Dietary Nitrate Supplementation Reduces Circulating Platelet-Derived Extracellular Vesicles in Coronary Artery Disease Patients on Clopidogrel Therapy: A Randomised, Double-Blind, Placebo-Controlled Study

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Abstract
Extracellular vesicles (EVs) are implicated in the pathogenesis of cardiovascular disease (CVD). Specifically, platelet-derived EVs are highly pro-coagulant, promoting thrombin generation and fibrin clot formation. Nitrate supplementation exerts beneficial effects in CVD, via an increase in nitric oxide (NO) bioavailability. Clopidogrel is capable of producing NO-donating compounds, such as S-nitrosothiols (RSNO) in the presence of nitrite and low pH. The aimof this studywas to assess the effect of nitrate supplementationwithversus without clopidogrel therapy on circulating EVs in coronary artery disease (CAD) patients. In this randomized, double-blind, placebo-controlled study, CAD patients with (n ¼ 10) or without (n ¼ 10) clopidogrel therapy received a dietary nitrate supplement (SiS nitrate gel) or identical placebo. NO metabolites and platelet activation were measured using ozonebased chemiluminescence and multiple electrode aggregometry. EV concentration and origin were determined using nanoparticle tracking analysis and time-resolved fluorescence. Following nitrate supplementation, plasma RSNO was elevated (4.7 _ 0.8 vs 0.2 _ 0.5 nM) and thrombin-receptor mediated platelet aggregation was reduced (_19.9 _ 6.0 vs 4.0 _ 6.4 U) only in the clopidogrel group compared with placebo. Circulating EVs were significantly reduced in this group (_1.183e11 _ 3.15e10 vs_9.93e9 _ 1.84e10 EVs/mL), specifically the proportion of CD41þ EVs (_2,120 _ 728 vs 235 _ 436 RFU [relative fluorescence unit]) compared with placebo. In vitro experiments demonstrated clopidogrel–SNO can reduce platelet-EV directly (6.209e10 _ 4.074e9 vs 3.94e11 _ 1.91e10 EVs/mL). In conclusion, nitrate supplementation reduces plateletderived EVs in CAD patients on clopidogrel therapy, increasing patient responsiveness to clopidogrel. Nitrate supplementation may represent a novel approach to moderating the risk of thrombus formation in CAD patients.

Introduction
Extracellular vesicles (EVs) are small, spherical structures (0.1–1-μm diameter) enclosedwithin a phospholipid bilayer, generated by multiple cell types including platelets, leukocytes and endothelial cells. EVs represent a novel way of communication between cells, transporting both protein and genetic material. Recently, EVs have been implicated as a potential biomarker of cardiovascular disease (CVD), and are considered to play a crucial role in the promotion of coagulation, 1 inflammation2 and cell survival.3 Elevated levels of EVs have been observed in a range of CVD states,4 and have been shown to correlate with poor outcomes in coronary artery disease (CAD) patients.5 Therefore, EVs represent an attractive target for pharmacological and/or dietary intervention.
Platelet-derived EVs have received the most attention in the field, primarily due to their abundance and reactivity.6 The surface of platelet-derived EVs is approximately 100-fold more pro-coagulant than the surface of activated platelets themselves.7 Platelet-derived EVs express phosphatidylserine

that stimulates thrombin generation and stabilizes fibrin clot formation.1 Therefore, a method of reducing platelet-EVs might be of clinical benefit in CVD patients. Thienopyridines, such as clopidogrel, act via irreversible inhibition of the adenosine diphosphate (ADP) receptor, preventing platelet activation. In addition to their antiplatelet actions, additional properties have been reported, particularly with clopidogrel, including improvement in nitric oxide (NO) bioavailability, anti inflammatory effects and reduced endothelial injury after percutaneous coronary intervention (PCI).8 Previous studies have shown that clopidogrel reduces EV production in parallel with suppression of platelet activation in patients with acute coronary syndromes (ACSs).9 NO plays an essential role in maintaining both endothelial and platelet homeostasis; however, NO levels are compromised in CVD, resulting in the progression of atherosclerosis and vascular dysfunction.10 In platelet physiology, NO, acting via the cyclic guanosine monophosphate (cGMP) pathway, offers an additional inhibitory pathway to thienopyridine mediated inhibition. When activated together, ex vivo studies have shown that they produce a synergistic anti-platelet effect, culminating in a reduction in intracellular calcium.11 Ingestion of dietary nitrate elevates circulating levels of nitrite via bioconversion by commensal bacteria in the enterosalivary circulation.12 The benefits of nitrate supplementation in CVD are well documented; it has recently been shown to improve vascular function in hypercholesterolemia patients, and reduce ex vivo platelet aggregation in response to ADP/collagen.13,14 Additionally, Lee et al have recently shown that sustained-release nitrates can act synergistically with clopidogrel to enhance platelet inhibition in patients undergoing PCIs.15 S-nitrosothiols (RSNO) are a class of compound formed by S-nitrosation of reduced sulphydryl groups, and are able to act as NO donors, with potent anti-platelet effects.16 Our group has previously demonstrated that thienopyridines readily form RSNO,17 dependent on a low pH and nitrite(NO_2^-) availability, both of which are present in the stomach. These thienopyridine–SNO molecules are able to mimic NO with the additional benefit that the NO "moiety" is protected and preserved from further reactive metabolism. We hypothesized that inorganic nitrate supplementation with clopidogrel therapy would increase plasma RSNO levels and subsequently reduce circulating EVs in CAD patients.

Methods

Subjects and Protocol

Twenty patients with established CAD volunteered and consented to take part in this proof-of-concept, randomized, double-blind, placebo-controlled, crossover study. Patients were split into those receiving clopidogrel 75 mg daily (n ¼ 10) and those receiving no anti-platelet therapy, referred to henceforth as "naïve" (n ¼ 10). Patients attended the Cardiology Day Case Unit, University Hospital of Wales (UHW), as part of their normal clinical care. All patients were randomized to receive either 2 _ 60 mL (67.2 mM, confirmed by ozone-based chemiluminescence) of dietary nitrate supplement (Science in Sport GoþNitrates), followed by a placebo (of identical appearance), or vice versa. We have previously shown that from ingestion of this dose of inorganic nitrate, plasma nitrate and nitrite values peak at 2 hours and have returned to baseline within 24 hours.18 We adopted a washout period of 7 days, in agreement with several other groups.19,20 ►Fig. 1 summarizes the study design. Blood samples were taken from the antecubital vein through an 18-g Venflon intravenous (IV) cannula into EDTA (ethylenediaminetetraacetic acid), citrate and hirudin vacutainers. Samples were taken both before ingestion ingestion of the supplement and 2 hours post-supplementation. Patients took all prescribed medication at least 1 hour prior to the study. Patients were included if they were male, over 18 years of age with stable CAD and had been fasted for >6 hours. Patients in the clopidogrel group must have been receiving clopidogrel for >1 month prior to commencement of the study. Patients were excluded if they had a clopidogrel intolerance or contraindication, were on other long-term oral anti-coagulant drugs or receiving IV or subcutaneous anti-thrombin therapy. Patients were also excluded if they had any ischemic event (ACS, stroke or transient ischemic attack) or revascularization procedure (PCI or CABG) within the preceding 3 months, chronic renal or liver disease, or an inability to give informed consent. This study conformed to the ethical principles contained in the Declaration of Helsinki. Ethical approval was provided by the South East Wales Research Ethics Committee (IRAS Project ID 102427).

