

# Mild-tourniquet induced ischaemia-reperfusion injury results in changes to haematological, haemostatic and inflammatory parameters.

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## ABSTRACT

**Background:** Ischaemia-reperfusion injury (IRI) is an underlying condition in cardiovascular disease such as atherosclerosis and stroke, and occurs during surgery that involves the application of a tourniquet. These clinical conditions are extremely prominent in the United Kingdom. This pilot-study aimed to determine the effects of mild tourniquet induced IRI on specific haematological, haemostatic and inflammatory parameters.

**Patients and Methods:** An *in-vivo* model of mild tourniquet induced IRI was performed on 15 volunteers (n=15). Tourniquet pressure was set between 20-40 mmHg for 10 minutes and rendered the arm temporarily ischaemic. Baseline venous blood samples were taken prior to ischaemia, then following the release of the tourniquet at 7 minutes and 48 hours reperfusion. The parameters investigated included: full blood count, von Willebrand factor (vWF), sE-selectin, prothrombin time (PT), Interleukin-6 (IL-6), IL-8 and IL-10.

**Results:** The results demonstrated a significant increase in vWF following reperfusion (p=0.005), and increasing trends of IL-6, IL-8 and sE-selectin concentrations (p=>0.05). Decreasing PT, white blood cell and platelet counts were observed following IRI but were not significant (p=>0.05).

**Discussion and Conclusion:** The study demonstrated that brief periods of IRI caused changes to haematological, haemostatic and inflammatory parameters. Specifically, a significant increase in vWF concentration was observed following tourniquet induced IRI. This suggests that changes to vascular integrity and that of endothelial activation may be occurring.

The results of this pilot-study provide a basis for further exploration of haematological, haemostatic and inflammatory parameters following IRI, which may increase our knowledge and understanding of a subject area that is not fully understood. Ultimately, further studies may highlight areas of therapeutic intervention for the underlying occurrence of IRI in pathological conditions, such as cardiovascular disease (CVD) and surgeries that involve the application of a tourniquet. These predictors, however, need further work to validate reliability in a clinical setting.

**Keywords:** IRI, vWF, cytokine, inflammation, endothelium

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## 1. INTRODUCTION

Organs and tissues require oxygenated blood to support cellular viability but the restriction or disruption of this nutritional blood supply is deemed as ischaemia, which can result in cellular dysfunction and necrosis [1]. Short term ischaemia causes only mild, reversible cellular damage if blood flow is returned promptly [2]. Yet peculiarly restoring blood flow to prevent permanent injury can result in greater injury to tissues and cells than that of the original ischaemia. This event is known as ischaemia-reperfusion injury (IRI) and can produce damage at a local and systemic level [3]. IRI is a common underlying clinical process that occurs in diseases such as stroke, myocardial infarction and atherosclerosis whereby blood passage is restricted and then reperfused during treatment [4]. Cardiovascular diseases (CVD) are the leading cause of death in the United Kingdom, accounting for one in three of all deaths totaling 191,000 each year [5]. Other occurrences of IRI include surgical procedures that involve the use of a tourniquet to create a bloodless field, such as orthopaedic knee and hip surgeries, and organ transplant whereby the ischaemic donated organ is reperfused once positioned within the recipient.

The factors causing IRI can be divided between biochemical changes during the period of ischaemia and those that occur upon reperfusion of the oxygenated blood. The disruption of oxygenated blood to tissues and organs alters their metabolic activity, causing biochemical changes at the cell surface, within the cytosol and in mitochondria [6, 7]. These prior biochemical changes are important factors that predispose tissues to undergo free radical damage upon reperfusion of oxygenated blood. As the oxygenated blood comes into contact with the vascular endothelium, superoxide is produced which stimulates changes. Nitric oxide (NO) is an endothelium derived product that provides protective measures such as reducing reactive oxygen waste and inhibiting the production of pro-inflammatory cytokines. During IRI, the imbalance of superoxide radicals reduces NO and removes the protective buffer, creating an environment appropriate for a pro-inflammatory response to occur.

Previous research investigating the effects of IRI on various haematological, haemostatic and inflammatory changes has encompassed some of the cell adhesion molecules, the cytokine cascade and endothelium derived molecules [4, 8, 9, 10]. Specifically, interleukin-6 (IL-6) and IL-8 are inflammatory cytokines which have been reported to be up-regulated following IRI as described by Moro et al (2007) and Huda, Solanki & Mathru (2004) in a clinical setting [11,12]. von Willebrand Factor (vWF) and sE-selectin have also been reported to increase in concentration as a response to endothelial activation, a key concept of IRI [13, 4, 8]. However, these papers largely focus on one of these areas, rarely exploring the causal relationship between haematology, haemostasis and inflammation in response to IRI.

