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Review

Effect of vitamin C on endothelial function in health and disease: A systematic review and meta-analysis of randomised controlled trials☆

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ABSTRACT

Background: Observational studies indicate that higher vitamin C intake is associated with reduced risk for cardiovascular diseases. However, randomised controlled trials (RCT) examining the effect of vitamin C on endothelial function (EF) have reported inconsistent results. The aims of this systematic review and meta-analysis were to determine the effect of vitamin C supplementation on EF and to investigate whether the effect was influenced by health status, study duration, dose and route of vitamin C administration.

Methods: We searched the Medline, Embase, Cochrane Library, and Scopus databases from inception to May 2013 for studies that met the following criteria: 1) RCT with adult participants, 2) vitamin C administered alone, 3) studies that quantified EF using commonly applied methods including ultrasound, plethysmography and pulse wave analysis.

Results: Pooling the data from 44 clinical trials showed a significant positive effect of vitamin C on EF (SMD: 0.50, 95% CI: 0.34, 0.66, $P < 0.001$). Stratification of the analysis by health outcome revealed improved EF in atherosclerotic (SMD: 0.84, 95% CI: 0.41, 1.26, $P < 0.001$), diabetic (SMD: 0.52, 95% CI: 0.21, 0.82, $P < 0.001$) and heart failure patients (SMD: 0.48, 95% CI: 0.08, 0.88, $P < 0.02$) after vitamin C supplementation. The effect size appeared to be unaffected by study design, duration, baseline plasma vitamin C concentration or route of administration of vitamin C. The meta-regression showed a significant positive association between vitamin C dose and improvement in EF (β: 0.00011, 95% CI: 0.00001, 0.00021, $P = 0.03$).

Conclusions: Vitamin C supplementation improved EF. The effect of vitamin C supplementation appeared to be dependent on health status, with stronger effects in those at higher cardiovascular disease risk.

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1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of mortality world-wide [1]. Endothelial dysfunction appears in the early stages of the pathogenesis of vascular disorders and it is closely related to the progression of severe clinical complications (i.e. stroke, pulmonary embolism and myocardial infarction) [2]. Endothelial dysfunction is also commonly observed in metabolic conditions associated with increased atherosclerotic risk such as diabetes mellitus, metabolic syndrome and hypercholesterolaemia [3–7].

The imbalance created by over-production of pro-inflammatory free radicals and reduced bio-availability of nitric oxide (NO) alters endothelial function (EF) and activates mechanisms (i.e. vasoconstriction, monocyte activation, smooth muscle cell proliferation and hyper- coagulation) which are causally linked to the formation of atherosclerotic plaques [8].

Endothelial function can be assessed in humans by measuring vascular responses to endothelial-specific physiological and pharmacological stimuli (post-occlusion hyperaemia or acetylcholine) or by determining circulating concentrations of endothelial-derived molecules. The most commonly applied methods include ultrasound, plethysmography and applanation tonometry, which provide information on EF through the assessment of flow mediated dilatation, forearm blood flow and pulse wave velocity, respectively [9].

Vitamin C (ascorbic acid) is a cofactor for several enzyme-catalysed reactions including the hydroxylation of proline and lysine, essential for the synthesis of collagen, radical scavenger activity and NO-sparing function [10]. Observational studies indicate that high vitamin C intake is associated with reduced CVD risk [11,12]. However, randomised controlled trials examining the effects of vitamin C supplementation on EF have reported apparently contradictory results; some studies reported improvement in EF [3,13] whilst others showed no effect of vitamin C supplementation [14,15].

To help resolve this controversy, the present study aimed to conduct a systematic review and meta-analysis of randomised clinical trials investigating the effect of supplemental vitamin C on EF. The secondary aim of the study was to determine whether the effects were modified by health status, duration of therapy, and dose and route of vitamin C administration.

2. Methods

The present systematic review was conducted according to the Cochrane guidelines [16] and it is reported according to PRISMA guidelines [17].

2.1. Literature search

Four databases (PubMed, Embase, Scopus, and Cochrane Library) were used to search for articles from inception to May 2013. In addition, a manual search of reference list of relevant reviews and articles included in the systematic review was performed. The search was conducted based on pre-defined search terms and using specific building blocks (Boolean terms, truncation) in searching each database: [ascorb* OR "vitamin C"] And [Endotheli*, Endotheli* dysfunction, vasoacti*, vasodilat*, microcirculat*, FMD, hyperaemia, plethysmography, flow mediated, endothelium-dependent, vasomotor, blood flow, brachial, vasodilation, circulation, micro-circulation, vascular resistance, wave, blood supply, arterial stiffness, digital volume pulse, iontophoresis, pulse amplitude tonometry, intracoronary angiography]. Full details of our search criteria are reported in the online Supplementary materials (Supplementary Table 1) and the characteristics of the excluded studies are presented in Supplementary Table 2.

2.2. Study selection

The following criteria were applied to identify the articles to be included in this systematic review and meta-analysis: 1) randomised controlled clinical trials (no exclusion criteria were applied in relation to study design or blinding); 2) studies involving adults aged 18 years or more and no exclusion criteria were applied for health status, smoking history or body size; 3) vitamin C administration alone i.e. not combined with other drugs or nutritional interventions; 4) studies were not excluded because of the dose, duration or route of administration of vitamin C; 5) studies reporting changes in EF measured using ultrasound, venous-occlusion plethysmography, pulse wave velocity, iontophoresis, pulse amplitude tonometry; 6) no language or time restrictions were applied in searching the databases. Two investigators (AA, MS) independently screened the titles and abstracts of the articles to evaluate eligibility for inclusion. If consensus was reached, articles were either excluded or moved to the next stage (full-text). If consensus was not reached the article was moved to the full-text stage. The full-texts of the selected articles were critically appraised to determine eligibility for inclusion in the systematic review. Disagreements were resolved by discussion between the reviewers until consensus was reached.

2.3. Data extraction and quality assessment

The following information was extracted from the eligible articles: 1) authors, journal details and year of publication; 2) participants (total umber, male/female ratio, age, health status and use of prescribed drugs); 3) study characteristics (design, inclusion/exclusion criteria, description of measurement protocols); 4) vitamin C intervention (dose, duration, route of administration, and type of control); 5) EF measurement (instrument, position, duration of cuffing) and 6) circulating concentrations of vitamin C before and after intervention.

In addition, we adopted the modified Jadad score to assess the risk of bias of the included studies; possible scores ranged from 0 to 5 and a score of ≤ 3 indicates high risk while a score of >3 indicates low risk of bias [18].

