

VEGF is indirectly associated with NO production and acutely increases in response to hyperglycaemia¹

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ABSTRACT

Background Increased levels of vascular endothelial growth factor (VEGF) have been observed in patients with metabolic syndrome (MetS). Nitric oxide (NO) formation is reduced in MetS, but its relationship to VEGF production remains poorly defined. We evaluated the association between VEGF/NO synthesis and insulin sensitivity in obese subjects and investigated the secretory response of VEGF to an acute elevation of glucose.

Materials and methods Seven healthy normal-weight subjects, seven obese subjects without MetS and seven obese subjects with MetS were recruited. Anthropometry, body composition and cardiometabolic functions (blood pressure, glucose, insulin, triglycerides, total cholesterol, HDL-C and VEGF) were measured, and a novel stable isotope method was used to assess *in vivo* rates of NO production. A frequent sampling intravenous glucose tolerance test was performed to study the dynamics of VEGF release.

Results Fasting VEGF levels were significantly higher in the two obese groups compared to the control group (P for trend = 0.02), but the difference was not significant after adjustment for age. Vascular endothelial growth factor levels were associated with systolic blood pressure ($\rho = 0.54$; $P = 0.01$) and NO production ($\rho = -0.44$; $P = 0.04$). Vascular endothelial growth factor levels increased in response to acute hyperglycaemia in normal-weight and obese subjects ($P < 0.001$).

Conclusions Vascular endothelial growth factor levels rapidly increase during hyperglycaemia and are inversely related to NO production at steady state. The potential link between the acute secretion of VEGF and atherosclerotic risk in subjects with poorly controlled glycaemia as well as the potential of lowering elevated VEGF levels by increasing NO production and/or availability warrants further investigation.

Keywords Hyperglycaemia, nitric oxide, obesity, vascular endothelial growth factor.

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Introduction

Angiogenesis is involved in the formation of new vascular networks in physiology (embryo/organogenesis, pregnancy, wound healing) and pathology (cancer, psoriasis) [1], and *de-novo* formation and remodelling of blood vessels are important events in the atherosclerotic process. Current research is investigating the role of angiogenesis as a risk factor for atherosclerosis and excess adiposity [2,3].

Vascular endothelial growth factor levels were directly associated with body mass index (BMI) but not with insulin

sensitivity in obese and normal-weight subjects [4]. Plasma VEGF levels were significantly associated with features of the metabolic syndrome (MetS) such as BMI, waist circumference, blood pressure and inflammation [5,6].

In vivo rates of nitric oxide (NO) synthesis are lower in metabolic disorders associated with insulin resistance [7]. In addition, we have recently demonstrated a reduction in NO production in obese subjects with MetS [8] and a direct association with insulin sensitivity [9], suggesting that VEGF may be inversely associated with NO production and insulin sensitivity. Here, we investigated this hypothetical association by comparing levels of VEGF in obese subjects with and without MetS to normal-weight controls. In addition, the acute secretory

¹The material presented in this manuscript is original and has not been submitted for publication elsewhere while under consideration for European Journal of Clinical Investigation.

response of VEGF to an intravenous glucose bolus was investigated in controls and obese subjects.

Methods

Subjects

Fourteen obese subjects (BMI: 30–40 kg/m²) and seven normal-weight subjects (BMI: 18.5–25.0 kg/m²) were recruited. Seven obese subjects had three or more criteria for the metabolic syndrome. All subjects gave written informed consent before participating in the study. The study was approved by the Cambridgeshire four Research Ethics Committee. Participants were excluded if they had any condition or were taking any medication that may have interfered with the analyses. A full description of the exclusion criteria is reported in the Online Supporting Information. Reporting of the study conforms to STROBE statement along with references to STROBE and the broader EQUATOR guidelines [10].

Screening visit

Body mass index was calculated, with weight and height measured to the nearest 0.1 kg/cm. Waist circumference was measured in triplicate. Body fat was measured using a leg-to-leg bio-impedance analyser (Tanita Corporation, Japan). Resting blood pressure was measured in triplicate using an automated sphygmomanometer (Dynamap, Critikon, UK), with a larger cuff for obese subjects. Blood and kidney function tests were performed after an overnight fast to ensure that participants met the inclusion criteria. The diagnosis of MetS was made according to the recent criteria proposed in a joint statement by the major diabetes, obesity and heart organisations [11], which requires at least three of the following criteria be fulfilled: waist circumference (WC) ≥ 102 cm (man), ≥ 88 cm (woman); triglycerides (TG) ≥ 1.7 mM; high-density lipoproteins (HDL-C) < 1.3 mM (man), < 1.1 mM (woman); high blood pressure $\geq 130/85$ mmHg; fasting plasma glucose ≥ 5.6 mM.

