Oxidative Medicine and Cellular Longevity

Polyphenols, Oxidative Stress, and Metabolic Syndrome

Lead Guest Editor: Chong Tian
Guest Editors: Lei Hao, Weijie Yi, Shibin Ding, and Fangyi Xu

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Editorial

Polyphenols, Oxidative Stress, and Metabolic Syndrome

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Metabolic syndrome (MetS), referring to a series of metabolic abnormalities, including central obesity, hyperglycemia, dyslipidemia, and hypertension, is common soil for many kinds of chronic diseases. Oxidative stress, which is commonly associated with MetS, has been proven to contribute to diabetes, atherogenesis, hypertension, and other cardiovascular diseases. Increasing evidence showed that polyphenols, a group of antioxidative phytochemicals, could protect cells against oxidative damage and, therefore, reduce the risk of associated diseases.

The objective of this special issue is to present how polyphenols protect against MetS and related disorders. Five among the many papers submitted were included after a thorough peer review process.

Nuts are polyphenol-rich but also hypercaloric food. L. Di Renzo et al. conducted a prospective pilot clinical trial and showed that extra calorie intake through hazelnuts leads to favorable blood lipid changes but not weight gain. Investigation of genomic response demonstrated that upregulation of genes implied in oxidative reactions and inflammation may be the mechanism underneath. This pilot study implied that nuts, although calorie dense, could be included in weight loss and anti-inflammatory diets.

Due to the wide-spanning and diverse distribution of polyphenols in foods, self-reported dietary intake suffers from errors. Meanwhile, validated *in vivo* biomarkers of polyphenols are still lacking. Precise assessment of polyphenol intake remains a challenge in epidemiological and clinical researches in this area. R. Yang et al. developed and validated a targeted metabolomics method by LC/MS/MS for the measurement of twenty-two polyphenol biomarkers in urine samples. The levels of the biomarkers were inversely associated with overweight and obesity. The research offered alternative polyphenol biomarkers and a complementary addition to self-reported survey for the evaluation of polyphenol intake.

Indoxyl Sulfate (IS), a uremic toxin derived from tryptophan metabolite by gut microbiota, is capable of exerting preoxidant effects on the cardiovascular system and accelerate vascular dysfunction. E. Getachew Assefa et al. investigated the protective effect of resveratrol against endothelial hyperpermeability induced by IS. The mechanism study showed that blockade of IS-aryl hydrocarbon receptor mediates Src activation and downregulates the activity of VE-cadherin and endothelial hyperpermeability.

Abnormal systemic oxidative stress, which is closely related to systemic inflammation, endothelial dysfunction, metabolic abnormality, and DNA damage, is proposed to be the most important pathophysiology of MetS. K. Liu et al. summarized evidences of the bioprotective effects of polyphenols against MetS and reviewed related antioxidative mechanisms, which involves oxygen scavenging, oxidoreductase enzyme system balancing, antioxidant responsive signaling pathway regulating, and mitochondrial function

restoring effects. These data deepens our understanding of polyphenolic compounds and may help provide new insight in the development of future antioxidant therapeutics.

Intestinal health, especially the integrity of intestinal barrier, plays a crucial role in the whole organism. Y. Zhuang et al. demonstrated that resveratrol could directly protect IPEC-J2 cells against oxidative stress through the PI3K/Akt-mediated Nrf2 signaling pathway in an oxidative stress model induced by H_2O_2 *in vitro*, which provided important evidence of a potential cytoprotective effect of resveratrol in the intestinal barrier.

All of these studies provide readers new insight regarding the evidences and mechanisms of polyphenols reducing oxidative stress-induced damage in the contexts of MetS. We are confident that these findings have contributed to researches concerning polyphenols, oxidative stress, and metabolic syndrome.

Conflicts of Interest

The editors declare that they have no conflict of interest regarding the publication of the special issue.

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Research Article

Resveratrol Attenuates Oxidative Stress-Induced Intestinal Barrier Injury through PI3K/Akt-Mediated Nrf2 Signaling Pathway

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Oxidative stress is implicated in a wide range of intestinal disorders and closely associated with their pathological processes. Resveratrol (RSV), a plant extract, plays a vital role in protecting various organs *in vitro* and *in vivo*. However, the benefits of RSV are controversial, and underlying mechanisms for its antioxidant effects on intestinal epithelial cells remain unclear. In this study, we evaluated the effects of RSV on oxidative stress induced by H_2O_2 in IPEC-J2 cells. We found that pretreatment with RSV significantly increased cell viability; increased expression levels of tight junction (TJ) proteins (claudin-1, occludin, and ZO-1); improved activities of superoxide dismutase-1 (SOD-1), catalase (CAT), and glutathione peroxidase (GSH-Px); and decreased intracellular reactive oxygen species (ROS) levels and apoptosis induced by H_2O_2 ($P < 0.05$). In addition, RSV upregulated Akt phosphorylation, Nrf2 phosphorylation, and expression levels of antioxidant genes HO-1, SOD-1, and CAT in a dose-dependent manner ($P < 0.05$) under oxidative stress. Knockdown of Nrf2 by short-hairpin RNA (shRNA) abrogated RSV-mediated protection against H_2O_2-induced apoptosis, RSV-induced increase of TJ protein levels, and antioxidant gene expression (SOD-1, CAT, and GSH-Px) ($P < 0.05$). Consistent with Nrf2 knockdown, the PI3K/Akt inhibitor LY294002 significantly suppressed RSV-induced Nrf2 phosphorylation and RSV-induced increase of TJ protein levels and antioxidant gene expression under H_2O_2 treatment ($P < 0.05$). Collectively, these results demonstrate that RSV can directly protect IPEC-J2 cells against oxidative stress through the PI3K/Akt-mediated Nrf2 signaling pathway, suggesting that RSV may be an effective feed additive against intestinal damage in livestock production.

1. Introduction

The intestine not only is a major digestive and absorptive organ for nutrients but also functions as a selective barrier against toxins, pathogens, and antigens from the luminal environment [1]. The intestinal barrier primarily consists of tight junction proteins (TJs), enterocyte membrane, antibacterial peptides, and the mucous layer and immune system. When the intestinal barrier is disrupted, the luminal toxins and antigens will penetrate subepithelial tissues through the barrier, causing a mucosal oxidative stress and systemic inflammatory response [1]. Meanwhile, overproduction of reactive oxygen species (ROS) and proinflammatory cytokines disrupts the intestinal barrier and dysfunction.

Table 1: Sequence of hairpin RNA.

Names	Sequence of hairpin RNA
shNRF2-sense	CCGGCATACTTTGGAGGCAAGATATCTCGAGATATCTTGCCTCCAAAGTATGTTTTTG
shNRF2-antisense	AATTCAAAAACATACTTTGGAGGCAAGATATCTCGAGATATCTTGCCTCCAAAGTATG

However, due to the complex physiological and/or chemical environment of the intestine, it is extremely susceptible to oxidative stress.

Oxidative stress, defined as the imbalance between the antioxidant systems and oxidative system causing overdose of ROS, can disrupt cellular signaling and function. It is believed that oxidative stress is involved in the development of intestinal diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome, and colon cancer [2–5]. Under the physiological condition, ROS is maintained at certain levels and excessive free radicals are usually scavenged by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPH-Px). However, under the imbalance between the antioxidant and the oxidative system, overdose of ROS can disturb epithelial cell integrity and intestinal barrier by decreasing tight junctions and cell quantity [6]. Recent studies have shown that ROS or other free radicals can disturb cell functions by influencing transcription factors and the redox-sensitive signaling pathway. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that has an important regulative effect on oxidative statues through induction of the expression of the antioxidant and phase 2 detoxifying enzymes and related proteins [7, 8]. In terms of the possible importance of ROS in intestinal injury, it is essential for cells to effectively upregulate antioxidants, decrease ROS production, and scavenge free radicals, which may contribute to increased intestinal permeability and epithelial apoptosis.

Plant extracts are considered as a potential source of antioxidant and anti-inflammatory molecules which have been identified and proposed as therapeutic agents to counteract oxidative stress-related disease [9]. Resveratrol (RSV) is a plant-derived stilbene (polyphenol) associated with a wide range of health benefits [10–13]. Many studies have suggested that RSV acts on multiple cellular targets such as Nrf2, NF-Kβ, Sirt1, and AMPK to control processes and signaling pathways related to reduction of oxidative stress, apoptosis, and anticancer effects [1, 14–16]. However, scientific research on healthy effects of dietary RSV has been controversial because of low bioavailability of RSV *in vivo* [17]. The doses of RSV in target tissues are extremely low and hardly reach the level of pharmacological concentration in *in vitro* studies [18]. Even though the function of RSV is still controversial, we hypothesize that RSV can directly protect intestines from oxidative stress through its rapid metabolism in intestinal cells. Therefore, we used an *in vitro* oxidative stress model induced by H_2O_2 to investigate whether RSV can prevent intestinal impairment. Our results provide insights for the future application of RSV as feed additives against intestinal damage in livestock production.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) required for cell culture were obtained from Gibco (Carlsbad, CA, USA). Resveratrol (RSV) and LY294002 (the PI3K/Akt inhibitor) were obtained from Selleckchem (Houston, United States). The antibodies against Nrf2, Keap1, and β-actin were obtained from Abcam, the antibodies against claudin-1 and occludin from Selleckchem (Houston, United States), and the antibody against ZO-1 from Proteintech (Wuhan, China). The plasmids pLKO.1, pLKO-scramble, pCMV-DR8.9, and pCMV-VSVG were kind gifts from Prof. Pinghui Feng (University of Southern California, USA).

2.2. Cell Culture. The porcine intestinal epithelial cells (IPEC-J2 cells) and human embryonic kidney 293 (HEK293T) cells were kindly provided by Prof. Guoyao Wu (Texas A&M University, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco) and maintained at 37°C with 5% CO_2.

2.3. Establishment of Nrf2-Knockdown IPEC-J2 Cell Line. Short-hairpin RNAs (shRNAs) were designed using the online shRNA design tool (https://portals.broadinstitute.org/gpp/public/) and cloned into the lentiviral vector pLKO.01 via Age I/EcoR I. The plasmids pLKO-shNrf2 or pLKO-scramble, pCMV-DR8.9, and pCMV-VSVG were transfected into 293T cells to produce lentiviruses. At 48 hours posttransfection, the lentiviruses were harvested and used to infect IPEC-J2 cells in the presence of polybrene (8 μg/mL). At 48 hours postinfection, the infected cells were selected and maintained with puromycin (2 μg/mL). The sequences of shRNAs are shown in Table 1.

2.4. Cell Viability Analysis. Cell viability was evaluated using the CCK-8 assay (Dojindo, Kumamoto) according to the manufacturer's instructions. Briefly, IPEC-J2 cells were cultured in 96-well plates at a density of 8×10^4 cells per well. On the following day, the cells were treated with medium containing different concentrations of H_2O_2 for 4 hours and then assayed for cell viability. RSV was treated for 6 hours before H_2O_2 treatment, and LY294002 (the PI3K inhibitor) at a concentration of 25 μM was pretreated for 2 hours before H_2O_2 and RSV treatment [19].

2.5. Analysis of the Activities of T-SOD, CAT, and GSH-Px. IPEC-J2 cells were pretreated with RSV at the concentration of 0 μM, 25 μM, and 50 μM for 6 h and then treated with or without 400 μM H_2O_2 for 4 h. The cells in each

Table 2: Sequence of target gene primer.

Gene names	PubMed no.	Sequence of primer	Length (bp)
Claudin-1	XM_005670262.3	CTAGTGATGAGGCAGATGAA AGATAGGTCCGAAGCAGAT	250
Occludin	NM_001163647.2	GAGTGATTCGGATTCTGTCT TAGCCATAACCATAGCCATAG	181
ZO-1	XM_021098896.1	TTGATAGTGGCGTTGACA CCTCATCTTCATCATCTTCTAC	126
Keap1	NM_001114671.1	TCTGCTTAGTCATGGTGACCT GGGGTTCCAGATGACAAGGG	158
NFE2L2	MH101365.1	TGCAGCTTTTGGCAGAGACA AGGAGCAATGAAGACTGGGC	119
SOD-1	NM_001190422.1	AAGGCCGTGTGTGTGCTGAA AGTGGCCACACCATCTTTGC	279
HO-1	NM_001004027.1	TACCGCTCCCGAATGAACAC GTCACGGGAGTGGAGTCTTG	209
CAT	NM_214301.2	CCTGCAACGTTCTGTAAGGC GCTTCATCTGGTCACTGGCT	72
GSX-1	NM_214201.1	CCTAGCAGTGCCTAGAGTGC CGCCCATCTCAGGGGATTTT	143
β-Actin	XM_003357928.4	TGCGGGACATCAAGGAGAAG AGTTGAAGGTGGTCTCGTGG	216

Figure 1: Effects of RSV and H_2O_2 on the viability of IPEC-J2 cells. (a) IPEC-J2 cells were incubated with increasing concentrations of RSV for 6 h (a) and H_2O_2 for 4 h (b), and then cell viability was measured by CCK-8 assays. Results are presented as the percentage of cell viability compared with the control (0 μM). Values are the mean ± SE; $n = 8$. Asterisks indicate a significant difference compared to the control group ($P < 0.05$).

group were lysed to determine the activities of T-SOD, CAT, and GSH-Px using commercial assay kits (Jiancheng, Nanjing, China) according to the instructions. The activities of T-SOD, CAT, and GSH-Px were calculated and expressed as U/mg protein.

2.6. Measurement of ROS Production. The relative levels of intracellular ROS were measured using a commercial ROS detection kit (Beyotime, China). Briefly, IPEC-J2 cells were cultured in 6-well plates for 16 h before treatment, pretreated with RSV (0 μM, 25 μM, and 50 μM) for 4 hours, and then exposed with H_2O_2 (500 μM) for 4 h to induce ROS production. Cells were washed twice with PBS and then incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFHDA) for 30 min. Fluorescence intensity was measured using a fluorescence microplate reader (Tecan, Sunrise) at excitation/emission wavelengths of 525/610 nm.

2.7. Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection. Apoptosis was determined using the Annexin V-FITC cell apoptosis detection kit (KeyGEN, Nanjing, China). Briefly, IPEC-J2 cells were cultured in 6-well plates for 16 h

Figure 2: Protective effects of RSV against H_2O_2-induced oxidative injury in IPEC-J2 cells. IPEC-J2 cells were pretreated with the indicated concentrations of RSV and then cocultured with 200 μM or 500 μM H_2O_2 for 4 hours. (a) The CCK-8 assay was performed on IPEC-J2 cells after incubation with RSV (6 h) and H_2O_2 (4 h). (b, c) Apoptosis cells were analyzed by flow cytometry using Annexin V and propidium iodide (PI) double staining. Results are presented as the percentage of cell viability compared with the control (0 μM). Values are the mean ± SE. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

before treatment, pretreated with Ly294002, and then treated with RSV (0 μM, 25 μM, and 50 μM) or H_2O_2 according to the experimental design. Following each specific treatment, cells were collected, washed twice with ice-cold PBS, and then centrifuged at 2000 rpm for 5 min. Cells were resuspended in 500 μL of 1x binding buffer and transferred to sterile flow cytometry glass tubes. 5 μL of Annexin V-FITC and 5 μL of PI were added, and

Figure 3: Effects of resveratrol and/or H_2O_2 on the levels of TJ proteins. IPEC-J2 cells were pretreated with the indicated concentrations of RSV and then cocultured with 500 μM H_2O_2 for 4 hours. (a) The expression of claudin-1, occludin, and ZO-1 was detected by quantitative real-time PCR (qRT-PCR). Data are shown as ratios of abundance of target gene transcripts in the treated cells to those in the control cells after normalization to β-actin. (b) Protein levels of claudin-1, occludin, and ZO-1 were detected by Western blot with β-actin as the loading control. Values are the mean ± SE; $n = 3$. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

samples were incubated at room temperature (25°C) in dark conditions for 10 min. Finally, flow cytometric analysis was performed according to the manufacturer's instructions (Beckman, Fullerton, California, USA).

2.8. Quantitative Real-Time PCR (qRT-PCR). After the extraction of total RNA in treated cells, reverse transcription and qRT-PCR were performed as described previously. Primers were designed with the online primer design tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) according to the gene sequences of the pig, and the sequences of the primers are shown in Table 2. β-Actin was used to normalize the levels of target gene transcripts, and the relative abundance of gene transcripts was normalized to the values of the control treatment.

2.9. Western Blot Analysis. Cells were treated with the indicated agents according to the experimental design and lysed with RIPA buffer (Beyotime) to extract total cellular proteins. The concentration of proteins was quantified using a BCA protein assay kit (Beyotime, China) according to the

Figure 4: Effects of resveratrol and/or H_2O_2 on ROS levels and the activities of T-SOD, CAT, and GSH-Px in IPEC-J2 cells. IPEC-J2 cells were pretreated with RSV at the concentration of 0 μM, 20 μM, and 50 μM for 6 hours and then cocultured with 500 μM H_2O_2 for 4 hours. (a) The fluorescence intensity of ROS was measured by a fluorescence microplate reader. (b–d) Cells were pretreated with or without RSV at the indicated concentrations for 6 h and then incubated in the presence of H_2O_2 at the concentration of 500 μM, and the activities of T-SOD (b), CAT (c), and GSH-Px (d) in cell lysates were detected by a spectrophotometer using commercial kits. Values are the mean ± SE; $n = 6$. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

instructions. Western blot analysis was performed as described previously. The indicated proteins were normalized to β-actin and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

3. Statistical Analyses

Results from a representative of three independent experiments were expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the LSD test was used for multiple comparisons, and a value of $P < 0.05$ was accepted as statistically significant. The statistical analyses were performed by GraphPad Prism 7.

4. Results

4.1. Concentration-Dependent Effects of H_2O_2 and RSV on Cell Viability. To determine suitable concentrations of H_2O_2 and RSV for subsequent experiments, we treated IPEC-J2 cells with different concentrations of H_2O_2 or RSV, and measured the viability of the treated cells by CCK-8 assays. As shown in Figure 1, a high concentration of RSV showed slight inhibition on IPEC-J2 cells, and RSV significantly decreased the viability of IPEC-J2 cells at both 200 μM and 400 μM for 6 h (Figure 1(a)). Treatment with H_2O_2 for 4 h resulted in significant cell shrinkage compared to the control group. We found that a concentration of 500 μM H_2O_2 sharply decreased cell viability to 40% compared to the control (Figure 1(b)). Pretreatment of RSV (20 μM, 50 μM) significantly attenuated the decreased cell viability and cell apoptosis caused by 500 μM H_2O_2 in a dose-dependent manner (Figure 2). Based on these results, we used 500 μM H_2O_2 to induce oxidative stress and used 20 μM or 50 μM RSV in subsequent experiments.

4.2. Resveratrol Elevates the Expression of TJ mRNAs in IPEC-J2 Cells. Intestinal integrity relies on tight junctions. Tight junctions (TJs) are the principal determinants of intestinal barrier function that seal the paracellular space between neighboring epithelial cells. Here, we examined the expression of TJ mRNAs in IPEC-J2 cell monolayers treated with or without RSV and/or H_2O_2. As shown in Figure 3, qRT-PCR results revealed that the expression of claudin-1, occludin, and ZO-1 was significantly decreased in IPEC-J2 cells

Figure 5: Effects of resveratrol and/or H_2O_2 on the expression of SOD-1, CAT, GSX-1, and HO-1 in IPEC-J2 cells. IPEC-J2 cells were pretreated with the indicated concentrations of RSV for 6 hours and then cocultured with 500 μM H_2O_2 for 4 hours. The expression of SOD-1 (a), CAT (b), GSX-1 (c), and HO-1 (d) was detected by qRT-PCR. Data are shown as ratios of abundance of target gene transcripts in the treated cells to those in the control cells after normalization to β-actin. Values are the mean ± SE; $n = 6$. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

treated with H_2O_2 compared with the control group (Figure 3(a)), whereas cotreatment with RSV reversed H_2O_2-induced downregulation of TJ proteins (claudin-1, occludin, and ZO-1). The protein levels (claudin-1, occludin, and ZO-1) examined by Western blot were consistent with the mRNA levels of TJ proteins (Figure 3(b)).

4.3. Resveratrol Elevates Antioxidant Ability and the Expression of Antioxidant Genes in IPEC-J2 Cells. The activities and expression of antioxidant enzymes are the main response for relieving oxidative stress damage in organs or cells. As shown in Figure 4, no significant ROS difference was found between cells treated with RSV alone, whereas RSV significantly decreased levels of ROS in the H_2O_2-treated cells in a dose-dependent manner (Figure 4(a)). It was shown that RSV significantly elevated the activities of T-SOD, CAT, and GSH-Px in the cells treated with or without H_2O_2 (Figures 4(b)–4(d)). qRT-qPCR results revealed that high concentration of H_2O_2 significantly decreased the expression of SOD-1, GSX-1, catalase (CAT), and HO-1 genes, whereas RSV reversed the H_2O_2-induced downregulation of SOD-1, CAT, GSX-1, and HO-1 genes (Figures 5(a)–5(d)).

4.4. Resveratrol Protects IPEC-J2 Cells against H_2O_2-Induced Barrier Dysfunction via the Nrf2/Keap1 Pathway. Given that RSV is a potent antioxidative agent promoting the expression of antioxidant genes, we investigated whether RSV relieves oxidative stress through the Akt and Nrf2/Keap1 signaling pathway, which is a key cellular cascade involved in cell survival and oxidative stress response. qRT-qPCR results revealed that H_2O_2 treatment significantly decreased the expression of Nrf2 and Keap1 genes, whereas RSV significantly reversed H_2O_2-induced downregulation of Nrf2 and Keap1 genes (Figure 6(a)). Western blot analysis showed that RSV enhanced the phosphorylation of Nrf2 and Akt in a

Figure 6: Effects of resveratrol and/or H_2O_2 on the Akt and Nrf2/Keap1 pathways in IPEC-J2 cells. IPEC-J2 cells were pretreated with the indicated concentrations of RSV for 6 hours and then cocultured with 500 μM H_2O_2 for 4 hours. (a) The expression of Nrf2 and Keap1 was detected by qRT-PCR. Data are shown as ratios of abundance of target gene transcripts in the treated cells to those in the control cells after normalization to β-actin. (b) Protein levels of Keap1, Nrf2, Akt, p-Nrf2, and p-Akt were detected by Western blot with β-actin as the loading control. (b) The levels of proteins were quantified, and data are presented as the means + SD. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

dose-dependent manner ($P < 0.05$), whereas no significant changes were found in the protein levels of Nrf2, Keap1, and Akt (Figure 6(b)).

4.5. Role of Nrf2 on RSV-Induced Cytoprotection against Oxidative Stress. To confirm the roles of Nrf2 in protecting IPEC-J2 cells against H_2O_2-induced oxidative stress, we silenced the Nrf2 gene expression by shRNA in IPEC-J2 cells, then treated Nrf2-knockdown cells with RSV and/or H_2O_2, and then determined cell viability, apoptosis, and expression of antioxidant genes. Knockdown of Nrf2 by shRNA largely eliminated the beneficial effects of RSV pretreatment based on the changes in cell viability and the expression of antioxidant genes. qRT-PCR analysis showed that knockdown of Nrf2 in IPEC-J2 cells sharply decreased cell viability (Figure 7(a)) and mRNA expression of SOD-1, CAT, claudin-1, occludin, and ZO-1 (Figures 7(b) and 7(c)) and abrogated protective effects of RSV on cellular apoptosis (Figure 7(d)) under oxidative stress. Western blot analysis showed that knockdown of Nrf2 significantly decreased the phosphorylation of Nrf2 (p-Nrf2/β-actin) compared with the control (SC group) under oxidative stress. However, the phosphorylation of Akt was not affected in Nrf2-knockdown cells compared with the control group (Figure 7(e)).

4.6. Role of PI3K/Akt on RSV-Induced Cytoprotection against Oxidative Stress. To examine the role of Akt in RSV-induced cytoprotection against H_2O_2-induced oxidative stress, we pretreated cells with LY294002, an inhibitor of PI3K/Akt, and then treated these cells with RSV and/or H_2O_2. We found that LY294002 abolished the protective effects of RSV against H_2O_2-induced downregulation of cell viability (Figure 8(a)), antioxidant genes (Figure 8(b)), and TJ protein levels (Figure 8(c)) and H_2O_2-induced apoptosis (Figure 8(d)). In addition, LY294002 treatment decreased the protein levels of Akt, Nrf2, p-Akt, and p-Nrf2 compared with the control (Figure 8(e)).

Figure 7: Continued.