2.4. Statistical analysis

Several methods were used to assess EF in humans including flow mediated dilation (FMD), forearm blood flow (FBF), and pulse wave analysis (PWA) with the results obtained from these methods reported on different scales. Therefore, to allow comparison of the effect sizes between studies, standardised mean differences (SMDs) were used as a summary statistic. SMD calculated by estimating the difference in the mean outcome values of the intervention and control groups and then dividing that difference by the standard deviation of the outcome values [19]. Meta-analysis was performed using the RevMan software (version 5.2, the Cochrane's collaboration, Oxford, UK) whilst publication bias and meta-regression were conducted by using Comprehensive meta-analysis software (version 2, Biostat, Englewood, New Jersey, USA). Calculation of effect sizes with 95% confidence intervals was accomplished by using inverse variance weighting. Random effect models were used to take into account between-study heterogeneity for study design and methods used to assess EF. Forest plots were generated for graphical presentation of the EF outcomes. For this purpose, the mean and SD of the EF measure before and after the intervention period (for both vitamin C intervention and control) were extracted and used in the analysis. For studies that reported changes in EF at two or more time-points (e.g. acute and chronic effects of vitamin C supplementation), the last EF measurement (chronic effect) was used in the meta-analysis. Data not provided in the main text or tables were extracted from the figures. For crossover trials, we used the mean and SD separately for the intervention and control conditions. This is regarded as a conservative approach that will reduce the power of these studies to show the true effect of the intervention [20].

Subgroup analyses were performed to identify possible sources of heterogeneity. Further sensitivity analyses were undertaken to investigate and identify the causes of heterogeneity and the variables which influenced the effect of vitamin C on EF. These factors included: health status (healthy vs chronic diseases) study design (randomised, double-blind, crossover), duration of intervention (acute vs. chronic), route of administration (oral vs. parenteral) and quality of the included studies (high vs. low quality according to Jadad Score). Meta-regression analysis was used to determine whether baseline vitamin C concentrations were associated with significant changes in EF. Furthermore, meta-regression analysis was used to estimate the dose–response relationship between vitamin C supplementation and EF. We investigated the lowest dose of vitamin C associated with significant changes in EF. We categorised the studies into 4 groups of vitamin C dose viz. <500, 500–1000, 1000–2000 and 2000+ mg/d and performed a meta-analysis for each sub-group.

Publication bias was evaluated by visual inspection of the funnel plot and by Begg's rank correlation test [21]. Furthermore, trials with the largest effect size were omitted and a meta-analysis conducted to evaluate the influence of those trials on the overall

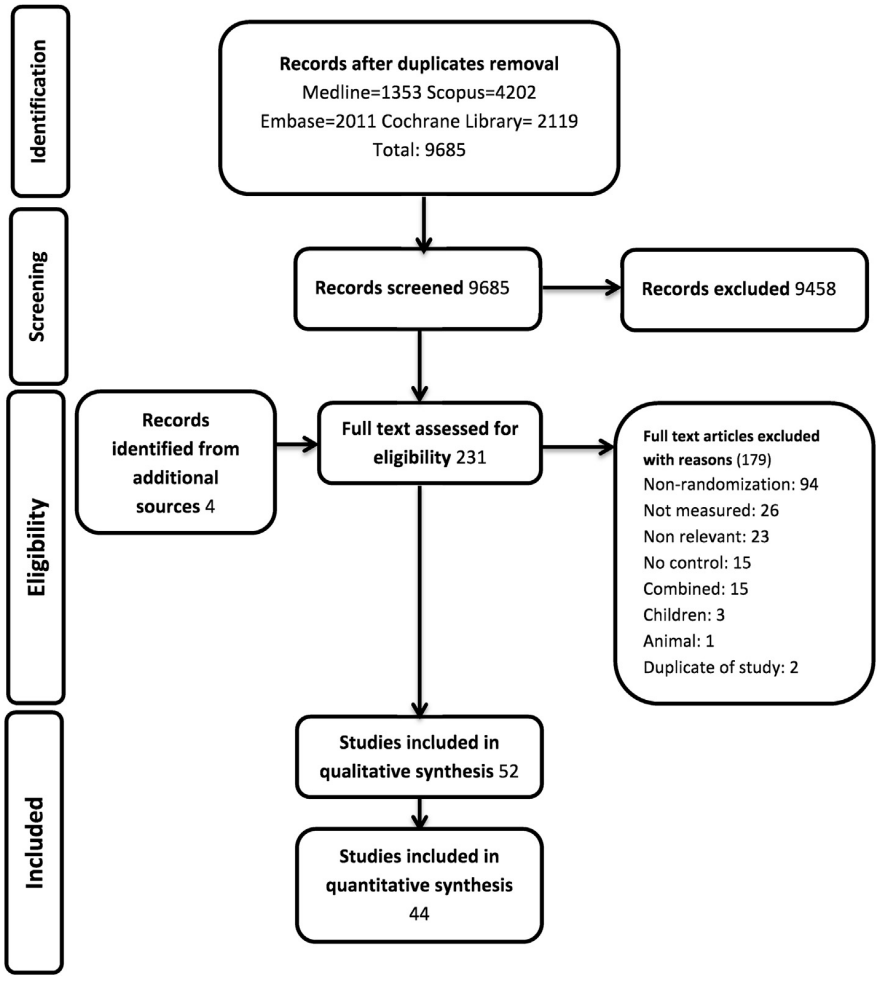

Fig. 1. Flow diagram of the trials selection process.

Table 1
Characteristics of the studies included in the systematic review.

