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# The effect of increased dietary fruit and vegetable consumption on endothelial activation, inflammation and oxidative stress in hypertensive volunteers

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**Abstract** *Background and aims:* Public health campaigns recommend increased fruit and vegetable (FV) consumption as an effective means of cardiovascular risk reduction. During an 8 week randomised control trial among hypertensive volunteers, we noted significant improvements in endothelium-dependent vasodilatation with increasing FV consumption. Circulating indices of inflammation, endothelial activation and insulin resistance are often employed as alternative surrogates for systemic arterial health. The responses of several such biomarkers to our previously described FV intervention are reported here.

*Methods and results:* Hypertensive volunteers were recruited from medical outpatient clinics. After a common 4 week run-in period during which FV consumption was limited to 1 portion per day, participants were randomised to 1, 3 or 6 portions daily for 8 weeks. Venous blood samples for biomarker analyses were collected during the pre and post-intervention vascular assessments. A total of 117 volunteers completed the 12 week study. Intervention-related changes in circulating levels of high sensitivity C-reactive protein (hsCRP), soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) did not differ significantly between FV groups. Similarly, there were no significant between group differences of change in homeostasis model assessment (HOMA) scores.

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*Conclusions:* Despite mediating a significant improvement in acetylcholine induced vasodilatation, increased FV consumption did not affect a calculated measure of insulin resistance or concentrations of the circulating biomarkers measured during this study. Functional indices of arterial health such as endothelium-dependent vasomotion are likely to provide more informative cardiovascular end-points during short-term dietary intervention trials.


## Introduction

A wealth of prospective epidemiological evidence has linked increased fruit and vegetable (FV) consumption with reduced rates of ischaemic heart disease [1] and stroke [2]. Although observational nutritional studies are often criticised [3], both British [4] and American [5] guidelines recommend increased dietary FV content as an effective means of cardiovascular risk reduction. In a recent dietary intervention trial, we described improved endothelium-dependent vasodilatation among hypertensive volunteers randomised to increased FV consumption [6]. While forearm blood flow response to intra-arterial acetylcholine is an established predictor of cardiovascular morbidity [7], the technique remains invasive, labour-intensive and unsuitable for use during larger, multicentre trials.

Measurement of relevant, informative biomarker concentrations within venous blood and/or urine may be a potentially useful alternative. The American Heart Association (AHA) has recognised high sensitivity C-reactive protein (hsCRP) as a risk stratification tool [8] and recent data suggest that hsCRP reduction by statin therapy is an effective primary prevention strategy among individuals with favourable lipid profiles [9].

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are thought to play important roles in mediating leukocyte infiltration of the evolving atherosclerotic plaque [10]. Activated or injured endothelial cells shed ICAM-1 and VCAM-1 into the circulation and plasma concentrations of these molecules independently predict incident coronary heart disease in healthy populations [11,12].

In addition to its vital role in normal haemostasis, the circulating glycoprotein Von Willebrand factor (vWF) is recognised as a useful marker of endothelial integrity [13]. During a 16 year follow-up, healthy men with higher serum vWF concentrations were significantly more likely to suffer a myocardial infarction [14].

The endothelium-derived serine protease plasminogen activator inhibitor-1 (PAI-1) inhibits endogenous fibrinolysis and elevated plasma levels have been associated with an increased incidence of heart attack and stroke [15]. A significant negative correlation between plasma PAI-1 levels and brachial flow-mediated dilatation among healthy volunteers has also been described [16].

Oxidation both contributes to and follows on from the continuous cycle of low-grade vascular inflammation which characterises atherosclerotic arterial degeneration [17]. Isoprostanes are derived from the non-enzymatic peroxidation of arachidonic acid and have become a popular, widely employed measure of systemic oxidative stress in cardiovascular disease [18].

Using stored blood and urine samples we examined the effects of an 8 week FV intervention on these markers of inflammation, endothelial activation and oxidative stress. In addition, since cross-sectional data has linked dietary patterns characterised by high fruit and vegetable consumption with improved insulin sensitivity [19,20], a measure of insulin resistance was calculated pre and post-intervention.

