

Review Article

The assessment of vascular function during dietary intervention trials in human subjects

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Abstract

The potential to reduce cardiovascular morbidity through dietary modification remains an area of intense clinical and scientific interest. Any putatively beneficial intervention should be tested within a randomised controlled trial which records appropriate endpoints, ideally incident CVD and death. However, the large sample sizes required for these endpoints and associated high costs mean that the majority of dietary intervention research is conducted over short periods among either healthy volunteers or those at only slightly increased risk, with investigators using a diverse range of surrogate measures to estimate arterial health in these studies. The present review identifies commonly employed techniques, discusses the relative merits of each and highlights emerging approaches.

Key words: Endothelium: Pulse wave velocity: Pulse wave analysis: Dietary intervention

Observational evidence linking particular dietary factors with a reduced incidence of cardiovascular morbidity and mortality has been used extensively to support various public health promotion strategies^(1,2). Increasingly, however, investigators are designing randomised controlled trials to confirm whether those diets or food groups may offer vascular protection. While hard clinical endpoints such as myocardial infarction, stroke and death are the ideal in such work, their use would necessitate prohibitively large, prolonged studies. The Dietary Approaches to Stop Hypertension (DASH) trial typifies much of the research in this field in that it recruited mildly hypertensive, but otherwise healthy, volunteers to an 8-week intervention⁽³⁾. While DASH was large enough to successfully employ a clinically relevant endpoint (brachial blood pressure), most dietary intervention studies rely on surrogate measures that will sensitively detect much earlier changes in arterial physiology. To underline this point, a recent meta-analysis considered randomised controlled trials which examined the link between flavonoids/flavonoid-rich foods and cardiovascular

risk⁽⁴⁾. Of the 133 studies included, none had cardiovascular morbidity or mortality as endpoints.

A range of vascular function methodologies is available to clinical researchers, and the choice for any one study is usually governed by local expertise and experience. However, it is vital that nutrition researchers have a clear understanding of the metric which their chosen technique will generate, and how applicable this is to their overall research question.

Here we consider endothelial vasodilator function, pulse wave mechanic analysis and biomarker measurement in the evaluation of arterial health during nutrition intervention studies conducted among human volunteers.

Endothelial dysfunction

All blood vessels are lined by an active cellular monolayer, known as the endothelium, which is responsible for many vital aspects of vascular homeostasis⁽⁵⁾. Endothelial cells produce a wide range of paracrine mediators, with multiple beneficial actions including anti-thrombotic, anti-platelet,

Abbreviations: CFPWV, carotid-femoral pulse wave velocity; CRP, C-reactive protein; DASH, Dietary Approaches to Stop Hypertension; EMP, endothelial microparticles; EPC, endothelial progenitor cells; FMD, flow-mediated dilatation; ICAM-1, intercellular adhesion molecule-1; PWV, pulse wave velocity; VCAM-1, vascular cell adhesion molecule-1.

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anti-inflammatory and vasodilatory effects⁽⁶⁾. Established cardiovascular risk factors are known to encourage the evolution of an atherosclerotic plaque by unfavourably altering endothelial cell physiology⁽⁷⁾. Thus, assessing the status of endothelial cells *in vivo* through their ability to produce NO, and thus mediate arterial dilatation, is common in cardiovascular research⁽⁸⁾. Provocation of NO production can be either mechanical (flow-mediated) or pharmacological.

Flow-mediated dilatation

Flow-mediated dilatation (FMD) describes arterial dilatation in response to increased intra-luminal shear stress. In humans, this phenomenon has been described using a forearm technique, in which reactive hyperaemia following release of an arm cuff at suprasystolic pressures mediates increased brachial artery diameter⁽⁹⁾. The vasodilatation can be quantified using B-mode ultrasound, and agreed protocols have been published to guide investigators using this procedure^(10,11). Since the technique does not involve needles, it has proven popular with researchers and less daunting for potential volunteers.

A growing body of research has not only demonstrated impaired brachial artery FMD among patients with recognised cardiovascular risk factors^(12–14), but also suggests that the measure may serve as an independent prognostic indicator in both high-risk populations^(15–17) and healthy volunteers⁽¹⁸⁾.

Brachial FMD has been employed by several groups as an endpoint during diet-related intervention trials. The effect of *n*-3 fatty acids on endothelial function has recently been reviewed⁽¹⁹⁾, as has the effect of fruit polyphenols⁽²⁰⁾, berries⁽²¹⁾ and green tea^(22,23) on vascular health, and these reviews include studies using FMD endpoints. In Table 1^(24–34), the intervention studies that have examined the effect of chocolate or cocoa on FMD are shown. Consumption of dark chocolate has been shown to improve brachial FMD, both acutely (six out of seven studies), and chronically (five out of six studies).

While it provides a minimally invasive option for vascular function assessment, the measurement of brachial FMD does rely on considerable skill in ultrasound image acquisition and concerns about this technique's reproducibility have been expressed⁽³⁵⁾. De Roos *et al.* report substantial within-subject variability for FMD among healthy volunteers, with a CV estimated at approximately 50%⁽³⁶⁾. Although much better CV figures (7–10%) have been published by Deanfield's group^(37,38), there remains a concern that, when employed in smaller, less experienced centres, the technique's inherent variability precludes adequate study power. In addition, the NO dependency of FMD has been questioned⁽³⁹⁾, such that this response is better considered a consequence of interplay between competing dilator and constrictor influences⁽⁴⁰⁾.

