	2 (33)

VT: ventricular tachycardia; VF: ventricular fibrillation; $^*P < 0.05$ compared with the model group.

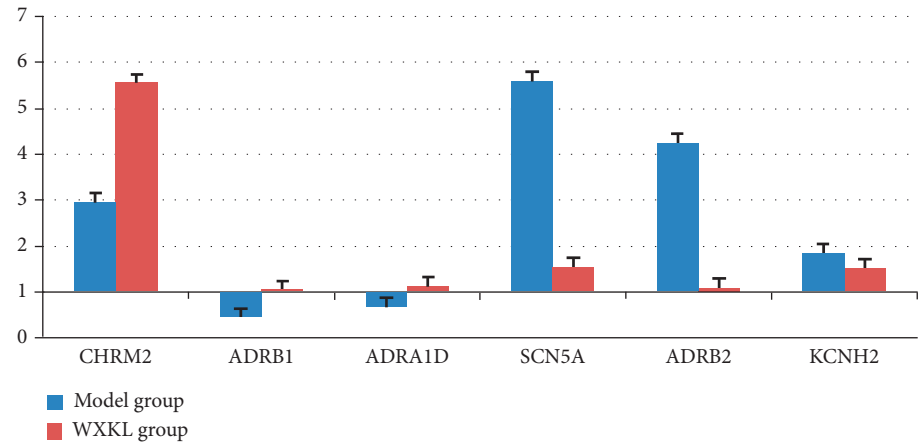

Figure 2: Effects of WXKL on *CHRM2*, *ADRB1*, *ADRA1D*, *SCN5A*, *ADRB2*, and *KCNH2* mRNA expression levels during MI.

[13, 14]. A large retrospective study demonstrated that beta blockers and ACE inhibitors are associated with improved secondary survival in patients surviving ventricular arrhythmias on admission [15]. KCNH2 mediates the rapid activation of delayed rectifier potassium current (IKr), which is important for normal ventricular repolarization [16]. *SCN5A* encodes voltage-gated sodium channel 1.5 (Nav1.5), which mediates inward sodium current (INa) and induces rapid depolarization. *SCN5A* mutations can impair the function of Nav1.5 and induce various arrhythmias [17]. The cardiomyocyte gap junction is the structural basis for the diffusion of action potentials in myocardial tissue and plays an important role in cell electrical coupling and action transmission [18]. The major gap junction of ventricular myocytes is GJA1 (*Cx43*). Cardiac pathological conditions affect the expression, translocation, and distribution of the gap junction, interfere with communication between cardiomyocytes, increase the cardiac potential decoupling rate, and induce arrhythmia [19]. Wen et al. reported that WXKL prevented ventricular arrhythmias induced by myocardial ischemia-reperfusion by upregulating the expression of *Cx43* [5].

The QT interval represents the depolarization and repolarization time of the ventricle, and the prolongation of the QT interval is important for malignant arrhythmias and sudden cardiac death [20]. WXKL is an effective alternative to prevent potentially fatal arrhythmias after myocardial infarction in an animal model [21]. In this study, ECG showed that WXKL could significantly shorten the QT interval, slow down the heart rate after MI, and reduce the incidence of arrhythmia, consistent with the results summarized in a recent review [22].

An early study reported that WXKL exerted antiarrhythmic effects by selectively inhibiting sodium current [23]. *SCN5A* gene mutation in patients with long QT syndrome delayed the sodium channel closure and increased myocardial cell potential, which prolonged the 2-phase plateau of action potential, leading to prolongation of the QT interval [24]. These results indicate that WXKL may lead to downregulation of the expression of *SCN5A* and inhibition of sodium inward currents.

Adrenaline may increase myocardial repolarization dispersion by acting on the $\beta 2$ receptor, triggering arrhythmia [25]. The use of $\beta 2$ receptor antagonists significantly reduced the incidence of ventricular fibrillation [26]. Consistent with these results, in this study, we found that WXKL selectively decreased the expression of the $\beta 2$ receptor, in which WXKL may reduce the incidence of

arrhythmia after MI by downregulating the $\beta 2$ receptor. Activation or overexpression of the $\beta 2$ receptor stimulates the L-calcium channel, resulting in a significant increase in L-type Ca^{2+} current. Within a few minutes from coronary occlusion, the initial decrease in the duration and amplitude of the cardiac electrical potential at rest occurs. This is due to ischemia-induced decrease of inward sodium currents with upregulation of calcium inward currents, causing initially prolonged and finally shorter QT interval. Moreover, some "border zones" between ischemic and nonischemic areas create areas with different refractory periods, which, along with acidosis (caused by ischemia), damage of the "gap junctions," and impaired conduction, lead to possible reentry circuits which, actually, may account for the most clinical relevant arrhythmias in ischemic heart disease. Indeed, Wang et al. found that WXKL may attenuate myocardial ischemia-induced arrhythmias by inhibiting L-calcium current and transient outward potassium current [27]. In addition, the QT interval can be affected by inhibiting L-calcium current [28]. We supposed that, after myocardial ischemia, WXKL may inhibit L-type calcium channels by downregulating the expression of the $\beta 2$ receptor, which in turn is involved in the regulation of the QT interval.

Timely correction of tachycardia during myocardial ischemia is important for preventing arrhythmias, and blocking the acetylcholine M receptor leads to faster heart rates [29]. CHRM2 is involved in the regulation of the heart rate [30]. Our results showed that WXKL significantly increased the mRNA expression of CHRM2, suggesting that WXKL could slow down the heart rate by upregulating CHRM2.

In conclusion, the network diagram showed that SCN5A and ADRB2 were the main targets for most active ingredients of WXKL, which are consistent with the important role of SCN5A and ADRB2 in the regulation of cardiac function. In summary, we screened potential targets of active ingredients of WXKL involved in the regulation of arrhythmia through TCMSP and confirmed that WXKL could shorten the QT interval and slow down the heart rate by downregulating *SCN5A* and *ADRB2* and upregulating *CHRM2* during MI. These findings provide novel insight into potential molecular mechanisms of WXKL in affecting cardiac electrical activation. Further investigation is actually required for a better definition of the role of WXKL in ischemia-induced changes in the different ion channels, as well.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

ZZ designed the experiments. YY, TL, YL, RC, and RZ performed the experiments and analyzed the data. All authors read and approved the manuscript.

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Review Article

Platelet Responses in Cardiovascular Disease: Sex-Related Differences in Nutritional and Pharmacological Interventions

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Cardiovascular diseases (CVD) represent one of the biggest causes of death globally, and their prevalence, aetiology, and outcome are related to genetic, metabolic, and environmental factors, among which sex- and age-dependent differences may play a key role. Among CVD risk factors, platelet hyperactivity deserves particular mention, as it is involved in the pathophysiology of main cardiovascular events (including stroke, myocardial infarction, and peripheral vascular injury) and is closely related to sex/age differences. Several determinants (*e.g.*, hormonal status and traditional cardiovascular risk factors), together with platelet-related factors (*e.g.*, plasma membrane composition, receptor signaling, and platelet-derived microparticles) can elucidate sex-related disparity in platelet functionality and CVD onset and outcome, especially in relation to efficacy of current primary and secondary interventional strategies. Here, we examined the state of the art concerning sex differences in platelet biology and their relationship with specific cardiovascular events and responses to common antiplatelet therapies. Moreover, as healthy nutrition is widely recognized to play a key role in CVD, we also focused our attention on specific dietary components (especially polyunsaturated fatty acids and flavonoids) and patterns (such as Mediterranean diet), which also emerged to impact platelet functions in a sex-dependent manner. These results highlight that full understanding of gender-related differences will be useful for designing personalized strategies, in order to prevent and/or treat platelet-mediated vascular damage.

1. Introduction

Noncommunicable diseases (mostly, cardiovascular disease (CVD), cancer, diabetes, and chronic respiratory disease) are the leading cause of mortality worldwide: with 41 million deaths, they were responsible for 71% of all global deaths in 2016 [1]. Among them, CVD remains the biggest cause of death globally in the last 20 years: according to the last World Health Organization Report, ischemic heart disease and stroke collectively accounted for 15.2 million deaths in 2016 [1].

Genetic, metabolic, and environmental aspects interact together, leading to metabolic and/or physiological changes (overweight and obesity, rise in blood pressure, and increase in blood glucose and cholesterol levels), which represent key CVD risk factors. Some of the risk behaviours (tobacco use, physical inactivity, unhealthy diet, and alcohol abuse) can be deeply modified, in order to lower CVD prevalence.

Remarkably, CVD prevalence, aetiology, and outcome are also strictly related to differences in sex (based on biological characteristics, such as gene expression, hormones, anatomy, and immune system) and gender (based on social and structural determinants). Usually, life expectancy is greater for women than men, but CVD, including myocardial infarction, stroke, cardiomyopathy, and hypertensive heart disease, displays sex- and age-related incidence: indeed, it accounts for 40% of all deaths in men and up to 49% of all deaths in women over the age of 65 years [2]. This finding may be due to increased prevalence of CVD risk factors in women with respect to their male counterparts [3, 4]. In this context, it should also be recalled that CVD prevalence, outcome, prevention, and treatment in women are often underestimated, due to underrepresentation of women in CVD clinical trials [5, 6].

Among biological risk factors, platelets are emerging as new players, since increased platelet aggregation is a major

determinant for heart attacks, stroke, and thrombosis: indeed, activated platelets are major components of thrombi occluding arteries and play a role in plaque formation within blood vessels during atherogenesis [7]. As a consequence, either antiplatelet therapy or other interventional strategies (such as those to promote consumption of foods rich in antioxidant and phytochemical compounds, fiber, and mono- and polyunsaturated fatty acids (PUFA)) are becoming increasingly relevant for preventing and treating vascular events in high-risk patients [8–16]. Accidentally, also in this context, a sex and gender disparity can be identified, in terms of aggregation response capacity and susceptibility to platelet aggregation inhibitors [17–19].

Based on this background, in this review, we will examine the state of the art concerning the main differences in platelet function between men and women, in order to establish their relationship with specific cardiovascular events and with responses to primary and secondary interventional strategies.

2. CVD Risk, Platelet Function, and Gender

Platelet count and mean platelet volume, usually linked to markers of platelet activation, are significantly associated with increased risk (as well as with outcome and mortality) of stroke, myocardial infarction, and coronary artery disease [20–22]. As a consequence, platelet number and size are commonly used tools for diagnosing and monitoring thromboembolic disorders [23, 24]. Activated platelets are also main players in atherogenesis, as they secrete proinflammatory chemokines that promote expression of adhesion molecules in atherosclerotic endothelial cells [25]. In atherosclerosis animal models, platelet/endothelium interaction precedes the onset of atherosclerotic lesions and inhibition of platelet adhesion decreases endothelium dysfunction and leucocyte recruitment in the atherosclerotic plaque [25]. Accordingly, currently used antiplatelet therapy has been proven to be effective in reducing thrombotic events (associated with a marked risk reduction of atherothrombotic events in high-risk settings, including patients with acute coronary syndromes), not only by inhibiting platelet activation but also by downregulating endothelial dysfunction and inflammation [26].

In 1999, Miller and collaborators highlighted that a gender-specific release of vasoactive factors from platelets could be found. In particular, they found that secretion of cyclooxygenase 1 (COX-1) metabolites of the ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (20 : 4 ω-6, AA) ((*i.e.*, thromboxane A2 (TxA2) and prostacyclin (PGI_2)), as well as secretion of serotonin from platelets, was higher in male with respect to female pigs, but platelets from ovariectomized females had the highest concentration of all vasoactive compounds, with respect to male counterparts. This pioneering study highlighted, therefore, that sex differences in platelet activity might explain differences in response to injury in the coronary circulation, usually observed in males and females [27]. Since then, several studies have been undertaken to unravel sex-related dissimilarities and to understand how these differences can affect platelet biology and CVD onset.

It is well established that traditional CVD risk factors (including obesity, dyslipidaemia, inflammation, diabetes, hypertension, and smoking) are greater in women than in men and that CVD risks are more age-dependent in females than in males [28, 29]. Nonetheless, prevalence of CVD is greater in men than in women [30, 31]; this sex disparity persists until women reach menopause, when CVD incidence rapidly rises, until it overtakes that of men [32–35]. Accordingly, a study enrolling 59 men (58.6 ± 9.9 years) and 75 postmenopausal women (61.4 ± 10.6 years) with angina and nonobstructive coronary artery disease (ANOCA) found significant differences between the two groups in relation to lipid profile and thrombogenicity [36]. Indeed, females display higher levels of total cholesterol, total LDL-C, HDL-C and their subtypes and IDLs, as well as elevated thrombin-induced platelet-fibrin clot strength, clotting index, and fibrinogen activity. As no differences were seen on inflammatory markers (including OxLDL, OxLDL/β2GPI, and urinary 11-dehydrothromboxane B_2), adverse cardiovascular events more frequently observed in females may be ascribed to the basal prothrombotic phenotype occurring in women with ANOCA [36–38].

The cardioprotective effects exerted by female hormonal levels produced during menstrual cycle (and lost after menopause) may explain the observed differences between sexes. Although the present review does not focus on molecular and cellular mechanisms of sex hormones, some details about their action (especially concerning platelets) need to be highlighted. Human platelets from both sexes express receptors for 17β-oestradiol (ERα and ERβ) and ER-regulatory proteins, as well as androgen and progesterone receptors [39–42]. However, available literature data regarding interactions among sex, steroid hormones, and platelet functions are controversial. Indeed, either positive or negative and even no effects on platelet aggregation, in response to different agonists, have been reported in relation to sex [27, 43–47]. Additionally, a proaggregatory effect of 17β-oestradiol has been reported in both healthy male and female platelets [48]; on the contrary, Coleman et al.'s group [44] has recently reported that female platelets have both increased aggregation and activation potential, and 17β-oestradiol pretreatment feminizes male platelets leading to similar platelet behaviour in response to platelet-activating factor (PAF). Accordingly, as emerged by a large, population based, case-control study on risk factors for venous thrombosis (MEGA study), women taking oral contraceptives have a significant thromboembolic risk, with a positive association with estrogen dose used [49]. As recently reviewed, results concerning the impact of menopausal hormonal therapy on platelet activation in women are also largely contradictory, much likely due to heterogeneity in experimental settings among studies [44, 50]. This rather complex puzzle is further complicated by the evidence that testosterone can enhance TxA2 receptor density and platelet aggregability [51, 52].

Beside hormonal changes, other several factors related to platelet biology can account for the prothrombotic state; some of them, highlighted by several experimental and clinical evidence, will be described in the next paragraphs.

2.1. Platelet Fatty Acid Composition of Plasma Membrane. The main feature distinguishing male and female platelets is the fatty acid composition of plasma membrane, especially concerning ω-6 (AA) and ω-3 (eicosapentaenoic (20 : 5 ω-3, EPA) and docosahexaenoic (22 : 6 ω-3, DHA) acids) PUFAs. A study enrolling healthy 40 men and 34 women (age 20-50 years) of Caucasian origin showed that (albeit same daily income of these lipids, as well as ω-6/ω-3 PUFA ratio) women show greater incorporation of DHA and EPA into phosphatidylcholine and phosphatidylethanolamine, compared to men, who, on the contrary, show higher levels of AA and other ω-6 PUFAs [53]. Conversely, a controlled, randomized, double-blind study reported no significant sex differences in EPA and DHA incorporation into platelet membrane after 12-month supplementation with oily fish [54], although the too wide age range of subjects enrolled (*i.e.*, 20-90) might represent an important confounding factor not to be overlooked.

Higher ω-3 PUFA proportion in female phospholipids might be related to sex-dependent differences in conversion of the essential α-linolenic acid (18 : 3 ω-3, ALA) to EPA and DHA. Humans, indeed, can endogenously synthesize EPA and DHA from ALA through a series of desaturations and elongations, but young women show a better capacity to produce long-chain PUFAs which is dependent on estrogen effects [55]. In particular, in healthy young females, about 21% of dietary ALA is converted to EPA, and 9% is converted to DHA [56], whereas only 8% of dietary ALA is converted to EPA and 0-4% is converted to DHA in healthy young men [57]. As a consequence, women have greater circulating plasma DHA concentrations, thus increasing DHA supply for incorporation into platelet membranes [55, 58, 59]. Replacement of AA with EPA and/or DHA in female circulating platelets alters the phospholipid bilayer, thus modifying the activity of membrane-associated molecules (*i.e.*, phosphatidylserine, GPIIb/IIIa exposure, and P-selectin) [60, 61]. It also reduces generation of proaggregatory and proinflammatory eicosanoids, such as TxA2 (through competitive inhibition of COX-1) and 12-hydro(pero)xyeicosatetraenoic (12-H(P)ETE) acids (by competition for 12-lipoxygenase (12-LOX)) [62–64]. Consequently, the differential incorporation in membrane phospholipids leads to a different degree of platelet aggregation and vessel occlusion, thus contributing to the protective effects of ω-3 PUFAs on cardiovascular risk [64].

2.2. Platelets Receptors and Platelet-Derived Microparticles. Platelet receptors and downstream signaling cascades are affected by sex (and age), depending on the receptor engaged. A study carried out on healthy donors (53 men and 56 women; age range: 19-82 years in men and 21-70 years in women), indeed, showed sex- and aging-dependent decrease of platelet glycoprotein (GP) Ib/von Willebrand factor (vWF) interaction, with age-related changes more profound in women than in men [65]. Conversely, Sestito and coworkers [47] reported no changes in relation to sex (and age) in 62 healthy subjects (11 men and 17 women < 55 years and 22 men and 12 women > 55 years) in platelet response to collagen and ADP, although platelets from older men had higher tendency towards aggregability with respect to younger ones. Based on these findings, it should be considered that different experimental settings and sample stratification may explain different platelet behaviours.

Although no gender difference in total number of GPIIb-IIIa (fibrinogen receptor) was expressed on platelet surface, nonetheless, women show higher receptor reactivity: in response to ADP and thrombin receptor activating peptide (TRAP), indeed, the amount of fibrinogen/GPIIb-IIIa complexes was significantly greater in healthy women (especially in fertile subjects) than in men [66]. Sex-specific difference in GPIIb/IIIa activity also seems to emerge from the CRUSADE study, where female patients with non-ST-segment elevation acute coronary syndromes (NSTE ACS) and treated with GPIIb/IIIa antagonists (eptifibatide, tirofiban) showed more haemorrhagic events than males [67]. Although female gender is recognized as a risk factor for bleeding, especially following medical or surgery [68–70], nonetheless several confounding factors can be identified in the study, in particular, (i) difference in mean age (women: 65 ± 10 years, men: 60 ± 12 years), (ii) presence of other risk factors in women (obesity, hypertension) and, importantly, (iii) excessive dose administration in women compared to men could have biased results. Finally, it has been showed that platelets from males generally respond stronger to activation of the α2-adrenergic receptor by epinephrine and serotonin signaling pathways [71], while showing stronger TxA2 receptor-related aggregation responses [51].

During activation, platelets release microparticles (pMPs), a heterogeneous population of membrane vesicles with distinct functional properties: based on their cargo of molecules and antigenic composition, indeed, these pMPs can modulate several biological functions, such as coagulation, inflammation, and transfer of bioactive molecules to other cells [72–74]. High prothrombotic activity has extensively been reported for circulating pMPs [75, 76], which can be considered specific candidate biomarkers for CVD diagnosis and prognosis in early and late disease processes. Indeed, plasma pMP levels are high in healthy subjects showing high coronary heart disease risk score [77], and their number and phenotype (*i.e.*, surface expression of P-selectin and phosphatidylserine) positively correlate with recurrent CVD events [78, 79].

According to their parental origin, a significant gender-specific difference has been found, with the amount of pMPs significantly greater in healthy women than in the corresponding counterparts [80]. A menstrual cycle-dependent difference in pMPs also exists: a case-control study enrolling 27 healthy women and 18 healthy men demonstrated increased pMP release in females, especially in the luteal phase [81]. This finding suggests, therefore, that higher circulating pMPs (together with other risk factors, including pregnancy, oral contraceptives, and hormone therapy) may contribute to higher risk of developing venous thromboembolism observed in women < 45 years [49, 82, 83].

2.3. Platelet and CVD Risk in Metabolic Syndrome. Metabolic syndrome is a cluster of cardiometabolic risk factors, including central obesity, hypertension, impaired glucose

metabolism, and dyslipidaemia [84, 85]. Several meta-analyses have shown that CVD risk in metabolic syndrome is higher in females than in males and sex differences in adiposity and insulin resistance may partly account for this increased risk [86, 87]. A Korean cross-sectional study, carried on 3827 participants (2169 men and 1658 women), showed a positive correlation for platelet count and an inverse correlation for mean platelet volume in women with metabolic syndrome, but such trend was not observed in men [88]. Moreover, a recent prospective longitudinal, observational, cohort study (the Framingham Heart Study) evaluated protein biomarker profiles in 3289 men and 3895 women (mean age 49 years), in order to identify key biological pathways differing between sexes [89]. Of 71 biomarkers analyzed, 86% were significantly different in the two groups; in particular, women showed upregulation of pathways involved in inflammation, immune response, and adiposity, while platelet homeostasis and fibrosis pathways were enriched in men [89]. According to available literature data, these sex differences in circulating CVD biomarkers were attenuated in postmenopausal women, confirming that CVD risk dramatically increases following menopause [89]. Given these gender-related divergences, physicians are encouraged to take care of sex-specific risks in primary cardiovascular prevention and for design of personalized therapeutic strategies [90].

3. Intervention Studies

Several interventional strategies have been established to reduce incidence of clinical events related to coronary heart, cerebrovascular, and peripheral vascular diseases. World guidelines are drawn up for primary (for lowering risk in people without clinical symptoms) and secondary (for people with clinically manifest CVD) prevention, and concern both lifestyle changes and drug use. Herein, we will focus on dietary habits and antiplatelet therapy since these are the interventions where gender differences are most evident.

3.1. Nutritional Strategies. Until few years ago, in studies concerning correlations between nutrition and CVD, the traditional research orientation was to identify harmful foods (e.g., unprocessed red and processed meats, sugar-sweetened beverages) and nutrients (such as *trans*- and saturated fats, cholesterol, and sodium), whose consumption is now strictly prohibited [15, 91]. As it is recently emerging, instead, the importance of the so-called "positive food/nutrients," whose diet reduction or absence plays an equally crucial role in increasing cardiovascular risk [15, 91]. Coherently, diets based on foods particularly rich in antioxidants, phytochemicals, fiber, vitamins, monounsaturated fatty acids, and PUFAs, such as Mediterranean diet (MD) and vegetarian diet, are recognized worldwide as protective against CVD and its risk factors [8–15]. Also, in this context, it has been recognized a sex-related difference in individual responses to specific dietary habits (*e.g.*, adherence to MD), with men displaying more favorable specific cardiometabolic changes, with respect to premenopausal women [92–95].

Some food patterns and bioactive components appear particularly interesting, as, besides their beneficial cardiovascular effects, they also act on platelets with a sex-depending impact.

3.1.1. Mediterranean Diet. MD, a typical eating pattern of the Mediterranean basin, is characterized by wide consumption of fruits, vegetables, cereals, legumes, fish, olive oil as main fat source, and moderate red wine intake. Due to the consumption of these food items, subjects adhering to MD assume significant amounts of main nutrients of a healthy diet [96].

Effects of MD adherence on platelets have been investigated in the Moli-sani population-based cohort study that enrolled 14586 Italian healthy men and women [97]. Food intake was determined by food frequency questionnaire, while adherence to the MD was analyzed by using the MD Score (MDS) that evaluates intake of specific MD items and the Italian Mediterranean Index (IMI), a score particularly related to typical products consumed in Italy. What emerged from this study is that (i) greater adherence to MD was significantly associated with reduction in platelet count; (ii) subjects with greater adherence had reduced odds of being in the highest platelet count group; and (iii) hypercholesterolemia and increase in C-reactive protein (marker of inflammation) were prevalent in high-platelet count individuals. However, the most intriguing finding was that, although the mean platelet count of all individuals was within normal-range values, nonetheless, it directly correlates with predicted CVD risks in men, but not in women [97]. In line with this finding, a previous Moli-sani population-based cohort study demonstrated that specific healthy MD foods, particularly rich in antioxidant and phytochemicals, protected men much more than women against hypertension and inflammation [96]. This finding, therefore, suggests that healthy dietetic habits represent a valid strategy for primary CVD prevention that, however, requires particular attention to sex-related responses.

3.1.2. ω-3 PUFA. Beyond different efficiency of ω-3 PUFA biosynthesis in men and women, the rate of conversion is however low to satisfy physiological needs [55–57]; therefore, nutritional guidelines recommend to take EPA and DHA from fish (particularly, cold-water fatty fish, such as salmon, mackerel, tuna, herring, and sardine fish) and other seafood. Sometimes, also EPA and DHA supplementation, in the form of oily fish or fish oil (often fish liver oil) or krill oil capsules, is advised, although content of EPA and DHA varies in each of these preparations [98].

Several population studies have shown that dietary fish intake (as part of a healthy eating pattern) is inversely associated with stroke incidence and mortality, and therefore, EPA and DHA are counted among those nutrients that benefit cardiovascular health [99, 100]. As emerged from epidemiological studies, among mechanisms underlying the EPA and/or DHA cardiovascular protective role, reduction of platelet activation deserves special mention. The Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT) study, where over 8000 high-risk

patients were enrolled and followed for five years, demonstrated that supplementation with EPA-ethyl ester (providing more EPA per gram of oil, with respect to other supplements) significantly reduced major CVD events by 25% [101]. The beneficial effect much likely derives from the ability of EPA to compete with AA as a substrate for platelet COX-1, thus counterbalancing production of proaggregatory thromboxane A2 [102].