## Methods

Details of this randomised dietary intervention trial have been published elsewhere [6]. Briefly, volunteers with brachial blood pressure in the range 140—190/90—110 mmHg were recruited from medical outpatient clinics. Following a common 4 week run-in period during which FV consumption was limited to 1 portion/day, participants were randomised to 1, 3 or 6 portions daily for the next 8 weeks. Compliance with the prescribed allocation was assessed using a series of contemporaneously completed 4-day food diaries. Vascular health was assessed immediately before and after this 8 week period. Patients attended a dedicated clinical research facility between 8 and 9 am having fasted from midnight the previous evening. They were also asked to refrain from caffeine, alcohol and smoking for at least 12 h pre-testing. Following 15 min supine rest, brachial blood pressure was measured and a 30 mL venous blood sample collected from the dominant arm. Volunteers then provided a spot urine sample before arterial cannulation and venous occlusion plethysmography.

All blood and urine samples were processed and frozen at −80 °C within 2 h of collection.

High sensitivity CRP levels were determined in serum using an immunoturbidimetric assay (Randox, Crumlin, Northern Ireland) on the ILab-600 biochemical analyser (Instrumentation Laboratories, Warrington, UK). Plasma concentrations of soluble ICAM-1 (sICAM-1) and (sVCAM-1) were determined using enzyme linked immunoassay (ELISA) kits (ImmunoDiagnostic Systems Ltd, Boldon, England). A Triturus analyser with dedicated software (Grifols Diagnostics, Barcelona, Spain) was used for all ELISA analyses. An immunoturbidimetric assay (STA-Liatest, Diagnostica Stago, Asnieres, France) was used in the Regional Coagulation Laboratory (Belfast City Hospital), to quantify plasma levels of vWF antigen. Intra and inter-assay CVs were <15%. PAI-1 was assessed using a sandwich ELISA assay (Trinity Biotech, Wicklow, Ireland) on an automated ELISA system.

Total urinary $F_2$-isoprostane concentrations were measured as an estimate of systemic oxidative stress using the method outlined by Roberts and Morrow [21]. Following extraction, derivatized spot urine samples were analysed

by gas chromatography-mass spectrometry using a Trace GC Ultra − DSQ II MS system (Thermo Fisher Scientific, Massachusetts, USA). The intra-assay CV for this technique was <12%. Total $F_2$-isoprostane content was standardised for urinary creatinine. The latter was measured using an enzymatic method (Randox, Crumlin, Northern Ireland) by automated colorimetric laboratory analysis (ILab-600, Instrumentation Laboratories, Warrington, UK).

Glucose was measured in serum by enzymatic colorimetric assay on a Roche modular analyser (Roche Diagnostics Ltd, West Sussex, UK). Insulin was measured in serum using Micro particle Enzyme Immunoassay (MEIA) technology (Abbott Laboratories, Berkshire) on an Abbott IMX analyser.

A measure of insulin resistance was derived from fasting glucose and insulin using the homeostasis model assessment (HOMA, fasting glucose (mmol/l) × fasting insulin (μmol/l)/22.5) in which higher scores denote low insulin sensitivity [22].

### Statistical analysis

The principle analysis for each variable was a between intervention group comparison of change using 1-way analysis of variance (ANOVA). The relationship between endothelium-dependent vasodilatation as defined by maximum response to acetylcholine and each biomarker measured at baseline was assessed using Pearson's correlation coefficients. The same approach was used to compare change in maximum response to acetylcholine with change in each biomarker. Non-parametric variables were log-transformed prior to analysis. All tests were 2-tailed and $p < 0.05$ was considered significant. Analyses were performed using SPSS version 17.0.1 (SPSS Inc. Chicago, IL).

As outlined previously a power calculation was performed based on the primary endpoint, endothelium-dependent vasodilatation [6].

## Results

A total of 147 eligible individuals initially agreed to take part in this study. There were 29 dropouts during the common 1 portion/day run-in phase. Only 1 volunteer failed to complete the 8 week intervention meaning that a total of 117 participants attended for vascular assessment on 2 occasions. Five individuals had pre-intervention serum hsCRP concentrations >10 mg/L which in accordance with AHA guidelines [8] were interpreted as consistent with active infection or inflammation, prompting exclusion of those volunteers from further analyses.

Throughout the 12 week trial period, participants were asked to minimise changes in other health and lifestyle behaviours. Both they and their general practitioners were asked to inform study investigators about any newly prescribed medications.