Another potential concern is that not all prospective studies have identified brachial FMD as an independent predictor of cardiovascular morbidity. Fathi *et al.* report no relationship between FMD and the risk of death, acute coronary syndrome or stroke among patients with established risk factors⁽⁴¹⁾. Austrian investigators note similar findings among patients undergoing coronary angiography⁽⁴²⁾ while in almost 3000 older adults, brachial artery diameter and FMD proved equally

effective predictors of vascular outcome⁽¹⁸⁾. However, although not always shown to be an independent predictor of cardiovascular events, this does not preclude its use as an endpoint in dietary intervention studies, as long as interpretation of the result is appropriate.

Despite these reservations, brachial FMD is widely regarded as the 'gold standard' technique by which to assess conduit vessel function in cardiovascular research⁽⁴³⁾. However, before adopting FMD as the endpoint of choice, trial investigators should consider whether conduit vessel function is the best metric with which to detect any intervention-related effect. The response quantified in standard FMD protocols depends upon forearm ischaemia-induced hyperaemic flow, which is itself a function of reduced vascular resistance. The latter is determined largely by endothelium-dependent microvascular tone and thus measures of reactive hyperaemia have been suggested as novel arterial descriptors⁽⁴⁴⁾. Analysis of brachial ultrasound recordings from over 2000 Framingham volunteers showed that Doppler-derived indices of hyperaemic shear stress correlated better with established cardiovascular risk factors than did FMD⁽⁴⁵⁾. Huang *et al.* have shown that lower hyperaemic flow velocities in the brachial artery following a standard period of forearm ischaemia independently predicted postoperative morbidity and mortality among patients with peripheral arterial disease undergoing elective vascular surgery⁽⁴⁶⁾.

It has been speculated that the microvascular dysfunction implied by reduced hyperaemic flow responses may be more sensitive to early atherosclerotic change than are disturbances in conduit artery vasomotion as quantified by FMD⁽⁴⁷⁾. This has particular relevance for the design of dietary intervention trials which often seek to detect subtle changes in healthy volunteers. It would therefore be considered best practice to record FMD and reactive hyperaemia concurrently, as reactive hyperaemia can be measured simultaneously with FMD, using the same equipment.

Pharmacological provocation of endothelium-dependent vasomotion

Where FMD uses mechanical shear stress to provoke arterial endothelial vasodilator production, the local infusion of appropriate agonists can produce a similar effect. While based on well-established physiological and pharmacological principles, this approach does rely on successful arterial puncture.

Furchgott & Zawadzki's description of acetylcholine-mediated endothelium-dependent vasodilatation using isolated arterial segments *in vitro* was soon extended to *in vivo* human work⁽⁴⁸⁾. Within the coronary circulation, direct intra-arterial injection of acetylcholine mediated vasoconstriction among patients with significant atherosclerotic lesions but dilated angiographically normal vessels⁽⁴⁹⁾. Several prospective studies among patients with clinical indications for cardiac catheterisation have established an abnormal coronary response to endothelium-dependent agonists as a powerful independent predictor of cardiovascular morbidity^(50–53). The potential risks associated with invasive cardiac assessment limit this

Table 1. Effect of cocoa or chocolate interventions on flow-mediated dilatation (FMD): evidence from randomised controlled trials

Study and year	Population	Intervention	Duration	Effect on FMD?	Effect size
Balzer <i>et al.</i> (2008) ⁽²⁴⁾	Diabetic patients	Acute (cocoa containing 371 or 963 mg flavanols) Chronic (cocoa 963 mg flavanols/d)	Acute and chronic (30 d)	Acute ↑ (at both doses) Chronic ↑	Acute +1.8% (at 2 h; 963 mg flavanols) Chronic +1.0%
Berry <i>et al.</i> (2010) ⁽²⁵⁾	Overweight or obese men and postmenopausal women	Cocoa containing 701 mg flavanols	Acute	↑	6.1% (at 2 h). No baseline measure taken. Significantly different from low-flavanol group
Davison <i>et al.</i> (2008) ⁽²⁶⁾	Overweight and obese adults	High-flavanol cocoa (902 mg flavanols/d)	Acute and chronic (12 weeks)	Acute ↑	Acute +2.4% (at 2 h)
Engler <i>et al.</i> (2004) ⁽²⁷⁾	Healthy adults	High-flavonoid dark chocolate bar (213 mg procyanidins, 46 mg epicatechin)	2 weeks	Chronic ↑ ↑	Chronic +1.6% +1.3%
Faridi <i>et al.</i> (2008) ⁽²⁸⁾	Healthy adults	Dark chocolate (22 g cocoa powder) Sugar-free cocoa (22 g cocoa powder)	Acute	Dark chocolate ↑ Sugar-free cocoa ↑	Dark chocolate 4.3% (at 2 h) Sugar-free cocoa 5.7% (at 2 h)
Farouque <i>et al.</i> (2006) ⁽²⁹⁾	CAD patients	Flavanol-rich chocolate bar and cocoa beverage (total flavanols 444 mg/d)	Acute and chronic (6 weeks)	Acute ↔	Acute –
Grassi <i>et al.</i> (2005) ⁽³⁰⁾	Essential hypertension patients	100 g dark chocolate/d (containing 88 mg flavanols)	15 d	Chronic ↔ ↑	Chronic – +1.5%
Heiss <i>et al.</i> (2003) ⁽³¹⁾	Out-patients with at least one cardiovascular risk factor	Cocoa drink containing 176 mg flavan-3-ols	Acute and after 2 d supplementation	Single dose ↑	Single dose +2.7% (at 2 h)
Heiss <i>et al.</i> (2007) ⁽³²⁾	Healthy male smokers	Flavanol-rich cocoa drink (918 mg/d)	Acute and chronic (7 d)	After 2 d ↑ Acute ↑	After 2 d +2.9% Acute +2.4%; dose-dependent magnitude of 2 h response also demonstrated
Heiss <i>et al.</i> (2010) ⁽³³⁾	CAD patients	High-flavanol cocoa drink containing 750 mg/d	30 d	Chronic ↑ ↑	Chronic +2.9% +3.8%
Hermann <i>et al.</i> (2006) ⁽³⁴⁾	Male smokers	40 g dark chocolate	Acute	↑	+2.6%