Figure 7: Effects of resveratrol on H_2O_2-induced cytotoxicity in Nrf2-knockdown IPEC-J2 cells. The Nrf2-knockdown (shNRF2-IPEC-J2) or control (SC-IPEC-J2) cells were pretreated with or without 50 μM RSV for 6 hours and then cocultured with 500 μM H_2O_2 for 4 hours. (a) Cell viability was measured using the CCK-8 assay. Results are presented as the percentage of cell viability compared with the control (0 μM). (b, c) The relative expression of SOD-1, CAT, claudin-1, occludin, and ZO-1 was detected by qRT-PCR. Data are shown as ratios of abundance of target gene transcripts in the treated cells to those in the control cells after normalization to β-actin. (d) Apoptotic cells were analyzed by flow cytometry using Annexin V-PI double staining. (e) Protein levels of Nrf2, Akt, p-Nrf2, and p-Akt were detected by Western blot with β-actin as the loading control. Values are the mean ± SE. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

5. Discussion

The intestine is a major digestive and absorptive organ for nutrients and provides a selective barrier against endogenous and exogenous antigens. Numerous studies have revealed that intestinal health, especially the integrity of the intestinal barrier, plays a crucial role in maintaining an organism's healthy state. Oxidative stress is a critical factor involved in the intestinal barrier disruption in intestinal diseases. Therefore, identification of appropriate plant extracts as therapeutic agents to counteract oxidative stress-related diseases is a research hotspot at present. In this study, IPEC-J2 cells were exposed to H_2O_2 to induce oxidative stress. We found that H_2O_2 treatment resulted in the reduction of cell viability and TJ protein expression, overproduction of ROS, disturbance of oxidation and antioxidation, and, finally, induced apoptosis. Moreover, the results also showed that RSV significantly attenuated H_2O_2-induced cell injury through increasing cell viability and upregulation of the expression of antioxidant genes.

As a dietary supplement, RSV is a polyphenolic compound contained in various fruits and herbs. Ever since RSV was identified to have multiple biological functions (antioxidant, anti-inflammatory, anticancer, etc.), it has been paid great attention for practical applications [10, 11, 20–22]. In the present study, our results showed that resveratrol potently protected IPEC-J2 cells against oxidative stress-induced intestinal injury using lower toxicity. The small intestinal has been found to be the major site of RSV absorption and metabolism; thus, we hypothesized that the intestinal epithelium may be a potential target for the beneficial effects of RSV though the first-pass effect by the intestine and microbe. Meanwhile, the anti-inflammatory and anti-oxidative effects of RSV were studied in many cases, such as models of ischemia/reperfusion injury and LPS induction [23, 24]. However, according to modern medical perspectives, oxidative stress plays a crucial role in the progression of many diseases such as the intestinal inflammatory bowel. Intestinal integrity is the basic guarantee for intestinal function. Intestinal integrity relies on TJs, which are the principal determinants of intestinal barrier function that seal the paracellular space between neighboring epithelial cells [25, 26]. Occludin, claudins, and ZO-1 are major intestinal barrier proteins [27]. In the present study, the expression of ZO-1, occludin, and claudin-1 showed a significant reduction after H_2O_2 treatment, whereas RSV attenuated dysfunction of the intestinal barrier by upregulating the expression of TJ proteins under H_2O_2-induced oxidative stress. Moreover, the results showed that RSV significantly attenuated H_2O_2-induced cell damage through increasing cell viability and decreasing apoptotic rate in IPEC-J2 cells. It is easy to speculate that RSV attenuates intestinal injury and especially barrier disruption mainly by upregulating the oxidative status.

Oxidative stress is due to the imbalance between pro- and antioxidant factors. Organisms or cells have antioxidant systems, which involve SOD, catalase, and GSH peroxidase, to scavenge overproduced ROS. This study showed that RSV could upregulate the activities of catalase (CAT), SOD, and GSH peroxidase and maintain intracellular ROS homeostasis under H_2O_2-induced oxidative stress or normal condition. On the one hand, RSV can directly clear free radicals by

Figure 8: Continued.

(e)

Figure 8: Effects of RSV and the selective inhibitor LY294002 on H_2O_2-induced cytotoxicity in IPEC-J2 cells. IPEC-J2 cells were pretreated with 25 μM LY294002 for 2 h and then treated with 50 μM RSV for 4 h before incubation with 500 μM H_2O_2 for 4 hours. (a) Cell viability was measured using the CCK-8 assay. Results are presented as the percentage of cell viability compared with the control (0 μM). (b, c) The relative expression of SOD-1, CAT, claudin-1, occludin, and ZO-1 was detected by qRT-PCR. Data are shown as ratios of abundance of target gene transcripts in the treated cells to those in the control cells after normalization to β-actin. (d) The apoptotic cells were analyzed by flow cytometry using Annexin V-PI double staining. (e) Protein levels of Nrf2, Akt, p-Nrf2, and p-Akt were detected by Western blot with β-actin as the loading control. Values are the mean ± SE. The columns with the same superscript capital letters and with different superscript capital letters mean no significant difference ($P > 0.05$) and significant difference ($P < 0.05$), respectively.

upregulating antioxidant enzyme activity *in vitro* and *in vivo* [21, 28]. Wang and his colleagues showed that RSV can protect the Caco-2 cell against H_2O_2-induced oxidative damage by reducing the malondialdehyde level and intracellular ROS accumulation. RSV has been reported to improve mitochondrial biogenesis and redox status by upregulating SOD and GSH-Px activity in suckling piglets [29]. On the other hand, RSV can also upregulate the expression of antioxidant genes in many studies, which is consistent with our results. In the present study, RSV increased the expression of SODs, GSXs, and HO-1. In the intracellular antioxidant system, HO-1, SODs, CAT, and GPXs are phase 2 genes which are known as target genes mediated by the Nrf2/Keap1 signaling pathway. Meanwhile, many studies have revealed that RSV attenuates oxidative stress-induced intestinal dysfunction by upregulating HO-1 expression [30]. Upregulation of antioxidant genes is cellular adaptation to oxidative stress and is mainly or partly regulated by the Nrf2 pathway [31].

Nrf2, a pivotal sensor of oxidative stress in cells, is a transcription factor that plays a central role in the regulation of antioxidant and phase 2 detoxifying enzymes and related proteins [32]. Keap1 binds to Nrf2 in the cytoplasm and facilitates Nrf2 ubiquitination which can prevent Nrf2 translocation into the nucleus under physiological conditions [33]. When the levels of intracellular ROS sharply increase, overproduced ROS modifies the reactive cysteine residues on Keap1, and then Keap1 dissociates from Nrf2, leading to the translocation of Nrf2 into the nucleus and the expression of its target genes [33, 34]. Recent studies have revealed that the phosphorylation of Nrf2 facilitates Keap1/Nrf2 dissociation and Nrf2 nuclear translocation. In the present study, RSV upregulated the levels of p-Nrf2 and p-Akt in a dose-dependent manner, suggesting that RSV attenuates oxidative status likely by activating the Nrf2 signaling pathway. Many studies have revealed that RSV treatment dramatically upregulates the expression of Nrf2 and HO-1 *in vivo* and *in vitro* [35, 36]. Furthermore, RSV-induced upregulation of antioxidant genes and the intestinal epithelial cell barrier was inhibited by Nrf2 knockdown, suggesting that Nrf2 may be a major target of RSV in relieving oxidative stress. However, the phosphorylation levels of the Akt protein were increased upon RSV treatment in our study. The PI3K/Akt signaling pathway is commonly involved in the Nrf2-dependent transcription in diverse cell types of responding ROS insults [37, 38]. Han's study showed that chlorogenic acid protects MC3T3-E3 cells against oxidative stress through the PI3K/Akt-mediated Nrf2 signaling pathway to induce HO-1 expression, suggesting that Nrf2 may be a downstream signal target of PI3K/Akt [19]. Therefore, Western blot analysis showed that RSV-induced increase of the Nrf2 phosphorylation level was inhibited by the specific PI3K/Akt inhibitor LY290042. In addition, RSV-induced changes of cell viability, apoptosis, antioxidant genes, and TJ expression were also inhibited by the inhibitor. These results were similar to effects of Nrf2 knockdown on IPEC-J2 cells. Moreover, the phosphorylation level of Akt was not affected when Nrf2 was knocked down. It indicates that the PI3K/Akt pathway plays a pivotal role in the cytoprotective effects of RSV-induced Nrf2 activation against oxidative stress.

Collectively, the present findings provide important evidence of potential cytoprotective effects of RSV against H_2O_2-induced dysfunction in the intestinal barrier. Pretreatment with RSV protects IPEC-J2 cells against oxidative stress by promoting antioxidant systems' activities, decreasing

intracellular ROS and the apoptotic rate, and upregulating the intestinal barrier. These cytoprotective effects of RSV at least partly depend on the PI3K/Akt-mediated Nrf2 signaling pathway.

Data Availability

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

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Yu Zhuang and Huirong Wu contributed equally to this study.

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Research Article

Role of Resveratrol on Indoxyl Sulfate-Induced Endothelial Hyperpermeability via Aryl Hydrocarbon Receptor (AHR)/Src-Dependent Pathway

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Resveratrol (RES), a dietary polyphenol compound, has been shown to possess health benefits due to its anti-inflammatory, antioxidative, and antiatherosclerosis properties. Tryptophan metabolite-derived indoxyl sulfate (IS) is identified as one of the uremic toxins and physiological endogenous ligand/activator of aryl hydrocarbon receptor (AHR), associated with atherosclerosis in chronic kidney disease (CKD) patients. Studies have shown that a high serum level of IS causes deleterious effects on health primarily by inducing oxidative stress and endothelial dysfunction. However, the precise mechanisms are still unclear. Here, we investigated the underlying mechanism of IS effect on endothelial permeability and the role of RES on IS-induced endothelial hyperpermeability via the AHR/Src-dependent pathway. Bovine aorta endothelial cells (BAECs) were cultured and incubated with IS in the presence or absence of RES, and transendothelial electrical resistance (TEER) and permeability of cells were measured. Alongside, AHR, Src kinase, and Vascular Endothelial Cadherin (VE-Cadherin) activation were examined. Our data showed that IS reduced TEER of cells resulting in increased permeability. VE-Cadherin, a vital regulator of endothelial permeability, was also significantly activated in response to IS, which appeared to be associated with changes of endothelial permeability and AHR/Src kinase. Interestingly, in this setting, RES reversed the effect of IS and inhibited the increased activation of Src induced by IS-activated AHR and modulated VE-Cadherin and permeability. CH223191, an inhibitor of AHR, significantly inhibits IS-induced endothelial hyperpermeability. Further analysis with treatment of PP2, an inhibitor of Src abolishing Src activation, suggests downstream factors. All our data indicated that IS upregulated the AHR/Src kinase pathway, and increased endothelial permeability and phosphorylation of VE-Cadherin may be represented and provide new strategies for addressing protective properties of RES against Src kinase involved in AHR-mediated endothelial hyperpermeability. The findings may be crucial for managing diseases in which endothelial permeability is compromised, and the dietary polyphenols are involved.

1. Introduction

The endothelium has exponentially been identified as a semipermeable barrier and an essential regulator of blood flow in both micro- and macrovascular beds. The endothelium lining the intima of the blood vessels plays crucial roles in the homeostasis of a variety of functions such as vascular smooth muscle tone, angiogenesis, host-defense reactions, and tissue fluid hemostasis [1]. The maintenance of a semipermeable barrier by the endothelium is remarkably essential in controlling the passage of fluid along with macromolecules between interstitial space and blood. It has been documented that loss of this function results in increased permeability [2]. Endothelial hyperpermeability shared characteristics of many diseases, including atherosclerosis trauma, sepsis, diabetes mellitus, uremic syndrome, and tissue inflammation, and hallmark of inflammatory diseases such as the acute respiratory distress syndrome [3–6]. The permeability characteristics of transported macromolecules are associated with their molecular radii together with the barrier properties of the specific endothelium [7].

Due to lack of renal clearance in CKD patients, retention/accumulation of uremic solutes is increased in proximal tubular cells and causes most of the renal injury and also contributes to elevated serum levels of uremic toxins [8]. Uremic toxin accumulation in serum under pathological condition is a primary event during the formation of atherosclerosis [9]. IS is a uremic toxin derived from a metabolite of dietary protein (tryptophan) and a risk factor for aggravating cardiovascular disease (CVD) in CKD patients mainly due to inducing oxidative activity and endothelial dysfunction [10, 11]. Intestinal bacteria form the precursor of IS via tryptophan degradation before absorption [12, 13]. Following ingestion of dietary protein, part of protein-derived tryptophan is cleaved into indole by the action of tryptophanase colonic microbes such as Escherichia coli and the indole is absorbed from the intestine into the blood and oxidized to indoxyl in the liver by the action of cytochrome p450 enzyme, and the indoxyl is further sulfonated in the liver and forms IS [14]. Recent clinical studies revealed that IS, a protein-bound indole derivatives uremic toxin, is one of risk factor for cardiovascular death in CKD patients (stage 2 to hemodialysis stage 5) [15] and considered major causes with an estimated 20-fold in cardiovascular mortality [16]. High serum concentration of IS is increased in CKD patients which primarily affect renal function and contributes to CKD progression which may also be recognized as the major cause of impairment of endothelial function, an initial step for atherosclerosis that accelerates CVD progression in CKD patients [11, 15]. The increased concentration of IS may result in an upsurge in the generation of free radicals and activate proinflammatory factors, leading to the progression of CVD. Studies revealed that IS-enhanced vascular injury exhibits inflammatory effects and impaired endothelial healing ability [17, 18]. In addition, IS is one of endogenous physiological ligands of AHR among indole derivatives such as tryptophan, indole, indoxyl-3-sulfate (I3S), indoxyl-3-acetate (I3A), and indole-3-methanol and a photoproduct of tryptophan through structural and chromatographic studies and characterized as potent agonists and activator of AHR [19, 20], and studies suggest that ligand-activated AHR induces a response in cardiovascular cells, although there are other potential agonists that directly stimulate AHR [21].

Ligand-AHR activation has been involved in the development of impaired endothelial function leading to endothelial cell dysfunction and injury, followed by the development of CVD [22]. AHR belonging to the basic helix-loop-helix/-Per-ARNT-Sim (bHLH-PAS) superfamily is activated by numerous endogenous and exogenous ligands and characterized as a ligand-dependent transcription factor [23]. Prior to ligand binding, AHR is located in the cytoplasm, a complex with different proteins such as heat-shock protein 90 (Hsp90), Hsp90-interacting protein P23, immunophilin-like protein XAP2 (AIP or ARA9), and Src kinase, and could control cellular processes along with other transcriptional factors in the cytoplasm [24–26]. Previous studies revealed that AHR function is mediated by different signaling pathways such as genomic and nongenomic pathways. The AHR genomic pathway is also called AHR canonical pathway. In AHR genomic pathway, the ligand enters the plasma membrane via passive diffusion and binds/activates AHR in the cytoplasm. Upon binding, ligand-AHR complex is exposed to nuclear translocation and forms a heterodimeric complex with its partner AHR nuclear translocator (ARNT also called HIF-1b) [27]. The heterodimer (AHR-ARNT) binds to DNA specific sequence named xenobiotic response elements (XREs) or dioxin response elements (DRE) located in the promoter region of target genes and induces the transcription of target genes (CYP1A1) and inflammatory response [23, 28, 29]. In classical AHR genomic pathway, the AHR activation is a ligand-dependent transcription factor. However, AHR also regulates gene expression through nongenomic, ARNT-independent genomic, as well as nonclassical, signaling pathway [26, 30]. Recently, it was reported that AHR regulated the number of genes without direct binding of the AHR to DRE consensus sequence [31].

In AHR nongenomic pathways, ligand-activated AHR leads to Src kinase activation and allows the dissociation from the AHR receptor complex in the cytoplasm and translocates to the membrane [32]. In the normal and pathological processes, Src kinase is known to regulate intracellular signal transduction in vascular biology, including endothelial cell permeability [33]. The integrity of cell to cell contact barrier function is disturbed by the activation of the Src kinase induced following the exposure to ligands [22]. Src kinase plays a role in regulating cadherin-dependent cell-cell contact accomplished by VE-Cadherin endothelial permeability [34]. In endothelial cells, VE-Cadherin is the most crucial cell-specific member of the cadherin protein family, implicated in this process by regulating and maintaining the structure of endothelial cell barrier function, and is vital in permeability changes [35]. VE-Cadherin mediates endothelial barrier dysfunction that contributes to the majority of CVD-related deaths associated with arteriosclerosis [36] by increasing endothelial permeability leading ultimately to endothelial failure. Reported studies indicated that TCDD,

exogenous ligand of AHR, increased inflammatory responses through a nongenomic AHR function [37, 38] and led to functional activation of the tyrosine kinase Src by releasing from AHR complex. In search of potential AHR ligand, IS has been common characterstics with TCCD; however, response after IS activation of AHR on the physiological consequences and the underlying mechanisms are underexploited. Therefore, in this study, we provided evidence in IS activation of AhR and the effect on endothelial permeability using bovine aorta endothelial cells (BAECs). In our data, we found that IS increased AHR activation and endothelial cell permeability, which may contribute to developments of a cardiorenal syndrome and disease related to endothelial hyperpermeability pathologies under uremic condition. Taken together, the findings indicate that IS plays a pivotal role in the disruption of endothelial permeability via AHR/Src-dependent pathway and the pathologies mentioned above, and therefore, there is a pressing need for researchers to investigate compounds that can alleviate the deleterious effects of IS.

Resveratrol (trans-3,4,5-trihydroxystilbene, RES) is a natural polyphenolic phytoalexin compound found abundantly in grapes, berries, red wine, and various other dark skin fruits [39] and has potential uses in human medicine since it modulates numerous physiological functions and exerts multiple effects including cancer, neurological, hepatic, and cardiovascular disease management [40–42] and has pharmacological antagonism property of AHR [43]; it possesses antiatherosclerosis effect [44], ameliorates hyperpermeability induced by high glucose-mediated caveolae via VEGF/KDR [45], protects intestinal hyperpermeability, upregulates heme oxygenase-1 expression by lessening oxidative stress [46], and protects breakage of blood-brain barrier properties induced oxidized LDL [47]. Furthermore, RES has been documented as an inhibitor of Src tyrosine kinase activity and proved to decrease endothelial hyperpermeability [48]. Reported studies suggested that RES reduce aortic fatty streak accumulation, initial lesion for the progression of atherosclerosis [49]. RES may be effective as a protective agent against AHR ligands. Shreds of evidence from previous studies have shown that RES can compete with the ligand for AHR binding and efficiently block the induction of CYP1A1 by AHR ligand. Despite the fact that RES was shown to inhibit TCDD-activated AHR-induced CYP1A1 expression in HepG2 cells [50], its role in IS-activated AHR-induced endothelial permeability was rarely reported. As both IS and RES could interact with AHR, the quite different roles on health or diseases are of concern. Therefore, in this study, we explored IS effect on endothelial permeability and how RES can rescue IS-induced endothelial hyperpermeability using bovine aorta endothelial cells (BAECs) with and without RES. Our data showed that IS affect cell viability, disrupt TEER of the cell, and increased permeability by increasing the activity of AHR complex/Src signaling mediated VE-Cadherin. However, RES inhibit IS-induced activated Src signaling and VE-Cadherin and downregulated endothelial hyperpermeability via AhR/Src-dependent pathway. This study is of crucial importance in disease condition where endothelial permeability is compromised.

2. Materials and Methods

2.1. Reagent and Chemicals. We purchased bovine aortic endothelial cells (BAECs) from the Health Science Research Resources Bank (Osaka, Japan, no. C-003-5C). Dulbecco's modified Eagle's medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco Inc. (Grand Island, NY, USA). FITC-dextran, PP2 (4 - Amino - 5 -(4 - chlorophenyl)- 7 -(dimethylethyl) pyrazolo [3 , 4 - d]), (an inhibitor of Src), pSrc (phosphorylated Src) and Src, AHR inhibitor (CH223191), indoxyl sulfate (IS), and CCK-8 Kit were from Sigma-Aldrich China Inc. (Shanghai, China). AHR, ARNT, pVE-Cadherin (phosphorylated VE-Cadherin), and VE-Cadherin antibody were purchased from ABclonal (Wuhan, China); β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol (3,5,4′-trihydroxy-trans-stilbene) was purchased from Nanjing Zelang Biotechnology Inc., China.

2.2. Cell Culture. BAECs were maintained at 37°C in 5% CO_2 in DMEM/low glucose containing 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were used for experiments between passages 2 and 9. Confluent BAECs were treated with IS in the presence and absence of RES. Confluent cells without IS were used as a control group.

2.3. Cell Viability Assay. Cell viability was analyzed by the cell counting kit-8 (CCK-8) assay. First, the experiment was performed based on reported maximum serum IS concentration (about 200-250 μg/ml) in uremic patients and exposed to the cells for different times to examine time-dependent effect of IS on the cells, and then, we make used of a wide range of concentration of IS for 24 h to determine the dose for further analysis. The cells were seeded in a 96-well plate at a density of 5×10^3 cells per well.

After being treated by different concentrations, cells were washed with PBS three times and incubated with 90 μl of fresh medium with 10 μl CCK-8 solution at 37°C for 2 h. The optical density was measured by an enzyme-linked immunoassay analyzer at 450 nm.

2.4. Transepithelial Electrical Resistance (TEER) Measurement. Transepithelial/transendothelial electrical resistance (TEER) of the cell monolayer was determined by a Millicell ERS-2 system. TEER measurement is a widely recognized quantitative method to measure the resistance of cell monolayer and integrity of close-fitting junctional dynamics in cell culture models of endothelial and epithelial cells. BAECs were seeded in the upper chambers of polycarbonate Transwell inserts (0.4 μM pore, 6.5 mm diameter, Millipore, USA) at 1.25×10^5 cells/ml and cultured until confluent for 4 days. Groups of cells were treated with IS in the presence and absence of RES, AHR inhibitor (CH223191), and PP2 (Src inhibitor) for different times and compared with the control and treatment groups. The resistance of the monolayer was determined daily by measuring the TEER with a Millicell ERS-2 system. Cell resistance values of multiple Transwell inserts of the experimental group were measured sequentially, and the mean was expressed in Ω (Ohms) after subtraction of

the value of a blank cell-free filter. The TEER of the monolayers was recorded when a stable resistance reading was achieved with triplicate measurements that were taken for each Transwell. The Millicell ERS-2 meter functionality was adjusted to ensure proper operation. The experiments were performed at 37°C with the passage cell numbers of 2 and 3. Resistance of the cell monolayer was calculated as per manufacturer instruction and considered as the area of the membrane used:

$$\text{Resistance Calculation}: R_{\text{cell monolayer}} = R_{\text{sample}}\text{R sample} - R_{\text{blank}}, \quad (1)$$

where $R_{\text{cell monolayer}}$ is resistance of the cell monolayer, R_{sample} is resistance reading across the cell (cell culture with cell), and R_{blank} is resistance reading across the blank cup (cell culture without cells).

Unit area resistance was also calculated as the value independent of the area of the membrane used; in this study, we used a 24-well plate: wall diameter 10 mm and membrane area 0.6 cm2:

$$\begin{aligned}&\text{Unit Area Resistance}\\&\quad = \text{Resistance}\,(\Omega) \times \text{effective membrane area}\left(\text{cm}^2\right) \quad (2)\\&\quad = (\Omega\text{cm})^2.\end{aligned}$$

2.5. Endothelial Permeability. Endothelial permeability was determined by the flux of FITC-labeled dextran tracers through the Transwell system. Briefly, BAECs were plated on the upper chambers of 0.4 μm pore size Transwell at 1.25×10^5 cells/ml and cultured until confluent. After treatment, the medium was replaced with fresh phenol red-free DMEM and FITC-dextran tracers (40 kDa, final concentration 1 mg/ml) were added into the upper chamber and incubated at 37°C for 2 h. After incubation, the amount of tracer in the lower chamber was determined by a microplate reader (Biotech, Highland Park, USA) at 494 nm excitation and 521 nm emission. All the data were recorded as the means of three experiments.