The main finding emerging from different studies is that ω-3 PUFAs counterbalance CVD risk factors in a gender-specific manner, both in primary and secondary prevention [103]. A meta-analysis indicated that dietary intake of fish and ω-3 PUFAs was correlated with lower incidence and mortality of stroke, especially in women and those with body mass index (BMI) $< 24\,kg/m^2$ [104]. Phang and collaborators [105] have shown that $1.0\,\mu M$ EPA, DHA, and docosapentaenoic acid (DPA) reduced aggregation of platelets isolated from healthy subjects. When data from male and female populations were combined together, according to already published literature [106–108], all tested ω-3 PUFAs reduced platelet aggregation, but with significant differences in terms of efficaciousness: EPA resulted to be the most effective showing a significantly higher percentage inhibition with respect to both DHA and DPA. When data were separated by sex, the same pattern of inhibition of platelet aggregation by ω-3 PUFAs was observed only in the male group, while differences were lost in females. The most pronounced anti-aggregatory effect of EPA observed only in men might, therefore, suggest a positive interaction between sex hormones and ω-3 PUFAs in modulating platelet activation cascades [105]. The same group [109] confirmed these findings also *in vivo*: in a blinded placebo-controlled intervention trial (enrolling 15 male and 15 female participants), the effects of a single acute oral dose of EPA- (providing 1 g EPA; EPA/DHA ratio = 5 : 1) or DHA- (providing 1 g DHA; EPA/DHA ratio = 1 : 5) rich oils on the aggregation response were investigated. Again, a gender-specific response was seen: DHA, but not EPA, significantly reduced platelet aggregation in women, whereas EPA, but not DHA, exerted an inhibitory action only in men. Accordingly, an inverse relationship between testosterone levels and platelet aggregation following EPA supplementation was observed. What emerges is, therefore, that males may benefit more from EPA supplementation and this may partly be explained considering that, among w3-PUFAs, EPA is more efficiently incorporated into male platelets [59]. Moreover, the finding that females are more responsive to DHA seems to be coherent with the evidence that, independently of dietary intake, females have higher circulating DHA concentrations, compared to males [59]. In the same population, authors also observed a gender-dependent response in the procoagulant activity of circulating pMPs. In male subjects, the single dose of EPA-rich oil inhibits pMP activity (-22%), in parallel with inhibition of platelet aggregation; on the contrary, DHA-rich oil reduces platelet aggregation, independently of pMP activity, in female subjects [110], thus pointing out to pMPs as one of the potential mechanistic pathways whereby ω-3 PUFAs might differentially modulate platelet activity and, therefore, yield cardiovascular benefits.

However, the study has some limitations, including differences in baseline characteristics (females were older and of postmenopausal age, while males had greater BMI and high testosterone levels) and in platelet-related parameters (longer lag time in males; higher platelet count and baseline platelet aggregation in females).

Thus, although available evidence highlights gender-specific effects of ω-3 PUFAs on platelet function, further work is needed to establish exact mechanisms underlying the interactions between sex hormones and this class of nutrients and future well-powered studies should be assessed to justify dietary recommendations for distinctive ω-3 PUFAs in men and women.

Although there are several data on beneficial effects of ω-3 PUFAs (taken from fish or supplements) in high-risk CVD patients, nonetheless, their therapeutic value, up to now, is not clear, as results are not conclusive and sometimes controversial [101]. Moreover, several confounding, often perplexing, factors should be considered, such as (i) differences in taking PUFAs from fish (which also is a source of other important nutrients, like selenium, iodine, zinc, calcium, and proteins), fortified foods (*e.g.*, enriched margarine), or supplements; (ii) harmful effects related to high-PUFA intake, especially through supplements, *i.e.*, high concentrations of toxic compounds (namely, mercury, dioxins, and polychlorinated biphenyls) in fish oils; (iii) other negative events dependent on ω-3 PUFAs themselves, such as prolonged bleeding time, increased lipid peroxidation, and abrogation of normal immune responses.

Although dietary modifications may help in preventing pathological conditions, all these elements point out that we are far from a solid, scientific-based knowledge for development of individualized PUFA supplementation regimens to prevent and manage CVD, and further studies are required to better define precise dietary indications.

3.1.3. Flavonoids. Flavonoids are a large family of over 5,000 hydroxylated polyphenolic compounds, which encompass six major subclasses of dietary significance, named anthocyanidins, flavan-3-ols (also referred as flavanols), flavonols, flavanones, flavones, and isoflavones. These phytochemicals are abundantly found in fruits, vegetables, and cocoa, as well as in beverages, such as tea and wine. Several factors may affect flavonoid content in food, among which are agricultural practices, environmental conditions, ripening, storage, and food processing; consequently, reported value contents in plants should be considered approximate [54, 111].

Flavonoids are often present as glycosides (such as isoflavones and anthocyanins), and this chemical feature, together with other factors (including some other chemical characteristics, interactions with other components of food matrix, composition of colonic microbiota, and gut and liver metabolism), influences their metabolic fate and bioavailability [54, 111]. For example, anthocyanins and galloylated catechins are poorly absorbed, while isoflavones seem to be the most bioavailable flavonoids [111].

Beyond these evidences, flavonoids have received particular attention for potential health benefits of fruit- and vegetable-rich diets, especially in relation to the cardiovascular

system [111]. Most (but not all) epidemiological studies, indeed, greatly suggest that high intake of dietary flavonoids (approximately 200 mg/day of total flavonoids) is inversely related to CVD risk and mortality [112–117]. Nonetheless, it must not be overlooked that some of their beneficial effects may also be attributed to other bioactive constituents, (including other polyphenols, vitamins, and minerals), synergizing with flavonoids.

If initially flavonoids were believed to mainly act as antioxidants, nowadays, it is well established that they positively impact cardiovascular health by exerting other biological activities, such as (i) induction of vascular endothelium relaxation, (ii) inhibition of endothelial dysfunction, (iii) stimulation of nitric oxide release, (iv) inhibition of platelet aggregation, and (vi) downregulation of proinflammatory mediators [116, 118]. There is, indeed, the consistent view that these compounds directly act on various signaling pathways, among them, those related to $P2Y_1/P2Y_{12}$ (ADP receptors), GPVI (collagen receptor), protease-activated receptor 1 (PAR1; thrombin receptor), and COX-1 signaling, through which flavonoids (especially, those extracted from cocoa, tea, pigmented rice, chokeberry, and oat) mitigate platelet adhesion, degranulation, and aggregation [119].

In this context, interventional studies have shown that flavanols, which include catechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate monomers; their dimers (theaflavins, thearubigins); and polymers (proanthocyanidins), appear the most efficacious in attenuating platelet hyperactivation. Most of the studies are focused on two of principal dietary sources of flavanols, *i.e.*, cocoa-based products and green tea [120]. Just an example, a double-blind randomized placebo-controlled trial, enrolling twenty patients with congestive heart failure, evaluated acute and chronic effects of commercially available flavanol-rich chocolate on platelet and endothelial functions and compared it with a chocolate-free cocoa liquor, as control. The authors reported that, shortly after ingestion, only flavanol-rich cocoa led to peripheral vasodilatation, endothelial function improvement, and reduction in platelet activation [121]. The exact contribution of flavanols in the beneficial effect of cocoa has further been assessed by Ostertag and coworkers [122], who evaluated potential sex differences in platelet responses. In their blinded randomized, controlled trial, the researchers compared flavanol-enriched dark chocolate (containing 907.4 ± 22.75 mg of flavan-3-ols) with both standard dark (containing 382.3 ± 45.20 mg of flavanols) and white (with no flavanols) chocolates, in relation to effects on platelets derived from healthy men and women. By pooling data from male and female groups, they found that acute consumption of the two types of dark chocolates reduced ADP- and thrombin-dependent activation and aggregation of platelets and increased the collagen/epinephrine-induced *ex vivo* bleeding time; these effects were time-dependent and more evident with flavanol-enriched dark chocolate. According to gender-related differences in platelet signaling cascades [51, 66, 71], ADP-triggered pathways were significantly inhibited in men, while thrombin-dependent signaling was exclusively attenuated in women, after consumption of flavanol-enriched dark chocolate. Analysis of plasma or urine concentrations of flavanols and their metabolites revealed gender-related absorption or metabolism of flavanols that might partially explain the different efficacy by which these phytochemicals can affect platelet functions [122]. However, it should be underlined that also white chocolate improved platelet profile in males [122], thus indicating the presence of other compounds, not yet identified, capable of exerting antiplatelet effects in a sex-dependent manner.

Besides flavanols, isoflavones (such as daidzein and genistein) deserve to be mentioned. These flavonoids, mainly found in soybeans and soy foods, show both estrogenic and antiestrogenic effects; therefore, they are also classified as phytoestrogens [111]. Accordingly, the effects derived from their intake (*via* foods and supplements) are object of extensive investigations, especially in the hormone-sensitive cancer research field. Moreover, it is well known that their assumption ameliorates some symptoms of menopause, such as hot flashes [123]. This evidence, together with the finding that isoflavones ameliorate lipid profile and endothelial function in a gender-specific manner, strengthens the idea that these phytoestrogens are also beneficial for cardiovascular health [124–126], especially for menopausal women, missing estrogen-dependent protection. For example, three prospective cohort studies have found positive correlation between higher intake of isoflavones and tofu (but not soy drinks) and moderately lower risk of developing coronary heart disease in both men and women; nonetheless, in women, the favorable association of tofu was more pronounced in young subjects or postmenopausal women without hormone use [127].

The capability of isoflavones to inhibit *in vitro* platelet activation induced by collagen or AA, through a mechanism dependent on their ability to act as TxA2 receptor antagonists, seems noteworthy [128]. A double-blind, randomized study has clearly underlined that supplementation with soy-derived isoflavones reduced the risk of thrombogenesis, by decreasing platelet TxA2 signaling [129]. Twenty-nine healthy postmenopausal women (aged 45-60 years) were randomly assigned to two groups, receiving either 100 mg/day soy isoflavone extract or placebo, for 3 months; what emerged is that supplementation had no significant effect on common CVD risk factors (lipid profile, blood pressure, and anthropometric measures), while significantly decreasing TxA2 receptor density (from 181.9 ± 30.9 to 115.2 ± 16.2 fmol/10^8 platelets) [129]. Conversely, a previous study evaluating the chronic effect of soy protein supplements (that are rich in isoflavones) in healthy young males showed that, although soy supplementation critically increased plasma concentration of isoflavones, nonetheless, such increase was not sufficient either to significantly inhibit *ex vivo* platelet aggregation or to ameliorate lipid profile [130].

In conclusion, although the impact of diet and gender on platelets is suggestive (Table 1), dietary manipulation of platelet function is still far from being realized, since gaps in our knowledge (especially concerning sex differences on bioavailability, metabolism, and activity of food components) persist and more research is required.

Table 1: Nutritional studies aimed at investigating gender-related differences in platelet responses.

Dietary factors	Experimental protocol	Main findings	Refs
Mediterranean diet (MD)	Population-based cohort study: 6975 males and 7611 females (mean age: 54.2 ± 11.5 yrs) adhering to MD and subdivided into 3 groups according to their PLT count: high-, medium-, and low-PLT count groups (2.5%, 95.6%, and 1.9% of the population, respectively).	In both sexes: PLT count was inversely associated with both MDS and IMI scores. Subjects with very high MD adherence had lower odds of having high-PLT count compared with individuals with poor adherence (OR 50.50; 95% CI: 0.31-0.80 and OR 5 0.73; 95% CI: 0.52-1.02 for MDS and IMI, respectively). In males: the mean PLT count increased with increasing of predicted CVD risk (low CVD risk: 236.5 ± 54.7, medium CVD risk: 239.6 ± 57.1, and high CVD risk: 247.1 ± 58.7; P for trend > 0.027 in multivariable analysis of variance). In females: no differences in PLT count within the predicted CVD risk.	[97]
ω-3 PUFA	Ex vivo study: PLT isolated from healthy 20 males (33.5 ± 2.1 yrs) and 22 females (35.7 ± 2.5 yrs) preincubated with 1 μM EPA, DHA, or DPA for 6 min at 37°C, before stimulation with 5 μg/mL collagen.	In both sexes: DHA and DPA equally reduced PLT aggregation (36.4% and 33.5% in men and women, respectively). EPA was the most efficacious PUFA (51.7%, $P < 0.01$ *vs.* DPA and $P < 0.004$ *vs.* DHA). In males: DHA (25.3%) and DPA (21.7%) were less effective, with respect to EPA (48.9%, $P < 0.002$ and $P < 0.001$, respectively). In females: DHA (46.5%), DPA (44.2%), and EPA (54.3%) equally reduced PLT aggregation.	[105]
	Blinded placebo-controlled trial: healthy 15 males (40.1 ± 2.1 yrs old) and 15 females (47.4 ± 1.9 yrs), alternatively receiving a single dose of 2 × 1 g capsules containing either (i) placebo or (ii) EPA-rich oil (providing 1 g EPA with EPA/DHA ratio = 5 : 1) or (iii) DHA-rich oil (providing 1 g DHA with EPA/DHA ratio = 1 : 5). Fasting blood samples collected for PLT aggregation assay at 0, 2, 5, and 24 hrs after supplementation.	In both sexes: EPA- and DHA-rich oils reduced PLT aggregation. EPA was the most effective at 2 (-3.6%, $P < 0.001$), 5 (-8.8%, $P < 0.001$), and 24 (-13.3%, $P < 0.006$) hrs postsupplementation. DHA was inefficacious at 2 and 5 hrs, but equally effective (-11.9%, $P < 0.016$) as EPA at 24 hrs. In males: only EPA reduced PLT aggregation at 2 (-11%, $P < 0.001$), 5 (-10.6%, $P < 0.003$), and 24 (-20.5%, $P < 0.008$) hrs. In females: only DHA reduced PLT aggregation at 24 hrs (-13.7%, $P < 0.05$).	[109]
	Blinded placebo-controlled trial: healthy 15 males (40.1 ± 2.1 yrs) and 15 women (47.4 ± 1.9 yrs), alternatively receiving a single dose of 2 × 1 g capsules containing either (i) placebo or (ii) EPA-rich oil (providing 1 g EPA with	In both sexes: neither oil affected pMP numbers, and only EPA-rich oil produced a decrease in pMP activity (−19.4%, $P = 0.003$). In males: EPA, but not DHA, increased the mean lag time (60 *vs.* 79 sec, +29.5%) and reduced ADP-induced PLT	[110]

Table 1: Continued.

Dietary factors	Experimental protocol	Main findings	Refs
	EPA/DHA ratio = 5 : 1) or (iii) DHA-rich oil (providing 1 g DHA with EPA/DHA ratio = 1 : 5). Fasting blood samples collected at 0 and 24 hrs after supplementation for PLT aggregation assay and measurement of pMP number and procoagulant activity.	aggregation (−20.5%, $P = 0.008$) and pMP activity (−22%, $P = 0.008$). Inverse relationship between PLT aggregation activity and testosterone levels ($r = -0.443$, $P = 0.04$). In females: DHA, but not EPA, was effective in reducing PLT aggregation (−13.7%), without affecting pMP number and activity.	
	Double-blind, randomized, controlled intervention trial: 79 men and 95 women aged 20–80 yrs receiving six 0.75 g capsules/day providing a total of 1.5 g EPA and 1.77 g DHA (i.e., 3.27 g EPA plus DHA), as TAG, equivalent to the amount in one portion of oily fish and six 0.75 g placebo capsules (high oleic sunflower oil), over 12 months. Fasting blood samples collected at 0 and 12 months after supplementation for lipid composition of platelet membrane.	In both sexes: no differences in basal content of EPA and DHA. Equal dose-dependent increases of EPA and DHA in platelet membrane between male and females after 12-month supplementation. In males: EPA increased in PLT membrane, but without statistical significance.	[131]
Flavanols	Blinded randomized, controlled acute trial: healthy 26 women (23–62 yrs; mean: 38 ± 2.4 yrs) and 16 males (25–65 yrs; mean: 46 ± 3.4 yrs), who acutely ingested 60 g of (i) flavanol-enriched dark chocolate (FDC; 907.4 ± 22.75 mg of flavan-3-ols), (ii) standard dark chocolate (SDC; 382.3 ± 45.20 mg of flavan-3-ols), and (iii) white chocolate (WC; not detectable). Fasting blood collected at 0, 2, and 6 hrs after supplementation for PLT activity assays.	Ex vivo bleeding time In both sexes: *ex vivo* bleeding time increased 6 hrs after consumption of FDC and SDC, but not of WC ($P = 0.011$), in both sexes. In females: *ex vivo* bleeding time increased 6 hrs after the consumption of FDC and SDC, but not with WC ($P = 0.016$). In males: *ex vivo* bleeding time increased 6 hrs after the consumption of FDC and WC ($P = 0.042$). PLT aggregation In both sexes: ADP-induced platelet aggregation reduced at 2 hrs, but not 6 hours, after consumption of FDC and SDC. In males: ADP-induced PLT aggregation was reduced at 2 and 6 hrs after consumption of FDC and SDC ($P = 0.008$ and $P = 0.020$ vs. women). In females: TRAP-induced PLT aggregation was reduced at 2 hrs, but not 6 hours, after consumption of FDC ($P = 0.010$, P value for interaction between treatment and gender: $P = 0.213$). PLT activation In both sexes: TRAP-induced fibrinogen binding decreased at 2 and 6 hrs after consumption of FDC and WC (respectively, $P = 0.014$ and $P = 0.021$ vs. SDC).	[122]

Table 1: Continued.

Dietary factors	Experimental protocol	Main findings	Refs
		In males: ADP-triggered P-selectin exposure decreased at 2 hrs, but not 6 hrs, after consumption of FDC and WB, but not with SDC ($P = 0.002$). In females: TRAP-induced fibrinogen binding was decreased at 2 hrs, but not 6 hours, after consumption of FDC ($P = 0.041$, P value for interaction between treatment and gender: $P = 0.304$).	
Isoflavones	Double-blind, randomized, placebo-controlled study: 29 postmenopausal women (45–60 yrs), who randomly received two daily capsules of a soybean isoflavone extract (23.4 ± 3.4 mg daidzein and 24.1 ± 4.6 mg genistein per capsule) or placebo for 12 weeks. Blood collected at 0 and 12 weeks after supplementation for PLT TxA2 receptor binding assay.	In females: PLT TxA2 receptor density decreased in isoflavone-treated subjects from 181.9 ± 30.9 to 115.2 ± 16.2 fmol/10^8 PLT ($P < 0.02$ vs. the placebo group). Decrease in TxA2 receptor density inversely correlated with serum concentrations of isoflavones.	[129]
	Double-blind, randomized, placebo-controlled study: healthy 10 men (25.8 ± 1.2 yrs) receiving 60 mg of soy proteins in the form of beverage powder and providing 131 mg of total isoflavones (80.3 mg genistein, 35.6 mg daidzein, and 15.1 mg glycitein) and 10 men (23.9 ± 0.9 yrs), receiving 60 mg of calcium caseinate powder (control), for 28 days. Blood was collected at 0, 28, and 56 days after supplementation for quantification of isoflavone content in plasma and PLT aggregation.	In males: plasma isoflavone content increased after 28 day in the supplementation group ($P < 0.05$ *vs.* basal values) and returned to baseline after 56 days (washout period). PLT aggregation was not affected by soy protein supplementation.	[130]

CI: confidence interval; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; IMI: Italian Mediterranean Index; MD: Mediterranean diet; MDS: Mediterranean Diet Score; OR: odds ratio; PLT: platelet; pMP: platelet microparticles; TAG: triglycerides; TRAP: thrombin receptor activating peptide; TxA2: thromboxane A2.

4. Antiplatelet Therapy

Current antiplatelet therapies mainly target enzymes (such as COX-1), receptors (such as thromboxane or ADP receptors), and glycoproteins (such as GPIb or GPVI) [132, 133]. Among antiplatelet drugs, the most widely used is aspirin that irreversibly inhibits COX-1, thus preventing conversion of AA into TxA2; nonetheless, it does not act on TxA2-independent signaling pathways and, moreover, long-term usage leads to increased risk of bleeding events [134]. To overcome these limitations, other drugs have been developed, such as the P2Y12 receptor inhibitors clopidogrel, prasugrel and ticagrelor. The first one is the most commonly used, but it shows a delayed therapeutic onset and may cause coagulation dysfunction; prasugrel inhibits platelet aggregation more rapidly than clopidogrel; the last antiplatelet drug exerts CVD protective effects without increasing overall bleeding and, being a P2Y12 receptor reversible inhibitor, loses pharmacological activity upon body clearance [135, 136].

Although women are less represented in cardiovascular clinical trials, nonetheless, numerous investigations have

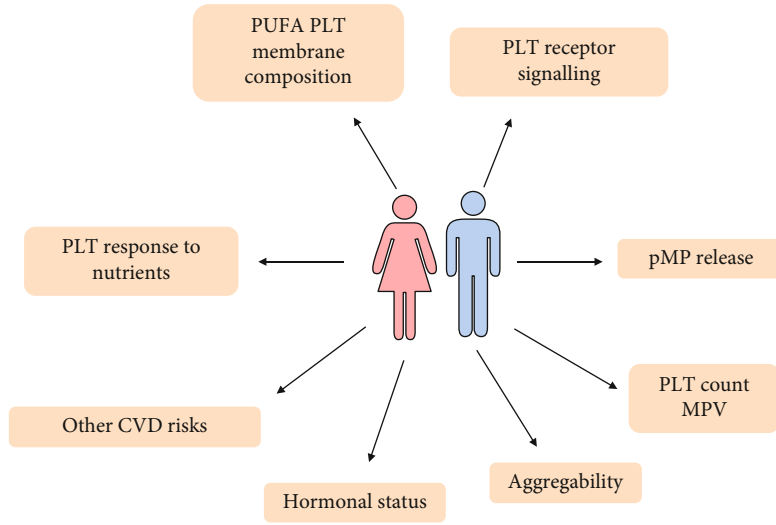

Figure 1: Schematic representation of the main sex-based differences in platelets in relation to cardiovascular disease. See text for details. CVD: cardiovascular disease; MPV: mean platelet volume; PLT: platelet; pMPs: platelet microparticles; PUFA: polyunsaturated fatty acids.

pointed out that some of the abovementioned female-related conditions (such as hormonal status and platelet biology) have to be taken into account in view of aspirin administration for primary prevention. Just an example, the Women's Health Study, evaluating the efficacy of aspirin in 39876 healthy women (≥45 years of age) monitored for 10 years, reported a significant prevention of ischemic stroke (RR = 0.83; $P < 0.04$); however, the drug also led to a parallel increment of gastrointestinal bleeding risk [137]. Other clinical trials and meta-analyses confirmed no significant benefit of aspirin treatment in women concerning cardiovascular events and CVD mortality, but a huge increase in risk of overall bleeding [134, 138]. Based on these findings, special attention should be paid when treating women with aspirin.

A meta-analysis of five randomized trials, involving 79,613 (of whom 23,533 are women) patients with cardiovascular heart disease, showed that clopidogrel (in addition to aspirin) significantly decreased cardiovascular events in both men and women. Although gender differences in the absolute benefit are not striking, during long-term antiplatelet therapy, risk of events was higher in women than in men and clopidogrel therapy seemed to be effective only in men. As documented by the *post hoc* subanalysis of the BleeMACS study, collecting data from fifteen centres in Europe (Germany, Greece, Italy, Netherlands, Poland, and Spain), Asia (China and Japan), North America (Canada), and South America (Brazil), the increased bleeding rates observed only in females were associated with prasugrel-/ticagrelor-based dual antiplatelet therapy [139]. Finally, the multicentre, Italian START ANTIPLATELET registry investigated the choice of antiplatelet treatment and its impact on one-year clinical outcome, in 625 males and 215 females presenting with acute coronary syndrome. In this study, what emerged is that dual antiplatelet therapy was more commonly prescribed in men and, when administered in both sexes, clopidogrel was the best choice for women, while prasugrel was preferentially used in men. However, gender-related differences in terms of therapy did not lead to different outcomes. Therefore, P2Y12 inhibitor choice in dual antiplatelet therapy is gender-specific (in order to counteract the increased bleeding risk usually observed in females), but it has a similar clinical outcome irrespective of sex [140].

High percentage of individuals usually experiences antiplatelet therapy resistance that impairs successful prevention of cardiovascular events, and some determinants of resistance to antiplatelet therapy are gender responsive [141–146]. A prospective study on 160 patients with stable coronary heart disease (118 men and 42 women, aged 65.2 ± 7.8 years), indeed, showed a sex-related response to long-term double antiplatelet therapy (75 mg/day aspirin and clopidogrel for three months): female gender was more predisposed to resistance to both aspirin and clopidogrel compared to men [19]. Two main factors may account for the worst responsiveness: women possess (i) greater aggregation capacity, maybe because of higher density of platelet receptors able to bind fibrinogen, and (ii) increased inflammatory status, as highlighted by higher concentrations of the proaggregatory C-reactive protein (CRP) and number of leukocytes and granulocytes.

5. Conclusions

Evidence to date has revealed sex-based differences in CVD prevention, diagnosis, and management. Among modifiable and nonmodifiable risk factors, platelet hyperactivity deserves particular mention, as activation and aggregation of platelets, as well as their interaction with endothelial cells and crosstalk with immune cells, play a major role in the pathophysiology of main cardiovascular events, including stroke, myocardial infarction, and peripheral vascular injury. Moreover, platelet biology is profoundly modulated by several elements, including sex hormones, nutrients, and inflammatory mediators (Figure 1). Consequently, men and women not only display a different platelet functionality but also distinctively respond to common antiplatelet drugs, as well as to specific dietetic habits. In conclusion, full

understanding of gender-related differences is the final goal in order to design tailored strategies for preventing and treating platelet-mediated vascular damage.

Abbreviations

12-H(P)ETE: 12-Hydro(pero)xyeicosatetraenoic acid
12-LOX: 12-Lipoxygenase
ANOCA: Angina and nonobstructive coronary artery disease
AA: Arachidonic acid
BMI: Body mass index
CRP: C-reactive protein
CVD: Cardiovascular disease
COX-1: Cyclooxygenase 1
DHA: Docosahexaenoic acid
DPA: Docosapentaenoic acid
EPA: Eicosapentaenoic acid
GP: Glycoprotein
PAF: Platelet-activating factor
pMP: Platelet microparticle
PGI_2: Prostacyclin
PUFA: Polyunsaturated fatty acid
RR: Relative ratio
TxA2: Thromboxane A2
TPA: Thrombin receptor activating peptide
vWF: von Willebrand factor.

