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# Role of whey protein in vascular function: a systematic review and meta-analysis of human intervention studies

Fatemeh Hajizadeh-Sharafabad[1,2], Elham Sharifi Zahabi[3] and Ali Tarighat-Esfanjani[1]*

[1]*Nutrition Research Center, Department of Clinical Nutrition, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*
[2]*Student Research Committee, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*
[3]*Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Tehran, Iran*

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## Abstract

Whey protein (WP) has been heavily appreciated as a rich source of bioactive peptides, with potential benefits for cardiovascular health. This study constitutes a systematic review and meta-analysis summarising the effects of WP consumption on vascular reactivity, arterial stiffness and circulatory biomarkers of vascular function. We searched electronic databases, including PubMed, SCOPUS and Web of science for relevant articles from inception to July 2020. Original clinical trials published in English-language journals that investigated the effects of WP on vascular function were eligible. A total of 720 records were identified in the initial search; from these, sixteen were included in our systematic review and thirteen in meta-analysis. The pooled analysis of six studies showed a significant increase in flow-mediated dilation (FMD) after WP consumption (weighted mean differences (WMD): 1·09 %, 95 % CI: 0·17, 2·01, $P = 0·01$). Meta-analysis of available data did not show any significant reduction in arterial stiffness measures including augmentation index (effect sizes: 7, WMD: −0·29 %, 95 % CI: −1·58, 0·98, $P = 0·64$) and pulse wave velocity (effect sizes: 4, WMD: −0·72 m/s, 95 % CI: −1·47, 0·03, $P = 0·06$). Moreover, the pooled analysis of six effect sizes showed no significant effects on plasma levels of nitric oxide following WP supplementation (WMD: 0·42 μmol/l, 95 % CI: −0·52, 1·36, $P = 0·38$). The overall results provided evidence supporting a protective effect of WP on endothelial function measured by FMD, but not for arterial stiffness measures and circulatory biomarker of vascular function. Further research is required to substantiate the benefits of WP on vascular function.

**Key words:** **Whey protein: Vascular function: Endothelial function: Cardiovascular health: arterial stiffness**

Endothelial dysfunction is considered the first reversible stage in the initiation of atherosclerotic lesions[1]. Endothelium, as a metabolically active organ, balances vascular tone, vascular growth and coagulation function and thereby regulates vascular homoeostasis[2,3]. The balance between endothelial-derived vasodilatory factors such as endothelial nitric oxide (eNO), prostacyclin, bradykinin, and hyperpolarising factor and vasoconstrictory factors such as endothelin-1 (ET-1), angiotensin II, and thromboxane A determines endothelium-dependent vasodilation[4,5]. Of these factors, eNO is the strongest endogenous vasodilator in the body that additionally inhibits vascular inflammation, platelet adherence and aggregation/proliferation of smooth muscle cells[6]. Damage to the endothelium impairs vascular homoeostasis and initiates a number of processes that progressed endothelial dysfunction to atherosclerosis and eventually CVD[6]. Therefore, from a pathophysiological viewpoint, improvement of vascular disorders and restoration of vascular homoeostasis should be the main focus of therapeutic strategies to prevent and manage CVD.

Epidemiological studies supported the inverse relationship between consumption of dairy products and CVD risk or mortality[7,8]. Dairy products contain valuable bioactive compounds which may mediate particular biological actions beyond nutrient supply, such as anti-hypertensive effects[9]. However, it remains to fully elucidate the compounds underlying these effects, milk-derived proteins and bioactive peptides may be responsible for the observed activities[10]. Amongst these proteins, milk-derived whey protein (WP), as a biologically active protein which may counteract cardiomatabolic disorders such as hypertension (HTN), diabetes mellitus, dyslipidemia, obesity and oxidative stress, has attracted a great deal of attention[11–13]. As evidence shows, WP exerts the angiotensin-converting enzyme (ACE)

**Abbreviations:** ACE, angiotensin-converting enzyme; AI, augmentation index; DVP-RI, digital volume pulse-reflection index; DVP-SI, digital volume pulse-stiffness index; eNO, endothelial nitric oxide; ET-1, endothelin-1; FMD, flow-mediated dilation; HTN, hypertension; ICAM-1, intercellular adhesion molecule; PWV, pulse wave velocity; RCT, randomised controlled trial; VCAM-1, vascular adhesion molecule-1; WMD, weighted mean differences; WP, whey protein.

* **Corresponding author:** Ali Tarighat-Esfanjani, email tarighat45@gmail.com

inhibitory behaviours and thereby modulates blood pressure and vascular reactivity[14,15]. Indeed, ACE inhibitors block the renin–angiotensin system which has principal role in the regulation of blood pressure[16]. In spite of ACE inhibitory and the apparent anti-hypertensive effects of WP[17,18], questions still remain in elucidation of the vascular effects of WP.

A recent meta-analysis reported that WP intake was significantly associated with improvement in some cardiovascular risk factors, including body weight, blood pressure and lipid profile[19]. However, the systematic review and meta-analysis by Wirunsawanya *et al.* did not include vascular parameters such as endothelial function, arterial stiffness and circulatory biomarkers of vascular function as study outcomes which are key factors in development and progression of CVD[19]. To the best of our knowledge, there is no complete overview of the evidence of the associations between WP consumption and markers of vascular function. Therefore, the objectives of this study are to systematically review and analyse all available randomised controlled trials (RCT) investigating the relationship between WP consumption and vascular function measurements and to identify existing research gaps. Common non-invasive methods to evaluate vascular function can be categorised as measures of endothelial function and arterial stiffness. The main techniques used to quantify endothelial function include flow-mediated dilation (FMD), laser Doppler imaging and peripheral artery tonometry. Arterial stiffness also is assessed by pulse wave velocity (PWV), augmentation index (AI) and digital volume pulse-stiffness index (DVP-SI)[20].

## Methods

This systematic review and meta-analysis was conducted according to the guidelines of the preferred reporting items for systematic reviews and meta-analysis (PRISMA) statement. The review protocol has been registered at PROSPERO database of Systematic Reviews (registration number: CRD42020180068).

### *Eligibility criteria*

Studies were considered eligible if they fulfilled following criteria: (1) original studies published in English language and (2) randomised, controlled trials investigating the effect of WP on vascular function in humans. Reviews, letters, case reports or case series, abstracts as well as studies in which WP was combined with other supplements were excluded.

### *Search strategy and study selection*

The study was designed in accordance with the PICOS criteria: the population was human model; the intervention was a treatment with WP; the comparator was no treatment, a placebo or standard treatment; the outcomes were vascular responses to WP; the study design was clinical trial. A systematic search was carried out in PubMed, SCOPUS and Web of science electronic databases for clinical trials investigating the relationship between WP and vascular function published before 30 July 2020. Key search terms were 'whey' OR 'whey proteins' OR 'whey protein' AND 'arterial stiffness' OR 'vascular function' OR 'endothelial function' OR 'flow-mediated dilation' OR 'peak wave velocity' OR 'augmentation index' OR 'carotid thickness' OR 'intima-media thickness' OR 'carotid plaque' OR 'nitrate-mediated dilation' OR 'IMT' OR 'endothelial' OR 'endothelium' OR 'blood flow' OR 'vascular resistance' OR 'arterial stiffness' OR 'FMD' OR 'PWV' OR 'AI' OR 'inflammation' OR 'inflammatory' OR 'adhesion molecules' OR 'intercellular adhesion molecule-1' OR 'ICAM-1' OR 'vascular adhesion molecule-1' OR 'VCAM-1' OR 'nitric oxide' OR 'endothelin-1' OR 'ET-1'. Initially, the titles and abstracts of all identified studies were screened according to the selection criteria and then retrieved and assessed full-text versions of potentially relevant articles for eligibility criteria. Any discrepancies between reviewers were resolved through discussion to reach a consensus. The following data were extracted from included studies: author/date/country, study design, characteristics of participants, type and dosage of WP, duration of the intervention, vascular parameters and other studied outcomes.