2.6. Western Blot Analysis. The expression level of proteins was analyzed using Western blot. Following IS treatment on BAECs in the presence and absence of RES and inhibitors, cells were washed with PBS and homogenized in 4°C radioimmunoprecipitation assay (RIPA) lysis buffer. Briefly, the cells were lysed in RIPA lysis buffer with phenylmethylsulfonyl fluoride (PMSF), placed on ice for 30 min, and centrifuged at 13500 rpm at 4°C for 15 minutes. The supernatant was collected, and protein concentration was quantified using a protein assay reagent (BCA Assay Kit). For the collection of cytosolic fractions of AHR, BAECs were suspended in ice-cold phosphate-buffered saline (PBS), homogenized for 10 strokes using a glass Dounce homogenizer, and centrifuged (12000 g, 30 s, 4°C). The pellet was collected and resuspended in 200 μl of ice-cold buffer A (10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin). After incubation on ice for 10 min, 10 μl of 10% IGEPAL was added, and the mixture was mixed thoroughly for 30 s and centrifuged (12000 g, 30 s, 4°C), and then, the supernatant was collected as the cytosolic fraction. Proteins were denatured and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene fluoride) (PVDF) membranes. Then, membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. After blocking, membranes were washed with Tris-buffered saline with Tween 20 (TBST) three times for 10 minutes each time and incubated at 4°C overnight with specific primary antibodies such as VE-Cadherin (1 : 1000), pVE-Cadherin (1 : 1000), AHR (1 : 1000), Src (1 : 1000), pSrc (1 : 1000), β-actin (1 : 5000), and ARNT (1 : 1000). Then, membranes were washed with TBST for three times and incubated with secondary antibody for 1 h at room temperature. After washing in TBST, proteins were visualized by an enhanced chemiluminescence (ECL, Immobilon, USA) Western blotting system. The relative concentration of target protein expression was determined by computer analysis using ImageJ and normalized to an internal standard (β-actin).

2.7. Immunofluorescence Staining. BAECs were seeded and grown to confluence on fibronectin-coated glass chamber slides. After treatment, the medium was removed, and the monolayers were washed with PBS containing 100 ml-glycine, fixed with 4% paraformaldehyde, and washed three times with PBS for 10 min and blocked with 5% (w/v) BSA in PBS for 30 min. Immunofluorescence was performed by staining with primary antibody against AHR at a dilution of 1 : 400 overnight at 4°C. Specific binding was detected using conjugated goat anti-rabbit antibody followed by tyramine labeling with red fluorescent. The fluorescence images were picked up using a cell imaging system. The slides were photographed using an Olympus LCX200 Imaging System (Olympus Corporation, Tokyo, Japan).

2.8. Statistical Analysis. Data were presented as the *mean* ± *SEM* (*Standard Error* of the *Mean*) and analyzed using GraphPad Prism 7.0 for Windows-statistical software (GraphPad Prism software, La Jolla, California, USA, http://www.graphpad.com), and the statistical difference was determined by one-way ANOVA procedure followed by the Student-Newman-Keuls post hoc test with 95% confidence and unpaired t-test. The level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effect of IS and RES on Cell Viability. The viability of BAECs was measured by using a CCK-8 uptake assay. First, BAECs were cultured and incubated with the maximum IS serum level under uremic condition (200 μg/ml) for different times (12-72 h), and the result showed that the viability/-survivability of BAECs was gradually declined in a time-dependent manner compared to the control (Figure 1(a)). Then, we have used a different concentration of IS (25-250 μg/ml) and incubated for 24 h to determine the effect

Figure 1: Effect of IS and RES on cell viability. (a) BAECs were incubated with IS (200 μg/ml) for different times (12-72 h); cell viability was decreased in a time-dependent manner. (b) Cells were stimulated with various concentrations of IS (25-250 μg/ml) for 24 h; the cell survivability also declined at high concentration. (c) RES treatment did not affect the viability of BAECs. Data are shown as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

of IS, and cell survivability was declined significantly at high concentration of IS (200/250 μg/ml) (Figure 1(b)); thus, we make used of 100 μg/ml IS for subsequent experiments. Coincubation of different concentrations (25, 50, and 100 μM) of RES did not affect the viability of BAECs (Figure 1(c)).

3.2. RES Supplementation Improved Impaired TEER Induced by IS. TEER of BAECs was measured by a Millicell ERS-2 system. TEER value is a strong indicator of cellular monolayer health and integrity in endothelial and epithelial monolayer cell culture models, which play a crucial role in regulating permeability. IS-induced disruption of endothelial cell monolayer integrity resulted in a sharp decrease in TEER of cells and increased permeability. Following incubation of cells with different concentrations of IS (25-200 μg/ml) for 24 h, TEER value of cell monolayer was weakened and significantly reduced at 100 μg/ml IS-incubated cells compared to the control (Figure 2(a)). RES supplementation at different concentrations (25, 50, and 100 μM) significantly increased TEER of cells ($P < 0.05$) which was declined in response to IS (100 μg/ml) exposure (Figure 2(b)). TEER value of cells also remained similar with the control following RES (50 μM), CH223191 (10 μM) pretreatment, and incubated cells with IS (100 μg/ml) for different times, whereas a gradual decrease of TEER of cells was observed in IS (100 μg/ml) treatment group ($P < 0.05$) (Figure 2(c)). Similar phenomena occurred in coincubation of PP2 (10 μM) and IS (100 μg/ml), and PP2 could restore the decreased TEER of cells (Figure 2(d)). Incubation of cells with CH223191 and PP2 did not affect the TEER of BAECs.

3.3. RES Attenuated IS-Increased Endothelial Permeability. We then measured the fluorescence intensity flux of FITC-dextran, which passes through endothelial cells (Figure 3) to the lower chamber demonstrating the permeability. BAEC permeability was gradually increased with the increase in IS concentration (25-200 μg/ml), and statistical significance reduction ($P < 0.05$) was observed in 100 μg/ml IS treatment group (Figure 3(a)). Similarly, endothelial cell permeability was increased after incubation of cells (100 μg/ml IS) for a different time in a time-dependent manner (Figure 3(b)). To determine the ameliorate property of RES on IS-induced permeability, BAECs were coincubated with different concentrations of RES (25, 50, and 100 μM), and as expected, RES lead to a significant decrease in permeability (Figure 3(c)). Furthermore, pretreatment of cells with RES (50 μM), CH223191 (10 μM), and PP2 (10 μM) decreased the hyperpermeability of cells induced by IS and brought close to the control level (Figures 3(d) and 3(e)), respectively. CH223191 and PP2 incubation on cells did not affect BAEC permeability.

3.4. IS Triggered AHR Activation and Increased Activation of Src and VE-Cadherin. Previous studies suggested that IS-induced activation of AhR leads to subsequent stimulation of downstream signal transduction to modulate protein and stimulates multiple pathways which lead to dysfunction of the endothelial cell [21, 51]. Since our previous data show that IS (100 μg/ml) impaired TEER of cells and induced endothelial permeability, in this research, we investigated the underlying mechanism by making use of Western blot which revealed that incubation of cells with IS (100 μg/ml) for 24 h triggered the activation of AHR and significantly decreased the cytosolic AHR protein expression level (Figures 4(a) and 4(b)) compared to the control which contributed to the increased activity of Src (phosphorylation of Src) (Figures 4(a) and 4(c)). Treatment of IS (100 μg/ml) also significantly increased phosphorylation of Vascular Endothelial Cadherin protein (pVE-Cadherin) (Figures 4(a) and 4(d)), which may promote endothelial cell hyperpermeability.

Despite the fact that the AHR nongenomic pathway is ARNT-independent, in the presence of ligand, the AHR has been activated (translocated) and regulate gene expression through a nongenomic signaling pathway [26, 30] as AHR regulated the number of genes without direct binding of the AHR to DRE consensus sequence [31]. Thus, in order to

Figure 2: RES supplementation improved the impaired TEER induced by IS. (a) Millicell ERS-2 meter measurement result indicated that the increase in the concentration of IS treatment causes a gradual decrease in TEER value of cells compared to the control group. (b) RES pretreatment (25, 50, and 100 μM) restored TEER of cells significantly. (c) TEER value of cells significantly decreased following IS treatment at 24 h, whereas there was no significant difference between the IS treatment group with CH223191 and RES compared with the control group. (d) Coincubation of IS with PP2 (10 μM) also restored the decreased TEER of cells. Data are presented as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. IS-treated group.

confirm the potential of IS on AHR activation, we determined the expression level of ARNT using Western blot; at the same time, we make used of immunofluorescence to observe the nuclear expression of AHR where BAECs were stained with anti-AHR antibody (red) and DAPI (blue) and visualized by fluorescence microscopy revealing a highly stained nuclear expression of AHR in IS treatment compared with the control while treatment with CH22319, an inhibitor of AHR, prevented the activation of AHR (Figure 5(a)). Simultaneously, we carried out Western blot analysis and observed a significantly increased ($P < 0.05$) ARNT (aryl hydrocarbon receptor nuclear translocator) protein expression level in response to IS treatment compared with the control (Figures 5(b) and 5(c)) which confirmed the direct consequence of IS on cytosol AhR activation.

3.5. Involvement of Src Kinase in AhR-Mediated Modulation of Permeability in response to IS Exposure. Several studies that reported using prototypic AhR agonists such as TCDD have led to the introduction of AHR-dependent Src kinase activation pathways, and recently, some of the toxic effects of these environmental pollutants are known [22]. Evidence indicated that Src protein kinase is located in the cytosol of cells complexed with AhR, and upon ligand binding with the AHR subunit, the Src kinase is released from the AHR complex and translocated to the cell membrane and regulates permeability. Our present data also revealed that IS activated AHR (decreased cytosolic AHR expression level) ($P < 0.001$); however, coincubation with AHR blocker, CH223191 (10 μM), inhibits the activation of cytosolic AHR (Figure 6(a)). IS causes a considerable rise in phosphorylation of Src

Figure 3: RES attenuated IS-increased endothelial cell permeability. (a) The permeability of endothelial cells gradually increased in response to different concentrations of IS for 24 h and reached significant at 100 μg/ml of IS-treated cells. (b) Different incubation periods of IS (100 μg/ml) increased cell permeability. (c) Cotreatment with RES (25, 50, and 100 μM) decreased the hyperpermeability induced by IS. (d) Pretreatment of CH223191 and RES (50 μM) markedly decreased permeability, respectively. (e) Coincubation of PP2 also decreased permeability. Data were expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$ vs. IS-treated group.

($P < 0.01$), and by making use of AHR blocker, CH223191 (10 μM), we also provided evidences that the activation and involvement of Src in permeability change in response to IS exposure are directly correlated with activated AHR by IS because CH223191 significantly eliminated the increased activity (phosphorylation of Src) (Figure 6(b)) ($P < 0.05$) and VE-Cadherin; the phosphorylation also decreased after incubation of the cell with CH223191 ($P < 0.05$)

Figure 4: IS triggered AHR activation and increased activation of Src and VE-Cadherin. (a, b) Western blot analysis result showed that IS (100 μg/ml) activated cytosolic AHR protein corresponding to the decreased cytosolic AHR ($P < 0.001$), (a, c) increased phosphorylation of Src, and (a, d) increased phosphorylation of VE-Cadherin protein, respectively ($P < 0.01$ and $P < 0.05$). Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control.

(Figure 6(c)) which may contribute to decreasing endothelial permeability induced by IS. Src inhibitor, PP2 (10 μM), also markedly abolished phosphorylation of Src (Figure 6(d)), and decreased VE-Cadherin phosphorylation induced by IS-activated AHR (Figure 6(e)) may lead to a decrease in endothelial permeability.

3.6. RES Inhibits Src Kinase Activity Mediating VE-Cadherin Induced by IS-Activated AHR and Modulates Permeability via AHR. In our study, first, we examined the role of RES on cytosolic AHR with or without the administration of IS. The Western blot analysis result demonstrated that the cytosolic AHR protein expression level was not affected by RES treatment at different concentrations (25, 50, and 100 μM) (Figure 7(a), a1). However, the loss of the cytosol AHR protein expression level was observed in the coincubation of RES with IS (100 μg/ml) (Figure 7(a), a2). Treatment of cells with different concentrations of RES (25, 50, and 100 μM) significantly reduced the Src phosphorylation induced by IS ($P < 0.05$) (Figures 7(b) and 7(c)). RES also significantly reduced VE-Cadherin phosphorylation with a statistical significance ($P < 0.05$) increased by IS and modulate endothelial permeability (Figures 7(b) and 7(d)).

4. Discussion

Our findings illustrated that the upregulation of IS activated the AHR/Src/VE-Cadherin pathway in hyperpermeability of bovine aorta endothelial cells ameliorated by RES and this is of relevance in CDK condition where upregulation of AHR/Src/VE-Cadherin activity in the pathogenesis of endothelial hyper-permeability-like pathology under uremic condition. Moreover, due to multiple biological function of RES and good permeation characteristics in endothelial cell with low toxicity even in high dose showed promising virtue. Previous studies demonstrated that due to deterioration of renal filtration in CKD patients, IS level in serum is elevated and develops a major risk factor for atherosclerosis [52, 53]. Since endothelial hyperpermeability is the result of endothelial dysfunction which is a marker of the early stage of atherosclerosis, inhibition of IS-induced endothelial hyperpermeability will play beneficial roles in maintaining endothelial cell function and prevent the development of atherosclerosis under uremic condition. Research studies reported that RES improves endothelial function and has beneficial effects on vascular tone and vessels in human and animal models [54]. Therefore, we investigated the effect of IS on BAECs and the role of RES against IS-induced endothelial hyperpermeability in this study.

When the cytotoxicity test for BAECs was investigated, pernicious effects of IS on cell barrier dysfunction were observed in cultured human umbilical vascular endothelial cells (HUVECs) [52]; in humans, they have also been associated with various adverse outcomes such as renal disease and vascular and bone diseases [55]. Even under the pathological

Figure 5: IS promotes AHR nuclear translocation and increased the level of ARNT expression level. (a) Immunofluorescence analysis using 200x objective results revealed the highly stained nuclear expression of AHR in the IS treatment group while treatment with CH223191 eliminated the effect of IS. (b, c) Western blot analysis also indicated that IS increased the expression of ARNT level while treatment with CH223191 decreased the expression of ARNT induced by IS. Data are expressed as the mean ± SEM. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. IS-treated group.

condition, the increased plasma level of IS induced oxidative stress, and this event leads to cellular toxicity by [48]. In this perspective, our present study highlights cytotoxicity of IS, which indicates a time- and dose-dependent pattern suggesting its toxicity (Figure 1). Since the cell viability demonstrated a gradual decrease, IS inhibit the viability of cells significantly at high concentration (200/250 μg/ml)-incubated cells for 24 h (Figure 1(a)). Our data are comparable with multiple studies about IS renal and nonrenal effects [9, 55]. Thus, we used 100 μg/ml of IS for the remaining experiments which is compatible with the serum concentration level in uremic condition. Treatment of different concentrations of RES (25, 50, and 100 μM) did not affect the viability of BAECs (Figure 1(c)).

Subsequently, the Millicell ERS-2 meter and flux of FITC-labeled dextran tracer assay were used to examine IS effect on TEER and permeability of BAECs, respectively, and evaluated the effect role of RES supplementation. The greater TEER value of cells is an indicator of cell monolayer health, confluence, structural barrier integrity, and the lesser endothelial cell hyperpermeability. In line with previous studies where IS mediated increase in endothelial permeability by inducing endothelial barrier dysfunction, disrupting intercellular junctions, and inducing intercellular gap formation [56], similar phenomena were observed in the present study (Figure 2) where IS decreased TEER value of BAECs (Figure 2) suggesting the increase in concentration of IS incubation that resulted in weakened cell monolayer which leads to an increased endothelial permeability. Treatment with RES (25, 50, and 100 μM) rescued TEER of cell impairment and restored significantly suggesting its potential of repairing injured cell monolayer and maintaining endothelial cell membrane disruption induced by IS and decreasing endothelial hyperpermeability. The longer incubation period of cell with 100 μg/ml of IS also decreased TEER value of cells and contributed for increasing endothelial permeability while in the opposite, TEER value of cells coincubated with RES (50 μM), CH223191 (10 μM), and PP2 (10 μM), respectively, had been comparable with the control group suggesting their ameliorative property. The findings are in agreement with

Figure 6: Src kinase involved in AhR-mediated modulation of permeability in response to IS exposure. (a) Cytosolic AHR expression level significantly decreased in response to IS exposure; cotreatment with CH223191 increased cytosolic AHR expression level. (b) CH223191 decreased Src phosphorylation significantly induced by IS. (c) CH223191 also reduced the increased activity of VE-Cadherin induced by IS. (d) PP2 abolished Src phosphorylation induced by IS, and (e) PP2 significantly decreased VE-Cadherin phosphorylation induced by IS. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$, and $^{\#\#\#}P < 0.01$ vs. IS-treated group.

the previous study showing its potential. It has been reported that RES has the potential to improve the release of nitric oxide (NO) and prostacyclin (PGI), thus playing a vital role in the maintenance of endothelial function [54]. RES also, as documented by some studies, acts as an inhibitor of Src tyrosine kinase activity and has been proved to decrease endothelial hyperpermeability [48]. The potential of PP2 to inhibit the increase in pulmonary microvascular leakage was also reported implying its action as a potent downregulator of endothelial hyperpermeability [57]. Furthermore, studies revealed that CH223191 also could inhibit migration of cells in response to TCDD-activated AhR in [58]. To further support our findings, we carried out flux FITC-labeled dextran tracer assay and measured the endothelial permeability, which revealed similar phenomena with the result found in TEER (Figure 3). CH223191 and PP2 treatment did not affect TEER of BAEC monolayer and permeability. Given the effect mentioned above of IS on endothelial permeability and ameliorative property of RES, AHR, and Src blockers in alleviating endothelial hyperpermeability, we make use of Western blot to assess the potential mechanisms of AHR/Src pathways being involved in endothelial permeability.

In nongenomic AHR pathway, ligand binding to AHR promotes Src kinase dissociation from AHR complex in the cytoplasm and leads to cell membrane translocation; thus, Src activity is increased and promoting disruption of cell-cell contact. Several studies have reported that the activity of Src can be upregulated by phosphorylation at Tyr 416,

Figure 7: RES inhibits Src kinase activity mediating VE-Cadherin activity induced by IS-activated AHR and modulates permeability via AHR: (a1) RES (25, 50, and100 μM) did not affect cytosolic AHR protein expression level; (a2) cytosolic AHR protein decreased significantly following coincubation of IS with RES (b, c). RES could significantly decrease the Src phosphorylation (b, d) RES also decreased the activity of VE-Cadherin induced by IS (100 μg/ml). Data were expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. IS-treated group.

situated present in the catalytic domain in response to stimulation of a variety of cellular receptors, and regulate different signaling pathways and control different receptor-induced biological activities [59]. In the present study, our Western blot analysis result demonstrated that loss of cytosolic AHR protein expression was observed following IS treatment suggesting that IS could trigger the activation of AHR and nuclear translocation in the meantime and promote the release of Src from its receptor complex in the cytoplasm and increased Src activity; in line with this, IS causes a significant increase of Src phosphorylation which mediates the most significant permeability-associated protein (VE-Cadherin) activity and may lead to the increased endothelial hyperpermeability (Figure 4). VE-Cadherin is known to mediate endothelial permeability modulation [57], and numerous stimuli may cause VE-Cadherin phosphorylation, in which Src kinase involves and acts as a key pathway mediator. Here, in this setting, we noticed increased phosphorylation of VE-Cadherin following IS treatment suggesting an increase in endothelial permeability.

As shown in Figure 5(a), the immunofluorescence analysis result further supported the activation and AHR nuclear translocation in response to IS exposure of cells stained with anti-AHR antibody (red) and DAPI (blue) and visualized by fluorescent microscopy which demonstrated highly stained nuclear expression of AHR and an increase in fluorescence intensity in the IS treatment group compared with the control whereas pretreatment of cells with AHR inhibitor (CH223191) blocked the activation and AHR nuclear translocation induced by IS (Figure 5(a)). Western blot analysis also indicated that an increased expression of ARNT is associated with activation and AHR nuclear translocation while treatment with AHR inhibitor (CH223191) decreased the expression level of ARNT induced by IS (Figures 5(b) and 5(c)), and this further confirmed the direct consequence of IS on AHR activation and promoting AHR nuclear translocation from the cytosol compartment to the nucleus and may lead to forming heterodimeric with ARNT and contribute to the increased expression level of ARNT.

Studies indicated that the increased activation of Src kinase following ligand-activated AhR has been noted in several different cell lines and hence may be essential for AhR-mediated regulation of AR activity [60]. Evidence suggesting that activation of AHR may result in deregulation of cell-cell contact has been put forth, hence evoking unbalanced proliferation and dedifferentiation, as well as enhanced motility [61]. Src kinase which has been associated with AHR complex is important in the disruption of cadherin-dependent cell-cell contact. Here, to confirm the involvement of Src kinase on AHR mediating endothelial permeability in response to IS exposure, we incubated cells with AHR blocker (CH223191) with IS (100 μg/ml) and employed Western blot.

The result demonstrated that IS increased AHR activation and CH223191 abolished AHR activation (Figure 6(a)). IS also triggered Src activation; however, CH223191 inhibit its activation (Figure 6(b)) and reduced phosphorylation of VE-Cadherin induced by IS (Figure 6(c)). From this event, we suggested that the inhibitions of Src phosphorylation are AHR-dependent because CH223191 significantly eliminated phosphorylation of Src and may contribute to the reduction of VE-Cadherin activity and suggested that Src kinase activity is essential for regulating endothelial permeability via AHR. The result is comparable with studies demonstrated where Src activation could also contribute to VE-Cadherin phosphorylation by stimulation of H_2O_2 [2]. From previous studies, it was reported that Src mediates vascular endothelial permeability due to TNF, reactive oxygen species (ROS), angiogenesis, and vascular leakage [59]; therefore, in this manner, the IS acts AHR activation and lead to upregulate the activity of Src which mediate the activity of VE-Cadherin, therefore increasing endothelial hyperpermeability. Similar phenomena were observed where treatment of cells with Src blockers abolished Src phosphorylation (Figure 6(d)) and VE-Cadherin phosphorylation (Figure 6(e)) induced by IS implying that PP2 has a potential to inhibit the increased activity of Src mediating VE-Cadherin and reduce hyperpermeability induced by IS. These results are also in agreement with studies which reported that phosphorylation of Src gives rise to endothelial hyperpermeability due to stimulation by Advanced Glycation End-products (AGEs) and PP2 downregulated Src expression illustrating that inactivation of Src decreased AGE-induced HUVEC proliferation, migration, and tube formation [62]. Next, we observed the effect of RES on cytosolic AHR and then evaluated coincubation with IS (Figure 7). The data demonstrated that treatment of cells with RES did not affect the cytosolic AHR protein level. However, loss of cytosolic AHR was observed following coincubation of IS with RES implying that IS aggravated the activation of AHR and promote nuclear translocation from the cytosolic compartment. In this setting, RES did not inhibit or interfere IS binding/activating AHR in the cytoplasm. In the present study, the role of RES illustrating inhibition of Src activation induced IS-activated AHR and modulated VE-Cadherin and permeability. Controversial study results have been reported with regard to RES inhibitory property of ligands activating AHR. Few reports revealed that RES prevent ligand AHR activation by interfering ligand binding with AHR in the cytoplasm whereas the activity of RES may occur in the nucleus after nuclear translocation AHR induced by ligands. In the present study, our finding is correlated with the previous study, where RES interferes with Src tyrosine kinase activity [47]. Our data is comparable with studies reported on the inhibitory property of resveratrol on TCDD-induced CYP1A1 expression in HepG2 cells and the inability of RES to bind AHR and interfere the binding of AHR with TCCD [50]. Here, our data (Figure 7) also revealed that RES markedly decreased phosphorylation of Src induced by IS-activated AHR and downregulate the activity of VE-Cadherin and decreased hyperpermeability significantly thus implying that AHR/Src kinase is necessary for IS-mediated stimulation of permeability-associated protein, VE-Cadherin activity. These observations strongly indicate a possible connection between ligand-activated AHR, Src kinase, and VE-Cadherin. Indeed, our current data show that blockage of Src activity by resveratrol would IS-induced tyrosine phosphorylation of VE-Cadherin and the subsequent decreased endothelial cell permeability. The inhibition of RES on ligand-AHR activation and its biological effect in the nucleus and cytoplasm (genomic and nongenomic AHR pathway) require further investigation.

5. Conclusions

All our data demonstrated that AHR/Src-dependent-driven pathways are involved in IS-induced hyperpermeability of BAECs. TEER of BAECs were weakened in response to IS stimulation which leads to increased permeability, and the possible underlying mechanism could be that IS activated AHR/Src signaling which mediates endothelial permeability via the most important permeability-associated protein, VE-Cadherin. RES improved TEER of cells, suppressed IS-stimulated endothelial hyperpermeability, and downregulated the increased activity of VE-Cadherin, possibly by inhibition of the activation Src induced by IS-activated AHR. Altogether, our findings show in part evidence of the protective property of RES via blockade of IS-AHR mediating Src activation and downregulating the activity of VE-Cadherin and hyperpermeability.

