

# openheart Targeting aspirin resistance with nutraceuticals: a possible strategy for reducing cardiovascular morbidity and mortality

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## To cite:

DiNicolantonio JJ, O'Keefe JH, McCarty MF. Targeting aspirin resistance with nutraceuticals: a possible strategy for reducing cardiovascular morbidity and mortality. *Open Heart* 2017;**4**:e000642. doi:10.1136/openhrt-2017-000642

Accepted 15 August 2017

## CLINICAL IMPACT OF ASPIRIN RESISTANCE

The failure of daily aspirin therapy to achieve an adequate or 'normal' suppression of platelet aggregation, as assessed *ex vivo*, is known as 'aspirin resistance'.<sup>1,2</sup> A substantial fraction of patients classified as aspirin-resistant are in fact poorly compliant.<sup>3</sup> In other cases, an increase in platelet turnover, often seen in association with systemic inflammation, as found in smokers and patients with diabetes, may render a once-daily administration schedule inadequate.<sup>2,4-6</sup> (Administering aspirin twice daily can result in greater platelet inhibition but may increase the risk for gastrointestinal bleeding.) When adverse pharmacokinetic factors impede the delivery of aspirin to platelets, an increase in dose can be helpful.<sup>7,8</sup> Concurrent administration of ibuprofen or other cyclooxygenase-1 (COX-1) inhibitors may prevent aspirin from acetylating the active site of COX-1.<sup>9</sup> But in some patients, even when platelet cyclooxygenase is fully inhibited, platelet aggregation remains anomalously high; this might be described as inherent aspirin resistance. Inherent aspirin resistance presumably reflects genetic or metabolic factors that alter the expression or function of platelet proteins such that platelets can aggregate effectively in the absence of thromboxane.

Although low-dose daily aspirin regimens reduce the risk for cardiovascular events by about 25% in patients with cardiovascular disease,<sup>10</sup> meta-analyses found that subjects who were resistant to ongoing aspirin therapy, as opposed to those who were sensitive, are about three times more likely to experience cardiovascular events.<sup>11,12</sup> This greatly increased risk is disproportionate to the benefit achievable with aspirin treatment, and evidently reflects the fact that aspirin resistance is serving as a marker for

metabolic factors, which themselves greatly increase cardiovascular risk. Nonetheless, there is strong evidence that intensified platelet-stabilising therapy can markedly improve outcomes in patients diagnosed with aspirin resistance. A number of controlled trials have defined groups of patients who are resistant to aspirin-clopidogrel therapy, and have randomised them to either continue with this standard care or to receive tailored platelet-stabilising regimens intended to achieve better control of platelet aggregation (entailing dosage increases or addition of additional agents such as integrin alpha-IIb beta-3 antagonists). A recent meta-analysis of such trials found that risk for subsequent death or stent occlusion was only one-quarter as great in patients receiving tailored therapy (OR=0.25, 95% CI 0.13 to 0.49), and risk for total vascular events was only 40% as high (OR=0.40, 95% CI 0.20 to 0.77).<sup>13</sup> Hence, additional or intensified measures for stabilising platelets appear to have important life-saving efficacy in aspirin-resistant patients.

The possibility of employing safe nutraceutical measures for this purpose should be considered. Agents that may have potential in this regard include the following:

### Spirulina/Phycocyanin: targeting NADPH oxidase

The Nox2-dependent form of NADPH oxidase is markedly activated when platelets interact with collagen via their chief collagen receptor, glycoprotein VI (GPVI). This event is the initial stimulus to thrombus formation when arterial plaque bursts and platelets are thereby exposed to collagen in the subendothelial ground substance. Interaction of collagen with GPVI leads to a series of intracellular tyrosine phosphorylation reactions, catalysed by an Src-like kinase and Syk, that induce formation of a signalling