↑, Significant increase; CAD, coronary artery disease; ↔, no significant change.

technique's applicability to trials conducted in healthy volunteers. However, the forearm is an accessible and relatively safe vascular bed, in which pharmacological challenges analogous to those described for epicardial vessels can be performed.

While coronary procedures have relied upon quantitative angiography, forearm studies mainly employ venous occlusion plethysmography to measure the efficacy of intra-brachial vasodilators. Concordant, proportionate responses to acetylcholine have been described in synchronously infused coronary and brachial arteries^(54,55). Poor forearm dilator responses to acetylcholine have been shown to predict increased rates of cardiovascular morbidity among hypertensive volunteers⁽⁵⁶⁾ and patients with coronary artery disease^(57,58).

It is important to appreciate that, when infused into the brachial artery, vasoactive agents exert their influence on forearm blood flow by modulating small vessel tone and this technique is thus an assessment of microvascular function⁽⁵⁹⁾.

Forearm blood flow response to locally infused endothelium-dependent vasodilators has been used as an endpoint in several dietary intervention trials. Healthy adults who consumed a Mediterranean-style diet for 6 weeks showed significant improvements in endothelium-dependent forearm hyperaemia^(60,61); however, Ambring *et al.* subsequently reported negative findings for a similar 4-week intervention conducted among younger volunteers using the same endpoint⁽⁶¹⁾. Investigators have also employed this technique to document the deleterious effects of increasing salt consumption. It was shown that 5 d of salt loading significantly reduced forearm blood flow responses to intra-brachial acetylcholine⁽⁶²⁾. Our group recently reported a significant dose-dependent relationship between increased dietary fruit and vegetable consumption and improved microvascular endothelial function, as quantified using this method⁽⁶³⁾.

However, the applicability of forearm blood flow manipulation through local vasodilator infusion to large, multi-centre clinical trials is limited by its reliance on arterial puncture, and the prospect of needle insertion is likely to prove inherently unattractive to many.

The most serious risks of brachial artery cannulation include occlusion of the artery leading to limb ischaemia, and median nerve damage due to direct trauma or compression by haematoma or infection. In reality, complications are rare, and usually involve minor bruising or local discomfort that resolve quickly, without intervention. It is, however, essential that this procedure is performed by an experienced researcher (with a background in vascular access and aseptic techniques) such as an intensivist, cardiologist or surgeon. This therefore restricts the use of this methodology to research centres with access to the aforementioned skilled operators, and also limits its use to smaller studies. The requirement for needle insertion may also deter participants, and those on oral anticoagulation or with significantly increased BMI must be excluded^(59,64,65).

An alternative, less invasive, approach to pharmacological manipulation of the microvascular endothelium involves transdermal drug delivery by iontophoresis⁽⁶⁶⁾. A small electrical current is applied to the forearm and mediates movement of polar drugs such as acetylcholine into cutaneous vessels. Changes in skin blood flow are then quantified using laser

Doppler flowmetry⁽⁶⁷⁾. In a recent study, a strong correlation between this measure of skin microvasculature and FMD of the brachial artery was reported⁽⁶⁸⁾. Therefore, this may offer an alternative endpoint for future intervention trials, although the clinical relevance is, as yet, less established than for other methods.

A small number of trials have already used this endpoint, including a trial of fruit and vegetable purée-based drinks (a trend towards an effect shown on this endpoint in both acute and chronic settings)⁽⁶⁹⁾, a trial of a green tea polyphenol extract (no effect in the tested chronic setting)⁽⁷⁰⁾, a study of acute fish oil consumption (effect on endpoint demonstrated)⁽⁷¹⁾ and a chronic study of weight reduction and exercise (no effect demonstrated)⁽⁷²⁾. A recent study of orange juice demonstrated an acute postprandial effect of orange juice or hesperidin consumption on microvascular reactivity, but no effect on fasting reactivity after 4 weeks of consumption⁽⁷³⁾.