Abbreviations

AHR:	Aryl hydrocarbon receptor
ARNT:	Aryl hydrocarbon nuclear translocator
BAECs:	Bovine aorta endothelial cells
BSA:	Bovine serum albumin
CCK-8:	Cell counting kit-8
CKD:	Chronic kidney disease
CVD:	Cardiovascular disease
DMEM:	Dulbecco's modified Eagle's medium
ECL:	Enhanced chemiluminescence
FBS:	Fetal bovine serum
IS:	Indoxyl sulfate
OD:	Optical density
PBS:	Phosphate-buffered saline
PMSF:	Phenyl-methane-sulfonyl fluoride
PP2:	4-Amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine
PVDF:	Polyvinylidene difluoride
RES:	Resveratrol
RIPA:	Radioimmunoprecipitation assay SDS-PAGE
Electrophoresis:	Sodium dodecyl sulfate polyacrylamide gel
SEM:	Standard error of the mean
pSrc:	Phosphorylated Src
TBST:	Tris-buffered saline with Tween
TCDD:	Tetrachlorodibenzo-p-dioxin
TEER:	Transendothelial electrical resistance
VE-Cadherin:	Vascular endothelial cadherin
pVE-Cadherin:	Phosphorylated vascular endothelial cadherin.

Data Availability

The data used to support the finding of this study are included in the article and can be acquired from the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Eskedar Getachew Assafa and Chenjiang Ying conceptualized and designed the study. Eskedar Getachew Assafa and Qiaoqiao Yan worked on methodology. Eskedar Getachew Assafa, Siameregn Berhe Gezahegn, and Maibouge Tanko Mahamane Salissou analyzed data and wrote the manuscript. Nannan Wu and Shuiqing He revised the manuscript. Chenjiang Ying and Xuezhi Zuo worked on conceptualization, supervision, and funding.

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Review Article

The Bioprotective Effects of Polyphenols on Metabolic Syndrome against Oxidative Stress: Evidences and Perspectives

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Polyphenols are the general designation of various kinds of phytochemicals, mainly classified as flavonoids and nonflavonoids. Polyphenolic compounds have been confirmed to exhibit numerous bioactivities and potential health benefits both *in vivo* and *in vitro*. Dietary polyphenols have been shown to significantly alleviate several manifestations of metabolic syndrome, namely, central obesity, hypertension, dyslipidemia, and high blood sugar. This review is aimed at discussing the bioprotective effects and related molecular mechanisms of polyphenols, mainly by increasing antioxidant capacity or oxygen scavenging capacity. Polyphenols can exert their antioxidative activity by balancing the organic oxidoreductase enzyme system, regulating antioxidant responsive signaling pathways, and restoring mitochondrial function. These data are helpful for providing new insights into the potential biological effects of polyphenolic compounds and the development of future antioxidant therapeutics.

1. Introduction

Polyphenols are compounds derived from plants, which exert protective effects against various environmental stresses in plants [1]. Polyphenols represent the largest group of phytochemicals that possess aromatic rings and ligand groups (mainly hydroxyl combined with different carbon sites). Natural polyphenols are secondary metabolites, and more than ten thousand natural polyphenols have been identified and evaluated thus far [2]. Depending on their structural features, polyphenols can be classified into flavonoids and nonflavonoids [3]. Flavonoids share a common structure of a 15-carbon skeleton, composed of two aromatic rings, connected through a heterogeneous pyrone C ring. This carbon bridge structure can be denoted as C6-C3-C6. As heterocyclic ring-linked groups, flavonoids harbor several subgroups, namely, flavonols, flavones, isoflavones, flavanones, anthocyanidins, flavan-3-ols, and minor subclasses of flavonoids, such as chalcones [3]. In contrast, nonflavonoids refer to phenolic acids, hydroxycinnamic acids, stilbenes, lignans, and coumarins [4, 5]. Representative chemical structures of flavonoids and nonflavonoids are shown in Figure 1. The classifications, compositions, and sources of polyphenols are shown in Table 1.

Although polyphenols do not belong to the category of essential nutrients, ingestion of a certain amount of polyphenolic compounds shows a positive influence on body growth and suppresses the occurrence and development of diseases. Several *in vivo* and *in vitro* studies have reported that polyphenolic compounds exhibit multiple bioactivities and potential health benefits, such as protection of cardiovascular diseases, disruption of oxidation and inflammatory response, growth inhibition of tumor cells and bacteria, and retardation of the aging process [2]. Metabolic syndrome (MetS) is a common clinical syndrome manifested as several abnormal medical phenotypes, such as central obesity, hypertension, dyslipidemia, as well as high blood sugar. MetS is a major trigger for the occurrence and progression of type II diabetes (T2D) and cardiovascular disease. With great advances in basic redox biology, the most important pathophysiology of MetS is considered to be abnormal systemic oxidative stress (OS) via increased production of free radicals and redox imbalance. Increased OS in the body triggers several diseases, such as diabetes [6], cardiovascular diseases like

Figure 1: Representative chemical structures of major groups of polyphenols, generally classified as flavonoids and nonflavonoids.

atherosclerosis [7], Alzheimer's disease [8], and even cancer [9]. OS is closely related to systemic inflammation, endothelial dysfunction, metabolic abnormality, and DNA damage [10]. Nowadays, the preferred management of MetS involves maintenance on a regular and standard diet with increased intake of fruits and vegetables, with reducing the consumption of high-fat, high-salt, and high-sugar substances and optimizing the diet structure, improving physical exercise training, and reducing alcohol consumption [11]. Therefore, a new preventive solution is urgently needed to reduce the morbidity and development of MetS. Until now, several studies have confirmed that supplementation with single/-multiple polyphenolic compounds or specific phenolic extracts exhibits protective effects on MetS in cell and animal models. Tian et al. indicated that polyphenols that are extracted from green tea (GT) cut down fat deposition in high fat-fed rats by inhibition of extracellular signal-regulated kinase (ERK) 1/2-peroxisome proliferator-activated receptor (PPAR) γ-adiponectin pathway [12]. They also reported that intake of green tea polyphenols (GTP) reduces the prevalence of coronary heart disease in the middle-aged and elderly Chinese populations to some extent [13]. Resveratrol (RSV, a form of nonflavonoid) treatment was demonstrated to attenuate the hyperpermeability and the overexpression of cav-1 induced by high glucose in a dose-dependent manner. RSV was confirmed to downregulate the increased expressions of vascular endothelial growth factor (VEGF) and kinase insert domain receptor (KDR or VEGF receptor-2) induced by high glucose [14]. In a randomized, controlled, double-blinded clinical trial, Faghihzadeh et al. found that RSV supplementation for 12 weeks significantly decreased the level of alanine aminotransferase (ALT) and arrested hepatic steatosis in nonalcoholic fatty liver disease (NAFLD) patients compared to the control group ($P < 0.05$) [15]. Therefore, polyphenols, as powerful antioxidants commonly found in fruits and vegetables, are considered to be a potential therapeutic candidate for MetS [16].

The purpose of this review is to renew and supplement information about the effects of polyphenols on MetS and related antioxidative mechanisms, derived from cell studies, animal studies, and especially, interventional human studies.

2. The Potential Roles of Polyphenols in MetS

Polyphenols are biomolecules derived from plant origin and have been proven to exhibit antioxidant and anti-inflammatory activities. Several lines of evidence have suggested that polyphenols, at certain doses, might delay or prevent MetS onset by decreasing body weight, blood pressure (BP), and blood glucose, and by improving abnormal lipid metabolism [17].

2.1. Hyperglycemia and Diabetes Mellitus. The International Diabetes Federation (IDF) has predicted that there were 451 million diabetes patients globally in 2017, increasing with an alarming rate, and by 2045, diabetes patients are estimated to be drastically increased up to 693 million. Majority of those patients were classified as type 2 diabetes (T2D) patients [18]. The main clinical characteristics of T2D involve systematic insulin resistance and relative insufficiency of insulin secretion [19].

Table 1: Summary of the classification, composition, and main sources of polyphenols.

Classifications	Subclasses	Compositions	Main sources
Flavonoids	Flavonols	Flavonols, kaempferol, quercetin, isorhamnetin, myricetin	Onions, shallots, spinach, green and black tea, dark chocolate, various fruits, vegetables, nuts
	Flavones	Apigenin, luteolin, tangeretin, nobiletin, wogonin, baicalein	Celery, parsley, some herbs, rooibos tea, citrus species, onion, garlic, pepper, Thai chili, citrus fruits, scutellaria, passiflora
	Isoflavones	Daidzein, genistein, glyciten	Leguminous plants, soybeans and soy products
	Flavanones	Naringenin, hesperetin, naringin, neohesperidin, rutinosides, narirutin, hesperidin	Flavedo of citrus fruits, bitter oranges, grapefruit, tomatoes, lemon, mandarin and grapefruit
	Anthocyanins	Pelargonidin, cyanidin, delphinidin, peonidin, petunidin, malvidin	Berries, cherries, red grapes, currants, red wines, oranges, the black varieties of soybeans, rice, beans, onions, potatoes, cabbage
	Flavan-3-ols	(+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epiafzelechin, (−)-epigallocatechin-3-O-gallate	Green tea, fruits, berries, cereals, nuts, chocolate, red wine
	Minor subclass of flavonoids	Chalcones	Tomatoes, licorice, shallots, and bean sprouts
		Dihydrochalcones (phloridzin, aspalathin, nothofagin)	Apples and apple products, rooibos tea
		Aurones	Vegetables and fruits
		Dihydroflavonols, flavan-3,4-diols	Biosynthetic intermediates of the flavonols and anthocyanins
Nonflavonoids	Phenolic acids	Hydroxybenzoic acids, gallic acid, protocatechuic acid	Gallotannins, raspberries, strawberries, blackberries, pomegranate, persimmon, walnuts, hazelnuts, grapes, wine, green and black teas, mangoes
	Hydroxycinnamic acids	Caffeic acid, ferulic acid, p-coumaric acid, sinapic acid	Flesh of grapes, blueberries, kiwis, plums, cherries, apples, coffee
	Stilbenes	Resveratrol	Red wines, grapes, peanuts, peanut products, plums, pine nuts
	Lignans	Syringaresinol, ecoisolariciresinol, matairesinol, medioresinol, pinoresinol, lariciresino	Linseed, algae, leguminous plants, cereals, vegetables, fruits
	Other polyphenols	Curcumin	Turmeric

In a clinical trial, 50 participants exhibiting more than one abnormal medical phenotypes of MetS were recruited and randomly administered 8 g dried grape pomace (containing mainly catechins and proanthocyanidins) or placebo daily. After six weeks of supplementation with grape pomace, basal insulin was decreased from to 8.5 to 5.5 μU/mL, showing a significant improvement in fasting insulinemia. No significant change was found in both fasting and postprandial glucose between the pre- and postintervention period. Moreover, homeostatic model assessment-insulin resistance (HOMA-IR) was decreased from 2.1 to 1.4 while quantitative insulin sensitivity check index (QUICKI) was increased from 0.35 to 0.42. Previous clinical studies have revealed that anthocyanins and RSV prevent incidence of diabetes, by reducing the levels of blood glucose and hemoglobin A1c (HbA1c), promoting secretion efficiency of pancreatic β cells, and ameliorating systematic insulin resistance [20]. In another randomized, controlled, crossover study, 12 healthy men participated in interventions with either 1.562 g gallic acid equivalent (GAE) beverage or a placebo beverage without polyphenols (CD) randomly, followed by a standard meal after 3 hours. Compared to the CD group, a 36% increase of insulin sensitivity index (SI) was found in the GAE group, while a 31% and 18% decrease was observed in the postprandial insulin incremental area (iAUC0-5h) and insulin secretion index, respectively, after polyphenol supplementation. Among the phenolic metabolites, gallic acid was positively related to the SI index ($r = 0.588$) but negatively with insulin secretion and response ($r = -0.604$) [21]. Polyphenol, added to the usual diet, could contribute to improve blood sugar metabolism but could have different influences in different individuals. In obese participants, with supplementation of mangoes that are rich in gallotannin (polymeric form of gallic acid) for 6 weeks, HbA1c was decreased by 18% [22]. Rienks et al. have systematically analyzed eight studies and the relationship between polyphenols and T2D by using a meta-analysis. In this analysis, most flavonoids, especially flavanones and anthocyanidins, exhibited nonlinear dose-response correlations with T2D. However, phenolic acids, belonging to nonflavonoids, showed a linear relationship. This difference might be attributed to the difference in their structures. The researchers concluded that additional dietary

polyphenols, especially flavonoids, might exert an important influence on the improvement of T2D [23].

This section summarizes the results from various clinical trials of polyphenolic compounds as antidiabetic agents. It has been identified that natural polyphenols show antidiabetic effects, by reducing the levels of blood glucose and HbA1c, increasing the secretion efficiency of pancreatic β cells, and ameliorating systematic insulin resistance. Dietary supplements with polyphenols have been confirmed as an indispensable part of diabetes diet therapy in clinical trials. However, how diabetes alters the bioavailability and bioactivity of polyphenols is not well understood. It might contribute to an increase in their inherent effects and clinical consequences.

2.2. Obesity. In the past few decades, the global incidence rate of obesity has dramatically increased. Epidemiological survey results show that, from 1975 to 2016, the incidence rate of obesity (defined as body mass index (BMI) ≥ 25 kg/m^2) was raised by affecting 40% more adults (21% in men and 24% in women). The morbidity rate of obesity (BMI ≥ 30 kg/m^2) in men is growing faster than that in women, with the rate increasing for men from 3% to 12% and for women only doubling more than twice (from 7% to 16%) [24]. It has been predicted that more than 1 billion people will be obese by 2030 [25].

Marranzano et al. designed a cohort trial to elucidate the relationship between obesity and estimated dietary flavonoid intake. Intake of flavonoids was negatively correlated with BMI ≥ 25 and the odds ratio (OR) was about 66%. Surprisingly, among the subclasses of flavonoids, only flavanones showed this similar correlation (OR = 0.68) after adjustment for confounding factors. These data indicated that higher intake of flavonoid resulted in the loss of body weight [26]. In a controlled and double-blind study, 95 overweight individuals were randomly administered 900 mg of a citrus-based polyphenol (mainly catechin and naringin) extract or a placebo taken daily with meals. Compared with the placebo group, waist and abdominal fat and hip circumference in the intervention arm were significantly decreased (waist -5.71% vs. -1.56%, hip -4.71% vs. -1.35%, and fat -9.73% vs. -3.18%) compared to those in the control arm. Furthermore, OS was also lowered by increasing superoxide dismutase (SOD) and glutathione (GSH) and reducing serum malondialdehyde (MDA) levels [27]. Consistent with other clinical trials, 33 participants were recruited with either BMI of more than 25 kg/m^2 or a waist circumference beyond 94 cm in men or 80 cm in women. They were randomly distributed into two groups that were administered a diet supplemented either with 500 mg C. fimbriata extract (gallic acid) or placebo. Waist to hip ratio (WHR) were measured to be decreased by 3% in the intervention group, compared to 1% in the control ($P < 0.05$). In addition, body weight, hip circumference, blood pressure, triglyceride levels, and fat accumulation were all found to be markedly decreased in the polyphenol-intake group compared to the placebo group ($P < 0.05$). These results indicated that the extract of C. fimbriata combined with diet control and adequate exercise might significantly reduce central obesity, one of the vital phenotypes of MetS [28].

Of course, there were also some studies that reported inconsistent or controversial results. The accretion of green tea (GT, mainly flavan-3-ols) for 12 weeks in sixty subjects (BMI > 18 kg/m^2) did not exert a significant influence on body weight, BMI, fat deposition, and resting energy expenditure [29]. Another 12-month randomized, double-blind, placebo-controlled clinical trial recruited 937 postmenopausal females (BMI ≥ 25.0 kg/m^2). These individuals were randomized into different groups, the GT extract group, containing individuals administered 843 mg (-)-epigallocatechin-3-gallate, and the remaining in the placebo group. The changes in BMI, overall fat content, percentage of body fat, or bone mineral density in 12 months were not statistically different [30].

In conclusion, this section reviewed the advances of the benefit of polyphenol supplementation in clinical trials. Consumption of polyphenols has been proposed to result in favorable improvement in fat deposition and extenuates inflammation, abnormal blood glucose metabolism, and oxidative unbalance status in overweight subjects, although some negative results were reported. In addition, polyphenol supplementation was well tolerated with no adverse effect [31–34]. Therefore, future researches are warranted to confirm these results over a longer period and discover the potential mechanisms of action of the polyphenols.

2.3. Hypertension. Elevated BP in MetS is a predominant risk factor contributing to cardiovascular damage. With an increase in blood pressure, multiple target organ dysfunctions can occur. Many studies have found that different polyphenols have different degrees of effects on BP.

Daily intake of 25 g chocolate, containing high content of polyphenols, resulted in significant decrease of about 5.93 mmHg and 6.4 mmHg in systolic and diastolic BP, respectively, after administration for 8 weeks [35]. Taubert et al. confirmed that even low habitual 6.3 g dark chocolate intake (equivalent with 30 mg polyphenols) can have a favorable clinical effect on individuals with untreated prehypertension or stage 1 hypertension not accompanied with targeted organ damage risks. From the baseline to end of trial for 18 weeks, mean diastolic BP dropped by 1.9 mmHg and systolic BP declined more prominently by 2.9 mmHg accompanied by a decreasing trend in hypertension prevalence from 86% to 68% in the experimental group. Moreover, the decline in BP was associated with a progressive elevation of S-nitrosoglutathione concentration at about 0.23 nmol/L, indicating that OS played an important role in this process [36].

It should be noted that not all polyphenols have the same effect on systolic and diastolic BP. A systemic review and meta-analysis of twenty-four randomized-controlled clinical trials (RCTs) recruited 1106 participants to explore the efficacy of flavonoid-rich cocoa (FRC) on alleviating potential cardiovascular damage and to evaluate their possible correlation. When administered with FRC for up to 2 weeks, systolic BP dropped by 1.63 mmHg (95%CI = 0.13, 3.12; $P = 0.033$), while diastolic BP exhibited no changes [37]. Nevertheless,

a randomized, double-blind crossover study showed the purveyance of orange juice, abundant in various kinds of polyphenols (mainly anthocyanins), reduced both systolic and diastolic BP. This intervention decreased systolic BP from 128 ± 1 mmHg to 124 ± 2 mmHg ($P < 0.05$) and decreased diastolic BP from 79 ± 1 mmHg to 76 ± 1 mmHg ($P < 0.05$) [38]. Nevertheless, some studies have reported controversial results. Koli et al. evaluated the potential functions of regular ingestion of flavonoid-abundant dark chocolate on BP, while restricting snack intake intervention, for 8 weeks. Intake of dark chocolate exhibited no significant benefits on 24 h resting BP (142 ± 11.5/89 ± 8.4 mmHg in initial vs. 142 ± 14.2 /88 ± 9.4 mmHg later, $P > 0.05$). The authors concluded that dark chocolate supplement showed no significant changes in BP or other cardiovascular risk factors during a reduced snack period [1]. As for nonflavonoids, in a randomized-controlled clinical trial, 37 middle-aged and older participants were enrolled and were either provided with 480 mL of tart cherry beverage (high in gallic acid) or control drink (only energy and sugar content) daily for 12 weeks. At the beginning, individuals with high polyphenols retained superior mean systolic BP than the control group (141.4 mmHg vs. 133.4 mmHg). At the end of this study, individuals consuming high polyphenolic compounds showed lower systolic BP than the control group (137.3 mmHg in the test group vs. 138.8 mmHg in the control group). However, no statistically significant change in diastolic BP was observed in either treated or control group [39]. Fang et al. conducted a clinical study showing that mango (full of gallotannin-derived polyphenols) supplementation for 6 weeks significantly decreased systolic BP (but not diastolic BP) in the individuals; however, no expected change in BP occurred in obese individuals [22].

Taken together, daily polyphenol-rich food intake lowers the blood pressure in adults, and it is a plausible intervention for improving cardiovascular health. However, some clinical studies have shown inconsistent results. Therefore, further clinical trials are urgently needed in a larger population and for longer durations to further elucidate the protective role of polyphenols in hypertension.

2.4. Dyslipidemia. There is no doubt that hyperlipidemia is one of the major risk factors for cardiovascular disease. In the USA, about 45.0% and 32.0% of females were found to have increased levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDLC), respectively [40].

Erlund et al. showed that, compared to the control, serum high-density lipoprotein cholesterol (HDLC) level significantly increased in the berry-eating group (abundant flavan-3-ols, flavonols, and flavanones) compared to the control group (5.2% vs. 0.6%, $P < 0.05$), while TC and triacylglycerol (TG) levels remained unchanged [41]. Another RCT indicated that green tea extract consumption in individuals for 1 year led to a marked decrease in circulating TC (-2.1% vs. 0.7%; $P < 0.01$), LDLC (-4.1% vs. 0.9%; $P < 0.0001$), and non-high-density lipoprotein cholesterol (-3.1% vs. 0.4%; $P < 0.01$) compared with placebo. The dose of serum HDLC remained unchanged, whereas TG concentration increased to 3.6% in the experimental group compared to the control group (3.6% vs. -2.5%, $P < 0.05$) [42]. Another clinical study showed that intake of 122 mg Goishi tea polyphenols daily increased HDLC level and decreased TC level in the individuals, exhibiting the potential beneficial effect of reducing risks of arteriosclerosis and cardiovascular diseases [43]. A systematic review including 17 clinical trials ($n = 1356$) concluded that intake of green tea epigallocatechin gallate (EGCG) from 107 to 856 mg/d apparently decreased LDLC by 9.29 mg/dL (95% CI, -12.27 to -6.31), and this protective effect mainly depends on the lipid level at baseline [44]. With consumption of 500 mg aronia extract, containing anthocyanins, proanthocyanidins, and hydroxycinnamic acids, the aronia-interventional group presented a favorable effect in microlipid metabolism. After 12 weeks of treatment, in the peripheral circulating bloodstream, TC concentration dropped by 8%, while LDLC level was increased up to 11%, compared to the placebo. Even LDL receptor protein in mononuclear cells was detected to be reduced by 56% from the baseline, whereas the placebo group showed no significant changes [45].

In conclusion, favorable changes were revealed in TC, LDLC, and HDLC levels after the administration of polyphenols in several clinical trials. These findings can guide us to suggest people to consume more fruits and vegetables containing abundant polyphenols that might improve blood lipid profiles, and thereby, prevent dyslipidemia. More studies are needed to further identify the favorable effects of these polyphenolic compounds on dyslipidemia and explore their underlying molecular mechanisms.

2.5. Nonalcoholic Fatty Liver Disease. Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. NAFLD is closely related to the abnormal metabolism of carbohydrates and lipids and triggers insulin resistance and T2D. The favorable effects of polyphenols on NAFLD have been investigated in several clinical trials.

The supplementation of 500 mg RSV for 12 weeks significantly decreased the alanine aminotransferase (ALT) concentration and hepatic steatosis in 50 NAFLD patients compared to the placebo ($P < 0.05$); however, no expected improvements in anthropometric measurements, insulin resistance (IR), lipid profile, and BP were observed ($P > 0.05$) [15]. This group also found that RSV (at the same concentration for the same period) consumption combined with lifestyle change exhibited superior effects compared to lifestyle modification alone [46]. The reduction of AST, plasma glucose, LDLC, TC, insulin resistance index, and TNF-α was also observed in NAFLD patients after administration of edible RSV capsules [47]. A short-term (8-week) daily intake of curcumin also extenuated liver fat and serum levels of ALT and AST in patients diagnosed with NAFLD [32]. Daily consumption of 70 mg of curcumin was found to alleviate liver fat content by 78.9% in NAFLD patients, while only 27.5% improvement was observed in placebo group patients. Most physiological and biochemical features of NAFLD were improved, including levels of TC, LDLC, ALT, and AST. During the trial, curcumin was found to be safe and well tolerated in the patients [48].

In summary, increasing evidence has shown the satisfactory effectiveness of polyphenolic compounds in NAFLD. In addition, it has been shown that polyphenol (flavonoids and nonflavonoids) supplements combined with lifestyle changes ameliorated both occurrence and progression of NAFLD, which exhibited a more favorable effect than lifestyle changes alone. Therefore, polyphenol supplement plus healthy lifestyle might be a feasible choice for patients with NAFLD.

3. Antioxidant Effects of Polyphenols on MetS

The detailed mechanism underlying the protective effects of polyphenol is not yet understood; however, oxidative stress, as one of the vital pathogeneses of MetS, suggests many targeted therapies for these diseases. For the purpose of establishing a clearer and deeper understanding of the roles of various kinds of polyphenols in MetS, we summarized the results of several *in vitro* and *in vivo* studies on antioxidant effects and related mechanisms in different models, as shown in Table 2 [49–64]. We also summarized the results of studies on the protective effects of polyphenols against MetS in clinical trials (as shown in Table 3) [15, 21, 22, 27, 28, 36, 38, 39, 41, 42, 46, 47, 65–70]. Figure 2 summarizes the schematic representation of the principal antioxidative mechanisms of polyphenols in various cellular models.

