Effects of a beverage rich in (poly)phenols on established and novel risk markers for vascular disease in medically uncomplicated overweight or obese subjects: a four week randomized trial

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ABSTRACT

Objective: To determine if **(**poly)phenols alter cardiovascular risk factors, we assessed the potential of a high (poly)phenol beverage drink, rich in hydroxycinnamates and flavonoids, to modify vascular function in middle aged, overweight or obese subjects without medical co-morbidity in a randomized placebo controlled pilot study. *Methods:* Randomly assigned active 250ml beverages containing 361mg of (poly)phenols and 120mg of vitamin C or placebo (no polyphenol / vitamin C) were taken twice daily for 4 weeks. Both beverages contained 40 kcals/250 ml. The primary end-points were pulse wave velocity (PWV) and cutaneous microvascular responses to sodium nitroprusside (SNP) and acetyl choline (ACh) laser doppler iontophoresis. A range of established and novel plasma markers were also measured. *Results:* Twenty subjects received active beverage and 19 placebo; all completed the study. There was no difference in cutaneous vascular response to either SNP or ACh with mean group differences (logΔ area under perfusion curve) of 0.30 (-0.65, 1.26) and 0.35 (-0.11, 0.81) respectively. Nor was there evidence of a change in log PWV with a mean group difference of 0.029m/s (-0.042, 0.10). No significant differences were seen in plasma leptin, apolipoproteins, cystatin C, insulin, adiponectin, CRP, ICAM-1, E-Selectin or t-PA, but IL-6 increased in active versus placebo recipients (0.32 vs – 0.18 pg/ml; p=0.010). *Conculsion:* There was no evidence for a short-term beneficial effect of (poly)phenol intervention on microcutaneous vascular response or pulse wave velocity, and no evidence for a benefit on established or novel risk factors in overweight or obese subjects. Our results do not support a short-term benefit of (poly)phenol supplementation on cardiometabolic risk.

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Abbreviations:

PWV pulse wave velocity; SNP sodium nitroprusside; ACh acetyl choline, HPLC-MS^2 high performance liquid chromatography-tandem mass spectrometry

1. Introduction

Epidemiological data support consumption of fruits and vegetables in the reduction of cardiovascular risk (1-5) leading to supportive recommendations by the World Health Organisation (6), European Society of Cardiology (7) and jointly the American College of Cardiology and American Heart Association (8). Many components may contribute to benefits including potassium, non-starch polysaccarides, carotenoids, vitamin C and E and phenolic compounds with bioactive phytochemical constituents having a C_6-C_3-C_6 flavonoid structure. The potential benefits of dietary poly(phenolics) have in particular, received intense research interest. (9) The effects of nutritional intervention on "hard" cardiovascular clinical outcomes such as myocardial infarction, time to coronary reperfusion, and death, are difficult to assess in prospective clinical studies. Effects on surrogate risk markers for cardiovascular disease can give an early indication of future potential. In addition to traditional modifiable cardiovascular risk markers such as lipid profile, smoking, diabetes and blood pressure, a burgeoning number of novel plasma biomarkers (10) are joined by non-invasive measurements of vascular function such as pulse wave analysis / velocity and cutaneous microvascular responses measured by laser doppler iontophoresis (LDI).

A combination of (poly)phenols from fruits in a single beverage may provide administration and compliance benefits when compared to consuming primary fruit and vegetable sources. This pilot study evaluates the potential for such a beverage to modify a range of traditional cardiovascular risk markers along with novel plasma biomarkers and vascular function measurements. The primary end-point of the study was to evaluate vascular function using pulse wave studies, and LDI after intervention with the study beverage rich in

(poly)phenols compared to a matched drink without polyphenols. Secondary end-points addressed changes in established and range of novel risk factors.