Non-invasive assessment using pulse wave mechanics and pulse contour analysis

A range of commercially available devices offers clinical investigators the opportunity to rapidly acquire arterial descriptors, usually by non-invasively recording pulse pressure tracings through a device, which then computes one or more output variables. While user friendly and therefore popular, these techniques rely on important biomechanical assumptions which often complicate their applicability and interpretation.

Translating intermittent ejection of blood from the heart to smooth end-organ perfusion is a complex biomechanical process that relies on optimal ventricular–vascular coupling. A variety of mechanical arterial descriptors has been used to quantify unfavourable disease-related changes in this interaction⁽⁷⁴⁾. Since these parameters are derived largely from non-invasive techniques, they have proved popular surrogate endpoints during intervention trials in cardiovascular medicine⁽⁴³⁾. Popular methods include measuring pressure pulse wave velocity (PWV) across a particular arterial segment and calculating indices of vascular compliance by mathematical pulse contour analysis. The effects of dietary and nutrient interventions on these endpoints have recently been systematically reviewed⁽⁷⁵⁾.

Pulse wave velocity

The velocity with which pressure pulse waves travel along an arterial segment can be mathematically related to that vessel's mechanical properties, by either the Moens–Kortweg or Bramwell–Hill equations⁽⁷⁶⁾. These formulae predict that pressure pulse waves will travel faster in less distensible arteries and thus PWV is a commonly cited descriptor of 'arterial stiffness'⁽⁷⁷⁾. A pressure transducer or tonometer is used to detect passage of the pulse wave between two anatomical locations. This can be done sequentially⁽⁷⁸⁾ or simultaneously⁽⁷⁹⁾, with gating to a contemporaneously recorded electrocardiogram. The carotid and femoral arteries are commonly chosen tonometry sites, as this allows estimation of aortic PWV.

Prospective data are now available to suggest that carotid-femoral PWV (CFPWV) is an independent predictor of cardiovascular morbidity among healthy individuals^(80,81). While tonometry at the radial site is much more convenient for both volunteer and investigator, carotid-radial PWV did not predict coronary events or strokes among a group of patients with end-stage renal failure in whom CFPWV did have independent prognostic value⁽⁸²⁾. This finding reflects structural distinctions between muscular forearm arteries and larger elastic vessels such as the aorta where medial cells have ectodermal rather than mesodermal origins⁽⁸³⁾.

As the intra-luminal pressure within an artery increases, progressively more collagen fibres are recruited and thus its mechanical characteristics change⁽⁸⁴⁾. An intervention which reduces blood pressure is, therefore, also likely to reduce PWV independent of any pleiotropic effect on the arterial wall⁽⁸⁵⁾. This is an important caveat to the interpretation of any trial which employs PWV as an endpoint. Several recent trials have reported significant reductions in CFPWV following interventions including weight reduction⁽⁸⁶⁾, DASH⁽⁸⁷⁾, low carbohydrate⁽⁸⁸⁾ and low-glycaemic index⁽⁸⁹⁾ diets. However, in each case, significant blood pressure reductions are also recorded. Similarly, in a Na-loading study among hypertensive volunteers, changes in CFPWV were positively correlated with changes in brachial blood pressure⁽⁹⁰⁾, whilst 6 months' supplementation with conjugated linoleic acid⁽⁹¹⁾ or 2 weeks of dietary salt restriction⁽⁹²⁾ failed to alter either blood pressure or CFPWV. However, an isoflavone intervention over 6 weeks did mediate significantly slower CFPWV in healthy volunteers without 24 h ambulatory blood pressure reduction⁽⁹³⁾.

In summary, if an intervention mediates a significant effect on arterial blood pressure, a concordant effect on PWV will be observed. This does not imply an alteration of vascular structure or function and can be predicted from arterial physiology.

Before choosing it as endpoint, investigators should postulate a mechanism by which their proposed intervention could change CFPWV. Through an *in vitro* model, impairment of local endothelial NO production has been shown to increase PWV across predefined arterial segments^(94,95). Furthermore, brachial artery FMD has been independently associated with CFPWV in cross-sectional technique comparison studies among healthy volunteers⁽⁹⁶⁾ and patients with isolated systolic hypertension⁽⁹⁷⁾. A significant, positive correlation between carotid-radial PWV and microvascular function in the forearm has also been described⁽⁹⁸⁾.

However, during the relatively brief trials that typify much human dietary intervention work it is questionable whether subtle alterations in levels of an endothelium-derived paracrine mediator could significantly change CFPWV which principally reflects aortic medial elastin:collagen ratios. Thus, this parameter is not ideal for use in short-term studies which aim to evaluate the possible endothelial effects of an intervention.

Pulse contour analysis

Palpation and analysis of the radial pulse as a means by which to assess systemic arterial health is a long-established practice in cardiovascular medicine⁽⁹⁹⁾. Algorithm-based

reconstruction of the aortic pressure pulse from tonometer-derived radial waveforms has fuelled renewed interest in this approach⁽⁸⁵⁾. A commercially available device has been widely used in cardiovascular research to estimate the aortic augmentation index, a measure of arterial wave reflection^(100,101). Since it is non-invasive, requires minimal training and generates an easily interpretable numeric output, this technique has proved popular in cardiovascular research.