Author	Year	Health status	Sample size (male %)	Age (mean ± SD)	Study design	Outcome	Duration	Vitamin C dose	Route	Control	Baseline measurement of EF[d]	Significant effect on EF[c]
Anderson et al. [52]	2006	Type 2 Diabetes	20 (70)	38–68 (53.3)	CO, DB	FMD	3 days	1 g	Oral	NR	3.8 ± 0.9	Yes
Antoniades et al. [49]	2003	Smokers	21 (57.1)	37.7 ± 2.2; 34.1 ± 2.4	Parallel, UB	FBF	4 weeks	1 g	Oral	Non antiox. Vit.	5.99 ± 0.6	No
Antoniades et al. [36]	2004	Type 2 Diabetes + CAD[1]	37 (81.1)	64.2 ± 11.7	Parallel, UB	FBF	4 weeks	2 g	Oral	Non antiox. Vit.	54.35 ± 30.2	Yes
		Type 2 Diabetes	17 (76.5)	61.36 ± 10.2		FBF					54.27 ± 30.4	No
		Healthy	21 (71.4)	59 ± 9.5		FBF					114.7 ± 51.3	No
Arcaro et al. [46]	2002	Healthy	7 (42.8)	22 ± 1	CO, UB	FMD	1 day	2 g	I.V	None	2.82 ± 0.67	Yes
Basili et al. [28][b]	2010	Angina Pectoris	56 (83.9)	50–84 (67)	Parallel, DB	cTFC	1 day	1 g	I.V	Saline	36.1 ± 8.90	Yes
Bayerle-Eder et al. [42]	2004	Healthy	10 (100)	20–29 (25)	CO, DB	FBF	1 day	24 mg/min	I.V	NaCl (0.9%)	316.7 ± 33.3	Yes
Cangemi et al. [3]	2007	Metabolic syndrome	18 (100)	(33–73) 51.9 ± 10.8	CO, DB	FMD	1 day	1 g	I.V	NaCl (0.9%)	5.2 ± 1.6	Yes
Ceriello et al. [38]	2008	Healthy	10 (60)	50.3 ± 2.5	CO, UB	FMD	1 day	3 mg/min	I.V	None	11.7 ± 0.7	Yes
		Type 2 Diabetes	10 (50)	50.2 ± 4.5–51 ± 5.6	CO, UB	FMD	1 day		I.V	None	5.9 ± 0.6	Yes
Ceriello et al. [53]	2013	Type 1 Diabetes	15 (NR)	23.2 ± 3.1	CO, UB	FMD	1 day	30 mg/min	I.V	None	6.8 ± 0.8	Yes
Chen et al. [54]	2006	Type 2 Diabetes	32 (40.6)	47±3–49 ± 2	Parallel, DB	FBF	4 weeks	800 mg	Oral	Citric acid	206 ± 31	No
Darko et al. [14]	2002	Type 2 Diabetes	35 (65.7)	56.6 ± 1.2/55.5 ± 1.8	Parallel, DB	FBF	3 weeks	500 mg	Oral	NR	2.1 ± 0.4	No
De Marchi et al. [39]	2012	Healthy	34 (50)	29–36	Parallel, UB	FBF	10 days	1 g	Oral	Na Bicarbonate	285.7 ± 23	Yes
Duffy et al. [60]	2001	Hypertension	39 (48.7)	46±4–48 ± 12	Parallel, DB	FMD	1 day	2 g	Oral	NR	8.9 ± 6.1	No
					Parallel, DB		4 weeks		Oral	NR		No
DuPont et al. [23][b]	2011	Chronic Kidney Diseases	8 (62.5)	48±5–52 ± 6	Parallel, UB	CVC	1 day	20 mM	Iontoph	Ringers Solution	13 ± 3	Yes
Ellis et al. [63]	2001	Heart Failure	10 (80)	29–74 (52)	CO, DB	FMD	1 day	2 g	I.V	Saline	1.9 ± 0.6	Yes
Gokce et al. [61]	1999	CAD	46 (91.3)	54 ± 9/56 ± 12	Parallel, DB	FMD	1 day	2 g	Oral	NR	6.6 ± 3.5	Yes
							4 weeks		Oral	NR		Yes
Gori et al. [30]	2007	Healthy	22 (100)	22–46	Parallel, DB	FMD	1 day	2 g	I.V	Saline	2.6 ± 0.1	Yes
Guazzi et al. [55]	2006	Atrial Fibrillation (AF)	12 (75)	64 ± 2	CO, DB		1 week	2 g	Oral	NR		Yes
		AF + Hypertension	12 (75)	61 ± 3								Yes
		AF + Type 2 Diabetes	12 (75)	63 ± 4								No
Hamabe et al. [62]	2001	Variant Angina	17 (64.7)	58 ± 6	CO, SB	FMD	1 day	1 g	I.V	Saline	3.92 ± 0.7	Yes
Holowatz et al. [26][b]	2006	Healthy (Young)	11 (63.6)	22 ± 1	Parallel, UB	CVC	1 day	10 mM	I.V	Ringers Solution	8.8 ± 0.8	No
		Healthy (Old)	10 (50)	68 ± 1		CVC					11.8 ± 1.9	Yes
Holowatz et al. [24][b]	2007	Hypertension	9 (66.6)	57 ± 4	Parallel, UB	CVC	1 day	10 mM	I.V	Ringers Solution	14.2 ± 1.6	No
		Normotensive	9 (66.6)	57 ± 3		CVC					15.3 ± 1.4	Yes
Holowatz et al. [25][b]	2011	Hypercholesterolaemia	9 (66.6)	53 ± 3	Parallel, UB	CVC	1 day	10 mM	I.V	Ringers Solution	87.4 ± 1.2	No
		Hypercholesterolaemia	9 (55.5)	49 ± 2		CVC					88.2 ± 1.1	Yes
Hornig et al. [64]	1998	Heart Failure	10 (NR)	58±5–61 ± 3	Parallel, UB	FMD	1 day	25 mg/min	I.A	Saline	2.79 ± 0.1	No
						FMD	4 weeks		Oral	NR	2.85 ± 0.1	Yes
Johnson et al. [31]	2012	Healthy	12 (100)	26.3 ± 0.9	CO, DB	FMD	1 day	500 mg	Oral	Sucrose Capsule	4.9 ± 0.63	Yes
Kanani et al. [47]	1999	Healthy	10 (80)	31 ± 3	CO, DB	FMD	1 day	2 g	Oral	NR	3.9 ± 0.2	Yes
Kelly et al. [32]	2008	Healthy	26 (69.2)	21–26	CO, DB	FBF	1 day	2 g	Oral	Flavoured Water	2.4 ± 0.8	No
Lisi et al. [33]	2013	Healthy	10 (70)	25–28	CO, UB	FMD	1 day	2 g	I.V	0.9% Saline	0.241 ± 0.03	Yes
LU et al. [56][b]	2005	Type 2 Diabetes	17 (70.6)	35–76[a] (54)	CO, DB	TtP	2 weeks	1 g	Oral	Semper Food	12 ± 3.3	No
Magen et al. [15]	2004	Hypertension	33 (51.5)	52.6 ± 12.3–54.4 ± 15.2	Parallel, UB	FMD	8 weeks	500 mg	Oral	NR	8.7 ± 6.4	No
Mullan et al. [57]	2002	Type 2 Diabetes	30	45–70	Parallel, DB	PWA	4 weeks	500 mg	Oral	NR	26.8 ± 5.5	Yes
Mullan et al. [40]	2004	Healthy	12 (100)	20–34 (25.2)	CO, DB	PWA	1 day	2 g	I.V	NR	9.4 ± 11.3	Yes
Mullan et al. [41]	2005	Healthy	9 (100)	21–34 (26)	CO, DB	FBF	1 day	2 g	I.V	Saline	2.2 ± 1.1	Yes
Nightingale et al. [65]	2003	Healthy	22 (86.4)	19–36	CO, DB	PWV	1 day	2 g	I.V	0.9% Saline	7.45 ± 1.02	No
		Heart Failure	50 (68)	54.8 ± 11.1–57.4 ± 9.5	Parallel, DB	PWV	1 day		I.V	NR	7.78 ± 1.86	No
		Heart Failure	38 (73.6)	57.8 ± 9.9–60.9 ± 6.3	Parallel, DB	PWV	4 weeks		Oral	NR	7.44 ± 1.12	No
Nightingale et al. [66]	2007	Heart Failure	37 (83.8)	49–78 (63.5)	Parallel, DB	PWV	4 weeks	4 g	Oral	NR	9.77 ± 0.58	Yes
Pellegrini et al. [50]	2004	Smokers	8 (100)	43 ± 3	CO, DB	FBF	5 weeks	1 g	Oral	NR	4.1 ± 0.5	No
Pleiner et al. [43]	2002	Healthy	8 (100)	26 ± 3	CO, DB	FBF	1 day	24 mg/min	I.A	Saline	3.9 ± 0.1	Yes
Pleiner a et al. [44]	2002	Healthy	10 (100)	24 ± 3	CO, DB	FBF	1 day	24 mg/min	I.A	NaCl (0.9%)	4.5 ± 0.1	Yes
Pleiner et al. [45]	2003	Healthy	16 (100)	21–38	CO, DB	FBF	1 day	24 mg/min	I.A	Saline	3.9 ± 0.1	Yes
Pleiner et al. [48]	2008	Healthy	20 (100)	20–37	Parallel, DB	FBF	1 day	60 mg/min	I.A	NR	6 ± 0.6	Yes
		Peripheral arterial disease	8 (100)	62 ± 2	CO, DB	FBF	1 day		I.A	NR	3.5 ± 0.3	Yes
Protogerou et al. [4]	2002	Hypercholesterolaemia	46 (86.9)	26–78 (51.3 ± 8)	Parallel, UB	FBF	1 day	2 g	Oral	Effervescent Tab.	5.2 ± 0.4	No
Raitakari et al. [5]	2000	Smokers	20 (40)	18–50	CO, DB	FMD	1 day	2 g	Oral	NR	2.8 ± 2	Yes