Biochemical Measurements

A full blood count was measured on an ABX Pentra X120

A full blood count was measured on an ABX Pentra X120 haematology blood analyser (Horiba, Northampton, UK). Serum cholesterol, high-density lipoprotein cholesterol and triglycerides were assessed using an Aeroset automated analyser (Abbott Diagnostics, Berkshire, UK). Low-density

lipoprotein cholesterol was calculated using Friedewald's formula. C-reactive protein was assayed by nephelometry (BN-II system, Milton Keynes, UK). All biochemical measurements were performed by the Department of Medical Biochemistry,UHW.

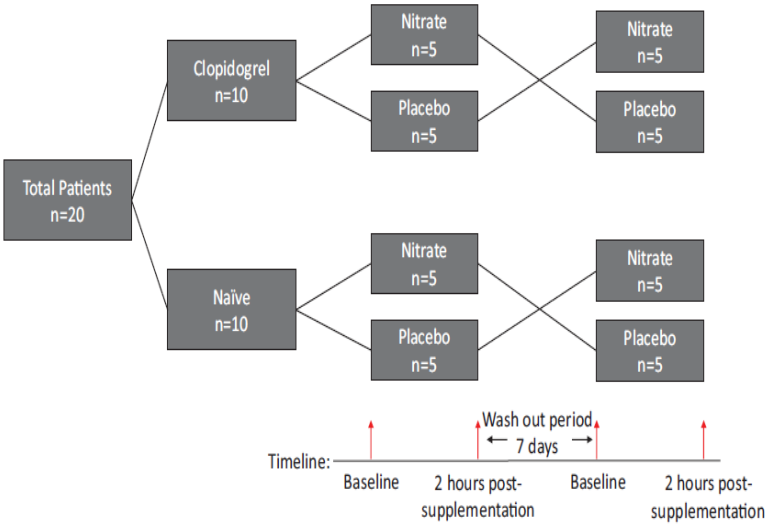


Fig. 1 Study design. Patients were split into those receiving clopidogrel 75 mg daily ($n = 10$) and those receiving no anti-platelet therapy (naïve, $n = 10$). Patients were randomly allocated to either nitrate supplementation (Science in Sport Go^{+} Nitrates gel), followed by a placebo of identical appearance, or vice versa. A washout interval of a minimum of 7 days separated the two treatment periods. Blood samples were taken immediately before ingestion of the supplement and 2 hours post-supplementation.

Measurement of Plasma NO Metabolites

Blood samples were collected into EDTA vacutainers and promptly centrifuged (2,500g, 15 minutes, 4°C) to yield plasma which was subsequently snap frozen in liquid nitrogen and stored at _80°C until analysis. NO metabolites were assessed using ozone-based chemiluminescence. Briefly, for plasma nitrite and RSNO analysis, 5-mL tri-iodide reagent (I3 _) was placed in a custom-built glass purge vessel and heated at 55°C via a water bath thermostatically controlled by a hotplate. The inert carrier gas (O2-free N2) purging I3 _ was linked to a sodium hydroxide trap (1 M), connected to an NOanalyser (Sievers NOA 280i; Analytix, Boldon, UK). Plasma samples (200 µL nitrite, 400 µL RSNO) were directly injected in to the purge vessel through a rubber septum injection inlet. To differentiate between nitrite and RSNO, samples were run before and after pre-treatment with 5% acidified sulphanilamide (290 mM), which removes NO2 _, thus allowing the selective measurement of the residual plasma RSNO. Increased accuracy of RSNO measurement was obtained by a 50-point adjacent signal averaging algorithm, improving the signal-to-noise ratio, using Origin 7.0 (OriginLab, Massachusetts). For nitrate analysis, plasma samples (20 µL) were injected into vanadium chloride heated at 90°C before detection via the NO analyser. Results were compared with a sodium nitrite/nitrate standard curve performed daily to account for day-to-day temperature fluctuations. Room temperature was 19 _ 2°C. In our hands, the limit of sensitivity for these plasma RSNO, nitrite and nitrate assays are >1, >10 and >500 nmol/L, respectively.

Platelet Aggregation

Whole blood aggregation was assessed via multiple electrode aggregometry, using the Multiplate analyser (Roche Diagnostics, Forrenstrasse, Switzerland). The adhesion and aggregation of platelets on the sensor surface enhances the electrical resistance between the two sensor electrodes within a test cell.Whole blood (300 µL) collected in a hirudin vacutainer was diluted 1:1 with saline solution and incubated at 37°C for 3minutes andmeasuredwithin 30minutes of blood drawing. Following this, platelet activation was initiated by the addition of either ADP (6.5 µM) or thrombin receptor-activating peptide (TRAP; 32 µM). The subsequent increase in impedance due to attachment of platelets to the

electrodes is transformed to arbitrary aggregation units. The aggregation measured is quantified as the area under thecurve (AUC) and expressed in units (U).

Extracellular Vesicle Isolation

Blood was collected into sodium citrate vacutainers, as recommended by the Scientific Standardisation Committee of the International Society on Thrombosis andHaemostasis.21,22 Blood was immediately centrifuged (2,500g, 15 minutes, 4°C)to isolate plasma, which was subsequently spun (2,500g,15 minutes, 4°C) to obtain platelet-poor plasma (PPP). PPP was ultracentrifuged (100,000g, 60 minutes, 4°C) and the resulting EV pellet was resuspended in phosphate-buffered saline (PBS) and slow-frozen overnight at _80°C. All EV analysis was completed within 7 days of isolation. This is an established protocol that we have utilized in several clinical studies to date.23–25EV Size and Concentratio nEV concentration and size distribution were determined using nanoparticle tracking analysis (NTA; NanoSight LM10 system, UK) as described previously. NTA is a laser-illuminated microscopic technique equipped with a 642-nmlaser and a high-sensitivity digital camera system (Orca-Flash2.8, Hamamatsu, NanoSight Ltd) that determines the brownian motion of nanoparticles in real time to assess sizea nd concentration. Sixty-second videos were recorded and particle movement was analysed using NTA software (version 2.3). Camera sensitivity and detection threshold were 11 to 14 and 4 to 6, respectively. EV samples were diluted in EV-free sterile PBS. Samples were run in quintuplicate, from which EV distribution, size and average concentration were calculated. EV concentrations were expressed as EVs/mL plasma.