2. Methods

A randomized, double blind, placebo controlled design was employed with a 1:1 allocation of subjects to parallel active and placebo arms. The beverages were manufactured and supplied by the Coca-Cola Company. The active drink contained 28% beverage, water and non-nutritive sweeteners (sucralose and acesulphame-K). The polyphenolic and hydroxycinnamate content was derived from green tea, grape seed, grape pomace, ruby red grape juice, lemon and apple extracts. The predominant active constituents in the 250ml beverage portion comprised the green tea derived flavan-3-ols (348µmol), mainly epigallocatechin (134µmol) and gallic acid (37µmol); 5-O-caffeoylquinic acid (33µmol) and dihydrochalcones (59µmol) from apples; citrus flavanones (36µmol); a range of flavonols (7µmol) and grape anthocyanins (5.6µmol). The grape seed component provided 72mg of procyanidins. The placebo beverage was coloured, flavoured and sweentened to be as similar as possible to the active beverage, but rendered (poly)phenol free, without beverage or extracts. The colouring and flavouring were felt highly unlikely to confer any biological activity. Properties of the active and placebo beverages are shown in Table 1. Bioavailability of a range of plasma and urinary metabolites including those related to the flavan-3-ols, flavanones, dihydrochalcones and 5-*O*-caffeoylquinic acid, was confirmed in 10 healthy volunteers following a diet low in flavonoids for 48 hrs (11). The blended active beverage delivered a similar range of flavonoid and phenolic compounds as if several different plant-derived fruits and tea were consumed independently.

Following local ethics committee approval and advertising in regional newspapers, subjects were initially telephone screened and then invited to a formal screening visit at which informed consent was obtained. Screening measurements included weight, height and blood pressure and 12 lead ECG. Biochemistry and haematology profiles were analysed by the routine National Health Service laboratory at the Western Infirmary, Glasgow and included urea and electrolytes for estimated glomerular filtration rate (eGFR), liver function tests (LFT), full blood count and glycosylated haemoglobin (HbA_1C). Suitable subjects included those over 55 years with a body mass index greater than 25 kg/m^2. Exclusion criteria included current and ex-smokers of less than 2 years, $HbA_1C > 5.5\%$, $eGFR<60ml/min/1.73m^2$, LFTs $\geq$ 3 times upper limit of normal, on medication for hypertension or BP >160mm Hg systolic, >90mm Hg diastolic, chronic disease associated with increased cardiovascular risk (cardiorespiratory, rheumatological, neoplastic), vitamin supplementation within the previous 14 days, and on medication likely to influence vascular function (eg corticosteroids). The study entry criteria were designed to include subjects with increased cardiovascular risk purely on the basis of their age and being overweight or obese. Following enrollment by the study team, subjects were randomized to receive active or placebo beverage, consecutively in groups of 10 balanced for gender. Randomization and subject assignment was arranged by the University of Glasgow School of Mathematics and Statistics; the study team was blinded to the allocation sequence. Previous studies using nutritional interventions to modify vascular markers were consulted for power estimations and a target of 40 completed subjects determined (12-16). There were no changes to the study protocol entry criteria or outcome measures following commencement.

The study was registered with Clinical Trials.gov, identifier NCT00795834. Guidelines issued by the National Patient Safety Agency (Research Ethics Service) were consulted to

confirm the status of the intervention as a food grade product. The principles of Good Clinical Practice were applied to all aspects of the study. Participants received a payment of £100 on completion as compensation for time spent on study activity, and transport costs were refunded.

All measurements were carried out at the British Heart Foundation Glasgow Cardiovascular Research Centre at the University of Glasgow. Subjects were advised to continue their normal diet and attend for baseline measurements fasted, in the morning, having completed a detailed food diary over the preceding 72 hours. The primary end-points were pulse wave velocity (PWV) and cutaneous microvascular responses to sodium nitroprusside (SNP) and acetyl choline (ACh) laser doppler iontophoresis. Secondary outcomes included anthropometric measurements and a range of established and novel plasma markers. A defined sequence was followed: 1. Laser doppler iontophoresis (LDI): The protocol used for assessment of microcutaneous skin response by LDI has been validated (17) and described in detail in clinical studies (12, 13). In brief: in a temperature controlled room and with skin temperature acclimitisation, cutaneous microvascular responses to topically applied vasoactive acetyl choline (ACh) and sodium nitroprusside (SNP) were assessed using a laser Doppler imager (Moor LDI-2; Moor Instruments, Axminster, UK). Iontophoresis was contained within 2 topically applied wells (ACh in the anodal chamber and SNP in the cathode) and a total of 20 scans were conducted with incremental current delivery. Doppler images were then interrogated to calculate skin perfusion. Sigma Chemicals, Poole, UK provided the iontophoresis chemicals which were dissolved fresh for daily use in 0.5% NaCl stock solution to a concentration of 1%. 2. Anthropometry: Height was measured using a Seca® stadiometer; weight and bioelectrical impedance were jointly measured using a Tanita® BC-418MA Body Composition Analyser, and waist was determined using a non-stretch tape. 3. Blood Pressure recordings were made using a