This pilot-study aimed to investigate the effects of mild-tourniquet IRI on haematological, haemostatic and inflammatory markers. Full blood counts were used to determine if IRI caused any significant changes to haematological parameters. The haemostatic response was measured by investigating vWF, sE-selectin and prothrombin time (PT), whilst the cytokines IL-6, IL-8 and IL-10 were monitored to measure the inflammatory response following IRI.

## 2. METHODOLOGY

### 2.1 Subject Volunteers

Ethical approval (Re: 771/13/RE/BS) for this study was permitted from the Faculty of Life Sciences Research Committee (FREC), University of Chester. All recruited volunteers initially completed a health questionnaire and their blood pressure (BP) recorded. Any

68 individuals with a history of diabetes or cardiovascular disease were excluded from the  
69 study, as were individuals with either low or high BP readings. 15 healthy volunteers were  
70 recruited for the study after informed consent (n=15). The volunteers participating in this  
71 study were aged between 20 and 45 years old (mean age  $28.07 \pm 7.25$  years; gender 13  
72 males and 2 females).

## 73 **2.2 Blood Samples**

74 Venous blood samples were collected into vacutainers containing di-potassium ethylene  
75 diamine tetra-acetic acid (EDTA), tri-sodium citrate and serum clot activator. Subject plasma  
76 was obtained by centrifuging whole blood samples at 450g for 15 minutes, following which  
77 all plasma samples were stored ( $-40^{\circ}\text{C}$ ), until required for the ELISA assays or semi  
78 automated analysis.

## 79 **2.3 Model of Ischaemia-Reperfusion Injury (IRI)**

80 This model employed an adapted method of mild tourniquet induced forearm ischaemia-  
81 reperfusion injury [4, 8, 14]. Venous blood samples were taken prior to commencing the  
82 investigations from the contra-lateral arm, which stood as a control measurement (baseline)  
83 for that particular individual. A sphygmomanometer was then placed around the upper  
84 experimental arm and inflated to approximately 20–40 mmHg for ten minutes, as described  
85 by others [14, 4, 8]. This procedure reduced blood flow to the arm (ischaemia). The cuff was  
86 then removed to allow full blood flow to the arm (reperfusion). Further blood samples were  
87 then collected at 7 minutes and 48 hours reperfusion.

## 88 **2.4 Measurement of Haematological Parameters (WBC, RBC, MCV, Hb, HcT &** 89 **Plts)**

90 Full blood counts were performed using a Coulter® MicoDiff18 automated cell counter  
91 (Beckman Coulter, U.K.).

## 92 **2.5 Measurement of Endothelial and Haemostatic Function (sE-selectin, vWF** 93 **& PT)**

94 Measurement of sE-selectin was performed using commercially available kits supplied by  
95 R&D Systems Europe, and involved using ELISA assay as described by the manufacturer  
96 (R&D Systems, Catalogue # SSLE00).

97 Plasma vWF concentration was measured as described previously by a sandwich-type  
98 ELISA technique, using rabbit anti-human vWF and rabbit anti-human vWF peroxidase  
99 conjugate (Dako, UK), [15, 16, 4].

100 PT was measured using a Randox Monza semi-automated system as described by the  
101 manufacturer's instructions (Randox RX Monza Method Sheet: PTH 2752). Citrated samples  
102 were used to measure PT, which is a haemostatic test that measures the extrinsic  
103 coagulation pathway.

## 104 **2.6 Measurement of Inflammatory Markers (IL-6, IL-8, IL-10)**

105 Measurement of inflammatory markers (IL-6, IL-8, IL-10) was performed using commercially  
106 available kits supplied by R&D Systems Europe, and involved using ELISA assays as  
107 described by the manufacturer (R&D Systems, Catalogue # S6050; S8000C; S1000B).

## 2.7 Statistical Analysis

During this study, all results were presented as mean  $\pm$  standard errors (SE) or median  $\pm$  Iqr. Where data were normally distributed, repeated measures one-way analysis of variance (ANOVA) between samples test was employed adopting a 5% level of significance. Post hoc testing was conducted using the Tukey test for pairwise comparisons between means. Data that did not comply with normality were analysed using the Friedman test. Where the Friedman test resulted in statistical significance, subsequent tests were performed using the Wilcoxon test. Statistical significance was accepted when  $p \leq 0.05$ .

## 3. RESULTS AND DISCUSSION

### 3.1. Measurement of Haematology (WBC, RBC, MCV, Hb, Hct and Plts) Parameters

Following mild tourniquet induced ischaemia-reperfusion injury changes were observed in several haematological parameters (Table 1). WBC, RBC and Hct demonstrated a decreasing trend from baseline at both 7 minutes and 48 hours reperfusion ( $p > 0.05$ ). MCV, Hb and Plts showed very little change from baseline values after ischaemia-reperfusion injury ( $p > 0.05$ ).