Among patients undergoing cardiac catheterisation, those in the highest quartile of augmentation index had significantly more coronary disease⁽¹⁰²⁾. Similarly, patients with end-stage renal failure who had higher augmentation indices were more likely to die during an 8-year follow-up period⁽¹⁰³⁾. However, a recent review of arterial stiffness by The Framingham Heart Study found that whilst a higher aortic PWV was associated with a 48% increase in cardiovascular events, the aortic augmentation index, central pulse pressure and pulse pressure amplification showed no such correlation⁽¹⁰⁴⁾. Like PWV, the aortic augmentation index is dependent on distending arterial blood pressure^(105,106), but additional variables, including height⁽¹⁰⁷⁾ and heart rate⁽¹⁰⁸⁾, must be factored in to its interpretation.

A number of nutrition intervention studies have used pulse contour analysis as an intermediate measure of vascular function. In an acute feeding study, food and water, but not water alone, reduced the aortic augmentation index 2h after consumption⁽¹⁰⁹⁾. This finding is confounded by concomitant decreases in arterial blood pressure and could be argued to reflect mean blood pressure change and therefore altered vessel haemodynamics, rather than an intrinsic alteration of vascular function. After 8 weeks, both low-fat and low-carbohydrate hypoenergetic diets significantly decreased brachial blood pressure among overweight volunteers, but only the former mediated a significant reduction in aortic augmentation⁽¹¹⁰⁾.

Again, investigators should have a clear hypothesis about the mechanism through which their intervention is likely to alter pulse wave morphology before choosing aortic augmentation as the endpoint. It has been shown that pharmacological manipulation of systemic endothelial NO production significantly changes aortic pressure pulse wave morphology and thus the augmentation index⁽⁹⁶⁾. In a situation analogous to the intra-brachial infusion of vasodilators and subsequent forearm blood flow measurement, systemic salbutamol (endothelium-dependent) and nitroglycerine (endothelium-independent) are administered with resulting changes in tonometry-derived aortic augmentation quantified to arrive at a 'global endothelial function index'⁽⁹⁶⁾. Such an approach may prove more sensitive to altered vascular health than resting measurements of aortic augmentation.

Biomarkers of vascular function

Biomarker measurement remains a popular endpoint in clinical research, as it is minimally invasive and samples can be stored for future analysis. A wide variety of biochemical species has been employed to quantify inflammation, oxidative stress, endothelial activation and arterial injury. Since no single 'gold

standard' measure has emerged, it is common practice during dietary research trials to employ a panel of such markers.

Biochemical measures

Since atherosclerosis is characterised by ongoing vascular inflammation, the acute-phase reactant C-reactive protein (CRP) has been proposed as a useful tool for improving disease prediction models⁽¹¹¹⁾. A high-sensitivity assay is used to accurately measure the lower CRP concentrations encountered among healthy individuals. Recent data suggest that statin-mediated reductions in CRP are associated with lower rates of cardiovascular morbidity⁽¹¹²⁾.

The evidence regarding dietary interventions and CRP is equivocal. For example, as summarised in Table 2^(113–128), only five out of sixteen fruit and vegetable randomised controlled trials (including Mediterranean diet and DASH trials, as fruit and vegetables are key components of these diets) have demonstrated a lowering in CRP levels. The duration of these five studies varied from 4 weeks in three studies, to 3 months in one study and 2 years in another. Of the studies, two were juice-based, one was a carotenoid-rich FV intervention and two were Mediterranean diet interventions. We refer the reader to a review by Giugliano *et al.*⁽¹²⁹⁾ for a broader discussion of the association between diet and inflammation.

In mediating leucocytic infiltration of the arterial intima, glycoprotein membrane components such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) promote oxidative stress and ongoing arterial inflammation⁽¹³⁰⁾. Activated endothelial cells are thought to shed these molecules into the circulation, allowing levels of their soluble form to be quantified as an estimate of ongoing arterial injury⁽¹³¹⁾. Adipocytes also express ICAM-1, and the soluble form of ICAM-1 is elevated in obese patients, being expressed in the stromal-vascular fraction of adipose tissue⁽¹³²⁾. This is likely to contribute to the link between obesity and inflammatory complications such as atherosclerosis.

Supplementation with isoflavones for 6 weeks has been reported to significantly reduce serum levels of VCAM-1 in healthy subjects⁽⁹³⁾, while additional dietary α -linolenic acid had a similar effect among dyslipidaemic male patients⁽¹³³⁾. Over a 24-month period, daily consumption of the phytoestrogen genistein significantly reduced circulating levels of both ICAM-1 and VCAM-1⁽¹³⁴⁾. While additional cherry consumption for 4 weeks lowered CRP levels among healthy volunteers, it had no effect on ICAM-1 concentrations⁽¹³⁵⁾ and similar negative results have been noted for brief fruit and vegetable interventions⁽¹²⁵⁾.