EV Cellular Origin

Time-resolved fluorescence was used to assess the origin of isolated EVs, as described previously.23 EVs (5e10/well) were loaded into a high protein binding 96-well plate (Greiner Bio-One, Germany) overnight at 4°C, before non-specific sites were blocked with 1% bovine serum albumin for 2 hours. EVs were incubated overnight at 4°C with mouse anti-human antibodies against CD9, CD41, CD11b, CD235a and CD144 as markers of exosomes, platelets, leukocytes, erythrocytes and endothelial cells, respectively (all antibodies from Abcam, Cambridge, UK). Markers were detected using a biotinylated anti-mouse IgG secondary antibody and a streptavidin:europium conjugate (PerkinElmer, Buckinghamshire, UK).

In Vitro Platelet EV Production

The in vitro model was designed to confirm the capability of clopidogrel–SNO to inhibit platelet EV release in response to a pharmacological stimulus (ADP). Platelet-rich plasma (PRP) was isolated from the blood of healthy volunteers via sodium citrate vacutainers (200g, 20 minutes). PPP was also isolated (200g, 20 minutes, followed by 2 _ 2,500g, 15 minutes) for use as a control. Platelets were stimulated with ADP (6.5 μM) and incubated for 5 minutes before $NaNO_2$, clopidogrel, S-nitrosoglutathione (GSNO) or clopidogrel–SNO (all 10 μM) were added and incubated for 1 hour at 37°C. This concentration was chosen to compare directly with in vitro aggregation studies in healthy blood showing the IC50 for GSNO/ clopidogrel–SNO as approximately 7.5 μM. Clopidogrel–SNO was produced as described in detail previously by our group.17

Statistics

A power calculation based on prior results from EV samples from CAD patients showed that nine subjects would provide 90% power for detecting a 20% difference in circulating EVs between placebo and nitrate supplementation, assuming10% variation, with α ¼ 0.05. Data were analysed using GraphPad Prism version 5.0. Data are expressed as mean _ standard error (SE). All data were assessed for both a period effect and treatment–period interaction and checked for normality using the Kolmogorov–Smirnov test. The change in measurement before and after nitrate supplementation or placebo was calculated and compared directly using a paired t-test or a Wilcoxon matched pairs test, for normally or non-normally distributed data, respectively.

Results

Subject Characteristics

Of the 20males that participated in the study, 10were taking clopidogrel (>1 month) and 10 were not receiving antiplatelet therapy (naïve group). The mean ages of the groups were 63.2 _ 3.6 and 62.7 _ 3.2 years, respectively. Biochemical measurements are summarized in ►Table 1. Importantly, no differences were observed in platelet count at baseline or at the follow-up visit. No differences were

seen in age, body mass index, biochemical or haematological parameters between groups. No significant period effects and treatment–period interaction were observed for any of the parameters determined in this study.

Plasma NO Metabolites

Baseline plasma nitrate, nitrite and RSNO measurements were not significantly different between naïve and clopidogrel groups (nitrate [NO3 _]: 31.14 _ 2.5 vs 31.34 _ 5.1 μM; NO2 _: 92.3 _ 9.0 vs 118.9 _ 15.7 nM; RSNO: 6.4 _ 0.5 nM vs 11.9 _ 3.4 μM). Nitrate supplementation significantly elevated plasma NO3 _ and NO2 _ compared with placebo in both naïve (ΔNO3 _: 252.1 _ 17.4 μM vs 3.7 _ 1.9 μM, $p < 0.001$; ΔNO2 _: 167.8 _ 40.1 vs 18.5 _ 22.6 nM, $p < 0.05$) (►Fig. 2a,c) and clopidogrel (ΔNO3 _: 256.6 _ 20.5 vs_2.0 _ 3.4 μM, $p < 0.001$; ΔNO2 _: 164.8 _ 68.5 vs _5.2 _ 12.2 nM, $p < 0.05$) (►Fig. 2b, d) groups. Increases in plasma nitrate and nitrite were similar between naïve and clopidogrel groups (ΔNO3 _naïve: 252.1 _ 17.4 μM vs clopidogrel: 256.6 _ 20.5 μM, $p > 0.05$; ΔNO2 _: naïve: 167.8 _ 40.1 nM vs clopidogrel: 164.8 _ 68.5 nM, $p > 0.05$). Plasma RSNO did not change following nitrate supplementation compared with placebo in the naïve group (ΔRSNO: 0.9 _ 1.3 vs _1.3 _ 0.6 nM) (►Fig. 2e). However, plasma RSNO was significantly elevated after receiving nitrate supplementation when compared with placebo in the clopidogrel group (ΔRSNO: 4.7 _ 0.8 vs 0.2 _ 0.5 nM, $p < 0.001$) (►Fig. 2f). Additionally, RSNO in the clopidogrel group was significantly elevated in comparison to the naïve group (4.7 _ 0.8 vs 0.9 _ 1.3 nM, $p < 0.05$), respectively.

Platelet Aggregation

TRAP-mediated platelet aggregation was not significantly different between clopidogrel and naïve groups (94.8 _ 7.0 vs 99.0 _ 6.3 U, respectively). Patients receiving clopidogrel had markedly lower ADP-mediated platelet aggregation compared with the naïve group at baseline (37.9 _ 3.80 vs 60.0 _ 4.4 U, $p < 0.001$). Nitrate supplementation failed to significantly reduce both ADP- and TRAP-mediated platelet aggregation in the naïve group when compared with placebo (ΔADP: _7.8 _ 5.8 vs 2.1 _ 4.9 U, ΔTRAP: _21.5 _ 8.4 vs _3.8 _ 9.2 U) (►Fig. 3a). Nitrate supplementation had no effect on ADP-mediated platelet aggregation compared with placebo in patients receiving clopidogrel (ΔADP: _2.2 _ 2.2 vs 0.1 _ 1.8 U) (►Fig. 3b). However, nitrate supplementation did significantly reduce TRAP-mediated platelet aggregation in the clopidogrel group compared with placebo (ΔTRAP: _19.9 _ 6.0 vs 4.0 _ 6.4 U, $p < 0.05$) (►Fig. 3b). The reduction in ADP- and TRAP-mediated aggregation was similar within both the naïve and clopidogrel groups (ΔADP: naïve: _7.8 _ 5.8 U vs clopidogrel: _2.2 _ 2.2 U, $p > 0.05$. ΔTRAP: naïve: _21.5 _ 8.4 U vs clopidogrel: _19.9 _ 6.0 U, $p > 0.05$).