**Table 1: Effect of IRI on various haematological parameters.** *The points represent mean/median  $\pm$  SE/Iqr, as determined by ANOVA or Friedman respectively. Significance accepted  $p < 0.05$ , (n=15).*

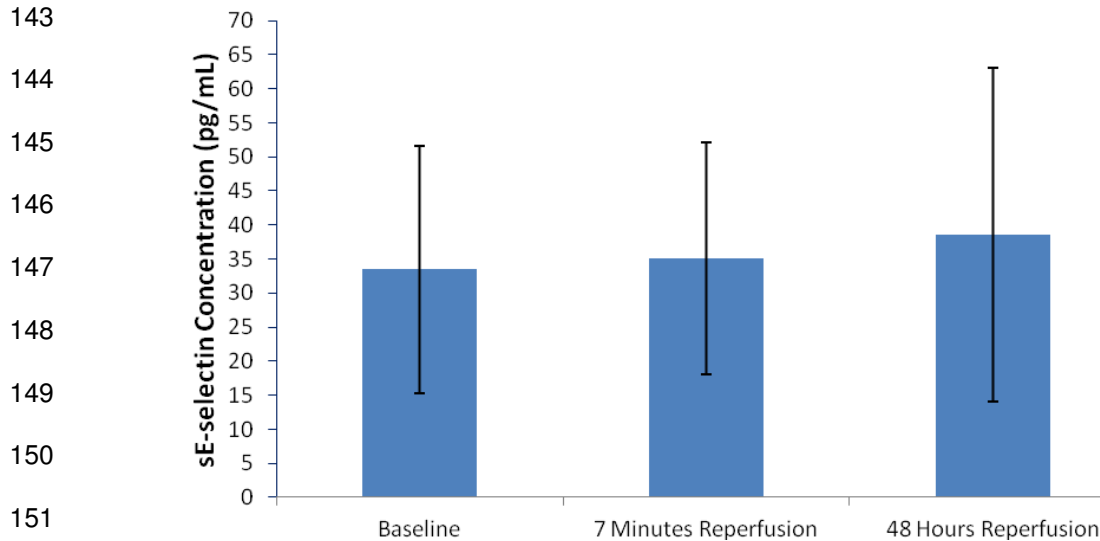
**Legend:** WBC – white blood cells; RBC – red blood cells; MCV – mean cell volume; Hb – Haemoglobin; Hct – haematocrit; Plts - platelets

Parameter	Baseline	7 minutes reperfusion	48 reperfusion	p-value (Significance $p < 0.05$ )
WBC ( $\times 10^9/L$ )	6.43 $\pm$ 1.66	6.37 $\pm$ 1.64	6.11 $\pm$ 1.58	$p = 0.439$
RBC ( $\times 10^{12}/L$ )	5.12 $\pm$ 1.32	4.98 $\pm$ 1.29	4.97 $\pm$ 1.28	$p = 0.298$
MCV (fL)	91.4 $\pm$ 81.3	90.9 $\pm$ 81.8	91.2 $\pm$ 81.6	$p = 0.06$
Hb (g/dL)	15.1 $\pm$ 12.6	14.9 $\pm$ 11.7	15 $\pm$ 12.3	$p = 0.692$
Hct (%)	46.06 $\pm$ 11.89	44.87 $\pm$ 11.57	44.52 $\pm$ 11.5	$p = 0.115$
Plts ( $\times 10^9/L$ )	215 $\pm$ 162	214 $\pm$ 164	211 $\pm$ 161	$p = 0.819$

### 3.2 Measurement of Endothelial and Haemostatic Function (sE-selectin, vWF and PT)

#### 3.2.1 sE-selectin concentration

137 The results are expressed as pg/ml and represent changes in sE-selectin concentration  
 138 following mild tourniquet induced ischaemia-reperfusion injury (Figure 1). This parameter  
 139 was measured as marker of endothelial activation. Following ischaemia-reperfusion a trend  
 140 of increasing sE-selectin was observed ( $p>0.05$ , as determined by the Friedman test).  
 141 Specifically, sE-selectin increased from baseline ( $33.46 \pm 18.12$ ), at 7 minutes reperfusion  
 142 ( $35.13 \pm 17.06$ ) and peaking at 48 hours reperfusion ( $38.55 \pm 24.48$ ).