3.1. Antioxidant Properties of Polyphenols. The antioxidant properties of polyphenols and secondary metabolites depend on the chemical construction of attached functional groups, more specifically, the permutation of functional groups about the nuclear structure. The number of hydroxyl residues greatly affects antioxidant activity by scavenging radicals and disrupting metal ion chelation [71]. In addition, other structures like O-methylation, 2–3 double bond, and 4-oxo, carbohydrate moieties, and degree of polymerization play roles in antioxidant activity [71]. Antioxidant activities of polyphenols are associated with their capacity to eliminate high levels of reactive oxygen species (ROS).

Bai et al. performed a study to investigate the biological characteristics of polyphenolic extracts from apple pomace using antioxidant assays and high-performance liquid chromatography (HPLC) analysis. The study identified that the antioxidant ability among apple polyphenolic extracts was highest in procyanidins B2, followed by chlorogenic acid, hyperin, quercetin, caffeic acid, syringin, cinnamic acid, and phloridzin [72]. Another study showed that O-methylation of the hydroxyls of the catechol B ring resulted in a decrease of the antioxidant activity of the parent compounds. Antioxidant activity from strong to weak is as follows: quercetin>epicatechin>catechin. In addition, pH value also affected antioxidant activity of polyphenols in this assay; lower antioxidant activity was observed at acidic pH. It suggested that the same polyphenols might exhibit different antioxidant capacities in different parts of the body and also provided a possible strategy to enhance the efficacy of dietary polyphenolic compounds [73]. Lemańska et al. reported that O-methylation modification of the quercetin and luteolin undermined the radical scavenging capability by affecting the donating property of electron and hydrogen atom. This study also provided a new insight into the mechanisms of catechol O-methylation on radical scavenging [74].

Cellular antioxidant activity (CAA) assay, combined with *in vitro* digestion, is notably more effective in detecting antioxidant activity than a simple chemical assay alone [75]. Sun et al. reported that flavonoids and phenolic acids in fresh citrus fruits significantly increased CAA values in a human intestinal HepG2 cell model [56]. In *ex vivo* studies, the order of ability to donate electron and antioxidant activity was gallic acid>quercetin>rutin>vanillin>acetylsalicylic acid. These compounds were considered to play roles in the antioxidant properties of *S. aegyptiaca* [57].

Increasing evidence has shown that polyphenols have strong antioxidant properties. Currently, it has been speculated that the antioxidant efficiency of polyphenols suppressed ROS generation by means of inhibiting the catalytic activity of related synthetic enzymes, scavenging of ROS, and upregulating the protective antioxidant defensive network [76, 77].

3.2. Antioxidant Signal Pathway Regulation. The antioxidant response elements (AREs), namely, cis-acting regulatory elements, participate in regulating the expression of special gene encoding enzymes involved in antioxidant and phase II detoxifying reactions [78]. The nuclear transcription factor, erythroid 2-like 2 (Nrf2), a central conditioner of AREs, is commonly associated with the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, and subsequently, degraded by the proteasome. However, in the stimulation of oxidative stress, Keap1 cysteine residues were oxidized, which led to Nrf2 dissociation and translocation into the nucleus, where it interacted with AREs, and thus, upregulated expression of downstream genes [79].

Polyphenols, as powerful antioxidants, were confirmed to promote the Nrf2 nuclear translocation under oxidative stress. On one hand, polyphenols protected against tert-Butyl hydroperoxide- (t-BHP-) induced oxidative injury via upregulation of the expression of primary and phase II detoxifying genes, especially enzyme-encoding genes. Heme oxygenase-1 (HO-1), genetically encoded by *Hmox-1*, is a rate-limiting enzyme of heme catabolism, which gets transformed into carbon monoxide (CO), biliverdin, and ferrous iron. The interaction of biliverdin and bilirubin constituted an effective antioxidant, namely, biliverdin reductase (BVR), to reduce OS [80]. The polyphenol-rich extract of the *Nymphaea nouchali* flower (NNF) was shown to reduce the adverse effects of t-BHP by attenuating oxidative DNA damage and reducing cellular ROS formulation. This process was accompanied by phosphorylation of MAP kinase (p38 kinase and ERK) as a downstream effect of upregulating Nrf2 nuclear translocation [81]. Polyphenol-rich *Nymphaea nouchali* leaf extract (NNLE) was also observed to act against OS by increasing the mRNA and protein level of antioxidant and phase II detoxifying enzymes, especially HO-1. NNLE supplement promoted Nrf2 translocation into the nucleus and the level of phosphorylated p38 kinase and ERK [82]. These results were consistent with other *in vitro* results reporting that quercetin [83], flavon-3-ols [84, 85], puerarin

Table 2: The antioxidant activity of polyphenols in both *in vitro* and *in vivo* models.

Authors, year	Sources	Main polyphenols	Cell models or animal models	Antioxidant activity changes
(Baird and Dinkova-Kostova 2011) [49]	L. coromandelica bark	Gallic acid, caffeic acid, (-)-epigallocatechin-3-gallate, chlorogenic acid, catechin	RAW 264.7 cells	Cellular ROS production↓, SOD, CSH-Px, and CAT activities↑
(Kim, Kim et al. 2011) [50]	Pycnogenol	Procyanidins, phenolic compounds	High glucose-treated renal tubular cells	Lipid peroxidation↓, total reactive species↓, superoxide↓, nitric oxide (NO(·))↓, peroxynitrite (ONOO(-))↓, iNOS↓, COX-2↓, NF-κB nuclear translocation↓.
(Tsai, Hsu et al. 2017) [51]	Chemical hemisynthesis	Resveratrol	Human fibroblast-like synoviocytes	NADPH oxidase activity ↓, ROS generation↓
(Adefegha, Oyeleye et al. 2018) [52]	African crocus and wonderful kola seeds	Phenolic acids, flavonoids	Rat penile homogenate	$FeSO_4$- and SNP-induced lipid peroxidation↓
(Lunder, Roskar et al. 2018) [53]	Coniferous	ND	Mouse C2C12 myoblast cells	Intracellular ROS production↓
(Oliveira, Dare et al. 2018) [54]	The leaves of Nectandra hihua	Flavonoids quercitrin, avicularin, juglalin, afzelin, astragalin	L929 fibroblasts	ROS production↓, lipid peroxidation inhibition↓
(Acero, Gradillas et al. 2019) [55]	Spanish local varieties of Prunus avium (L.)	ND	HepG2 cells	XOD↓, ROS↓
(Sun, Tao et al. 2019) [56]	Fresh citrus fruits	Flavonoids, phenolic acids	Intestinal HepG2 cells	CAA values ↑
(Nauman, Kale et al. 2018) [57]	Chemical hemisynthesis	Gallic acid, quercetin, rutin, acetylsalicylic acid	The ex-liver of mice	Peroxidative damage in microsomes↓, protein carbonyl in cytosolic fraction↓
(Liu, Ren et al. 2014) [58]	ND	Resveratrol	CK-exposed mice	The MDA activity↓, SOD, CSH-Px, and CAT activities↑
(Auberval, Dal et al. 2017) [59]	Red wine	Resveratrol	Wistar rats	Lipid peroxides↓, oxidative proteins↓.
(Jian, Ding et al. 2018) [60]	Loquat leaf	Flavonoids	$PM_{2.5}$-induced NAFLD mice	Oxidative MDA↓, SOD↑
(Li, Chen et al. 2018) [61]	D. loddigesii, Dendrobium	Bibenzyls, phenanthrenes	Diabetic mice	The MDA activity↓, SOD, CSH-Px, and CAT activities↑
(Nauman, Kale et al. 2018) [57]	Chemical hemisynthesis	Gallic acid, acetylsalicylic acid	C57BL/6 mice	SOD, CSH-Px, and CAT activities↑, lipid peroxidation↓
(Song, Park et al. 2018) [62]	Walnut, chokeberry	Anthocyanins	Balb/c mice	MDA↓, lipid peroxidation↓, SOD and CSH-Px activities↑, antioxidant enzyme gene expression↑
(Zyzelewicz, Bojczuk et al. 2018) [63]	Cocoa bean	Flavan-3-ols, flavonols, phenolic acids	Male Wistar rats	GSH↑, GSSG↓
(Qian, Wang et al. 2019) [64]	Bilberry and black currant	Anthocyanins	Male ICR mice	SUA level↓, XOD activity↓, XOD mRNA and protein expressions↓

(↓): decrease; (↑): increase; ND: not detected; ROS: reactive oxygen species; NADPH: nicotinamide adenine dinucleotide phosphate; SNP: sodium nitroprusside; CAA: cellular antioxidant activity; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; NF-κB: nuclear factor-kappa; XOD: xanthine oxidase; SOD: superoxide dismutase; CSH-Px: glutathione peroxidase; GSH: glutathione; GSSG: oxidized glutathione; CK: cigarette smoke; MDA: malondialdehyde; CAT: chloramphenicol acetyl transferase; SUA: serum uric acid; NAFLD: nonalcoholic fatty liver disease.

Table 3: Summary of results from clinical trials of polyphenols in individuals with MetS.

Authors, year	Sources	Main polyphenols	Individuals	Duration	Bioactivities	Improvements
(Faghihzadeh, Adibi et al. 2015) [15]	Resveratrol capsules	Resveratrol	50 NAFLD patients	12 weeks	ND	ALT↓, hepatic steatosis↓
(Costabile, Vitale et al. 2018) [21]	Red grape pomace	Anthocyanins, flavan-3-ol, procyanidins	12 healthy men	1 week	ND	Postprandial insulin incremental area↓, insulin secretion index↓, IS index↑
(Fang, Kim et al. 2018) [22]	Mango	Gallic acid, gallotannin, galloylglycosides, flavonoids	12 lean and 9 obese participants	6 weeks	Anti-inflammation: IL-8↓ and MCP-1 ↓.	In lean participants: systolic BP↓ In obese participants: HbA1c and PAI-1↓
(Dallas, Gerbi et al. 2014) [27]	Sinetrol-XPur	Catechin, naringin	95 overweight subjects	12 weeks	Antioxidation: MDA↓, SOD↑, GSH↑ Anti-inflammation: CRP↓, fibrinogen↓	Waist and hip circumference↓, abdominal fat↓, body weight↓
(Astell, Mathai et al. 2013) [28]	C. fimbriata extract	Gallic acid	43 overweight and obese subjects	12 weeks	ND	Waist circumference↓, WHR↓ palatability of the test meal↓, sodium intake↓, body weight↓, BMI↓, hip circumference↓, systolic BP↓, HR↓, TG↓, total fat and saturated fat intake↓
(Taubert, Roesen et al. 2007) [36]	Cocoa-containing foods	Ericatechin, procyanidin dimer, flavonols	44 untreated upper-range prehypertension or stage 1 hypertension	18 weeks	Antioxidation: fasting plasma levels of S-nitrosoglutathione↑	Systolic BP↓, diastolic BP↓, hypertension prevalence↓
(Rangel-Huerta, Aguilera et al. 2015) [38]	Orange juice	Anthocyanins	100 obese or overweight adults	12 weeks	Antioxidation: 8-OHdG↓, GR↓, erythrocyte catalase↓, 8-isoPGF2α↓, SOD↑	Body mass index↓, waist circumference↓, leptin↓, systolic and diastolic BP↓
(Chai, Davis et al. 2018) [39]	Tart cherry juice	Gallic acid	17 men and 20 women	12 weeks	ND	Systolic BP↓, LDLC↓, TC↓
(Erlund, Koli et al. 2008) [41]	Berry	Quercetin, caffeic acid, vanillic acid,	72 unmedicated subjects with cardiovascular risks	8 weeks	ND	HDLC↑, systolic BP↓
(Samavat, Newman et al. 2016) [42]	Green tea	Catechins	936 women	12 months	ND	Circulating TC↓, LDLC↓ Non-HDLC↓.
(Faghihzadeh, Adibi et al. 2014) [46]	Resveratrol capsules	Resveratrol	50 NAFLD patients	12 weeks	Anti-inflammation: IL-6↓, NF-κB activity↓, serum CK-18 ↓	Weight↓, body mass index↓, waist circumference↓, ALT↓, hepatic steatosis grade↓

Table 3: Continued.

Authors, year	Sources	Main polyphenols	Individuals	Duration	Bioactivities	Improvements
(Chen, Zhao et al. 2015) [47]	Resveratrol capsules	Resveratrol	60 NAFLD patients	3 months	Anti-inflammation: NF-κB activity ↓, serum CK-18 ↓	AST↓, glucose↓, LDLC↓, ALT↓, TC↓HOMA-IR↓TNF-α↓, CK-18 fragment↓, FGF21↓, adiponectin level↑
(Martinez-Maqueda, Zapatera et al. 2018) [65]	Dried grape pomace	Anthocyanins, flavan-3-ol	50 participants with at least one phenotype of MetS	6 weeks	ND	IR↓, fasting insulinemia↓, Is↑
(Tynkkynen, Mursu et al. 2012) [66]	Chocolate	Epicatechin	45 nonsmoking volunteers	3 weeks	Antioxidation: oxidation susceptibility of serum lipids↓	HDL↑
(Mursu, Voutilainen et al. 2005) [67]	Polyphenol-rich phloem	Catechins	75 nonsmoking hypercholesterolemic men	4 weeks	Antioxidation: Oxidation resistance of total serum lipids↑	Lipid peroxidation↓
(Espinosa-Moncada, Marin-Echeverri et al. 2018) [68]	Vaccinium meridionale Swartz (agraz)	Anthocyanins	40 women with MetS	4 weeks	Antioxidation: serum antioxidant capacity↑, urinary 8-OHdG ↓ Anti-inflammation: Hs-CRP levels ↓	Serum antioxidant capacity↑, DNA oxidative damage↓
(Vetrani, Vitale et al. 2018) [69]	ND	Flavonoids, flavan-3-ols, phenolic acids, flavonols	78 individuals with at least one features of the MetS	8 weeks	Antioxidation: urinary isoprostanes↓	Postprandial lipid response↓, VLDL↓, early insulin secretion↑
(Chiva-Blanch, Urpi-Sarda et al. 2013) [70]	Red wine	Catechin, epicatechin, malvidin-3-glucoside, gallic acid	73 male moderate alcohol consumers	4 weeks	ND	Plasma insulin↓, HOMA-IR↓, HDLC↑, Apo A-I↑, Apo A-II↑, lipoprotein↓

(↓): decrease; (↑): increase; ND: not detected. MetS: metabolic syndrome; IR: insulin resistance; IS: insulin sensitivity; IL: interleukin; MCP-1: monocyte chemotactic protein-1; HbA1c: glycosylated hemoglobin; PAI-1: plasminogen activator inhibitor 1; BP: blood pressure; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione; CRP: C-reactive protein; WHR: waist to hip ratio; BMI: body mass index; HR: heart rate; TG: triacylglycerol; LDLC: low-density lipoprotein cholesterol; 8-OHdG: 8-hydroxydeoxyguanosine; GR: glutathione reductase; HDLC: high-density lipoprotein cholesterol; TC: total cholesterol; VLDL: very low-density lipoprotein; NAFLD: nonalcoholic fatty liver disease; ALT: alanine aminotransferase; NF-κB: nuclear factor-kappa B; CK: cytokeratin; FGF: fibroblast growth factor; 8-isoPGF2α: 8-iso-prostaglandin F2α; AST: aspartate aminotransferase; HOMA-IR: homeostasis model assessment insulin resistance index; TNF-α: tumor necrosis factor-alpha; hs-CRP: high-sensitivity C-reactive protein; Apo: apolipoprotein.

Figure 2: Schematic representation of the main antioxidative mechanisms responsible for MetS regulated by polyphenols.

[86], and phenolic acids [87] activate HO-1 by suppressing the MAPK pathway to mediate antioxidant activity.

However, multiple potential mechanisms have been hypothesized for the separation of Keap1 and Nrf2 and Nrf2 nuclear translocation. According to the first mechanism, polyphenols were able to increase cellular Nrf2 levels. Alam et al. had shown that NNF extract supplement increased the protein level of Nrf2 in a time-dependent manner, reaching the maximum level at 12 h after treatment [81]. In addition, quercetin also suppressed the ubiquitination and proteasomal turnover of Nrf2 to maintain its stability [83]. Secondly, NNF extract supplement dose-dependently decreased the transcriptional and translational levels of Keap1 [81]. Quercetin was also found to significantly reduce posttranslational levels of Keap1 protein [83]. However, the main bioactive polyphenols, agrimonolide (AM) and desmethylagrimonolide (DM), have been proved to facilitate Keap1 degradation to further accelerate the release of Nrf2 [88].

3.3. Regulation of Oxidoreductase Enzyme System

3.3.1. Induction of Antioxidant Enzymes. Cellular ROS levels are regulated by an enzymatic antioxidant system, such as SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). SOD can transform superoxide into H_2O_2, and then, H_2O_2 is catalyzed by CAT and GSH-Px. The Nrf2-ARE pathway was found to activate the endogenous protective system, facilitating antioxidant capacity in cells [88]. Song et al. found that anthocyanin supplement upregulated the expressions of SOD and GSH-Px, and subsequently, attenuated lipid peroxidation in the serum, kidney, and liver in an aging mouse model [62]. Psotova et al. had detected the inhibitory ability of some flavonoids on lipid peroxidation. This study showed that the order of the inhibitory ability of flavonoids on lipid peroxidation was as follows: quercetin>baicalein>-kaempferol>luteolin>apigenin [89]. RSV supplementation significantly restored the activities of SOD, GSH-Px, and CAT. When administered with resveratrol at the dose of 1 mg/kg, the activities of the above enzymes increased 1.4-, 1.5-, and 1.3-fold, respectively, compared to the control group [58]. Glutathione, as a major antioxidant, exists in almost every cell in reduced (GSH) and oxidized (GSSG) forms. The above enzymes play key protective functions against reactive species. Pereira et al. compared the structure-antioxidant activity of GSH combined with different kinds of flavonoids. All of tested flavonoids exhibited antioxidant properties; however, those carrying the catechol in B ring showed synergistic effects with GSH, except those with -OH group at C6, such as quercetin, (+)-catechin, fisetin,

luteolin-7-O-glucoside, and taxifolin. In addition, adducts formed at C′2 and C′5 of the B ring exhibited a notably stronger antioxidant activity than those at C′6 and C′8 of the A ring [90].

3.3.2. Inhibition of Oxidant Enzymes. Oxidant enzymes, a large collection of multiple enzymes, include nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase (XOD), nitric oxide synthase (NOS), cyclooxygenase (COX), and lipoxygenase (LOX). They not only play essential roles in redox reactions but also promote cellular reactive oxygen and nitrogen (ROS/RNS) [91]. The NOX family members exhibit multiple cellular localizations, activated patterns, and types of produced ROS. However, as major producers of ROS, all those members could generate hydrogen peroxide and superoxide [92].

RSV has been found to attenuate particular matter-enhanced COX-2/prostaglandin E_2 (PGE2) expression, NOX activity, and ROS generation by inhibiting the activation of oxidative stress signaling pathways (NF-κB/NOX/-ROS) in human fibroblast-like synoviocytes [51]. Nuclear factor κB (NF-κB) is a nuclear transcription factor participating in the regulation of various inflammation-related genes, which can be activated by several stimulations through different forms of radiation to oxidative stress. Resveratrol was shown to impair p65 NF-κB translocation into the nucleus, and subsequently, inhibit binding with DNA in a dose-dependent manner [51, 58]. Resveratrol was confirmed to protect from oxidative stress by downregulation of protein kinase C-α (PKC-α) activation and NOX in A549 cell line and isolated human neutrophils [93, 94]. Gang et al. found that puerarin decreased NOX activity by suppressing activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) and membrane translocation of oxidase subunits, and then, reduced ROS production [86]. RSV was shown to suppress NOX activity, including an O-methylation group, a 4′-OH group, and an extra -OH group. RSV was confirmed to inhibit 11β-hydroxysteroid dehydrogenase type 1 directly by decreasing cortisol production [95]. With supplementation of anthocyanins, both mRNA and protein level of XOD as well as XOD activity in the serum and liver were inhibited significantly in a dose-dependent manner [64]. Consistent with previous reports, polyphenol extracts of sweet cherry, containing hydroxycinnamic acids and flavonoids, were found to inhibit the XO system to reduce intracellular ROS [55]. Lin et al. compared the effects of a series of phenolic acids on XOD activity. A biochemical method was utilized to study the inhibitory ability of phenolic acids on XOD, and the order of inhibition was as follows: sinapic acid>ferulic acid>syringic acid>p-coumaric acid>chlorogenic acid>caffeic acid [96]. Based on previous studies, flavonoids inhibit XOD activity not only in a dose-dependent manner but also in a structure-activity manner. Hydroxyl attached to C′5 and C′7 and the double bond nitric oxide, as a common type of reactive nitrogen, participated in several physiological and pathological processes. RSV was found to be able to disrupt the formation of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production in macrophages [94, 97]. Another polyphenol, quercetin, was also shown to reduce the production of NO by downregulating the NF-κB signaling pathway [98].

3.4. Restoration of Mitochondrial Function. Mitochondria, as one of the important organelles, are considered to play a vital role in energy production and the regulation of cellular homeostasis and energy production. However, extra ROS produced by impaired mitochondria, especially superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2), contributes to a burden of oxidative stress. In turn, extra ROS leads to rapid depolarization of mitochondrial inner membrane potential and impairment of oxidative phosphorylation, further accelerating ROS generation and damage.

Cocultured with catechin-rich cocoa flavanol, both rat islets and pancreatic β cells have been demonstrated to exhibit enhanced insulin secretion and mitochondrial respiration. The activities of mitochondrial components, III, IV, and V, were also increased, along with induced production of adenosine triphosphate (ATP) [99]. Catechins were demonstrated to promote Nrf2 nuclear translocation, initiate the expression of related target genes, like nuclear respiratory factor 1 (Nrf1) and GA-binding protein transcription factor alpha subunit (GABPA), improve the cell's mitochondrial function, and reduce OS [99]. Using electron microscopy, (-)-epicatechin(EPI) was shown to alleviate the decline in quantity and volume densities of mitochondria induced by ischemia/reperfusion compared to sham-operated animals [100]. EPI was also proved to significantly increase the stability of mitochondrial membranes and defend calcium-triggered mitochondrial deformation and swelling by activating δ-opioid receptor (DOR) [101]. DOR was confirmed to activate extracellular signaling-regulated kinase (ERK) signaling and decrease the cytochrome c release, protecting neurons against oxidative damage [102]. At the microscopic level, the protection of EPI on the mitochondrial membrane was prominent when exhibiting less mitochondrial respiration suppression, lowering Ca^{2+} accumulation in mitochondria, along with a certain amount of NADPH [100]. Ca^{2+} has been confirmed to enhance the activity of the mitochondrial respiratory chain by activating a Ca^{2+}-dependent dehydrogenase in the tricarboxylic acid (TCA) cycle, thus producing ATP by oxidative phosphorylation and stabilizing the mitochondrial membrane potential. However, upon excess accumulation of mitochondrial Ca^{2+}, the permeability of the mitochondrial inner membrane increased significantly, impairing inner and outer membrane potential difference and mitochondrial respiration, eventually leading to failure of mitochondrial function [103].

RSV has been investigated in several studies, showing a significant protective effect on mitochondrial function. RSV was demonstrated to enhance the production of ATP and phosphocreatine in T2D female rat hearts in the reperfusion period. Biochemical analysis confirmed that RSV improved the levels of indicators of mitochondrial function, including an increase in total adenine nucleotide, creatine, and citrate synthase activity [104]. It should be noted that the effect of RSV on mitochondria depends very strictly on drug concentration. At low concentration (<50 μM), RSV promoted

cellular antioxidant ability, activating AMP-activated protein kinase- (AMPK-) and sirtuin 1- (SIRT1-) related signaling pathways to enhance the network formation of mitochondria against toxins and disease-related stress. However, when its concentration increased to more than 50 μM, RSV destroyed cellular Ca^{2+} homeostasis, impaired mitochondrial membrane potential, and selectively activated caspases to induce cell death [103].

Taken together, this section provided an overview of these findings and is aimed at evaluating the potential medical feasibility of mitochondrial manipulation by polyphenols. We believe that the main pharmacological potential of polyphenols is closely associated with mitochondria, thus highlighting the advantage of these special cellular organelles as promising future therapeutic targets.