### Baseline characteristics

Pre-intervention characteristics of the remaining 112 participants according to FV allocation are summarised in Table 1. There were no statistically significant differences between groups at baseline. Data from 4-day food diaries completed during the common 1 portion/day run-in period suggest good compliance with the washout.

### Intervention-associated changes in markers of inflammation, endothelial activation and oxidative stress

Concentrations of hsCRP, ICAM-1, VCAM-1, vWF, PAI-1, urinary $F_2$-isoprostane and HOMA scores are summarised in Table 2. Despite increases in reported FV consumption and accompanying changes in biomarkers of FV consumption [6], indicating good compliance with the intervention, there

**Table 1** Pre-intervention phase characteristics of 112 participants according to daily fruit and vegetable allocation.

| | 1/day ($n$ = 33) | 3/day ($n$ = 39) | 6/day ($n$ = 40) |
|---|---|---|---|
| Age (years) | 52.4(7.9) | 56.1(8.4) | 53.7(7.1) |
| Men (%) | 70 | 62 | 63 |
| BMI (kg/m$^2$) | 29.7(4.4) | 28.2(3.2) | 28.8(3.3) |
| Systolic blood pressure (mmHg) | 139.4(15.0) | 144.6(18.1) | 145.3(15.7) |
| Diastolic blood pressure (mmHg) | 82.0(11.9) | 81.1(11.1) | 86.3(11.0) |
| Current smokers (%) | 39 | 3 | 15 |
| Former smokers (%) | 36 | 44 | 35 |
| Antihypertensive therapy (%) | 36 | 46 | 63 |
| Lipid-lowering therapy (%) | 52 | 67 | 55 |
| Triglycerides (mmol/l)* | 2.10(0.53) | 1.75(1.54) | 1.56(1.43) |
| Total cholesterol:HDL ratio | 4.37(1.35) | 4.18(1.45) | 4.31(1.31) |
| Serum glucose (mmol/l)* | 6.02(1.40) | 5.98(0.95) | 5.82(0.97) |
| Serum insulin (μmol/ml)* | 10.67(15.60) | 8.27(3.90) | 8.76(8.67) |
| Fruit and vegetable intake (portions/day)* | 0.9(0.3) | 1.0(0.5) | 1.1(0.6) |

For all variables $n$ = 33; 39; 40 for the 1-;3-;6-portions/day groups respectively, except for triglycerides, cholesterol:HDL ratio and CRP, where $n$ = 32; 39; 38. Continuous variables are summarised as mean ± standard deviation except for * where data is skewed and a geometric mean with interquartile range is quoted.

**Table 2** Biomarker concentrations following the run-in (pre) and intervention (post) phases with *p* values from between group comparisons of change by one-way ANOVA for each variable.

| | 1/day $n = 29$ | | 3/day $n = 35$ | | 6/day $n = 34$ | | *p* for ANOVA |
|---|---|---|---|---|---|---|---|
| | Pre | Post | Pre | Post | Pre | Post | |
| hsCRP (mg/l) | 2.03(1.29) | 1.90(1.87) | 1.71(1.56) | 1.39(1.04) | 1.49(1.08) | 1.47(1.42) | 0.95 |
| ICAM-1 (ng/ml) | 692(358) | 698(318) | 605(203) | 583(202) | 545(189) | 572(189) | 0.44 |
| VCAM-1 (ng/ml) | 779(343) | 713(306) | 803(341) | 672(253) | 696(368) | 653(268) | 0.25 |
| vWF (IU/ml) | 0.92(0.46) | 0.93(0.57) | 1.00(0.27) | 1.00(0.40) | 0.91(0.31) | 0.93(0.30) | 0.99 |
| PAI-1 (ng/ml) | 58.30(28.19) | 58.58(35.29) | 50.20(29.05) | 50.33(21.09) | 48.43(34.86) | 53.06(35.68) | 0.35 |
| $F_2$-Isoprostane: creatinine (ng/mg)[a] | 1.63(1.09) | 1.40(1.65) | 1.09(0.93) | 1.07(1.39) | 1.44(1.24) | 1.31(1.22) | 0.29 |
| HOMA Score | 2.86(3.70) | 2.90(3.00) | 2.20(1.29) | 2.52(1.81) | 2.41(2.55) | 2.64(2.79) | 0.27 |
| Fruit and Veg consumption (portions/day) | 0.9(0.3) | 1.1(0.5) | 1.0(0.5) | 3.2(0.8) | 1.1(0.6) | 5.6(0.9) | <0.01 |
| β-cryptoxanthin (μmol/l) | 0.05(0.05) | 0.05(0.04) | 0.06(0.05) | 0.07(0.09) | 0.07(0.07) | 0.11(0.10) | <0.001 |
| Lutein (μmol/l) | 0.13(0.07) | 0.14(0.07) | 0.15(0.08) | 0.17(0.11) | 0.16(0.08) | 0.20(0.12) | 0.002 |
| VitaminC (μmol/l) | 23.7(18.8) | 25.8(18.9) | 25.7(15.8) | 38.9(24.3) | 27.9(25.8) | 42.3(28.1) | 0.06 |