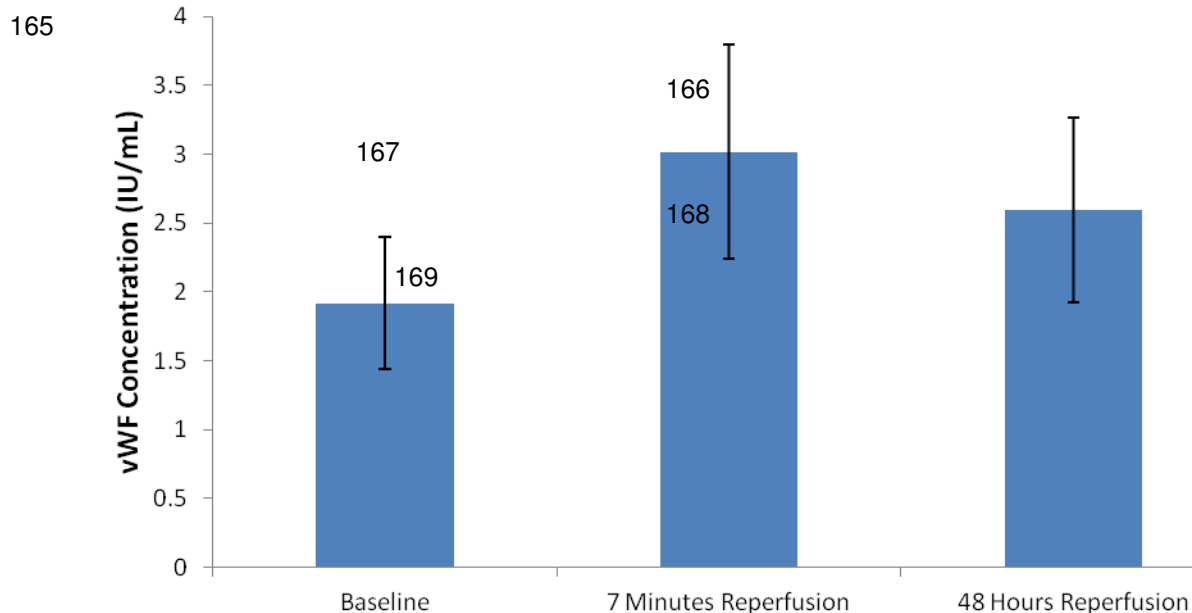


152 **Figure 1: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on**  
 153 **sE-selectin concentration.** The points represent median  $\pm$  Iqr,  $p>0.05$  as determined by the  
 154 Friedman test. (n=15).

155

### 156 **3.2.2 vWF**

157 The results are expressed as IU/ml and represent the changes in vWF concentration  
 158 following mild tourniquet induced ischaemia-reperfusion injury (Figure 2). This parameter  
 159 was measured as marker of endothelial activation. Following ischaemia-reperfusion a  
 160 significant change in vWF was observed ( $p=0.005$ ), as determined by ANOVA). Specifically,  
 161 vWF concentration increased from baseline ( $1.92 \pm 0.48$ ) and during 7 minutes reperfusion  
 162 ( $3.02 \pm 0.78$ ). Following 48 hours reperfusion, vWF concentration decreased but remained  
 163 higher than those of basal values ( $2.59 \pm 0.67$ ). Upon further analysis, pairwise comparisons  
 164 showed significant differences between baseline vs 7 minutes reperfusion ( $p=0.004$ ).

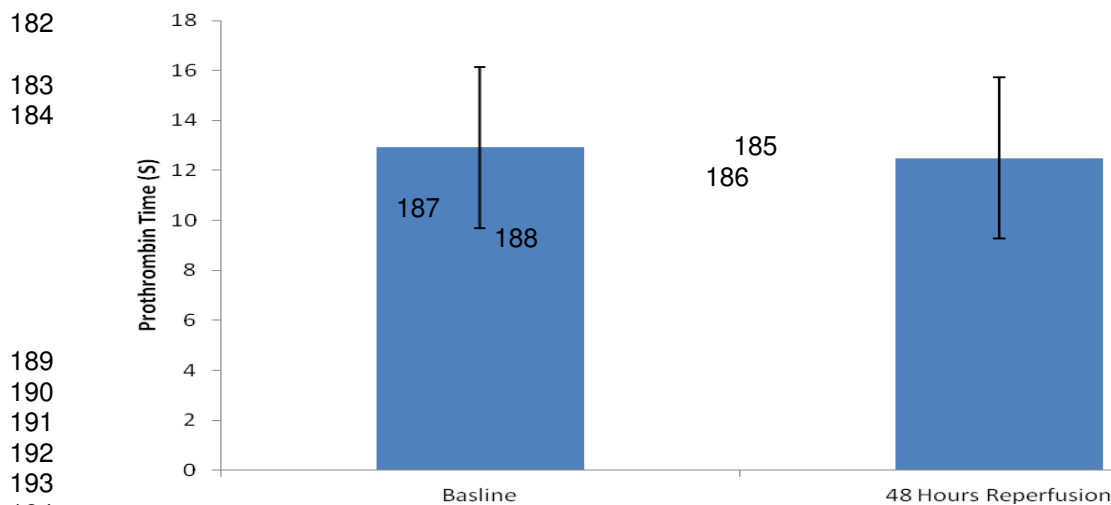


170 **Figure 2: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on**  
 171 **vWF concentration.** *The points represent mean  $\pm$  SE,  $p=0.005$  as determined by ANOVA. Upon*  
 172 *further analysis, pairwise comparisons showed significant differences between baseline vs 7 minutes*  
 173 *reperfusion ( $p=0.004$ ), ( $n=15$ ).*