DinaMap® Pro 200 according to the guidelines of the American Heart Association (18). 4. Pulse wave measurements were made using a SphygmorCor® tonometer device and immediate data analysis software (AtCor Medical Pty Ltd, West Ryde, NSW, Australia. Pulse wave analysis at the wrist determined aortic augmentation index (AAI), and pulse wave velocity (PWV) was measured using carotid and femoral arterial waveforms. Using the live quality control analysis, a minimum of 2 acceptable recordings of AAI and PWV were obtained. 5. Phlebotomy provided 20ml of venous blood which was immediately centrifuged with plasma aliquots stored at -80ºC. Batch analysis was conducted at completion of the study by the Department of Biochemistry at Glasgow Royal Infirmary. Enzymatic colorimetric assays were conducted for cholesterol and triglyceride; immunoturbidimetric assays were used for ApoB, A1 and sCRP. Analysis was carried out on a Hitachi Modular P analyzer using kits supplied by Roche Diagnosics, Germany. VLDL and LDL Cholesterol results were calculated using the Friedwald Equation. IL-6, ICAM, sE-Selectin and Adiponectin were analysed using a Multiskan Ascent Plate reader and software (Thermolife Sciences, Basingstoke, UK) and kits provided by R&D Systems Europe Ltd, Abingdon, UK. Insulin was measured using the Mercodiaultrasenstive insulin ELISA. Leptin analysis was performed using an in-house assay; the methodology has been previously published (19). t-PA was measured with an ELISA (Biopool AB, Umea, Sweden).

Following baseline measurements, subjects commenced their allocated study beverage, consumed as 250ml doses twice daily for 4 weeks and were advised to continue their normal diet. A 2 week supply of beverage was provided at the baseline visit. Subjects were advised to store the beverage in their domestic refrigerator. Blinded reserve batches were available for substitution in the event of damage or loss to the supplements. The properties of the placebo and active beverages are described in Table 1. The individual (poly)phenolic components in the

active beverage were analysed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS^2) and the amounts detected were in keeping with the quantities found in an earlier batch of the drink used in an acute feeding study (11). The (poly)phenolic compounds were not present in detectable amounts in the placebo drink.

Subjects attended a mid-point visit, timed to occur 60 minutes after administration of the study beverage with subjects otherwise fasted. The first voided urine in the morning prior to administration of the beverage was collected, along with a second urine sample approx. 120 min following administration. Venous blood was drawn at 60 - 90 minutes post administration. Plasma and urine samples were frozen at -80 degrees (plasma being archived for possible future analysis). Urine was analysed by HPLC-MS^2 as described by Borges et al. (11) in order to assess the presence of flavan-3-ol and dihydrochalcone metabolites derived from intake of the (poly)phenol-rich drink. Compliance was also checked by reconciliation of returning packaging; tolerance was noted including any adverse events and the second 2 week supply provided. The end-point visit was conducted on completion of the study beverage, with the final dose taken on the morning of the visit. To achieve dietary replication before the 2 main testing points a detailed food diary over the preceding 72 hours was again completed, referring to the diary completed prior to the baseline visit. Measurements were identical to the baseline visit. The study procedure is summarized is Figure 1.

Baseline characteristics were compared using 2 sample t-tests or rank-sum for non-parametric data. LDI perfusion data was extracted from the scan images using Moor software and corrected for skin resistance, an important factor in interpretation of LDI data (20, 21). Perfusion data for each of the 20 scans per visit in anode and cathode wells were converted to an area under curve (AUC) result. The AUC data were examined for normality and log-

transformed where appropriate. Differences in the log transformed AUC data between follow-up and baseline visits were calculated for SNP and ACh studies in the active and placebo groups and comparisons made using 2 sample t-tests. For PWV and AAI data, and all secondary outcomes, paired differences were calculated - a positive value meaning the measure increased over time. Two-way ANOVA on paired differences was determined with an additive model: Gender + Group. If residuals appeared normal, analysis proceeded; otherwise, differences of logs were taken. The p-value for Gender effect was noted: if Gender effect not significant, groups were compared using 2-sample t test (pooled SD); if Gender effect significant, groups were compared adjusting for Gender in ANOVA. Statistical analysis was carried out using Minitab 15 with the significance level set at $p<0.05$ and no adjustment for multiple testing. Specific analysis of the dietary records was not intended: they were included as an aide memorie to allow replication of the diet before the 2 testing points.