Frequent sampling intravenous glucose tolerance test (FSIVGTT)

Subjects arrived in the morning to the research unit after they had fasted for at least 12 h and followed the low NO₃⁻ diet for 24 h as part of the Salivary Oral Nitrate Test (S-ONT) used for the measurement of NO production [8]. Two cannulae were inserted in the antecubital vein, one in each arm. Three blood samples were taken before the administration of glucose to measure baseline insulin and glucose levels. An intravenous glucose dose (1.75 g of [6,6]-²H₂-glucose to 19 g unlabelled glucose in the form of a 50% aqueous solution) was then given over 1 min. Twenty minutes after glucose administration, 201 mU of Human Actrapid insulin was given. A total of 30 blood samples were taken at -10, -5, -1, 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, 20, 25, 30,

35, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 210 and 240 min relative to the administration of glucose. Insulin sensitivity (Si) was characterised by the minimal model as defined by Bergman *et al.* [12]. The plasma glucose and insulin concentrations during the FSIVGTT are showed in Figure S1 of the online Data S1. After the FSIVGTT, subjects were served a very low NO₃⁻ meal as part of the oral nitrate test protocol (ONT). A description of the meals and the low NO₃⁻ diet provided have been previously described [8] and it is reported in the Online Supporting Information.

Salivary oral nitrate test (S-ONT)

After the low NO₃⁻ meal, each participant was instructed not to eat and to drink only low NO₃⁻ bottled water (Buxton) until the following morning (18-h fasting period). Four hours after the meal, a pre-dose saliva sample was collected and a dose of 4 mg of Na¹⁵NO₃⁻ was given as a drink in 100 mL of distilled water. Each subject was asked to provide another saliva sample 2 h later. All subsequent saliva samples were collected by the volunteer at home using pre-labelled containers. The protocol for the collection of saliva samples has been previously described [8]. The final saliva sample was collected 18 h after the meal on the following morning. NO production was estimated by analysing the isotopic decay in saliva of an oral dose of labelled NO₃⁻ using an exponential function for a single compartment. Data were described using a semi-logarithmic plot, and the slope and intercept of the regression line were used to derive NO synthesis, as previously described [8].

Materials

Trifluoroacetic anhydride (TFAA), sodium hydroxide and mesitylene were obtained from Sigma-Aldrich Company Ltd, Poole, Dorset, UK. All the stable isotopically labelled compounds, ¹⁵N-sodium nitrate (98%+), [6,6]-²H₂-glucose (98%+) were obtained from Cambridge Isotope Laboratories Inc, Andover, MA, USA.

Nitrate measurements

Salivary nitrate concentrations were measured using a commercial 96-well kit (Cayman Chemical Co, Ann Arbor, MI, USA). ¹⁵N enrichment of nitrate was measured by gas chromatography mass spectrometry (GC/MS) following derivatisation of saliva samples with mesitylene and TFAA as catalyst to give a single product, nitromesitylene (1,3,5-trimethyl nitrobenzene), as previously described [13].

Glucose enrichment measurement

Plasma glucose enrichments were analysed by GC/MS after derivatisation with fluorinated methyl boronic acid, as previously described [14].

Clinical biochemistry

Blood (full blood count, glucose, insulin, Hb1Ac), lipid profile (HDL-C, LDL-C, triglycerides and total cholesterol), C reactive protein (CRP) and kidney function tests (urea, creatinine) were measured using validated methods. Plasma asymmetric dimethylarginine (ADMA) levels were measured by competitive ELISA (Immunodiagnostik, Belsham, Germany). Plasma VEGF concentrations were measured using an enzyme-linked immunosorbent assay (Quantikine™; R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Some of the results presented in this manuscript have been reported elsewhere [8]. Data are shown as mean and standard deviation (SD). The Kruskal–Wallis and the Mann–Whitney test were used to test for differences between groups. The Friedman test was used to test within-group differences in VEGF levels during acute hyperglycaemia. Analysis of covariance for repeated measures was used to assess VEGF changes during acute hyperglycaemia after including age in the model as covariate. Time (T) was the repeated measure factor, and group (G) was entered as the between-subject factor. The interaction term (T × G) was built to assess between-group differences in VEGF responses during hyperglycaemia. Variables entered into the model were transformed using the rank transformation method to account for the small sample size and deviation from normality distribution [15]. The Spearman rank correlation (unadjusted and age-adjusted) was used to assess the association between VEGF and cardiovascular and metabolic parameters. All statistical analyses were carried out using SPSS 16 for Windows (SPSS Inc., Chicago, IL, USA). The significance level was set at 0.05.