Conflicts of Interest

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

Authors' Contributions

M. Valeria Catani and Isabella Savini are equally senior authors.

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Research Article

Cardioprotective Effect of (*Z*)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid: Inhibition of Apoptosis in Cardiomyocytes

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Background. Although many studies have been performed to elucidate the molecular mechanisms of heart failure, an effective pharmacological therapy to protect cardiac tissues from severe loss of contractile function associated with heart failure after acute myocardial infarction (MI) has yet to be developed. *Methods*. We examined the cardioprotective effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid, a new compound with potent antioxidant and antiapoptotic activities in a rat model of heart failure. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid was systemically delivered to rats 6 weeks after MI at different doses (15, 30, and 60 mg/kg). Cardiac function was assessed by hemodynamic measurements. The expression of proinflammatory cytokines, apoptosis-related molecules, and markers of adverse ventricular remodeling was measured using RT-PCR and Western blot. *Results*. Treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid significantly improved cardiac function, in particular by increasing dP/dt. Simultaneously, the expression of the proinflammatory cytokines TNF-α and IL-1β was markedly reduced in the treatment group compared with the MI group. In addition, (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated tissues displayed decreased expression of Bax, caspase-3, and caspase-9 and increased expression of Bcl-2, which was in part due to the promotion of Akt phosphorylation. *Conclusion*. These data demonstrated that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid possesses potent cardioprotective effects against cardiac injury in a rat model of heart failure, which is mediated, at least in part, by suppression of the inflammatory and cell apoptosis responses.

1. Introduction

A severe loss of contractile function is the main characteristic of end-stage heart failure. Pathological analyses of the myocardium from patients with end-stage heart failure indicate a loss of cardiomyocytes as well as fibrosis and cardiomyocyte hypertrophy [1]. The collective evidence now demonstrates that a defect in the myocardium is a main reason for heart failure. Therefore, recent studies have focused on identifying chemical agents that can prevent cardiac damage following MI. Caspase-9 appears to play an important role in cell apoptosis. Activation of caspase-9 can initiate a proteolysis cascade by cleaving other caspases, such as caspase-3, which are effectors that carry out the cellular death program [2, 3]. Meanwhile, protein kinase (Akt), a key effector in the antiapoptosis survival pathway, can inactivate glycogen synthase kinase 3β (GSK-3β) by phosphorylation, thereby blocking caspase-3 activation [4]. Additionally, an imbalance of proapoptotic versus prosurvival members in the Bcl-2 family plays a major role in the apoptosis of cardiomyocytes and heart failure [5, 6].

Furthermore, recent evidence from both animal and human studies suggests that increased production of inflammatory cytokines and cardiac cell oxidative stress are associated with a poor prognosis following MI and may play a critical role in the pathogenesis and progression of heart failure. Indeed, myocardial cell death is reported to trigger an acute inflammatory reaction that leads to chemokine and cytokine production and the recruitment of macrophages that results in leukocyte infiltration of the infract area [7–10]. In addition, it is also known that after myocardial ischemic injury, cytokines such as TNF-α and IL-6 are also

released and that these molecules can promote cell death signaling cascades in cardiac tissues [11]. Taken together, these findings indicate that inhibiting cardiomyocyte apoptosis and reducing inflammatory responses in cardiac tissues may prevent the loss of the myocardium and thereby reduce the adverse effects associated with heart failure.

In the present study, we assessed the therapeutic effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid in a rat model of heart failure. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid is a compound with two phenolic hydroxyl groups. It is reported that caffeic acid phenethyl ester, which also has two phenolic hydroxyl groups, has antiviral, anti-inflammatory, antioxidant, and immunomodulatory properties, in addition to being a potent and specific inhibitor of NF-κB activation [12–17].

In one of our previous studies, we demonstrated that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid mediates potent protective effects against hypoxia-induced cellular damage by blocking oxidative stress and apoptosis [18]. These findings encouraged us to investigate the hypothesis that administration of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid could modulate local myocardial inflammation, inhibit cardiomyocyte apoptosis, attenuate adverse ventricular remodeling, and subsequently improve cardiac performance in post-MI chronic heart failure.

2. Methods

2.1. Experimental Heart Failure and Administration of (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid. The rats used in the current study were supplied by the experimental animal center of Fudan University. Adult male Sprague-Dawley rats weighing 230-250 g (8-10 w) were used for the study. The animals were housed with a 12 h light-12 h dark cycle and had free access to standard pellet food and water throughout the experiment. All of the experiments were approved by the local ethics committee of Fudan University.

For the experiments, the adult male Sprague-Dawley rats were randomly divided into five groups: a sham operation group, MI group, and MI with drug treatment groups (15, 30, and 60 mg/kg). The drug was dissolved in DMSO (dimethyl sulfoxide). The drug was injected intraperitoneally 6 weeks after surgery once daily. The sham operation group and the MI group were injected only with DMSO according to animal weight. Prior to the induction of MI, the animals were intubated and artificially ventilated with a rodent ventilator (DHX-150, China) under anesthesia with 7% chloral hydrate (60 mg/kg i.p.). The left anterior descending coronary artery was ligated with a 5-0 suture, 1-2 mm below the left atrial appendage [19]. Six weeks after surgery, after measuring the hemodynamic parameters, the hearts from rats representing each group were excised and washed with PBS. The whole LV was stored at -80°C.

2.2. Assessment of Cardiac Function Hemodynamic Measurements. Left ventricular pressures were measured via a saline-filled cannula that was inserted through the right carotid artery and connected to a pressure transducer. The cannula was inserted into the left ventricle to monitor left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP), as well as to measure the maximum rate of the rise of the left ventricular pressure rising rate (dP/dt).

2.3. Enzymatic Activity Assays. Six weeks after surgery, the whole blood of the rats was centrifuged (3000 r/min, 10 min, 4°C) to obtain serum. The antioxidant enzyme activities of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), and glutathione S-transferase (GST) were measured in the serum. MDA, CAT, SOD, GSH, GSH-Px, and GST levels were measured by using a commercially available kit according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China).

MDA activity was measured by adding 1 ml of the standard agent to the standard tubes. Next, 1 ml of absolute ethyl alcohol was added to the blank tubes. Then, 1 ml of the sample was added to the sample tubes and control tubes. The tubes were shaken for a long period. Then, 3 ml of reagent 2 was added to all the tubes, and then, 1 ml of reagent 3 was added to the standard tubes, blank tubes, and sample tubes. Next, 1 ml of 50% glacial acetic acid was added to the control tubes. The tubes were mixed and covered with plastic wrap. A small hole was made in the plastic wrap. The tubes were incubated at 95°C for 40 minutes and cooled in flow water. The tubes were centrifuged at 3000 to 4000 rpm for 10 minutes. The absorbance of the supernatant was measured using a plate reader at 532 nm.

CAT activity was measured by first preparing positive control tubes and sample tubes. Then, 0.1 ml of serum was added to the sample tubes, and 1.0 ml of reagent 1 and 0.1 ml of reagent 2, both kept at 37°C, were added to each tube. They were incubated on a shaker at 37°C for precisely 1 min. After that, 1.0 ml of reagent 3 and 0.1 ml of reagent 4 were added to all tubes. Then, 0.1 ml of the serum was added to the positive control tubes. Finally, the absorbance was read at 405 nm using a plate reader.

To measure GSH activity, the preparation of the sample was as follows: 0.5 ml of the sample and the buffer of reagent 1 were mixed and centrifuged at 3000 to 4000 rpm for 10 minutes. Then, 1 ml of the sample supernatant was added to sample tubes, while 1.0 ml of reagent 1 and 1.0 ml of standard 20 μmol/l aqueous GSH were added to the blank tubes and the positive control tubes. Then, 1.25 ml of reagent 2, 0.25 ml of reagent 3, and 0.05 ml of reagent 4 were added to each tube. The absorbance was read at 420 nm using a plate reader.

SOD activity was measured as follows: 0.5 ml of the sample and the buffer of 1.0 ml reagent 1 were mixed; meanwhile, 0.5 ml of water and the buffer of 1.0 ml reagent 1 were mixed as the positive control. Then, 0.1 ml of reagents 2, 3, and 4 was added to the sample tubes and the positive control tube. Then, all of the tubes were incubated at 37°C for precisely 40 minutes. 2.0 ml of color reagents were added to each tube. Finally, all the tubes were placed at room temperature for 15 minutes. The absorbance was read at 550 nm using a plate reader.

GSH-Px activity was measured by first adding 0.2 ml of aqueous 1 mmol/l GSH to the control tubes and the sample tubes. Then, 0.1 ml of the sample was added to the sample tubes. All of the tubes and reagent 1 were kept at 37°C for 5 minutes. Then, 0.1 ml of reagent 1 was added to each tube. All of the tubes were incubated at 37°C for precisely 5 minutes. Two milliliters of reagent 2 were added to every tube, and 0.1 ml of the sample was added to the control tubes. The tubes were centrifuged at 3000 to 4000 rpm for 10 minutes. One milliliter of the supernatant from the control tubes and the sample tubes was transferred to new tubes labeled the same as the old tubes. Simultaneously, 1 ml of the GSH standard buffer was added to the blank tubes and 1 ml of the aqueous 20 μmol/l GSH was added to the standard tubes. Then, 1 ml of reagent 3, 0.25 ml of reagent 4, and 0.05 ml of reagent 5 were added to all of the tubes. The plate was covered and incubated on a shaker for 15 minutes at room temperature. Finally, the absorbance was read at 412 nm using a plate reader.

GST activity was measured by first adding 0.3 ml of aqueous 1 mmol/l GSH to the control tubes and the sample tubes. Then, 0.1 ml of the sample was added to the sample tubes. The tubes were mixed and incubated at 37°C for 30 minutes. Then, 2 ml of reagent 2 was added to all the tubes. Next, 0.1 ml of the sample was added to the control tubes. The tubes were centrifuged at 3000 to 4000 rpm for 10 minutes. One milliliter of the supernatant from the control tubes and the sample tubes was transferred to new tubes labeled the same as the old tubes. Then, 2 ml of the GSH buffer and 2 ml of aqueous 20 μmol/l GSH were added to the blank tubes and standard tubes. Finally, 2 ml of reagent 3 and 0.5 ml of reagent 4 were added to all the tubes. The tubes were incubated for 15 minutes at room temperature, and the absorbance was read at 412 nm using a plate reader.

2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cardiac myocytes by using TRIzol (Invitrogen, Carlsbad, CA) as previously described. The RNA concentration was determined by measuring the absorbance at 260 nm. Reverse transcription (RT) was conducted by using a PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacturer's recommended protocol. The primers for RT-PCR were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, China. The primers used for amplification were synthesized as follows: TNF-α forward 5′-ATGAGC ACGGAAAGCATGATCCGA-3′, reverse 5′-CCAAAG TAGACCTGCCCGGACTC-3′; IL-1β forward 5′-ATGG CAACTGTCCCTGAACTCAACT-3′, reverse 5′-CAGGAC AGGTATAGATTCAACCCCTT-3′; Bcl-2 forward 5′-CGGGAGAACAGGGTATGA-3′, reverse 5′-CAGGCT GGAAGGAGAAGAT-3′; Bax forward 5′-GCAGGGAGG ATGGCTGGGGAGA-3′, reverse 5′-TCCAGACAAGC AGCCGCTCACG-3′; caspase-3 forward 5′-CTGGACTGC GGTATTGAG-3′, reverse 5′-GGGTGCGGTAGAGTAA GC-3′; TGF-β forward 5′-CGCAACAACGCAATCTATG-3′, reverse 5′-AGCCCTGTATTCCGTCTCC-3′; collagen-1 forward 5′-CATAAAGGGTCATCGTGGCT-3′, reverse 5′-TGTTCTCAATCTGCTGGCTCA-3′; and GAPDH forward 5′-TTCAACGGCACAGTCAAGG-3′, reverse 5′-CGGCATGTCAGATCCACAA-3′. The PCR products were analyzed by electrophoresis in 1.5% agarose gels. The intensity of each band was photographed and quantified by using a Bio-Rad iQ5 system (Bio-Rad Laboratories, CA, USA) as a ratio of a target gene over GAPDH.

2.5. Western Blot Analysis. Frozen LV specimens were dispersed mechanically in the lysis buffer. The lysate was centrifuged at 10,000 r/min for 10 min at 4°C, and the supernatant was collected. The protein concentrations were quantified using an enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China). After the protein concentrations were determined, 30-50 μg of protein was separated by SDS-PAGE and electrophoretically transferred onto PVDF membrane and blocked overnight with 1% skim milk in TBS at 4°C. The blots were incubated with specific primary antibodies against Bcl-2, Bax, Akt, and p-Akt for 2 h at room temperature, washed, and then incubated with appropriate peroxidase-conjugated secondary antibodies. Bcl-2, Bax, Akt, and phosphor-Akt antibodies and goat anti-rabbit IgG conjugated with peroxidase were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA, and Cell Signaling technology, Inc., USA, respectively. The immune complexes were visualized by using ECL detection reagents following the manufacturer's protocol. Densitometric analysis was carried out with a Western blotting detection system (Alpha Innotech, USA). The band intensity for each sample was analyzed, and protein expression was normalized to GAPDH.

2.6. Statistical Analysis. The data are presented as mean ± SD. Statistical analysis was performed using SPSS version 15.0. The differences between groups were determined using one-way ANOVA for repeated measures. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid Improves Cardiac Function. The synthetic route of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid was performed as described in detail previously in our laboratory [18].

Table 1 lists the effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid on rat physiological and hemodynamic parameters 6 weeks after MI. Compared with the sham operation group, the MI group presented significantly increased LV end-diastolic Pressure (LVEDP), in addition, decreased Left ventricular systolic Pressure(LVSP) and maximum rate of dP/dt. In the MI group, dP/dt as an index of myocardial contractility was significantly reduced compared with the sham operation group. In addition, in the 15 mg/kg (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated group, a significant improvement of dP/dt was observed ($P < 0.05$). These data indicated that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid promotes cardiac function at a lower concentration.

Table 1: Cardiac parameter changes following (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid injection at 15, 30, and 60 mg/kg concentrations six weeks after MI. Data are expressed as mean ± SD. $^{\#}P < 0.05$ versus sham, $^{*}P < 0.05$ versus MI, and $n = 6$ in each group.

Variable	Sham	MI	MI+acrylic acid (15)	MI+acrylic acid (30)	MI+acrylic acid (60)
Body weight (g)	396 ± 62.12	310 ± 38.72	343 ± 41.43	354 ± 27.33	352 ± 18.18
Heart weight (mg)	940 ± 67	936 ± 164	963 ± 111	923 ± 90	896 ± 110
Heart/body weight (mg/g)	2.6 ± 0.1	3.1 ± 0.2	2.7 ± 0.3	2.6 ± 0.1*	2.7 ± 0.1
Left ventricular systolic pressure (LVSP) (mmHg)	112 ± 32	101 ± 5$^{\#}$	107 ± 8	105 ± 10	101 ± 13
LV end-diastolic pressure (LVEDP) (mmHg)	2.9 ± 0.67	13.2 ± 7.5$^{\#}$	9.65 ± 1.6	8.2 ± 2.0	13.1 ± 4.8
$+dP/dt$ max (mmHg)	5649 ± 1860	2564 ± 131$^{\#}$	3407 ± 239*	3083 ± 92	3232 ± 378

3.2. Dose-Dependent (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid Protection against Oxidative Stress in Failing Hearts. The antioxidant effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid were implicated by the enhanced activities of CAT, SOD, and GSH-Px and the levels of GSH in the serum, as well as the protein levels of CAT, SOD-1, and GPx in the left ventricle (Figures 1(a)–1(e)). On the other hand, (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment reduced the levels of serum MDA in a dose-dependent manner (Figure 1(f)). In addition, (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid also increased the total antioxidant capacities and in parallel decreased the lipid peroxidation levels in the serum and left ventricle in a dose-dependent manner. The GSH content in the (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment (15, 30, and 60 mg/kg) group was lower than that in the control group ($P < 0.05$). After treatment with three different concentrations of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment, GSH levels were higher in the treatment group than in the MI group ($P < 0.05$). The higher the concentration of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid (15, 30, and 60 mg/kg), the higher the GSH concentration was. In addition, the levels of GSH-Px and GST were higher in the treatment group than in the MI group. Third, the levels of many additional enzymes, including CAT, MDA, and SOD, were also significantly different in the treatment group compared with the MI group. The CAT levels in the treatment group were significantly higher than those in the model group ($P < 0.05$). Similarly, the SOD levels were similar to the CAT levels. Lastly, the MDA concentration in the treatment group of HF rats was lower than that detected in the MI group. The posttreatment values for GSH, GSH-Px, CAT, SOD, and MDA all implied that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid had the ability to protect antioxidant effects from a failing heart.

3.3. (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid Attenuates Inflammatory Cytokine Expression in Failing Hearts. The protective effect of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid was evaluated by analyzing the expression levels of proinflammatory genes in the peri-infarct area using RT-PCR. In the MI group, an upregulation in TNF-α mRNA levels was observed compared with the sham group. This effect was partially reversed by (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment, especially at the 15 mg/kg dose (Figure 2(a)). Similarly, a significant increase in IL-1β mRNA expression was detected in the MI group, which was attenuated by treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid at a 15 mg/kg dose (Figure 2(b)). These data suggested that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid is a potent inhibitor of the inflammatory process.

3.4. (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid Reduces Cardiomyocyte Apoptosis and Increases Akt Phosphorylation in Failing Hearts. We next determined the inhibitory effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid on cardiomyocyte apoptosis. We measured the protein and mRNA expression levels of several apoptosis-related molecules in the peri-infarct area. RT-PCR revealed that the expression level of Bcl-2 was increased in the MI group and the (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated group compared with the sham operation group. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment resulted in a significant increase in Bcl-2 mRNA in the treatment group compared with the MI group (Figure 3(a)). In contrast, the mRNA levels of the proapoptotic molecules Bax, caspase-3, and caspase-9 were significantly reduced in the (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated group compared with the MI group (Figure 3(b)). Western blot analysis confirmed the RT-PCR results. Indeed, a significant increase in Bcl-2 protein levels and a reduction in Bax protein levels were detected in the (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated group compared with the MI group (Figures 3(a) and 3(b)). Moreover, these changes appeared to be associated with an increase in the phosphorylation of Akt in the MI and (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated animals.

Western blot analysis revealed that Akt was activated in the hearts of MI animals after 6 weeks. In the (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid-treated group, a significant increase in the phosphorylation of Akt was observed compared with the MI group (Figure 4). The combined findings of this study indicate that the activation of Akt mediated the expression of apoptosis-related molecules.

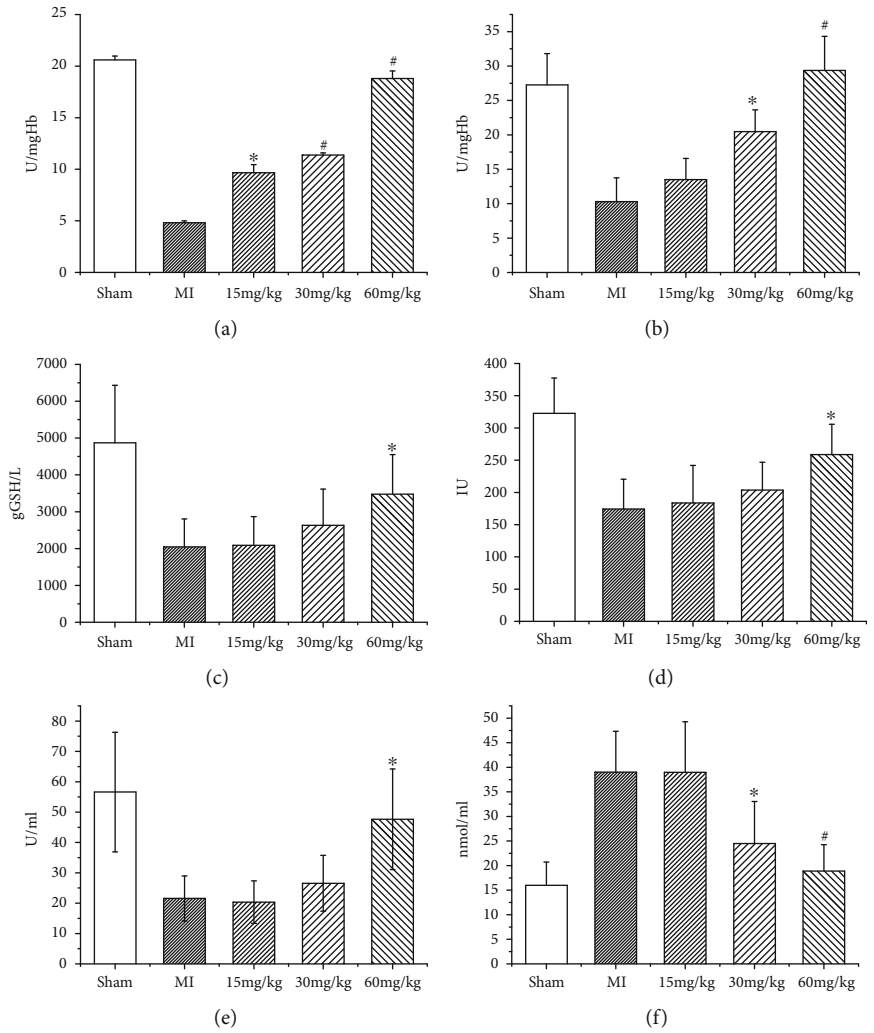

Figure 1: The antioxidant effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid were implicated by the enhanced activities of catalase (CAT) (a), superoxide dismutase (SOD) (b), glutathione (GSH) (c), glutathione peroxidase (GSH-Px) (d), and GST and the levels of glutathione S-transferase (GST) (e) in the serum. On the other hand, (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment reduced levels of serum malondialdehyde (MDA) (f) in a dose-dependent manner. $^{\#}P < 0.05$ versus sham, $^{*}P < 0.05$ versus MI, and $n = 6$ in each group.

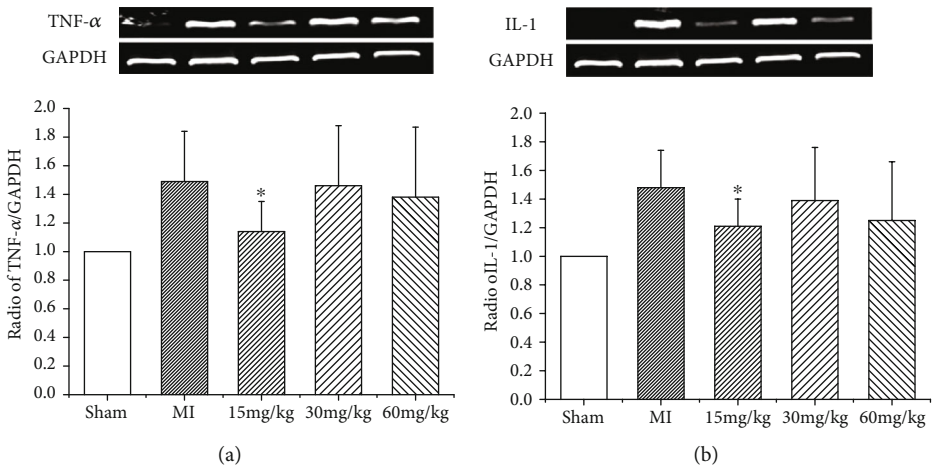

Figure 2: Expression of TNF-α and IL-1β mRNA. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment (15, 30, and 60 mg/kg) reduces inflammatory cytokine expression in failing hearts. (a) TNF-α mRNA level. (b) IL-1β mRNA level. Values are expressed as mean $\pm$ SD, $^{*}P < 0.05$ versus MI. $n = 6$ in each group.

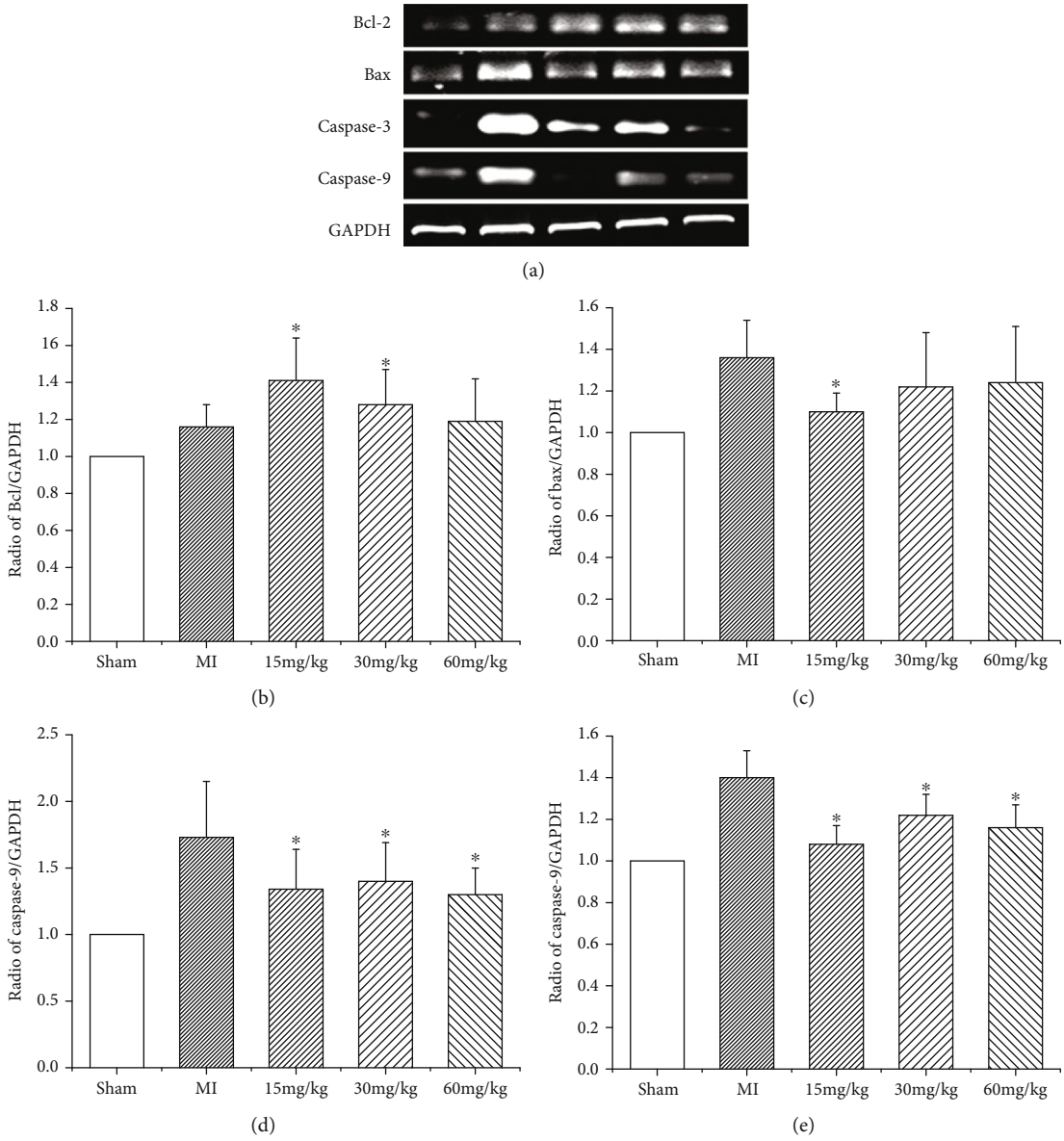

Figure 3: (a) Expression of Bcl-2, Bax, caspase-3, and caspase-9 mRNA. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment (15, 30, and 60 mg/kg) reduces the mRNA expression levels of apoptosis-related molecules in heart tissue. (b–e) Relative Bcl-2, Bax, caspase-3, and caspase-9 mRNA levels. Values are expressed as mean ± SD, $^*P < 0.05$ versus MI. $n = 6$ in each group.

