

1 **The *ex vivo* antiplatelet activation potential of fruit phenolic metabolite**  
2 **hippuric acid**

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26 **Abstract**

27 Polyphenol-rich fruit and vegetable intake has been associated with reduction in platelet  
28 hyperactivity, a significant contributor to thrombus formation. This study was undertaken to  
29 investigate the possible role of hippuric acid, a predominant metabolite of plant cyclic  
30 polyols, phenolic acids and polyphenols, in reduction of platelet activation-related  
31 thrombogenesis. Fasting blood samples were collected from 13 healthy subjects to analyse  
32 the effect of varying concentrations of hippuric acid (100  $\mu$ M, 200  $\mu$ M, 500  $\mu$ M, 1 mM and 2  
33 mM) on activation-dependant platelet surface-marker expression. Procaspase activating  
34 compound-1 (PAC-1) and P-selectin/CD62P monoclonal antibodies were used to evaluate  
35 platelet activation-related conformational changes and  $\alpha$ -granule release respectively using  
36 flow cytometry. Platelets were stimulated *ex vivo* via the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> – adenosine diphosphate  
37 (ADP) pathway of platelet activation. Hippuric acid at a concentration of 1 mM and 2 mM  
38 significantly reduced P-selectin/CD62P expression ( $p=0.03$  and  $p<0.001$  respectively)  
39 induced by ADP. Hippuric acid at 2 mM concentration also inhibited PAC-1 activation-  
40 dependant antibody expression ( $p=0.03$ ). High *ex vivo* concentrations of hippuric acid can  
41 therefore significantly reduce P-selectin and PAC-1 expression thus reducing platelet  
42 activation and clotting potential. However, although up to 11 mM of hippuric acid can be  
43 excreted in the urine per day following consumption of fruit, hippuric acid is actively  
44 excreted with a recorded C<sub>max</sub> for hippuric acid in human plasma at 250-300  $\mu$ M. This is  
45 lower than the blood concentration of 1-2 mM shown to be bioactive in this research. The  
46 contribution of hippuric acid to the protective effects of fruit and vegetable intake against  
47 vascular disorders by the pathways measured is therefore low but could be synergistic with  
48 lowered doses of antiplatelet drugs and help reduce risk of thrombosis in current antiplatelet  
49 drug sensitive populations.

50 **Keywords:** Platelet activation, polyphenol, hippuric acid, thrombosis, P-selectin

## 51 INTRODUCTION

52 According to a recent World Health Organisation report, 17.5 million people died from  
53 cardiovascular disease in 2012, representing 31% of all global deaths <sup>1</sup>. Amongst several risk  
54 factors including smoking, hypertension, genetic association and obesity, poor nutrition has  
55 also been one of the important contributors. Several studies have reported the benefits of a  
56 healthy diet of fruit and vegetables in reducing the risk of vascular disorders <sup>2-5</sup>. Interest in  
57 plant derived antioxidants and polyphenols such as anthocyanins and quercetin continues to  
58 grow as a result of their reported antithrombotic and cardiovascular benefits <sup>6-13</sup>. Analysis of  
59 the pharmacokinetics and bioavailability of plant derived compounds after  
60 polyphenol/anthocyanin rich food intake has shown that the concentrations of anthocyanins  
61 and structurally related metabolites only reach micro-molar concentrations *in vivo* (less than 5  
62  $\mu\text{M}$ ) <sup>14</sup>. However hippuric acid is a major metabolite post polyphenol consumption that is  
63 excreted in urine in up to millimolar concentrations *in vivo* <sup>15, 16</sup>. Consumption of prunes <sup>16</sup>,  
64 black and green tea solids <sup>17</sup>, cranberry juice <sup>18</sup>, red wine, grape juice <sup>19</sup>, and fruits/vegetables  
65 in general <sup>15</sup> have been shown to increase urinary hippuric acid levels. According to a dietary  
66 intervention study by Toromanovic and colleagues <sup>16</sup>, urinary excretion of levels of hippuric  
67 acid reached up to 11 mM per 24 hours after consumption of 300 g of edible fruits <sup>16</sup>. The  
68 increased levels are believed to be due to plant derived simple phenolic compounds and the  
69 microbial degradation of anthocyanins, chlorogenic acids and quinic acid in the colon and  
70 their catabolism to benzoid type compounds <sup>20-22</sup>. In spite of hippuric acid being a consistent  
71 biomarker/metabolite that can be excreted via the urine in grams per day, there are no well-  
72 documented cardiovascular benefits associated with it.

