Clopidogrel results in favourable changes in Nitric Oxide metabolism in patients undergoing percutaneous coronary intervention.

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Short running title: Clopidogrel favourably affects nitric oxide metabolites

Clopidogrel has been shown to have a morbidity and mortality benefit in the prevention of ischaemic complications in patients with acute coronary syndrome and/or after percutaneous coronary intervention (PCI)[1]. 600mg of clopidogrel can not only significantly inhibit platelet inhibition acutely (within 2 hours) but also improve endothelial dysfunction in stable CAD patients via a mechanism independent of platelet function[2]. A reduction in endothelial injury after PCI also occurs in the presence of clopidogrel[3]. Clopidogrel has also been associated with improved systemic endothelial nitric oxide (NO) bioavailability in patients with coronary artery disease[2]. Endothelial nitric oxide synthase (eNOS) is the main source of NO in the vasculature[4] and its activity directly proportional to plasma nitrite concentration and reflects cardiovascular NO bioavailability[5]. 70% of resting plasma nitrite is derived from eNOS activity in humans. NO metabolites including nitrite, nitrate and RSNO are now considered a direct measure of a physiological activity of NO and also a biologically active NO reservoir. We examined the effect of acute and chronic clopidogrel on these NO metabolites, the vasodilatory effect (cGMP) and oxidative/nitrosative stress markers in the plasma in patients undergoing PCI.

Methods: A prospective, single centre study was undertaken with 58 patients undergoing PCI with stent implantation for stable angina enrolled after informed consent. All patients were fasted for 6 hours and maintained on aspirin (75mg/day). 36 patients studied were loaded with 600mg of clopidogrel. 22 patients already on 75mg clopidogrel maintenance dose for ≥3 days were also studied. Samples were collected pre and 2h post clopidogrel. The platelet poor plasma was isolated from each sample and snap frozen in liquid nitrogen and stored at -80°C. Plasma nitrite, nitrate and S-nitrosothiols (RSNO) were measured using ozone-based chemiluminescence (NO analyzer, NOA280i, Sievers) as it has been described previously[6]. Commercial ELISA kits were used to measure Cyclic guanosine 3', 5'-monophosphate cGMP (R&D Systems), and 3-NT (Hycult) according to the manufacturer's instructions. Measurement of plasma total antioxidant capacity known as oxygen radical absorbance capacity assay has been described elsewhere[7] and plasma reduced thiols were measured using ThioStar Fluorescent Thiol Detection Reagent (Bioquote). **Statistical analysis:** Results are shown as mean±SD, where "N" represents the number of assayed blood samples. Pre and post loading clopidogrel differences were analysed by paired 2-tailed student *t*-test. Differences between pre clopidogrel and

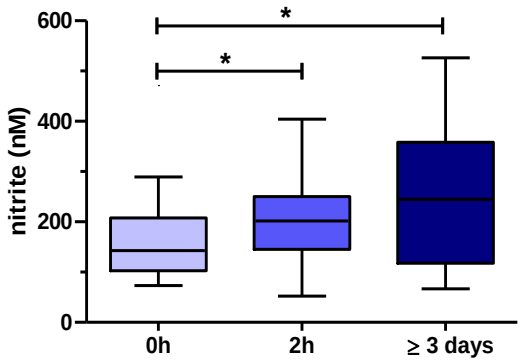
chronic were analysed by non-parametric 2-tailed Mann Whitney test. The association between variables was determined from Pearson's correlation coefficients, $p<0.05$ was considered statistically significant.

Results: After 2 hours of clopidogrel 600mg loading dose there was a significant increase in plasma nitrite levels from 160+/-78.9 to 202.6+/-84.7, $p<0.05$. Plasma nitrate and RSNO levels did not change respectively after acute loading 30.2+/-11.5 to 28.6+/-11 (p=ns) and 15.5+/-8.7 to 14.1+/-5.4 (p=ns) (Figure1a). There was a further time dependent increase in plasma nitrite (254.3±139.4 nM (p=0.002) with chronic therapy. As a downstream effector of vasodilatation cGMP was significantly greater after a loading dose (215.2 +/-127 to 234.6+/-109) (p=0.039) and after chronic therapy (281.7+/-70.8) (p=0.009) (Figure1b). The rise in plasma nitrite levels correlated positively with elevation in plasma cGMP levels (r=0.12, n=67, p=0.004). The rise in plasma nitrite levels inversely related to the total antioxidant capacity of plasma (r=0.07, n=67, p=0.03). Both nitrite and cGMP correlated positively with reduced thiols (r=0.37, p=0.01 and r=0.43, p=0.002, respectively (See also online supplementary data).