### *Study quality and risk of bias within the studies*

The risk of bias for each study was assessed using the Cochrane collaboration tool which encompasses domains of selection bias, performance bias, detection bias, attrition bias, reporting bias and other bias. The quality of evidence for each outcome was classified as low risk, high risk or unclear risk of bias in each study. The overall risk of bias was assigned, with each study being rated as high (high risk of bias for one or more key domains), low (low risk of bias for all key domains) or unclear (low or unclear risk of bias for all key domains). Any disagreement regarding the risk of bias was resolved by discussion.

### *Statistical analysis*

Focusing on main vascular outcomes, a meta-analysis was performed to evaluate the strength of the association of vascular outcomes with WP supplementation. Any outcomes that were examined by at least four studies were included in the meta-analysis. For trials that examined the changes in outcomes at multiple time points, only the last measurement was included in the meta-analysis. For each included studies, the pooled effects for FMD (%), AI (%) and PWV (m/s), and eNO (μmol/l) were determined as weighted mean differences (WMD; final values – baseline values) and 95 % CI using the random-effects model. If not provided by the author, standard deviation was calculated using the following formula: SD = SEM × square root of the sample size. Also, data were extracted from the figures, if outcomes were not expressed in the main text or tables. Forest plots were produced to graphically present the means along with corresponding 95 % CI for each study. To quantify statistical inconsistency of measurements across studies, the heterogeneity index ($I^2$) was used and $I^2 > 50\,\%$ or $P < 0{\cdot}01$ were considered to represent substantial heterogeneity. In addition, subgroup analyses according to health condition, participants' age and intervention duration were used to explore possible sources of heterogeneity. Publication bias was determined by Begg's and Egger's regression test. All analyses were conducted using



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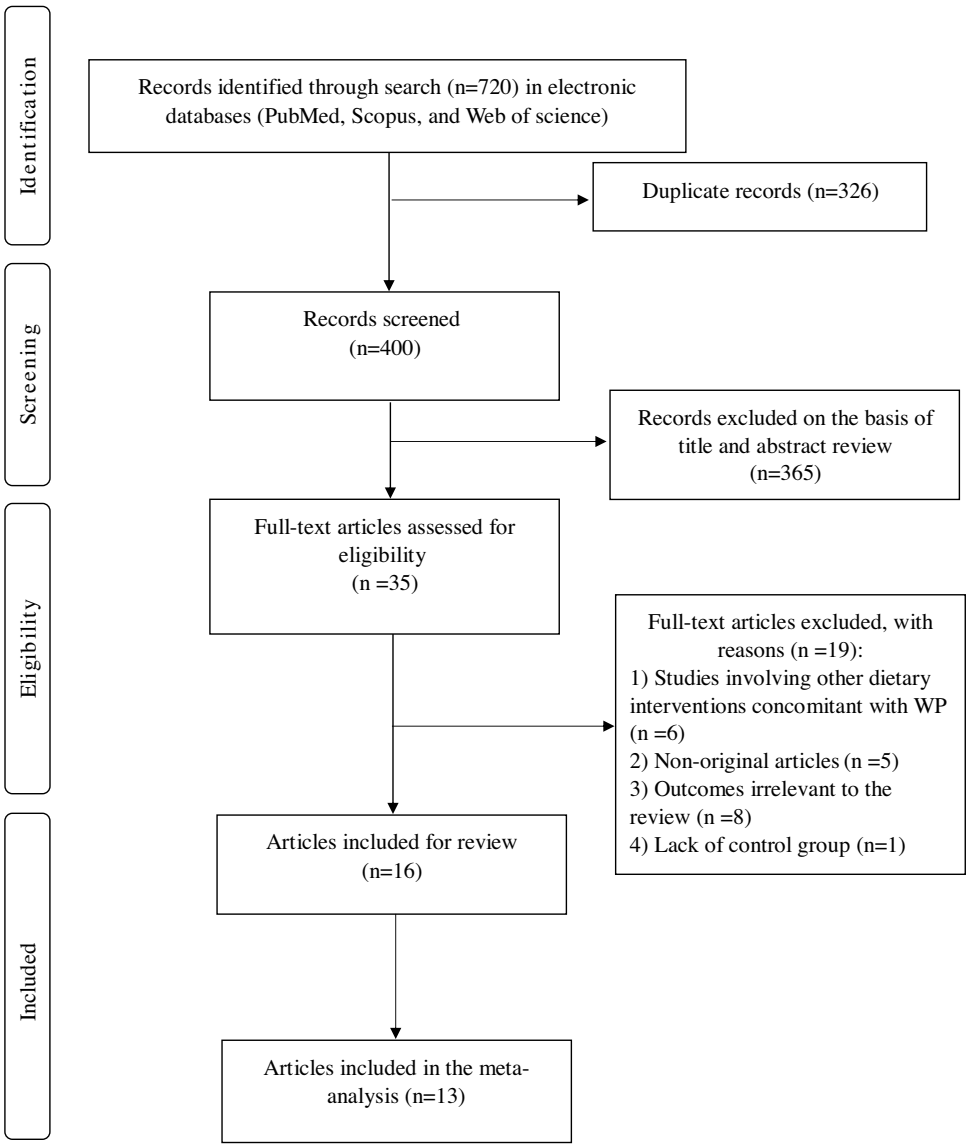


**Fig. 1.** Flow diagram of the literature search and study selection process. WP, whey protein.

Comprehensive Meta Analysis V2, and a $P < 0{\cdot}05$ indicated statistical significance.

## Results

Figure 1 presents the flow diagram of study selection process. A total of 720 records were identified from the database search, of which 326 duplicate papers were removed, 365 of the remaining 400 records were excluded on the basis of title and abstract. Finally, thirty-five full-text articles were screened for eligibility, and sixteen studies were identified as being RCT of WP on vascular function and included in the systematic review. Moreover, only thirteen RCT provided sufficient data to be included in the meta-analysis. Remaining three studies were excluded from quantitative assessment due to insufficient data. We tried to contact the authors of the trials with insufficient data by email to access full data on related outcomes, but no response was received from authors.

### *Study characteristics*

Tables 1 and 2 detailed the main characteristics of the sixteen studies of WP interventions. Seven of the included studies involved acute follow-up and eight evaluated the effect of chronic WP on study outcomes. In addition, one study had reported both acute and chronic effects of WP on different outcomes[21]. All included studies were RCT, of which nine had a crossover design. The sample size of the acute studies ranged from nine to twenty-five subjects, while in the chronic studies, the number of participants that completed each study varied from 20 to 173. Based on BMI values, six studies included obese population[22–27], seven included overweight[17,28–33] and other three studies included normal-weight participants[21,34,35]. Most

