

1 **TITLE:** Role of cyclooxygenase in the vascular response to locally delivered
2 acetylcholine in Caucasian and African descent individuals

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16 **LIST OF ABBREVIATIONS**

17 ACh Acetylcholine

18 AFD African descent

19 AUC Area under curve

20 CAU Caucasian

21 COX Cyclooxygenase

22 ED50 Half-maximal effective dose

23 IQR Interquartile range

24 LDU Laser Doppler units

25 MAP Mean arterial pressure

- 1 Mdn Median
2 NFCI Non-freezing cold injury
3 SD Standard deviation
4 TXA₂ Thromboxane A₂

5

6 **ABSTRACT**

7 **INTRODUCTION:**

8 Individuals of African descent (AFD) are more susceptible to non-freezing cold injury
9 (NFCI) compared with Caucasian individuals (CAU). Vasodilatation to acetylcholine
10 (ACh) is lower in AFD compared with CAU in the non-glabrous foot and finger skin
11 sites; the reason for this is unknown. Prostanoids are responsible, in part, for the
12 vasodilator response to ACh, however it is not known whether the contribution differs
13 between ethnicities.

14 **METHODS:**

15 12 CAU and 12 AFD males received iontophoresis of ACh (1 w/v %) on non-
16 glabrous foot and finger skin sites following placebo and then aspirin (600 mg, single
17 blinded). Aspirin was utilised to inhibit prostanoid production by inhibiting the
18 cyclooxygenase (COX) enzyme. Laser Doppler flowmetry was utilised to measure
19 changes in skin blood flow.

20 **RESULTS:**

21 Not all participants could receive iontophoresis charge due to high skin resistance;
22 these participants were therefore excluded from the analyses.

23 *Foot:* ACh elicited greater maximal vasodilatation in CAU than AFD following
24 placebo (P=0.003) and aspirin (P<0.001). Aspirin did not affect blood flow responses
25 in AFD, but caused a reduction in the area under curve for CAU (P=0.031).

1 *Finger*: ACh elicited a greater maximal vasodilatation in CAU than AFD following
2 placebo (P=0.013) and aspirin (P=0.001). Aspirin tended to reduce maximal
3 vasodilatation in AFD (P=0.053), but did not affect CAU.

4 **CONCLUSIONS:**

5 CAU have a greater endothelial reactivity than AFD in both foot and finger skin sites
6 irrespective of COX inhibition. It is concluded that the lower ACh-induced
7 vasodilatation in AFD is not due to a compromised COX pathway.

8

9 **KEY WORDS:** Non-freezing cold injury; ethnicity; skin blood flow; endothelial-
10 dependent vasodilatation; acetylcholine; cyclooxygenase.

11

12 **HIGHLIGHTS**

- 13 • ACh-induced cutaneous dilatation is attenuated in African individuals versus
14 Caucasians.
- 15 • COX inhibition attenuated the dilatation in the foot skin site for Caucasians.
- 16 • COX is not responsible for the lower vasodilator responses in African
17 individuals.

18

1 INTRODUCTION

2 Non-freezing cold injury (NFCI) is a preventable clinical injury that affects the
3 peripheral skin sites (particularly fingers and toes) of individuals who experience
4 prolonged exposure to local cold tissue temperatures (0 °C to 20° C) (Ungley and
5 Blackwood, 1942). Symptoms of this injury may last for many years and often
6 include pain, numbness and hyperhidrosis which, combined with cold
7 hypersensitivity of the injured limb, can lead to increased susceptibility to further cold
8 injuries (Golden et al., 2013; Ungley et al., 1945). This type of injury is a concern for
9 those involved in outdoor work (e.g. agriculture or forestry work, military) or
10 recreational activities (e.g. skiing, mountaineering) that take place in cold conditions
11 which may also elicit freezing cold injuries (Hashmi et al., 1998; Mäkinen et al., 2009;
12 Morrison et al., 2015).

13
14 Individuals of black African descent (AFD) are more susceptible than Caucasian
15 (CAU) individuals to NFCI (Burgess and Macfarlane, 2009; DeGroot et al., 2003).
16 The reason for this is not known but it is thought that sustained skin blood flow in the
17 extremities in low environmental temperatures can prevent local cold injuries
18 (Daanen and van der Struijs, 2005; Lewis, 1941; Wilson and Goldman, 1970). During
19 hand immersion in cold water (8 °C) for 30 minutes and subsequent rewarming of
20 dry skin in 30 °C air, AFD experienced greater finger vasoconstriction and slower
21 rewarming compared with CAU (Maley et al., 2014) indicating AFD received a
22 greater “dose of cold”. We investigated whether this was due to alterations in the
23 control of the microcirculation of the extremities and demonstrated that endothelial-
24 dependent (ACh), but not -independent (SNP), vasodilatation was significantly

1 attenuated in AFD compared with CAU in non-glabrous finger and toe skin sites
2 (Maley et al., 2015).