3.5. (Z)-2-Acetoxy-3-(3,4-Dihydroxyphenyl) Acrylic Acid Reduces Adverse Ventricular Remodeling. Six weeks after MI, collagen deposition was detected by measuring the expression levels of TGF-β and collagen type I mRNA in the noninfarct area. RT-PCR revealed that TGF-β and collagen type I mRNA were significantly upregulated in the MI group. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment could reduce the expression of these two molecules, although the differences were not statistically significant (Figure 5).

4. Discussions

Our data indicated that treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid improved cardiac function, particularly by increasing *dP*/*dt*, an important marker of left ventricular function in chronically failing hearts. Among the doses of 15, 30, and 60 mg/kg, using the lowest dose (15 mg/kg) provided the best protective effects. Although doses lower than 15 mg/kg were not used, the present results suggested that treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid at lower doses may provide better benefits. Thus, the optimal dose remains to be determined.

The pathological mechanism of myocardial infarction is multifactorial. Previous studies have shown that a large number of reactive oxygen species are generated; studies also have confirmed that the production of a large number of oxygen free radicals after ischemia is one of the main mechanisms of cardiomyocyte injury. Our results show that (*Z*)-2-

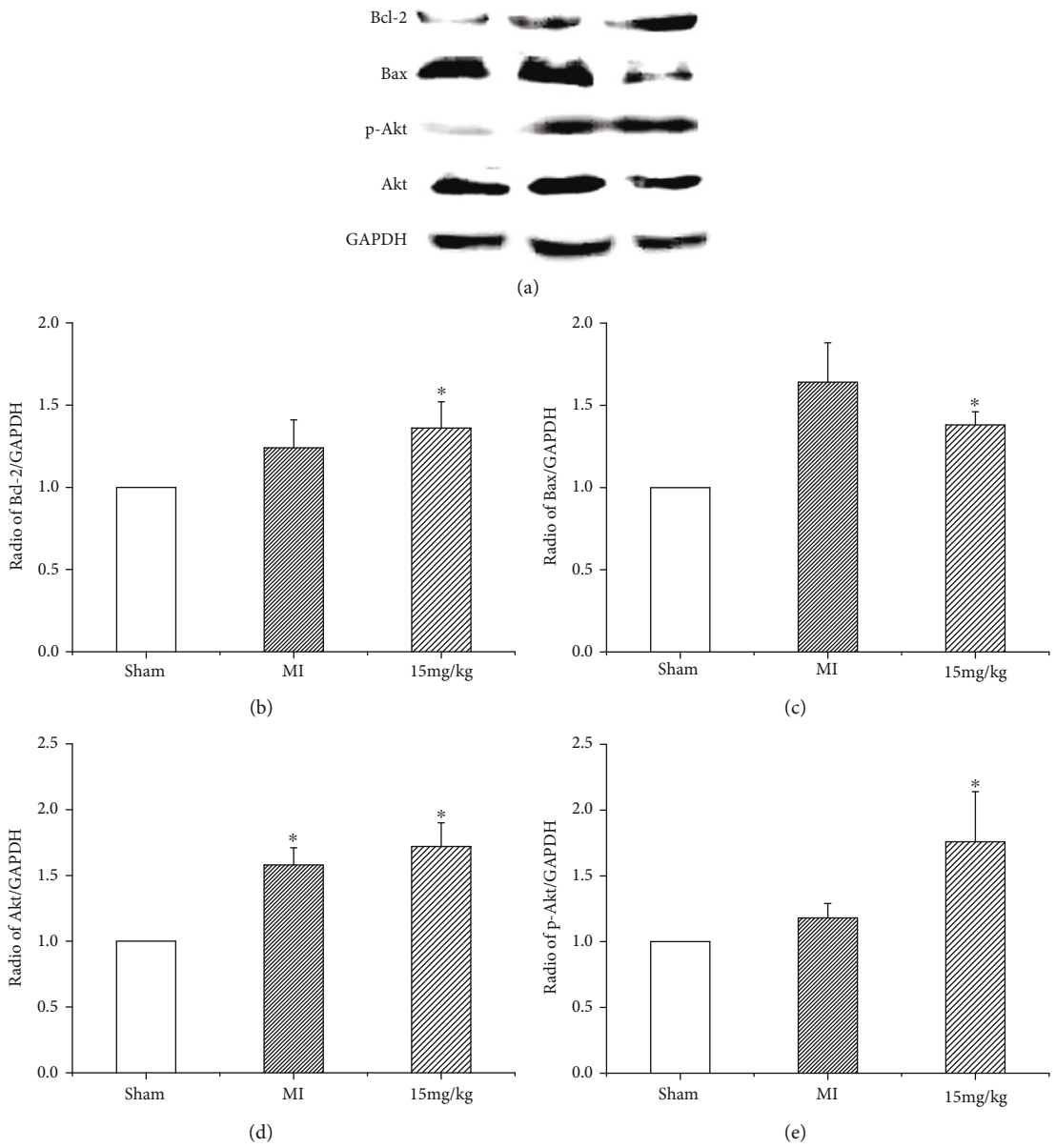

Figure 4: (a) Expression of Bcl-2, Bax, Akt, and p-Akt protein. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment reduces the protein expression levels of apoptosis-related molecules in failing hearts. (b–e) Representative Western blots of Bcl-2, Bax, Akt, and p-Akt protein levels. Values are expressed as mean ± SD, $^*P < 0.05$ versus MI. $n = 6$ in each group.

acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid can reduce the leakage rate of LDH, reduce the production of MDA, inhibit the expression of apoptosis-related factors, and show good myocardial protection. Those endogenous antioxidant enzymes, SOD and catalase, can detoxify reactive oxygen species and thus rescue cells from oxidative damage. The protective effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid appear to be associated with the attenuation of inflammatory cytokine expression and the inhibition of myocardial apoptosis. Expression of the TNF-α and IL-1β genes was significantly reduced. It is known that inflammatory responses and cytokine release play an active role in heart damage following myocardial infarction [20, 21]. A large number of reports have demonstrated that the expression of proinflammatory cytokines is directly related to the degree of heart failure and inversely related to survival [20, 22]. Indeed, results obtained from several animal studies and some clinical trials suggest that suppression of inflammatory cytokines may improve cardiac performance [23]. Interestingly, studies from Kurrelmeyer et al. demonstrated that transgenic mice with knocked-out TNF receptors showed an increase in apoptosis induced by acute coronary occlusion, suggesting a protective TNF-α effect in the myocardium [24]. However, the protective role of TNF-α in heart failure models remains unclear. In our study, treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid significantly improved dP/dt, which was related to the reduction of the gene expression levels of TNF-α and IL-1β. It is conceivable that (*Z*)-2-

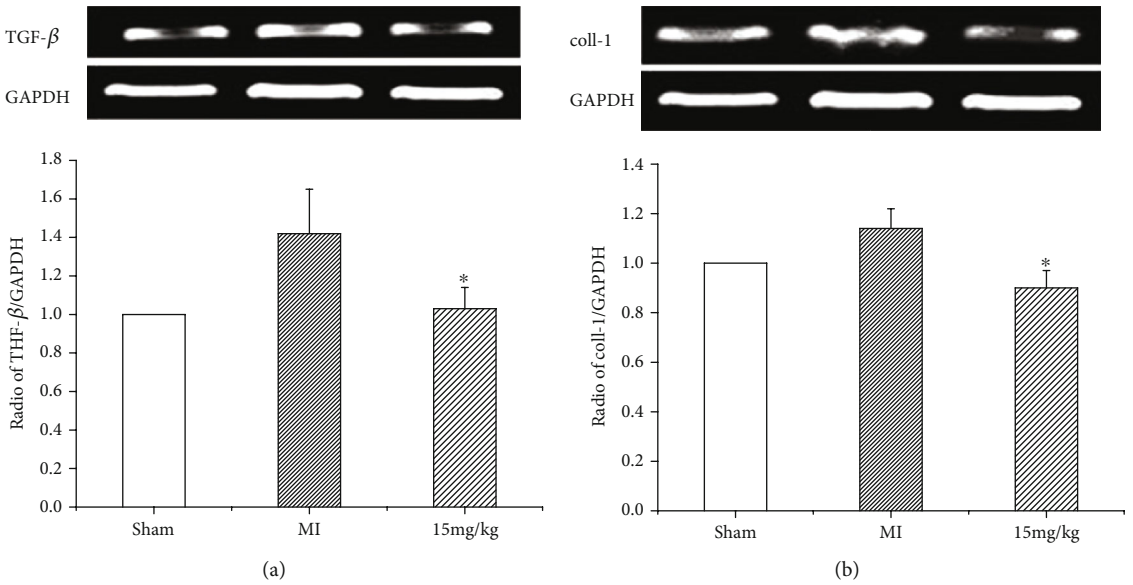

Figure 5: (a) Expression of TGF-β mRNA. (Z)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid reduces adverse ventricular remodeling in failing hearts. (b) Expression of collagen type I mRNA. (Z)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid reduces adverse ventricular remodeling in failing hearts. Values are expressed as mean ± SD, $^*P < 0.05$ versus MI. $n = 6$ in each group.

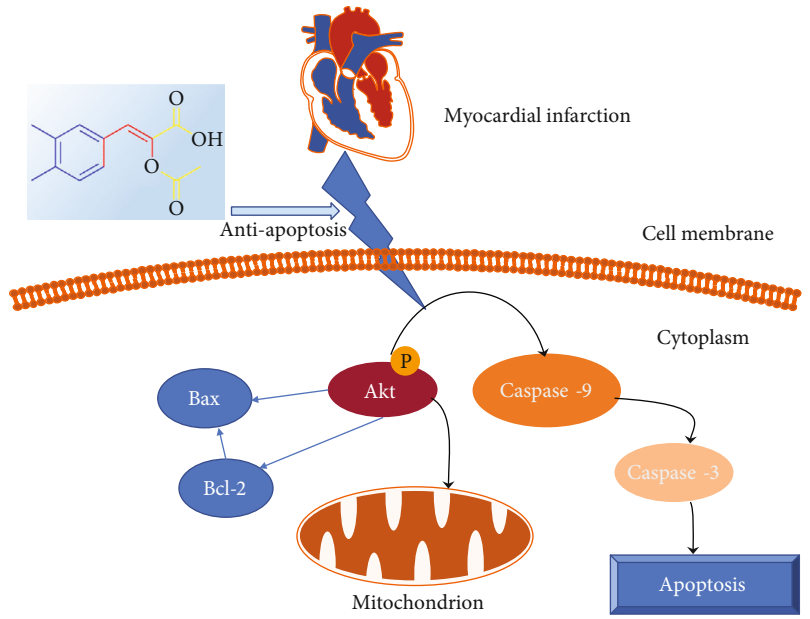

Figure 6: Potential mechanisms involved in the cardioprotective effect of (Z)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid by inhibition of apoptosis in cardiomyocytes.

acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid plays a beneficial role in cardiac repair after MI by significantly reducing the inflammatory response in the infracted myocardium.

In addition to the potent anti-inflammatory effects, the protective effects of (Z)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid were supplemented by inhibiting cell apoptosis via the promotion of Akt phosphorylation and modulation of the gene expression of apoptosis-related molecules. Cytotoxic cytokines and tissue-damaging agents are two major inducers of apoptosis. Cytotoxic cytokines induce apoptosis by binding to their receptors. Activation of these receptors causes activation of caspase-8, which can then activate other caspases as well as the proapoptotic members of the Bcl-2 family. The activated caspase-8 leads to the release of cytochrome c from the mitochondria to the cytoplasm, which activates caspase-3, leading to inevitable cell death [25]. Activation of caspase-3 has been reported in the heart tissues of a number of species during end-stage heart failure, including humans, sheep, and rabbits [26]. Several studies have demonstrated the feasibility of suppressing caspases to prevent heart failure. IDN-1965, a small molecule nonpeptide caspase inhibitor, inhibits a broad spectrum of

caspases and has been shown to be effective in inhibiting apoptosis and liver injury induced by cytokines, suggesting a new basis for developing pharmaceutical agents against heart failure [27]. In our study, treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid reduced the mRNA expression levels of caspase-3 and caspase-9, indicating (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid may be a potent caspase inhibitor.

It is well known that an imbalance of proapoptotic versus prosurvival members in the Bcl-2 family plays an important role in apoptosis of various cell types [28]. The prosurvival factors Bcl-xL and Bcl-2 have been observed in patients with end-stage heart failure and in cultured cardiomyocytes upon exposure to cytotoxic cytokines [29–31]. The proapoptotic factor Bax has also been found in cultured ventricles in a chronic pressure-overloaded rabbits' heart model [32]. Although Bax gene expression was not found to be altered in failing human hearts, there is much evidence supporting the role of the Bcl-2 family in apoptosis. For example, the overexpression of Bcl-2 or Bcl-xL prevents mitochondrial membrane permeability transition and Bax-mediated release of cytochrome c [33]. Our study demonstrated that Bcl-2 and Bax gene expression increased in the MI group six weeks after surgery. (*Z*)-2-Acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment significantly decreased Bax gene expression and further improved mRNA expression of Bcl-2, which is associated with improved heart function. Akt is a key modulator in the apoptosis survival pathway. Increased Akt/GSK-3β phosphorylation plays an important role in promoting cell survival. Akt inactivates GSK-3β and then blocks cytochrome c release and caspase-3 activation. Our results indicated that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid increased phosphorylated Akt levels compared to the untreated animals in the MI group, further indicating that the protective effect of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid was achieved by inhibiting myocardial cell apoptosis.

Ventricular remodeling is another characteristic of heart failure. Many studies have shown that failure to prevent cardiomyocyte apoptosis and suppression of proinflammatory cytokines are involved in mediating cardiomyocyte hypertrophy and ventricular remodeling. Our study indicated that treatment with (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid decreased TGF-β and collagen type I gene expression, which was not the result that we expected. This phenomenon may be associated with differences between animal species of animals or sample amount. Our next study will further confirm the effects of (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid on other animal models, such as murine, rabbit, or porcine models.

5. Conclusions

The present study demonstrates that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid treatment was useful in treating post-MI heart failure by preferentially modulating the local inflammatory response and inhibiting myocardial apoptosis as evidenced by decreasing TNF-α, IL-1β, caspase-3, and capase-9 expression levels. In addition, the treatment improved outcomes by modulating the Akt prosurvival pathway. Taken together, these results suggest that (*Z*)-2-acetoxy-3-(3,4-dihydroxyphenyl) acrylic acid may be a potent chemical agent that can be used to protect failing hearts (Figure 6).

Data Availability

The Cardiac parameter changes data, enhanced activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and the levels of glutathione (GSH) in the serum, the protein and mRNA levels of signals in the left ventricle used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

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Review Article

Is Extra Virgin Olive Oil an Ally for Women's and Men's Cardiovascular Health?

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Noncommunicable diseases are long-lasting and slowly progressive and are the leading causes of death and disability. They include cardiovascular diseases (CVD) and diabetes mellitus (DM) that are rising worldwide, with CVD being the leading cause of death in developed countries. Thus, there is a need to find new preventive and therapeutic approaches. Polyphenols seem to have cardioprotective properties; among them, polyphenols and/or minor polar compounds of extra virgin olive oil (EVOO) are attracting special interest. In consideration of numerous sex differences present in CVD and DM, in this narrative review, we applied "gender glasses." Globally, it emerges that olive oil and its derivatives exert some anti-inflammatory and antioxidant effects, modulate glucose metabolism, and ameliorate endothelial dysfunction. However, as in prescription drugs, also in this case there is an important gender bias because the majority of the preclinical studies are performed on male animals, and the sex of donors of cells is not often known; thus a sex/gender bias characterizes preclinical research. There are numerous clinical studies that seem to suggest the benefits of EVOO and its derivatives in CVD; however, these studies have numerous limitations, presenting also a considerable heterogeneity across the interventions. Among limitations, one of the most relevant in the era of personalized medicine, is the non-attention versus women that are few and, also when they are enrolled, sex analysis is lacking. Therefore, in our opinion, it is time to perform more long, extensive and lessheterogeneous trials enrolling both women and men.

1. Introduction

The Mediterranean diet (MedDiet) includes high consumption of legumes, cereals, fruits, and vegetables; moderate fish and wine consumption; and low consumption of red meat ([1] and cited literature). The MedDiet also includes the consumption of 25–50 ml/day of extra virgin olive oil (EVOO), which seems to have health benefits [2, 3].

Cardiovascular diseases (CVD) are the main cause of deaths, accounting for >17 million deaths annually [4]. The beneficial effect of MedDiet on CVD is suggested by several randomized clinical trials, although some recent papers stated that the evidence is still uncertain [5, 6]. For example, the Oslo Diet-Heart Study and the Finnish Mental Hospital Study [7–9] tested the effectiveness of low-cholesterol diets, enriched in polyunsaturated fatty acids, showing a decrease in coronary heart diseases (CAD) and blood cholesterol (Chol). Moreover, the Seven Countries Study, enrolling 11,579 middle-aged men from eight nations of seven Mediterranean and non-Mediterranean countries, shows a lower mortality from ischemic heart disease (IHD) in Mediterranean populations compared to those of Northern Europe and America [10]. PREDIMED study proves that EVOO is linked to lower risk of cardiovascular (CV) events [11]. However, a Cochrane Systematic Review proves that elevation in polyunsaturated fatty acids (PUFA) assumption has a small effect, if any, on all-cause mortality or CV deaths although it slightly decreases Chol and probably triglycerides

(TG), leaving practically unaltered high density lipoprotein (HDL) [12].

Beneficial effects of EVOO are also associated with the presence of minor polar compounds (MPCs) that have antioxidant, anti-inflammatory, anti-aggregating, and antimicrobial activities and regulate serum insulin/glucose response [13–21]. A claim of the European Food Safety Authority (EFSA) declared that "consumption of olive oil polyphenols contributes to the protection of blood lipids from oxidative damage" at a daily dose of 5 mg of hydroxytyrosol (HTyr) and its derivatives (e.g., oleuropein complex and tyrosol) [22].

Actually, botanicals are largely used [23, 24], especially by women [25, 26], but rigorous findings regarding their efficacy and safety profiles are still lacking [27]. Besides, the influence of sex on botanicals including EVOO, VOO, OO, and MPCs is also lacking; nevertheless, the individual's sex and gender is one of the most important modulators of CV health [28–39] and the numerous sex and gender differences at CV level are summarized in Table 1. Previously, we reviewed the sex-gender effect on polyphenols of various origins [25, 26]; here we focus on EVOO and its MPCs because, as already mentioned, EFSA declares their utility in ameliorating low-density lipoproteins (LDL) oxidation and their importance in MedDiet [22].

2. MedDiet and Sex Differences

The Mediterranean Region includes about 20 nations with different ethnic, historical, and cultural backgrounds; religions (Muslims, Orthodox Christians, Catholic Christians, Jews); and economic status [56], and the UNESCO declared that MedDiet is an intangible cultural heritage [57]. Importantly, MedDiet also includes social aspects (social integration) and a peculiar way of life (sleeping and nutrition) that may play a role in reducing age-related diseases [58, 59]. However, the transferability of the benefit of MedDiet outside of Mediterranean Region decreases the importance of social aspects [60, 61]. In particular, it has been found that, in US women who are adherent to MedDiet, the CV risk reduced by about 25% over 12 years, having a reduction in myocardial and cerebral infarcts and vascular death [62].

Mediterranean populations have the lowest prevalence of chronic inflammatory disease and have very high life expectancy [63]. Actually, adherence to this diet is decreased [56] nevertheless many authors declare that adherence to the MedDiet has beneficial effects on diabetes mellitus (DM), obesity, and CVD [11, 64–70].

High adherence to the MedDiet reduces the overall mortality [71–73] and the risk of CVD (10%) and neoplastic diseases (4%) [71]. Adherence to MedDiet induces small favorable changes in some risk factors for CVD, but its effect on hematic lipids is generally weak [74]. Low-carbohydrate MedDiet reduces glycosylated haemoglobin (HbA1c) levels and delays the use of oral antidiabetic drugs when compared with a low-fat diet [75–77]. Recently, it has been shown that MedDiet can influence the genetics. However, there is not univocal data on health benefits [5, 6]. Importantly, investigating the rs7903146 polymorphism in the transcription factor 7-like 2 gene, Corella and coworkers [78] proved that in the homozygotes the hypercholesterolemia and hypertriglyceridemia are reduced by MedDiet.

Low adherence to MedDiet and smoking are independent predictors of 10-year CV events in women and in men, respectively [79]. The adherence to the MedDiet, nonsmoking, normal weight, and regular physical activity reduce the mortality in men and in women, but the statistical significance is reached only in women [72, 73, 80]. However, the response to the MedDiet seems to be greater in men than in premenopausal women when cardiometabolic changes are considered [81–84]. MedDiet ameliorates plasma lipid profile and diastolic blood pressure (DBP) without impacting on leptin levels and the leptin-to-adiponectin ratio in both sexes [84]. Only in men, it ameliorates the insulin homeostasis and redistribution of LDL subclasses from smaller to larger LDL, while an opposite trend is observed in women [81]. Finally, MedDiet increased telomere length, a marker of biological age, in women [85], although no consensus is found about this effect [86]. Finally, men are less adherent to MedDiet than women [87].

3. EVOO, VOO, OO, and MPC

OO is produced from the fruits of *Olea europaea* L. evergreen trees, a plant cultivated worldwide, but it is typical cultivation of the Mediterranean area [88]. It mainly contains monounsaturated fats (98-99% of total weight of EVOO), such as oleic acid, followed by a low amount (1-2%) of phenols, phytosterols, tocopherols, and squalene [89]. Importantly, in EVOO only, fatty acids are stabilized by MPCs, with antioxidant activities [90].

EVOO composition and concentration in MPCs are extremely variable either qualitatively or quantitatively (200–600 mg/kg) [91]. MPCs are dependent on the tree cultivar, the climate, growing, and production procedure [92]. The phenolic cluster of EVOO can be subdivided into several subclasses. In particular, EVOO contains saponifiable compounds (triacylglycerol, partial glycerides, esters of fatty acids or free fatty acids, and phosphatides) and unsaponifiable compounds (hydrocarbons (squalene), phytosterols (β-sitosterol, stigmasterol, and campesterol), tocopherols, carotenoids, pigments (chlorophylls), aliphatic and triterpenic alcohols, triterpenic acids (oleanolic acid), volatile compounds, and polyphenols) [93].

In general, secoiridoids are the most representative followed by phenolic alcohols such as Tyr and HTyr, flavonoids, lignans, and phenolic acids [89, 92]. In general, HTyr, Tyr, and conjugated forms of secoiridoids like oleuropein (which are hydrolyzed to HTyr and Tyr in the stomach) are the most representative [94]. HTyr also originates by the hydrolysis of oleuropein during olive ripening or/and during the storage and elaboration of table olives [95]. It can be found in a free form, such as acetate form, or as part of oleacein, verbascoside, and oleuropein [93]. Also ligstroside, oleacein, and oleocanthal are sources of HTyr and Tyr [96].

Table 1: Examples of sex and gender differences in CVD and risk factors.

Diseases or risk factors	Sex differences	References
Myocardial infarction	Women are 10 years older than males and have higher mortality in younger ages and have more atypical symptoms. Women have less anatomical obstructive CAD than men; it is estimated a 20% or greater excess of normal or nonobstructive arteries in women vs men	[40–42]
Heart failure	Lower incidence in women but the prevalence is similar in both sexes, with diastolic heart failure being more common in women. Lower mortality rate in women than in men	[40, 41]
Hypertension	Lower incidence in premenopausal women	[40]
Cardiac hypertrophy	Premenopausal women are better protected than men; men have more cardiac hypertrophy	[40, 43]
Ischemia-reperfusion injury	Studies evidenced that females have lower ischemia-reperfusion injury	[40]
Diabetes	Higher increased risk of CVD in women vs men	[40]
Endothelial dysfunction	More frequent in women vs men	[44, 45]
HDL	Higher levels in women vs men; the difference declines with age	[46]
TG	Higher increased risk of CVD in women vs men. In women, they increase after menopause	[47]
Chol	Levels rise in menopausal transition period	[47]
LDL	Levels rise in menopausal transition period	[46]
Lp (a)	Levels rise in menopausal transition period	[46]
Smoking	Less women smoke vs men, but smoking has more negative effects on women	[48]
Social economicus status	In women, it is inversely associated with increased risk of CAD, stroke, and CVD. In particular, for CHD, it is associated with lower education	[49]
Psychological factors	Women had higher contributions from psychosocial risk factors (45.2% vs 28.8% in men)	[50, 51]
Unique for women		
Gestational diabetes, pre-eclampsia, syndrome of polycystic ovary	Higher increased risk of CVD in women	[48, 52]
Oral contraceptives	A large cohort study (1.6 million of women, 15 to 49 years old) shows that ethinylestradiol (20 μg or 30 to 40 μg) is associated with an increased risk of MI. The risk is not significantly varied by progestin	[53]
	OC should not be prescribed for women over the age of 35 years and smokers (American College of Obstetricians and Gynecologists) and should be prescribed with caution in case of CV risk factors such as hypertension, diabetes, and dyslipidemia	[54]
Hormone replacement therapy	A large cohort study shows that ethinylestradiol is associated with an increased risk of MI that is not significantly changed with progestins	[55]

Some of MPCs such as HTyr, Tyr, and their secoiridoid derivatives (oleuropein, oleuropein aglycone, and elenolic acid dialdehydes) are hydrophilic [97], while other MPCs are lipophilic [89]. Lignans belong to the family of phytoestrogen [98] and in general the predominant lignan is (+)-1-acetoxypinoresinol [98]. The leaves of the *Olea Europaea* L. contain higher concentrations of phenols than the olive fruit and derived oils [99–101]. The predominant MPCs in the leaves are verbascoside, apigenin-7-glucoside, luteolin-7-glucoside, HTyr, Tyr, and oleuropein [102]. Notably, a single MPC may possess distinct biological activity [103, 104]. Thus, it is impossible to extrapolate the result of the single EVOO, VOO, and OO to another. For example, Chetoui and Blanqueta cultivars (rich in linoleic acid) induce higher total triacylglycerol (TAG) incorporation into THP-1 cells than Buldiego and Picual (rich in oleic acid), promoting foam cells formation [104]. Further, extracts of Taggiasca and Seggianese, which have different amounts and composition of MPCs, have a different antioxidant activity being higher in Seggianese extract [103].