3. Results

Recruitment and study procedures were conducted over a 6 month period from July 2008 to January 2009. 95 subjects responded to advertisements and were checked for eligibility by telephone resulting in 53 invitations for formal screening and 49 attendances. Entry criteria resulted in 10 exclusions (BMI: 1, Blood Pressure: 2, Medical history/ECG: 2, HbA1c: 5) yielding 39 subjects for enrolment. The aim to reach the recruitment target of 40 subjects was balanced with time constraints for study completion considering the shelf-life of the investigative product. Baseline characteristics were compared for placebo and active groups (Table 2) and demonstrated equivalence in the 2 groups.

All 39 enrolled subjects completed the study. There were no serious adverse events and tolerability was good. Compliance with study beverage determined by reconciliation of returned packaging was excellent (99% in active, 98% in placebo). In addition, HPLC-MS^2 analysis detected ten flavan-3-ol and three phloretin metabolites, principally glucuronides and sulfates, that had previously been shown to be indicative of intake (11), in the urine of 19 of the 20 participants in the active arm but none of those who consumed the placebo drink. The results in Table 3 demonstrate no significant change in the primary end-points of PWV, or microcutaneous skin vascular responses measured by LDI in active versus placebo recipients. There was a trend ($p=0.078$) towards reduction in AAI. Similarly, no significant differences in adiposity (weight, waist or plasma leptin) or blood pressure are noted although systolic blood pressure trended ($p=0.097$) towards reduction in active drink recipients.

Results in Table 4 show no significant changes in any lipid, renal or metabolic parameter. However, a significant ($p=0.01$) increase in plasma IL-6 was noted in active versus placebo recipients. None of the outcomes were altered by repeat analysis excluding data from two subjects with likely acute inflammation indicated by elevations in CRP beyond 20mg/l at any measurement point during the study.

4. Discussion

The results of this study did not demonstrate evidence for improvement in microvascular function measured by LDI, or in vascular stiffness measured by PWV following 4 weeks supplementation with a (poly)phenol-rich fruit and tea-derived beverage in overweight or obese

subjects otherwise free from medical comorbidity and possibly considered “metabolically healthy obese”, although, as discussed below many were at medium cardiovascular risk by modern standards. Plasma markers of adiposity, lipid profiles, renal and metabolic markers and markers of inflammation and endothelial activation did not demonstrate any significant change following ingestion of the active supplement. The exception to this was IL-6 which exhibited a statistically significant increase in subjects receiving the active beverage. IL-6 increases are thought to reflect a heightened inflammatory state. This result was rather unexpected and is not easily explained, unless as a Type I statistical error, or represents a regression to the mean. Nevertheless, it is important to note that IL-6 signaling is potentially causally linked to cardiovascular risk (22); the intriguing possibility that an intervention rich in polyphenols might exert a pro-oxidant effect is worthy of examination in future relevant trials.

The number of subjects was similar to those in other studies of this type, but inevitably the power constraints, in particular the between-subject variability, raise the possibility of a type II statistical error. However other factors may explain the results. Cardiovascular (CV) risk assessment at baseline using the Joint British Society risk calculators suggested that the majority of subjects were at medium risk with six (15%) having a CVD risk above 20% risk threshold. Nevertheless, all were overweight and the majority were in the obese category; obesity is a strong risk factor for diabetes and other cardiometabolic outcomes and known to lessen life expectancy (23). Other researchers using subjects at greater CV risk have found improvements in brachial artery flow mediated dilatation (FMD) measurements following isoflavone supplementation for 12 weeks in patients with prior ischaemic stroke (24) and similar findings have been reported with black tea (25) and purple grape juice (26) in subjects with angiographically proven coronary artery disease, though the latter was not a randomized trial.

Green tea extracts in Japanese subjects with visceral obesity led to reductions in systolic blood pressure however the CV risk status of the included subjects is not clear (27). Conversely, increases in systolic blood pressure were seen with a combination of vitamin c and grape-seed polyphenols in a study of treated hypertensives (28). Thus, in totality, including the results from the present study, one cannot infer a consistent effect of short term supplements on cardiovascular risk surrogates in properly conducted randomized trials of which there are only but a few such high quality studies.