Results

The baseline characteristics of the study participants are shown in Table 1. Subjects in the control group were significantly younger and had a better overall metabolic profile than those in either obese group. There were no differences between groups for kidney function, glucose, diastolic blood pressure and HDL-C. Plasma levels of insulin, triglycerides and total cholesterol were significantly higher in the obese group with MetS. The control group had higher values of Si compared to the two obese groups. In contrast, rates of NO synthesis were significantly lower in the obese group with MetS compared to the control and obese without MetS groups. Differences in NO production remained significant after age adjustment ($P = 0.006$). Fasting VEGF levels were significantly higher in the two obese groups compared to the control group (P for trend = 0.02), but the differ-

ence was no longer significant after adjustment for age (Table 1).

Vascular endothelial growth factor concentrations were directly correlated with age, BMI, WC, fat mass (FM), SBP and fasting insulin and inversely related to insulin sensitivity and NO production. After age adjustment, only SBP and NO synthesis remained significantly associated with VEGF levels. Vascular endothelial growth factor levels were not significantly associated with biomarkers of endothelial dysfunction (ADMA) and inflammation (CRP) (Table 2).

Plasma VEGF levels rapidly increased in response to rising plasma glucose levels during the initial phase of the FSIVGTT. The increase in VEGF levels remained significant after age adjustment ($P < 0.001$), and the proportional changes relative to baseline after 19 min were 67% for the control group ($P = 0.003$), 70% for the obese without MetS ($P = 0.003$) and 39% for the obese group with MetS ($P = 0.54$). The interaction term (T × G) in the adjusted model was not significant ($P = 0.09$), which indicates a similarity in VEGF responses to hyperglycaemia between the three groups (Fig. 1).

Discussion

Obese subjects with MetS had significantly lower levels of NO production and insulin sensitivity compared to obese subjects without MetS and normal-weight subjects. Fasting NO and SBP were significantly correlated with VEGF levels, which may suggest interplay of these factors in the development of endothelial dysfunction in obesity. Hyperglycaemia induced a rapid increase in VEGF levels, and the responses were greater in normal-weight and obese subjects without MetS. The difference between the two obese groups may be related to the higher degree of endothelial dysfunction associated with MetS, potentially translating into slower signal transduction and a delayed VEGF secretory response. This assumption could not be experimentally tested in our protocol as an insulin bolus was administered at 20 min, confounding the effects of hyperglycaemia on VEGF secretion (no VEGF levels were measured past 20 min).

This study revealed a reciprocal association between NO production and VEGF levels using for the first time a validated stable isotope method for the measurement of *in vivo* rates of whole-body NO production [8]. A reciprocal relationship between NO and VEGF production has been observed in cellular models [16] but these results are not consistent with human studies [17,18]. Specifically, two studies have reported lower plasma nitrate levels, used as an index of NO production and decreased VEGF levels in obese subjects with MetS [17] and hypertension [18]. The decrease in VEGF levels observed in these two studies contrasts with the increase in VEGF levels observed in subjects with obesity [19], MetS [5], hypertension

Table 1 Baseline characteristics of the control group and of the two obese groups divided according to the diagnosis of metabolic syndrome (MetS)