73 Several *in vitro* and *in vivo* trials have demonstrated the prospective bioactivity of  
74 natural dietary phenolics rich in antioxidants, such as anthocyanins <sup>6, 23, 24</sup> and quercetin  
75 derivatives <sup>25</sup> in reducing thrombotic risk by alleviating platelet hyperactivity/aggregation.

76 Hippuric acid, however, occurs in very high concentrations *in vivo* versus very low levels of  
77 most other phenolics<sup>26</sup>. Similar to the action of current anti-platelet drugs such as aspirin and  
78 clopidogrel, polyphenols have been demonstrated to block platelet activation pathways  
79 (cyclooxygenase-1 – COX-1 and P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub>) consequently reducing the risk of thrombosis<sup>9</sup>,  
80<sup>27, 28</sup>. We hypothesize that, active metabolites occurring *in vivo* at high concentration such as  
81 hippuric acid, could also be partially responsible for the claimed anti-platelet properties of  
82 plant derived phenolic acids and polyphenols.

83 This *ex vivo* trial aimed to evaluate the effect of hippuric acid, a potential active  
84 metabolite, in targeting the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> pathway of platelet activation. We have analysed the  
85 effect of various concentrations of the metabolite on activation-dependant platelet surface  
86 receptor P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> induced by adenosine diphosphate (ADP), a physiological platelet  
87 activator, by evaluating the expression of platelet activation-related conformation change and  
88 degranulation.

89

## 90 **MATERIALS AND METHODS**

### 91 ***Subject recruitment and sample collection***

92 The study protocol was approved by the Griffith University Human Research ethics  
93 committee (GU Protocol no. MSC/13/11/HREC) and was performed in compliance with the  
94 relevant laws and institutional guidelines. Thirteen healthy subjects were recruited from the  
95 general population by means of advertisements placed around Griffith University, Gold Coast  
96 campus. Subjects were healthy, non-smokers with no history of metabolic or cardiovascular  
97 diseases and were not on any anti-inflammatory, anti-platelet medications or health  
98 supplements at least 7 days prior to blood collection. Informed consent was obtained from all

99 volunteers prior to commencement of the study. Fasting whole blood samples were collected  
100 from the median cubital vein by a trained phlebotomist at the School of Medical Science,  
101 Griffith University. Care was taken to ensure minimal sample handling or agitation to prevent  
102 platelet activation. No samples were obtained from traumatic phlebotomy procedures or  
103 contained obvious clots. Blood was drawn into tri-potassium EDTA (1.8 mg/mL)  
104 anticoagulant tubes (used for full blood examination - FBE) before collection into tri-sodium  
105 citrate (28.1 g/mL) tubes (used for platelet surface-marker expression) to avoid risk of  
106 collecting venepuncture activated platelets. Baseline FBE and biochemical profile was  
107 carried out for initial subject screening and recruitment using a Coulter® Ac.T™ 5diff CP  
108 haematology analyser (Beckman Coulter, Inc., Lane Cove, NSW, Australia) and Cobas  
109 Integra 400® plus biochemistry analyser (Roche diagnostics, Basel, Switzerland)  
110 respectively.

### 111 ***Hippuric acid***

112 Hippuric acid used in the analysis was purchased from Sigma-Aldrich, Australia. Working  
113 solutions of 100 µM, 200 µM, 500 µM, 1 mM and 2 mM were prepared from a stock solution  
114 of 20 mM concentration diluted in 1x concentration of phosphate buffered saline (PBS).  
115 Aliquots of different concentrations of hippuric acid were stored at 4°C for a maximum of 2  
116 weeks.

### 117 ***Activation dependant platelet surface-marker expression***

118 Venous blood collected into tri-sodium citrate tubes were used to evaluate platelet surface  
119 marker expression. This analysis was performed and interpreted using the BD LSRFortessa  
120 cell analyser (BD Biosciences, North Ryde, NSW, Australia) and BD FACSDiva software  
121 (version 6.1.3, BD Biosciences, North Ryde, NSW, Australia) respectively. Platelet  
122 activation dependant monoclonal antibodies (mAb) were used to evaluate the expression of  
123 positive activated platelets induced by ADP agonist. CD42b-peridinin chlorophyll protein

124 (CD42b-PerCp-Cy5.5) conjugated mAb was used to identify the GPIb-IX receptors, the most  
125 abundant receptor on the platelet population. Platelet activity was assessed by activation-  
126 dependant platelet surface marker expression using PAC-1-fluorescein isothiocyanate (PAC-  
127 1-FITC recognises activated conformational changes in the fibrinogen binding receptor  
128 GPIIb-IIIa) and P-selectin/CD62P- (expressed in activated de-granulated platelets)  
129 conjugated mAb. The antibodies and their respective isotype controls were purchased from  
130 BD Biosciences (BD Biosciences, North Ryde, NSW, Australia).