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complex centred around the protein 'linker for activated T cells' (LAT).<sup>14</sup> This complex confers an activating phosphorylation on phospholipase C-gamma, which, by generating diacylglycerol and inosine-1,4,5-trisphosphate, induces a spike in intracellular free calcium as well as activation of protein kinase C (PKC), key triggers for platelet aggregation.<sup>15</sup> The concurrent activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase)—likely downstream from PKC activation—serves to potentiate this signalling pathway by generating hydrogen peroxide in the microenvironment of the GPVI-LAT signalling complex; this hydrogen peroxide oxidises active site cysteine groups in the tyrosine phosphatase SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase), reversibly inhibiting it, and thereby prolonging the half-lives of the tyrosine phosphorylations which SHP-2 targets.<sup>16–18</sup> Studies show that agents that inhibit Nox2 activity decrease the aggregatory response of platelets to collagen exposure; moreover, platelets that are genetically deficient in Nox2 are less responsive to collagen.<sup>19–21</sup> Conversely, platelets deficient in peroxiredoxin II or glutathione peroxidase activity are hyper-responsive to collagen.<sup>16–22</sup> In C57BL/6J mice, susceptibility to induced carotid or venous thrombosis increases during ageing, a phenomenon associated with increased expression of NADPH oxidase components. When these mice are bioengineered to overexpress glutathione peroxidase, this age-related increase in thrombotic activity is abolished; treatment with the NADPH oxidase inhibitor apocynin has a similar effect.<sup>23</sup> Platelets from patients determined to be aspirin-resistant showed greater expression of NADPH oxidase components and greater NADPH oxidase activity when stimulated; the NADPH oxidase inhibitors apocynin and diphenyleneiodonium (DPI) diminished the aggregatory responses of these platelets to collagen and epinephrine, whereas they had little effect on platelets from aspirin-sensitive patients.<sup>24</sup>

Much remains to be learnt regarding the full impact of NADPH oxidase activation on platelet function. Other physiological agonists that promote platelet aggregation—thrombin, thromboxane, epinephrine and (adenosine diphosphate (ADP)—signal via G-protein mechanisms (as opposed to tyrosine phosphorylation); they provoke at most a mild activation of NADPH oxidase.<sup>25</sup> Nonetheless, some studies report that NADPH oxidase inhibition blunts the response of platelets to thrombin or epinephrine; there seems to be agreement that such inhibition fails to influence the proaggregatory impact of ADP.<sup>21–26–27</sup> How superoxide generation influences thrombin and epinephrine signalling remains unclear. Potentially, NADPH oxidase-generated superoxide could influence platelet function by interfering with the antiaggregatory impact of nitric oxide (NO) (by scavenging NO or promoting uncoupling of NO synthase, as discussed below). Also, superoxide can interact spontaneously with arachidonic acid to generate F<sub>2</sub>-isoprostanes, which can promote platelet aggregation by serving as agonists for

the thromboxane receptor (thereby bypassing the inhibitory impact of aspirin on thromboxane synthesis).<sup>28–30</sup>

These considerations suggest that targeting Nox2-dependent NADPH oxidase complexes might be a worthwhile strategy for stabilising platelets, particularly in patients with aspirin resistance.<sup>19</sup> In that regard, the administration of potent doses of lipophilic statins such as atorvastatin or rosuvastatin can exert a platelet-stabilising effect within 2 hours, and this effect may reflect NADPH oxidase inhibition; statins can downregulate NADPH oxidase activity by suppressing isoprenylation and membrane association of Rac1, an essential component of Nox2-dependent NADPH oxidase.<sup>31–35</sup>

Nutraceutical strategies that target Nox2 may also represent a practical option for platelet stabilisation. Free bilirubin functions physiologically within cells to inhibit certain NADPH oxidase complexes, including those that are Nox2-dependent<sup>36–39</sup>; it therefore is not surprising that exposure to high physiological levels of free bilirubin has been reported to decrease collagen-triggered platelet aggregation.<sup>40</sup> (As might be expected, bilirubin did not influence the aggregatory response to ADP.) This phenomenon may contribute to the relative protection from cardiovascular events enjoyed by individuals with high-normal plasma levels of free bilirubin, including people with Gilbert syndrome.<sup>41–43</sup> Phycocyanobilin (PhyCB), a protein-bound chromophore in cyanobacteria such as spirulina, is a close chemical relative of bilirubin, and has been found to share bilirubin's ability to inhibit NADPH oxidase complexes, likely explaining the profound antioxidant/anti-inflammatory effects of orally administered spirulina or PhyCB-enriched spirulina extracts in rodent studies.<sup>44–47</sup> Hence, adequate intakes of spirulina or of PhyCB-rich extracts may have clinical potential as a platelet-stabilising strategy. Indeed, exposure of platelets to phycocyanin, the spirulina protein, which features PhyCB as a covalently attached chromophore, has been reported to reduce their responsiveness to collagen.<sup>48–49</sup>