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**Table 1.** Overview of the characteristics and main findings of the acute RCT included in the systematic review

| Author/date/ Country | Study design | Study participants | Nutritional intervention | | | | Main outcomes | Results |
|---|---|---|---|---|---|---|---|---|
| | | | Groups | Form of WP | Dosage | Duration | | |
| Ballard, *et al.*/ 2009/ USA[21] | Double-blinded, crossover RCT | 20 healthy volunteers aged 25 years, BMI 24·3 kg/m$^2$ | WP packets *v.* placebo | Peptide isolated from a WP hydrolysate | 5 g | Up to 120 min after ingestion (haematological factors were assessed after 2 weeks of ingestion) | FBF, FMD, VCAM-1, ICAM-1 and eNO | –Significant increase in FMD.<br>–Significant increase in FBF.<br>–No significant differences in VCAM-1 and ICAM-1.<br>–Significant inhibition of eNO decline. |
| Pal, *et al.*/ 2011/ Australia[23] | 3-arm cross-over RCT | 20 overweight/ obese postmenopausal women aged 57 years, BMI 32·5 kg/m$^2$ | WP meal *v.* casein meal *v.* placebo | WP isolate | 45 g | Up to 6 h | AI | –No significant differences in AI. |
| Ballard, *et al.*/ 2012/ USA[29] | Double-blinded, crossover RCT | 21 individuals with impaired FMD aged 55 years, BMI 27·8 kg/m$^2$ | WP packets *v.* placebo | Peptide isolated from a WP isolate | 5 g | Up to 120 min after ingestion | Artery diameter, FMD, eNO and ET-1 | –Significant increase in FMD only at 120 min.<br>–No significant differences in absolute FMD and artery diameter.<br>–No significant differences in plasma levels of eNO and ET-1. |
| Mariotti, *et al.*/ 2015/ France[25] | 3-arm, cross-over RCT | 10 healthy overweight men, aged 35 years, BMI, 30·2 kg/ m$^2$ | WP *v.* casein *v.* LAC | WP isolate | 45 g | Up to 6 h after ingestion | Salbutamol-induced endothelium-dependent dilation, glyceryl trinitrate-induced endothelium-independent dilation, artery diameter, DVP-SI, ICAM-1 and VCAM-1 | –No significant differences in endothelium-dependent and -independent dilation.<br>–No significant differences in DVP-SI and artery diameter.<br>–No significant differences in plasma level of ICAM-1 and VCAM-1. |
| Rontoyanni, *et al.*/ 2015/ USA[34] | Crossover RCT | 14 healthy men, aged 26 years, BMI 22·7 kg/m$^2$ | WP drink *v.* carbohydrate drink *v.* placebo | WP isolate | 44 g | Up to 30 min after ingestion | RI, DVP-SI and systemic vascular resistance | –Significant decrease in RI.<br>–No significant differences in DVP-SI.<br>–No significant differences in systemic vascular resistance. |
| Fekete, *et al.*/ 2018/ UK[30] | Double-blinded, crossover RCT | 25 adults with pre-HTN/mild HTN, aged 50 years, BMI 27·3 kg/m$^2$ | WP *v.* casein *v.* placebo | WP isolate | 28 g for breakfast and 28 g for lunch | Up to 8 h after ingestion | FMD, AI and DVP-SI | –Significant time × treatment increase in FMD.<br>–Significant reduction in AI both after 5 and 8 h.<br>–No significant differences in DVP-SI. |

**Table 1.** (*Continued*)

| Author/date/ Country | Study design | Study participants | Nutritional intervention | | | | Main outcomes | Results |
|---|---|---|---|---|---|---|---|---|
| | | | Groups | Form of WP | Dosage | Duration | | |
| McDonald *et al.* 2018/ USA[27] | 4-arm, cross-over RCT | 23 adults with obesity and pre-diabetes, aged 33 years, BMI 31·6 kg/m$^2$ | WP drink *v.* non-fat milk *v.* casein drink *v.* placebo | WP isolate | 16·5 g | Up to 180 min after ingestion | FMD, asymmetric dimethylarginine/arginine, symmetric dimethylarginine/ arginine, eNO, ET-1 and di/tetrahydrobiopterin | –Significant inhibition of decline in FMD at 30–90 min post-ingestion.<br>–Significant attenuation in asymmetric dimethylarginine/arginine and symmetric dimethylarginine/ arginine.<br>–Significant increase in eNO and arginine.<br>–No significant differences in plasma levels of ET-1 and di/tetrahydrobiopterin. |
| Oliveira, *et al.* 2019/ Brazil[32] | Double-blinded, crossover RCT | 9 healthy adults, aged 22 years, BMI 25·9 kg/m$^2$ | WP drink *v.* fish protein hydrolysate drink *v.* placebo | WP hydrolysate | 20 g | Up to 120 min after ingestion | FMD | –No significant differences in artery diameter.<br>–Significant increase in FMD only after 30 min. |

RCT, randomised controlled trial; WP, whey protein; FBF, forearm blood flow; FMD, flow-mediated dilatation; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; eNO, endothelial nitric oxide; AI, augmentation index; ET-1, endothelin-1; LAC, α-lactalbumin-enriched whey protein; DVP-SI, digital volume pulse-stiffness index; RI, reflection index; HTN, hypertension.

of included RCT were carried out in the individuals who had at least one cardiovascular risk factor, including overweight, obesity and HTN. FMD responses to WP were assessed in eight RCT, of which four were carried out on pre-HTN/mild HTN[17,30,33,35], two on healthy volunteers[21,32], one on obese individuals[27] and one on subjects with impaired FMD[29]. Also, ten RCT evaluated the effect of WP on arterial stiffness[17,22–26,28,30,31,34], of which four were conducted on overweight/obese individuals[22,23,25,28], three on pre-HTN/mild HTN[17,24,30], one on haemodialysis patients[26] and two on healthy volunteers[31,34]. Effects of WP on the plasma levels of , intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) were evaluated in three studies of which two were conducted on healthy[21,25] and one on HTN/ mild HTN individuals[17]. There were six studies that examined the impacts of WP on eNO. Three studies were conducted on pre-HTN/mild HTN[17,33,35], one on healthy subjects[21], one on obese individuals[27] and one on subjects with impaired FMD[29]. In addition, three studies evaluated the effects of WP on plasma levels of ET-1[27,29,35] of which one study conducted on obese subjects[27], one on patients with impaired FMD[29] and one on pre-HTN/mild HTN individuals[35].

Thirteen of the included studies used isolate type of WP to determine the effect of WP on vascular endothelial function. Remaining RCT also utilised a peptide isolated from a WP isolate (*n* 2), WP concentrate (*n* 1) and WP hydrolysate (*n* 1). Chronic studies used various dosages of WP from 0·7 to 56 g/d during a time frame ranging from 2 weeks to 12 months. In the acute studies, participants had consumed 5–45 g of WP during 0·5 to 8 h. These sixteen studies originated from the USA (*n* 7), UK (*n* 2) and Australia (*n* 2), and single studies from Denmark, France, China, Russia and Brazil. Finally, of sixteen eligible documents that were described in qualitative review, thirteen provided sufficient data on some outcomes and were included in the meta-analysis.

## *Studies of WP' association with vascular function: a qualitative review*

***Acute studies.*** Main results obtained in acute studies of WP intervention have been summarised in Table 1. These studies mainly used FMD[21,27,29,30,32], DVP-SI[25,30,34], AI[23,30], digital volume pulse-reflection index (DVP-RI)[25,34], adhesion molecules[21,25], eNO[21,27,29] and ET-1[27,29] to assess vascular function. Ballard *et al.* reported that the consumption of 5 g/d of a WP-derived peptide (NOP-47) mixed in water significantly increased FMD of healthy volunteers at 30 (8·87 %), 60 (9·94 %) and 90 (9·02%) min post-ingestion compared with the same time points following consumption of placebo[21]. NOP-47 also markedly inhibited plasma eNO reduction at 120 min compared with placebo. However, no significant effects of NOP-47 on ICAM-1 and VCAM-1 were obtained[21]. In addition, the authors examined eNO-independent vasodilation using venous occlusion strain gauge plethysmography from 20 to 120 min post-NOP-47 ingestion and observed that NOP-47 significantly increased forearm blood flow (29·9%) at 120 min[21]. In another study, the same authors found that acute ingestion of 5 g of NOP-47 in the individuals with impaired FMD significantly enhanced

FMD at 30 (4·6%) and 120 (5·1%) min post-intervention compared with the baseline, although, between-group changes in FMD reached to significance only at 120 min. Also, compared with placebo, absolute FMD responses, brachial artery diameter and plasma levels of eNO and ET-1 during the NOP-47 ingestion did not change significantly[29]. In the overweight or obese postmenopausal women, Pal *et al.* did not observe significant changes in AI (the indicator of arterial stiffness) after 6 h of the ingestion of a breakfast meal in conjunction with 45 g WP isolate[23]. Mariotti *et al.* did not find significant changes in the vascular endothelial-dependent reactivity (based on the salbutamol-induced decrease in DVP-RI), endothelial-independent vascular reactivity (based on the glyceryl trinitrate-induced decrease in DVP-RI), DVP-SI and plasma levels of ICAM-1 and VCAM-1 in overweight men during 6 h after oral consumption of 45 g WP compared with the control group[25]. Similarly, in healthy men, drinks containing 44 g WP did not make significant changes in DVP-SI, and systemic vascular resistance up to 30 min post-ingestion compared with isoenergetic drinks containing carbohydrate or water; however, it significantly prevented an increase in DVP-RI[34].