131 Whole blood was incubated with respective concentrations of hippuric acid and  
132 control (PBS) for 15 min at room temperature (25°C). After incubation, the samples were  
133 then diluted in filtered modified tyrode's buffer (pH 7.2) and incubated with CD42b, PAC-1,  
134 P-selectin mAb's for 15 mins in the dark at room temperature. ADP (5  $\mu$ M) was incubated  
135 with the blood-antibody mixture for 10 min in the dark at room temperature to stimulate the  
136 platelets via the P<sub>2</sub>Y<sub>12</sub>/P<sub>2</sub>Y<sub>1</sub> platelet activation pathway. The suspension was then fixed with  
137 1% paraformaldehyde solution (pH 7.2), incubated in the dark at room temperature for 15  
138 min and analysed in the flowcytometer for antibody expression. The setup and optimal  
139 fluorescence compensation of the flowcytometer was validated using BD Cytometer Setup  
140 and Tracking beads and BD CompBead compensation particles (BD Biosciences, North  
141 Ryde, NSW, Australia). Ten thousand platelet events were acquired, gated on the basis of  
142 light scatter and CD42b MAb expression. Activation dependant-MAb (PAC-1 and P-selectin)  
143 expression by activated platelets was articulated as mean fluorescence intensity (MFI).

#### 144 *Statistical analysis*

145 A two-way ANOVA following Tukey's post comparison test was performed using GraphPad  
146 Prism version 6.0 for Windows (GraphPad Software, La Jolla, California, USA). A minimum  
147 sample size of 12 subjects in each group is required for 80% power to detect a 5% variation  
148 in the laboratory parameters measured, where a 3-5% standard deviation exists in the

149 population, assuming an alpha error of 0.05. All the data is expressed as mean  $\pm$  standard  
150 deviation. Differences between the groups were considered to be significant when  $p < 0.05$ .  
151 Any significant statistical interactions were included in the analysis where applicable.

152

## 153 **RESULTS**

154 The baseline full blood cell counts, inflammation marker and biochemical profile of the  
155 subjects under study were within normal reference ranges (Table 1) as established by the  
156 Royal College of Pathologists Australasia<sup>29</sup>.

157 It was observed that whole blood *ex vivo* treatment with 1 mM and 2 mM  
158 concentrations of hippuric acid reduced ADP-induced P-selectin/CD62P expression by 7.5%  
159 ( $p=0.03$ ) and 14% ( $p < 0.001$ ) respectively (Figure 1). Lower concentrations of hippuric acid  
160 (100  $\mu$ M, 200  $\mu$ M or 500  $\mu$ M) did not have a statistically significant effect on P-selectin  
161 platelet surface marker expression but demonstrated a trend in inhibition of the activation-  
162 dependant monoclonal antibody expression (Figure 1).

163 Hippuric acid at a concentration of 2 mM inhibited the initial platelet activation phase,  
164 by 11% ( $p=0.03$ ), involving its conformational change targeting the GPIIb-IIIa receptor,  
165 evident from PAC-1 activation-dependant monoclonal antibody expression (Figure 2). Lower  
166 concentrations of hippuric acid did not affect PAC-1 expression.

167

## 168 **DISCUSSION**

169 In this trial we have demonstrated the potential of hippuric acid in alleviating platelet  
170 activation stimulated by an exogenous agonist, ADP, which is responsible for thrombus  
171 formation and platelet adhesion *in vivo*. This potent platelet stimulant was used to activate

172 resting platelets *ex vivo* and target the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> receptor of platelet activation. Similar to  
173 the action of the anti-platelet drug clopidogrel, i) hippuric acid at 1 mM and 2 mM  
174 concentrations reduces P-selectin/CD62P mAb expression indicating its ability to inhibit  
175 platelet degranulation consequently platelet activation ii) 2 mM concentration of hippuric  
176 acid reduces PAC-1 mAb expression therefore suppressing the initial phase of activation –  
177 GPIIb-IIIa platelet receptor-related conformational change induced by ADP platelet agonist.  
178 However we did not observe inhibition of platelet activation by lower concentrations of  
179 hippuric acid in relation to the expression of activation-dependant conformational change or  
180 degranulation markers.