	Controls	Obese without MetS	Obese with MetS	P value
N	7	7	7	
M/F	3/4	1/6	3/4	
Age (years)	27.4 (10.9)	48.0 (8.0)	48.0 (10.7)	0.01^{a,b}
Weight (kg)	65.2 (16.2)	95.7 (11.8)	103.9 (10.7)	0.001^{a,b}
Height (cm)	171.0 (14.0)	166.4 (9.8)	176.1 (5.4)	0.24
Body mass index (kg/m ²)	21.9 (2.2)	34.5 (2.3)	33.4 (2.9)	0.001^{a,b}
Waist circumference (cm)	78.0 (10.7)	109.9 (8.5)	111.3 (5.1)	0.001^{a,b}
Systolic blood pressure (mmHg)	106.7 (8.3)	120.3 (13.1)	130.1 (11.6)	0.01^b
Diastolic blood pressure (mmHg)	64.4 (10.2)	74.2 (10.2)	76.0 (7.7)	0.16
Glucose (mmol/L)	4.3 (0.4)	4.7 (0.4)	4.9 (0.5)	0.20
Insulin (pmol/L)	28.8 (6.8)	48.8 (18.1)	56.5 (12.5)	0.004^{a,b}
Triglycerides (mmol/L)	0.8 (0.3)	1.1 (0.3)	1.8 (0.4)	0.006^b
HDL-C (mmol/L)	1.5 (0.3)	1.5 (0.1)	1.2 (0.1)	0.07
Total cholesterol (mmol/L)	4.4 (0.7)	5.9 (1.2)	6.1 (1.4)	0.05 ^b
Creatinine (mmol/L)	74.7 (11.9)	63.0 (6.8)	78.7 (17.4)	0.09
Urea (mmol/L)	4.0 (1.3)	4.5 (0.6)	5.2 (0.1)	0.16
CRP (mg/L)	0.98 (0.63)	3.37 (1.66)	2.97 (1.90)	0.01^{a,b}
ADMA (μmol/L)	0.44 (0.07)	0.48 (0.06)	0.59 (0.08)	0.02^{b,c}
Insulin sensitivity (pmol/h/L)	0.012 (0.008)	0.0057 (0.0026)	0.0052 (0.0031)	0.05^{a,b}
Nitric oxide synthesis (μmol/kg/h)	0.63 (0.29)	0.49 (0.22)	0.21 (0.13)	0.009^{b,c}
VEGF (pg/mL)	11.01 (4.09)	20.70 (9.35)	18.86 (4.70)	0.02^{a,b}

Mean (SD) is shown. Significant results are shown in bold. Kruskal–Wallis test was used to test for differences across the three groups (trends). The Mann–Whitney test was utilised in a post hoc analysis to compare the individual groups. Letters indicate statistical significance between groups ($P < 0.05$). VEGF, vascular endothelial growth factor; CRP, C reactive protein; ADMA, asymmetric dimethylarginine.

^aControl vs. Obese without MetS.

^bControl vs. Obese with MetS.

^cObese with MetS vs. Obese without MetS.

[20], type 2 diabetes with and without microvascular complications (nephropathy, retinopathy)[21], coronary heart disease [22] and peripheral artery disease [22]. In diabetic and nondiabetic obese subjects [23] and in hypertensive patient with and without retinopathy [20], flow-mediated dilation of the brachial artery in response to reactive hyperaemia was inversely associated with the levels of VEGF. Valabhji *et al.* [24] showed that elevated VEGF levels in patients with type 1 diabetes correlated with decreased carotid artery distensibility. Moreover, VEGF directly correlated with carotid-to-intima media thickness in 909 healthy subjects (BMI = 26.5 ± 4.2 kg/m²) from the Salzburg Atherosclerosis Prevention Program in Subjects at High Individual Risk (SAPHIR)[25]. Thus, our results are in agree-

ment with a robust direct association between VEGF and endothelial dysfunction, suggesting potential new therapeutic avenues targeting elevated VEGF levels by enhancing NO production and/or availability.

The VEGF response to controlled hyperglycaemia in the obese without MetS and normal-weight controls suggests that endothelial cells are sensitive to acute changes in metabolic fluxes, which may be a physiological response to cellular changes in redox status and related to increased nutrient uptake. Insulin stimulates VEGF expression [26], and a recent study has demonstrated a role of VEGF-B in endothelial fatty acids uptake [27]. A significant direct correlation between plasma VEGF concentration and HbA1c has been observed in

Table 2 Unadjusted and age-adjusted correlation between vascular endothelial growth factor (VEGF, pg/mL) and the main measures of metabolic and cardiovascular function