174

### 175 **3.2.3 Prothrombin Time (PT)**

176 The results are expressed as seconds and represent the changes in PT following mild  
 177 tourniquet induced ischaemia-reperfusion injury (Figure 3). This parameter was measured as  
 178 marker of haemostatic function, specifically investigating the extrinsic pathway. Following  
 179 ischaemia reperfusion, a decrease in PT was observed from baseline ( $12.93 \pm 3.23$ ) and at  
 180 48 hours reperfusion ( $12.49 \pm 3.23$ ). This change was not significant ( $p>0.05$ , as  
 181 determined by paired t-test).



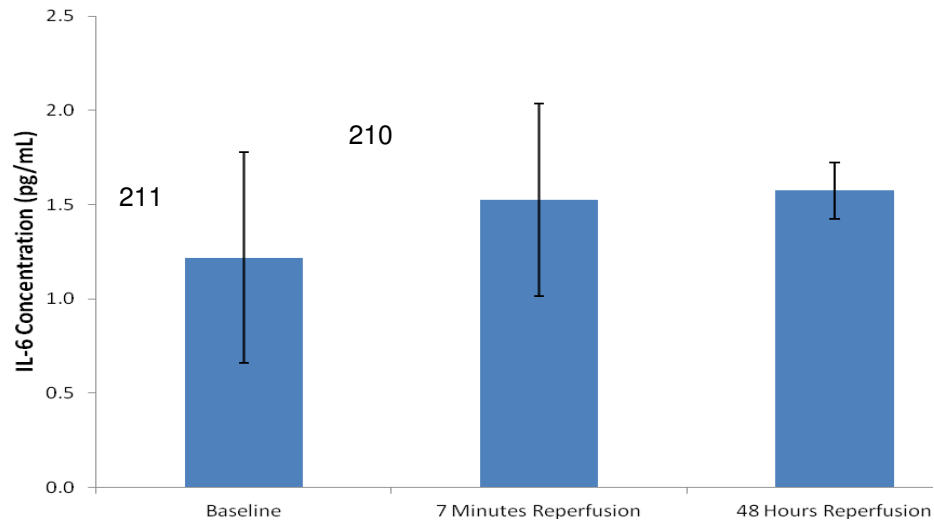
195 **Figure 3: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on**  
 196 **prothrombin time.** *The points represent median  $\pm$  Iqr,  $p>0.05$  as determined by the Friedman test.*  
 197 *( $n=15$ ).*  
 198

## 199 **3.3 Measurement of Inflammatory Markers (IL-6, IL-8 and IL-10)**

### 200 **3.3.1 IL-6**

201 The results are expressed as pg/ml and represent changes in IL-6 concentration following  
 202 mild tourniquet induced ischaemia-reperfusion injury (Figure 4). This parameter was  
 203 measured as marker of inflammatory response. Following ischaemia-reperfusion a trend of  
 204 increasing IL-6 was observed ( $p>0.05$ , as determined by the Friedman test). IL-6 increased  
 205 from baseline ( $1.22 \pm 0.56$ ), during 7 minutes reperfusion ( $1.52 \pm 0.51$ ) and peaking at 48  
 206 hours reperfusion ( $1.58 \pm 0.15$ ).

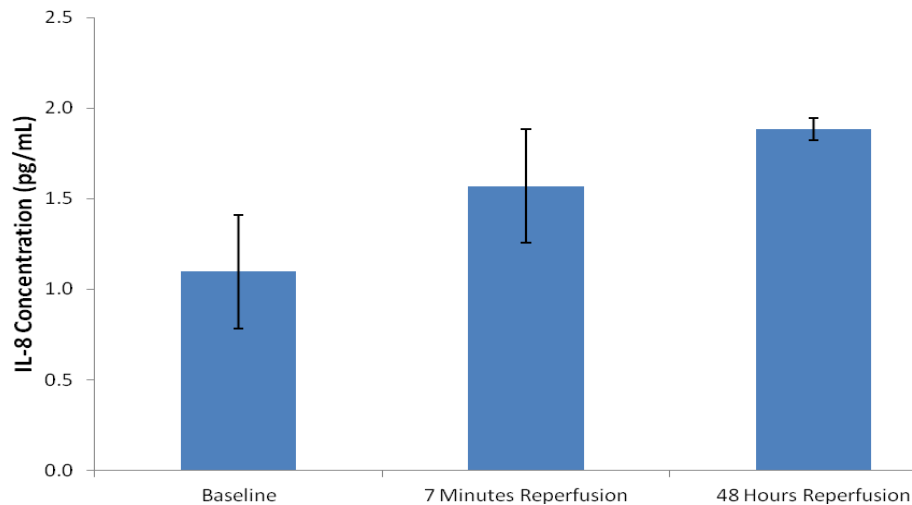
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**Figure 4: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-6 concentration.** The points represent median  $\pm$  Iqr,  $p=>0.05$  as determined by the Friedman test. ( $n=15$ ).