### High-dose biotin: mimicking NO

NO produced by healthy vascular endothelium has a platelet-stabilising effect. Moreover, platelets express the endothelial isoform of nitric oxide synthase (eNOS), which is activated by the increase in cytoplasmic free calcium as well as the PI3K/Akt activation associated with platelet aggregation; hence, the NO produced within stimulated platelets acts as a feedback brake on aggregation.<sup>50</sup> The effect of NO in this regard is mediated by direct stimulation of the soluble guanylate cyclase (sGC), resulting in increased synthesis of cyclic guanosine monophosphate (cGMP). The consequent rise in platelet cGMP boosts the activity of cGMP-dependent protein kinase I (cGKI), which then phosphorylates a protein (IRAG) associated with the inositol trisphosphate receptor in the platelet endoplasmic reticulum (ER), diminishing the ability of inositol trisphosphate to provoke release of calcium from the ER, and thereby

opposing aggregation.<sup>51 52</sup> Additionally, cGKI may work in an additional, still poorly understood way to oppose ADP-mediated activation of Rap1b, the G-protein that enables platelet aggregation by activating the fibrinogen-binding activity of integrin alpha-IIb beta-3.<sup>53</sup>

Not surprisingly, agents capable of mimicking and potentiating NO's activation of sGC have platelet-stabilising activity.<sup>54–57</sup> In this regard, supraphysiological concentrations of the vitamin biotin can directly stimulate sGC activity, and orally administered high-dose biotin exerts antihypertensive effects in rats that reflect this stimulation of sGC.<sup>58–60</sup> Since biotin is well tolerated in daily doses as high as 300 mg, it may have clinical potential as a platelet-stabilising agent.<sup>61–63</sup>

### Citrulline and high-dose folate: restoring coupling of eNOS

When vascular endothelium is under chronic oxidative stress—as it frequently is in patients at increased cardiovascular risk—eNOS can become ‘uncoupled’ owing to oxidation of its cofactor tetrahydrobiopterin (BH4) and/or increased production of asymmetric dimethylarginine (ADMA); the uncoupled enzyme produces superoxide rather than NO.<sup>64–67</sup> Peroxynitrite, which evolves from the spontaneous interaction of superoxide and NO, is a key mediator of BH4 oxidation.<sup>66</sup> This uncoupling of eNOS might also occur in platelets, either owing to elevated plasma levels of ADMA or oxidation of platelet tetrahydrobiopterin.<sup>68–70</sup> Oxidation of platelet BH4 may be common in patients with unstable angina, possibly reflecting repeated episodes of oxidative stress triggered by interaction of platelets with the coronary subendothelium.<sup>70 71</sup> The platelets of smokers and patients with diabetes and metabolic syndrome may also be under chronic oxidative stress. In these conditions, platelet production of NO has been reported to be subnormal and superoxide production elevated, likely reflecting eNOS uncoupling.<sup>72–76</sup> Hyperglycaemia can boost mitochondrial production of superoxide in diabetic platelets.<sup>77</sup> The excess exposure to free fatty acids and glucose typically seen in type 2 diabetes and metabolic syndrome may activate PKC via increased diacylglycerol synthesis; PKC, in turn, can stimulate NADPH oxidase activity.<sup>78 79</sup> Likewise, semistable toxins in cigarette smoke such as acrolein can stimulate PKC, boosting oxidative stress.<sup>80–82</sup> Not surprisingly, patients with diabetes, metabolic syndrome or tobacco addiction are more likely to be classified as aspirin-resistant, and aspirin therapy appears to have a limited impact on risk for coronary events in patients with diabetes.<sup>83–93</sup> In oxidatively stressed platelets, aspirin therapy leads to an increase in isoprostane production that would be expected to partially offset the benefit stemming from inhibition of thromboxane synthesis.<sup>94</sup>