	Unadjusted		Adjusted for age	
	Rho coefficient	P value	Rho coefficient	P value
Age (years)	0.56	0.008	–	–
Body mass index (kg/m ²)	0.56	0.008	0.40	0.07
Waste circumference (cm)	0.63	0.002	0.42	0.06
Systolic blood pressure (mmHg)	0.72	< 0.001	0.54	0.01
Diastolic blood pressure (mmHg)	0.24	0.28	–0.15	0.54
Glucose (mmol/L)	0.32	0.14	0.02	0.93
Insulin (pmol/L)	0.54	0.01	0.36	0.11
Cholesterol (mmol/L)	0.04	0.83	–0.30	0.18
Triglycerides (mmol/L)	0.22	0.31	–0.05	0.83
HDL-C (mmol/L)	–0.22	0.31	–0.14	0.55
CRP (mg/L)	0.32	0.15	0.12	0.62
ADMA (μmol/L)	0.17	0.46	–0.14	0.55
NO synthesis (μmol/h/kg)	–0.50	0.02	–0.44	0.04
Insulin sensitivity (pmol/h/L)	–0.49	0.02	–0.28	0.22

Spearman rank correlation was used to test the strength of the association between VEGF and the other variables. Significant results are highlighted in bold.

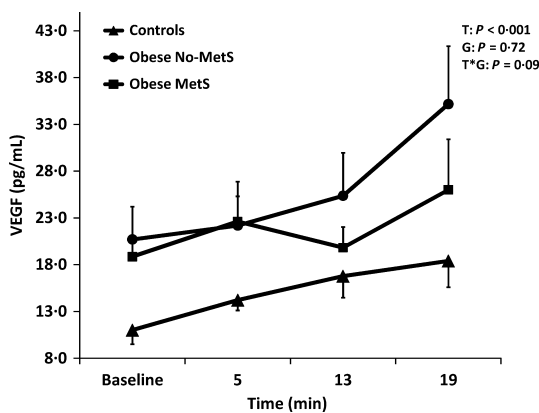


Figure 1 Acute response of vascular endothelial growth factor (VEGF) to controlled hyperglycaemia in controls and obese subjects with and without metabolic syndrome (MetS). Analysis of covariance for repeated measures was used to assess VEGF changes during acute hyperglycaemia after including age in the model as covariate. Time (T) was the repeated measure factor, and group (G) was entered as the between-subject factor. The interaction term (T × G) was built to assess between-group differences in VEGF responses during hyperglycaemia. Error bars are SE.

patients with diabetes [21], suggesting that chronic hyperglycaemia may increase VEGF secretion and that reduction in elevated levels of VEGF may be possible with improved glycaemic control [28]. However, Loebig *et al.* [4] showed a lack of association of VEGF with insulin sensitivity in normal-weight and obese subjects, and VEGF levels remained unchanged in patients with type 1 diabetes after 210 min of hyperglycaemia (12.0 mM) and normoglycaemic (5.0 mM) clamps with high (120 mU/kg/h) and standard insulin infusions (30 mU/kg/h), respectively [29].

The cross-sectional design and the small sample size were important limitations of this study, and the results should therefore be interpreted with caution. In addition, a significantly younger control group may have confounded the association. We purposely chose this group as the reference population to remove potential age-related changes in hormonal and cardiometabolic functions to evaluate the deviation of NO production measured in obese, insulin-resistant individuals from optimal, physiological rates. The analyses were adjusted for age to account for the potential confounding effect. Nevertheless, ours is the first report of a reciprocal relationship between NO production, as measured using stable isotope tracers, and plasma VEGF levels and the magnitude of acute increases in VEGF secretion during hyperglycaemia. These

results warrant further investigation in larger clinical studies, in particular with regard to their relevance for an enhanced atherosclerotic risk in obesity and in patients with diabetes with poorly controlled glycaemia.

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Conflicts of interest

None declared.