In another study by Fekete *et al.*, adults with pre-HTN or mild HTN ingested a high-fat, isoenergetic breakfast and lunch with either WP (28 g for breakfast and 28 g for lunch), casein or maltodextrin and then they were examined for vascular function at time 0, 180, 300 and 420 min after meals[30]. The researchers found a significant increase in FMD (0·60 %) at 5 h after breakfast meal in conjunction with WP compared with maltodextrin. In addition, AI reduced after WP consumption compared with maltodextrin both after 5 (18·2 %) and 8 (30·9 %) hours; however, the changes in DVP-SI were not significant between groups[30]. In a study by McDonald *et al*,. the adults with obesity and pre-diabetes were randomised to WP isolate (16·5 g), non-fat milk, sodium caseinate and placebo in which all groups consumed glucose in addition to dietary interventions to induce hyperglycemia-induced impairments in endothelial function and then % FMD was assessed at 30 min intervals for 180 min post-prandially[27]. The authors observed that compared with the placebo, both WP and milk significantly inhibited the declines in FMD at 120–150 min, while in the placebo group, %FMD significantly decreased up to 2 % post-prandially. Moreover, WP significantly increased post-prandial eNO, with no significant changes in plasma levels of ET-1[27]. Oliveira *et al.* examined the acute effect of WP on vascular function in healthy adults up to 120 min post-ingestion and reported a significant increase in FMD (9·02 %) only at 30 min after consumption. No significant differences were observed for brachial artery diameter[32].

*Chronic studies.* Outcomes of the included chronic RCT are detailed in Table 2. Chronic studies mainly used FMD[17,21,33,35], arterial stiffness parameters[17,22,24,26,28,31], adhesion molecules[17,21], eNO[17,21,33,35] and ET-1[21,35] to measure the impact of WP on vascular function. Pal *et al.* found a significant reduction in arterial stiffness measured by AI (14 %) in overweight/obese individuals who consumed 54 g/d WP isolate mixed with 250 ml water for 12 weeks[22]. In contrast, the consumption of 35 g/d WP for 12 weeks did not make significant changes in arterial stiffness obtained from AI measurement in overweight adolescents[28]. In another study, the intake of 30 g/d WP over 4 weeks significantly decreased arterial stiffness measured by aortic AI (8·2 %) and brachial–ankle PWV (57 cm/s) in obese and pre-HTN women[24]. Fekete *et al.* found a significant increase in FMD (1·31 %) of adults with pre-HTN or mild HTN following 8 weeks of 56 g/d WP supplementation[17]. However, the differences in DVP-SI, AI (measures of arterial stiffness) and DVP-RI (measure of vascular tone) were not statistically significant[17]. The authors also reported a significant reduction in ICAM-1 level with no significant changes in eNO and VCAM-1 concentrations compared with the controls[17]. Ballard *et al.* in another study showed that the administration of 5 g/d of NOP-47 for 2 weeks had no significant effects on serum levels of ICAM-1 and VCAM-1 in healthy subjects[21]. In another study by Petyaev, *et al.* in which subjects with pre-HTN consumed 70 mg/d WP isolate for 4 weeks, no significant changes were detected in FMD or plasma level of eNO[33]. In a recent study by Jeong *et al.*, intradialytic WP supplementation (30 g, three d/week) did not make significant changes in arterial stiffness which was measured by AI and PWV after 6 and 12 months of supplementation in haemodialysis patients[26]. Yang *et al.* showed that the consumption of 30 g/d of WP for 12 weeks led to a significant increase in FMD (5·2 %) in adults with pre-HTN/mild HTN compared with the controls[35]. In the overweight and obese individuals, also the FMD showed a significantly higher increase in the WP group than in the controls (7·3 %). In contrast, differences in FMD change were not significant in the normal-weight individuals. The changes in the plasma levels of ET-1 and eNO were not significant, as well[35]. In another study, the consumption of 50 g/d of WP isolate for 12 weeks in elderly participants made a significant decrease in the aortic stiffness, assessed via gold standard carotid-femoral PWV (4 %), without significant changes in AI and pulse pressure[31].

## Meta-analysis

*Effect of WP on FMD and arterial stiffness measures.* Of included trials, 6 (4 acute studies and 2 chronic study) with 153 individuals provided adequate data on FMD (Table 3). The pooled estimate showed a significant improvement in FMD after WP intake (Fig. 2; WMD: 1·09 %, 95 % CI: 0·17, 2·01, $P = 0·01$). There was a high degree of heterogeneity between the studies ($I^2$ index 99·85 %, $P = 0·000$). Subgroup analysis was conducted to find the causes of high heterogeneity. This analysis showed no detectable effect of WP on FMD in individuals aged less than 45 years or healthy individuals ($P = 0·33$), whereas the subjects aged ≥ 45 years old or individuals with impaired health showed a significant increase in FMD (WMD: 1·41, 95 % CI: 1·05, 1·78, $P = 0·000$). Subgroup analysis based on the study duration, showed no significant effect on FMD following chronic supplementation with WP ($P = 0·12$), while acute ingestion of WP significantly increased FMD (WMD: 1·59, 95 % CI: 1·45, 1·72, $P = 0·000$). A sensitivity analysis conducted to test the effect of individual study on the overall results and indicated that one study on adults with pre-HTN/mild HTN had the largest influence on FMD[30]; as after exclusion of this study from analysis, the pooled