Table 1 Participant characteristics

Abbreviations: ACEi/ARB, angiotensin converting enzyme inhibitors/angiotensin II receptor blocker; BMI, body mass index; CABG, coronary artery bypass grafting; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; GTN, glyceryl trinitrate; HDL, high-density lipoprotein;LDL, low-density lipoprotein; MI, myocardial infarction; NSAIDs, non-steroidal anti-inflammatory drugs; TIA, transient ischemic attack.

Note: Summary of patient characteristics including age, BMI, SHIM score, cardiovascular risk factors, medications, biochemical and haematological measurements. Haematological measures were taken at the beginning of both patient visits, prior to any treatment. No statistically significant differences between the two groups were observed ($p > 0.05$).

	Naïve group ($n = 10$)		Clopidogrel group ($n = 10$)	
Age	62.7 ± 3.19		63.2 ± 3.66	
BMI (kg/m^2)	29.9 ± 1.49		28.2 ± 1.18	
Cardiovascular risk factors				
Diabetes mellitus	1 (10%)		2 (20%)	
Past/current smoking	3 (30%)		6 (60%)	
Hypertension	7 (70%)		8 (80%)	
Dyslipidaemia	10 (100%)		8 (80%)	
Family history of premature				
Heart disease (<65 y)	4 (40%)		2 (20%)	
Stroke/TIA	1 (10%)		2 (20%)	
Peripheral vascular disease	1 (10%)		1 (10%)	
History of MI	4 (40%)		7 (70%)	
Previous revascularization				
PCI	4 (40%)		7 (70%)	
CABG	2 (20%)		4 (40%)	
Respiratory disease (asthma/COPD)	0 (0%)		3 (30%)	
Medications				
Aspirin	7 (70%)		4 (40%)	
Clopidogrel	0 (0%)		10 (100%)	
Proton-pump inhibitor	5 (50%)		7 (70%)	
Beta blockers	4 (40%)		6 (60%)	
ACEi/ARB	6 (60%)		8 (80%)	
Statins	10 (100%)		8 (80%)	
GTN	1 (10%)		1 (10%)	
Thyroxin	1 (10%)		0 (0%)	
NSAIDs	0 (0%)		2 (20%)	
Oral anti- coagulants	1 (10%)		0 (0%)	
Calcium channel blockers	2 (20%)		1 (10%)	
Diuretics	2 (20%)		2 (20%)	
Biochemical measures				
CRP (mg/L)	5.3 ± 4.27		4.90 ± 4.63	
Total cholesterol (mmol/L)	4.58 ± 0.77		4.30 ± 0.82	
Triglycerides (mmol/L)	2.1 ± 1.80		1.47 ± 0.90	
HDL (mmol/L)	1.11 ± 0.26		1.07 ± 0.13	
LDL (mmol/L)	2.61 ± 0.54		2.57 ± 0.68	
Total cholesterol:HDL ratio	4.13 ± 1.69		4.02 ± 0.57	
Haematological measures	Visit 1	Visit 2	Visit 1	Visit 2
White cell count (×10^9/L)	6.0 ± 0.50	6.3 ± 0.6	6.7 ± 0.6	6.4 ± 0.3
Haemoglobin (g/L)	149.1 ± 3.1	151.5 ± 3.6	147.5 ± 3.4	144.9 ± 3.4
Platelet count (× 10^9/L)	232.8 ± 25.0	219.4 ± 16.1	234.7 ± 13.4	237.8 ± 18.2

Fig. 2 NO metabolite measurements. Thechangein plasmaNO metabolites 2 hours post-nitrate supplementation or placebo. (a) Naïve group plasma NO3□,μM. (b) Clopidogrel group plasma NO3□,μM. (c)Naïve group NO2□,nM. (d) Clopidogrel group plasma NO2□,nM. (e) Naïve group plasma RSNO, nM. (f) Clopidogrel group plasma RSNO, nM. Data are expressed as mean □ SEM (n ¼ 10). $p < 0.05$ and □□□$p < 0.001$

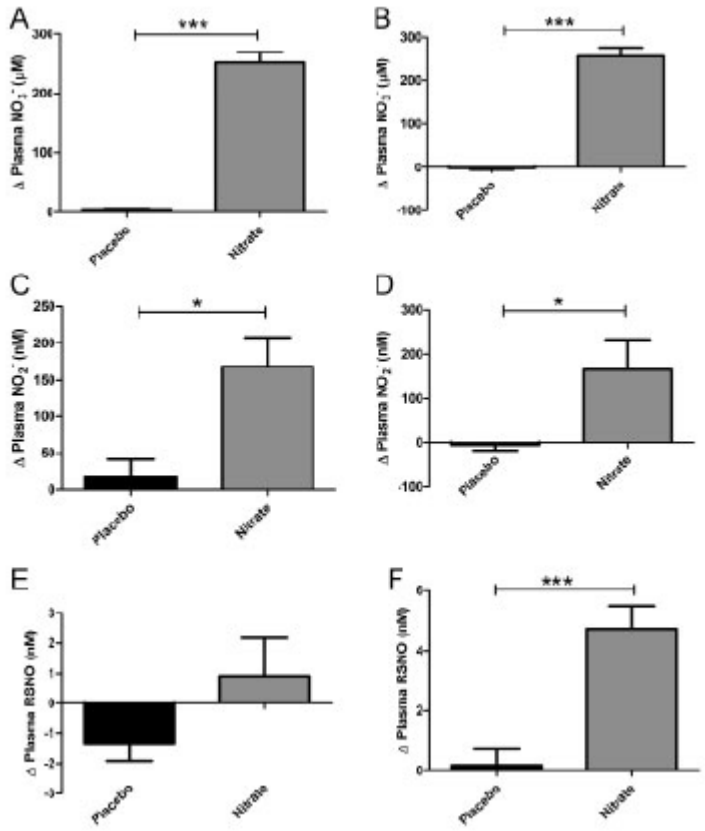
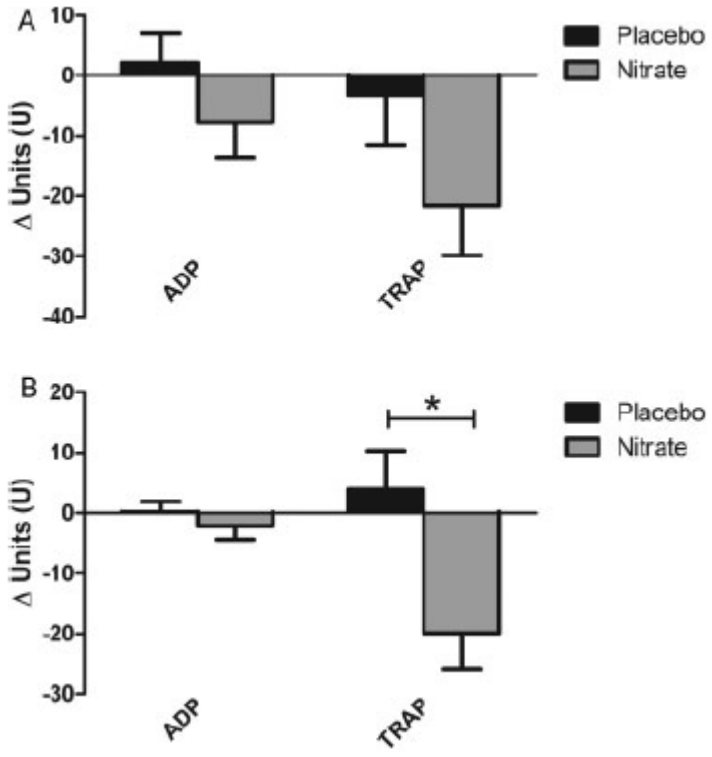