4. Pharmacokinetics of MPCs and Influence of Sex

The influence of sex and gender on pharmacokinetics of phenols was recently reviewed [25]. Briefly, in humans, MPCs are well adsorbed (~40%–95%, using HTyr and Tyr as proxy) [105, 106]. It is important to recall here that, in humans, there is an endogenous synthesis of HTyr during the metabolism of dopamine with its formation being favored by ethanol [107]. In addition, HTyr is a product of oleuropein hydrolysis that can occur in the stomach. Besides, gut microbiota generates HTyr from oleuropein [108].

In the intestinal tract (both ileum and colon), more than 40% of HTyr is absorbed by bidirectional passive transport [108], which depends on numerous factors such as food matrix or vehicle. The absorption of HTyr and Tyr is higher when administered as an OO solution than as aqueous solution [108]. In the gastric and intestinal tract MPCs are hydrolyzed [109], with some exceptions. In particular, oleuropein is degraded by the colon microbiota to HTyr that is then absorbed [109]. HTyr bioavailability seems to be

influenced by sex [110]. The maximum plasmatic concentration of HTyr is reached 5–30 min after administration of EVOO and VOO [108]. HTyr and its derivatives cross the blood brain barriers [111]. Finally, HTyr is incorporated in HDL, which is higher in women than in men [108].

HTyr and Tyr are extensively metabolized by phase I enzymes, such as CYP2D6 and CYP3A4, and by phase II enzymes both at intestinal and hepatic levels [108, 112]. Numerous phase I and II enzymes present numerous sex differences both in animals and in humans [33]. Thus, the metabolism of MPCs can be sex divergent at least in rats [110]. In humans, the biotransformation of HTyr and Tyr mainly occurs through glucuronidation and sulphation, and the main circulating metabolites are both HTyr sulfate and HTyr acetate [108]. HTyr is also metabolized by catechol-O-methyl transferases that are more expressed in men than in women [33] forming 3-hydroxy-4-methoxyphenyl ethanol (homovanillyl alcohol) [113]. Globally, HTyr and Tyr have lower bioavailability than their metabolites [107]. Inside the cells, the conjugated forms can be deconjugated and thus HTyr and Tyr metabolites can be reformed. Finally, the intestinal microorganisms metabolize HTyr into hydroxylated phenylacetic acid, acetic acid, and benzoic acid [114]. In plasma and urine, 98% of HTyr is recovered as glucuronide form and only 2% is free [115]. Usually, the complete elimination of HTyr and metabolites occurs approximately in 4 and 6 h in rats and humans, respectively [116]. HTyr is mainly excreted by the renal route where it is present both in conjugated and nonconjugated form [108]. Urinary HTyr levels (adjusting for ethyl glucuronide) are higher in men than in women [107]. In addition, through the biliary route they reach the small intestine where they can be retransformed and reabsorbed [116]. Despite the enterohepatic recycling, a small amount (about 5%) of total HTyr is excreted by feces [116] and the consumption of MPC-rich OO elevates the free HTyr levels in feces of men [114]. Notably, Tyr, HTyr acetate, 3,4-dihydroxyphenylacetic acid, and homovanillyl alcohol administration changes urinary excretion of catecholamines (dopamine, normetanephrine, norepinephrine, and 3-methoxytyramine) in male and female rats, with the excretion being significantly higher in male than in female rats [110].

Oleocanthal constitutes about 10% of the olive's MPCs (100–300 mg/kg EVOO) [117]. Oleocanthal, as other MPCs, is stable at acid pH and at 37°C and it is biotransformed by phase I and II enzymes, with glucuronidation being the prevalent way [117]. Oleocanthal and other secoiridoids and their metabolites are mainly eliminated by renal route and they are found in human urine 2–6 h after the intake [117].

Little and nonunivocal data are available on sex influence on bioavailability of chlorogenic acids ([118] and cited literature) and lignans. After long flaxseed lignan secoisolariciresinol diglycoside exposure, female rats have higher lignan concentrations in heart and thymus than male rats [119]. A strong association between dietary lignan intake and prevalent obesity exists only for boys [120].

Importantly, pharmacokinetic interactions with other botanicals and prescription drugs have been described. For example, bioavailability of HTyr is enhanced when co-administered with the thyme extracts [121].

Considering the role of gut microbiota in sex healthcare paradigm [122, 123] and their ability to expand metabolic activity of the host [124], it important to recall that they could be a modifier of the activity and kinetic of all compounds present in olive and leaves and other matrixes [125]. In turn, OO derivatives may influence the gut microbiome. For example, the dialdehydic form of decarboxymethyl oleuropein aglycone, oleocanthal, HTyr, and Tyr may inhibit the growth of bacteria [126], including the beneficial ones [127]. Sex-gender differences in the microbiota are recently reviewed by Kim et al. [128]. Here, it is important to recall that microbiota modifications may participate in the pathophysiology of CVD [129]. For example, some metabolites of gut microbiota such as short-chain fatty acids and trimethylamine N-oxide may participate in the modulation of blood pressure through G protein receptors [129]. Further gut microbiota may inhibit HDL-coordinated reverse cholesterol transport [129].

Globally, the effects of MPCs on microbiota appear to be compound and sex specific, and in consideration of sex differences that characterize the human microbiota, its effects on MPC fate and activity should be accurately studied.

5. Effect of EVOO, VOO, OO, Leaf Extracts, and MPCs on Endothelial Dysfunction: Influence of Sex

Endothelial function is a barometer of vascular health [130] and it is a predictor and a pathogenic mechanism of atherosclerosis [131], being also related to the prognosis and severity of CVD [50, 132]. Endothelial dysfunction is more precocious than atherosclerotic plaques and it is a more prominent risk factor in women than in men (Table 1). It is related to oxidative stress, inflammation, platelet activity, an alteration of glucose metabolism, and uric acid levels [133–136], and all these processes present sex differences [34, 136–140].

5.1. Effect of EVOO, VOO, OO, Leaf Extracts, and MPCs on Oxidative Stress: Influence of Sex. The influence of sex on oxidative stress is widely reviewed [34, 137]. However, no univocal results are obtained and this could depend on species, tissues, and cells used and on donor age. For example, Brunelli et al. [141] report no differences in the plasma antioxidant barrier, although women present a higher oxidative status than men. Moreover, they suggest that premenopausal and postmenopausal women are similar [141]. By contrast, Vassalle and coworkers [47] report that menopause is a condition that elevates oxidative stress. Further, young men have lower levels of malondialdehyde (MDA) in comparison to fertile women and older men [142]. After correction for body weight (BW), both pre- and postmenopausal women have higher amounts of carbonylated proteins *vs* men of similar age [142]. Others show that lipid and protein oxidation are increased in peri- and postmenopausal women, whereas superoxide dismutase (SOD) and catalase (CAT) activities are decreased and increased in postmenopause and in perimenopausal women, respectively [143]. Glutathione (GSH) and glutathione

peroxidase (GPx) are lower in women aged 32–39 years than in women aged 20–25 years. Meanwhile, 20–25-year old men have higher GSH and lower glutathione disulfide (GSSG) than women of the same age. The SOD and CAT activities are higher in women aged 32–39 years than in men and women of younger age [144]. Moreover, women with CAD seem to have higher oxidative stress than men [145]. Another study shows that African American women with symptomatic peripheral artery disease produce more ROS than men, while Caucasian men and women do not diverge indicating that ethnicities could play a role in sex and gender differences [146–150]. Others report the opposite trend and others do not find any significant sex difference [151–153].

The antioxidant activity of EVOO, VOO, and MPCs is extensively reviewed [154, 155] (Table 2). It is based on their scavenger, chain breaking, and chelating activities [116]. Moreover, they favor the resistance over oxidation [266]. High dose of oleuropein and HTyr may exert prooxidant activity [267, 268], and this paradoxically could be one of the mechanisms of their antioxidant activity because it can activate the translocation of nuclear factor E2-related factor 2 (Nrf2) to the nucleus [269] in a sex-specific manner [270, 271] that leads to modifications of proteins expression and activity such as γ-glutamylcysteine ligase, which is expressed less in female rat livers than in male ones [272]. After trauma and hemorrhage, HTyr elevates liver Nrf2 modulating heme oxygenase-1 (OH-1) especially in rat females (proestrous phase) compared to males [273]. Through Nrf2, MPCs can also activate phase II detoxifying enzymes and mitochondrial biogenesis, two critical pathways in reducing the negative effect of oxidative stress [271]. Oleuropein and HTyr seem to be scavengers of HOCl [274], which starts LDL lipid peroxidation and oxidizes the apolipoprotein (Apo) B-100 [275]. However this is not a univocal result [213]. Finally, in animals and in humans, HTyr may interact with several microRNAs [218, 276] that regulate numerous cellular function including DICER function that is relevant to the redox state [277, 278].

5.2. Effect of EVOO, VOO, OO, Leaf Extracts, and MPCs on Inflammatory Response: Influence of Sex. The effect of sex on inflammatory response has been recently reviewed [138, 139, 279]. Women and men have a different immune system [281] and arachidonic acid (AA) cascade [281]. This last generates numerous compounds with proinflammatory and anti-inflammatory activities. Interestingly, females seem to be protected against endothelial dysfunction induced by systemic inflammation [282]. In particular, COX2 and COX1 female knockout mice have less inflammatory edema and joint destruction than male mice [283]. Consistently, expression of COX2 is more elevated in male than in female cells [284]. More PGE_2 is produced by human male neutrophils *vs* female ones [284]. In male coronary rat arteries, $PGF_2\alpha$ exerts a major contraction in male arteries than in female ones for the presence of more PG receptors [285]. Also the lipoxygenase (LOX) system presents some sexual dimorphism. 5-LOX and its 5-lipoxygenase-activating protein (FLAP) are downregulated by androgens [286]. Thus, the bigger production of leukotrienes in monocytes and neutrophils of women is not surprising [286]. In human neutrophils and monocytes, the synthesis of lipoxin A_4 (LXA_4), a proresolving molecule [287], is reduced by estradiol [281]. Further, a positive and a negative correlation exist between age and aspirin triggered 15-epi-LXA_4 in women and men, respectively [288]. Resolvins, protectins, and maresins activities may be influenced by sex [289]. For example, D-resolvin is higher in women exudate whereas chemoattractant leukotriene B_4 is higher in men [282]. The precursors of oxylipins are higher in the female urine than in male one [290].

Also the nuclear factor-kappa b (NF-*k*B) pathway, which is crucial for inflammatory response [291], is sex-dependent with its activation being mediated by the adaptor molecule MyD88, which interacts with cytoplasmic estrogen receptor-α [292]. The NF-*k*B activation is higher in female human umbilical cord vein endothelial cells (HUVEC) than in male ones, under hyperoxic conditions [293]. Also the tumor necrosis factor-α (TNF-α) pathway exhibits sex differences. For example, the human female adult cardiac progenitor cells appear to be more responsive to TNF-α when migration and cell cycle progression are considered [294]. Young men have lower levels of TNF-α when compared to fertile women [142]. Also the interleukin systems present some sex differences, with IL-6 being significantly higher in postmenopausal women than in premenopausal women [142], and in young women with CAD either in basal condition or after stress than men [295]. The anti-inflammatory effects of OO and its derivatives are summarized in Tables 2 and 3. In general, female animals and women are less studied and OO with a high content of MPCs is more active in the control inflammation, redox status, and lipid metabolism than OO with low content of MPCs. For example, EVOO with high MPCs reduces peripheral blood mononuclear cells (PBMC) activation of the CD40/CD40 ligand (CD40L) and LDLox and modifies numerous genes [313]. Some MPCs like HTyr exert anti-inflammatory activity with multiple mechanisms attenuating iNOS, COX2, and IL-1β expression and TNF-α and inhibiting the activation of granulocytes and monocytes [116]. Also oleocanthal and Tyr inhibit COX [246, 360].

5.3. Effects of EVOO, VOO, OO, Leaf Extracts, and MPCs on Platelets Function: Influence of Sex. Human platelets are sexually divergent; women have more platelets, longer bleeding time, and more activatable glycoprotein IIb/IIIa than men whereas platelet spreading and adherence are higher in men than in women [135]. The already described sex differences in AA pathways may induces sex differences in platelet aggregation. Adenosine diphosphate (ADP) and collagen-induced aggregation are higher in women, and women and men respond differently to antiaggregating agents [135, 361]. Both preclinical and clinical studies (Tables 2 and 3) show that EVOO and some of its MPCs (HTyr, oleuropein aglycone, luteolin, and oleocanthal) reduce platelet aggregation [13, 180], interfering either with

Table 2: Some CV effects of EVOO, VOO, OO, leaf extracts, and MPCs.

EVOO, VOO, OO, leaf extracts, and MPCs	Activity	References
Acetoxypinoresinol	Using DPHH test, it exerts antioxidant effects	[156]
Caffeic acid	It inhibits 5-LOX and exerts an antioxidant effects in *male* rat peritoneal leukocyte triggered by calcium ionophore and PMA	[157]
	It decreases IL-1β in human blood cultures (sex not reported) stimulated with LPS	[158]
EVOO	*In healthy men*, EVOO reduces urinary excretion of urinary 8-oxo-deoxyguanosine by 13%	[159]
	In *30 hamster males*, it reduces atherosclerosis	[160]
	In ApoE deficient mice *(14 females and 22 males),* the antiatherogenic effect of EVOO is reduced by dietary cholesterol	[161]
	In ApoE deficient mice (*54 females*), EVOO from different cultivars reduces atherosclerotic lesions, plaque size, and macrophage recruitment if compared to diets containing palm oil. EVOO also induces a cholesterol-poor, ApoA-IV-enriched lipoparticles with enhanced arylesterase and antioxidant activities	[162]
	In *male* STZ-diabetic rats, it raises BW and HDL and decreases glycaemia, TG, Chol, being ineffective in healthy rats	[163]
	In STZ-diabetic rats (*sex not reported*), it elevates HDL and reduces Chol, TG, and LDL	[164]
	In human platelets obtained from *3 male and 2 female* healthy subjects, it reduces NOX2 activation and H_2O_2 production	[165]
	In vitro, it inhibits ACE, α-glucosidase, and α-amylase being more active vs α-glucosidase; the richest MPC EVOO is also the most active	[166]
	Seggianese EVOO extract (rich in secoiridoids) is more active in preventing human LDL oxidation than Taggiasca EVOO extract (rich in lignans) (*sex not reported*)	[103]
	In vitro, Spanish EVOO inhibits α-glucosidase, α-amylase, and 5-LOX	[167]
	LDL and HDL obtained from treated healthy *14 women and 10 men* are less oxidizable and are more resistant to lipid peroxidation. Both EVOO and EVOO extract enhance the Chol efflux	[168]
	In *male hypertensive rats*, EVOO + olive + leaf rich in HTyr, 3,4 dihydroxyphenylglycol, and oleuropein decreases BP, angiotensin II, and endothelin-1 *vs* low MPC oil. There are no significant differences in plasma Na^+, urea, HDL, and LDL	[169]
	In an acellular model, HTyr rich extracts have a higher antioxidant and antimutagenic activity than Tyr-rich extract. In HELA cells, the Tyr-rich extract is more effective in increasing GSH whereas ROS levels are not changed by tested EVOO extracts. All extracts upregulate Keap1/Nrf2 pathway	[170]
	In male mice, high-fat EVOO diet improves glycaemia, insulinemia, glucose tolerance, insulin sensitivity, and insulin secretion. It reduces β-cell apoptosis and normalizes islet glucose metabolism vs high fat lard diet	[171]
	EVOO extract inhibits p50 and p65 NF-kB translocation in both stimulated and unstimulated PMA-challenged human monocytes and monocyte-derived macrophages (*sex not reported*)	[172]
	In ECV304 cells (*sex not reported),* EVOO extract partially prevents the increase of NO/ET-1 levels induced by high glucose/FFA	[173]
	In *male rats*, a bolus of EVOO changes the phospholipids of HDL	[174]
	Serum obtained from 6 healthy *males* and 6 *females* treated with EVOO extract rich in oleuropein and ligstroside reduces the VEGF-stimulated increase in NOX, Nox4, and MMP-9 activities, migration, and invasiveness. It also regulates VEGF-induced morphological differentiation capacity of HUVEC *(sex not reported)* into capillary-like structures. In human microvascular endothelial cell line, it reduces the VEGF-induced angiogenesis	[175]
	In *male* rats, subacute administration of both EVOO rich in MPC and native EVOO with low MPCs reduces ADP platelet aggregation, but acutely only MPC-rich extract reduces ADP induced aggregation	[176]
	In vitro unfiltered EVOO extract with peptide of low molecular weight inhibits ACE angiotensin converting enzymes in vitro, and in hypertensive *male* rats, it reduces SBP and DBP	[177]
	In ApoE deficient mice (*sex not reported*), extracts (EVOO vs EVOO + polyphenols green tea) enhance macrophage Chol efflux but only EVOO + polyphenols green tea reduces lipid peroxidation	[178]
	In vitro, Galician EVOO with high level of oleuropein and ligstroside derivatives inhibits the α-amylase and α-glucosidase, being more effective in inhibiting α-glucosidase than acarbose	[167]
EVOO vs sunflower oil, sunflower oil + oleic acid, MPC-deprived EVOO, sunflower oil enriched with the MPC of EVOO, and sunflower oil + oleic acid + MPC of EVOO	In all *male* rats fed with a high-Chol diet, GSH and IL-6 do not vary. EVOO, sunflower oil + MPC of EVOO, and sunflower oil + oleic acid + MPC of EVOO decrease the elevation in MDA and TNF-α levels induced by high-Chol diet	[179]
OC-rich EVOO with 1 : 2 oleacein/oleocanthal, 2 : 1 ($D2_i2$) rich in Tyr; EVOO 1 : 2 oleacein/oleocanthal ($D2_i0.5$) rich in Tyr	In healthy men (20 and 50 years), 40 ml of enriched EVOO for one week reduces collagen-stimulated platelet aggregation	[180]
OO	*In ApoE deficient mice (77 males 63 females),* all treatments reduce TG being ineffective versus Chol and vs the number of lesions; however, their dimensions are reduced in females by palm and olive II oils	[181]
	In *40 male* new Zeeland rabbits, dietary supplementation with 15% OO reduces the thrombogenic factors and elevates antithrombotic factors	[182]
	In *male rats*, OO reduces and prevents the growth of urinary stones	[183]
VOO	In *24 male* new Zeeland rabbits, it reduces atherosclerosis	[184]
	In *40 male* new Zeeland rabbits, it reduces atherosclerosis	[182]
	In human PBMC *(sex not reported)* and HL60 cells *(sex not reported*), it inhibits H_2O_2 and PMA induced DNA damage, being HTyr and Tyr, respectively (extract without verbascoside)	[185]
Extract of olive cake vs extract of thyme and vs extract of olive cake + thyme extract	In *male* rats, single oral administration of the three extracts regulates plasma antioxidant status (DPPH and FRAP) in a time and extract dependent way. In red cells, extracts decrease SOD but increase GPx and CAT	[186]

Table 2: Continued.

EVOO, VOO, OO, leaf extracts, and MPCs	Activity	References
	In vitro experiments, HTyr and many other phenolic compounds added to standard cell culture media (such as DMEM, MEM, or RPMI) produce H_2O_2 in the one- to three-digit micromolar range	[187, 188]
	In alloxan-diabetic *male rats*, it lowers glycaemia, TG, Chol, alkaline phosphatases, AST and ALT, aspartate and lactate transaminases, lipid peroxidation, total and direct bilirubin, creatinine, urea and increases HDL and hepatic and renal SOD, CAT, and GPx	[189]
	In alloxan-*diabetic male rats*, it decreases glycaemia, Chol, and oxidative stress	[190]
	In STZ-diabetic *male rats*, it reduces plasma lipid peroxidation, nerve conduction velocity, and thermal nociception and attenuates the decline of sciatic nerve Na^+K^+ ATPase activity	[191]
	In STZ-diabetic *male rats,* it lowers oxidative, nitrosative, and inflammatory biomarkers and platelet aggregation	[192]
	In STZ-diabetic *male rats*, it reduces retinopathy, lipid peroxidation, nitrosative stress, TBX2, 6-keto-PGF1α, and IL-β1	[193]
	In STZ-diabetic *male rats*, it lowers retinal ganglion cell number, retinal thickness, and cell size	[193]
	In STZ-diabetic *male rats*, it reduces brain lipid peroxidation and inflammation, nitrosative stress, cell death, IL-1β, PGE_2	[194]
	In STZ-induced diabetic and triton WR-1339 induced hyperlipidemic *male mice*, it reduces plasma glucose, TG, Chol, lipid peroxidation, TNF-α, CRP and elevates, glucose tolerance, antioxidants, and atherosclerotic index	[195]
	It prevents metabolic syndrome and inhibits the hepatic and muscular SREBP-1c/FAS pathway reducing oxidative stress and mitochondrial abnormalities and improving lipid and glucose metabolism in *db/db C57BL/6J male mice*	[196]
	In the brain of diabetic *db/db C57BL/6J male mice*, it activates AMPK, SIRT1, and PPARγ coactivator-1α and reduces oxidative stress	[197]
	In LPS-stimulated human monocytic cells (*sex not reported*), it suppresses NO release and attenuates the transcription and expression of TNF-α, iNOS, and COX2 in a dose-dependent way	[198]
	In HUVEC (*sex not reported),* HTyr and its metabolites suppress TNF-α-induced phosphorylation of NF-κB, ROS production, depletion of GSH, adhesion molecules and downregulate genes encoding antioxidant enzymes. They also reduce the adhesion of human monocytes (cell line) to HUVEC. Finally, they reduce carrageenan induced paw edema and TPA-induced ear edema *in male* mice	[199]
	The HTyr pretreatment of HUVEC (*sex not reported*) suppresses inflammatory angiogenesis induced by PMA and ameliorates mitochondrial function	[200]
	In *male* mice, it ameliorates the impact on body adiposity induced by the obesogenic diet	[201]
	In *male rats fed* fed with high-fat diet, it reduces AST, ALT, Chol, liver inflammation, and nitrosative/oxidative stress. It improves glucose tolerance, insulin sensitivity, and intestinal barrier integrity and functions and increases hepatic PPARα and its downstream-regulated genes	[202]
	In *male* mice fed with diet-induced obesity, it improves glucose homeostasis, insulin signaling markers, chronic inflammation, hepatic steatosis, and endoplasmic reticulum stress	[203]
	In *male* rats fed with a diet-induced metabolic syndrome, it reduces adiposity and ameliorates impaired glucose, insulin tolerance, and endothelial dysfunction. It also decreases SBP, left ventricular fibrosis, and resultant diastolic stiffness and markers of liver damage. Notably, the diet used for induction of metabolic syndrome alters HTyr metabolism	[204]
	In endothelial cells obtained from porcine pulmonary arteries (*sex not reported*), it increases AMPK, CAT activities, forkhead transcription factor, and cytoprotection against TNF-α-induced damage through the suppression of caspase-3 and NF-kB activation. It also promotes wound healing via Nrf2 synthesis and stabilization	[205, 206]
	In rat aorta VSMC (*sex not reported*), it exerts a proapoptotic effect through NO production and protein phosphatase 2A activation with subsequent inactivation of AKT	[207]
HTyr	In *male* rat peritoneal leukocytes triggered by calcium ionophore, it inhibits 5-LOX and exerts antioxidant effects in leukocytes triggered by PMA	[157]
	In a *female mice* model for accelerated aging, it induces the expression of SIRT1	[208]
	In vitro, it inhibits human platelet (*sex not reported*) aggregation induced by ADP and collagen being more active than other MPCs and TBX2 production induced by collagen and thrombin	[209]
	In pooled human liver microsomes (*sex not reported*), it inhibits androstenedione 6β-hydroxylase and reductive 17β-HSD activity, whereas it is inactive *vs* oxidative 17β-HSD	[210]
	In white adipose of *male mice* fed with high-fat diet, it reduces the increase in oxidative stress, lipid, and protein oxidation and increases the antioxidant defenses	[211]
	In adult *male* rats, it reduces myocardial infarction area, necrosis and apoptosis, the release of LDL and CPK, probably through upregulation of PI3K/AKT pathway	[212]
	It is a scavenger of hydroxyl radicals, with peroxynitrite and O_2^- being inactive *vs* HOCl and H_2O_2. It protects LDL against oxidation but is not effective vs the oxidation of LDL isolated from humans after HTyr intake (*sex not reported*)	[213]
	It inhibits α-glucosidase and α- amylase, being more effective vs α-glucosidase	[214]
	In human aortic endothelial cells (*sex not reported*) stimulated with TNF-α, it significantly reduces the secretion of P-selectin, ICAM-1, VCAM-1, and MCP-1	[215]
	In human HUVEC *(sex not reported),* it reduces the stimulated tube-like differentiation and the stimulated locomotion, MMP-9 secretion induced by PMA, PMA-stimulated COX2 activity and expression. Pretreatment with HTyr before PMA decreases intracellular ROS and nuclear translocation of the p65 NF-κB subunit and NF-κB transactivation	[216]
	In *male rats*, HTyr, 3,4-DHPEA-EA and 3,4-DHPEA-EDA reduce the increase in intracytoplasmic Ca^{2+} induced by vasopressin. Further, higher concentration of HTyr exerts an endothelium-independent effect. 3,4-DHPEA-EA and 3,4-DHPEA-EDA exert an endothelium-dependent vasodilation in aorta increasing the production of NO	[217]
	It regulates expression of numerous miRNA in the mice gut (*sex not reported)* being less effective in other tissues.	[218]
	HTyr administration increases TG	[219]
	In male mice, it lowers Chol	
	In human monocytes (*sex not reported*) stimulated with PMA, it reduces the expression of mRNA and protein of COX2 decreasing PGE_2 and O_2-production and increases TNF-α production. In human neutrophils (*sex not reported*) stimulated with PMA, or chemotactic peptide FMLP or opsonized zymosan particles, it does not influence the production of O_2– and NOX activity whereas it inhibits the production of H_2O_2	[220]
	In human PBMC (*sex not reported*) and in human monocytic cell line U937 stimulated with PMA, it reduces the secretion of MMP-9, PGE_2 production, COX2 protein expression, and COX2 mRNA without modifying COX1. It inhibits both PGE_2 and MMP-9 release from human monocyte-derived macrophages. It suppresses NF-κB activation in human monocytoid cells and reduces PKCα and PKCβ1 activation. Notably, it does not affect MMP-9 and COX2 in basal conditions	[221]
	In LPS-stimulated human monocytic THP-1 cells (*sex not reported*), it reduces LPS-stimulated NO and ROS formation in a concentration-dependent way, increases GSH levels, and suppresses the of NF-kB activation	[222]
	In young *male* C57BL/6 mice treated with MPC does not modify BW, food intake, and TG but it lowers plasma Chol, leptin. In murine 3T3-L1 preadipocytes, it positively modulates the glutathione-driven antioxidant enzymatic machinery reducing GSSG/GSH ratio, through the modulation of genes related to oxidative stress	[223]
	In *male rats* with diet-induced metabolic syndrome, it decreases glucose tolerance, lipids, ALT, AST activity, insulin, weight gain, fat mass, liver steatosis, and ventricular fibrosis	[204]
	It prevents COX2, TNF-α, DNA damage, and oxidative stress in Balb/c mice treated with LPS (*sex not reported)*	[224]
	It increases the TNF-α mRNA level in LPS-activated human monocytes (*sex not reported)*	[225]
HTyr, oleuropein, EVOO extract, homovanillyl alcohol	In HUVEC (*sex not reported)*, EVOO extracts decrease cell surface expression and mRNA of ICAM-1 and VCAM-1. Olea and HTyr are the main actors for these effects. Homovanillyl alcohol inhibits cell surface expression of adhesion molecules, but the effects on mRNA are small	[226]

Table 2: Continued.