**Table 2.** Overview of the characteristics and main findings of the chronic RCT included in the systematic review

| Author/date/ Country | Study design | Study participants | Nutritional intervention | | | | Main outcomes | Results |
|---|---|---|---|---|---|---|---|---|
| | | | Groups | Form of WP | Dosage | Duration | | |
| Pal, *et al.*/ 2009/ Australia [22] | Single-blinded, parallel RCT | 70 overweight/obese individuals aged 48 years, BMI 31·3 kg/m$^2$ | WP sachet *v.* casein sachet *v.* placebo | WP isolate | 54 g/d | 12 weeks | AI | Significant decrease in AI. |
| Ballard, *et al.*/ 2009/ USA[21] | Double-blinded, crossover RCT | 20 healthy volunteers aged 25 years, BMI 24·3 kg/m$^2$ | WP packets *v.* placebo | Peptide isolated from a WP hydrolysate | 5 g/d | 2 weeks | VCAM-1 and ICAM-1 | No significant differences in VCAM-1 and ICAM-1. |
| Petyaev, *et al.*/2012/ Russia [33] | Parallel RCT | 40 subjects with pre-HTN, aged 56 years, BMI 27·0 kg/m$^2$ | WP *v* placebo | WP isolate | 70 mg/d | 4 weeks | FMD and eNO | –No significant differences in FMD compared to baseline.<br>–No significant differences in plasma level of eNO compared to baseline. |
| Arnberg, *et al.*/ 2013/ Denmark [28] | Double-blinded, parallel, RCT | 173 overweight adolescents aged 13 years, BMI 23 to 27 kg/m$^2$ | WP drink *v.* skimmed milk *v.* Casein drink *v.* Placebo | WP isolate | 35 g/d | 12 weeks | Aortic PWV and AI | No significant differences in aortic PWV and AI. |
| Figueroa, *et al.*/ 2013/ USA [24] | Double-blinded, parallel, RCT | 33 obese and pre-HTN women aged 30 years, BMI 35·2 kg/m$^2$ | WP *v.* Casein *v.* placebo (all combined with moderate exercise) | WP isolate | 30 g/d | 4 weeks | Brachial PWV and AI | Significant decrease in brachial PWV and AI. |
| Fekete, *et al.*/ 2016/ UK[17] | Double-blinded, 3-arm, cross-over RCT | 38 adults with pre-HTN/ mild HTN, aged 53 years, BMI 27·1 kg/m$^2$ | WP *v.* casein *v.* placebo | WP isolate | 56 g/d | 8 weeks | FMD, AI, DVP-SI, RI, ICAM-1, eNO and VCAM-1 | –Significant increase in FMD.<br>–No significant differences in AI, DVP-SI and RI.<br>–Significant decrease in ICAM-1.<br>–No significant differences in eNO and VCAM-1. |
| Jeong, *et al.*/ 2019/ USA[26] | Double-blinded, parallel, RCT | 101 haemodialysis patients, aged 55 years, BMI 31·4 kg/m$^2$ | WP *v.* placebo *v.* WP+ exercise training | WP isolate | 30 g, 3 d/ week | 12 months | Aortic PWV and AI | No significant differences in aortic PWV and AI. |
| Yang, *et al.*/ 2019/ China[35] | Double-blinded, parallel, RCT | 54 adults with pre-HTN/ mild HTN, aged 43 years, BMI 24·2 kg/m$^2$ | WP sachet *v.* placebo | WP concentrate | 30 g/d | 12 weeks | FMD, ET-1 and eNO | –Significant increase in FMD.<br>–No significant differences in ET-1, eNO. |
| Lefferts *et al.*/ 2020/ USA [31] | Double-blinded, parallel, RCT | 99 older adults aged 67 years, BMI 27·2 kg/m$^2$ | WP *v.* placebo | WP isolate | 50 g/d | 12 weeks | Aortic PWV, AI, artery diameter and intima-media thickness | –Significant decrease in aortic PWV.<br>–No significant differences in AI, artery diameter and intima-media thickness. |

RCT, randomised controlled trial; WP, whey protein; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; FMD, flow-mediated dilatation; eNO, endothelial nitric oxide; PWV, pulse wave velocity; AI, augmentation index; HTN, hypertension; DVP-SI, digital volume pulse-stiffness index; RI, reflection index; ET-1, endothelin-1.

**Table 3.** The effects of WP supplementation on vascular function in the participants of included studies

| Variable | Number of effect sizes | WMD | 95 % CI | *P* (between group) | Heterogeneity | |
|---|---|---|---|---|---|---|
| | | | | | $I^2$ (%) | *P* heterogeneity |
| FMD | 6 | 1·09 | 0·17, 2·01 | 0·01 | 99·85 | 0·000 |
| AI | 7 | –0·29 | –1·58, 0·98 | 0·64 | 72·25 | 0·001 |
| PWV | 4 | –0·72 | –1·47, 0·03 | 0·09 | 86·46 | 0·000 |
| eNO | 6 | 0·42 | –0·52, 1·36 | 0·38 | 59·86 | 0·02 |

WP, whey protein; WMD, weighted mean difference; FMD, flow-mediated dilation; AI, augmentation index; PWV, pulse wave velocity; eNO, endothelial nitric oxide.

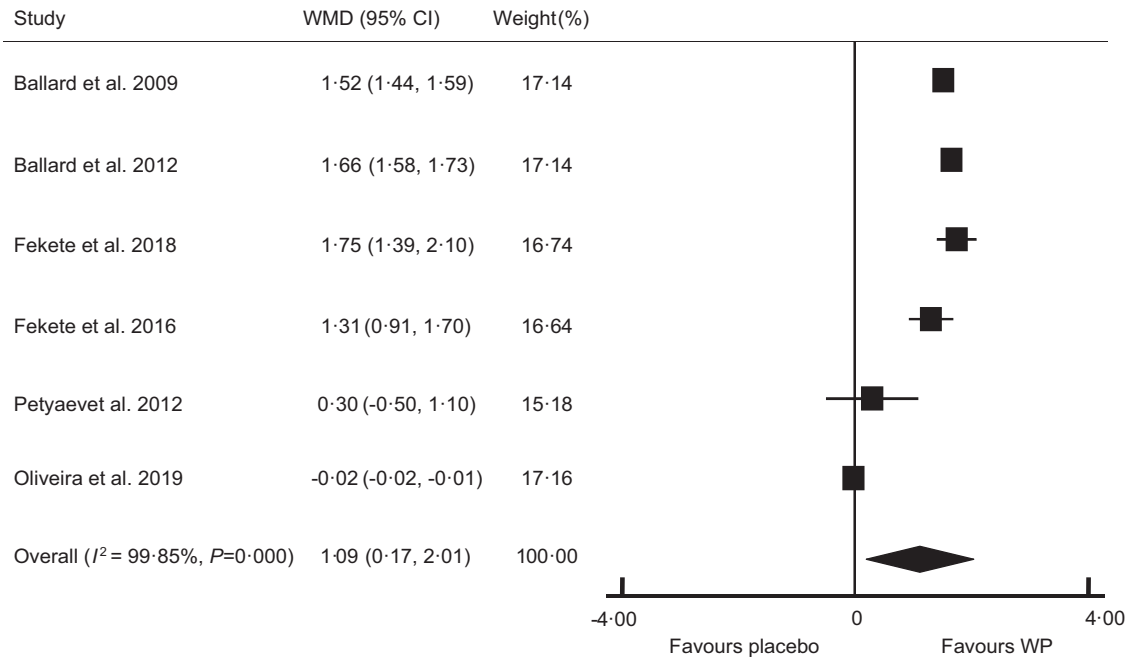



**Fig. 2.** Forest plot of the effect of whey protein on flow-mediated dilation. WMD, weighted mean difference; WP, whey protein.

WMD became insignificant (WMD: 0·96 %, 95 % CI: −0·03, 1·97, $P = 0·06$) compared with that from the main analysis. Begg's ($P = 0·45$) and Egger's test ($P = 0·10$) did not confirm the presence of publication bias.

Of included studies, 7 (6 chronic studies and 1 acute study) with 534 participants provided adequate data on AI (Table 3) and revealed a non-significant decrease in AI following WP consumption (Fig. 3; WMD: −1·29 %, 95 % CI: −1·58, 0·98, $P = 0·64$). The $I^2$ index (72·25 %) and Cochrane Q test ($P = 0·001$) revealed a high inter-trial heterogeneity. This finding did not change after subgroup analyses based on health condition, participants' age and intervention duration (Table 4). The pooled WMD from sensitivity analysis was similar to that from the main analysis. The results from Begg's ($P = 0·54$) and Egger's test ($P = 0·31$) did not confirm the likely of publication bias across studies.