181 In platelet related thrombogenesis, platelets adhere to damaged endothelium; undergo  
182 a conformational change followed by activation and degranulation <sup>30</sup>. This activation results  
183 in the binding of fibrinogen to platelet surface receptors consequently leading to thrombus  
184 formation <sup>30</sup>. Current antiplatelet drugs target different receptors on the surface of platelets or  
185 their activation pathways in order to reduce platelet hyper-activation or hyper-aggregability.  
186 For example, aspirin, the most commonly used antiplatelet drug and salicylate derived from  
187 fruit and vegetables in the diet <sup>31</sup>, inhibit platelet activity by targeting the cyclooxygenase-1  
188 (COX-1) enzyme hence blocking the production of thromboxane A<sub>2</sub> (TxA<sub>2</sub>), a pro-thrombotic  
189 agent <sup>32</sup>. Furthermore, clopidogrel binds irreversibly to the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub>-ADP platelet surface-  
190 receptor consequently reducing ADP-induced platelet activation <sup>33</sup>. In the current *ex vivo* trial  
191 it was observed that 1 mM and 2 mM concentrations of hippuric acid efficiently target the  
192 P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> platelet surface-receptor induced by ADP. In an *in vitro* study performed by  
193 Ostertag and colleagues, 100 μM hippuric acid did not inhibit collagen induced P-selectin  
194 expression but reduced platelet aggregation <sup>26</sup>. Furthermore, Rechner et al. demonstrated that  
195 lower concentrations of hippuric acid (10 μM) did not exhibit an inhibitory effect on  
196 thrombin receptor activating peptide induced P-selectin expression in an *in vitro* trial

197 evaluating the effect of anthocyanins and colonic metabolites of polyphenols on platelet  
198 function<sup>23</sup>. The action of hippuric acid on platelet activation inhibition could also potentially  
199 be due to the internalization and metabolism by enzymes in platelets capable of modifying  
200 molecules by the addition of a methyl, sulphate or a glucorinide group. These functional  
201 groups, conjugated to polyphenols or their metabolites within the platelets, might impact the  
202 regulation of activation<sup>34</sup>. Our results are in agreement with the above *in vitro* trials, where  
203 low concentrations of hippuric acid did not effectively block platelet activation dependant  
204 receptors. Studies had not previously been extended into the higher concentrations of 1-2  
205 mM. Consumption of a 300 g portion of blueberries, cranberries and prunes resulted in  
206 excretion of 11 mM hippuric in 24 hours and other fruits such as raspberry, melon and  
207 blackberry resulting in excretions of 5-6 mM hippuric in 24 hours peaking at 10-15 hours<sup>16</sup>.  
208 Children and adolescents eating fruit and vegetable averaged 2 mM hippuric acid per day in  
209 urine<sup>15</sup>. Since this millimolar excretion of hippuric acid occurs via the portal circulation it is  
210 believed that high concentrations could also be detected in plasma post polyphenol rich fruit  
211 or vegetable consumption. Hippuric acid, following a bolus dose of benzoic acid, has a  
212 recorded maximum concentration ( $C_{max}$ ) in human plasma of 250-300  $\mu$ M with elimination of  
213 hippuric acid occurring at a first-order rate or in a non-saturable manner with the maximum  
214 urinary excretion rates of hippuric acid independent of the dose size<sup>35</sup>. Serum hippuric acid  
215 concentrations were in the range 1.1 – 6.1 mM when 5g (28 mM) hippuric acid was bolus  
216 dosed for antibacterial effects<sup>36</sup>. While the *in vivo* levels of hippuric acid in plasma that can  
217 be reached following fruit consumption have not been tested they are not likely to reach the  
218 recorded  $C_{max}$  of 250-300  $\mu$ M but could be higher than the 100  $\mu$ M hippuric acid shown by  
219 Ostertag and colleagues to reduce platelet aggregation *in vitro*<sup>26</sup>. Although several dietary  
220 intervention and *in vivo* studies have shown the mechanism of inhibition of platelet function  
221 and anti-clotting activity by polyphenols, the prospective of active metabolites such hippuric

222 acid being a possible reason for the observed anti-thrombotic effect has not been  
223 demonstrated. We have shown the potential of the metabolite in reducing platelet activation  
224 related conformational change and inhibition of  $\alpha$ -granule release although at a level at least  
225 three times higher than what is currently known to occur *in vivo* as a result of fruit and  
226 vegetable consumption.