EVOO, VOO, OO, leaf extracts, and MPCs	Activity	References
HTyr HTyr- acetate (HTyr-Ac) HTyr ethyl hydroxytyrosol ether (HTyr-Et)	In *male* rats fed with high-fat diet, the compounds improve glucose, insulin, leptin levels, lipid peroxidation, and antioxidant capacity status, with HTyr-Ac being the most active. They also reduce the release of inflammatory biomarkers. HTyr-Ac and HTyr-Et improve adipose tissue distribution and adipokine production, decreasing MCP-1 and IL-1β levels	[227]
HTyr and homovanillic alcohol	In PBMC obtained by *healthy men and women*, they inhibit the increase of IL-1β, MIF, and RANTES induced by oxysterols	[228]
HTyr-acetate (HTyr-Ac)	In TNF-α- stimulated HUVEC (*sex not reported*), it reduces the inflammatory response partly through the TNFRSF1A/SIRT6/PKM2-mediated signaling pathway	[229]
HTyr and oleuropein	Both compounds inhibit oxidative burst in human granulocytes and monocytes obtained from healthy individuals (*sex not reported*) stimulated with PMA. HTyr attenuates the generations of NO and PGE_2. In LPS triggered RAW264.7, it reduces NRf2 nuclear translocation and miR-146a expression	[230]
HTyr and HTyr-NO	In vascular ring obtained from *male rats*, it releases NO while HTyr is ineffective. HTyr NO decreases Chol, TG, lipid peroxidation and increases SOD and NO in the serum of STZ-diabetic *male mice*. Both HTyr-NO and HTyr upregulate SIRT1 expression in the thoracic aorta of *male* diabetic mice. In HUVEC triggered by hyperglycaemia (*sex not reported*), HTyr-NO increases cell viability and reduces oxidative stress trough SIRT1	[231]
HTyr, dialdehydic form of elenolic acid linked to HTyr, oleuropein aglycon, oleuropein, Tyr, the dialdehydic form of elenolic acid linked to Tyr, caffeic acid, and verbascoside	In human PBMC and HL60 cells *(sex not reported)*, they inhibit H_2O_2-induced DNA damage	[185]
HTyr + nicotinate	It inhibits α-glucosidase, and in healthy *male* mice fed with high-fat diet, it has hypoglycemic, antioxidant, and hypolipidemic activities	[232]
HTyr + eicosapentaenoic acid (EPA)	In *male* mice fed a with high-fat diet, it reduces the steatosis and elevates the hepatic levels of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), resolvins and attenuates proinflammatory markers	[233]
Leaf extract	In INS-1 cells (*sex not reported*), leaf ethanolic extract and oleuropein improve the damage induced by H_2O_2. The leaf extract is more potent than oleuropein in preventing the cytotoxic effects and only leaf extract preserves GPx	[234]
	In STZ-diabetic *male* rats, the extract ameliorates diabetic alterations	[235]
	In STZ-diabetic *male* rats, it decreases glycaemia and HbA1c and increases insulin. It also inhibits α-amylase and α-glucosidase	[236]
	In acellular model, it inhibits DPPH radical generation. In STZ-diabetic *male* rats, the extract increases CAT activity, GSH and lowers lipid peroxidation, Chol, TG, histological pancreas, and hepatic damage	[237]
	In *male* alloxan-diabetic rats, it shows a hypoglycemic effect and reduces the damage of islets of langerhans	[238]
	In cultured neonatal rat cardiomyocytes *of both sexes*, it decreases maximum I (Ca,L) in a reversible manner	[239]
	Male rats fed with a high-fat diet develop signs of metabolic syndrome. Comparing rats with high-fat diet *vs* those with high-fat + leaf extract enriched with MPCs, it emerges that leaf extract improves the signs of metabolic syndrome and decreases MDA and uric acid while it is not effective on BP	[240]
	In human coronary artery endothelial cells (*sex not reported*) stimulated with serum amyloid A, it reduces the release of IL-6, IL-8, mRNA expression of E-selectin, the phosphorylation of p65 of NF-κB, DNA damage and stabilizes microRNA-146a and let-7e	[241]
	In *male rats*, leaf extract containing 20% of HTyr decreases the paw edema induced by carrageenan and IL-1β and TNF-α release. It does not affect the anti-inflammatory cytokine IL-10	[242]
	In diet-induced hypercholesterolemic *male rats,* olive leaf extracts enriched with oleuropein enzymatic and acid hydrolysates rich in oleuropein aglycone and HTyr decrease Chol, TG, and LDL and elevate HDL and serum antioxidant potential. In livers, hearts, kidneys, and aorta lipid peroxidation decreases while liver CAT and SOD increase	[243]
Luteolin	It is antioxidant in chemical test and prolongs the lag phase of LDL oxidation. It protects the cells against H_2O_2 induced damage but it is ineffective *vs* platelet aggregation (*sex not reported*)	[209]
Oleacein	In vitro, it inhibits angiotensin converting enzyme	[244]
	It stabilizes atherosclerotic plaque in samples obtained from 20 hypertensive individuals of *both sexes*	[245]
Oleocanthal	It is a nonselective inhibitor of COX1 and 2 and attenuates iNOS and human recombinant 5-LOX, being ineffective *vs* 15-LOX. Regarding 5-LOX, it is less active than oleuropein and oleacein. In addition, it inhibits TNF-α, IL-1β, IL-6, and GM-CSF	[117, 246, 247]
	In rat and mouse trigeminal ganglia (*females and males used in equal ratio*), it acts as agonist of TRPA1	[248, 249]
	In *male* adult rats, it decreases the traumatic injury reducing the inflammatory response by reducing the eNOS and iNOS	[250]
	In murine chondrogenic ATDC-5 cells and in mouse macrophage J774A.1, it inhibits the LPS-mediated upregulation of NOS2 and LPS induced release of cytokines (*sex not reported*)	[251]
	In human monocytes (*sex not reported*), it reduces the release of O_2^-, PGE_2 and the expression of COX2 and inhibits NAPH-oxidase	[220]
	In vitro, it inhibits α-glucosidase and α- amylase	[214]
	In C2C12 cells (*sex not reported*), it protects against H_2O_2 induced damage; further it increases glucose consumption and the phosphorylation of AMPK/ACC and MAPK, but not PI3 kinase/Akt. It improves the insulin sensitivity via insulin-dependent (PI3 kinase/Akt) and insulin independent (AMPK/ACC)	[252]
	In bovine VSMC *(sex not reported)*, it inhibits cell proliferation in the G1-S phase probably by inhibition of ERK1/2	[253]
	In caco cells *(sex not reported)*, it inhibits maltase, human sucrose, glucose transport across Caco-2 monolayers, and uptake of glucose by GLUT2 in Xenopus oocytes; it is a weak inhibitor of human α-amylase	[254]
	In vitro, it inhibits platelet aggregation being less active than HTyr. In whole blood, collagen platelet aggregation is not modified (*sex not reported*)	[209]
	It is antioxidant both in chemical assay and in the lag phase prolonging of LDL oxidation. However it is less active than homovanillic alcohol	[255]
Oleuropein	In samples of pooled human liver microsomes (*sex not reported*), it inhibits CYP3A	[256]
	J774A.1 cells (*sex not reported*) and in peritoneal macrophages from *male* mice, it increases the production of NO that is blocked by NOS inhibitor	[257]
	In *male* rat peritoneal leukocytes triggered by calcium ionophore, it inhibits 5-LOX and exerts antioxidant effects when leukocytes are stimulated by PMA	[157]
	In human HUVEC *(sex not reported)*, oleuropein and HTyr reduces the stimulated tube-like differentiation and stimulates locomotion, the increase in MMP-9 secretion induced by PMA without affecting tissue inhibitors of MMP, with this activity being mediated by pretranslation process. It inhibits PMA-stimulated COX2 activity and expression. HTyr before decreases intracellular ROS and nuclear translocation of the p65 NF-κB and its transactivation	[216]
	In pooled human liver microsomes (*sex not reported*), they inhibit androstenedione 6β-hydroxylase and 17β-HSD	[210]
Oleuropein glycoside	In diluted human blood cultures (*sex not reported*) stimulated with LPS, it decreases IL-1β	[158]
Oleuropein, caffeic acid, Tyr HTyr	In acellular models, they scavenger reactive nitrogen species, with Tyr being the less active; however they do not inhibit the nitrergic transmission in the nerve-stimulated anococcygeus preparation of *male* rats	[258]
Oleuropein-containing supplement OPIACE	In DM2 model (Tsumura Suzuki obese diabetes *male*) mice, the diet attenuates hyperglycaemia and impairs glucose tolerance and oxidative stress but has no effect on obesity	[259]
Olive water methanol extract	In normotensive anaesthetized and atropinized rats (*sex not reported*), the intravenous administration of extract reduces the BP. In isolated atria of Guinea pig *of both sexes*, it reduces the spontaneous beating. In isolated thoracic artery of *male* and *female* rabbits it reduces K^+ and/or phenylephrine induced contraction	[260]
Pinoresinol	Using DPHH test, it exerts antioxidant effects being more active than acetoxypinoresinol	[156]
	In PMA-stimulated RAW 264.7 macrophages (*sex not reported*), Tyr decreases the O_2^- and H_2O_2 generation induced by PMA and scavenges the O_2^-. These effects seem to be linked with the impairment of (3H)AA release, COX2 expression, PGE_2/B4 synthesis, and NO release	[261]

Table 2: Continued.

EVOO, VOO, OO, leaf extracts, and MPCs	Activity	References
Tyr	In RAW 264.7 macrophages (*sex not reported*), triggered by oxLDL-stimulated Tyr reverts H_2O_2 generation and the AA release and PGE_2 production	[262]
	In human monocytes (*sex not reported*) stimulated with PMA, it reduces the production of O_2^- and the expression of mRNA and protein of COX2, dose-dependently decreasing PGE_2 production	[220]
	In RAW 264.7 macrophages (*sex not reported*), it reduces the activation of iNOS and COX2 gene expression, NF-κB, interferon regulatory factor-1 (IRF-1), and activator of transcription-1α (STAT-1α) induced by gliadin + IFN-γ	[263]
	In *male* rat peritoneal leukocytes triggered by calcium ionophore, it inhibits 5-LOX and exerts antioxidant effects when leukocytes are stimulated by PMA	[157]
	In human PBMC (*sex not reported)* and HL60 cells, it inhibits H_2O_2-induced DNA damage	[185]
	In PBMC obtained by *healthy men and women*, it inhibits the increase of IL-1β, MIF, and RANTES induced by oxysterols	[228]
Tyr, Tyr glucuronate (Tyr-GLU), and sulfate (Tyr-SUL)	In TNF-α treated-HUVEC *(sex not reported*), Tyr and Tyr-SUL prevent ROS generation and GSH decrease and downregulate GPx-1, GCL, and OH-1 genes. Tyr-SUL, Tyr, and Tyr-GLU prevent the phosphorylation of NF-κB signaling proteins. Tyr-GLU and Tyr-SUL prevent the increase of genes and proteins expression and secretion of adhesion molecules. *In vivo*, Tyr and Tyr-SUL, in a dose-dependent manner, ameliorate plantar and ear edemas in *male mice*	[264]
Tyr, oleuropein, and olive pomace	In anoxic EA.hy926 human endothelial cell line *(sex not reported*), both Tyr and oleuropein attenuate anoxia-induced expression of MMP-9 and MMP-2. Tyr is more efficient than oleuropein in reducing TNF-α. The olive pomace ameliorates all the above parameters and induces time-dependent phosphorylation of p38 MAPK and ERK1/2, and inhibits anoxia-induced NF-κB activation.	[265]
Verbascoside	In PBMC *(sex not reported)* and HL60 cells, it inhibits $H_2O_2^-$ induced DNA damage.	[185]

AA pathways [362] or with other mechanisms such as calcium mobilization and attenuating iNOS activity [247, 363]. In hypercholesterolemic patients, MPCs decrease platelet aggregation inhibiting procoagulant factors, such as plasminogen activator inhibitor-1 and factor VII [364]. Small crossover trial proves that oleocanthal is the most active in inhibiting collagen-induced aggregation at least in men [180], probably because it is a nonselective inhibitor of COX. HTyr antiaggregant activity seems to be agonist specific [209]. However, in vivo, it remains difficult to discriminate EVOO associated effects of specific MPCs and phenols. Tables 2 and 3 show that, globally, the majority of the studies are performed on males and even when females are recruited no sex analysis is performed.

5.4. Effect of EVOO, VOO, OO, Leaf Extracts, and MPCs on Glucose Metabolism: Influence of Sex. Their effects are summarized in Tables 2 and 3. Briefly, the antidiabetic actions may reside in the inhibition of α-amylase and α-glucosidase [166, 167, 214, 365], which might lead to less effective absorption of glucose [366]. Some authors suggest that HTyr is a better inhibitor of α-amylase than of α-glucosidase [214]. Also oleuropein inhibits these enzymes [214]. Beyond the inhibition of these enzymes, other mechanisms have been proposed for the antidiabetic activity including antioxidant and anti-inflammatory action (see above) and activation of AMP-activated protein kinase and of incretin release [197, 205–207, 341]. In particular, the antidiabetic activity of HTyr and oleuropein is recently reviewed [367, 368]. Again it emerges that the antidiabetic activity has been mainly studied in males; nevertheless, it clearly shows that DM presents numerous sex differences [39], including the relative risk for CVD associated with hyperglycaemia that is higher in women than in men (Table 1).

5.5. Effects of EVOO, VOO, OO, Leaf Extracts, and MPCs on Uric Acid: Influence of Sex. It is related to CV events both in women and in men [140, 369, 370], but it is a higher risk in women [371]. However, these are not univocal data because others sustain that this association is present only in women [372–374], who have lower plasma levels that men [375]. Leaf extracts of olive tree and HTyr inhibit xanthine oxidase reducing uric acid synthesis [376]. In male rats, HTyr also regulates transcription of some renal transporters that favor uric acid excretion [377].

6. Clinical Studies

Results of clinical studies are summarized in Table 3. The beneficial aspects of regular use of OO on CVD has been suggested by numerous authors [2, 154, 306, 310, 378–380], through the biological activities discussed above and summarized in Table 2. However, clinical studies have common limitations: (a) despite the numerosity of studies, the size of samples is very small and they do not take into account the high interindividual variability; (b) they are relatively limited or of questionable quality; (c) with some exceptions they are very short in duration; (d) they are mainly performed on Mediterranean populations; (e) they have heterogeneous designs, with variation in control diets and in the type of oil used. Therefore, to overcome these limitations we focus on meta-analyses.

Schwingshackl and Hoffmann [381] reported that the use of OO is associated with a 20–40% lower risk of stroke and CHD. Another meta-analysis of case-control, prospective cohort studies and randomized controlled trials proves a negative relationship between OO consumption and stroke (and stroke and CHD combined), but the association is not significant for CHD [348]. A successive meta-analysis proves that high EVOO MPCs ameliorate surrogate end points such as lipid peroxidation, oxLDL, Chol, and HDL [382]. In addition, the subgroup analysis indicates an improvement in inflammatory biomarkers and in BP [382]. After pooling oil interventions, PCR and IL-6 are lowered compared to baseline [380]. Others show that the regular dietary intake of OO reduces CRP, IL-6, and TNF-α [383]. The comparison of the effect of different types of OO (refined, mixed, low, and high MPC EVOO) shows no significant effects on Chol, HDL, TG, or DBP [3]. However, in secondary analyses, EVOO may reduce oxLDL *vs* refined OO in a dose-dependent manner. Finally, one meta-analysis that includes 1089 participants shows that OO increases HDL reducing LDL and TG, while ApoA1 and ApoB are not significantly changed [384].

Table 3: Clinical studies on the effect of EVOO, VOO, OO, leaf extracts, and MPCs.