Oxidation both contributes to and follows on from the continuous cycle of low-grade vascular inflammation which characterises atherosclerotic arterial degeneration⁽¹³⁶⁾. Markers of systemic oxidative stress in biological fluids have long been suggested as surrogates for vascular injury, and the isoprostanes, derived from non-enzymic arachidonic acid peroxidation, are commonly measured⁽¹³⁷⁾. A dietary intervention study has reported that urinary levels of 8-iso-PGF_{2 α} were significantly reduced among healthy women consuming nine or ten portions of fruit and vegetables daily for

8 weeks⁽¹³⁸⁾. However, investigators have largely reported negative findings when this endpoint has been employed to study the effects of black tea⁽¹³⁹⁾, a Mediterranean diet⁽⁶¹⁾, five or six portions of fruit and vegetables daily^(98,138) and pure dietary flavonoids⁽¹⁴⁰⁾.

Other potential biomarkers of the atherosclerotic process include the enzyme lipoprotein-associated phospholipase A₂^(141,142), tissue plasminogen activator^(143,144) and plasminogen activator inhibitor-1⁽¹⁴⁵⁾.

Novel biochemical approaches

The array of biomarkers available and lack of an agreed 'gold standard' often prohibits rational study design. To quantify the biological effects of an 'anti-inflammatory mix' dietary supplement among obese volunteers, Bakker *et al.* employed a novel 'nutrigenomics' approach⁽¹⁴⁶⁾. This involved measurement and integrated analysis of 120 plasma proteins, 274 plasma metabolites and peripheral blood cell transcriptomes. While such a comprehensive approach is labour intensive and statistically complex, it may represent a valuable means by which to define more subtle intervention-related changes.

More recently, circulating endothelial microparticles (EMP), endothelial progenitor cells and endothelial cells have also been used as indices of vascular health.

Endothelial microparticles. The endothelium is responsible for a diverse range of functions, including regulation of vascular vasomotor activity, coagulation activity, anti-inflammatory status, and therefore a comprehensive assessment of endothelial function or dysfunction is difficult, with available methods usually only providing information on one separate aspect of endothelial function. It has been proposed recently that EMP may fulfil the role of a more universal marker of vascular health⁽¹⁴⁷⁾. EMP are small non-nucleated phospholipid vesicles shed from injured endothelial cells in response to pro-inflammatory stimuli and vascular injury. They affect endothelial NO synthesis^(148,149), diminishing acetylcholine-induced vasorelaxation and NO production by endothelial cells *in vitro*⁽¹⁵⁰⁾, correlate with markers of inflammation⁽¹⁴⁷⁾, and have pro-coagulant potential^(147,151–153).

A number of studies have examined EMP in relation to vascular damage and CVD risk. A significant increase in EMP has been shown in patients with CHD, the metabolic syndrome, diabetes and heart failure^(154–158). A recent study by Wang *et al.* has shown that EMP count is associated with systolic blood pressure, being elevated even in patients with mild hypertension, and was also associated with arterial stiffness⁽¹⁵⁹⁾. Wang *et al.* suggest that EMP may be a useful parameter for monitoring the process of vascular repair in hypertensive subjects, whilst the accompanying Editorial calls for further studies to determine whether EMP quantification might be a useful marker of endothelial dysfunction⁽¹⁴⁷⁾. Their use in dietary intervention research has been limited to date, but circulating microparticle concentrations are known to increase after ingestion of a fatty meal⁽¹⁶⁰⁾, whilst a recent paper has shown that following a Mediterranean diet for 4 weeks in older subjects significantly decreased total microparticle, EMP and apoptotic EMP concentrations

Table 2. Effect of fruit and vegetable (FV) interventions on C-reactive protein (CRP): evidence from randomised controlled trials