Schindler et al. [27],[b]	2003	Hypertension	20 (60)	59 ± 3	Parallel, UB	CVR	8 weeks 1 day 2 years	3 g	Oral I.V Oral	NR NaCl (0.9%) NR	143 ± 48		No No Yes
Schneider et al. [37]	2005	Hypertension Healthy	15 (73.3) 15 (66.6)	40 ± 11 39 ± 10	CO, UB CO, UB	FBF FBF	1 day	18 mg/min	I.A	No treatment No treatment	3.8 ± 1.4 3.8 ± 1		Yes Yes
Silvestro et al. [6]	2002	Intermittent claudication	16 (87.5)	62 ± 3	Parallel, UB	FMD	1 day	50 mg/min	I.V	Saline	7.5 ± 1		Yes
Singh et al. [34]	2002	Healthy	36 (50)	63–72 (67.5)	Parallel, DB		6 weeks	1 g	Oral	NR	4.64 ± 0.35		No
Tousoulis et al. [51]	2003	Smokers	21 (66.6)	34.2 ± 2.2–38.6 ± 2.2	Parallel, UB	FBF	4 weeks	2 g	Oral	No treatment	5.97 ± 0.74		No
Tousoulis a et al. [58]	2003	Type 2 Diabetes	39 (87.2)	63.3 ± 2.7–67.4 ± 2.1	Parallel, UB	FBF	4 weeks	2 g	Oral	NR	51.8 ± 4.7		Yes
Tousoulis et al. [59]	2007	Type 2 Diabetes	26 (53.8)	59.1 ± 2.4–60.9 ± 3.1	Parallel, SB	FBF	4 weeks	2 g	Oral	No treatment	51.3 ± 6.02		No
Ward et al. [29],[b]	2005	Hypertension	37 (70.3)	59.5 ± 5.9–63.6 ± 8.2	Parallel, DB	FMD	6 weeks	250 mg	Oral	NR			No
Watanabe et al. [13]	1998	Healthy	24 (75)	32±6–36 ± 8	Parallel, DB	FBF	3 days	2 g	Oral	NR	2.62 ± 0.55		Yes
		CAD	24 (79.2)	64±8–65 ± 10		FBF	3 days		Oral	NR	2.54 ± 0.48		Yes
Wilkinson et al. [35]	1999	Healthy	8 (100)	20–42	Parallel, DB	PWA	1 day	2 g	Oral	NR	1.3 ± 3.5		Yes
Williams et al. [67]	2001	Renal allograft Recipients	13 (69.2)	30–64 (48 ± 12)	CO, DB	FMD	1 day	2 g	Oral	NR	4.5 ± 0.9		Yes

CAD, coronary artery diseases; CO, crossover; DB, double-blind; SB, single-blind; EF: endothelial function; UB, non-blinded; FMD, flow mediated dilation; FBF, forearm blood flow; cTFC, corrected TIMI frame count; CVC, cutaneous vascular conductance; TtP, time to peak; PWA, pulse wave analysis; CVR, coronary vascular resistance; I.V, intravenous; I.A, intra-arterial; Iontoph., Iontophoresis; NR, not reported.

[a] Range.

[b] Excluded from meta-analysis.

[c] Yes = $p < 0.05$, No = $p > 0.05$.

[d] Baseline measurements of EF are reported in different units according to the specific methodology (FMD = absolute (mm) or relative (%) changes in brachial artery diameter; FBF = absolute or percentage change of arm volume × ml/100 ml tissue/min; cTFC = frames/s; CVC = percentage of maximal CVC (%CVC_{max}); TtP = seconds; PWA = percent (%); PWV = m/s; CVR = mmHg/ml/g per min).

effect of vitamin C. Heterogeneity between studies was evaluated using Cochrane Q statistics; $P > 0.1$ indicates significant heterogeneity. The I^2 test was also used to evaluate consistency between studies where a value <25% indicates low risk of heterogeneity, 25–75% indicates moderate risk of heterogeneity, and >75% indicates high risk of heterogeneity [22].

3. Results

3.1. Search results

The process of screening and selection of the studies is summarised in Fig. 1. The primary search of the four databases produced 9685 articles after the removal of duplicates. Additionally, four studies were found by manually searching references of the relevant reviews and studies. After title and abstract screening, 231 full-text papers were retrieved for further evaluation and, from these, 52 publications were identified which met the inclusion criteria for entry in the systematic review. Studies were excluded either because they were not randomised or they provided insufficient information to determine their eligibility. Forty-four studies included in the systematic review were eligible for the meta-analysis. Seven trials were excluded from quantitative data synthesis because the methods used for EF measurement were not commonly applied. These methods include: cutaneous vascular conductance [23–26], coronary vascular resistance [27] and myocardial blood flow [28]. One study was excluded from analysis because we could not obtain informative data [29].