Fig. 3 Platelet aggregation. ADP- and TRAP-induced platelet ag-gregation as measured by MEA (multiple electrode aggregometry).(a) Agonist response in the naïve group. (b)Agonistresponse inthe clopidogrel group. Data are expressed as mean □ SEM (n ¼ 10). p < 0.05.



EV Size and Concentration

Nitrate supplementation had no effect on the mode size of EVs compared with placebo in both naïve (123 _ 9.2 vs 111 _ 7.3 nm) and clopidogrel (129 _ 11.4 vs 122 _ 16.2 nm) groups, respectively. EV concentration at baseline did not differ between naïve and clopidogrel groups, respectively (5.0e11 _ 4.2e10 EVs/mL vs 4.7e11 _ 3.7e10 EVs/mL). ►Fig. 4a shows the size distribution profile of EVs in both the naïve and clopidogrel group at baseline, split into 50-nm bin sizes. Nitrate supplementation did not reduce the circulating EV concentration compared with placebo in the naïve group (ΔEVs: _2.78e10 _ 4.22e10 vs 1.76e10 _ 1.23e10 EVs/mL). However, nitrate supplementation did significantly reduce EV concentration in the clopidogrel group compared with placebo (ΔEVs: _1.183e11 _ 3.15e10 vs _9.93e9 _ 1.84e10 EVs/mL, $p < 0.01$) (►Fig. 4b). The reduction in circulating EV concentration was similar between naïve and clopidogrel groups (_2.78e10 _ 4.22e10 vs _1.183e11 _ 3.15e10 EVs/mL, $p > 0.05$), respectively.

EV Cellular Origin

Comparison between the naïve and clopidogrel groups revealed that the clopidogrel group had significantly higher baseline levels of exosomal (CD9), platelet (CD41) and leukocyte (CD11b) markers compared with the naïve group

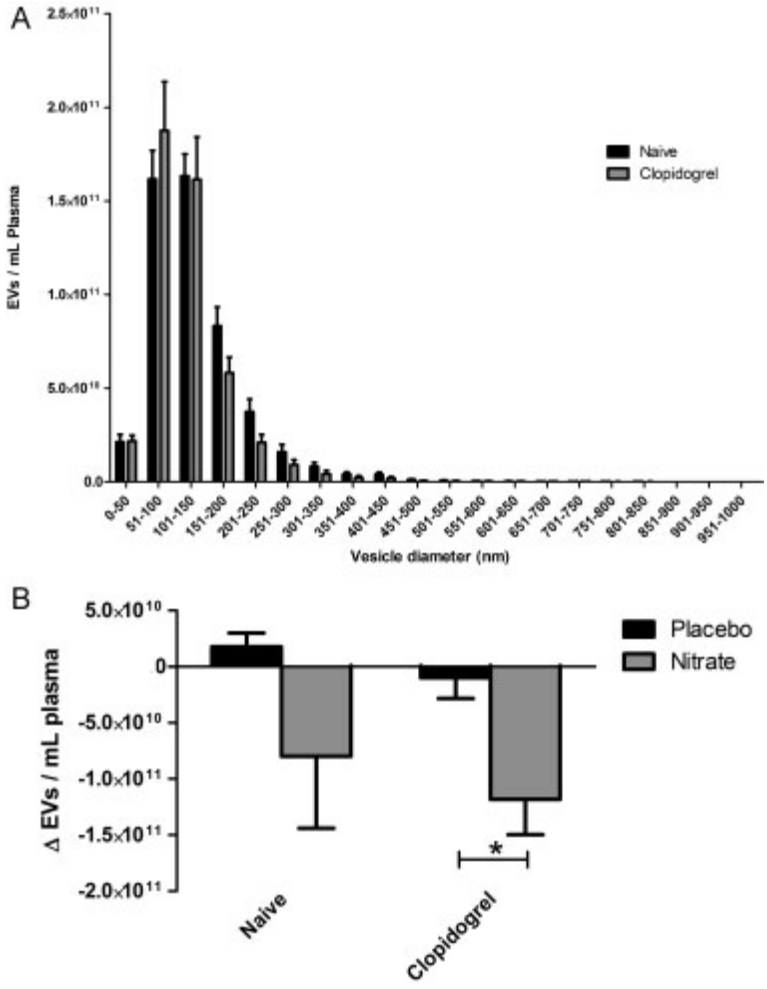


Fig. 4 Circulating EV concentration. (a) Size distribution profile for EVs in both the naïve and clopidogrel group, split into 50-nm bin sizes. (b) Change in circulating plasma EVconcentration 2 hours post-nitrate supplementation/placebo in naïve and clopidogrel groups determined by NTA (nanoparticle tracking analysis). Data are expressed as mean _ SEM (n ¼ 10). _$p < 0.05$.

(CD9: 32,899 _ 1,303 vs 23,812 _ 1,891 RFU [relative fluorescence unit], $p < 0.001$; CD41: 15,753 _ 1,372 vs 10,064 _ 705 RFU, $p < 0.01$; CD11b: 14,245 _ 1,512 vs 9,578 _ 1,250 RFU, $p < 0.05$) (►Fig. 5a). No significant differences were seen in surface protein markers for exosomes, platelets, leukocytes, erythrocytes (CD235a) and endothelial cells (CD144) of EVs in the naïve group following

nitrate supplementation compared with placebo (►Fig. 5b). CD41 was significantly reduced following nitrate supplementation compared with placebo in patients receiving clopidogrel (ΔCD41: _2,120 _ 728 vs 235 _ 436 RFU, p < 0.05) (►Fig. 5c). Changes in surface marker levels were similar