Alcohol ingestion was not specifically limited in study subjects: intake was minimal in most subjects however a small number recorded significant consumption in their food records. Studies have suggested a potentially protective effect of alcohol on vascular function (29) but it is more likely that this is a U-shaped relationship with detrimental effects at excessive levels (30). Subjects did attend visits fasted, including abstaining from alcohol from the preceding night, however longer term effects of alcohol on vascular reactivity would not be mitigated by this. Another confounding factor relates to the usual diet of subjects during the study phase. Ideally, to investigate the effect of a (poly)phenol-rich beverage, all other dietary (poly)phenols would be eliminated. Experience in the preliminary feeding study (11) highlighted how difficult a (poly)phenol-free diet is to adhere to rendering this impracticable in a short to medium term nutritional study. Diet records provided some insight but were primarily in place to ensure replication of diet in the days before visits – the only practical way to exert some control over dietary confounding. We did not intend to perform a detailed analysis of the diet records however a provisional review of the quality of the dietary recordings suggested that they were unlikely to provide meaningful results, or alter interpretation of the outcome. We elected not to ask subjects to keep a dietary record for the entire 4 week study period as it would have been

similarly unlikely to have contributed useful data and would have been particularly onerous to complete, threatening study compliance. Dietary records are notoriously inaccurate particularly in overweight and obese subjects who usually under-report intake. The absence of any weight change in subjects during the study period strongly suggests that there were no significant dietary fluctuations. Variations in diet and the inability to accurately control or record this is a significant confounding factor in studies of this type but there are few practical alternatives. An alternative approach, adopted by Plotnick et al assessed the ability of fruit and vegetable concentrates taken over 4 weeks, to mitigate the acute effects of a high fat meal on vascular reactivity. Those supplemented with the phytochemical concentrates exhibited blunting of the effect of high fat ingestion on brachial artery FMD measurement (31).

The administration of multiple fruit constituents in a single beverage offers compliance benefits and is an attractive means to assess the potential of phytochemicals to modify vascular risk markers. Our preliminary bioavailability study confirmed excellent (poly)phenol bioavailablity and tolerance. Recovery of urinary metabolites and plasma pharmacokinetics of flavan-3-ol, flavanone and dihydrochalcone phase II metabolites absorbed in the proximal and distal gastrointestinal tract were broadly comparable to other feeding studies with green tea, coffee, apple cider, orange juice and green tea (11). The active beverage delivered a daily polyphenol dose of 722mg; comparable to that reported in observational studies examining the effect of a Mediterranean Diet high in (poly)phenol foods on cardiovascular events (32).

Much of the research in this field involves small studies, relatively few of which are placebo controlled or indeed randomized or blinded. All of these interventional studies, including this research, depend on surrogate markers such as LDI, Pulse Wave studies, FMD or the plethora of proposed plasma biomarkers. These are convenient to measure at time-points before and after an

intervention but it should be stressed that they are not a replacement for true cardiovascular outcomes such as myocardial infarction and stroke. This pilot study has shown no convincing evidence for beneficial effects on cardiovascular risk markers and joins a very limited number of mainly negative studies in similar populations (33). That noted, pilot evidence for an effect on a urinary proteomic-derived coronary artery disease biomarker was noted after 2 weeks with the active beverage in this study (34), similar to another study in healthy volunteers taking olive oil for an equivalent duration(35), though the relevance of these changes for future risk is not yet established. Larger studies with longer follow up, possibly over several years, and including, where possible, cardiovascular risk markers such as carotid intima-media thickness measurements are necessary to better assess the potential for phytochemicals to modify vascular risk. We recognize that effects of diet on health may develop over longer time-spans, although of interest, some lifestyle interventions (e.g. increased activity) can show rapid benefit. Ingestion of flavan-3-ols such as cocoa has been associated with lower blood pressures in an observational study of elderly men (36) and improved coronary vasodilatation after acute consumption in heart transplant recipients (37), suggesting rapid changes in functional measures with flavan-3-ols. It remains possible however that, despite suggestive in vitro evidence and supportive prior trials, isolated (poly)phenols may not exert beneficial effects on CV risk. Positive reporting bias is a particular problem in interventional studies, particular in the field of nutritional supplementation, and as such our results are a timely reminder for the need for more detailed and robust trials in this area in the future.

For the purposes of this short-term study examining the effects of a nutritional interventional in isolation, it was necessary for subjects to maintain their usual diet and no significant weight loss was desired or observed during the study. It is highly possible that beneficial effects of a

nutritional intervention will be attenuated or negligible without concomitantly addressing activity levels and weight loss. In other words, our results could be interpreted as further highlighting the importance of the need to change usual lifestyle risk factors (diet and activity levels) to alter risk, rather than assuming supplements will lessen risk for which robust evidence is largely missing.