3.2. Studies characteristics

The total number of participants from 52 studies included in this systematic review was 1324 with a median of 16 (range 7–56) participants per study. Nearly three-quarters of participants were males (964 males, 360 females) and the overall median age was 51.1 (range 22–68) years. Fourteen of these trials had 2-3 independent subgroups and 5 studies measured the effect of vitamin C at two different time points (Table 1).

The designs used in the studies included: 25 crossovers and 27 parallel RCTs and, according to study blinding, there were 31 double-blind, 2 single-blind and 19 non-blinded studies. The duration of the interventions ranged from 1 day to 2 years but most of the trials (32) were “acute” (up to 2 weeks in duration) while the other 20 were “chronic” trials (more than 2 weeks).

Twelve studies investigated the effect of vitamin C in healthy volunteers [23–26,30–37]. In addition, 12 studies recruited healthy participants to test the protective effects of vitamin C supplementation on endothelial dysfunction induced experimentally by the administration of glucose [38–41], lipids [42,43], endotoxins [44,45], organic nitrate [13], insulin [46], methionine [47] or by experimental ischaemia-reperfusion [48]. Four studies investigated the effects of vitamin C in smokers [5,49–51].

The remaining studies were conducted in subjects with cardiovascular and metabolic conditions including diabetes [14,36,38,52–59], hypertension [15,24,27,29,37,55,60], coronary heart disease [13,28,55,61,62], peripheral vascular disease [6,48], heart failure [63–66], hypercholesterolaemia [4,25], chronic kidney disease [23,67] metabolic syndrome [3].

Different methods were used to assess changes in EF in the trials. The most commonly used methods were flow-mediated dilation (FMD), forearm blood flow (FBF) and pulse wave analysis (PWA). Some trials used cutaneous vascular conductance [23–26], positron emission tomography [27] coronary angiography [28] or capillaroscopy [56] for EF measurement (Table 1 and Supplementary Table 3).

3.3. Qualitative analysis

Two third of the studies included in the present systematic review reported a significant improvement in EF in response to vitamin C administration whereas the other third reported no effect of vitamin C. The quality of the included studies ranged from 1 to 5 (Jadad score) and thirty studies had a low risk of bias (Jadad score ≥4) (Supplementary Table 2, online material). Seven studies described the method of randomisation [3,28,38,45,49,51,57] and 6 studies stated the methods of allocation concealment [3,28,55–57,63]. The drug history of the participants was reported by all except six studies [28,32,33,35,37,44]. Seven studies reported and described the participant dropout [15,29,32,54,56,60,65]. For the crossover trials, most reported the washout period used (range 1–7 weeks) while 4 studies only did not report this information [23,38,42,44,50].

3.4. Meta-analysis

Forty-four studies (1129 participants) were included in the meta-analysis to examine the pooled effect of vitamin C supplementation on EF measured using FBF (20 studies), FMD (19 studies) and PWA (5 studies). The pooled estimate showed a significant improvement in EF after vitamin C administration (SMD: 0.50, 95% CI: 0.34, 0.66, $P < 0.001$). There was a moderate degree of heterogeneity between studies ($X^2 = 111.93$, $I^2 = 54\%$, $P < 0.001$).

Subgroup analysis was performed to investigate the causes of heterogeneity. This analysis demonstrated a significant difference in the effect size between participant groups ($X^2 = 33.9$, $I^2 = 82.3\%$, $P < 0.001$). There was no detectable effect of vitamin C supplementation on EF in healthy volunteers ($P = 0.72$) and healthy smokers ($P = 0.87$); whereas the healthy volunteers who underwent experimentally-induced endothelial dysfunction showed the largest improvement in EF (SMD: 0.87, 95% CI: 0.62, 1.13, $P < 0.001$) (Table 2, Fig. 2). Moreover, vitamin C supplementation improved EF significantly in diabetic ($P < 0.001$), atherosclerotic ($P < 0.001$), and heart failure ($P < 0.02$) patient groups. In contrast, there was no significant change in EF in response to vitamin C supplementation in hypertensive patients ($P = 0.66$) (Table 2, Fig. 3).

Sensitivity analyses (Table 2) detected no significant differences in EF response to vitamin C administration between groups stratified according to duration or route of vitamin C administration, study design, blinding, or risk of study bias. Omission of 3 studies with the largest effects [3,13,62] yielded a slightly lower effect size

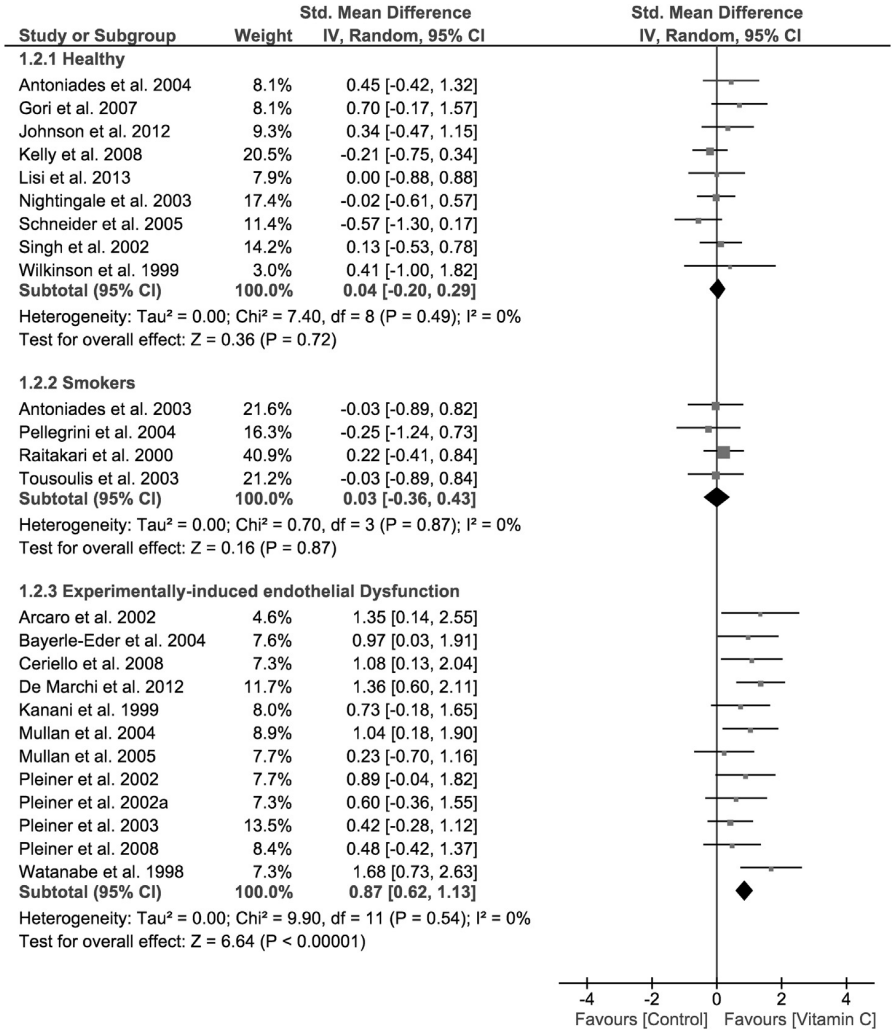

Fig. 2. Forest plot showing the effect of vitamin C supplementation on endothelial function in healthy individuals, smokers and healthy individuals with experimentally-induced endothelial dysfunction.