All variables are summarised as geometric mean (interquartile range).
hsCRP — High sensitivity C-reactive protein, ICAM-1 — Intercellular adhesion molecule-1, VCAM-1 — Vascular cell adhesion molecule-1, vWF — von Willebrand factor, PAI-1 — Plasminogen activator inhibitor-1, HOMA — Homeostasis model assessment.
[a] For spot urine samples $n = 28$, 35 and 34 for 1, 3 and 6-portions/day respectively.

were no significant between group differences in change for any of the biomarkers measured. Similarly, the intervention did not alter insulin resistance as assessed by HOMA scores.

### Relationship between biomarkers and endothelium-dependent vasodilatation

At baseline, a significant negative correlation was noted between HOMA score and maximum response to acetylcholine ($r = -0.247$, $p = 0.012$). No significant correlations were noted between change in maximum response to acetylcholine over the course of the intervention and change in any of the biomarkers measured (Table 3).

## Discussion

Despite mediating improved endothelium-dependent vasodilatation, increased FV consumption over an 8 week period did not significantly alter the circulating concentrations of hsCRP, ICAM-1, VCAM-1, vWF, PAI-1, change urinary $F_2$-isoprostane excretion or improve insulin sensitivity as assessed by HOMA score. Although this was not a controlled feeding study, 4-day contemporaneous food diary data and appropriate increases among selected circulating micronutrient concentrations suggested good compliance with the prescribed dietary intervention.

Cross-sectional studies, largely employing food frequency questionnaires, continue to associate higher FV intake with lower serum levels of hsCRP [23,24]. In a study similar to the current work, 64 healthy volunteers consumed either 2, 5 or 8 portions of carotenoid-rich FV daily for 4 weeks [25]. Between group-comparisons of change in serum hsCRP showed a significantly greater reduction among those eating 8 portions/day. Participants in Watzl et al's study were healthy, exclusively male, non-smokers who habitually consumed up 4—5 servings of fruit and vegetables daily and thus direct comparisons with the current study are difficult.

**Table 3** Relationship between baseline/intervention-associated change in maximum response to acetylcholine and baseline/change in biomarker.

| Variable[a] | Correlation coefficient at baseline | *p* | Correlation coefficient for change | *p* |
|---|---|---|---|---|
| hsCRP | −0.11 | 0.28 | 0.11 | 0.28 |
| sICAM-1 | −0.03 | 0.71 | −0.46 | 0.65 |
| sVCAM-1 | −0.10 | 0.31 | 0.01 | 0.89 |
| vWF | −0.10 | 0.31 | 0.08 | 0.42 |
| PAI-1 | 0.08 | 0.41 | −0.05 | 0.42 |
| Urinary $F_2$-isoprostane | −0.10 | 0.33 | 0.17 | 0.87 |
| HOMA score | −0.25 | 0.01 | −0.01 | 0.92 |

hsCRP — High sensitivity C-reactive protein, ICAM-1 — Intercellular adhesion molecule-1, VCAM-1 — Vascular cell adhesion molecule-1, vWF — von Willebrand factor, PAI-1 — Plasminogen activator inhibitor-1, HOMA — Homeostasis model assessment.
[a] In each case the differences between log-transformed variables were compared.