227         Microbial degradation of polyphenols especially anthocyanins and quinic acid or  
228 chlorogenic acid in the colon, followed by hepatic conjugation with glycine, is believed to be  
229 responsible for the observed increased levels of hippuric acid <sup>20, 21</sup>. It has also been suggested  
230 that majority of the hippuric acid seen in urine is due to synthesis from quinic acid present in  
231 antioxidant rich fruits <sup>21</sup>. Ronald W. Pero recently proposed that quinic acid and its end  
232 catabolite, hippuric acid may themselves not be efficacious but may lead to the production of  
233 increased levels of nicotinamide and tryptophan as antioxidants through the shikimate  
234 pathway <sup>21</sup>. The present study shows the anti-thrombotic effect of acute *in vitro* addition of  
235 varying concentrations of hippuric acid to whole blood directly. It is unclear whether a  
236 similar effect of active metabolites can be seen after prolonged consumption of large  
237 quantities of polyphenol rich food.

238         In addition to increased levels of hippuric acid post consumption of a polyphenol rich  
239 diet, it is also a metabolite of acetylsalicylic acid (aspirin). The other major metabolites of  
240 aspirin include salicyluric acid and gentisuric acid which have very similar structural moieties  
241 as hippuric acid <sup>37</sup>. Acetylsalicylic acid is converted to salicylic acid and conjugates with  
242 benzoic acid in the liver to result in the synthesis of hippuric acid. This pharmacokinetics of  
243 acetylsalicylic acid might partly explain the reason behind the anti-thrombotic activity of its  
244 potential metabolite, hippuric acid. Aspirin alleviates platelet activation by blocking the  
245 COX-1 pathway resulting in reduced arachidonic acid production hence preventing  
246 thromboxane synthesis consequently thrombus formation. Further mechanistic studies

247 evaluating the potential of hippuric acid in reducing arachidonic acid induced platelet  
248 activation by blocking the COX-1 pathway of platelet activation is warranted.

249 It has also been demonstrated that thiol derivatives of antiplatelet drugs such as  
250 clopidogrel and ticlopidine bind in a covalent manner to the P<sub>2</sub>Y<sub>1</sub>/P<sub>2</sub>Y<sub>12</sub> ADP receptor and  
251 block the activation of platelets<sup>38</sup>. Hippuric acid at concentrations attained *in vivo* post  
252 polyphenols rich food consumption could potentially help block the ADP receptor of  
253 activation thereby mimicking the action of anti-platelet drugs. We hence believe that  
254 consumption of polyphenols rich foods could be synergistic with lowered doses of  
255 antiplatelet drugs and help platelet activation inhibition and thus reduce risk of thrombosis in  
256 current antiplatelet drug sensitive populations.

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261

## 262 **CONFLICT OF INTEREST AND FUNDING DISCLOSURE**

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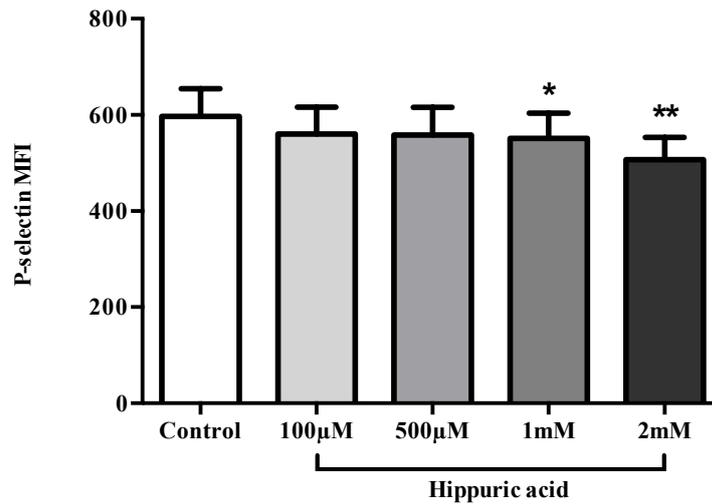
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344 **Figure 1:** The effect of varying concentrations of hippuric acid on P-selectin/CD62P surface  
 345 marker expression. Hippuric acid at 1 mM and 2 mM concentration inhibits P-selectin  
 346 activation dependant monoclonal antibody expression consequently platelet de-granulation/ $\alpha$ -  
 347 granule release ( $\downarrow 45.9 \pm 50.3$ ,  $p=0.03$  and  $\downarrow 89.6 \pm 74.6$ ,  $p<0.001$  respectively). N=13 and  
 348 the data is represented as mean fluorescence intensity (MFI) versus hippuric acid  
 349 concentrations. \*signifies statistical significance  $p<0.05$ . \*\*signifies greater statistical  
 350 significance  $p<0.001$ . Data expressed as Mean  $\pm$  SD.