Table 2
Sensitivity analyses of the standardized mean difference.

Group	No. of trials or subgroups	Effect size	95% CI	P
Health status				0.0001[a]
• Healthy	9	0.04	−0.20–0.29	0.72
• Smokers	4	0.03	−0.36–0.43	0.87
• Experimentally-induced ED	12	0.87	0.62–1.13	<0.001
• Atherosclerosis	7	0.84	0.41–1.26	<0.001
• Heart failure	4	0.48	0.08–0.88	0.02
• Diabetes	10	0.52	0.21–0.82	<0.001
• Hypertension	4	−0.10	−0.53–0.34	0.66
Duration				0.06[a]
• Acute (≤2 weeks)	32	0.57	0.37–0.78	<0.001
• Chronic (>2 weeks)	17	0.29	0.08–0.49	<0.01
Route of administration				0.09[a]
• Oral	27	0.39	0.20–0.59	<0.001
• Parenteral	18	0.68	0.41–0.95	<0.001
Study design				0.67[a]
• Parallel	21	0.46	0.24–0.69	<0.001
• Crossover	24	0.53	0.31–0.76	<0.001
Blinding				0.58[a]
Double-blind	28	0.47	0.28–0.65	<0.001
Single-Blind	2	1.02	−0.02–2.07	0.05
Non-blinded	14	0.50	0.19–0.80	0.001
Vitamin C dose (mg/d)				<0.001[a]
• 90–500 (333.7 ± 187.2)[b]	8	0.22	−0.05–0.49	<0.1
• 501–1000 (940 ± 134.9)	10	0.59	0.35–0.83	<0.001
• 1001–2000 (1941.2 ± 159.7)	31	0.46	0.32 to 0.60	<0.001
• 2001–4000 (3160 ± 763.2)	4	0.58	0.19–0.97	<0.01
Risk of bias (Jadad Score)				0.14[a]
• Low risk (>3)	30	0.59	0.41–0.78	<0.001
• High risk (≤3)	14	0.34	0.05–0.62	<0.001

ED, endothelial dysfunction.
[a] Difference between strata.
[b] Range (mean ± SD).

(SMD: 0.40, 95% CI: 0.26, 0.54, $P < 0.001$) and reduced the heterogeneity ($X^2 = 74.8$, $P < 0.006$, $I^2 = 37\%$).

The stratified meta-analysis according to vitamin C dose showed no significant effect of lower doses (0–500 mg/d) on EF whereas higher doses (>500 mg/d) elicited significant improvements in EF (Table 2).

Meta-regression analysis showed no significant correlations between baseline vitamin C concentrations (β: −0.00452, 95% CI: −0.01877, 0.00973, $P = 0.53$) and changes in EF. However, there was a positive, significant correlation between vitamin C dose and magnitude of effect on EF (β: 0.00011, 95% CI: 0.00001, 0.00021, $P = 0.03$).

3.5. Publication bias

Visual inspection of the funnel plot showed evidence of asymmetry (Supplementary Fig. 1, online material) but Begg's rank correlation test yielded a non-significant Kendall tau of 0.19 ($P = 0.06$).

4. Discussion

To our knowledge, this is the first systematic review and meta-analysis evaluating the effect of supplemental vitamin C on EF in human adults. Data synthesis from 44 RCTs demonstrated that supplemental vitamin C was associated with significant improvement of EF in subjects with cardio-metabolic disorders.

There is extensive evidence from observational studies demonstrating associations between higher vitamin C intake (or status) and better cardiovascular health [68,69]. Meta-analysis of data from 9 cohort studies showed that vitamin C supplementation at doses exceeding 700 mg/day was associated with 25% reduction in coronary heart disease risk [70]. Moreover, a six-year study conducted by Salonen et al. showed that vitamin C supplementation was associated with significant regression of atherosclerotic lesions in participants with low baseline plasma vitamin C concentration [11]. However, these positive results were not confirmed by RCTs and a small number of trials have been conducted to test the cardiovascular effect of vitamin C supplementation as a single intervention [71]. A non-significant effect of vitamin C supplementation on major cardiovascular outcomes was also observed in seminal trials such as the Women Antioxidant Cardiovascular Study (WACS) or the Physicians Health Study (PHS II) [72,73]. These two trials are an example of the negative results obtained from large-scale trials testing the efficacy of anti-oxidant therapies for the prevention and treatment of cardiovascular disorders. The debate around the inefficacy of vitamin C is still topical and possible explanations for this lack of effect have been attributed to the inability of the trials to account for vitamin C status and genotypic variation among individuals for genes controlling vitamin C metabolism and bioactivity (i.e., Sodium Dependent Vitamin C Transporters (SVCT1 and SVCT2), Haptoglobin, Glutathione S-Transferases) as well as differences in vitamin C intake and confounding effects of other medical treatments (i.e., statins, anti-hypertensive) [74,75].

Michels et al. [74] have recently highlighted major limitations in vitamin C research and proposed recommendations for the design and conductions of unbiased and robust trials including 1) targeting subjects with low vitamin C status, 2) providing evidence of the efficacy of the interventions by measuring the elevation of plasma ascorbate levels, 3) taking into consideration the distribution of the vitamin C in various organs and tissues and 4) assessing intermediate biomarkers of inflammation or oxidative stress closely linked to vitamin C bio-activity.