4. Challenges and Future Directions

This article reviews the classifications, chemical structures, sources, and potential roles of various kinds of polyphenols in metabolic disorders. Furthermore, acting against the oxidative stress is confirmed to be a major potential mechanism for the bioprotective effect of polyphenols in MetS. Polyphenols are naturally occurring drugs that rely on their various biological activities, not just antioxidant, and have gradually become an integral part of the dietary preventive treatment of many diseases, including MetS. However, various challenges still exist and are arduous. First, improving the bioavailability of polyphenols more effectively in order to promote their effectiveness is challenging. From several animal models and clinical trials, researchers have reached a consensus that polyphenols possess low bioavailability/high bioactivity paradox [3]. For example, the daily dosage of resveratrol in humans is much lower than that in mice (150 mg/day vs. 200-400 mg/kg/day) [105, 106]; however, the plasma resveratrol levels is about 2-23 folds higher in humans than in mice (231 ng/mL vs. 10-120 ng/mL) [106]. These differences may be attributed to different metabolic rates of resveratrol between humans and mice, suggesting that resveratrol may exert its effects at different concentration ranges in different mammals [107]. Due to these differences between humans and animals, we emphasize that clinical research is more desirable and informative. Second, a linear dose-response relationship existed in the polyphenol extract of *D. loddigesii* (DJP) and the improvement of diabetes [61], while a quadratic relationship was shown between flavonoid-rich cocoa (FRC) dose and flow-mediated vascular dilation (FMD) (P-nonlinearity = 0.004), and its maximum effect was observed at 500 mg/d [37]. Moreover, a quadratic relationship was also observed between polyphenolic dose and HDLC levels (P-nonlinearity = 0.06) [37]. Third, if the polyphenols are extracted as the medicine or as health supplements, attention should be paid to the activity loss and degradation of polyphenols during the extraction process. Fourth, the effects cannot be generalized for all kinds of polyphenols, because each polyphenol has its own unique features. In view of the fact that dietary polyphenols are a mixture, the research on potential mechanism is more complicated and difficult. Hence, it is difficult to compare doses achieved from polyphenolic supplements and polyphenol-rich food. The dose-response relationship between polyphenols and diseases is still largely unknown, requiring further studies to provide reasonable standards or critical doses for daily polyphenol intake.

Current lines of evidences suggest that polyphenols have the potential to alleviate phenotypes of MetS. However, further studies are required to identify the therapeutic potential of polyphenols in MetS progression. Safe doses also need to be determined, as the effects greatly vary among polyphenols and food sources, and no specific food or polyphenol is able to improve all phenotypes of MetS [17]. In addition, several undesirable properties of polyphenols have been disclosed, including genotoxicity [108], thyroid damage [109], hormone disorder [110], antinutritional activity [111], and interactions with other drugs [112]. Therefore, the potential cytotoxicity of polyphenols will become one of the important study directions in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kui Liu and Miao Luo contributed equally to this paper.

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Clinical Study

A Hazelnut-Enriched Diet Modulates Oxidative Stress and Inflammation Gene Expression without Weight Gain

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Introduction. Inflammation is associated with obesity condition and plays a pivotal role in the onset and progression of many chronic diseases. Among several nutraceutical foods, hazelnuts (Corylus avellana L.) are considered an excellent anti-inflammatory and hypolipidemic food being the second richest source of monounsaturated fatty acids among nuts and because they are rich in vitamins, minerals, and phenolic compounds. *Materials and Methods*. A prospective pilot clinical trial on 24 healthy volunteers who consumed daily, as a snack, 40 g of hazelnuts (261.99 kcal/1096.17 kJ) for six weeks was conducted. Anthropometric measurements, body composition analysis, and nutrigenomic analysis on 12 anti-inflammatory and antioxidant genes were evaluated at baseline (T0) and after hazelnut intervention (T1). *Results*. No significant changes were detected on body composition analysis after hazelnut consumption. Conversely, significant upregulation was detected for SOD1 ($2^{-\Delta\Delta Ct} = 2.42$), CAT ($2^{-\Delta\Delta Ct} = 2.41$), MIF ($2^{-\Delta\Delta Ct} = 4.12$), PPARγ ($2^{-\Delta\Delta Ct} = 5.89$), VDR ($2^{-\Delta\Delta Ct} = 3.61$), MTHFR ($2^{-\Delta\Delta Ct} = 2.40$), and ACE ($2^{-\Delta\Delta Ct} = 2.16$) at the end of the study. *Conclusions*. According to emerging evidences, hazelnut consumption does not lead to weight gain probably due to the improvement of the body's antioxidant capacity by the upregulation of genes implied in oxidant reactions and inflammation.

1. Introduction

Inflammation is a constant feature associated with the onset and progression of many chronic degenerative diseases, dramatically increasing in western countries, and one of the leading causes of its insurgence is the high adipose tissue content [1]. White adipose tissue (WAT) is a metabolic organ able to satisfy body functional demands through the storage, in case of an extra caloric intake, or the mobilization, in case of metabolic demands, of energy. WAT is composed of adipocytes, which has marked cellular heterogeneity, vascularisation, and innervation, with a complex hormonal homeostatic system [2]. The relationship between obesity and inflammation was firstly observed in the 1990s. The

metabolic dysfunction of adipocytes is the main cause of chronic inflammation in WAT, through the increasing expression of many biologically functional cytokines/chemokines considering the major mediators of inflammation in particular in obesity condition, like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-8 (IL-8), and interleukin-17D (IL-17D) [3]. The release of those inflammation cytokines determined the subsequent activation of tissue-resident macrophages [4]. In obesity, the excess of lipids and the consequent surplus of WAT can cause also an increase of reactive oxygen species (ROS) levels and the reduction of the antioxidant defenses, events that determine the induction of systemic oxidative stress [5].

Among several nutraceutical foods, tree nuts are edible dry fruits that, together with cereals and legumes, have characterized the human diet since preagricultural times, representing a high-energy and nutrient-dense food, rich in minerals, vitamins, and bioactive compounds [6]. The scientific evidence arises from both epidemiological observations and clinical trials, showing beneficial effects of nut intake on the health status. The reason why a diet rich in nuts has these health benefits is due to their high content in monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) [7], sterols, and fibers and to the presence of bioactive molecules like vitamin E (α-tocopherol), vitamin B, arginine, and polyphenols [8, 9]. In particular, the nut antioxidant capacity was widely discussed, highlighting the bioactivity potential of nut phenolic compounds [10]. There is also a suggestion of regulatory effects of nuts on inflammation, demonstrated by the change of circulating inflammatory markers and the expression of ligands for inflammatory molecules in circulating monocytes after two weeks of an enriched nut diet [11]. Furthermore, nuts are particularly rich in calories but their enriching in diets does not change the body mass index (BMI). Actually, a higher weight loss and a greater protection against cardiovascular risk factors in subjects who consumed nuts in their diet compared to who follow other dietary plans were shown [12–14]. These observations support the theory for which the introduction of nuts in weight loss or weight control diets determines a more favourable outcome.

For all these reasons, nuts are included in the American Heart Association dietary metrics for defining ideal cardiovascular health in their recent report on setting goals for health promotion and disease reduction for 2020 [15]. Furthermore, the World Health Organization (WHO) recommend a daily intake of nuts as a fundamental part of a cardioprotective diet [16].

Among nuts, hazelnuts (Corylus avellana L.) belong to the Betulaceae family and are considered excellent anti-inflammatory and hypolipidemic food [17], being the second richest source of MUFAs among nuts and because they are rich in vitamin E and tocopherols, phytosterols (mainly α-sitosterol), magnesium, copper and selenium, L-arginine, polyphenols, folate, and fibers. Among the phenolic compounds, gallic acid, p-hydroxybenzoic acid, epicatechin, caffeic acid, sinapic acid, and quercetin are found in hazelnuts, with the highest unsaturated/saturated fatty acid ratio among nuts [12].

To our knowledge, only one study defined the right intake of hazelnuts to consume in order to maintain body weight [18], and few works have had a nutrigenomic approach to study the effects of hazelnut consumption [19, 20]. However, nowadays, no studies have identified the right intake of hazelnuts to be able to maintain or improve body weight and/or body composition and enhance anti-inflammatory gene expression. The mechanisms behind weight and of body composition changes given by hazelnut consumption, as well as their modulatory effect on the inflammatory genomic upstream pathway, represent a new field to discover.

The aims of this pilot study were to investigate the effect of hazelnut treatment (HNT) on body composition and on genomic response of genes related to oxidative stress and inflammation.

2. Materials and Methods

2.1. Subjects and Study Design. For this prospective clinical trial, 30 healthy volunteers were recruited and analyzed from the staff of the Clinical Nutrition and Nutrigenomic Section, Department of Biomedicine and Prevention of the University of Rome Tor Vergata. Body composition data and blood samples for genomic analysis were collected at baseline (T0) and after 6 weeks of hazelnut intervention (T1) in order to evaluate nutritional, oxidative, and inflammation statuses. In this pilot study, every recruited subject was considered a control of himself. Lifestyle habits of healthy volunteers did not change during the study period. No abnormality was presented during the study period. All participants recruited in the study authorized their participation by reading and signing the informed consent, conducted in accordance with the provisions of the Ethics Committee of Medicine, University of Rome Tor Vergata, and with the Helsinki Declaration of 1975 as revised in 1983. This protocol has been registered with Trial Registration Number ClinicalTrials.gov. ID: NCT01890070.

2.2. Exclusion Criteria. In order to be included in the study, subjects had to respect the following exclusion criteria: active tobacco smoking; pregnancy; breastfeeding; type 1 and type 2 diabetes; past or active cardiovascular diseases; metabolic, endocrine, liver, kidney, and autoimmune disorders; chronic viral (hepatitis C and B and HIV) and cancer diseases; corticosteroid and chronic anti-inflammatory therapy; and participation in other dietary trials.

2.3. Dietary Intervention. During the study period, all the recruited subjects consumed daily 40 g of hazelnuts (261.99 kcal/1096.17 kJ), cultivar Tonda Gentile Romana, provided by Coopernocciola in Vico Matrino (Viterbo, Italy). A 24 h dietary recall was performed at the baseline to all participants. All subjects were administered a standard isocaloric diet according to the following Mediterranean diet criteria: 55% of carbohydrates, 20% of proteins (>50% of vegetable derivation), <30% of lipids (on total kcal: saturated

fat <10%, 6–10% polyunsaturated fatty acids (PUFA), n-6/n-3 PUFA ratio of 3 : 1, 15% of monounsaturated fatty acids (MUFA), and <1% transfatty acids), and 30 g of fiber. Caloric intake calculation of all standard isocaloric diets was based on a 24 h dietary recall evaluation for each subject. Standard diets were elaborated with a proper software (Dietosystem, DS Medica, Milan, Italy). All subjects were asked to eliminate any other type of hazelnuts or nuts from their diet.

2.4. Anthropometric Measurements and Body Composition Analysis. All volunteers were subjected to an anthropometric evaluation after overnight fasting. Body weight and height were measured according to previously described methods [21]. Body weight was evaluated with balance scale to the nearest 0.1 kg (Invernizzi, Rome, Italy). Height was measured with a stadiometer to the nearest 0.1 cm (Invernizzi, Rome, Italy). BMI was calculated using the following formula: BMI = body weight (kg)/height $(\mathrm{m})^2$. Body composition analysis was assessed by Dual Energy X-Ray Absorptiometry (DXA) (i-DXA, GE Medical Systems, Milwaukee, WI, USA) [22].

2.5. Biochemical Analysis. Blood tests were carried out at the "Policlinico Tor Vergata (PTV)" of Rome, Italy. Analyses were performed at baseline after a 12-hour overnight fast. Blood samples (10 mL) were collected into EDTA tubes (Vacutainer®), placed in ice, and plasma was separated by centrifugation. Laboratory analysis included complete blood count, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (Tg). Complete blood count, except serum lipid and Tg analyses, was carried out with an ADVIA®1800 Chemistry System (Siemens Healthcare). Serum lipid profile components were determined by standard enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Serum Tg were measured by a coupled enzymatic method on the Beckman Synchron LX20 automated system.

2.6. Sample Collection. RNA Extraction and Analysis. Blood samples were collected and stabilized in PAX gene Blood RNA Tubes (Pre AnalytiX, Qiagen, Hombrechtikon, Switzerland) and then stored at -80°C until use. Blood samples were pooled according to intervention times (baseline and postintervention). Total RNA was purified with a PAX gene Blood miRNA Kit, following the manufacturer's instructions (Pre AnalytiX, Qiagen, Hombrechtikon, Switzerland), and quantified through spectrophotometry (Nanodrop, Wilmington, USA). Specific RT2 Profiler PCR Arrays (Qiagen, Netherlands) were used for human oxidative stress (PAHS-065ZA, Qiagen, Netherlands) and human inflammation (PAHS-097ZA, Qiagen, Netherlands) pathways. The gene expression of the following 12 genes was analyzed: superoxide dismutase 1 (SOD1) (NCBI Accession number: NM_000454.4), catalase (CAT) (NCBI Accession number: NM_001752.3), macrophage migration inhibitory factor (MIF) (NCBI Accession number: NM_002415.1), peroxisome proliferator-activated receptor gamma (PPARγ) (NCBI Accession number: NM_001354667), vitamin D receptor (VDR) (NCBI Accession number: NM_000367.2), methylenetetrahydrofolate reductase (MTHFR) (NCBI Accession number: NM_001330358.1), angiotensin I-converting enzyme (ACE) (NCBI Accession number: NM_000789.3), apolipoprotein E (APOE) (NCBI Accession number: NM_001302691.1), interleukin 6 receptor (IL6R) (NCBI Accession number: NM_000565.3), nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NFKB1) (NCBI Accession number: NM_003998.3), insulin-like growth factor 2 receptor (IFG2R) (NCBI Accession number: NG_011785.3), and upstream transcription factor 1 (USF1) (NCBI Accession number: NM_001276373.1). Each qRT-PCR experiment was performed in triplicate and repeated at least twice, in line with the manufacturer's instructions (Qiagen, Netherlands). β-Actin (ACTB) (NM 001101) was used as a housekeeping gene. A comparative threshold (CT) cycle was used to determine the gene expression level. A CT value was normalized using the formula ΔCT = CT (gene) − CT (housekeeping gene). The relative gene expression levels were determined according to the following formula: $\Delta\Delta$CT = ΔCT sample − ΔCT calibrator. The value used to plot relative gene expression was determined using the expression fold change (FC) = $2^{-\Delta\Delta CT}$.

2.7. Statistical Analysis. Statistical analysis was carried out using IBM SPSS 21.0 for Windows (Armonk, NY: IBM Corp, USA). Power calculation was evaluated on total cholesterol, with a 2-sided test and an $\alpha = 0.05$. After the Shapiro-Wilk test, a paired t-test or a nonparametric Wilcoxon test was performed to evaluate differences before and after hazelnut interventions. All tests were considered significant at $p \leq 0.05$. For genomic analysis, the value used to plot relative gene expression was determined using the expression fold change (FC) = $2^{-\Delta\Delta CT}$. Only genes with a FC ≥2 were considered significantly upregulated for differentially expressed genes. Conversely, genes with a FC≤0.5 were considered significantly downregulated for differentially expressed genes.

3. Results

3.1. Subjects Characteristics. Of the thirty subjects enrolled, one of them was excluded from the trial (subject declined to participate) (Figure 1). At the end, twenty-four subjects completed the trial. Any changes to trial outcomes after the trial commenced occurred. The power of the study was 0.61. The average age of subjects was 51.58 ± 9.37 years (58.3% male and 41.7% female) (Table 1).

3.2. Body Composition and Bioclinical Analysis. After 6 weeks of HNT (T1), a significant reduction of the abdominal circumference ($p = 0.04$; $\Delta\% = -0.53\%$) was observed, compared to baseline (T0) values. At the same time, in DXA measurements of bone mineral content (BMC), total body lean (TBLean), android body fat (ABFat), gynoid body fat (GBFat), and total body fat (TBFat), no significant changes were detected. The same observations were highlighted for the changes in the appendicular skeletal muscle mass index (ASMMI) and t-score parameters (Table 2). Furthermore,

Figure 1: Flow chart study design.

among the biochemical parameters, only TC ($p = 0.01$; $\Delta\% = -10.48\%$) and LDL-C ($p = 0.01$; $\Delta\% = -12.12\%$) had a significant reduction after 6 weeks of HNT, as well as the TC/HDL-C ratio ($p = 0.03$; $\Delta\% = -5.83\%$). No other changes were highlighted for serum lipid profile and inflammation markers (Table 2).

3.3. Gene Expression Data. Significant upregulation with a fold change exceeding the threshold set at 2 was detected for SOD1 ($2^{-\Delta\Delta Ct} = 2.42$), CAT ($2^{-\Delta\Delta Ct} = 2.41$), MIF ($2^{-\Delta\Delta Ct} = 4.12$), PPAR$\gamma$ ($2^{-\Delta\Delta Ct} = 5.89$), VDR ($2^{-\Delta\Delta Ct} = 3.61$), MTHFR ($2^{-\Delta\Delta Ct} = 2.40$), and ACE ($2^{-\Delta\Delta Ct} = 2.16$) after HNT (Figure 2). No significant gene expression changes were observed for APOE, IL6R, NFKB1, IFG2R, and USF1 ($0.5 < 2^{-\Delta\Delta Ct} < 2$).

4. Discussion

Hazelnuts have been demonstrated to be able to reduce atherogenic/cardiovascular risk and inflammation [12, 18, 23]. The beneficial effects of hazelnuts on inflammation are due to the content of various nutrients and bioactive substances, and their biochemical profile depends on the cultivar and the country of origin. Nowadays, Turkey is the main country producing hazelnuts in the world, followed by Italy, where Tonda Gentile Romana is found, a typical cultivar from the Latium region, which is particularly rich in stearic, oleic, and linoleic fatty acids [24]. However, the reason why a diet rich in hazelnuts has beneficial effects on health is because of their high content of MUFAs, PUFAs, fibers, α-tocopherol, phytosterols, phenolic compounds, magnesium, copper, and selenium [7, 12]. Some of these components have a potential antioxidant effect through their major nonenzymatic antioxidant proprieties [25]. Constitutive enzymes function or even through their ability to increase the expression of genes involved in anti-inflammatory and antioxidant processes and/or to reduce the expression of inflammatory genes [26–28]. It is well known that the imbalance of the ROS production and the body's antioxidant capacity determines oxidative stress,

Table 1: Anthropometric and clinical baseline characteristic of study subjects.

Parameter ($n = 24$) Frequency (n, %)	Male ($n = 14$; 53.8%)			Female ($n = 10$; 41.7%)		
	Median	P25	P75	Median	P25	P75
Age (years)	56.50	52.00	60.50	47.50	41.25	56.25
SBP (mmHg)	125.50	112.25	140.00	108.00	101.75	121.50
DBP (mmHg)	74.50	65.00	88.25	72.50	63.75	75.50
Height (cm)	172.00	165.00	173.25	161.00	155.75	162.00
Weight (kg)	75.00	69.50	82.95	63.95	58.83	78.68
BMI (kg/m^2)	26.23	24.34	27.36	25.37	22.99	30.07
Neck circumference (cm)	41.00	39.75	42.63	37.00	35.38	38.25
Waist circumference (cm)	88.50	82.75	92.25	81.50	72.00	85.88
Abdominal circumference (cm)	94.00	87.13	97.00	92.75	86.75	99.75
Hip circumference (cm)	97.50	94.00	99.25	100.50	96.00	114.13
Waist/hip ratio	0.91	0.87	0.96	0.79	0.73	0.84
TBFat (kg)	19.30	16.20	22.60	22.90	20.51	37.41
TBFat (%)	25.60	22.28	28.58	38.45	33.05	42.28
ABFat (%)	33.15	25.08	37.53	43.00	33.98	51.75
GBFat (%)	25.70	22.98	27.15	39.35	36.88	49.63
TBLean (kg)	53.94	48.04	58.36	38.39	36.44	40.93
BMC (g)	2896.00	2631.75	3108.50	2313.00	2059.50	2634.75
ASMMI	8.74	8.37	9.29	6.67	6.42	7.59
t-score	-0.40	-0.93	0.13	0.25	-1.13	1.60
Neutrophils (K/μL)	2850.00	2000.00	3530.00	3070.00	2190.00	3360.00
Lymphocytes (K/μL)	1800.00	1240.00	2000.00	2120.00	1160.00	2540.00
Platelets (K/μL)	191000.00	167000.00	204000.00	243000.00	173000.00	256000.00
TC (mg/dL)	193.00	149.00	245.00	214.00	159.00	270.00
HDL-C (mg/dL)	50.00	43.00	66.00	67.00	38.00	70.00
LDL-C (mg/dL)	131.00	80.00	159.00	146.00	75.00	178.00
Tg (mg/dL)	95.00	42.00	113.00	99.00	54.00	124.00
NLR	1.46	1.11	2.40	1.52	0.83	2.22
PLR	120.00	77.78	129.41	106.60	65.04	186.72
Tg/HDL-C	1.44	0.68	2.51	1.81	0.77	2.24
TC/HDL-C	3.86	2.40	4.00	3.87	2.34	4.07
kcal/diet	1960.00	1650.00	2050.00	1840.00	1230.00	2050.00

Anthropometric and clinical characteristic at baseline (T0) and after HNT (T1). Results are expressed as the median, minimum, and maximum for each parameter. A paired t-test (a) or a nonparametric Wilcoxon test (b) was performed to evaluate differences before and after hazelnut intervention. All tests were considered significant at $p \leq 0.05$. SBP: systolic blood pressure; DBP: diastolic blood pressure; TBFat: total body fat; ABFat: android body fat; GBFat: gynoid body fat; TBLean: total body lean; BMC: bone mineral content; ASMMI: appendicular skeletal muscle mass index; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Tg: triglycerides; NLR: neutrophil-lymphocyte ratio; PLR: platelet-lymphocyte ratio.

which is implicated in several pathological processes. Despite the fact that 30% of obese subjects are considered metabolically healthy, a condition where insulin sensitivity, visceral fat content, and intima media thickness of the carotid artery are similar to healthy normal weight, the majority of obese patients were metabolically unhealthy, with an increased risk of cardiovascular problems, metabolic disorders, arterial hypertension, and chronic heart disease development [3]. Adipocytes represent the main cause of chronic inflammation in WAT because of the increased expression of several cytokines/chemokines and ROS production, within a reduction of the antioxidant defenses [5, 29]. In particular, obesity-related disorders are mainly due to the increasing levels of visceral adiposity tissue (VAT), which are considered extremely dangerous because they are related to the high concentration of inflammatory cytokines and obesity-related cardiometabolic problems [3]. It is well known that nuts are particularly rich in fats and calories and usually considered a hypercaloric food, but it was observed that hazelnuts or nut-enriched diets do not change weight or BMI. In fact, several epidemiological studies detected an inverse or a null association between nut consumption and BMI [17–20]. More specifically, consumption up to 60 g of hazelnuts per

Table 2: Anthropometric and clinical characteristic at baseline (T0) and after hazelnut treatment (HNT) (T1).