Study and year	Population	Intervention	Duration	Effect on CRP
Berry <i>et al.</i> (2010) ⁽¹¹³⁾	Pre-hypertension	Cross-over: control level FV containing 15 mmol K per d or an additional 20 or 40 mmol K per d provided as FV or 40 mmol K per d as potassium citrate capsules	6 weeks (5-week washout)	↔
Blum <i>et al.</i> (2007) ⁽¹¹⁴⁾	Healthy adults	300 g tomatoes v. usual diet with tomatoes prohibited	4 weeks	↔
Dalgard <i>et al.</i> (2009) ⁽¹¹⁵⁾	Peripheral arterial disease	Cross-over: orange and blackcurrant juice (500 ml per d) and vitamin E (15 mg); juice + placebo; sugar drink + vitamin E; sugar drink + placebo	4 weeks (4-week washout)	↓*
Erlinger <i>et al.</i> (2003) ⁽¹¹⁶⁾	Hypertension	Control v. DASH diet (rich in FV, about nine servings per d)	12 weeks	↔
Esposito <i>et al.</i> (2004) ⁽¹¹⁷⁾	Metabolic syndrome	Mediterranean diet v. prudent diet	2 years	↓
Freese <i>et al.</i> (2004) ⁽¹¹⁸⁾	Healthy adults	810 g VBA + rich linoleic acid; 196 g VBA + rich linoleic acid; 810 g VBA + rich oleic acid; 196 g VBA + rich oleic acid	6 weeks	↔
Jin <i>et al.</i> (2010) ⁽¹¹⁹⁾	Healthy adults	Placebo; FV juice powder concentrate; FV powder concentrate with added berry powders	60 d	↔
Karlsen <i>et al.</i> (2010) ⁽¹²⁰⁾	≥ One risk factor for CVD	Bilberry juice v. water	4 weeks	↓
Lehtonen <i>et al.</i> (2010) ⁽¹²¹⁾	Healthy adults (female)	Lifestyle intervention v. lifestyle intervention with berry products (equalling 1138 g berries per week +3.5 g berry oils per week)	20 weeks	↔
McCall <i>et al.</i> (2010) ⁽¹²²⁾	Hypertension	Dose-response: 1 v. 3 v. 6 portions FV per d	8 weeks	↔
Mena <i>et al.</i> (2009) ⁽¹²³⁾	High-risk CVD	Low-fat diet v. Mediterranean diet with olive oil v. Mediterranean diet with nuts	3 months	↓†
Michalsen <i>et al.</i> (2006) ⁽¹²⁴⁾	CAD	Mediterranean diet v. written advice-only group	1 year	↔
Paterson <i>et al.</i> (2006) ⁽¹²⁵⁾	Healthy adults	Cross-over: carotenoid-rich or control vegetable soups and beverages	4 weeks (10-week washout)	↔
Rallidis <i>et al.</i> (2009) ⁽¹²⁶⁾	Abdominal obesity	Mediterranean diet v. Mediterranean diet with dietetic supervision	2 months	↔
Stull <i>et al.</i> (2010) ⁽¹²⁷⁾	Obese, non-diabetic	Smoothie containing 22.5 g blueberry bioactives twice daily v. smoothie of equal nutritional value without added blueberry actives	6 weeks	↔
Watzl <i>et al.</i> (2005) ⁽¹²⁸⁾	Non-smoking men	Dose-response: 2 v. 5 v. 8 servings per d carotenoid-rich FV	4 weeks	↓

↔, No significant change; ↓, significant decrease; DASH, Dietary Approaches to Stop Hypertension; VBA, vegetables, berries and apples; CAD, coronary artery disease.

* Orange and blackcurrant juice reduced CRP relative to sugar drink.

† CRP decreased only after Mediterranean diet with olive oil.

when compared with a SFA-rich diet or a low-fat, high-carbohydrate diet⁽¹⁶¹⁾. They therefore potentially offer a novel and informative endpoint.

Endothelial progenitor cells. Asahara *et al.* isolated and characterised a circulating angioblast which had the potential to form endothelial cells *in vitro*, thus allowing subsequent quantitative flow cytometry in samples of peripheral blood⁽¹⁶²⁾. Endothelial progenitor cells (EPC) have a constitutive vasoreparative function, but after acute vascular damage, such as stroke or myocardial infarction, these cells are mobilised into peripheral blood where they participate in endothelial repair, regenerative processes and neovascularisation^(163,164). It has been proposed that assessment of endothelial progenitor cells offers a dynamic, integrated index of systemic vascular damage and, as such, will offer more insight than any currently available biochemical markers⁽¹⁶⁵⁾. While a wide range of EPC subsets have been identified, most clinical studies have concentrated on CD34⁺ populations isolated from peripheral blood. An inverse relationship between circulating CD34⁺ EPC and cardiovascular risk factors has been demonstrated in both healthy subjects and patients with CVD^(166,167), whilst circulating EPC count may also act as a prognostic biomarker, being associated with worse outcome in patients with suspected or confirmed coronary artery disease⁽¹⁶⁸⁾. A recent study has shown that circulating CD34⁺ cells are inversely associated with obesity, and that weight loss results in an increase in circulating progenitor cells, including EPC⁽¹⁶⁹⁾.

Among healthy volunteers, short-term dietary interventions with green tea⁽¹⁷⁰⁾, vegetables⁽¹⁷¹⁾, red wine⁽¹⁷²⁾ and the Mediterranean diet⁽¹⁶¹⁾ have all been shown to significantly increase circulating concentrations of endothelial progenitor cells. Assessing EPC numbers and function may also be informative, as increasing red wine consumption in healthy volunteers has recently been shown to increase endothelial progenitor cell migration and proliferation and to decrease the extent of apoptosis⁽¹⁷³⁾, whilst a high-flavanol intervention has been shown to increase EPC number, but had no effect on their function (measured as ability to survive, differentiate, proliferate and to migrate)⁽³³⁾.

Circulating endothelial cells. Circulating endothelial cells are also recognised as markers of endothelial damage, and have been shown to be increased in acute coronary syndromes, heart failure, stroke and diabetes mellitus⁽¹⁷⁴⁾. Although no dietary intervention studies have examined effects on numbers of circulating endothelial cells, they may be an informative target in future studies.