The pooled analysis of 4 studies, with 406 participants showed that WP supplementation did not affect PWV significantly, while there was a trend towards a reduction in PWV (Fig. 3; WMD: −0·72 m/s, 95 % CI: −1·47, 0·03, $P = 0·06$). Considering $I^2$ index (86·46 %) and Cochrane Q test ($P = 0·000$), a high inter-trial heterogeneity was detected. The subgroup analyses indicated a significant decrease in this marker in the studies with participant's aged ≥ 45 years and duration of ≥ 12 weeks (Table 4). Moreover, a sensitivity analysis indicated that one study on overweight adolescents had the most effect on PWV[28]; as the pooled WMD was shifted to the significance after exclusion of this study from analysis (WMD: −1·00 m/s, 95 % CI: −1·61, 0·39, $P = 0·001$)[28]. In contrary to Begg's test ($P = 0·30$), Egger's test ($P = 0·003$) showed evidence of publication bias.

### *Effect of WP on circulating biomarkers of vascular function.*

The only circulating biomarker that met the meta-analysis criteria was eNO. There were five RCT with six effect sizes regarding the effect of WP on eNO. The pooled analysis of these trials involving 173 participants showed no significant effects on plasma levels of eNO following WP supplementation (Fig. 4; WMD: 0·42 μmol/l, 95 % CI: −0·52, 1·36, $P = 0·38$). The $I^2$ index (59·86 %) and Cochrane Q test ($P = 0·02$) revealed a moderate inter-trial heterogeneity. As the Table 4 shows, subgroup analysis indicated a significant increase in eNO level in healthy individuals, but not in subjects with impaired health. However, subgroup analyses based on participants' age or study duration indicated no significant effect on FMD after WP consumption. A sensitivity analysis showed that one chronic study on middle-aged pre-HTN subjects had the largest influence on eNO levels[33]; as after

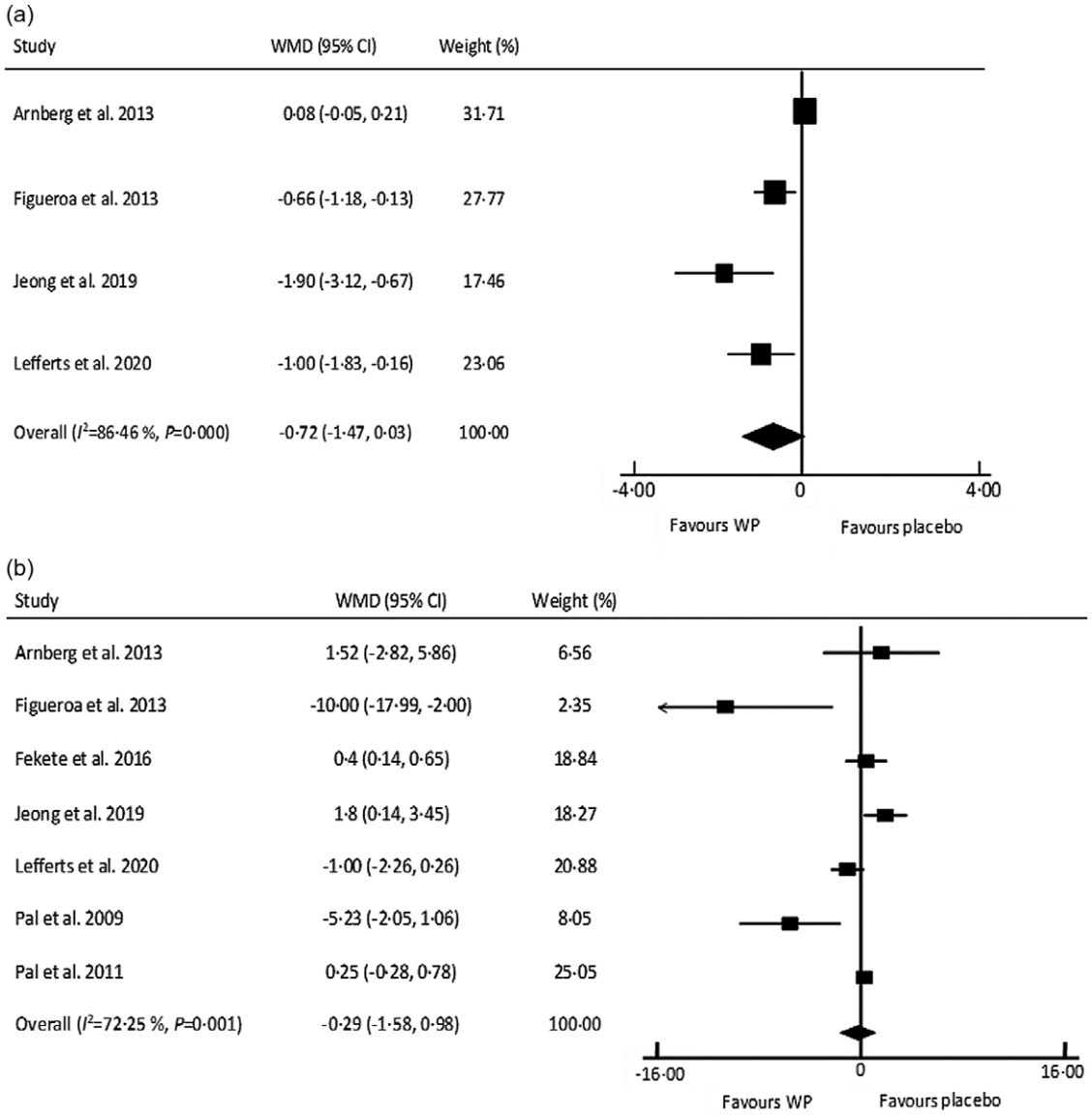



**Fig. 3.** Forest plot of the effect of whey protein on arterial stiffness measures: (a) pulse wave velocity and (b) augmentation index. WMD, weighted mean difference; WP, whey protein.

exclusion of this study from analysis, the pooled WMD reached the significance level (WMD: 0·64 μmol/l, 95 % CI: 0·01, 1·27, $P = 0·04$) compared with that from the main analysis. Based on Begg's ($P = 0·70$) and Egger's tests ($P = 0·54$), there was no evidence of publication bias.

### *Assessment of the quality and risk of bias*

Figure 5 shows the risk of bias in each individual studies. Although, all of sixteen RCT were randomised trial, only six of the sixteen RCT provided adequate information about the method of randomisation and three studies explained the methods of allocation concealment. Blinding of participants and researchers also did not conduct by six RCT or was unclear in six studies. Six RCT did not blind the outcome assessors and seven did not specify if they were blind to the trial groups. Regarding attrition bias, three did not explain the completeness of outcome data such as attrition and exclusions from the analysis. Fifth criterion was the only item that all RCT had low risk of bias. For the last criterion, five studies were at high risk of bias because of no consideration of baseline values for weigh and/or dietary habits or the variations in weight and/or nutritional habits during the chronic intervention in the statistical analyses. Two studies conducted by Fekete *et al.* could be considered as a high-quality trial, because of being all key domains at low risk of bias. Based on judgements of risk of bias for each item, ten RCT were at high risk of bias out of which six studies were at risk of performance and detection bias. Remaining four studies judged to be unclear risk of bias.