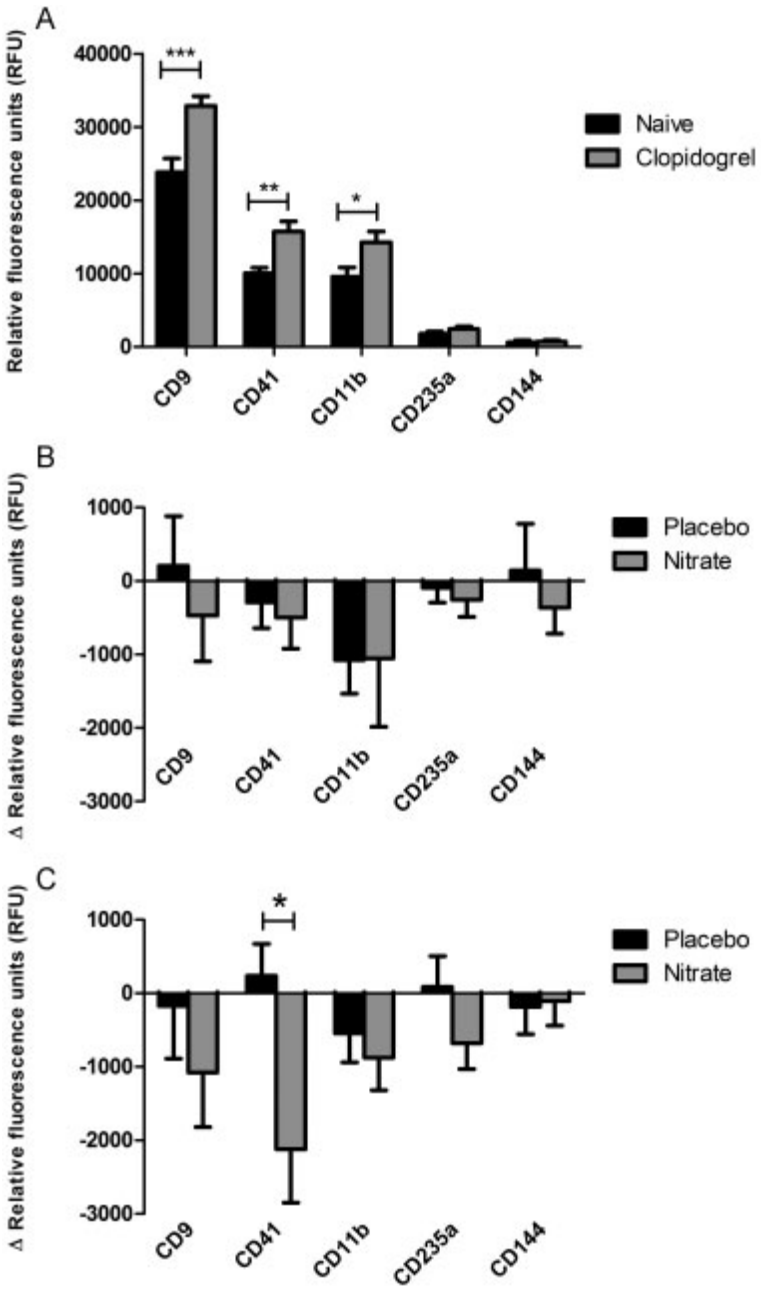


Fig. 5 Effect of nitrate supplementation on EV surface protein. The change in content of exosomes (CD9), platelet (CD41), leukocyte (CD11b), erythrocyte (CD235a) and endothelial (CD144) in plasma EVs after nitrate supplementation versus placebo. (a) Difference in baseline levels of protein expression between naïve and clopidogrel groups (n ¼ 10). (b) Change in protein expression after nitrate supplementation/placebo in the naïve group (n ¼ 10). (c) Change in protein content after nitrate supplementation/placebo in the clopidogrel group (n ¼ 10). Proteins were detected using a streptavidin– europium conjugate and measured using time-resolved fluorescence (relative fluorescence units [RFU]). Data are expressed as mean _ SEM. ___p < 0.001, __p < 0.01 and _p < 0.05. between naïve and clopidogrel groups (CD9: _471 _ 625 vs _1,079 _ 744, CD41: _498 _ 423 vs _2,120 _ 728, CD11b: _1,053 _ 934 vs _877 _ 441, CD235a: _259 _ 230 vs _678 _ 354, CD144: _364 _ 354 vs _106 _ 336, p > 0.05 for all comparisons).

In Vitro Experiments

Laboratory experiments were undertaken to investigate the direct effect of nitrite, clopidogrel and clopidogrel–SNO separately on platelet activation and EV generation. EV generation was increased 200% in response to addition of ADP. Following a dose–response, 10 μM of inhibitors (sodium nitrite, clopidogrel–SNO, GSNO) were added, which showed this concentration was effective at inhibiting platelet aggregation and thus platelet-derived EV release. Indeed, aggregation measured in response to ADP was significantly inhibited by both clopidogrel–SNO and GSNO, with a similar IC50 (6.56 _ 1.08 vs 8.42 _ 1.35 μM). Nitrite alone was ineffective at inhibiting platelet aggregation in response to ADP unless concentrations greater than 10 mM were applied (►Fig. 6a). The addition of sodium nitrite (NaNO2) or clopidogrel to stimulated PRP had no effect on EV production (NaNO2: 3.83e11 _ 1.707e10 EVs/mL, clopidogrel: 3.91e11 _ 1.805e10 EVs/mL, vs ADP alone as control 3.94e11 _ 1.91e10 EVs/mL). Similarly, no change in CD41 was observed (NaNO2: 20,093 _ 1,244 RFU, clopidogrel: 18,238 _ 2,824 RFU, vs ADP control: 19,703 _ 1,375 RFU, $p > 0.05$). Following the addition of clopidogrel–SNO, EV production significantly reduced compared with the ADP control (6.209e10 _ 4.074e9 vs 3.94e11 _ 1.91e10 EVs/mL, $p < 0.001$). GSNO also significantly reduced EV production compared with the ADP control (8.67e10 _ 8.63e9 EVs/mL, $p < 0.001$) (►Fig. 6b). Reductions were also seen in CD41 expression following addition of both SNO molecules compared with control (GSNO: 8,946 _ 1,460 RFU, clopidogrel–SNO: 9,713 _ 1,214 RFU, vs ADP control: 19,703 _ 1,375 RFU, $p < 0.01$) (►Fig. 6c).

Discussion

This study shows that acute dietary NO3 _ supplementation significantly reduces circulating EVs in patients with CAD already established on long-term clopidogrel therapy. Furthermore, it significantly reduced platelet activation via the thrombin receptor, accompanied by a reduction in the proportion of platelet-derived EVs. This reduction was associated with a rise in circulating RSNO only in the clopidogrel group. Previously thought of as a physiologically inert oxidative end product of NO metabolism, it is now widely accepted that NO3 _ and NO2 _ metabolism occurs in both blood and tissues to form NO and other bioactive NO metabolites, representing an alternative to the “classical” L-arginine pathway. 12 NO3 _ supplementation has been shown to have a variety of benefits in CVD including improving vascular function,13 decreasing blood pressure26 and attenuating oxidative stress.27

Baseline measurements of plasma NO2 _ prior to NO3 _ supplementation or placebo were noticeably lower in both the naïve (92.3 _ 9.0 nM) and clopidogrel (118.9 _ 15.7 nM) groups compared with the values typically observed in healthy individuals (200–300 nM) we have reported previously. 28 These low NO2 _ levels reflect the reduced NO bioavailability and degree of endothelial dysfunction seen in these patient cohorts.