### 3.3.2 IL-8

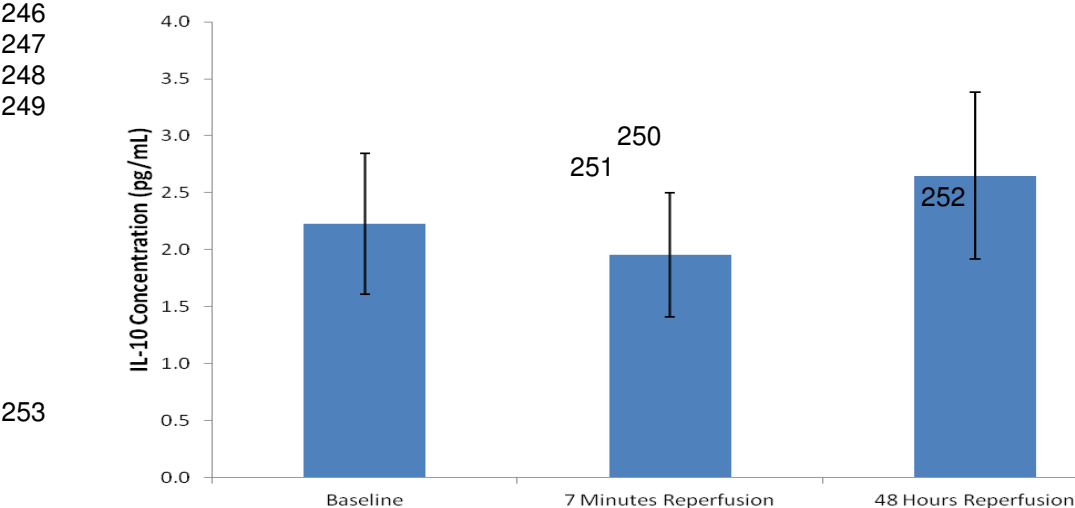
The results are expressed as pg/ml and represent changes in IL-8 concentration following mild tourniquet induced ischaemia-reperfusion injury (Figure 5). This parameter was measured as marker of inflammatory response. Following ischaemia-reperfusion a trend of increasing IL-8 was observed ( $p=>0.05$ , as determined by the Friedman test). IL-8 increased from baseline ( $1.1 \pm 0.31$ ), during 7 minutes reperfusion ( $1.57 \pm 0.31$ ) and peaking at 48 hours reperfusion ( $1.88 \pm 0.06$ ).



**Figure 5: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-8 concentration.** The points represent median  $\pm$  Iqr,  $p>0.05$  as determined by the Friedman test. ( $n=15$ ).

### 3.3.3 IL-10

The results are expressed as pg/ml and represent changes in IL-10 concentration following mild tourniquet induced ischaemia-reperfusion injury (Figure 6). This parameter was measured as marker of inflammatory response. IL-10 decreased from baseline ( $2.23 \pm 0.62$ ) and during 7 minutes reperfusion ( $1.96 \pm 0.54$ ). However, an increase of IL-10 to that above baseline ( $2.65 \pm 0.74$ ) was seen at 48 hours reperfusion. These changes observed in IL-10 concentration were not significant ( $p>0.05$ , as determined by the Friedman test).



**Figure 6: Effect of mild tourniquet induced forearm ischaemia-reperfusion injury on IL-10 concentration.** The points represent mean  $\pm$  SE,  $p>0.005$  as determined by ANOVA, ( $n=15$ ).

### 3.4 Discussion

This pilot-study aimed to determine whether ischaemia-reperfusion injury, using a mild tourniquet induced forearm model, resulted in changes to haematological, haemostatic and inflammatory parameters. Another aim was to explore whether any causal links between the parameters and IRI could be observed. The study demonstrated that vWF concentration changed significantly ( $p=0.005$ ) following IRI, whilst IL-6, IL-8 and sE-selectin also increased but were not significant. The reperfusion of oxygenated blood to ischaemic tissue is known to activate the endothelium creating a pro-inflammatory and pro-coagulation state [9, 17]. In agreement with other, changes to the inflammatory cytokines, IL-6 and IL-8, in addition to the observed changes to vWF and sE-selectin in our study, support the premise of endothelial activation following IRI.