Compounds	Individuals	Design	Main data	References
High MPC EVOO vs moderate and low MPC EVOO	200 healthy *men*	Multicenter RC crossover design	The negative association between the oleic/linoleic acid ratio and biomarkers of oxidative stress and improvement of LDL fatty acid profile	[296]
EVOO *vs* saturated fat diet	18 healthy postmenopausal *women*	Prospective, longitudinal, study	EVOO decreases the risk to develop the metabolic syndrome and CAD	[297]
EVOO *vs* soya oil	41 adult *women* with excess body fat	Double-blinded RC vs placebo	EVOO increases fat loss and reduces DBP and some biochemical parameters	[298]
High MPC EVOO *vs* low MPC EVOO	9 *men* and 11 *women* with metabolic syndrome	RC sequential crossover design	After EVOO-based breakfast, numerous inflammatory genes involved in factor NF-κB, AP-1, MAPK, and AA pathways are repressed in PBMC	[299]
High MPC VOO vs intermediate and low VOO	19 *men* and 30 *women* with metabolic syndrome	RC, crossover design	High MPC VOO-based breakfast attenuates plasma LPS, TLR4, and SOCS3 proteins, activation of NF-κB and the IL-6 vs low and intermediate oil. In PBMC, postprandial expression of IL-1B, IL-6, and CXCL1 is reduced especially by high MPC VOO	[300]
High MPC EVOO *vs* low MPC EVOO	*6 healthy men* and *6 healthy women; 6 men* and *6 women* with metabolic syndrome	Paired study	Acute high MPC EVOO transiently improves glycaemia and insulin sensitivity. It directly modifies the miRNA of PBMC. Acute EVOO poor in MPC is less effective	[278]
EVOO *vs* ROO	14 healthy and 14 hypertriacylglycerolemia *men*	Blind RC crossover design	EVOO has postprandial anti-inflammatory effects	[301]
EVOO	*26 male and 34 female* DM2 patients	RC trial	Both atorvastatin and EVOO reduce plasma lipids and increase HDL with a higher activity of atorvastatin	[302]
EVOO	17 *males* and 13 *females* with impaired fasting glucose	Blind RC crossover design	After EVOO meal, glucose, TG, ApoB-48, and DPP4 activity decrease, whereas insulin and GLP-1 increase *vs* meal without EVOO.	[303]
EVOO *vs* coconut oil *vs* unsalted butter	Healthy *women (67%)* and *men (33%)*	RC trial	Chol and HDL do not change after EVOO meal *vs* meal without EVOO No changes in BW, BMI, central adiposity, fasting blood glucose, SBP, and DBP for all diets. Butter increases LDL; coconut increases HDL	[304]
EVOO *vs* VOO	*41 males* and *females* (overweight or obese)	Single-blinded RC	EVOO decreases SBP and increases anti-CD3/anti-CD28 stimulated T cell proliferation *vs* VOO	[305]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
VOO rich in MPC *vs* ROO	*11 women* at stage 1 of essential hypertension or 13 with normal-high BP	Double-blind RC crossover design	VOO rich in MPC decreases SBP, DBP, CRP, LDL, ADMA and increases nitrites/nitrates and hyperemic area after ischemia	[306]
Diet enriched with VOO, walnuts, or almonds	*9 female and 9 male* hypercholesterolemic patients	RC crossover design	The VOO, walnut, and almond diets reduce LDL; They reduce LDL, Chol, and LDL/HDL ratio. Other lipid fractions, oxidation, and inflammatory biomarkers do not change	[307]
OO rich in MPC *vs* OO + EGCG	Patients with endothelial dysfunction, OO rich in MPC (13 *men* and 15 *women*) OO + EGCG (10 *men* and 14 *women*)	Double-blinded RC	They reduce endothelial dysfunction, but only OO reduces inflammatory biomarkers, white blood cells, monocytes, and lymphocytes	[308]
OO enriched with oleanolic acid (OA) *vs* OO	176 individuals of *both sexes* with impaired fasting glucose and impaired glucose tolerance	Multicenter double-blind RC trial	The intake of OO rich in OA reduces the risk of developing DM in individuals with impaired fasting glucose and impaired glucose tolerance	[309]
MedDiet + EVOO *vs* MedDiet + nut *vs* control	7447 old participants of PREDIMED *(43% men* and *57% women)* at risk for CVD	Observational study in primary prevention	Long intake of MedDiet + EVOO and MedDiet + nut reduces primary CV events	[11]
High MPC EVOO *vs* moderate and low MPC VOO	18 *healthy men*	Double-blind RC, crossover design	High PMC EVOO reduces SBP *vs* basal values and low PMC VOO. It maintains DBP values compared to low MPC VOO. Further, it reduces ACE and NR1H2 gene expressions *vs* basal and IL-8RA *vs* low PMC MPC	[310]
MeDiet + EVOO vs MeDiet + washed EVOO vs habitual diet	*26 healthy men* and *64 healthy women*	RC crossover design	In plasma, MedDiet + EVOO reduces oxidative and inflammatory status. In PBMC, it reduces oxidative stress, the gene expression of INF-γ, Rho GTPase-activating protein 15, IL-7 receptor, adrenergic β2 receptor and polymerase (DNA-directed) k. These effects with the exception of polymerase (DNA-directed) κ are more elevated when EVOO rich in polyphenols was added	[311]
High MPC EVOO *vs* low MPC EVOO	46 healthy subjects *(14 men* and *32 women)*	RC crossover design	No effect on fasting plasma lipids, oxLDL, and LPO	[106]
EVOO *vs* refined OO	24 *men*	RC crossover design	Only EVOO rich in MPCs lowers oxLDL being ineffective *vs* plasma lipids	[312]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
High MPC VOO *vs* moderate and low MPC VOO	18 healthy *men*	RC crossover design	High MPC VOO reduces oxLDL MPC-1, CD40L, IL-23A, IL-7R, IL-8RA, ADRB2, and OLR1 genes, whereas IFNG, IL-7R, IL-23A, CD40L, MCP-1, and IL-8RA decrease with low MPC VOO	[313]
High MPC VOO + triterpenes (OVOO) *vs* OVOO + higher MPC and triterpenes (FOO) vs low MPC and triterpenes (VOO)	27 healthy *men* and 26 healthy *women*	Double-blind RC, crossover design	Urinary 8-hydroxy-2′-deoxyguanosine, plasma IL-8, and TNF- α decrease more after FOO vs OVOO	[314]
High MPC VOO + triterpenes (OVOO) *vs* OVOO + higher amounts of MPC and triterpenes (FOO) vs low MPC and triterpenes (VOO)	27 healthy *men* and 26 healthy *women*	Double-blind RC, crossover design	After OVOO, HDL increases only in females. Chol increases after FOO and TG after VOO and OVOO. SBP decreases after the VOO and increases after the FOO. DBP and pulse pressure do not vary as well as LDL, sICAM-1, and sVCAM-1. Plasma ET-1 decreases after the VOO, OVOO, and FOO	[315]
VOO, VOO + MPC (FVOO), VOO + MPC + Thyme phenols (FVOOT)	Hypercholesterolemic *men* and *women*	Double-blind RC crossover design	Acute and sustained intake of VOO and FVOO attenuate PON1 protein and increase PON1-associated specific activities, while FVOOT has opposite effects. Only VOO increases PON3 protein	[316]
VOO *vs* VOO + MPC (FVOO) vs VOO + MPC + Thyme phenols (FVOOT)	Hypercholesterolemic volunteers: 5 women *and 7 men*	Double-blind RC, crossover design	FVOOT reduces serum oxLDL and elevates gut bifidobacteria vs VOO. FVOO does not change blood lipids and microbial populations but elevates the coprostanone vs FVOOT	[317]
VOO *vs* VOO + MPC (FVOO) VOO + MPC + Thyme phenols (FVOOT)	Hypercholesterolemic volunteers: *19 men* and *14 women*	Double-blind, RC crossover design	Urinary HTyr sulfate and thymol sulfate increase after FVOO or after FVOOT, respectively. FVOO and FVOOT do not change glycaemia, TG, LDL, HDL, ApoAI, and ApoB100 *vs* VOO with the exception of LDL that decreases after FVOO. FVOO and FVOOT change the lipoprotein subclasses profile and decrease insulin resistance index. BP and BMI do not change	[318]
VOO *vs* VOO + MPC (FVOO)	Prehypertensive or stage 1 hypertension participants (7 *men* and 6 *women*)	Double-blind RC crossover design	FVOO decreases ischemic reactive hyperemia, oxLDL, postprandial glycaemia, TG, PAI-I, and CRP *vs* VOO	[319]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
VOO *vs* VOO + MPC and VOO + Thyme	*8 men* and *14 women* hypercholesterolemic subjects	Double-blind, RC crossover design	In PBMC, the intake of enriched VOO and VOO + thyme increases the expression of proteins involved in Chol efflux and nuclear receptor-related genes	[320]
VOO *vs* VOO + MPC (FVOO) and VOO + Thyme (FVOOT)	Hypercholesterolemic subjects: *19 men* and *14 women*	Double-blind, RC crossover design	The 2 enriched oils elevate antioxidants in HDL, whereas α-tocopherol is elevated only after FVOOT	[321]
VOO *vs* VOO + MPC *vs* VOO + MPC + Thyme phenols	19 hypercholesterolemic *men* and 14 *women*	Double-blind RC crossover design	Their consumption of each oil affects the HDL proteome in a cardioprotective mode	[322]
Diets with VOO and refined OO vs sunflower or corn oil during washout period	24 young *women* with high-normal BP or stage 1 essential hypertension	Double-blind RC crossover design	Only VOO decreases SBP and DBP, serum asymmetric dimethylarginine, oxLDL, and CRP. It increases the plasma nitrites/nitrates ratio and hyperemic area after ischemia	[306]
High MPC OO enriched breakfast *vs* low MPC OO breakfast	5 hypercholesterolemic *men* and 16 *women*	RC design sequential crossover	After the high MPC breakfast, FVIIa increases less and PAI-1 activity decreases more than after the low MPC breakfast	[169]
OO rich in MPC *vs* refined OO	69 healthy participants of *both sexes*	Double-blind RC parallel design	Both OO improve the urinary proteomic CAD score but not chronic kidney disease or DM proteomic biomarkers. No differences are measured between the two OO	[99]
OO with high *vs* OO with moderate MPC	pre/hypertensive patients 17 *men* and *6 women*	RC crossover design	In white blood cells, high MPC OO increases gene expression of ATP binding cassette transporter-A1, scavenger receptor class B type 1, PPARα, PPARγ, PPAR δ, and CD36 *vs* moderate MPC OO	[323]
High MPC OO *vs* moderate MPC and low MPC OO	30 *healthy subjects of unknown sex*	Double-blind RC vs placebo- crossover design	The consumption of oil rich in MPCs increases MPCs in LDL-C and decreases oxLDL	[324]
High MPC OO *vs* moderate and low MPC OO	12 healthy *male* subjects	Double-blind RC, crossover design	All OO promote postprandial increase in F2-isoprostanes whereas the LDL oxidation is inversely linked with MPCs	[325]
High MPC OO *vs* moderate and low MPC OO	200 healthy *men*	RC crossover design	HDL and Chol increase and decrease linearly with the MPC amounts, respectively. OxLDL and MPC amount are inversely related. TG decrease is not influenced by MPC amount	[325]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
High MPC OO *vs* low MPC OO	10 menopausal healthy *women*	RC design crossover	MPC-rich OO diet reduces DNA damage vs low MPC OO whereas plasma antioxidant capacity does not diverge	[326]
High MPC OO *vs* moderate and low MPC OO	12 *male* healthy subjects	Double-blind, RC crossover design	Short-term consumption of MPC-rich OO decreases plasma oxLDL, urinary 8-oxo-dg and increases plasma HDL and GPx *vs* moderate and low MPC OO	[327]
High MPC OO	Patients with polymorphism in NOS3 Glu298Asp (rs1799983) of eNOS (*22 men, 35 women*)	RC sequential crossover design	Single administration seems to reduce the deleterious effect of the T allele carrier's condition	[328]
High MPC OO vs moderate and low MPC OO	30 healthy men from a religious center	RC, crossover design	MPC-rich OO is more effective in protecting LDL oxidation and in raising HDL than OO with lower quantities of MPCs	[15]
High MPC OO *vs* low MPC OO	22 mildly dyslipidemic subjects	RC crossover design	MPC-rich OO lowers plasma TXB_2 and elevates plasma antioxidant capacity *vs* low MPC OO. Urinary F2-isoprostanes and plasma lipids do not diverge between the two groups	[329]
High MPC OO *vs* low MPC OO enriched breakfast	21 hypercholesterolemic subjects *(5 men* and *16* postmenopausal women)	RC crossover design	High MPC OO protects against postprandial endothelial dysfunction and decreases lipid peroxide and F2-isoprostanes *vs* low MPC OO	[330]
High MPC OO vs low phenolic OO	28 individuals with CHD (*sex not reported)*	Double-blind RC placebo-controlled, crossover design	Enriched OO decreases IL-6 and CRP being ineffective on soluble sICAM-sVCAM-1 and lipid profile	[331]
High MPC OO vs low MPC OO *vs* corn oil	12 healthy men	The study has a Latin square design	Enriched OO decreases TXB2 and LTB4 and increases plasma antioxidant capacity	[332]
High MPC OO *vs* low MPC OO	40 *men* with stable CID	RC crossover design	MPC-rich OO decreases oxLDL and LPO and increases GPx	[333]
OO *vs* sunflower-seed *vs* and rapeseed	18 healthy *men*	Double-blind RC crossover design	Postprandial lipid and lipoprotein concentrations are not greatly affected versus rapeseed and sunflower-seed oil, while rapeseed and OO diets have the same effect on LDL oxidation	[334]
OO	18 healthy *men*	RC crossover design	OO may attenuate the acute procoagulant effects of fatty meals	[335]
OO	8 *men* and 5 *women* with type DM2	Single-blinded RC crossover design	It increases in GLP-1 and GIP	[336]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
OO (unrefined)	23 hypertensive patients *of both sexes*	Double-blind RC crossover design	Resting SBP and DBP are significantly lower at the end of the MUFA diet *vs* the PUFA diet. The cold pressor test and isometric exercise are similar. Daily drug dosage is significantly reduced during the MUFA *vs* PUFA diet	[337]
High MPC OO *vs* low MPC OO	Healthy smokers: 11 *men* and 14 *women*	Single-blind RC crossover design	Plasma antioxidant capacity and oxLDL do not differ significantly between the rich and low MPC OO	[18]
High MPC OO (HPCOO); low MPC VOO low-MPCOO (LPCOO), refined OO	25 healthy *men*	RC parallel, crossover, design	HPCOO decreases ApoB-100 and small LDL particles vs baseline and LPCOO. LPCOO increases previous parameters. HPCOO increases the lag time of LDL oxidation, which is not affected by LPCCO. LPL gene expression is not significantly changed by both OO	[338]
High MPC OO (HPCOO); VOO low MPC OO (LPCOO); refined OO	47 healthy men	RC crossover design	HPCOO increases HDL cholesterol efflux capacity *vs* the LPCOO and incorporation of MPC and their metabolites in HDL and HDL2. HPCOO intake decreases HDL3 and the HDL core becomes TG-poor, and HDL fluidity increased	[339]
HTyr	Healthy subjects (*12 men* and *16 women)*	Double-blinded, RC crossover design	Regular intake of HTyr improves the antioxidant defense and decreases nitrate and MDA	[340]
HTyr	21 healthy volunteers (*sex not reported*)	Double-blinded, RC crossover design	In PBMC, it induces miR-193a-5p, which leads to the generation of anti-inflammatory molecules	[218]
Oleuropein	24 healthy participants (*sex not reported*)	Double-blind RC Latin square design	No effect on postprandial glucose derived from bread, but in solution it attenuates postprandial blood glucose after 25 g sucrose, but has no effect after 50 g of sucrose or glucose	[254]
Oleuropein	Healthy 10 *men* and *10 women*	Double-blind RC crossover study	Its intake lowers glycaemia, DPP-4 activity, soluble NADPH oxidase-derived peptide activity, 8-iso-PGF2α, platelet $p47^{phox}$ phosphorylation and elevates insulin and GLP-1	[341]
Low-fat diet *vs* high in saturated fat (butter) *vs* high in monounsaturated fat (EVOO) diets	8 *women* and 5 *men* with type 1 DM	RCT crossover design	The addition of EVOO attenuates the early postprandial glucose response	[342]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
Lunch + EVOO	17 men and 13 women patients with impaired fasting glucose	RCT crossover design	Lunch + EVOO reduces glucose, TG, ApoB-48, and DPP4 activity and increases insulin and GLP1. Chol and HDL do not change	[303]
Lunch + EVOO	*12 healthy men* and *13 healthy women*	RC crossover design	Lunch + EVOO decreases postprandial glucose and LDL	[343]
Lunch + EVOO vs lunch + corn oil	Healthy subjects *(12 men and 13 women)*	RCT crossover design	Lunch + EVOO ameliorates postprandial oxidative stress and endothelial dysfunction being lunch + corn oil ineffective	[344]
Lunch + EVOO	30 patients with impaired fasting glucose	RC crossover design	Lunch+EVOO attenuates the increase of oxidative stress and in LPS	[345]
Lunch + EVOO	Subgroup of the PREDIMED study, *110 women* with metabolic syndrome	Multicenter, controlled parallel group	MedDiet + EVOO decreases urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine and prostanoids	[346]
MedDiet + EVOO *vs* MedDiet + nuts *vs* MedDiet with advice to use low fat	7477 individuals (57% women) at high CV risk	Randomized multicenter PREDIMED study testing the MedDiet in primary CV prevention	MedDiet + EVOO and MedDiet + nuts reduce the incidence of major CV events by approximately 30% *vs* the control diet	[347]
MedDiet + EVOO *vs* MedDiet + nuts *vs* MedDiet with advice to use low fat	2292 (*1343* women) patients with high CV risk 2210 (*1200* women) 2203 (*1323* women)	Post hoc analysis of the PREDIMED study	MedDiet + EVOO reduces the risk of atrial fibrillation	[348]
MedDiet + EVOO *vs* MedDiet + nuts *vs* MedDiet with advice to use low fat	351 men and women with DM2 or CV risk ≥3	A subgroup of PREDIMED study	MedDiet + EVOO decreases the BW and changes fat distribution	[349]
MedDiet + EVOO *vs* MedDiet + nuts *vs* MedDiet with advice to use low fat	*Men* and *women* (3541 patients) at high CV risk	PREDIMED study	The MedDiet + EVOO reduces DM2 risk among persons with high CV risk	[350]
MedDiet + EVOO *vs* MedDiet + nuts *vs* MedDiet with advice to use low fat	3230 *men* and *women* with DM2	PREDIMED study	MedDiet + EVOO may delay the introduction of glucose-lowering medications	[351]
MedDiet + EVOO *vs* MedDiet + nuts, low-fat diet	Old *men* and *women*	PREDIMED study	MedDiet especially if supplemented with EVOO changes the transcriptomic response of genes related to CV risk	[352]
MedDiet + EVOO *vs* MedDiet + nuts, low-fat diet	Old *men* and *women*	PREDIMED study	Both diets decrease IL-6, IL-8, MCP-1, and MIP-1β. MedDiet + EVOO decreases IL-1β, IL-5, IL-7, IL-12p70, IL-18, TNF-α, IFNγ, GCSF, GM-CSF, ENA78, E-selectin, and sVCAM-1 *vs* the MedDiet + nuts group	[353]

Table 3: Continued.

Compounds	Individuals	Design	Main data	References
MedDiet + EVOO *vs* MedDiet + nuts, low-fat diet	160 (*74 men* and *86 women*) with high CV risk	PREDIMED study subgroup	Both diets reduce CRP, IL-6, TNF-α, and MCP-1. After 3 years, both reduce CD49d and CD40 expressions in T lymphocytes and monocytes and increase HDL but decrease Chol, LDL, TG, and BP. At 5 y, low-fat diet increases glucose and glycated hemoglobin	[354]
MedDiet *vs* MedDiet + EVOO MedDiet + corn oil	12 *men* and 13 *women*	RC crossover design	EVOO but not corn oil counteracts the upregulation of NOX2 protecting from postprandial oxidative stress	[344]
MedDiet rich in OO	805 patients (*sex not reported*) with CHD, who had their last coronary event more than 6 months before enrolment, stratified in diabetes and prediabetes	Prospective, randomized, single-blind, controlled trial (CORDIOPREV)	MedDiet rich in OO improves endothelial function in patients with prediabetes and DM *vs* low-fat diet	[355]
Leaf extract	60 prehypertensive men	Double-blind, RC crossover design	It reduces plasma TC, LDL, TAG, HDL, Chol/HDL ratio, IL-8. It does not affect oxLDL, CRP, adiponectin, ICAM-1, VCAM-1, P-selectin, E-selectin, IL-6, IL-10, IL-1β, TNF-α, fasting glucose, insulin, fructosamine or calculated HOMA-IR or QUICKI indices, nitrites. It reduces SBP and DBP	[356]
Leaf extract	*9 male* and *9 female* healthy volunteers	Double-blind, RC crossover design	It modulates positively vascular functions and IL-8 production	[357]
Leaf extract	46 participants *(sex not reported)*	Double-blinded RC, placebo-controlled trial	It improves insulin secretion and sensitivity and increases IL-6, IGFBP-1, and IGFBP-2. It does not affect IL-8, TNF-α, CRP, lipid profile, BP, body composition, carotid intima-media thickness, or liver function	[358]
Leaf extract	152 patients with stage-1 hypertension (85.4% and 87.6% *women* in OO and captopril groups, respectively)	Double-blind RC	Leaf extract and captopril reduce SBP and DBP in a similar manner. Only leaf extract reduces TG	[359]

Another crucial risk factor for CVD is hypertension [385], a condition that presents numerous sex differences [386]. After 4 years of follow-up, results of interventional and randomized PREDIMED study show no significant variations in systolic blood pressure (SBP), whereas DBP is decreased in EVOO and EVOO + nuts MedDiet [387]. The 1-year trial that examines 235 subjects (56.5% women) proves that MedDiet supplemented with either EVOO or mixed nuts reduces SBP and DBP [388]. A meta-analysis, which includes primary and secondary prevention trials proves that high MPC OO slightly reduces SBP and oxLDL compared to low MPC OO, leaving Chol, TG, MDA, and DBP unchanged [389]. A very small decrease in blood pressure is observed in MedDiet + EVOO or nut *vs* a low-fat control group [390]. Finally, the meta-analysis of RTC of PREDIMED shows that the MedDiet lowers SBP by 3.02 mm Hg and DBP by 1.99 mm Hg [391]. Importantly, a systemic review that includes primary prevention proves the importance of pharmaceutical form because only liquid oil but not capsule with oil significantly reduces DBP [392].

OO impacts on glucose metabolism, two meta-analyses, which include cohort and interventional studies in prevention and care of DM2 [380, 393], prove that there is a 16% risk reduction in people that consume more OO with high

amount of MPCs *vs* those who consume OO with small amounts of MPCs. In patients with DM2, OO supplementation reduces HbA1c, fasting plasma glucose and inflammatory biomarkers, compared to controls [380]. In addition, MedDiet and MedDiet + EVOO + nuts reduce metabolic syndrome and insulin resistance in the postpartum [394, 395].

Indeed, a systemic review and meta-analysis, which includes RC trials that examine lipid profile, inflammation, and oxidative stress biomarkers in individuals that consume low MPC OO and high MPC OO, observed the improvement in MDA, oxLDL, Chol, and HDL. The subgroup analyses and individual studies measure additional improvements in inflammatory markers and blood pressure. Nevertheless, the authors conclude that there is a need for longer-term studies in non-Mediterranean populations because most studies were rated as having low-to-moderate risk of bias [382]. A recent meta-analysis, including RC trials for more than 3 weeks and examining at least two of the following OO: refined OO, mixed OO, low MPC EVOO, and high MPC EVOO, suggests that it is not possible to reach any clear conclusion for the beneficial effects [3]. Moreover, in line with what was observed with prescription drugs [39], a gender gap exists because the majority of clinical studies are performed mainly on males, and if they include females, results are not stratified for sex. This leads to low scientific value of the results in consideration of the numerous sex differences observed in CVD, DM, and hypertension (Table 1).

7. Conclusions

To have a clear conclusion, it is important to harmonize study design. For example, it will be important to declare whether the goal is the use of OO as a supplement or as a part of dietary pattern. If it is given as a supplement, it is important to consider the pharmaceutical form (liquid, capsule, and excipients) because this could modify both the pharmacokinetics and pharmacodynamics. Furthermore, considering the prevention and therapy of non-communicable diseases such as CVD and DM, there is a need for long-term studies that consider also a sufficient number of extra-Mediterranean people and low-risk populations (most of the trials are conducted on high-risk populations and this could result in underestimation of possible benefits on low-risk populations [396]).

Considering the great sex differences observed in CVD (Table 1) and in DM [32, 39, 397] and the possible sex-divergent effects of MPCs [25, 26, 398, 399], it is necessary to enroll males and females in studies, to overcome the sex and gender gap that pervades all the research in the field of the OO, VOO, EVOO, leaf extracts, and MPCs. In the era of personalized medicine, it is mandatory to consider the sex and gender aspects to answer a multiplicity of questions regarding the effects of diet and specific diet components on health and to relieve consumer uncertainty and promote health, comprehensive cross-demographic studies using the latest technologies, which include foodomics, integrated omics approaches, personomics, and appropriate study design.

Abbreviations

ACC: Acetyl-CoA carboxylase
ACE: Angiotensin converting enzyme
PI3 kinase: Phosphatidylinositol 3-kinase/Akt
ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs (aggrecanase)
AMPK: AMP-activated protein kinase
AP-1: Activator protein-1
AR: Androgen receptor
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
Chol: Cholesterol
COX: Cyclo-oxygenase
CRP: C reactive protein
CVD: Cardiovascular disease
DPP4: Dipeptidyl-peptidase-4
DPHH: 1,1-Diphenyl-2-picrylhydrazyl radical
ERK: Extracellular regulated mitogen-activated protein kinase
EDHF: Endothelium-derived hyperpolarization factor
EFSA: The European Food Safety Authority
eNOS: Endothelial nitric oxide synthase
ET: Endothelin
ET-1: Endothelin receptor-1
EGFR: Epidermal growth factor receptor
EET: Epoxyeicosatrienoic acid
ERK, PI3K/Akt/ FOXO3a: Phosphoinositide 3-kinase/Akt/ Forkhead box O3
FAS: Fatty acid synthase
FPPS: Farnesyl diphosphate synthase
GCL: Glutamate-cysteine ligase
GIP: Glucose-dependent insulinotropic polypeptide
GLP-1: Glucagon-like peptide-1
GM-CSF: Granulocyte-macrophage-colony-stimulating factor
GPx-1: Glutathione peroxidase 1
17-beta-HSD: 17-beta-hydroxysteroid dehydrogenase
HEL60: Promyelocytic leukemia cells
HMEC-1: Human microvascular endothelial cell line
HIF-1α: Hypoxia-inducible factor-1
ICAM: Intercellular adhesion molecule-1
iNOS: Inducible nitric oxide synthase
IL: Interleukin
JNK: c-Jun N-terminal kinase
LPS: Lipopolysaccharide
LPL: Lipoprotein lipase
LTB4: Leukotriene B4
IRF-1: Interferon regulatory factor-1
MDA: Malondialdehyde
MIF: Macrophage migration inhibitory factor
MMP: Matrix metalloproteinases
MAPK: Mitogen-activated protein kinases
MCP-1: Monocyte chemoattractant protein
MIP-1α: Macrophage inflammatory protein-1α

MPC: Minor polar compound
MPO: Myeloperoxidase
EGFR: Epidermal growth factor receptor
miRNAs: Micro-ribonucleic acids
NADPH oxidase: Nicotinamide adenine dinucleotide phosphate oxidase
NEP: Neutral endopeptidase
NO: Nitrogen oxide
NF-κB: Nuclear factor-kappa B
Nrf2: Nuclear factor E2-related factor 2
oxLDL: Oxidized low-density lipoprotein
OH-1: Heme oxygenase-1
PAI-I: Plasminogen activator inhibitor-1
PI3: Phosphatidylinositol 3-kinase/Akt
PMA: Phorbol myristate acetate
PGI_2: Prostacyclin
PPAR: Peroxisome proliferator activated receptor
PPARγ coactivator-1α: Peroxisome proliferator activated receptor coactivator γ-1α
ROS: Reactive oxygen species
mTOR: Mammalian target of rapamycin
TXA_2: Thromboxane A_2
TXB_2: Thromboxane B_2
TRPA1: Transient receptor potential cation channel subtype A1
SIRT: Sirtuin
SREBP-1c: Sterol regulatory element binding protein 1c
STZ: Streptomycin
TG: Triacylglycerol
VCAM-1: Vascular cell adhesion molecule-1
VEGF: Vascular endothelial growth factor
VSMC: Vascular smooth muscle cells
Akt: Protein kinase B
CBS: Cystathionine β-synthase
CD: Cluster of differentiation
CSE: Cystathionine γ-lyase
EGFR: Epidermal growth factor receptor
FMO3: Flavin containing monooxygenase 3
p-Akt: Phosphorylated Akt
p-ERK: Phosphorylated.

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

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Research Article

Isoliquiritigenin Inhibits Atherosclerosis by Blocking TRPC5 Channel Expression

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Isoliquiritigenin (ISL) is a flavonoid isolated mainly from the licorice plant, a traditional Chinese herb. ISL has shown anticancer, anti-inflammatory, antioxidant, and antidiabetic activities. However, the pharmaceutical effects of ISL on atherosclerosis are seldom explored. In this study, we used apolipoprotein *E* (ApoE) knockout mouse model and angiotensin II- (Ang II-) stimulated vascular smooth muscle cells (VSMCs) to elucidate the pharmacological mechanism of ISL to inhibit atherosclerosis. We found that in ApoE$^{-/-}$ mice ISL could attenuate atherosclerotic lesion, reduce serum lipid levels, and inhibit TRPC5 expression. In vitro, ISL inhibited Ang II-stimulated proliferation of VSMCs and suppressed Ang II-induced TRPC5 and PCNA expressions in a dose-dependent fashion. In conclusion, our findings provide novel insight into the pharmacological effects of ISL on atherosclerosis and suggest that ISL is beneficial for cardiovascular protection.

1. Introduction

Isoliquiritigenin (ISL) is a flavonoid compound from *Glycyrrhiza glabra*, the licorice plant of the traditional Chinese herb [1]. Studies have shown that isoliquiritigenin has a variety of activities such as anticancer [2], antibacterial [3], antivirus [4], antiasthma [5], anti-inflammatory [6], antidiabetic [7], and antioxidant activities [8]. Furthermore, ISL could reduce low-density lipid (LDL) via antioxidant activity [9]. In addition, ISL has been shown to inhibit the proliferation and induce the apoptosis of tumor cells [10–13].

Vascular smooth muscle cells (VSMCs) play an important role to maintain vascular tension and basic physiological functions in tunica media. In the development of atherosclerosis, VSMCs can transform to dedifferentiated phenotype after intimae damage and have increased the ability of proliferation and migration [14]. The phenotype change of VSMCs participates in the process of plaque forming and atherosclerosis acceleration [15]. However, whether ISL could suppress the proliferation of VSMCs remains obscure.

As an important second messenger, Ca^{2+} is controlled by different types of Ca^{2+} channels and transporters in the membrane of VSMCs. Among them, store-operated channels (SOCs) can activate the transcription of early response genes and affect the proliferation, migration, and synthesis of excessive extracellular matrix [16]. Transient receptor potential canonical 5 (TRPC5) channel is a representative of SOCs mainly localized in VSMCs and is activated by IP_3 (inositol 1,4,5-trisphosphate) to induce slow and continuous calcium influx [17]. Interestingly, a recent study reported that ISL induced vasodilation by activating Ca^{2+}-activated K^+ channels in VSMCs [18]. Therefore, we hypothesized that ISL may modulate the TRPC5 channel to regulate the proliferation of VSMCs. In this study, we used the apolipoprotein *E* (ApoE) model and angiotensin II- (Ang II-) induced VSMCs model to elucidate the pharmacological mechanism of ISL to inhibit atherosclerosis.

2. Materials and Methods

2.1. Animal Model. All animal experiments were approved by the Animal Care and Use Committee of Binzhou Medical University. C57BL/6J mice and ApoE knockout C57BL mice (male, 6-week-old) were purchased from the Laboratory Animal Center of Peking University (Beijing, China). The mice were bred and maintained in barrier facilities at 24°C–26°C with 12 h light/12 h dark cycle. All mice were treated following the Chinese Institutes of Health Guide for the Care and Use of Laboratory Animals. ApoE$^{-/-}$ mice were randomly divided into 2 groups ($n = 20$): model group and ISL group, and fed with a regular chow diet (21% fat + 0.15% cholesterol). ISL group mice were lavaged with ISL (20 mg/Kg/d). C57BL/6J mice were chosen to be the control group ($n = 20$), which were fed ordinary food. All the mice were killed by euthanasia after 12 weeks.

2.2. Reagents. ISL (MB2209, Dalian, China) was purchased from Melone and dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 0.25 mol/l. Then, it was diluted to the final concentrations in a culture medium, and DMSO final concentration was <0.1% (v/v) to avoid its toxic effect on the growth of cells. Primary antibodies for TRPC5 and MOMA-2 were from Abcam, α-SMA antibody was from Bioss (China), proliferating cell nuclear antigen (PCNA) antibody was from Affinity, and GAPDH antibody was from Epitomics.

2.3. Blood Lipid Analysis. The retro-orbital plexus method was used to obtain blood samples. Serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by using commercial enzymatic methods with the kits on RX-30 device (Nihon Denshi, Tokyo, Japan).

2.4. Histology and Morphometric Analyses. The aortic roots were collected and stored at −80°C. Samples were sliced into 25 sections (10 μm thick). The sections were stained with hematoxylin-eosin, and plaque area (PA), luminal area (LA), and the percentage of corrected plaque area (PA/LA) were measured using Image-Pro Plus software. In addition, the sections were subjected to immunohistochemical (IHC) staining with TRPC5 and integrated option density (IOD) of TRPC5 staining was measured using Image-Pro Plus software.

2.5. Cell Culture. VSMCs were isolated from C57BL/6J mice thoracic aorta as described (Rodriguez et al., 2007). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose (HyClone, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, USA) in a humid atmosphere with 5% CO_2 at 37°C. VSMCs were characterized by α-actin immunocytochemistry assay (Bioss, Beijing, China). Cells from generations 4–9 were used for the experiments. VSMCs were divided into 5 groups: control group (treated with 2% FBS), Ang II 10^{-6} mol/l group (treated with angiotensin II at 10^{-6} mol/l), Ang II 10^{-6} mol/l + 10 μmol/l ISL group, Ang II 10^{-6} mol/l + 20 μmol/l ISL group, Ang II 10^{-6} mol/l + 40 μmol/l ISL group, and Ang II 10^{-6} mol/l + 60 μmol/l ISL group.