5. Summary

In summary, in this randomized trial, we could not demonstrate any evidence for a short-term beneficial effect of a (poly)phenol-enriched drink on microcutaneous vascular response or pulse wave velocity, and no clear evidence for a benefit on established or novel plasma-based risk factors.

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Conflicts of Interest:

Dr William Mullen was involved in the formulation of the investigative beverage but has no financial interest in its development or in the sponsoring company.

Susan A Roberts is an employee of the Coca-Cola Company.

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Highlights

What Is New?

- Whilst there are supportive observational data, randomised placebo-controlled trials on polyphenol are sparse but urgently needed
- Our study which meets CONSORT criteria is one of the best in this area using a range of vascular and established and novel risk factors

What Is Relevant?

- (Poly)phenols are suggested to protect against cardiovascular risk but data quality are weak

Summary of the conclusions of the study

- We did not demonstrate any evidence for a short-term beneficial effect of a (poly)phenol-enriched beverage on a range of established or novel cardiovascular risk factors in predominantly obese subjects

Table 1: Key properties of Investigational Beverages

	Active	Placebo
Volume (single unit)	250ml	250ml
Total (poly)phenol content (mg)	361	Negligible
Vitamin C content (mg)	120	0.00
Caloric value (Kcal/250ml)	40.0	40.0
Vitamin E, carotenoids, cagnesium, potassium, sodium	Negligible	

Table 2: Baseline Characteristics:

Characteristic	**Active Group n=20**	**Placebo Group n=19**
	Mean; SD	**Mean; SD**
Demographics / non-invasive measures		
Sex (males)	10	10
Age, Years	60.30; 4.60	62.21; 4.26
BMI	33.08 6.68	31.49; 4.38
Waist cm	105.66;17.76	105.26; 13.69
%FAT	37.2	34.8
Family History Diabetes Mellitus* (y)	3	2
Family History CVD** (y)	11	8
Smoking Pack Years, Median (IQR)	4.81(18.38)	0.00 (6.00)
Alcohol Units / Week	11.35; 10.14	11.11; 14.37
Systolic Blood Pressure, mmHg	135.15; 14.56	136.68; 14.36
Diastolic Blood Pressure, mmHg	73.85; 8.70	79.11; 7.46
Ethnic Group: Caucasian	20	18
Blood results		
HBA1C %	5.43; 0.24	5.42; 0.21
Leptin, ng/ml	22.04; 13.02	20.24; 15.59
Cholesterol, mmol/l	5.50; 0.97	5.60; 0.87
Trigs, mmol/l	1.26; 0.54	1.40; 0.62
HDL-C, mmol/l	1.34; 0.36	1.20; 0.25
Apo AI, g/l	148.7; 27.0	140.2; 19.9
Apo B, g/l	104.0; 17.2	110.4; 20.6
Cystatin C, mg/l†	0.965; 1.13	0.960; 1.19
Fast. Glucose, mmol/l	5.32; 0.49	5.09; 0.24
Adiponect, ng/ml†	6701; 1.79	6836; 1.84
Insulin, pmol/l†	6.55; 1.55	5.70; 1.86
CRP, mg/l†	2.56; 2.03	2.20; 2.46
IL-6, pg/ml†	1.35; 1.92	2.03; 1.90
ICAM-1, ng/ml†	223; 1.19	213; 1.28
E-Selectin, ng/ml†	33.8; 1.82	32.8; 1.55
t-PA, IU/ml	9.21; 2.25	7.88; 2.26
Vascular function measures		
Log (ΔACh AUC)§	6.43; 1.27	7.04; 1.00
Log (ΔSNP AUC)§	6.22; 1.47	6.72; 1.03
Log (PWV) meters/sec	2.12; 0.16	2.20; 0.20
AAI	0.35; 0.094	0.36; 0.091
Log (ΔACh AUC)§	6.43; 1.27	7.04; 1.00

All results as Mean; SD unless otherwise stated; †Geometric Mean; Geometric SD.