Administration of citrulline in multigram daily doses can oppose ADMA-mediated uncoupling of eNOS by boosting intracellular levels of arginine.<sup>95–98</sup> High-dose folate promotes restoration of normal levels of tetrahydrobiopterin in oxidatively stressed endothelium. This

reflects the ability of intracellular reduced folates to scavenge peroxynitrite-derived radicals, thereby protecting BH4 from oxidation; moreover, high-dose folate boosts endothelial expression of dihydrofolate reductase, an enzyme that can reduce oxidised BH4 (dihydrobiopterin) back to BH4.<sup>99–103</sup> It is as yet unknown whether high-dose folate can induce dihydrofolate reductase in megakaryocytes (and hence increase its expression in platelets). In any case, when eNOS is uncoupled in vascular endothelium and/or platelets, administration of citrulline and of high-dose folate may help to restore physiologically appropriate production of NO,<sup>104</sup> although the direct impact of high-dose folate on platelets may hinge to some extent on its capacity to boost platelet dihydrofolate reductase activity. These agents would not be expected to influence platelet function in healthy subjects whose eNOS activity is properly coupled.

### N-acetylcysteine: boosting glutathione levels

Like reduced folates, reduced glutathione can scavenge peroxynitrite-derived radicals, thereby protecting BH4 from oxidation.<sup>105–107</sup> Indeed, in patients with type 2 diabetes, daily parenteral administration of glutathione was found to increase the eNOS activity of their platelets.<sup>108</sup> Moreover, through interaction with glutathione peroxidase and glutaredoxin, glutathione can oppose the signalling effects of hydrogen peroxide, either by eliminating hydrogen peroxide or by rereducing oxidised cysteinyl groups.<sup>109–111</sup> Cysteine availability is rate-limiting for glutathione synthesis, and tissue glutathione levels can be boosted by increased cysteine intake, most conveniently achieved with supplemental N-acetylcysteine (NAC).<sup>112–115</sup> Tissue glutathione levels tend to decline with ageing, reflecting inducibility of the rate-limiting enzyme for glutathione synthesis, gamma-glutamyl synthetase; youthful levels of glutathione can be restored with NAC supplementation.<sup>113 116–118</sup> Although the impact of NAC ingestion on platelet reactivity *in vitro* or *ex vivo* has received little study, NAC concentrations comparable to those achievable in plasma with oral administration have been reported to decrease oxidant stress in human platelets, and decrease their aggregability in response to thrombin and ADP; this effect is contingent on conversion of NAC to glutathione.<sup>119 120</sup> The impact of supplemental NAC on platelet function *ex vivo* merits study, most notably in patients such as those with diabetes, whose platelets may be under increased oxidative stress.

### Glycine: hyperpolarising platelet membranes

Glycine-gated chloride channels are expressed by various tissues, including platelets.<sup>121–126</sup> High-normal plasma levels of glycine boost the open probability of these channels, and in most tissues this induces an influx of chloride ions, leading to hyperpolarisation of the plasma membrane. Notably, platelets appear to express such channels, and activation of this channel with glycine reduces the responsiveness of platelets to both collagen and ADP.<sup>126</sup> Indeed, previous studies have shown that

plasma membrane potential can regulate platelet responsiveness; most such studies, although not all,<sup>127</sup> have concluded that depolarised platelets are sensitised to aggregation provoked by collagen, thrombin, ADP and epinephrine—although depolarisation per se does not increase calcium influx or elevate intracellular levels of free calcium.<sup>128–131</sup> Analogously, platelets are responsive to endothelium-derived hyperpolarising factor, which suppresses ADP-induced platelet adhesion to endothelial cells.<sup>132</sup> How membrane polarisation influences platelet activation remains unclear. When rats were fed glycine-enriched diets (2.5%–5.0%), the amplitude of platelet aggregation ex vivo in whole blood, in response to collagen or ADP, was reduced by approximately 50%.<sup>126</sup> Hence, dietary glycine supplementation, which has been found to be feasible and well tolerated, may have potential as a platelet-stabilising strategy.