Significant elevations in plasma RSNO levels following NO3 _ supplementation were seen only in patients taking clopidogrel. We have previously demonstrated in vitro that the low pH environment of the stomach can modify thienopyridines, to form thienopyridine–SNO molecules29 that exhibit NO-like biological effects, including platelet inhibition and vasodilation.30,31 The low pH exposes the free thiol group present in these drugs, before biotransformation in the presence ofNO2 _ to form RSNO compounds. These molecules provide an addition to the nitrate–nitrite–NO pathway and the effects seen upon nitrate/nitrite supplementation.32

Patients receiving proton-pump inhibitors (PPIs; and thus an increased pH in the stomach) exhibit reduced platelet inhibition in response to clopidogrel, although the mechanismof this is felt to be multifactorial and incompletelydefined.33 Recently, Pinheiro et al have shown that oralnitrite administration in rats was associated with an increase in RSNO and a decrease in blood pressure.34 Furthermore, treatment with both PPIs and the thiol-depleting agent buthionine sulfoximine attenuated the increase in plasma RSNO and blunted the anti-hypertensive effects of nitrite.34

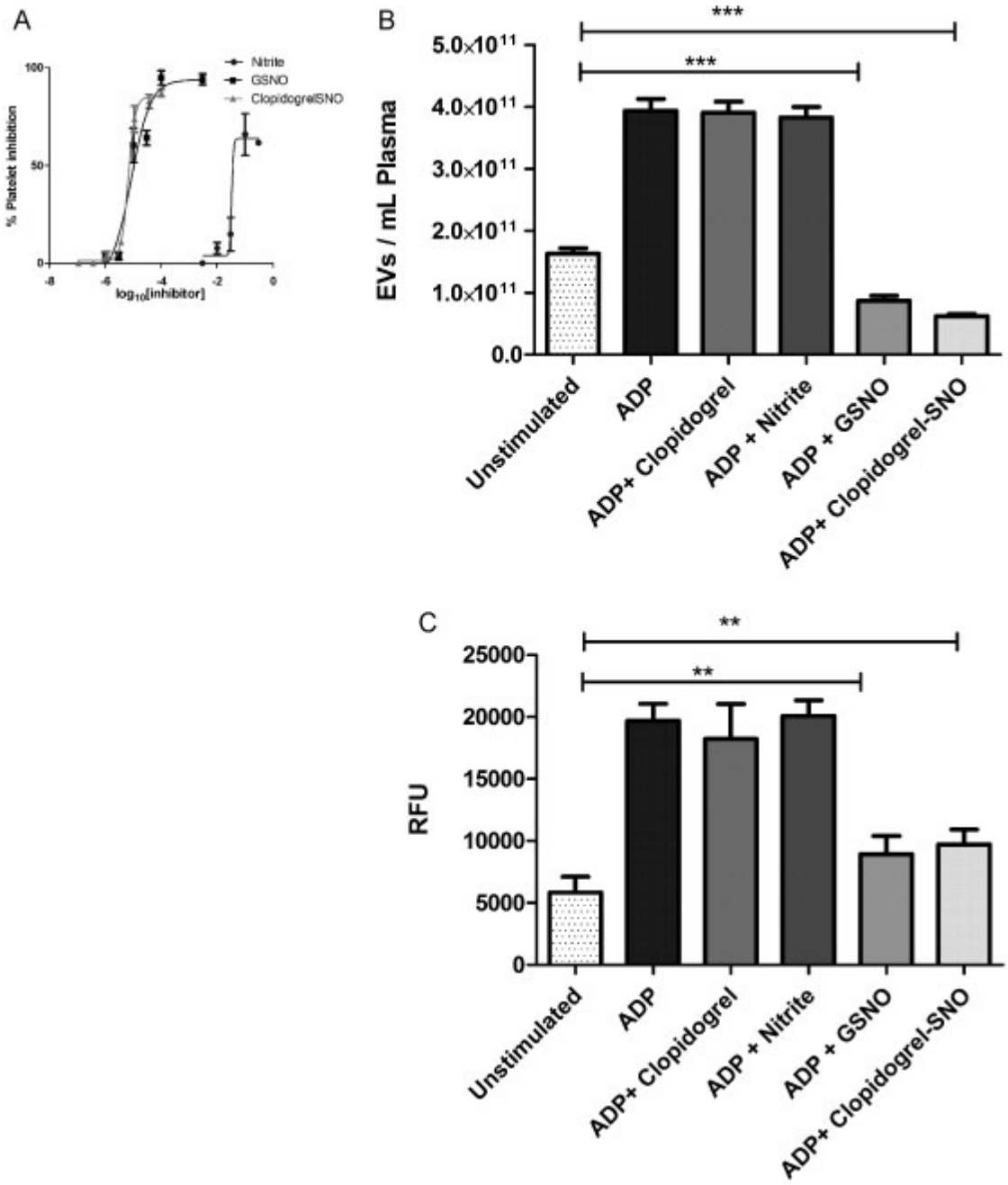


Fig. 6 In vitro platelet EV production. (a) Dose–response curve of GSNO, clopidogrel–SNO and nitrite on platelet inhibition. The IC50 of GSNO
and clopidogrel–SNO were similar (6.56 _ 1.08 vs 8.42 _ 1.35 μM). (b) The effect of various agents on EV production. Clopidogrel–SNO
severely attenuated EV production in platelet-rich plasma. An alternative nitrosothiol, GSNO, also inhibited EV production. (c) The reduction in
EV concentration was mirrored by a reduction in CD41 expression, measured by TRF. n ¼ 4, ___p < 0.001 and __p < 0.01.

However, the influence of stomach pH on RSNO formation inpatients is yet to be established.