Contributions

MS contributed to the design of the study and collection of the data, analysis of the samples and wrote the manuscript. VT contributed to the sample analysis and to the critical revision of the manuscript. BCM contributed to the statistical analysis and to the critical revision of the manuscript. MF contributed to the interpretation of the results and to the critical revision of the manuscript. LCJB designed the study and revised the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

ONLINE SUPPLEMENTARY MATERIAL

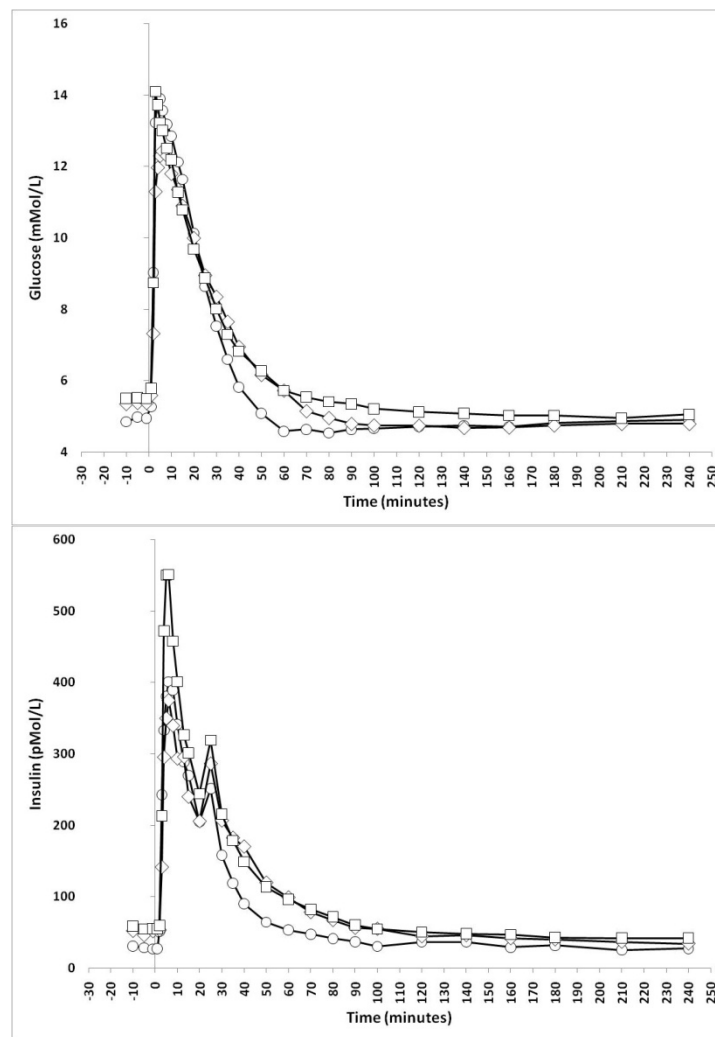


Figure S1: Plasma glucose and insulin concentrations during a FSIVGTT in normal weight and obese subjects divided according to the diagnosis of MetSyn. Symbols are: control group (O); obese NO-MetSyn (\diamond); obese-MetSyn (\square).

Exclusion Criteria

Participants were excluded if they had any of the following: history of substance abuse or alcoholism; allergy or intolerance to intervention foods; pregnancy or breastfeeding; professional athletes; weight change more than 3 kg in the last month; diagnosis of malignancy; chronic and acute metabolic and inflammatory conditions likely to interfere with the study outcome; severe anemia (Hb < 10 mg/dL); type 1 diabetes and subjects with T2D if treated with insulin and/or oral hypoglycaemic agents; weight loss medications; drugs having an effect on either NO production or on insulin sensitivity (corticosteroids, phenytoin, erythromycin, sildenafil, diuretics, laxatives, anticoagulants, antacids, nitrate derived agents, steroids, statins and any other anti-dyslipidaemic agent, including fibrates and nicotinic acid); Subjects taking antihypertensive agents, hormonal replacement therapies (estrogens, thyroxine, progesteron) and psychiatric drugs (antidepressants, sedatives, antipsychotics) were excluded if the dose had been started/changed in the previous six months.

Dietary Plan

Low Nitrate Diet (ONT method)

It was essential to modify the diet to restrict NO_3^- intake for of both methods. Since NO_3^- occurs in both plants and water it is a natural component of human diet. Nitrate is used as a preservative in cured meat but fresh meat contains little NO_3^- . Fruit and grains have a small impact on dietary intake of NO_3^- . The diet and the meals used in this study were prepared on the basis of a recent report on NO_3^- content in food products¹. Subjects were advised to avoid all vegetables, cooked and raw, and all tomato based products (spaghetti, pizza sauces, ketchup, tomato juice), potatoes, cured meat, seafood and fish, cheese, wines, beer, alcohol and tap water. Nitrate free water was provided during the study. On this protocol NO_3^- intake was expected to be $<30 \text{ mg/day}^2$.