Discussion: Loading doses and/or long-term clopidogrel results in a increase in plasma NO metabolites, specifically plasma nitrite, and indices of effective vasodilatation. This is apparent as little as 2 hrs post loading, and is significantly higher in the chronic setting (when compared to the naive state). An increased total anti-oxidant capacity of plasma acutely and augmented production of reduced thiols chronically also suggests an overall parallel positive influence on the anti-oxidant status of plasma. The linear relationship between the rise in plasma nitrite and cGMP also strongly implies a direct relationship between increases in endothelial NO production and vasorelaxation induced by clopidogrel. The significant rise in plasma nitrite acutely post clopidogrel loading and during chronic therapy has clear clinical relevance, as plasma nitrite in large reflects endothelial eNOS activity and is a marker of NO bioavailability. In addition, elevation of circulating nitrite levels in patients undergoing PCI is likely to have a number of secondary benefits. Nitrite confers marked protection against ischaemia/ reperfusion injury in myocardial, hepatic, renal, pulmonary and the cerebral vasculature[8]. The cytoprotective effect possibly due to reduction of nitrite to NO during ischaemia or hypoxia[9]. This is supported in our data by the linear relationship between the rise in plasma nitrite and cGMP and is consistent with data showing the degree of P_2Y_{12} blockade by clopidogrel being related to the release of circulating endothelial cells (a response to insult) at the time of PCI. The magnitude of the rise of plasma nitrite in our patients is similar to studies where direct infusion of nitrite demonstrated 'protection' against ischaemia in CAD patients, vasodilatatory effects in peripheral and central vessels in hypoxia in humans and changes in the pulmonary vasculature[10]. The acute improvement in antioxidant capacity may be due to an increase in bioavailable nitric oxide. This effect is likely to be blunted over time in chronic therapy due to the multiple pro/antioxidant mechanisms seen longer term. Consistent with this, we observed upregulation of plasma reduced thiols in a time dependent manner, reflecting improved redox balance. **Conclusion:** Beneficial changes in NO metabolism occur after clopidogrel loading that is maintained during chronic therapy. A direct correlation between nitrite and cGMP in plasma most likely reflecting increased bioavailable NO and effective vessel dilatation. Parallel improvements in antioxidant capacity of plasma, provides evidence of additional non-platelet pleiotropic benefits of clopidogrel in patients undergoing PCI.

Figure 1a. The influence of clopidogrel on NO metabolites.
(All figures * =p<0.05)

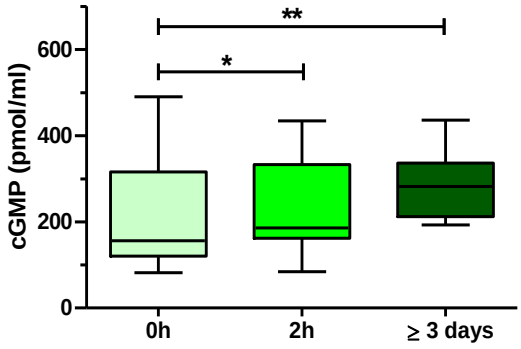
a)



Plasma levels of nitrite in CAD patients before (0h) and after (2h) receiving a loading dose of clopidogrel and after at least 3 days of a maintenance therapy (≥3 days).

Boxes and whiskers are presented according to Tukey method. Paired t-test (0 h vs. 2 h), n=36, *p<0.05; Mann Whitney test (0 h vs. ≥3 days), n=22, *p<0.05.

Figure 1b. The influence of clopidogrel on cGMP.



Plasma levels of cGMP in CAD patients before (0h) and after (2h) receiving a loading dose of clopidogrel and after at least 3 days of a maintenance therapy (≥3 days).

Boxes and whiskers are presented according to Tukey method. Paired t-test (0 h vs. 2 h), n=24, *p<0.05; Mann Whitney test (0 h vs. ≥3 days), n=19, **p<0.01.

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