* Type 1 or 2 DM in a first degree relative; **HT, IHD, PVD or Cerebrovascular disease in a first degree relative; §Results corrected for skin resistance. CVD – cardiovascular disease; IQR – interquartile range; HBA1C – glycosylated haemoglobin; HDL – high density lipoprotein; Apo A1- apolipoprotein A1; Apo B – apolipoprotein B; CRP – C reactive protein; IL-6 – interleukin-6; ICAM-1 –intracellular adhesion molecule 1; t-PA – tissue plasminogen activator; ACh – acetylcholine; SNP – sodium nitroprusside; AUC – area under curve; PWV – pulse wave velocity; AAI – aortic augmentation index

Table 3: Primary End Points: measures of Vascular Function / blood flow

	Active Group Mean diff (SD) N=20	Placebo Group Mean diff (SD) N=19	Group difference: Mean (95% CI)
	Primary end-points		
log(ΔACh AUC)*	0.00 (1.8)	-0.30 (0.89)	0.30 (-0.65, 1.26)
log(ΔSNP AUC)*	0.098 (0.77)	-0.25 (0.61)	0.35 (-0.11, 0.81)
$Log(PWV)_{meters/sec}$	0.013 (0.12)	-0.016 (0.10)	0.029 (-0.042, 0.10)
	Other vascular measure		
AAI	-0.022 (0.064)	0.011 (0.048)	-0.033 (-0.070, 0.004)

ACh – acetylcholine; SNP – sodium nitroprusside; AUC – area under curve; PWV – pulse wave velocity; AAI – aortic augmentation index

*Results corrected for skin resistance.

Table 4: Secondary End-Points

	Active Group Mean diff (SD) N=20	Placebo Group Mean diff (SD) N=19	Group difference: Mean (95% CI)
	Adiposity and blood pressure		
Weight, kg	0.40 (1.1)	0.63 (0.8)	-0.22 (-0.86, 0.42)
Waist, cm	0.01 (1.3)	-0.53 (1.5)	0.55 (-0.37, 1.47)
Leptin, ng/ml	0.86 (3.7)	0.42 (4.2)	0.44 (-2.13, 3.02)
%Body Fat	0.36 (1.8)	-0.68 (2.9)	1.05 (-0.55, 2.65)
Systolic BP, mmHg	-3.4 (13.3)	3.1 (10.2)	-6.5 (-14.2, 1.2)
Diastolic BP, mmHg	-1.6 (6.5)	-2.4 (4.5)	0.7 (-2.9, 4.4)
	Lipids and apolipoproteins		
Cholesterol, mmol/l	-0.044 (0.43)	0.044 (0.57)	-0.088 (-0.414, 0.239)
Trigs, mmol/l	0.003 (0.32)	0.099 (0.33)	-0.096 (-0.306, 0.114)
HDL-C, mmol/l	-0.017 (0.087)	0.034 (0.107)	-0.050 (-0.113, 0.013)
Apo AI, g/l	-1.05 (8.4)	3.20 (11.6)	-4.28 (-10.82, 2.26)
Apo B, g/l	0.34 (8.4)	0.40 (11.9)	-0.02 (-6.67, 6.64)
	Renal and metabolic markers		
log(Cystatin C, mg/l)	0.018 (0.074)	0.009 (0.053)	0.009 (-0.033, 0.051)

Fast. Glucose, mmol/l	-0.015 (0.43)	0.021 (0.40)	-0.036 (-0.308, 0.236)
log(Adiponect, ng/ml)	-0.007 (0.17)	0.063 (0.18)	-0.070 (-0.183, 0.043)
log(Insulin, pmol/l)	0.002 (0.37)	-0.015 (0.46)	0.024 (-0.230, 0.279)
Inflammatory and endothelial activation markers			
log(CRP, mg/l)	0.31 (1.01)	-0.13 (0.68)	0.44 (-0.12, 1.00)
log(IL-6, pg/ml)	0.32 (0.63)	-0.18 (0.48)	0.49 (0.13, 0.86)*
log(ICAM-1, ng/ml)	0.040 (0.090)	0.035 (0.090)	0.005 (-0.054, 0.063)
log(E-Selectin, ng/ml)	-0.006 (0.20)	-0.029 (0.25)	0.028 (-0.109, 0.164)
t-PA, IU/ml	0.36 (2.0)	0.34 (1.4)	0.02 (-1.10, 1.14)

*$p<0.05$

Apo A1- apolipoprotein A1; Apo B – apolipoprotein B; CRP – C reactive protein; IL-6 – interleukin-6; ICAM-1 – intracellular adhesion molecule 1; t-PA – tissue plasminogen activator

Figure 1:

Study Flow