Very low nitrate meals (ONT method)

The meals were served to each participant before the start of their 18-hr fasting period. The meals were formulated to have an energy content of $\sim 1400 \text{ kcal}$ and provide a NO_3^- intake $<5 \text{ mg/meal}$. The NO_3^- content of food was derived from a published report on NO_3^- content in food products¹. Ingredients with very low NO_3^- content ($<5 \text{ mg/100g}$) were chosen (pasta, eggs, rice, cereals, oil and spreads, fruit, milk). Low NO_3^- bottled mineral water (0.1 mg/l) was used for cooking the meals. Three meals were developed, and the subjects were allowed to choose the one the preferred as this was felt would improve compliance. The McCance-Widdowson food composition tables were used to calculate the energy content of the meals³. The nutritional composition of the three meals is described in **Table S1a, S1b and S1c**.

Table S1a: Meal 1 = Chicken Fried Rice					
Food	Grams	Nitrate (mg) /100g	Total Nitrate (mg)	Kcal/100g	Total Kcal
Chicken Breasts	100	1	1	155	155
Egg	80	0.49	0.392	151	120.8
Rice	90	2.5	2.25	383	344.7
Vegetable oil	20	0	0	900	180
Parmesan cheese	10	1.61	0.161	400	40
Yoghurt	150	0.05	0.075	82	123
Chocolate Mousse	120	0	0	300	360
Chocolate	40	0	0	520	208
Sliced Brown Bread	40	2.5	1	227	90.8
Cream Cheese	35	0	0	162	56.7
Butter	10	0	0	737	73.7
Water	Ad Lib	0	0		
Coffee/Tea	Ad Lib	0	0		
Salt	A bit	0	0		
Pepper	A bit	0	0		
Total	695	8.15	4.878	4017	1752

Table S1b: Meal 2 = Chicken Sandwich					
Food	Grams	Nitrate (mg) /100g	Total Nitrate (mg)	Kcal/100g	Total Kcal
Chicken Breasts	100	1	1	155	155
Sliced Brown Bread	80	2.5	2	207	165.6
Mayo	15	0	0	700	105
Yoghurt	150	0.05	0.075	82	123
Chocolate Mousse	120	0	0	300	360
Chocolate	40	0	0	520	208
Sliced Brown Bread	80	2.5	2	227	181.6
Cream Cheese	35	0	0	162	56.7
Butter	20	0	0	737	147.4
Water	Ad Lib	0	0		
Coffee/Tea	Ad Lib	0	0		
Salt	A bit	0	0		
Pepper	A bit	0	0		
Total	640	6.05	5.075	3090	1502.3

Table S1c: Meal 3 = Egg-Mayo Sandwich					
Food	Grams	Nitrate (mg) /100g	Total Nitrate (mg)	Kcal/100g	Total Kcal
Egg	160	0.49	0.784	151	241.6
Sliced Brown Bread	80	2.5	2	207	165.6
Mayo	15	0	0	700	105
Yoghurt	150	0.05	0.075	82	123
Chocolate Mousse	120	0	0	300	360
Chocolate	40	0	0	520	208
Sliced Brown Bread	80	2.5	2	227	181.6
Cream Cheese	35	0	0	162	56.7
Butter	20	0	0	737	147.4
Water	Ad Lib	0	0		
Coffee/Tea	Ad Lib	0	0		
Salt	A bit	0	0		
Pepper	A bit	0	0		
Total	700	5.54	4.859	3086	1589

Participants were invited to eat as much as they could. Food was weighted before and after consumption for the calculation of energy and nitrate intake. On average, volunteers ingested 1406 ± 322 kcal and 4.39 ± 0.56 mg of energy and nitrate, respectively.

References

1. European Prospective Investigation On Cancer, E. FOOD CONTENT OF POTENTIAL CARCINOGENS Nitrates, nitrites, nitrosamines, heterocyclic amines and polycyclic aromatic hydrocarbons. (EPIC, 2004).
2. L'Hirondel, J. *Nitrate and Man: Toxic, Harmless Or Beneficial?*, (CABI, Wallingford, UK, 2002).
3. Holland, B., Welch AA, Unwin ID, Buss DH, Paul AA, Southgate D. *McCance and Widdowson's the composition of food*, (The Royal Society of Chemistry, Cambridge, UK, 1991).