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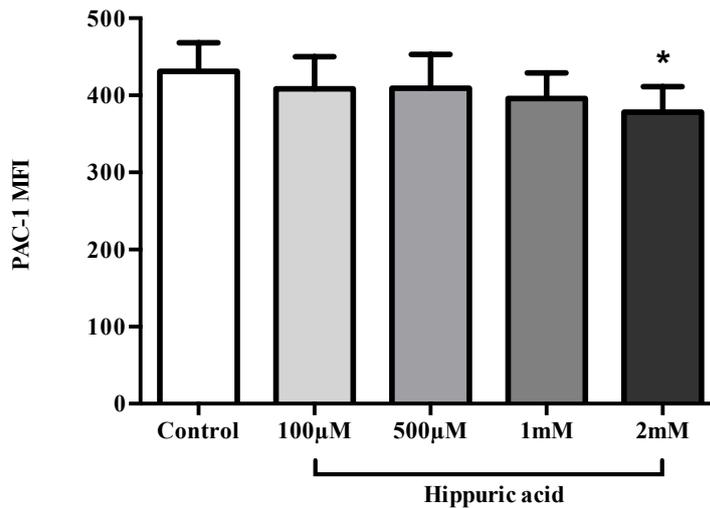
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359 **Figure 2:** The effect of different concentrations of hippuric acid on PAC-1 activation-  
 360 dependant surface marker expression. Hippuric acid at 2 mM concentration reduces  
 361 activation-dependant conformational changes in platelets ( $\downarrow 53.0 \pm 81.2, p=0.03$ ). N=13 and  
 362 the data is represented as mean fluorescence intensity (MFI) versus hippuric acid  
 363 concentrations. \*signifies statistical significance  $p<0.05$ . Data expressed as Mean  $\pm$  SD.

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**Table 1:** Baseline parameters of subjects under study

<b>Parameters</b>	<b>Mean± SD</b>	<b>Reference ranges</b>
<i>n</i> (M/F)	13	NA
Age (y)	30 ± 3	NA
Body mass (kg)	73 ± 8	NA
BMI (kg/m <sup>2</sup> )	23.6 ± 0.7	20 – 25
Haemoglobin (g/L)	140 ± 10	120 – 180
Haematocrit (%)	42 ± 2	40 – 54
RBC (×10 <sup>12</sup> /L)	4.9 ± 0.4	3.8 – 6.5
WBC (×10 <sup>9</sup> /L)	6.2 ± 1.2	4.0 – 11.0
Platelet (×10 <sup>9</sup> /L)	217 ± 13	150 – 400
MPV (fL)	9.0 ± 0.9	7.5 – 11.5
Total cholesterol (mmol/L)	4.40 ± 0.81	3.10 – 6.50
HDL (mmol/L)	1.52 ± 0.32	> 1.0
Triglycerides (mmol/L)	0.85 ± 0.35	0.20 – 1.90
LDL (mmol/L)	2.49 ± 0.72	< 6.00
Glucose (mmol/L)	4.87 ± 0.62	3.0 – 6.0
Uric acid (μmol/L)	274.7 ± 75.5	200 – 430
HS-CRP (mg/L)	0.85 ± 0.76	0.0 – 6.0

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375 BMI, body mass index; RBC, red blood cell; WBC, white blood cell; MPV, mean platelet  
 376 volume; HDL, high density lipoprotein; LDL, low density lipoprotein, HS-CRP, high  
 377 sensitivity C-reactive protein, NA, Not applicable.

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