Moreover, it should be noted that glycine is a substrate for glutathione synthesis, and supplemental glycine may amplify the impact of concurrent supplemental NAC on tissue glutathione levels.<sup>118 133</sup>

### Taurine: boosting hydrogen sulfide production

There are several reports that supplemental taurine can exert a platelet-stabilising effect in normally nourished humans and rats<sup>134–137</sup>; however, a null effect was seen in one clinical study.<sup>138</sup> How taurine might influence platelet function is unclear. A recent clinical study has found that plasma concentrations of hydrogen sulfide (H<sub>2</sub>S) are markedly enhanced by taurine supplementation (1.6 g daily); concurrent rodent studies suggest that this phenomenon reflects increased vascular induction of enzymes that generate H<sub>2</sub>S.<sup>139</sup> There is clear evidence that H<sub>2</sub>S has a stabilising effect on platelets; the basis of this effect remains unclear, and it does not appear to reflect an upregulation of NO bioactivity.<sup>140–144</sup> Future studies assessing the impact of dietary taurine on platelet function should evaluate the possible contribution of H<sub>2</sub>S production to any platelet-modulatory effects observed.

### Long-chain omega-3 fats: more than thromboxane antagonists

Decades ago, the prolonged bleeding times and superior cardiovascular health of Eskimos following their traditional lifestyle motivated the first studies demonstrating that diets high in long-chain omega-3s from fish oil could reduce the aggregability of platelets.<sup>145–147</sup> This phenomenon was first attributed to the ability of eicosapentaenoic acid (EPA) to compete with arachidonic acid for access to COX-1, diminishing thromboxane synthesis.<sup>145 147</sup> However, it was soon discovered that the antithrombotic impact of omega-3-rich fish is complementary to that of aspirin, implying that thromboxane antagonism is not the only mechanism responsible for the antiaggregatory impact of fish oil.<sup>148–151</sup> Enrichment of platelet membrane lipids with docosahexaenoic acid (DHA) has been reported to decrease the aggregatory response to collagen.<sup>152–154</sup> The clinical impact

of DHA supplementation per se on platelet function is the subject of conflicting reports.<sup>155 156</sup> How DHA might influence platelet function remains unclear. The impact of long-chain omega-3 on platelet response to collagen is notable—after 28 days of supplementation with 3.4 g EPA+DHA daily, the aggregatory response to collagen was inhibited by about 50%.<sup>150</sup>

### SUMMING UP

A significant proportion of patients at risk for thrombotic episodes fail to achieve an adequate control of platelet aggregability when placed on aspirin therapy. These individuals are at greatly increased risk for cardiovascular events—in large part because of the metabolic factors that destabilise their platelets—but clinical studies demonstrate some ancillary measures that promote greater platelet stability can notably decrease their risk. Nutraceutical measures, because of their relative safety, affordability and broader protective metabolic impacts, may have particular merit for this purpose.

Nox2-dependent superoxide production, which plays a key role in collagen-triggered GPVI signalling and acts in other ways to promote aggregation, may be suppressible with the spirulina chromophore PhyCB. Platelet exposure to episodic or chronic oxidative stress, associated with diabetes, metabolic syndrome, acute coronary syndrome and smoking, can uncouple platelet eNOS, diminishing the platelet-stabilising activity of NO while further adding to the burden of oxidative stress. Supplemental citrulline, high-dose folate and NAC may have potential for recoupling eNOS activity in platelets. High-dose biotin should mimic the platelet-stabilising actions of NO by directly stimulating sGC, and supplemental taurine may help to stabilise platelets by boosting production of H<sub>2</sub>S. The platelet-stabilising activity of glycine may reflect hyperpolarisation of platelet membrane potential, which downregulates platelet aggregation for unknown reasons. Long-chain omega-3 fatty acids, while decreasing platelets production of thromboxane, appear to work in additional ways to stabilise platelets. These measures can be expected to be safe and reasonably well tolerated. Functional foods providing at least several of the agents discussed here may ultimately represent a feasible and practical strategy for optimising platelet activity with nutraceuticals. Such foods moreover may contribute more generally to vascular and metabolic health.

**Contributors** All authors contributed to the final manuscript.

**Competing interests** JJDN declares no conflict. JHOK is an owner of CardioTabs, a nutraceutical company. MFMC is co-owner and science director of a nutraceutical company that sells a product containing glycine.

**Provenance and peer review** Not commissioned; internally peer reviewed.

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