Short-term dietary FV intervention trials in cohorts at an increased cardiovascular risk have generally reported negative findings when hsCRP is employed as an endpoint [26–28]. This lack of effect on CRP is also shown in other dietary intervention studies where significant improvements in a functional vascular measure are reported. Among volunteers with abdominal obesity, a 2 month Mediterranean diet, which included a significant increase in FV consumption, significantly improved flow-mediated dilatation (FMD) of the brachial artery but failed to change serum hsCRP [29]. Similar results have been reported for a 5 week soy-diet intervention in renal transplant patients [30]. While trials designed to produce weight loss describe associated reductions in plasma PAI-1 concentrations [31,32], more specific dietary interventions (for example, with fatty acids or Mediterranean diet) have generally failed to alter either fibrinolytic parameters or circulating vWF levels [33,34]. When 112 volunteers at increased cardiovascular risk were randomised to a low-fat or Mediterranean diet for 3 months, significantly reduced concentrations of plasma sICAM-1 and sVCAM-1 were observed in the latter group [35]. Investigators have also reported down-regulation of these adhesion molecules following 4 week red wine [36] and 8 week soy nut interventions [37]. Such positive findings contrast with those reported here and may be related to a number of factors. Mena and colleagues studied more comprehensive dietary revisions, for longer intervention periods, in a cohort where the prevalence of type 2 diabetes approached 70% [35]. The red wine and soy nut studies recruited medication-naive, healthy volunteers while again employing a more specific test-intervention.

Investigators have reported that urinary levels of $F_2$-isoprostane were significantly reduced among healthy women consuming 9–10 portions of fruit and vegetables daily for 8 weeks [38]. Since these investigators recruited non-smoking, medication-free volunteers from a womens' health group, extrapolation to the current cohort is difficult. It remains possible, however, that 6 FV portions daily is an insufficient quantity to significantly reduce systemic oxidative stress. While significant reductions in plasma isoprostane concentrations have been reported following a 14 day FV soup intervention among 12 healthy volunteers, these findings have not been replicated in larger, longer studies employing fruit [39], antioxidant [40], and flavonoid-rich food [41] supplementation.

A previous study examining the effects of regular black tea consumption among healthy volunteers noted improved brachial flow-mediated dilatation without any change in urinary isoprostane excretion [42]. This is perhaps unsurprising since cross-sectional research describes generally poor correlations between endothelium-dependent forearm blood flow and isoprostane concentrations in urine [43].

Italian investigators have reported that randomisation to a Mediterranean diet, which included higher FV content, significantly reduced HOMA scores among volunteers fulfilling 'metabolic syndrome' criteria [44]. This 24 month intervention was much longer than that presented here and also mediated significant improvements in weight and blood pressure. A recent 4 week plant-based low carbohydrate dietary intervention in dyslipidaemic patients was characterised by increased FV consumption but failed to significantly alter HOMA scores [45].

The relationship between reduced endothelium-dependent vasodilatation and impaired glucose tolerance has been well described [46] and thus our finding of a significant negative correlation between HOMA score and maximum response to acetylcholine is not surprising. Changes in these parameters were not related however, suggesting that FV-associated improvements in endothelium-dependent vasodilatation are not mediated by enhanced insulin sensitivity. Alternatively, a more sensitive test of glucose tolerance, such as the euglycaemic clamp technique, may be necessary to further explore this hypothesis.

The current work did not exclude volunteers prescribed regular antihypertensive or lipid-lowering therapy and it is possible that these agents may have masked any effect of FV consumption on biomarker concentrations. Equally, to increase the clinical and public health applicability of our findings, a controlled feeding approach was not employed and it remains possible that additional, non-prescribed, dietary or lifestyle intervention-associated changes occurred. Circulating salicylic acid concentrations have recently been shown to have a significant positive correlation with fruit and vegetable consumption among healthy volunteers who were not taking aspirin [47]. This interesting observation suggests that salicylic acid measurement may prove useful when considering future studies to examine the relationship between diet and systemic inflammation.

Short-term intervention studies which employ surrogate end-points to detect a biological effect are performed frequently in cardiovascular research. The current findings suggest that increased FV consumption does not alter circulating hsCRP, sICAM-1, sVCAM-1, vWF, PAI-1 or urinary isoprostane concentrations. These biomarkers appear dissociated from the observed improvements in endothelium-dependent vasodilatation and are thus unlikely to serve as informative measures for future similar studies.

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