	Baseline (T0)		HNT (T1)		Δ%	p
	Median	Min–Max	Median	Min–Max		
SBP (mmHg)	116.50	96.00-169.00	120.00	100.00-150.00	3.00	0.81[a]
DBP (mmHg)	73.00	57.00-93.00	75.00	60.00-86.00	2.74	0.28[a]
Weight (kg)	71.40	53.50-93.00	71.05	53.50-93.00	-0.49	0.46[a]
BMI (kg/m^2)	25.95	20.64-35.60	25.76	20.64-35.44	-0.71	0.55[a]
Neck circumference (cm)	39.50	33.00-44.00	40.00	34.00-43.00	1.27	0.71[b]
Waist circumference (cm)	86.25	66.50-101.00	85.00	66.00-103.00	-1.45	0.90[a]
Abdominal circumference (cm)	94.00	73.00-110.00	93.50	81.00-110.50	-0.53	0.04[a]
Hip circumference (cm)	98.25	92.00-117.00	99.00	91.00-116.00	0.76	0.44[b]
Waist/hip ratio	0.87	0.71-1.03	0.87	0.69-1.00	0.00	0.34[a]
TBFat (kg)	34.75	14.60-53.10	34.95	13.50-52.60	0.58	0.89[b]
TBFat (%)	29.65	16.3-54	29.05	18.00-53.30	-2.02	0.73[a]
ABFat (%)	28.75	16.10-48.30	28.80	15.40-48.00	0.17	0.15[a]
GBFat (%)	21.08	12.02-38.02	21.34	12.49-38.13	1.21	0.89[a]
TBLean (kg)	47.63	32.87-70.81	48.09	32.40-68.42	0.97	0.34[a]
BMC (g)	2703.00	1667.00-3742.00	2622.00	1692.00-3627.00	-3.00	0.29[a]
ASMMI	8.37	5.98-10.28	8.05	6.06-10.57	-3.83	0.11[a]
t-score	-0.1000	-1.90-1.90	-0.1000	-1.90-1.90	0.00	0.87[a]
Neutrophils (K/μL)	2970.00	2220-6860	3245	2170-8481	11.71	0.40[a]
Lymphocytes (K/μL)	1695.00	1160.00-2680.00	1660.00	754.00-2700.00	-7.11	0.18[b]
Platelets (K/μL)	194500.00	166000.00-293000.00	214000.00	148000.00-324000.00	6.01	0.14[a]
TC (mg/dL)	181.00	149.00-214.00	167.00	102.00-200.00	-10.48	0.01[a]
HDL-C (mg/dL)	51.50	35.00-79.00	47.50	37.00-80.00	-6.93	0.22[a]
LDL-C (mg/dL)	114.00	75.00-146.00	103.00	47.00-125.00	-12.12	0.01[a]
Tg (mg/dL)	100.50	42.00-170.00	82.50	44.00-151.00	-11.99	0.20[a]
NLR	2.08	0.83-4.48	2.14	0.95-6.68	28.87	0.18[b]
PLR	127.08	61.94-198.28	135.69	64.91-309.02	18.10	0.08[a]
Tg/HDL-C	2.02	0.68-3.55	1.87	0.61-3.02	-5.61	0.55[b]
TC/HDL-C	3.71	2.28-5.03	3.27	2.23-4.6	-5.83	0.03[a]
kcal/diet	1965.00	1230.00-2780.00	2226.99	1491.99-3041.99	11.38	0.03[a]

Anthropometric and clinical characteristic at baseline (T0) and after HNT (T1). Results are expressed as the median, minimum, and maximum for each parameter. A paired *t*-test (a) or a nonparametric Wilcoxon test (b) was performed to evaluate differences before and after hazelnut intervention. All tests were considered significant at $p \leq 0.05$. SBP: systolic blood pressure; DBP: diastolic blood pressure; TBFat: total body fat; ABFat: android body fat; GBFat: gynoid body fat; TBLean: total body lean; BMC: bone mineral content; ASMMI: appendicular skeletal muscle mass index; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Tg: triglycerides; NLR: neutrophil-lymphocyte ratio; PLR: platelet-lymphocyte ratio.

day did not affect weight and improve blood cholesterol levels [18].

Since 1 kg of fat contains 9000 kcal [30], an excess of 261.99 kcal per day for 6 weeks, due to the intake of 40 g of hazelnuts, would have led to a fat tissue gain of 1 kg, while no significant difference of weight and/or fat mass emerged from the results. Interestingly, as demonstrated by previous observations [12–14], although we noticed a small but significant reduction of abdominal circumference ($p < 0.05$), we did not observe any changes in weight, BMI, android, gynoid body fat, and total body fat measured by DXA after 6 weeks of a reported 1096.17 kJ/d increase in energy intake with the hazelnut-enriched diets. Furthermore, in this study, we observed a significant reduction of TC, LDL-C, and TC/HDL-C ratio according to Perna et al. [14].

There are several reasons why nut consumption does not determine weight gain and improve cholesterol profile. Firstly, nuts contain a good amount of proteins and fibers, and they have a low glycemic index, contributing to enhance satiety through the reduction of calorie intake from other food sources [31]. In addition, the high content of dietary fibers could reduce the bioavailability of cholesterol from food, and the antioxidant molecules contained in hazelnuts contribute to their antiatherogenic effect [14]. Moreover, the typical crunchy texture of nuts promotes satiety through the mechanical act of chewing, which determines the secretion of food intake regulation [32]. Secondly, previous works suggested that nut consumption leads to the energy expenditure and thermogenic effect increase, probably because of the high unsaturated/saturated fat ratio characteristic of the nuts

Figure 2: Gene expression after hazelnut treatment. Different levels of the fold change of 12 genes were analyzed: superoxide dismutase 1 (SOD1), catalase (CAT), macrophage migration inhibitory factor (MIF), peroxisome proliferator-activated receptor gamma (PPARγ), vitamin D receptor (VDR), methylenetetrahydrofolate reductase (MTHFR), angiotensin I-converting enzyme (ACE), apolipoprotein E (APOE), interleukin 6 receptor (IL6R), nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NFKB1), insulin-like growth factor 2 receptor (IFG2R), and upstream transcription factor 1 (USF1). Genes with a FC ≥ 2 were considered significantly upregulated for differentially expressed genes; genes with a FC ≤ 0.5 were considered significantly downregulated for differentially expressed genes.

[33]. Thirdly, lipids of nuts are not easily bioavailable [34], and then, a great part of them is excreted with feces and not accessible for metabolization [35]. Fourthly, the application of Atwater factors to nuts determined an overestimation of energy contents [36].

Another possible explanation about nut consumption and the maintenance of weight or BMI could be suggested by the antioxidant potential of hazelnuts. It is well known that some foods have peculiar antioxidant activities [37], which can affect some disturbances such as obesity. In fact, the relation between obesity and inflammation could be considered bidirectional. Few studies demonstrated that a previous inflammatory status is found before the overweight and obesity onset, becoming a possible risk factor of them [38, 39]. In the SUN study, Ramallal et al. [40] observed that subjects who follow a proinflammatory diet have a clinically relevant weight gain (3-5 kg), as compared with those in the anti-inflammatory diet group. The antioxidant capacity determined by nuts was widely discussed, highlighting the bioactivity potential of nut phenolic compounds [13].

Unfortunately, only few studies observed the effect of hazelnut consumption on inflammatory and/or anti-inflammatory gene expression, and the most relevant results were related to the upregulation of some antioxidant genes [25, 26]. In this pilot study, we confirmed that after hazelnut consumption there is an upregulation of the two of the major antioxidant enzymes, SOD1 ($2^{-\Delta\Delta Ct} = 2.42$) and CAT ($2^{-\Delta\Delta Ct} = 2.41$), which are two of the most important genes involved in the antioxidant pathway, thanks to their ability to catalyze the reaction that leads from superoxide (O_2^-) to oxygen and water production. Furthermore, during our trial, we observed an upregulation of MIF ($2^{-\Delta\Delta Ct} = 4.12$). MIF, a cytokine able to regulate innate and acquired immune responses, is usually associated to atherosclerosis progression, obesity, insulin resistance, and inflammatory diseases [41]. Recently, MIF was identified as a key regulator of antioxidant response element (ARE), a DNA enhancer that controls the expression of phase II detoxifying enzymes and cytoprotective proteins for redox homeostasis [42]. In this view, in accordance with other studies [43, 44], MIF seems to be a sensor for oxidative stress, and its upregulation, as shown in our work, could represent a new way to reduce oxidative stress, because of its role as a key regulator of ARE-mediated gene expression. Furthermore, MIF regulates the antioxidant system by binding and activating transcription factors such as Nrf2, which during oxidative stress translocate into the nucleus, inducing ARE activation. In our pilot study, hazelnut consumption is associated also with the upregulation of PPARγ ($2^{-\Delta\Delta Ct} = 5.89$), an enzyme that cooperates in the modulation of oxidative stress, antioxidant response, and inflammatory diseases. In fact, PPARγ has a reciprocal transcriptional regulation with Nrf2, acting synergically in the activation of antioxidant genes [45]. The upregulation of SOD1, CAT, MIF, and PPARγ observed in this study is in line with previous works, which have demonstrated the antioxidant proprieties of a diet rich in nuts, related to the regulation of cellular pathways of atherosclerosis, inflammation, and oxidative stress [46] (Figure 2). At the same time, PPARγ, which controls M2 macrophage activation, seems to be related to VDR expression. The reduction of VDR expression could determine the abolishment of PPARγ effects, establishing a possible VDR-PPARγ pathway that, along with vitamin D, regulates macrophage phenotype [47]. Furthermore, VDR inhibits the initiation of endothelial

inflammatory diseases, like atherosclerosis, and reducing inflammatory cytokine/chemokine production in macrophages, when it is activated by vitamin D [48]. The upregulation of VDR ($2^{-\Delta\Delta Ct} = 3.61$) observed in our study (Figure 2) amplified the antioxidant and anti-inflammatory potential of hazelnuts. In fact, the increased VDR gene expression overlaps PPARγ upregulation, suggesting a possible effect of HNT on the VDR-PPARγ pathway.

High blood concentrations of homocysteine (Hcy), an important intermediary in the metabolism of methionine and cysteine, are another potential inflammatory factor. Hcy induces the production of several proinflammatory cytokines, and at the same time, in endothelial cells, high levels of Hcy induce the oxidative inactivation of nitric oxide with the collaboration of several ROS. Furthermore, Hcy plays an important role in the conservation of intracellular glutathione pools and then in oxidative stress conditions. A full MTHFR expression and activity are strictly necessary for the proper recycle of Hcy. Its reduced gene expression could lead to mental disorders, developmental delays, cardiovascular diseases, and cancer [49]. The increased MTHFR gene expression ($2^{-\Delta\Delta Ct} = 2.40$) observed in this pilot study after HNT could reinforce the antioxidant effect of hazelnut consumption (Figure 2). In fact, if MTHFR gene is upregulated, we can speculate a reduction of ROS production and inflammation.

Ordinary nut consumption, maybe due to the polyphenol content, is associated with beneficial effects on blood pressure [47]. High blood pressure is one of the risk factors associated with oxidative stress and inflammation. Usually, increased levels of vascular ACE, the key regulator of the renin-angiotensin system and kallikrein-kinin system, are associated with increased blood pressure [50]; however, evidences have been reported between ACE gene expression and hypertensive condition [51]. In our study, hazelnut consumption leads to an increase of ACE gene expression ($2^{-\Delta\Delta Ct} = 2.16$) (Figure 2), but not to a significant change in SBP or DBP ($p > 0.05$), strengthening the observation of Mohammadifard N. et al. that maybe only mixed nut and pistachio consumption could be associated with better blood pressure outcomes [52].

5. Conclusions

In conclusion, the reasons why nuts do not determine weight gain are various, like satiety induction, energy expenditure, thermogenesis increase, and the low nutrient bioavailability. According to emerging evidences, hazelnut antioxidant capacity could be another reason. Although this pilot study was not without limitations, as the lack of biochemical analysis, serum protein levels of investigated genes, antioxidant markers, and the limited sample size, our results suggest that an ordinary consumption of small amount of hazelnuts could be a nutritional treatment for all chronic degenerative diseases underlying oxidative stress and inflammation reinforcing the WHO recommendation. Furthermore, the lack of weight gain and, more importantly, the lack of fat mass gain with the hazelnut consumption, which is still considered a high-calorie food, could represent an encouragement for the inclusion of this food not only in the anti-inflammatory dietary patterns but also in all weight loss diets. Our data should be confirmed on a larger number of subjects, with a prospective long-term controlled trial.

Abbreviations

ABFat:	Android body fat
ACE:	Angiotensin I-converting enzyme
ARE:	Antioxidant response element
APOE:	Apolipoprotein E
ASMMI:	Appendicular skeletal muscle mass index
BMI:	Body mass index
BMC:	Bone mineral content
CAT:	Catalase
CCL5:	Copper chaperone for superoxide dismutase
DBP:	Diastolic blood pressure
DXA:	Dual energy X-ray absorptiometry
FC:	Fold change
GBFat:	Gynoid body fat
GPX1, GPX2:	Glutathione peroxidases 1 and 2
GSR:	Glutathione reductase
HNT:	Hazelnut treatment
HSPA1A:	Heat shock protein 1A
Hcy:	Homocysteine
IFG2R:	Insulin-like growth factor 2 receptor
IL-17D:	Interleukin-17D
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
IL-8:	Interleukin-8
IL6R:	Interleukin 6 receptor
KRT1:	Keratin
MIF:	Macrophage migration inhibitory factor
MBL:	Mannose-binding lectin (protein C) 2 soluble
MTHFR:	Methylenetetrahydrofolate reductase
MUFAs:	Monounsaturated fatty acids
NFKB1:	Nuclear factor of kappa light polypeptide gene enhancer in B-cell 1
PPARγ:	Peroxisome proliferator-activated receptor gamma
PUFAs:	Polyunsaturated fatty acids
ROS:	Reactive oxygen species
SOD1:	Superoxide dismutase 1
SBP:	Systolic blood pressure
TXNRD1:	Thioredoxin reductase 1
TBFat:	Total body fat
TBLean:	Total body lean
TNF-α:	Tumor necrosis factor-α
USF1:	Upstream transcription factor 1
VAT:	Visceral adiposity tissue
VDR:	Vitamin D receptor
WAT:	White adipose tissue
WHO:	World Health Organization.

Data Availability

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

Disclosure

The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

LDR designed the study. SG, SB, LA, VA, MM, AC, IJ, and IC acquired the data. LDR and GC analyzed and interpreted the data. LDR and GC drafted the work and substantively revised it. SG had the primary responsibility of the study. All authors read and approved the submitted and final version of the manuscript. All authors agree to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Laura Di Renzo and Giorgia Cioccoloni contributed equally to this work.

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Supplementary Materials

Graphical Abstract: effects of 6 weeks of hazelnut administration on body composition and gene expression. Effects of hazelnut administration on body composition and gene expression of 7 genes belonging to the oxidative stress and the related involved pathway. Superoxide dismutase 1 (SOD1), catalase (CAT), macrophage migration inhibitory factor (MIF), peroxisome proliferator-activated receptor gamma (PPARγ), vitamin D receptor (VDR), methylenetetrahydrofolate reductase (MTHFR), angiotensin I-converting enzyme (ACE), interleukin-10 (IL10), RAS, M1 and M2 macrophages, nuclear factor- (erythroid-derived 2) like 2 (Nrf2), and antioxidant response element (ARE). *(Supplementary Materials)*

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Research Article

Application of Urinary Polyphenol Biomarkers Measured by Liquid Chromatography Tandem Mass Spectrometry to Assess Polyphenol Intake and Their Association with Overweight and Obesity in Free-Living Healthy Subjects

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Although some polyphenol biomarkers in serum or urine have been identified by untargeted metabolomics and proved to reflect dietary polyphenol intake, only a few of them have been validated in different studies and populations with simple and reliable targeted methods. In the present study, a targeted metabolomics method by LC/MS/MS for the measurement of twenty-two polyphenol biomarkers in urine samples was established and validated to effectively assess the habitual polyphenol intake in free-living healthy Chinese subjects. Multivariate logistic regression models were used to assess relationships of biomarkers with overweight and obesity after adjusting for potential confounders. The levels of urinary polyphenol biomarkers, especially gut microbial metabolites of polyphenols, were inversely associated with overweight and obesity, and this association was more pronounced in the inflammatory groups, suggesting that it is of great importance to maintain polyphenol biomarkers at high levels or intake-sufficient polyphenols in obesity with chronic inflammation than others. The measurement of these biomarkers may offer a valid alternative or complementary addition to self-reported survey for the evaluation of polyphenol intake and investigation into their relationships with chronic disease-related endpoints in large-scale clinical and epidemiologic studies.

1. Introduction

Polyphenols composed of a wide variety of phytochemicals abundant in plants, ranging from simple phenolic acids to flavonoid polymers, are commonly consumed through a diet rich in fruit, vegetables, tea, wine, and soy-based foods [1]. Habitual consumption of dietary polyphenols has been consistently linked with a wide spectrum of potential health benefits in humans, including anti-inflammatory, antiobesity, gastroprotective, and anticarcinogenic effects [2–4].

Despite advances in nutritional epidemiological study design and analytical strategies, precise estimation of polyphenol intake in free-living populations remains a major challenge [5], because polyphenols are abundant, wide-spanning, and diverse in the human diet, and their quantity in foods is heavily influenced by a food's growth and processing conditions [6]. Most epidemiologic studies rely on self-reported dietary assessment methods that might suffer from systematic and random errors related to the variability in the polyphenol content of foods, bias in dietary misreporting (especially underreporting of bias towards unhealthy foods and overreporting of bias towards fruits and vegetables), and differences in absorption and metabolism between individuals [7–9]. Moreover, underreporting of intake of unhealthy foods is particularly common in an obesity population [10], which is a major concern considering the increasing prevalence of obesity worldwide.

Therefore, there is a pressing need for dietary polyphenol biomarkers to precisely evaluate polyphenol intake. Dietary biomarkers have the potential to improve the measurement of dietary exposures by validating diet questionnaires [11] or by replacing inadequate dietary data [12]. Metabolomics,

the measurement of small molecules, including metabolic parent compounds, substrates, and products, in biospecimens, may more precisely quantify dietary exposures and thus provide better estimates of disease risk in epidemiologic studies [13]. Metabolites may better reflect "true exposure" and also capture exposure to nonnutritive substances, such as compounds generated by cooking or gut microbiota, which may play important roles in disease etiology [14, 15].

Recent studies have shown that metabolomics can be successfully applied to dietary research and can identify some novel potential dietary polyphenol biomarkers, but most studies were small dietary interventions [16–19]. Although most of these studies calculated the half-life using single doses of polyphenols or polyphenol-rich foods, in fact, the intake of polyphenol is usually chronic; thus, biomarkers identified may not perform well as proxies for usual food intake [7, 14]. A case in point is that a recent citrus feeding study identified more than 600 metabolites associated with acute citrus consumption; however, only 12 metabolites were related to regular dietary citrus consumption in a free-living population [19].

Considering that polyphenols or polyphenol-rich foods are generally consumed almost daily, it would be advantageous to identify biomarkers of chronic intakes and apply these biomarkers in epidemiological studies, with chronic nutritional doses, using the usual biofluids collected in epidemiological studies, such as plasma or morning urine. A recent untargeted metabolomics study proved that metabolic profiles of urine samples developed in a highly controlled environment reflect dietary intake and can be used to model and classify dietary patterns of free-living populations [20].

The untargeted metabolomics could discover new biomarkers and often offer qualitative concentrations (i.e., relative concentration), while the targeted metabolomics allows us to obtain a "real" quantification (i.e., absolute concentration) of these biomarkers. Accordingly, these biomarkers can be easily applied in epidemiological studies. Despite the relatively high number of polyphenol biomarkers, only a few of them have been validated in different studies and populations with simple and reliable targeted methods. Most of the related studies mainly involved North American and European people. Thus, the relationships need to be further validated in external independent populations, especially in Asian people of whose category and content of foods for dietary polyphenol intake and food cooking methods are very different from those in the West.

Therefore, we proposed to use a cross-sectional study of free-living Chinese subjects to investigate and validate the effect of urinary polyphenol biomarkers measured by liquid chromatography tandem mass spectrometry (LC/MS/MS) on the assessment of polyphenol intake and their association with overweight and obesity.

2. Materials and Methods

2.1. Study Population. This is a cross-sectional study conducted on Chinese subjects. A total of 123 apparently healthy participants (52 males and 71 females, 42.3 ± 15.2 years old) were recruited at random from a group of Beijing residents attending an annual physical examination at Beijing Hospital in December 2013. Participants were excluded if they had malignant tumors, blood system diseases, chronic obstructive pulmonary disease, autoimmune diseases, infectious diseases, or other clinically significant illnesses, if they were taking prescription medication or nutritional supplements, or if they presented any abnormalities on physical examination, electrocardiography, or screening blood tests. Women were ineligible if they were pregnant or breastfeeding. The participants were required to complete a questionnaire with the help of an investigator. The sera from fasting blood samples obtained from subjects were isolated and stored at -80°C until being analyzed. The first-void urine samples were collected in 0.5% HCl, and after centrifugation, ten 600 μL aliquots were stored at -80°C before analysis. This study was approved by the Ethics Committee of the Beijing Hospital of the Ministry of Health, and written informed consents were obtained from all participants.

2.2. Measurement of Urinary Total Polyphenol and 22 Polyphenol Biomarkers. Total polyphenols in urine (uTP) were measured by the Folin-Ciocalteu method modified by Medina-Remon et al. [21]. Briefly, urine samples were applied to the Plexa PAX solid phase extraction cartridge; after washing, the polyphenols were eluted, evaporated, and reconstituted. Total polyphenols were measured by Folin-Ciocalteu colorimetric assay, calculated by gallic acid standard curve, and corrected by urine creatinine levels. uTP was expressed as mg gallic acid equivalent (GAE)/g of Crea.

A targeted metabolomics method by LC/MS/MS was developed and applied to the measurement of 22 polyphenol biomarkers in urine samples. Standards of 22 polyphenol biomarkers, HPLC grade acetonitrile, and ammonium acetate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Generally, aliquots of 500 μL calibrators and urine samples were respectively applied to the Plexa PCX solid phase extraction cartridge. After extraction and purification, the reconstituted solutions were injected into an LC/MS/MS system. LC-MS/MS analysis was performed using an Applied Biosystems Sciex API 5500 QTRAP tandem mass spectrometer (Framingham, MA, USA) equipped with an Agilent 1260 Series HPLC system (Santa Clara, CA, USA). The LC separation was performed by an XTerra MS C18 (2.1 mm × 100 mm, 3.5 μm) column. The injection volume was 10 μL, and the flow rate was 300 μL/min. Gradient elution was carried out with 5 mmol/L ammonium acetate as mobile phase A and acetonitrile as mobile phase B. We applied a gradient profile with the following proportions (v/v) of phase B [t (min), %B]: (0, 0), (5, 15), (8, 50), (9, 100), (14, 100), and (15, 0). The column was reequilibrated for 20 min. MS detection was performed with negative electronic spray ionization (ESI) in a multiple reaction monitoring (MRM) mode at a source temperature of 600°C and a voltage of -4000 V. The dwell times were 0.08 s for MRM. Nitrogen was used as the curtain, nebulizer, and collision gas at pressures of 30, 80, and 80 psi, respectively. Ion transition of each polyphenol biomarker was presented in Supplementary Table 3). The calibration curve was established using a regression of peak areas (y) of each

polyphenol to the corresponding concentrations (x) of the calibrators. The calculated concentrations of the polyphenols were corrected by urine Crea levels. And by using the PCX extraction column, most of the 22 polyphenols had recoveries of >80%. All polyphenols were separated and analyzed within 14 min. Imprecision analysis showed that the established LC/MS/MS method had intra-assay and total imprecisions of 1.00%~10.62% and 3.70%~17.09%, respectively.

2.3. Dietary and Covariate Assessment. Covariate data of demographic and lifestyle variables were ascertained by a questionnaire. Usual dietary intake over the 6 months before the survey was measured by a short self-administered food frequency questionnaire (FFQ). The survey provided information on frequency of consumption and category of polyphenol-rich foods in Chinese dietary habits. The levels of consumption of fruits, vegetables, coffee, and tea were categorized as binary (low or high). The levels of polyphenol content in various consumption fruits were estimated by the Phenol-Explorer 3.6 database [22] and categorized as binary [low (bottom and middle tertiles) or high (top tertile)]. Subjects with four or more of the five dietary conditions, including consumption of high levels of fruits, vegetables, coffee, or tea and high level of polyphenol content of consumption fruits, were assigned to the polyphenol-rich diet group.

Height, body weight, and sitting blood pressure (BP) were measured. The serum samples were tested for fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and C-reactive protein (CRP), using assay kits from Sekisui Medical Technologies (Osaka, Japan) on a Hitachi 7180 chemistry analyzer. Serum concentrations of 8-isoprostane (IsoP) were determined by the commercial EIA kit (Cayman Chemical Company, USA). The diagnoses of overweight/obesity and other components of MetS were made according to the criteria provided by the Chinese Diabetes Society (2004) [23].

2.4. Statistical Analysis. The main continuous variables were all tested for normality. Normally distributed data were expressed as means ± SD, whereas variables obeying a skewed distribution were shown as the median (interquartile range). Count data were reported as frequencies and percentages. We constructed the mean SD score (z score) [24] of 22 urinary polyphenol biomarkers for subjects with complete data to reduce the large number of correlated variables into uncorrelated factors [25]. The z score of 22 urinary polyphenol biomarkers was computed by the summary of z score of each biomarker/22. For quantitative data, the variance analysis was conducted to compute the difference across the tertile of the z score of urinary polyphenol biomarkers, whereas for count data, the chi-square test was used. Correlation coefficients between levels of z scores of each urinary polyphenol biomarker and metabolic traits were calculated by Spearman's correlation. The nonparametric Mann-Whitney test was used to evaluate the differences of z scores of urinary polyphenol biomarkers between two diet groups or the normal and overweight/obesity groups. Principal component analysis (PCA) was also conducted for 22 urinary polyphenol biomarkers to determine the variation in attributes between polyphenol-rich and normal diet groups. Multivariate logistic regression models were used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs), which adopted the entry method and the maximum likelihood ratio test for overweight/obesity. Potential confounding variables, including age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, high levels of physical activity, and polyphenol-rich diet were controlled in the regression models. The performance of the z score of urinary polyphenol biomarkers for classifying overweight/obesity was assessed by logistic regression and receiver operating characteristic analysis. Stratified and interaction analyses were performed according to serum CRP levels [<0.7 (median) and ≥0.7 mg/L]. To further explore the nonlinear interaction of polyphenol biomarkers and serum CRP concentration for overweight/obesity, we treated the serum CRP by restricted cubic splines (RCS) with three freedom degrees in the age- and gender-adjusted logistic models. Two-sided p values < 0.05 were considered statistically significant. SPSS 21.0 software (SPSS Inc.) was used for the statistical analysis of the data, and R packages of "vegan 2.5-4" and "splines 0.2.8" were used for PCA and RCS analysis.