To our knowledge, this is the first study to demonstrate that dietary nitrate supplementation can reduce circulating EV concentration when administered with clopidogrel. The lack of RSNO produced in the naïve group and absence of a significant reduction in EV concentration suggest these effects are due to formation of clopidogrel–SNO molecules in the stomach. Conversely, in patients receiving clopidogrel and NO3_ clopidogrel–SNO is formed which can subsequently donate NO in the plasma as previously demonstrated.35 Our group have previously shown that nitrite-derived NO can reduce EV production in endothelial cells.36 This is consistent with previous reports that showed impairment of NO production increased EV production in endothelial cells.37 It is well established that NO elicits many of its effects, such as platelet activation, via a reduction in intracellular calcium. Conversely, many of the processes required for EV formation, such as lipid membrane remodelling and cytoskeleton disruption require increases in intracellular calcium.38 Our in vitro results confirm clopidogrel– SNO, derived from NO2 _, inhibits platelet EV generation directly, where NO2 _ and clopidogrel alone had no effect. This is consistent with inhibition of platelet aggregation we have observed at these concentrations and with previously established effects of RSNO on platelets.39

Interestingly, a significant reduction in EV concentration was only seen in the clopidogrel group. Whether this decrease can be attributed to the increased RSNO levels seen compared with the naïve group, or as a result of the combination of two independent effects of NO and clopidogrel, remains unclear. Clopidogrel acts via inhibition of the ADP/ P2Y12 receptor, preventing the inhibition of adenylate cyclase, consequently reducing platelet aggregation. The ADP receptor has previously been shown to have a potentiating role in dense granule secretion.40 Granule and vesicle release from platelets is mediated by common cellular machinery such as SNARE proteins, intracellular calcium levels and cytoskeletal organization.41 Thus, it is possible that the reduction in plasma EV seen in our patient cohort could be due to the combined, independent effects of NO and clopidogrel. However, given our data showing a significant reduction of platelet EV seen when clopidogrel–SNO is administered in vitro, it seems likely that the increased formation of RSNO in these patients causes the reduction in EV seen in this study.

Consistent with previous ex vivo studies, we provide evidence that nitrate supplementation can act synergistically with the P2Y12 inhibitor clopidogrel resulting in augmented platelet inhibition than clopidogrel alone.12 The significant reduction in TRAP-induced platelet activation suggests RSNO may act via elevation of cGMP, as shownpreviously.42

Measurement of surface protein content revealed that the proportion of platelet-derived EVs decreased in the clopidogrel group. This specific reduction in platelet-derived EVs is in keeping with the hypothesis that the combined effects of RSNO and clopidogrel are responsible for the reduction in EV concentration seen in this study. Both cGMP and cyclic adenosine monophosphate are established synergistic mediators of platelet inhibition, 43 and it has previously been established that combined NO and clopidogrel have a synergistic effect on platelet activation.11

Platelet aggregation plays a key role in the development of atherosclerosis, and anti-platelet therapy is well established in the treatment of CVD. Under physiological conditions, adhesion of platelets to the endothelium is inhibited by endogenous production of NO, highlighted by the reduced NO bioavailability observed in CVD states. Previous studies have also shown a reduction in platelet activation ex vivo following nitrate supplementation.26 These authors also showed that prevention of the enterosalivary bioconversion of NO3 _ toNO2 _ prevented the expected rise in plasma NO2 _, the decrease in blood pressure and the inhibition of platelet aggregation.26

This study highlights the ability of a simple dietary intervention, in combination with clopidogrel, to reduce platelet activation, as well as circulating, pro-coagulant EVs in a CVD cohort. Thus, inorganic nitrates are capable of increasing patient responsiveness to clopidogrel. High on-treatment platelet reactivity is common in approximately 15 to 40% of patients prescribed clopidogrel.44 As formation of clopidogrel– SNO does not require activation of the pro-drug, all patients on clopidogrel would benefit increases in NO bioavailability, regardless of their clopidogrel metabolism. This may explain the various pleiotropic effects of clopidogrel that have been observed in recent studies.8 Ultimately, reducing the circulating, pro-coagulant EVs in CAD patients offers a new approach to moderating of the risk of thrombus formation and thus a myocardial infarction. This may also be of therapeutic

interest in other CVD cohorts, as well as cancer patients, where cancer-associated thrombosis is the second leading cause of death.45

This study has several limitations. First, despite the robust design of a placebo-controlled, double-blind, crossover study, the small sample size of groups (n ¼ 10), powered to enable the study to detect significant changes in EV concentration, limits the overall power of the study in terms of association between factors. Thus, there are several nonsignificant trends which may have been significant in a larger sample population. Although not statistically significant, a trend to decreased platelet aggregation was observed in patients not receiving clopidogrel. A large-scale study will be required to fully investigate the effect of NO3 _ alone on platelets; however, this observation was not accompanied by a decrease in EV or increase in RSNO. Second, the concentration of circulating RSNO attained in patients receiving clopidogrel is considerably lower than those used in in vitro models. The intentions of the in vitro studies were not to mimic the in vivo concentrations of these molecules, but rather a proof-of-principle to demonstrate the capability of clopidogrel–SNO to inhibit platelet EV release in response to addition of a pharmacological agent. The authors also recognize small differences in both cardiovascular risk factors and medications between the naïve and clopidogrel groups, rendering it difficult to conclude that differences in outcomes seen in this study are due to clopidogrel treatment or other potential confounding factors. Patients in both groups had been prescribed PPIs, which may have affected increased pH in the stomach toward neutrality and thus may have interfered with clopidogrel–SNO formation. However, it is important to note that PPI raises stomach pH from approximately 2 to 3 to about 4 to 6.46–48 At this pH, RSNO formation is only marginally reduced, as shown previously.17 Furthermore, nitrate supplementation was given about 1 hour after patients took their clopidogrel. This may not be optimal timing to facilitate the formation of clopidogrel– SNO. However, studies have highlighted the delayed transit of clopidogrel in CVD patients, visualizing intact tablets within the stomach 1.5 to 2 hours following ingestion.49 Thus, significant levels of clopidogrel would remain in the stomach while simultaneous increases in nitrite within the stomach also occur, facilitating clopidogrel–SNO formation. Comparisons between naïve and clopidogrel groups showed a similar reduction in platelet aggregation, EV concentration and EV surface markers following nitrate supplementation. Thus, due to the lack of RSNO produced by the naïve group, it is unlikely that the formation of RSNO is the sole mechanism responsible for the beneficial effects of nitrite. Finally, our study investigated the acute effects of a single nitrate supplement. Future studies should address the effect of chronic nitrate supplementation on EV populations in relation to RSNO and platelet activity in patients.

In summary, this study shows that acute nitrate supplementation can reduce platelet-derived EVs and residual platelet activity only in patient's receiving clopidogrel and nitrate concomitantly. Increases in plasma RSNO are observed only in these patients. These results suggest that dietary NO3 _ supplementation could provide an additional adjunct to platelet inhibition with clopidogrel in patients at risk from CVD. Medical Biochemistry, UHW, for performing the biochemical measurements on the patient samples. Finally, the authors would like to thank the nursing team for overseeing the care of the patients at both the cardiology outpatients' clinic and the cardiac day care unit at the University Hospital of Wales.

Conflicts of Interests
None declared.

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