The diminution of oxidative damage and vascular inflammation is a core strategy to maintain a healthy EF and reduce the risk for CVDs. NO is a major player in preserving EF integrity by counteracting the effect of inflammatory mediators and eliciting smooth muscle relaxation, vasodilation, inhibition of adhesiveness of monocytes and platelets, whilst enhancing proliferation and reducing apoptosis of endothelial cells [8]. In addition to its direct reactive oxygen species (ROS)-quenching function, the beneficial effects of vitamin C on EF may be related to the increase in NO bioavailability as a result of the enhanced efficiency of the enzymatic and non-enzymatic synthetic NO pathways and reduced cross-reactivity with ROS [76]. Specifically, the primary mechanisms linking vitamin C to NO involve the 1) inactivation of superoxide and prevention of its interaction with NO to produce harmful peroxy-nitrite [77], 2) increase in the availability of the co-factor tetrahydrobiopterin (BH4), which is a vital cofactor for endothelial nitric oxide synthase (eNOS) in catalysing the synthesis of NO. BH4 deficiency causes uncoupling of eNOS and the production of the harmful superoxide and peroxynitrite free radicals [78,79], 3) increased release of NO from S-nitrosothiols and enhanced conversion of nitrite to NO in the circulation [76], 4) enhanced activity of eNOS enzyme to produce NO [10] and 5) stimulation of the enzyme guanylyl cyclase, which is responsible for the production of cGMP and relaxation of vascular smooth muscle cells [80]. Altogether, the pleiotropic actions of NO determine protective effects on EF by modulating mechanisms directly related to the pathogenesis of atherosclerosis such as maintenance of vascular tone and permeability, angiogenesis and vascular remodelling, leucocyte adhesion and transmigration and platelet aggregation. The use of cellular and animal models has been fundamental to advance the understanding of the molecular mechanisms linking vitamin C to the NO pathway. However, the results of these experiments have been challenged by the absence of vitamin C (scorbutic cells) and elevated oxygen concentrations

used in cell culture experiments and by the endogenous synthesis of vitamin C for most animals. Therefore, the extrapolation of the results obtained from these models requires a careful interpretation and, conceptually, it raises doubts about the validity of these cellular and animal models, particularly rats and mice, to investigate the relationship between vitamin C and NO physiology and the effects on human health and disease [74,81,82].

In the present analysis, the greatest protective effect of vitamin C on EF was observed in experimentally-induced endothelial dysfunction i.e. among healthy volunteers exposed to metabolic and inflammatory stressors (e.g. glucose, free fatty acids, methionine and endotoxins) to induce endothelial dysfunction. However it is important to highlight that the beneficial effect of vitamin C observed in this group was achieved by the use of supra-physiological doses of vitamin C (240–2600 mg) and the duration of the investigations was generally very short (≤1 day). Conversely, vitamin C did not have any significant effect on EF in trials recruiting healthy volunteers without any evidence of endothelial dysfunction. Previous reviews on the topic have also concluded that vitamin C supplementation in healthy people with normal vitamin C status has no effect on EF [83]. These results and the administration of oral vitamin C doses exceeding the current RDA (200 mg/day) have questioned the relevance of vitamin C supplementation in healthy individuals and suggested that greater benefits may be achieved in individuals with lower vitamin C status and increased oxidative stress [74]. In the present meta-analysis we found significant improvement in EF with doses greater than 500 mg/day. These doses are several folds higher than the daily recommended doses of vitamin C to the general population (40 mg in UK or 90 mg in the USA) [71], which seems to be adequate to approach maximal plasma levels of vitamin C (100–120 μM) [84]. Furthermore, some of the studies used doses higher than 2 g/day which is the upper

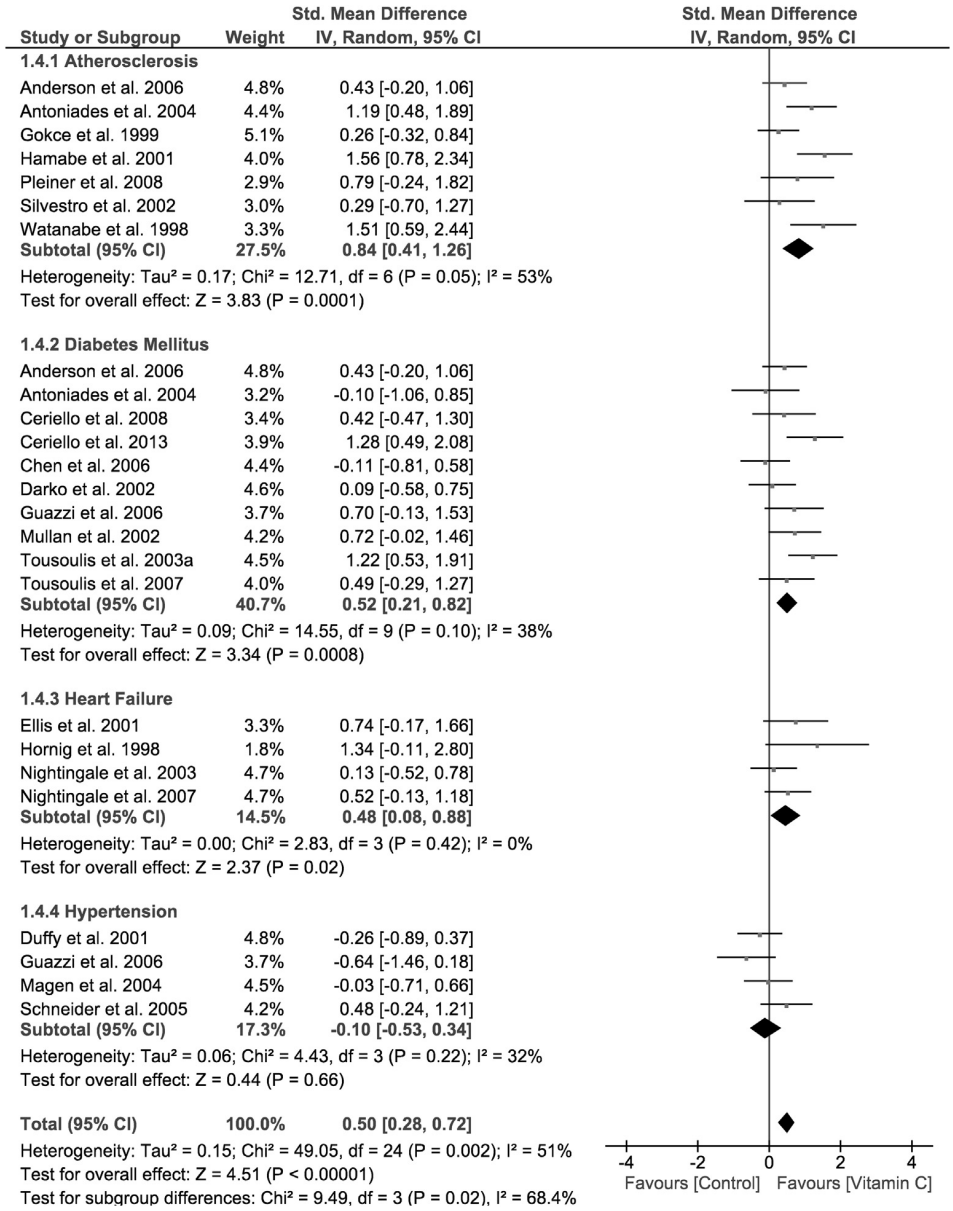

Fig. 3. Forest plot showing the effect of vitamin C supplementation on endothelial function in participants with cardio-metabolic disorders.

level recommended by the Institute of Medicine [85]. Sensitivity analysis conducted in this meta-analysis showed that doses higher than 2 g/day did not cause further improvement in EF. None of the studies that used vitamin C doses higher than 2 g/day reported any adverse effects.