The endothelial derived molecule sE-selectin demonstrated a trend of increasing concentration following IRI, which was in agreement with the report published by Domanski et al. (2006). Specifically, they found that upon renal reperfusion of the donated organ, sE-selectin increased significantly from baseline at 3 minutes reperfusion. Yu, Hu, Li & Wen

272 (2011) also demonstrated a significant increase in sE-selectin immediately following total hip  
273 replacement and up to 24 hours post operatively [13]. Whilst these two papers reported  
274 significant increases of sE-selectin following reperfusion, the trend observed in this report  
275 correlates with their pattern of results. Further evidence of endothelial activation was  
276 supported by the significant changes in vWF following during the present study. A similar  
277 observation has previously been reported by Hughes *et al.* (2007; 2010), who have also  
278 demonstrated an increase in vWF concentration in non-surgical models of IRI [4, 8].

279 The endothelium is the interface between blood and surrounding tissues, composed of a  
280 monolayer of endothelial cells [18]. The endothelial surface is covered by the glycocalyx  
281 (GCX), composed of heparin sulphate proteoglycans, which supports homeostasis of the  
282 blood vessel wall. The conditions that arise during ischaemia, and particularly reperfusion,  
283 cause this GCX layer to partially shed. Activation of the endothelium occurs upon GCX  
284 shedding, causing a conversion to a pro-inflammatory and pro-coagulation state, which  
285 disseminates injury [9, 17]. It is proposed that activation of the endothelium is aided by the  
286 increase of sE-selectin and vWF which was observed following ischaemia-reperfusion in this  
287 study. sE-selectin, an adhesion molecule responsible for recruitment of neutrophils,  
288 monocytes and lymphocytes, is exclusively expressed by activated endothelial cells, which  
289 are also the main source of vWF production [19, 20]. During IRI, the imbalance of  
290 superoxide radicals reduces nitric oxide, an endothelium derived product, upon which vWF  
291 stimulation is enhanced in humans [21, 22]. vWF possesses binding and bridging functions  
292 that can cause damage if present in plasma at high levels by increasing platelet aggregation  
293 and thrombus formation [23]. The findings of the present study support this notion, with  
294 circulating platelets decreasing from baseline at 7 minutes and 48 hours reperfusion (Table  
295 1), whilst the prothrombin time decreased (Figure 3). With regards to the present study,  
296 samples for vWF analysis were assayed in blood collected in EDTA rather than citrated  
297 tubes, which have previously been reported to provide higher results than blood collected in  
298 citrate tubes [24, 25]. However, the aim of the present study was to determine the effects of  
299 IRI on vWF and not to compare the effects of anti-coagulants on VWF, and thus was  
300 relevant to this study.

301 The inflammatory changes observed in the present study are in agreement with other  
302 research exploring the impact of IRI in a variety of clinical settings [9, 11, 26, 27]. Moro *et al.*  
303 (2007) performed coronary occlusion on rats, and demonstrated that IL-6 significantly rose  
304 upon reperfusion for several days after surgery [11]. Our results, although not significant,  
305 also demonstrated an increase in IL-6 following IRI up to 48 hours reperfusion and are in  
306 agreement with Moro *et al.* (2007). Other studies, exploring the effects of IL-6 in a clinical  
307 setting have demonstrated similar findings of increased IL-6 concentration [9, 26, 27]. Huda  
308 *et al.* (2004) demonstrated a significant increase of IL-8 after 4 hours reperfusion following  
309 elective knee surgery [12]. Although not significant, a similar pattern of results were seen in  
310 the present study, which demonstrated an increased IL-8 concentration following IRI up to  
311 48 hours. It can therefore be appreciated that following mild tourniquet induced IRI, changes  
312 to IL6 and IL-8 may be supporting a pro-inflammatory environment. In contrast to the pro-  
313 inflammatory cytokines (IL-6 and IL-10), the anti-inflammatory cytokine IL-10 was shown to  
314 decrease immediately following reperfusion in the present study. This finding is in contrast to  
315 Zhao *et al.* (2005), who demonstrated a rapid increase of IL-10 following liver transplant  
316 between identical twins [28]. This deviation may be because the model used in the present  
317 paper was too mild to induce an accurate IL-10 response.

318 The effects of ischaemia-reperfusion at a cellular level provide many mechanisms upon  
319 which an inflammatory response may be stimulated. Cytokines are released in a cascade,  
320 with earlier cytokines such as TNF- $\alpha$  causing subsequent inflammatory cytokines such as IL-  
321 6 and IL-8 to be released [29]. IL-6 and IL-8 both have common cells of origin; macrophages