3. Results

3.1. Characteristics of Population according to the Levels of Urinary Polyphenol Biomarkers. The concentration of 22 urinary polyphenol biomarkers of 123 subjects had skewed distribution, and they could not be normally distributed after natural logarithm, logarithmic transformation of 10, and square root (SQRT) transformation. The prevalence of overweight and obesity in our study was 28.5%. As shown in Table 1, according to the tertile of the z score of 22 urinary polyphenol biomarkers, the subjects with higher levels of urinary polyphenol biomarkers were more likely to be older and female (p trend = 0.014 and 0.008, respectively). With respect to metabolic traits, HDL-C significantly increased with increasing urinary polyphenol biomarker levels, which had a dose-response relationship ($p = 0.017$ for trend); in contrast, UA and CREA levels declined notably (p trend = 0.010 and 0.030, respectively). Additionally, with increasing urinary polyphenol biomarker levels, subjects tended to have habits of high consumption of fruits, tea, and polyphenol-rich diet (all p trend < 0.05) and have fewer current drinkers (p trend = 0.031). Expectably, the concentration of urinary total polyphenol by Folin-Ciocalteu colorimetric assay increased gradually with increment levels of the z score of 22 urinary polyphenol biomarkers (p for trend < 0.001).

3.2. Correlations between Levels of Urinary Polyphenol Biomarkers and Cardiometabolic Risk Factors. As shown in Figure 1, Spearman's correlation analysis demonstrated the correlation between metabolic characteristics and levels of urinary polyphenol biomarkers. Most of the urinary

Table 1: Characteristics of the study participants by the z score of 22 urinary polyphenol biomarkers.

Characteristic [a]	Tertile of z score of 22 urinary polyphenol biomarkers [b]			p trend[c]
	T1	T2	T3	
Number	41	41	41	
Age (y)	36.3 ± 14.3	46.4 ± 14.2	44.3 ± 15.5	0.014
Male, n (%)	24 (58.5)	16 (39.0)	12 (29.3)	0.008
BMI (kg/m^2)	23.0 ± 3.6	23.7 ± 3.4	22.4 ± 2.5	0.425
SBP (mmHg)	112.2 ± 16.4	119.0 ± 15.6	114.6 ± 13.3	0.482
DBP (mmHg)	73.4 ± 10.3	76.6 ± 7.5	74.1 ± 6.9	0.711
FBG (mmol/L)	5.0 ± 0.5	5.1 ± 0.6	4.9 ± 0.5	0.618
TG (mmol/L)	1.0 (0.8~1.5)	1.0 (0.6~1.5)	0.9 (0.6~1.3)	0.104
TC (mmol/L)	4.5 ± 0.9	4.9 ± 1.1	4.6 ± 0.8	0.646
HDL-C (mmol/L)	1.3 (1.1~1.5)	1.4 (1.3~1.7)	1.5 (1.2~1.8)	0.017
LDL-C (mmol/L)	2.6 (2.3~3.4)	3.1 (2.4~3.9)	2.7 (2.3~3.4)	0.911
UA (μmol/L)	309.4 ± 76.8	281.5 ± 62.8	268.5 ± 72.0	0.010
CREA (μmol/L)	69.1 ± 13.6	63.8 ± 11.9	62.4 ± 16.0	0.030
Overweight/obesity[d], n (%)	13 (31.7)	16 (39.0)	6 (14.6)	0.088
Hypertension[d], n (%)	6 (14.6)	4 (9.8)	3 (7.3)	0.373[e]
Dyslipidemia[d], n (%)	8 (19.5)	7 (17.1)	8 (19.5)	1.000
Impaired fasting glucose[d], n (%)	1 (2.4)	3 (7.3)	2 (4.9)	0.802[e]
MetS[d], n (%)	3 (7.3)	0 (0)	0 (0)	—
Current smoker, n (%)	8 (19.5)	4 (9.8)	9 (22.0)	0.884[e]
Current drinker, n (%)	14 (34.1)	6 (14.6)	6 (14.6)	0.031
High levels of physical activity, n (%)	15 (37.5)	17 (41.5)	17 (41.5)	0.719
High consumption of vegetables, n (%)	20 (48.8)	23 (56.1)	26 (63.4)	0.184
High consumption of fruits, n (%)	11 (26.8)	18 (43.9)	29 (70.7)	<0.001
High levels of polyphenol content of consumption fruits, n (%)	15 (36.6)	14 (34.1)	16 (39.0)	0.819
High consumption of coffee, n (%)	3 (7.3)	2 (4.9)	2 (4.9)	0.815[e]
High consumption of tea, n (%)	6 (14.6)	11 (26.8)	14 (34.1)	0.043
Polyphenol-rich diet, n (%)	7 (17.1)	10 (24.4)	19 (46.3)	0.004
CRP (mg/L)	0.6 (0.3~1.1)	0.8 (0.5~1.6)	0.5 (0.2~1.2)	0.609
IsoP (ng/L)	92.4 (57.8~138.1)	117.0 (50.0~164.1)	83.9 (45.3~123.1)	0.459
uTP (mg GAE/g Crea)	77.4 (63.7~98.4)	117.8 (95.4~136.7)	145.8 (120.0~211.1)	<0.001

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; TG: triglyceride; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; UA: uric acid; CREA: creatinine; MetS: metabolic syndrome; CRP: C-reactive protein; IsoP: 8-isoprostane; uTP: urinary total polyphenol. [a]Data are the mean ± SD, median (interquartile range) for continuous variables, or cases (percentage) for categorical variables. [b]The z score of 22 urinary polyphenol biomarkers was computed by the summary of the z score of each biomarker/22. [c]p values for trend. [d]Defined according to the criteria of metabolic syndrome from Chinese Diabetes Society (CDS) (2004) for Chinese. [e]p values for the Fisher exact test.

polyphenol biomarkers showed remarkable positive correlation with urinary total polyphenol and age. Significantly negative correlations were found respectively between most of urinary polyphenol biomarkers, especially gut microbial metabolites, and cardiovascular risk factors, such as TC, TG, LDL-C, FBG, UA, CRP, and IsoP ($p < 0.05$), while positive correlation existed between these biomarkers and HDL-C, respectively.

3.3. Comparison of Levels of Urinary Polyphenol Biomarkers according to the Presence of Polyphenol-Rich Diet. As shown in Figure 2(a), subjects with polyphenol-rich dietary habit have higher levels of most of urinary polyphenol biomarkers compared with the normal diet group. Ferulic acid ($p = 0.048$), coumaric acid ($p = 0.048$), syringic acid ($p = 0.008$), kaempferol ($p = 0.029$), (3,4)-dihydroxyphenylacetic acid ($p = 0.006$), sinapic acid ($p = 0.004$), hippuric acid ($p = 0.044$), and z score of the total 22 urinary polyphenols ($p = 0.004$) levels in the polyphenol-rich diet group were significantly increased in comparison with the other groups.

Principal component analysis and biplot (Figure 3) showed that 22 urinary polyphenol biomarkers can differentiate effectively the polyphenol-rich diet from the other one. Six of urinary polyphenol biomarkers, including

Figure 1: Correlation heatmap illustrating the relationship between urinary polyphenol biomarkers and cardiometabolic quantitative phenotypes in the study population. Spearman's correlation coefficients are presented in a blue-white-red color scheme. Dark red indicates a more positive correlation, and dark blue indicates a more negative correlation; white indicates no correlation. $^{*}p < 0.05$. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; TG: triglyceride; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; UA: uric acid; CREA: creatinine; CRP: C-reactive protein; IsoP: 8-isoprostane; uTP: urinary total polyphenol.

ferulic acid, hydroxyhippuric acid, sinapic acid, hippuric acid, 3-hydroxyphenylacetic acid, and 3-(4-hydroxyphenyl) propionic acid, contributed the most to distinguishing the two diet groups.

3.4. Association of Levels of Urinary Polyphenol Biomarkers with Overweight/Obesity. According to the definition of MetS, the population was divided into the control and case groups, respectively, based on whether the population had MetS, overweight/obesity, hypertriglyceridemia, dyslipidemia, and impaired fasting glucose. Regarding the patterns of dichotomous cardiometabolic traits, as shown in Figure 2(b), overweight and obesity subjects have lower levels of most of urinary polyphenol biomarkers compared with the normal group. Caffeic acid ($p = 0.002$), ferulic acid ($p = 0.002$), 3-hydroxybenzoic acid ($p = 0.040$), and z score of the total 22 urinary polyphenol ($p = 0.047$) levels in the overweight and obesity groups were significantly decreased in comparison with the normal group. There are no statistical differences of z score of urinary polyphenol biomarkers between different groups according to the presence of MetS and other components of MetS.

Furthermore, the associations between the levels of urinary polyphenol biomarkers and overweight/obesity were analyzed by multivariate logistic regression (Table 2). After adjusting for age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, high levels of physical activity, and polyphenol-rich diet, the ORs for overweight/obesity were all meaningful no matter whether z score levels of urinary polyphenol biomarkers were regarded as continuous variables or categorical variables (all $p < 0.05$). Each 1 score increase of z score levels of urinary polyphenol biomarkers was associated with approximately a 28-fold decreased risk of overweight/obesity [OR (95% CI): 0.036 (0.004~0.358), $p = 0.005$]. Besides, tertile analyses revealed linear decreases of overweight/obesity risk with an approximately 8-fold decrease in the risk of overweight/obesity in the top vs. bottom tertile of z score levels of urinary polyphenol biomarkers [OR (95% CI): 0.133 (0.027~0.666), p trend = 0.015].

Figure 2: Comparison of z scores of concentration of urinary polyphenol biomarkers according to the presence of (a) polyphenol-rich diet or (b) overweight/obesity. $^{*}p < 0.05$. Data are depicted by box plots extending from the 25th to the 75th percentile and whiskers ranging from the lower limit to the upper limit. The horizontal line in the box plot represents the median. The z score of total 22 polyphenol biomarkers was computed by the summary of the z score of each polyphenol biomarker/22.

Besides, by means of making an analysis of the ORs of each type of polyphenol biomarkers for overweight/obesity, we observed that urinary levels of gut microbial metabolites of polyphenol had the most remarkable correlation with overweight/obesity [OR (95% CI): 0.099 (0.019~0.006), p trend = 0.015] (Table 3).

Figure 3: Principal component analysis plot for polyphenol-rich and normal diets with urinary polyphenol biomarkers. Arabic numerals of 1-22 represent caffeic acid, ferulic acid, chlorogenic acid, coumaric acid, cinnamic acid, syringic acid, vanillic acid, protocatechuic acid, quercetin, quercetin 3-glucuronide, kaempferol, naringenin, hesperetin, (3,4)-dihydroxyphenylacetic acid, hydroxyhippuric acid, 3-hydroxybenzoic acid, homovanillic acid, 3-hydroxy-4-methoxycinnamic acid, sinapic acid, hippuric acid, 3-hydroxyphenylacetic acid, and 3-(4-hydroxyphenyl) propionic acid, respectively. The ellipses represent the 80% confidence intervals.

3.5. More Significant Association between Urinary Polyphenol Biomarker Levels and Overweight/Obesity in the Inflammatory Groups. The stratified analysis of the associations of urinary polyphenol biomarker levels and overweight/obesity was conducted according to a median concentration (0.7 mg/L) cut-off point of CRP. The results showed that the OR (95% CI) between urinary polyphenol biomarker levels and overweight/obesity was 0.000067 (0.00-0.017) in those participants with higher CRP levels, whereas in the lower CRP group, the OR (95% CI) was 0.425 (0.0069-29.547), in a multivariable logistic model as mentioned above. A significant interaction was observed between urinary polyphenol biomarker levels and CRP for overweight/obesity (p for interaction = 0.033). Furthermore, we used restricted cubic splines to explore the nonlinear interaction of polyphenol biomarkers and serum CRP concentration for overweight/obesity. In Figure 4, with the increase of serum CRP concentration, the inverse relationship of polyphenol biomarkers with overweight/obesity gradually enhanced, until reaching a state of almost infinitely large.

3.6. Assessment of ROC Curves for Overweight/Obesity. We used ROC analysis to assess the performance of the classifier models for overweight/obesity classification. ROC curves and measures of the area under the curve (AUC) were presented for three multivariable models: (1) clinical model inclusive of traditional risk factors (age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, and physical activity); (2) model 1 plus polyphenol-rich diet; and (3) model 2 plus the z score of 22 urinary polyphenol biomarkers. In Figure 5, the results showed greater discriminative capability for the clinical model containing urinary polyphenol biomarkers (AUC 0.884, 95% CI: 0.813~0.956) compared with the model containing clinical variables only (AUC 0.846, 95% CI: 0.775~0.917). The model of clinical factors containing urinary polyphenol biomarkers yielded a sensitivity of 82.8% and a specificity of 88.2%.

4. Discussion

In the present study, twenty-two polyphenol biomarkers were simultaneously quantified by LC/MS/MS analysis using a reverse-phase column and multiple reaction monitoring detection. These biomarkers, including hydroxycinnamic acids, hydroxybenzoic acids, flavonols, favanones, and gut microbial metabolites, were selected for their native forms abundant in Chinese dietary habits, for being representative of the main types of polyphenol metabolites reported to correlate with polyphenol intake by previous studies, and for being commercially available, on the basis of a literature survey [26–30]. The levels of urinary polyphenol biomarkers were proved and validated to effectively assess the habitual polyphenol intake and their associations with overweight/obesity in free-living healthy Chinese subjects. Taken together, the identification of biomarkers of dietary polyphenol exposure would provide an additional tool to investigate relationships with chronic disease-related endpoints.

A few previous studies [14, 20, 31] that related dietary polyphenol intake to metabolic profiles in free-living populations are consistent with our results. Garcia-Perez and colleagues [20] compared urinary metabolic profiles after consumption of different diets and found 19 urinary metabolites to be present in higher concentrations after consumption of healthy diet compared with unhealthy diet. The metabolites reflecting higher intake of fruits and vegetables included hydroxyhippuric acid and hippuric acid, which also showed the capability to distinguish two diets in our study. To the best of our knowledge, this is the first study to evaluate and verify the association between targeted urinary polyphenol biomarkers and polyphenol intake with Chinese dietary habits.

Urine, as a biospecimen, less invasively collected than the blood and more affordable to obtain, offers a greater collection of volumes and thus may turn out to be a potentially highly useful biological resource for large-scale clinical and epidemiologic studies. Ideal biomarkers are reliably measured, specifically exposed, and easily collected [32]. However, owing to the fact that urine metabolite concentrations can be highly variable because of ionic strength, pH, osmolarity, and variations in polyphenol pharmacokinetics, it is sometimes assumed that it may influence the correlations between polyphenol intake and urinary biomarkers [7]. Therefore, in the current study, the selected polyphenols are all of explicit pharmacokinetic characteristics [27], and their absolute concentration in urine is relatively high. Despite the fact that the half-life of some of them is short and the concentration in urine may generally reach the

Table 2: Odds ratios (95% confidence intervals) for overweight/obesity[a].

	Univariable		Multivariable[b]	
	OR (95% CI)	p	OR (95% CI)	p
Age	1.034 (1.007~1.062)	0.015	1.068 (1.016~1.123)	0.010
Female	1.347 (0.603~3.011)	0.468	1.459 (0.239~8.904)	0.682
FBG	5.722 (2.250~14.549)	<0.001	4.063 (1.299~12.710)	0.016
UA	20.619 (6.780~62.707)	0.001	1.007 (0.998~1.017)	0.114
CREA	0.993 (0.966~1.022)	0.646	0.992 (0.938~1.050)	0.791
CRP	1.220 (1.014~1.467)	0.035	1.129 (0.916~1.393)	0.255
IsoP	1.003 (0.999~1.007)	0.143	1.002 (0.998~1.007)	0.354
Hypertension[a]	2.394 (0.743~7.714)	0.144	0.653 (0.132~3.224)	0.601
Dyslipidemia[a]	4.609 (1.782~11.924)	0.002	8.216 (2.090~32.303)	0.003
Current smoker	0.365 (0.100~1.327)	0.126	0.206 (0.031~1.392)	0.105
Current drinker	0.703 (0.256~1.933)	0.495	1.008 (0.222~4.586)	0.992
High levels of physical activity	0.737 (0.324~1.677)	0.467	0.239 (0.064~0.891)	0.033
Polyphenol-rich diet	1.391 (0.600~3.226)	0.441	1.182 (0.329~4.241)	0.798
z score of 22 urinary polyphenol biomarkers as a continuous variable				
Per score increment	0.209 (0.056~0.776)	0.019	0.036 (0.004~0.358)	0.005
z score of 22 urinary polyphenol biomarkers as a categorical variable				
T1	1.0 (referent)	—	1.0 (referent)	—
T2	1.378 (0.555~3.421)	0.489	0.573 (0.158~2.076)	0.397
T3	0.369 (0.124~1.095)	0.073	0.133 (0.027~0.666)	0.014
p value for trend	0.090		0.015	

FBG: fasting blood glucose; UA: uric acid; CREA: creatinine; CRP: C-reactive protein; IsoP: 8-isoprostane. [a]Defined according to the criteria of metabolic syndrome from Chinese Diabetes Society (CDS) (2004) for Chinese. [b]Set of independent variables: age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, high levels of physical activity, polyphenol-rich diet, and z score of 22 urinary polyphenol biomarkers as a continuous or categorical variable.

Table 3: Odds ratios (95% confidence intervals) of each type of polyphenol biomarkers for overweight/obesity[a].

z score	Multivariable[b]	
	OR (95% CI)	p
Hydroxycinnamic acids[c]	0.291 (0.069~1.223)	0.092
Hydroxybenzoic acids[c]	0.576 (0.225~1.473)	0.249
Flavonols[c]	0.516 (0.149~1.792)	0.298
Favanones[c]	0.461 (0.178~1.193)	0.110
Gut microbial metabolites[c]	0.099 (0.019~0.510)	0.006

[a]Defined according to the criteria of metabolic syndrome from Chinese Diabetes Society (CDS) (2004) for Chinese. [b]Set of independent variables: age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, high levels of physical activity, polyphenol-rich diet, and z score of each type of polyphenol biomarkers as a continuous variable. [c]The z score of each type of polyphenol biomarkers was computed by the summary of the z score of each polyphenol biomarker/numbers of biomarkers of each type.

Figure 4: Adjusted odds ratios (OR) of levels of urinary polyphenol biomarkers for overweight/obesity by serum CRP concentrations. Serum CRP concentrations were modeled as restricted cubic splines with three freedom degrees. Multivariable logistic regression models (for overweight/obesity) were adjusted for age (continuous) and gender. The z score of urinary polyphenol biomarkers at the 25th percentile was used as reference. The dashed lines represent the 95% confidence intervals.

basic level in 48 h, some studies demonstrated that the concentration in urine may possibly achieve a detectable stable state when people intake polyphenol-rich foods daily on a regular basis [7, 28, 33].

The inverse associations between levels of urinary polyphenol biomarkers and overweight/obesity were also observed after being controlled for multivariates. ROC curves

Figure 5: Receiver operating characteristic (ROC) curves for overweight/obesity. ROC curves and measures of the area under the curve (AUC) are presented for three multivariable models: (1) clinical model inclusive of traditional risk factors (age, gender, FBG, UA, CREA, CRP, IsoP, hypertension, dyslipidemia, smoking and alcohol drinking status, and physical activity; black solid line); (2) model 1 plus polyphenol-rich diet (red line); and (3) model 2 plus the z score of 22 urinary polyphenol biomarkers (green line).

to assess the performance of the classifier models for overweight/obesity classification showed that using biomarkers in combination with self-reports could strengthen the discriminative capability of the clinical model. Through analyzing the relationship of each type of polyphenol biomarkers with overweight/obesity, we observed that urinary levels of gut microbial metabolites of polyphenol had the most significant and negative association with overweight/obesity. So far as we know, current thinking considers gut microbiota playing a potential role on the development of obesity and its related comorbidities [34]. And dietary habits are considered to be the main contributing factors to the diversity of the human gut microbiota by altering its composition [35]. A number of studies have shown long-term dietary patterns and short-term interventions induce changes in gut microbiota structure and function, and they have also revealed the fact that metabolic changes ultimately could influence host physiological health [36]. Our findings support these points of views on the relationship among diet, gut microbiota, and obesity.

The microbial polyphenol metabolism is kept to a general pattern, in which the extremely diverse group of plant polyphenols is transformed into a relatively small number of common metabolites [37]. Because of the complex determination of fecal flora, there is a need for rapid and sensitive analytical methods that can quantify such metabolites for a large number of samples with rapid and sensitive targeted metabolomics analysis [38]. A growing number of studies have shown concern on the determination of polyphenol microbial metabolites, especially in urine or plasma, but only a few have been committed to their accurate quantification in different biospecimens [39]. The developed method in the current study has brought about a possibility of using the simple method for quantifying or evaluating gut microbiota and their association with diet and disease or their interaction in the future.

In addition, a significant interaction was observed between urinary polyphenol biomarker levels and CRP for overweight/obesity. CRP is a marker of chronic inflammation and is an independent predictor of all-cause cardiovascular mortality. The negative association of urinary polyphenol biomarker levels with overweight/obesity was more pronounced in the inflammatory groups in the present study. What's more, with the increase of serum CRP concentration, the inverse relationship of polyphenol biomarkers with overweight/obesity gradually enhanced. Those consistent findings showed that the association of urinary polyphenol biomarker levels with overweight/obesity was more pronounced in participants with a chronic inflammatory status than others. The chronic low-grade inflammatory condition accompanying obesity has been implicated as a major player in both obesity and its associated pathophysiological consequences [40]. Thus, the current results demonstrate that it is of crucial importance to maintain polyphenol biomarkers at high levels or intake-sufficient polyphenols in obesity with chronic inflammation than others.

There are several limitations. Firstly, results were based on a short and simple self-reported FFQ that may likely attenuate the observed correlations. The FFQ provided information on the frequency of consumption of most common polyphenol-rich foods in Chinese dietary habit, but exclusive of weight and portion size, because there is no need to estimate the mean daily polyphenol intake as a continuous variable for the lack of a Chinese-specific food polyphenol composition database. However, it is because of the reasons mentioned above; our results proved that urinary polyphenol biomarkers could offer a valid alternative or complementary addition to self-reported survey for the evaluation of polyphenol intake while it is impossible for large-scale epidemiologic studies to collect details of dietary information. Secondly, metabolite concentrations account for the genetic effect modification of metabolic processes. We were unable to evaluate that, which could influence the extent to which a metabolite can act as a universal dietary biomarker. Thirdly, we cannot rule out the possibility that some findings may have been derived by chance, but we attempted to minimize this by controlling for multivariates. Finally, except for overweight/obese, there are no statistical differences of levels of urinary polyphenol biomarkers between different groups according to the presence of other components of MetS. The limitations of the cross-sectional study design and small sample size may account for this discrepancy. The findings need to be validated in future studies.

5. Conclusions

A targeted metabolomics method by LC/MS/MS for the measurement of 22 polyphenol biomarkers in urine samples was established and validated to effectively assess the habitual polyphenol intake in free-living healthy Chinese subjects. We also found that the levels of urinary polyphenol biomarkers, especially gut microbial metabolites of polyphenols, were inversely associated with overweight and obesity, and this association was more pronounced in the inflammatory groups suggesting that it is of great importance to maintain polyphenol biomarkers at high levels or intake-sufficient polyphenols in obesity with chronic inflammation than others. The measurement of these biomarkers may offer a valid alternative or complementary addition to self-reported survey for the evaluation of polyphenol intake and investigation into their relationships with chronic disease-related endpoints in large-scale clinical and epidemiologic studies.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

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

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Supplementary Materials

Supplementary Table 1: ion transitions for polyphenol biomarkers and instrumental parameters for their MS/MS detection in an MRM mode. *(Supplementary Materials)*

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