However, several factors may influence the complex network of mechanisms regulating plasma vitamin C bioavailability and transfer and the resulting physiological effects in individual tissues. For example, ageing appears to reduce the absorptive capacity for vitamin C and lower plasma vitamin C concentrations have been associated with smoking, aspirin use, alcohol consumption, obesity and genetic variation for SVCT, Haptoglobin and Glutathione S-Transferase [74,75,86]. In addition, transport rate and saturation point may vary between cells and tissues and the pharmacodynamic profile cannot be directly extrapolated from plasma vitamin C concentration [74]. The median age of participants included in the meta-analysis was 51.1 years and the majority of the trials recruited participants with cardio-metabolic disorders. This type of population may be characterised by lower vitamin C levels and greater cellular requirements and therefore more than the recommended vitamin C dose may be needed to evoke a physiological response [74]. Lower baseline vitamin C concentration was not associated with greater effects on EF in the meta-analysis. However, plasma vitamin C concentration was reported in ~50% of the trial (24 out of 44 trials) and therefore these results warrant a cautious interpretation and emphasise the importance of measuring baseline vitamin C status in future trials. Our meta-analysis showed that vitamin C was highly effective in improving EF in patients with coronary and peripheral vascular diseases which may be characterised by greater requirements for vitamin C to counteract the increased oxidative stress. Therefore, vitamin C may be an effective nutritional intervention to restore EF induced by NO deficiency and high concentration of ROS in individuals with cardiovascular and metabolic disorders. In other work, vitamin C was effective in reducing the formation, and neutralising the activity, of oxidised-low density lipoprotein (oxLDL) which is regarded as the trigger of the inflammatory process in the endothelial tissue and the initiator of atherosclerosis [87]. Vitamin C significantly improved EF in diabetic patients. Previous studies demonstrate that patients with diabetes had low circulating vitamin C concentrations, known as latent scurvy [88]. Since the vitamin C metabolite dehydroascorbate is transported via glucose transporters, hyperglycemia may competitively reduce ascorbate transport and potentially lead to intracellular vitamin C deficiency [89]. Heart failure patients were found to be at high risk of developing endothelial dysfunction due to ROS accumulation and NO deficiency [90]. Endothelial dysfunction in heart failure patients worsened the prognosis and increased their mortality rate [91]. Although only a small number of heart failure studies were included in this meta-analysis, these showed that vitamin C supplementation was effective in improving EF in these patients. The meta-analysis has also showed some unexpected findings. Specifically, we found no effect of vitamin C on EF in smokers. The lack of effect may be due to the high level of oxidative stress in chronic smokers which may need very high doses of vitamin C or other antioxidants to compete with the superoxide anion to prevent interaction with, and inactivation of, NO [10]. Additionally, chronic smoking leads to induction of cytochrome oxidase liver enzymes that may affect the bioavailability of vitamin C [92]. Further studies are required to clarify the role of vitamin C on EF in chronic smokers. Similarly, vitamin C supplementation did not improve in EF in hypertensive patients in this analysis. We interpret these results cautiously because of the small number of studies included in the data synthesis. Previous studies which demonstrated beneficial effects of vitamin C on the endothelium in hypertensive usually used parenteral, supra-physiological doses of vitamin C employed for short time periods [93,94]. A recent meta-analysis demonstrated that vitamin C reduced blood pressure significantly [95], an effect that can be explained by several mechanisms, other than improving EF, as suggested by Houston [96]. Overall, the results from our meta-analysis suggest that studies testing the effect of supplementation with vitamin C on EF should be focussed on population sub-groups who are most likely to benefit, in particular those at high risk of oxidative stress e.g. diabetic, atherosclerotic, or heart failure patients rather than healthy individuals.

The strength of the present systematic review and meta-analysis is largely dependent on the rigour of the study design and on the quality of the included studies. In addition, the critical appraisal of the evidence and transparent reporting of the main findings is supported by the adherence of the systematic review to the PRISMA guidelines [17]. However, this systematic review has some limitations. First, EF is a surrogate endpoint for cardiovascular diseases. Nevertheless, evidence from previous studies showed that coronary and brachial endothelial functions predict short- and long-term atherosclerosis progression and cardiovascular events rate [97]. Moreover, improvement in EF was associated with a more favourable prognosis for nonfatal cardiovascular events including acute pulmonary oedema and ischaemic stroke [98]. A recent meta-analysis demonstrated that a 1% reduction in FMD was associated with 8% increase in the risk of cardiovascular events [99]. Second, many of the studies included in the present analysis were relatively small with 43% of the studies having a sample size of <20. Third, the considerable variability in the design, duration, dose of vitamin C and the subject characteristics (age, sex, health status) may have contributed to the significant heterogeneity observed in our meta-analysis. Fourth, the asymmetry observed in the funnel plot may be an evidence of publication bias. However, this observation should be interpreted cautiously. Apparent asymmetry in such funnel plots may be due to factors other than the presence of publication bias including: 1) most of the included studies had small sample size (small study effect). The small study effect means that small trials tend to report greater treatment effect than large trials and therefore causes asymmetry of the funnel plot [100]; 2) heterogeneity in design and in outcome measures between studies (there is substantial clinical and methodological heterogeneity among the studies included in our meta-analysis), and studies involving different populations [101]. Fifth, we found too few studies to allow us to determine the effect of vitamin C in patients with metabolic syndrome, hypercholesterolaemia, and chronic kidney diseases.

In conclusion, vitamin C supplementation significantly improved EF in patients with diabetes, atherosclerosis, and heart failure. No effect of vitamin C was observed in healthy volunteers, smokers or hypertensive patients. There was a positive dose–response relationship between vitamin C dose and effect on EF and oral doses greater than 500 mg/d vitamin C were associated with beneficial effects on EF. Altogether, these results support the idea that vitamin C may be a useful nutritional intervention for the secondary prevention of cardiovascular diseases. However, future RCT to test the effect of supplementary vitamin C on major cardiovascular outcomes (morbidity and mortality) should recruit those likely to benefit i.e. non-smoking patients with diabetes, atherosclerosis or heart failure.

Authors' contributions

AA drafted the manuscript; AA, MS, and JM: conceived the idea for the study and developed the search strategy; AA, MS and JL: conducted the search and summarised the data; all authors contributed to the data analysis, verification, writing and revising the manuscript. All authors had full access to all the data in the

study and responsible for the integrity and accuracy of the data and its analysis.

Conflict of interest

None to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2014.04.004.

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