## Discussion

To the best of our knowledge, the present systematic review and meta-analysis is the first to assess the effects of WP on vascular function and provides a comprehensive synthesis of results from

available RCT. In most included studies, endothelial function was measured by FMD responses which is the gold standard measure of endothelial function and is capable of detecting small effects of interventions[36]. The key finding of this meta-analysis was the significant beneficial effect of WP on FMD. This finding could be of clinical value, because each 1 % increase in FMD, independently of confounding variables may decrease the risk of cardiovascular events up to 10–13 %[37–39]. In addition, further subgroup analyses revealed a remarkable improvement in FMD only in studies including individuals with impaired health or individuals aged ≥ 45 years old and in studies with acute follow-up. Although due to the limited number of trials available for subgrouping, such analyses should be interpreted conservatively. Our sensitivity analysis on FMD was also in line with subgroup analyses, where the exclusion of one study investigating acute effects of WP on adults with pre-HTN/mild HTN and aged 50 years significantly altered the results[30]. Accordingly, the individual's health status and age as well as the study duration may play important roles in determining the FMD response to WP. A lack of a significant effect of WP in young individuals may be due to a 'ceiling effect' in young adults with no space to ameliorate the function of a healthy vascular endothelium. This finding might indicate that WP was effective in restoring age-related declines in endothelial function in the middle-aged and older adults who are at high risk of developing CVD[40–42].

WP has high content of arginine, which is the precursor for eNO synthesis; therefore, increasing eNO production was speculated to be possible mechanism of WP to explain the observed improvement in the vascular function[21,43]. Nevertheless, we did not observe significant effects of WP on eNO concentrations. Therefore, this suggests that WP may employ eNO-independent mechanisms to augment vasodilation. Although, noteworthy is that eNO predominantly acts in an autocrine and paracrine manner, suggesting that the serum levels of eNO may not thoroughly reflect its bioactivity[29]. On the other hand, some WP-derived peptides such as *α*- and *β*-lactorphins exhibit opioid activities and may employ an analgesic impact on the nervous system[44–46]. The interaction of WP-derived peptides with opioid receptors presented in endothelial cells may stimulate the release of eNO into the underlying vascular tissues to dilate vessels in an autocrine or paracrine way, without affecting plasma levels of eNO[44–47]. An extract isolated from a WP hydrolysate, namely NOP-47 also may improve FMD through eNO-dependent and -independent pathways and needed to be more studied.

Growing evidence also shows that the ACE inhibitory activities as well as the release of other endothelial-derived vasoactive substances such as prostanoids and endothelial hyperpolarising factor are potential candidates for vasodilatory properties of WP. Intestinal digestion of WP has shown to generate bioactive peptides such as isoleucine-proline-alanine tripeptides which are subsequently passed to the circulatory system intact to exert physiological functions[48]. These WP-derived tripeptides show ACE inhibitory activities which not only reduce the production of the vasoconstrictor angiotensin II but also impede degradation of the vasodilatory molecule bradykinin and boost the release of endothelial-derived vasoactive substances and thereby decrease wave reflection magnitude[14,21,49,50]. Particularly, stimulation of prostanoids synthesis following WP consumption, as showed in several studies[51–53], may mediate endothelial-dependent vasodilation generated in response to WP. Nevertheless, the exact mechanisms by which WP may affect FDM responses are yet to be elucidated and needed to be more investigated.

Our meta-analysis demonstrated no significant decreases in AI or PWV. Further subgroup analysis showed that the individuals aged over 45 years would obtain the maximum benefit from WP consumption with respect to PWV reduction, but not AI. In this regard, sensitivity analysis also showed that the elimination of the only study on the adolescents[28] reached PWV to the significance level. Moreover, subgroup analysis based on study duration indicated that the interventions with longer periods are needed to occur significant changes in the PWV, as the gold standard index of arterial stiffness. Also, four studies used DVP-SI method to measure arterial stiffness, of which the number of studies providing adequate data on DVP-SI was less than that of our meta-analysis criteria. Therefore, we qualitatively reviewed the available RCT in which none of four studies detected significant changes in DVP-SI, after WP consumption[17,25,30,34]. Also, of two available studies investigating the effect of WP on reflection index, as a measure of vascular tone, only one reported significant decrease in comparison with controls[34]. It is worth mentioning that none of included studies considered average intake of WP supplied by foods, suggesting that the wide ranges of consumption of WP sources such as dairy products by participants may dilute the 'true' response of WP, thus lowering the definite effectiveness within meta-analyses.

This systematic review has also demonstrated that available RCT have mainly focused on studying arterial stiffness measures, FMD and eNO level; however, the evidence on the impact of WP on ET-1 and vascular inflammatory biomarkers such as ICAM-1 and VCAM-1 is scarce at present and requires more study. In this regard, three RCT assessed the effect of WP on ET-1, none of which detected significant changes in the plasma level of ET-1[27,29,35]. We found four human studies investigating the effect of WP consumption on serum levels of ICAM-1 and VCAM-1; however, the eligible studies were less than that to be included in the meta-analysis. Based on qualitative review of these studies, only one of four RCT reported significant decrease in ICAM-1 after WP consumption compared with controls[17]. In line with our results, other trials investigating anti-inflammatory effects of dairy products also did not report significant changes in adhesion molecules[54,55].

Overall, it seems that the exposure to WP results in the shift of properties of the endothelium towards a vasodilatory phenotype, while to occur remarkable variations in arterial stiffness indices, long-term interventions addressing older adults are required. Further studies with a focus on the possible mechanisms that orchestrate the communication between the WP and the vascular system are warranted.

### *Knowledge gaps and future directions*

In spite of methodological strength and comprehensive overview of the available research, caution should be taken in

**Table 4.** Subgroup analyses for the effects of WP supplementation on vascular function in the participants of included RCT

| Variables | Subgroups | | Number of effect sizes | Pooled WMD | 95 % CI | $I^2$ | P (between group) |
|---|---|---|---|---|---|---|---|
| eNO | Health status | Healthy individuals | 1 | 0·71 | 0·57, 0·84 | 0·00 | 0·00 |
| | | Individuals with impaired health | 5 | 0·22 | –1·33, 1·78 | 64·20 | |
| | Participants' age | < 45 years | 3 | 0·59 | -0·20, 1·40 | 54·09 | 0·17 |
| | | ≥ 45 years | 3 | –0·27 | –3·49, 2·94 | 72·38 | |
| | Intervention duration | Acute | 2 | 0·90 | –0·22, 2·04 | 20·96 | 0·22 |
| | | Chronic | 4 | –0·13 | –1·78, 1·51 | 66·82 | |
| FMD | Health status or participants' age | Healthy individuals or aged < 45 years | 2 | 0·74 | –0·76, 2·25 | 99·93 | 0·00 |
| | | Individuals with impaired health or aged ≥ 45 years | 4 | 1·41 | 1·05, 1·78 | 78·71 | |
| | Intervention duration | Acute | 3 | 1·60 | 1·48, 1·73 | 71·23 | 0·00 |
| | | Chronic | 3 | 0·53 | –0·45, 1·51 | 95·55 | |
| AI | Health status | Healthy individuals | 4 | –0·77 | –2·43, 0·88 | 73·02 | 0·41 |
| | | Individuals with impaired health | 3 | –0·07 | –2·98, 2·83 | 76·9 | |
| | Participants' age | < 45 years | 2 | –3·73 | –14·97, 7·51 | 83·75 | 0·78 |
| | | ≥ 45 years | 5 | –0·12 | –1·32, 1·07 | 73·45 | |
| | Intervention duration | Acute | 1 | 0·25 | –0·28, 0·78 | 0·00 | 0·48 |
| | | Chronic | 6 | –0·77 | –2·76, 1·20 | 76·11 | |
| PWV | Health status | Healthy individuals | 2 | –0·37 | –1·42, 0·66 | 84·00 | 0·07 |
| | | Individuals with impaired health | 2 | –1·15 | –2·34, 0·03 | 69·95 | |
| | Participants' age | < 45 years | 2 | –0·24 | –3·12, 0·67 | 86·04 | 0·01 |
| | | ≥ 45 years | 2 | –1·33 | –2·18, –0·48 | 29·35 | |
| | Intervention duration | < 12 years | 1 | –0·66 | –1·18, –0·13 | 0·00 | 0·005 |
| | | ≥ 12 years | 3 | –0·82 | –1·98, 0·34 | 87·43 | |

WP, whey protein; RCT, randomised controlled trial; WMD, weighted mean difference..