Considerations when choosing a method

Investigators should first consider the mechanism through which they propose that the intervention will act, alongside the locally available resources and skills. A decision tree outlining the main considerations and choices is shown in Fig. 1. A panel of approaches, assessing both conduit vessels and the microvasculature, would be most appropriate where

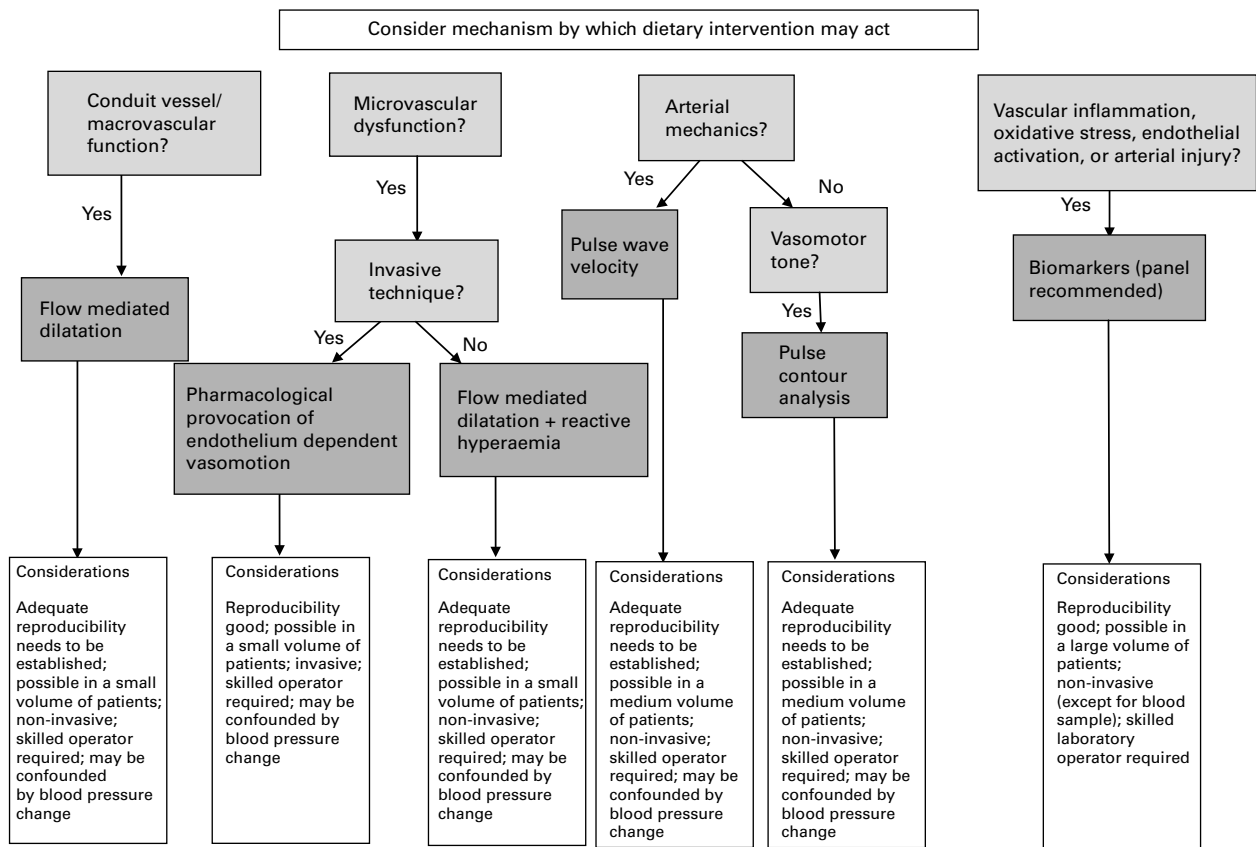


Fig. 1. Decision tree when considering method of vascular function assessment.

possible. Recent studies using different vascular function methodologies, although also with variation in study designs and populations, have demonstrated contrasting results, despite similar interventions^(63,113), and such a panel of endpoints may well have been informative in explaining these differing results. The choice of method may depend on whether investigators propose and are testing an acute or chronic effect of the dietary intervention. Investigators should also consider whether effects of the intervention are likely to be demonstrated in the fasting or postprandial state, as a recent study of *n*-3 fatty acids has demonstrated effects on postprandial macro- and microvascular function, but no effect on fasting measures⁽¹⁷⁵⁾.

The confounding effects of blood pressure must be considered when interpreting study results. Changes in blood pressure, and therefore blood flow, will also cause changes in the FMD of a conduit artery, and which do not necessarily reflect a change in the endothelial function of that vessel⁽¹⁰⁾. Forearm blood flow and PWV will also be affected by changes in blood pressure. In summary, if an intervention mediates a significant change in arterial blood pressure, a concordant effect on other vascular assessments will be observed. This does not imply an alteration of vascular structure or function and can be predicted from arterial physiology.

Conclusions

A wide variety of techniques are employed to provide surrogate vascular endpoints for short-term dietary trials in human subjects. FMD of the brachial artery and aortic PWV examine conduit and large elastic vessel properties respectively, and therefore do not estimate arterial function at a microvascular level where the bulk of endothelial cells are found.

The mechanism through which an investigator believes their intervention will act, combined with the resources and skill set of the investigating team, will most probably influence the choice of assessment method. At present no single, all-encompassing vascular function test exists, and perhaps a more useful approach would be to combine several methods to comprehensively assess arterial function and mechanics at multiple sites.

Potentially useful emerging techniques include the analysis of post-ischaemic arterial waveforms during FMD determination, quantifying pulse contour changes in response to vasodilator challenges and measuring circulating endothelial progenitor cell and microparticle concentrations.

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