2.6. Cell Proliferation Assay. VSMCs were seeded at 2,000 cells/well in 96-well plates overnight. After the treatment, cells were incubated with 10 μl/ml CCK8 for 4 h, and then the absorbance at 450 nm was measured using a reader.

2.7. PCR. Total RNA was extracted from aorta using Trizol. Real-time PCR was performed using SYBR Green on a Rotor-Gene 3000 Run StepOnePlus™ Real-Time PCR System (Corbett, Australia). The sequences of primers were as follows: TRPC5, forward 5′-ACAAAAAGGTCAACTACTCACCG-3′, reverse 5′-CAGTGGCATAGTCCCCCTTCT-3′; GAPDH, forward 5′-AACTGCTTAGCACCCCTGGC-3′, reverse 5′-ATGACCTTGCCCACACAGCCTT-3′. Quantitative measurements were determined using the ΔΔCt method and GAPDH was used as an internal control.

2.8. Western Blot Analysis. Proteins were extracted and 40 μg from lysates per lane was loaded on a 10% SDS-polyacrylamide electrophoresis gel and then electrophoresed and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blotted with specific antibodies against TRPC5, PCNA, and GAPDH at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactivity was detected by using the enhanced chemiluminescence (ECL) method. Protein content was calculated by densitometry using LabWorks software.

2.9. Statistical Analysis. All data are presented as mean ± SD and analyzed by SPSS 13.0 software. For comparison among groups, one-way ANOVA was applied, followed by LSD test. $P < 0.05$ was regarded as significant.

3. Results

3.1. ISL Improved the Health Condition in ApoE$^{-/-}$ Mice. The general condition of the control group was the best in all mice. ApoE$^{-/-}$ mice in the AS model group showed the lowest growth and the weight increased faster (Figures 1(a) and 1(b)). Compared to the AS model group, ApoE$^{-/-}$ mice in the ISL group had better body mass index (BMI) at 12–18 weeks (Figure 1(c), $P < 0.05$).

3.2. ISL Improved Blood Lipids and Atherosclerosis Lesion in ApoE$^{-/-}$ Mice. HE staining indicated that the atherosclerosis model group had unstable plaques which were diffusing all the lumen of aortas, but they were improved in the ISL group (Figure 2(a)). Serum levels of TC, TG, and LDL-C were the highest while HDL-C level was the lowest in atherosclerosis model group. TC, TG, and LDL-C levels significantly

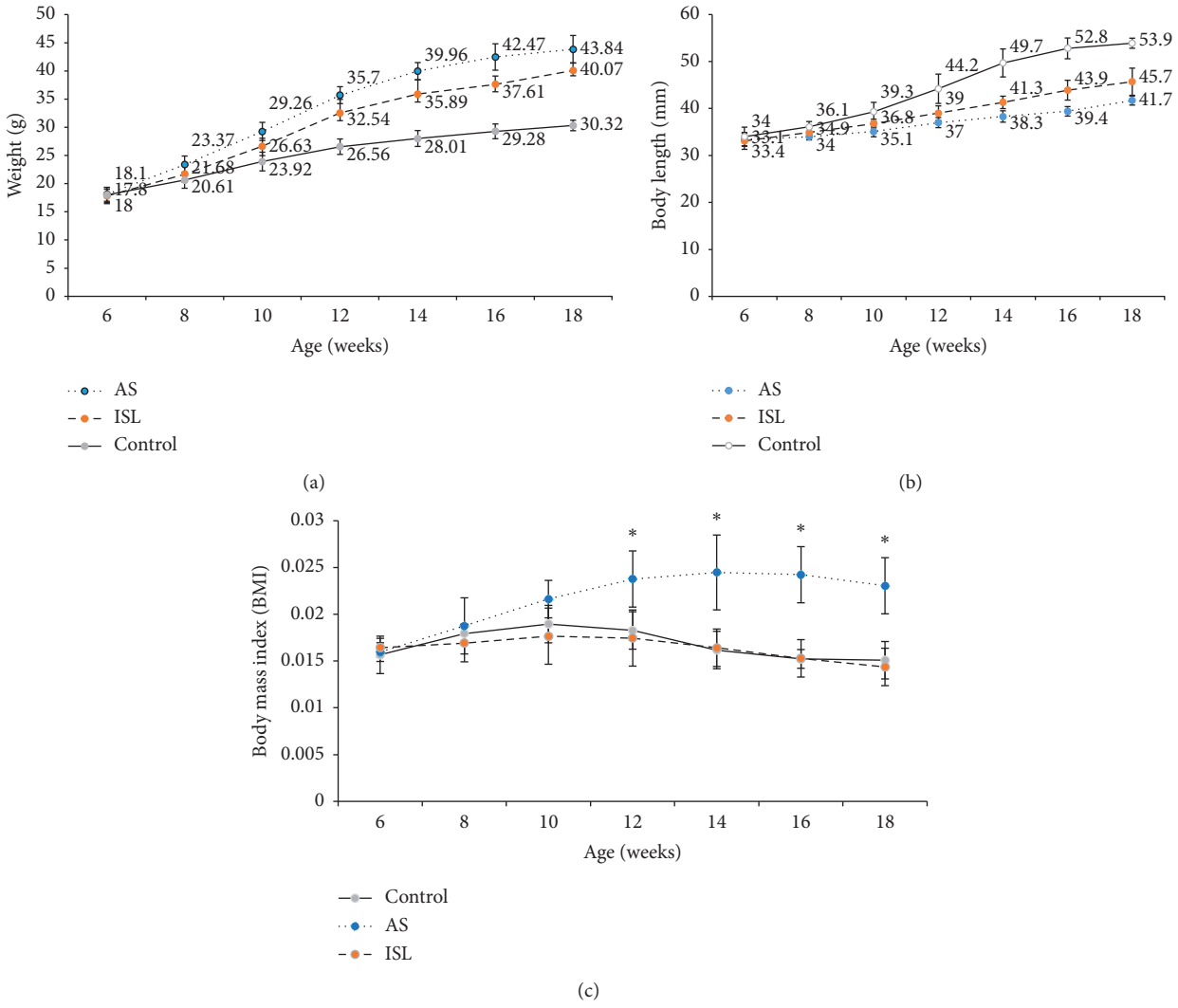

Figure 1: Body weight and length and body mass index of mice in three groups ($n = 20$). (a) Body weight in different groups of mice of different ages. (b) Body length in different groups of mice of different ages. (c) Body mass index (BMI) in different groups of mice of different ages. Control: control group; AS: atherosclerosis group; ISL: isoliquiritigenin group. $^*P < 0.05$ compared to AS at the age of 12, 14, 16, and 18 weeks.

declined and the HDL-C level increased in the ISL group compared with the model group (Figure 2(b)). The aortic intima of the model group was thickened, and plaque formation, significant luminal stenosis, more lipid pools, and meager fibrous caps within plaques were observed in the model group. In contrast, these lesions were slight, plaque areas were small, lipid pools were thin, and fewer foam cells and inflammatory cells were found in the ISL group, and plaque area was reduced (Figures 2(c)–2(e)).

3.3. ISL Inhibited TRPC5 Expression in Atherosclerosis Model Mice. Immunohistochemical analysis showed that TRPC5 was located both in tunica media and in VSMCs which migrated into artery intima (Figures 3(a) and 3(b)). TRPC5 staining was significantly stronger in the model group compared to the control group but was significantly weaker in the ISL group compared to the model group (Figure 3(c)). PCR analysis confirmed that the relative TRPC5 mRNA level was significantly higher in the model group compared to the control group but was significantly lower in the ISL group compared to the model group (Figure 3(d)).

3.4. ISL Inhibited Ang II-Induced Proliferation of VSMCs. We isolated VSMCs from C57BL/6J mice aorta and immunocytochemistry confirmed the identification of VSMCs (Figure 4(a)). CCK8 assay showed that VSMCs proliferation was activated by angiotensin II. However, Ang II-stimulated VSMCs proliferation was inhibited by ISL in a concentration-dependent manner (Figure 4(b)).

3.5. ISL Inhibited TRPC5 and PCNA Expression in Ang II-Stimulated VSMCs. Real-time PCR showed that Ang II

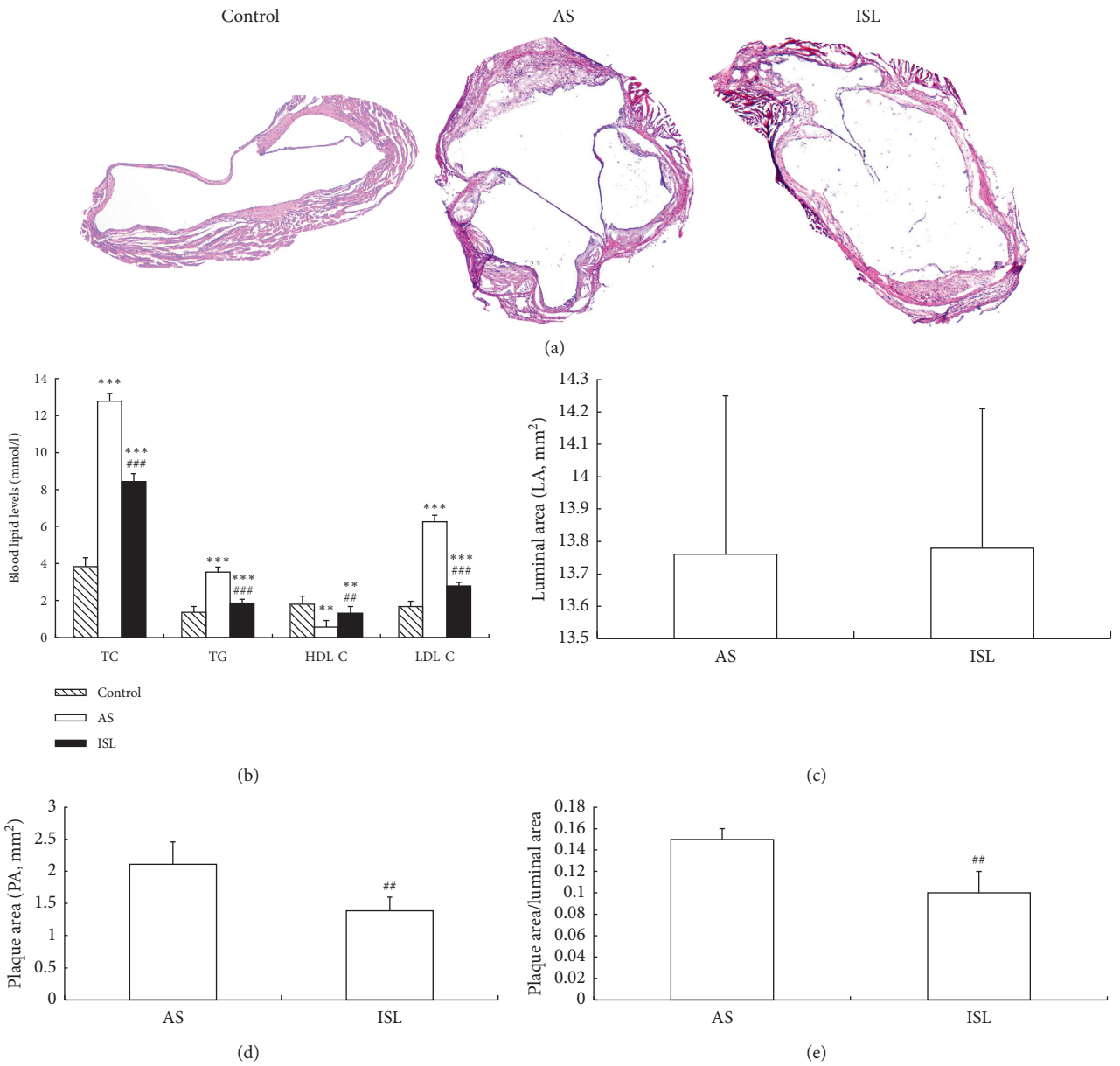

FIGURE 2: Histology and morphometric analyses of atherosclerotic lesion ($n = 20$). (a) HE staining of aortic tissues (×40). No obvious aortic lesion was observed in control group. AS group showed unstable plaques which were diffusing all the lumen of aortas, but they were improved in ISL group. (b) The levels of blood lipids (unit as mM) in different groups. (c) Comparison of luminal area (LA, unit as mm^2) in AS and ISL groups. (d) Comparison of plaque area (PA, unit as mm^2) in AS and ISL groups. (e). Comparison of the ratio of PA/LA in AS and ISL groups. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control group; $^{\#\#}P < 0.01$, compared to AS group. Control: control group; AS: atherosclerosis group; ISL: isoliquiritigenin group.

significantly induced the expression of TRPC5 mRNA in VSMCs, but ISL reduced Ang II-stimulated TRPC5 mRNA expression (Figure 5(a)). Western blot analysis showed that Ang II significantly induced the expression of TRPC5 protein in VSMCs, but ISL reduced Ang II-stimulated TRPC5 protein expression (Figures 5(b) and 5(c)). In addition, Ang II significantly induced the expression of PCNA protein in VSMCs, but ISL reduced Ang II-stimulated PCNA protein expression (Figure 5(d)).

4. Discussion

Glycyrrhiza glabra (Licorice) is a traditional medicinal herb widely used with many pharmaceutical effects [19]. ISL is a flavonoid compound isolated from this plant and has a variety of activities [20]. However, the pharmaceutic effects of ISL on atherosclerosis have not been explored. Therefore, in this study, we chose ApoE knockout mice as an atherosclerosis model to investigate the anti-atherosclerosis effects of ISL.

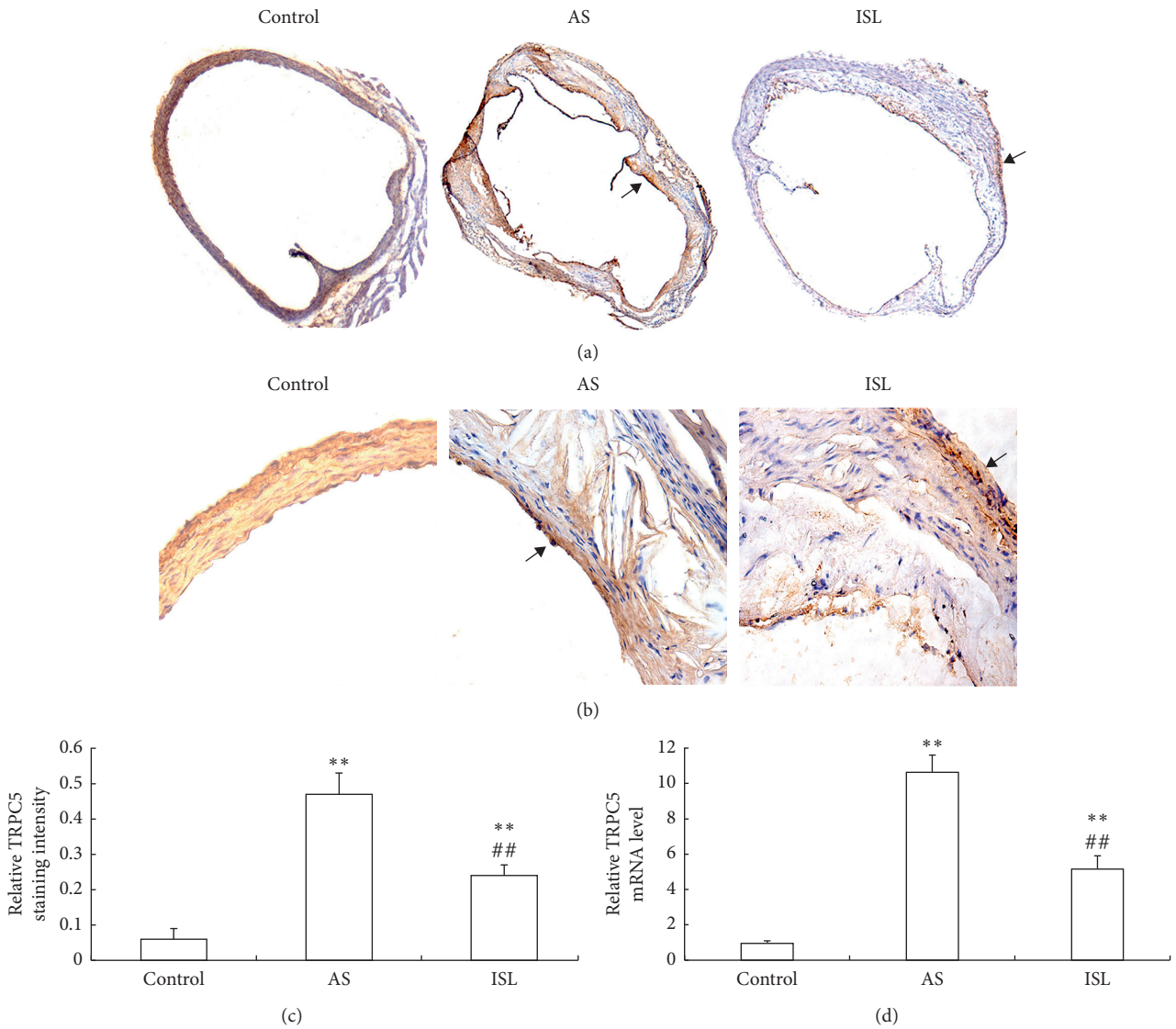

FIGURE 3: TRPC5 expression in mice of three groups. (a) TRPC5 staining in aortas of each group (×40). Staining area was indicated by the arrows. (b) TRPC5 staining in aortas of each group (×400). Staining area was indicated by the arrows. (c) Comparison of TRPC5 staining intensity in different groups. (d) Comparison of TRPC5 mRNA levels in different groups. $^{**}P < 0.01$, compared to control group; $^{\#\#}P < 0.01$, compared to AS group. Control: control group; AS: atherosclerosis group; ISL: isoliquiritigenin group.

In the present study, we found that ISL could regulate blood lipids of ApoE$^{-/-}$ mice. A previous study showed that licorice flavonoid oil could regulate the expression of lipid metabolism-related genes to ameliorate hyperlipidemia of C57BL/6J mice [21]. Our results showed that ISL significantly improved the weight and blood lipids in ApoE$^{-/-}$ mice fed with a high-fat diet. Furthermore, ISL could inhibit TRPC5 expression not only in high-fat diet-induced atherosclerosis model but also in primary VSMCs stimulated by angiotensin II.

It is known that phenotype changes in VSMCs promote the formation of atherosclerosis [22, 23]. Proliferation and migration of VSMCs are critical for plaque formation and atherosclerosis development. TRPC5 is the main subtype of store-operated channels in the aorta and can be activated by inositol 1,4,5-triphosphate (IP3), leading to continuous calcium influx [17]. TRPC5 could regulate the function of VSMCs [24, 25]. Oxidized low-density lipoprotein (ox-LDL), a risk factor accelerating atherosclerosis, was found to promote VSMCs proliferation and migration, and TRPC5 channels were sensitive to antioxidant [26]. In ApoE$^{-/-}$ mice, blood lipid deposits on the aorta intima increased ox-LDL to stimulate TRPC5 overexpression. In vitro, angiotensin II induced VSMCs proliferation, and internal Ca^{2+} could stimulate TRPC5 channels directly [27]. In our present study, we found that ISL significantly downregulated TRPC5 and PCNA expression in a dose-dependent manner. Thus, we speculate that ISL may inhibit VSMCs proliferation by downregulating TRPC5. However, our study has limitations in that we did not further investigate the signaling pathways which may be responsible for mediating the inhibitory effects of ISL on TRPC5 expression.

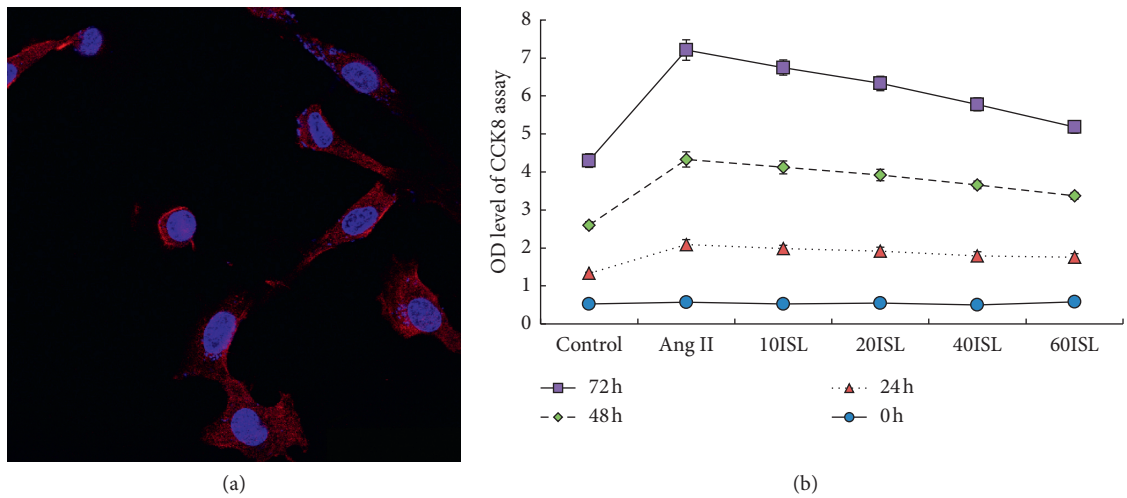

Figure 4: ISL inhibited the proliferation of VSMCs. (a) Identification of VSMCs isolated from C57BL/6J mice aortas (staining ×400). (b) CCK8 assay of the proliferation of VSMCs. Values are expressed as means ± SD ($n = 5$). Control: control group; Ang II: Ang II group; 10ISL: Ang II 10^{-6} mol/l + 10 μmol/l ISL group; 20ISL: Ang II 10^{-6} mol/l + 20 μmol/l ISL group; 40ISL: Ang II 10^{-6} mol/l + 40 μmol/l ISL group; 60ISL: Ang II 10^{-6} mol/l + 60 μmol/l ISL group.

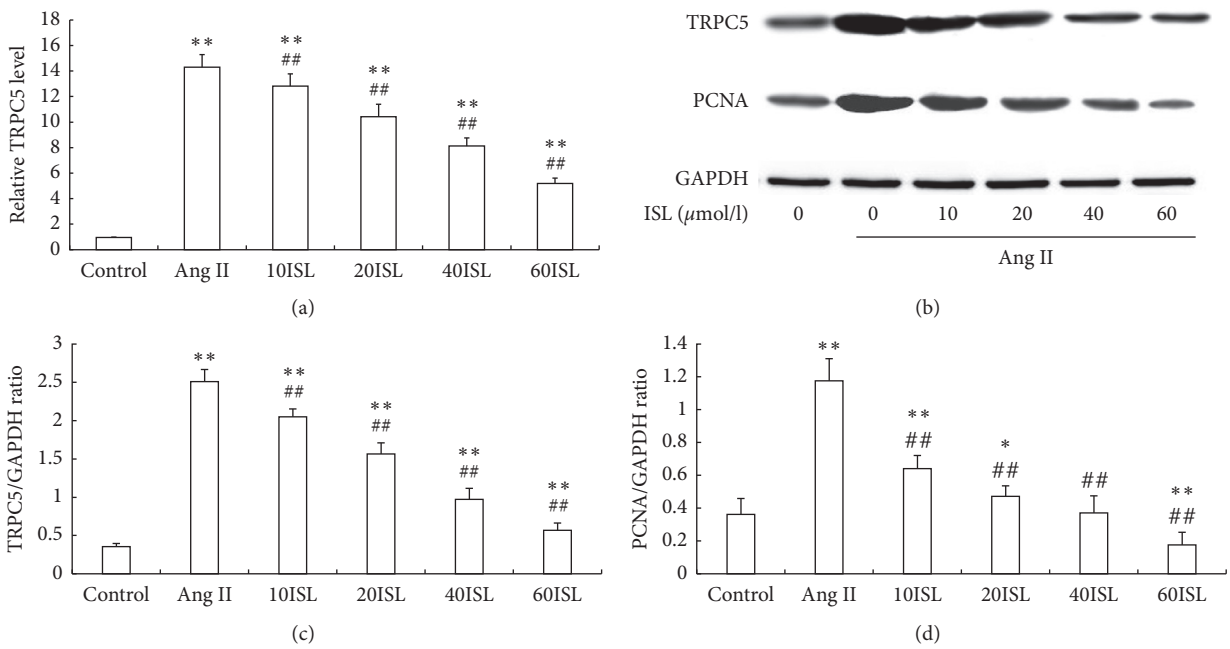

Figure 5: ISL inhibited TRPC5 and PCNA expressions in VSMCs. (a) The mRNA levels of TRPC5 in different groups. (b) The protein levels of TRPC5 and PCNA were determined by Western blot analysis. GAPDH was loading control. The representative images were shown from 5 independently performed tests. (c) Densitometry analysis of TRPC5 protein expression. (d) Densitometry analysis of PCNA protein expression. Values are expressed as means ± SD ($n = 5$). $^{**}P < 0.01$, compared to control group; $^{\#\#}P < 0.01$, compared to Ang II group. Control: control group; Ang II: Ang II group; 10ISL: Ang II 10^{-6} mol/l + 10 μmol/l ISL group; 20ISL: Ang II 10^{-6} mol/l + 20 μmol/l ISL group; 40ISL: Ang II 10^{-6} mol/l + 40 μmol/l ISL group; 60ISL: Ang II 10^{-6} mol/l + 60 μmol/l ISL group.

Interestingly, it was reported that ISL could inhibit NF-κB and mitogen-activated protein kinases (MAPK) signal pathways [28]. It has been confirmed that the activation of NF-κB and MAPK pathways accelerates the atherosclerosis process and promotes VSMCs proliferation and migration [29]. Oxidative stress and calcium influx may also affect TRPC5 expression. Therefore, further studies are needed to reveal the mechanisms by which ISL modulates TRPC5 expression and inhibits atherosclerosis.

In conclusion, we demonstrate that ISL inhibits TRPC5 overexpression not only in a high-fat diet-induced atherosclerosis mouse model but also in primary VSMCs stimulated by angiotensin II. Furthermore, ISL improved atherosclerosis in the mouse model and inhibited the

proliferation of primary VSMCs. These findings provide novel insight into the pharmacological effects of ISL on atherosclerosis and suggest that ISL is beneficial for cardiovascular protection.

Data Availability

All data are available upon request from the corresponding authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jie Qi and Jianguo Cui contributed equally to this work.

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