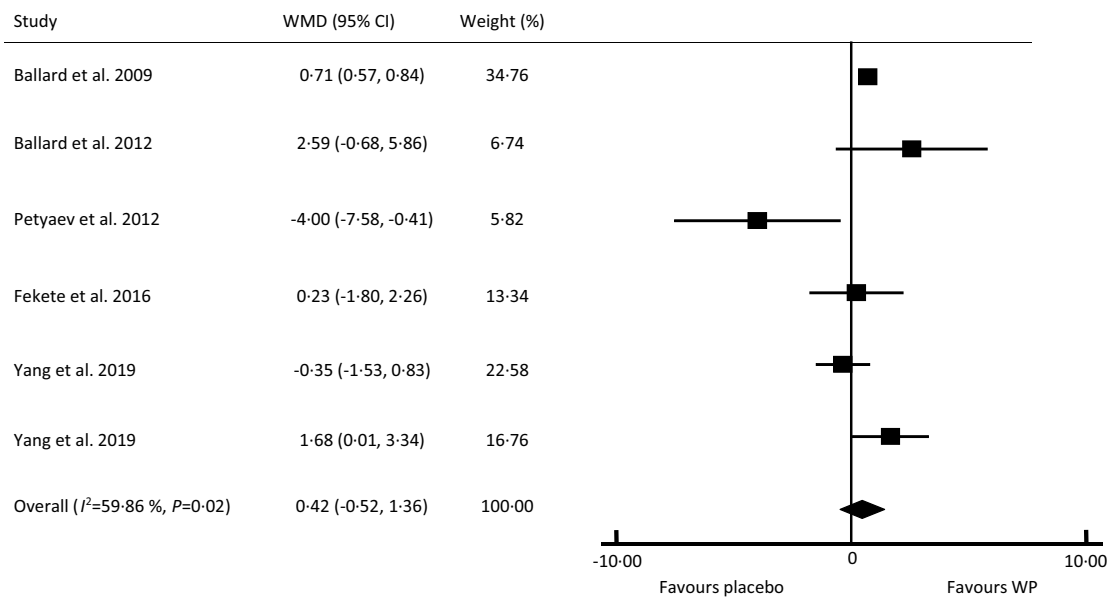
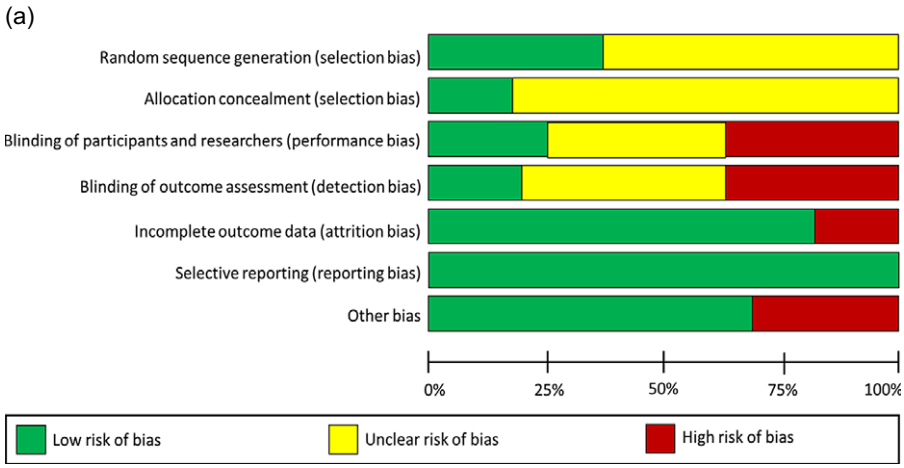



**Fig. 4.** Forest plot of the effect of whey protein on plasma level of endothelial nitric oxide. WMD, weighted mean difference; WP, whey protein.

interpreting the observed results, because high heterogeneity between studies was observed in terms of study design, study quality, assessment methodology, characteristics of subjects, the type and dose of WP, and the duration of the intervention. Given that most studies included participants without known CVD, the impaired parameters of vascular function that were seen in the patients with CVD were absent and the changes in some vascular parameters to WP were not significant. Therefore, future studies evaluating the effect of WP on vascular responses in subjects with vascular dysfunction are needed to allow more definitive conclusions to be obtained. Also, it seems that the short-term interventions are unlikely to induce




(b)

| Author/year | Random sequence generation (selection bias) | Allocation concealment (selection bias) | Blinding of participants and researchers (performance bias) | Blinding of outcome assessment (detection bias) | Incomplete outcome data (attrition bias) | Selective reporting (reporting bias) | Other bias |
|---|---|---|---|---|---|---|---|
| Ballard 2009 | ? | ? | ? | ? | − | + | − |
| Pal 2009 | ? | ? | − | − | + | + | + |
| Pal 2011 | ? | ? | ? | ? | + | + | + |
| Ballard 2012 | ? | ? | ? | ? | + | + | − |
| Petyaev 2012 | ? | ? | − | − | + | + | − |
| Arnberg 2013 | ? | ? | + | ? | + | + | + |
| Figueroa 2013 | ? | ? | ? | ? | − | + | + |
| Mariotti 2015 | ? | ? | − | − | − | + | − |
| Rontoyanni 2015 | + | ? | − | − | + | + | + |
| Fekete 2016 | + | + | + | + | + | + | + |
| Fekete 2018 | + | + | + | + | + | + | + |
| McDonald 2018 | ? | ? | ? | ? | + | + | + |
| Jeong 2019 | + | + | − | − | + | + | + |
| Oliveira 2019 | ? | ? | ? | + | + | + | − |
| Yang 2019 | + | ? | − | − | + | + | + |
| Lefferts 2020 | + | ? | + | ? | + | + | + |

**Fig. 5.** Risk of bias graph (a) and summary (b) of the included studies. + shows a low risk of bias, – shows a high risk, and ? shows an unclear risk. WMD, weighted mean difference; WP, whey protein.

remarkable changes in arterial stiffness in participants without vascular dysfunction. In addition, the effect size observed in vascular function in the acute studies may be affected by test meal which slow down gastric emptying and delay the effect of bioactive components. Therefore, acute studies should assay vascular response to WP only at fasting state. Meanwhile, future research is recommended to consider the intake of dairy products, as the dietary sources that contributes most to the WP intake. Future research is highly recommended to address potential components of WP (individual WP, peptide fractions or amino acids) that mediate the improvements in FMD observed in the present meta-analysis.

## Conclusion

The present systematic review and meta-analysis suggests a beneficial role for WP in the endothelial function, but not for arterial stiffness, and serum level of eNO. Moreover, qualitative review of studies investigating the effect of WP on adhesion molecules and ET-1 did not show definitive impact in these factors. Our study suggested that individuals with impaired health or aged over than 45 years old may be more likely to benefit from WP than other populations. Moreover, in contrary to FMD which is likely improved with acute consumption of WP, the effects on PWV may require longer interventions to take place. Taking these findings into account, the period of exposure to the dietary agent and participants' characteristics may play determinant roles in the effectiveness of WP on vascular outcomes. More research that is adequately powered, addresses current knowledge gaps and evaluates dose and time responses across different populations should be conducted to further enhance our understanding of the efficacy of WP on vascular function and to elucidate the mechanisms underlying the observed effects.

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F. H-S. contributed to the study conception, design and data collection, and interpretation and drafting the manuscript. E. S. Z. contributed to the data collection and drafting the manuscript. A. T-E participated in revising the paper critically and approving the version of the manuscript being submitted.

The authors declare no conflicts of interest.

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