and endothelial cells, which together cause endothelial activation, neutrophil chemoattraction and release. IL-6 is also responsible for up-regulation of adhesion molecules that contribute to neutrophil adhesion to the endothelium, thought to contribute to unsuccessful organ transplant [30]. The results of this paper demonstrate an increase in IL-6 over the course of reperfusion measurements, but also show a decrease in white blood cells (Table 1). During an inflammatory response the number of white blood cells would be expected to increase, yet the results of this paper indicate that leukocytes are becoming trapped and activated. Chemoattractants, such as IL-8, increase the adherence of neutrophils to the endothelium, which occurs within minutes of reperfusion [31]. Activated neutrophils release proteases such as human neutrophil elastase (HNE) from granules causing necrosis, whilst also impacting micro-vessels, endothelial permeability and capillary plugging. The loss of the endothelial permeability barrier causes haemorrhage, whilst platelet adhesion causes a loss in antithrombotic activity [32]. As vWF has already been implicated in the increase of thrombus formation, the combination of haemostatic and inflammatory changes may be the likely cause of IRI pathology, which is clinically relevant as excessive clot formation following surgery is a concerning post-surgical complication. In contrast to the inflammatory cytokines, IL-10 has been suggested to hamper endothelial activation, which in turn would reduce adhesion molecules [33]. In the present study IL-10 was seen to decrease upon early reperfusion, but increased above baseline at 48 hours (Figure 6). This may suggest that IL-10 does not play a role in the down-regulation of pro-inflammatory cytokines following early reperfusion, and could possibly be hampered by the significant increase in concentration of vWF, although, in order to confirm this more studies would need to be undertaken.

There were several limitations of this study, particularly the amount of reperfusion samples that were able to be obtained following tourniquet induced ischaemia. However, due to time constriction recruiting more subject volunteers for the study would have been beneficial and may have helped provide statistical significance to some of the parameters that were measured in the study. Whilst the parameters measured in this study provided information regarding the haematological, haemostatic and inflammatory response following IRI, there are several other parameters that could have been included. Specifically, TNF- $\alpha$ , which plays a predominant role in early inflammation, cell surface adhesion molecules, such as CD11b or CD62L, and other haemostatic parameters such as fibrinogen [34, 35]. The duration of rendering the arm ischaemic and the set tourniquet pressure employed in the present study was relatively short and very mild in comparison to a typical clinical setting. For example, during lower limb orthopaedic surgery tourniquet pressure is set to approximately 250-350 mmHg for periods of up to 2 hours [36, 37]. However, despite the acknowledged limitations of this study, the main aim was to determine the effects of effects of a non-surgical model of mild IRI on specific haematological, haemostatic and inflammatory parameters. Generally, the present study achieved this and provides a sound platform to continue research into this area.

#### 4. CONCLUSION

The study demonstrated that brief periods of IRI caused changes to haematological, haemostatic and inflammatory parameters. Specifically, a significant increase in vWF concentration was observed following tourniquet induced IRI. This suggests that changes to vascular integrity and that of endothelial activation may be occurring.

The results of this pilot-study provide a basis for further exploration of haematological, haemostatic and inflammatory parameters following IRI, which may increase our knowledge and understanding of a subject area that is not fully understood. Ultimately, further studies may highlight areas of therapeutic intervention for the underlying occurrence of IRI in pathological conditions, such as cardiovascular disease (CVD) and surgeries that involve the

372 application of a tourniquet. These predictors, however, need further work to validate  
373 reliability in a clinical setting.

374

## 375 **ACKNOWLEDGEMENTS**

376

377 The authors would like to thank all the volunteers who kindly agreed to participate in the  
378 study.

379

## 380 **COMPETING INTERESTS**

381

382 The author(s) declare that they have no competing interests.

383

## 384 **AUTHORS' CONTRIBUTIONS**

385

386 The design of the study and subject recruitment was carried out by RJE and SFH, whilst  
387 both SFH and PET were involved in the blood sampling procedures. RJE performed all of  
388 the analytical procedures, with help from SFH during cytokine ELISA and PET during vWF  
389 and PT testing. RCC provided advisement regarding ELISA optimisation and interpretation  
390 of results. SFH provided supervisory support during the study and RJE drafted the  
391 manuscript. All authors read and approved the final manuscript.

392

## 393 **ETHICAL APPROVAL**

394

395 Ethical approval (Re: 771/13/RE/BS) for this study was permitted from the Faculty of Life  
396 Sciences Research Committee (FREC), University of Chester.

397

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## 518 ABBREVIATIONS

- 519
- 520 IRI – Ischaemia-Reperfusion Injury
- 521 vWF – von Willebrand Factor
- 522 PT – Prothrombin Time
- 523 IL – Interleukin
- 524 CVD – Cardiovascular Diseases
- 525 NO – Nitric Oxide
- 526 EDTA - di-potassium ethylene diamine tetra-aceticacid
- 527 ELISA – Enzyme-Linked Immunosorbent Assay
- 528 WBC – White Blood Cell
- 529 RBC – Red Blood Cell
- 530 MCV – Mean Cell Volume

531 HcT – Haematocrit  
532 Plts – Platelets  
533 APTT – Activated Partial Thromboplastin Time  
534 ANOVA – One-Way Analysis of Variance  
535 GCX – Glycocalyx  
536 HNE – Human Neutrophil Elastase  
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