3

4 Local application of acetylcholine (ACh) increases prostanoid and nitric oxide
5 production eliciting vasodilatation (Holowatz et al., 2005; Kellogg et al., 2005).
6 Prostanoids are produced from arachidonic acid, released from the cell membrane,
7 metabolised by the enzyme cyclooxygenase (COX) (Vane et al., 1998) to produce
8 prostaglandin H₂ which is further metabolised by various synthase enzymes to
9 produce various prostanoids (Félétou, 2011; Hamberg et al., 1975; Moncada and
10 Vane, 1979; Moncada et al., 1976). The vascular wall synthesises each of these
11 prostanoids, the most abundant being prostacyclin (PGI₂), whilst platelets are the
12 main source of thromboxane A₂ (TXA₂) (Dubois et al., 1998; Félétou, 2011; Majed
13 and Khalil, 2012; Moncada and Vane, 1978; Tang and Vanhoutte, 2008). In young
14 healthy individuals TXA₂ and PGI₂ elicit vasoconstriction and vasodilatation,
15 respectively (Félétou, 2011; Majed and Khalil, 2012).

16

17 Blocking COX inhibits all vasodilator and vasoconstrictor prostanoid production (Roth
18 et al., 1975; Vane, 1971). The net action of COX inhibition varies between
19 populations. In young, healthy individuals, COX inhibition attenuates the vasodilator
20 response to ACh in the forearm circulation assessed with laser Doppler flowmetry
21 (Holowatz et al., 2005; Kellogg et al., 2005; Noon et al., 1998). However, the role of
22 COX in response to ACh appears compromised in certain populations. Normotensive
23 aged (>60 years) and hypertensive individuals (>46 years) exhibit similar endothelial
24 dysfunction in response to ACh, with COX inhibition (indomethacin) restoring the
25 vasodilator response as assessed by plethysmography (Taddei et al., 1997b). This

1 vasodilator restoration was due to an increase in nitric oxide bioavailability (Taddei et
2 al., 1997a). More recently, *in-vitro* studies performed on human small arteries noted
3 the antioxidant, ascorbic acid, and a non-selective COX inhibitor (indomethacin)
4 augmented the vasodilator response to ACh in hypertensive samples, although their
5 actions were not additive (Viridis et al., 2013). Collectively, this body of research
6 provides evidence that the mechanism of endothelial dysfunction in aged and
7 hypertensive individuals is due, in part, to COX activity diminishing the vasodilator
8 response to endothelial-dependent vasodilators through reductions in nitric oxide
9 bioavailability. Whether the endothelial dysfunction in AFD observed previously
10 (Maley et al., 2015) is caused by a differing contribution of the COX pathway
11 between ethnic groups is not known. Given that AFD experience greater levels of
12 oxidative stress (Fearheller et al., 2011; Kalinowski et al., 2004), and COX increases
13 reactive oxygen species (Kukreja et al., 1986; Viridis et al., 2013) as well as
14 producing TXA₂, it is possible that the COX pathway may contribute to the attenuated
15 ACh-induced vasodilatation compared with CAU.

16

17 Therefore, the aim of the present study was to establish the contribution of COX to
18 ACh-induced vasodilatation in both CAU and AFD. As we have previously observed
19 an attenuated ACh-induced vasodilator response in AFD compared to CAU, it was
20 hypothesised that AFD would experience a lower vasodilator response to ACh
21 compared with CAU, and COX inhibition would augment endothelial reactivity in
22 AFD.

23

24 **METHODS**

25 *PARTICIPANTS*

1 This study was given a favourable ethical opinion from the University of Portsmouth
2 Science Faculty Ethics Committee. The participants were made aware of the
3 purpose, procedures and risks of the study prior to giving their informed written
4 consent. 12 CAU and 12 AFD male volunteers participated in the study. All CAU
5 were born in the UK. Eight AFD were born in the UK whilst four were born in Africa
6 (Zimbabwe, Ghana, Kenya and Uganda) and had resided in the UK for an average
7 of 11 years with a minimum of seven years. CAU and AFD were of similar age
8 (mean [SD], 22 [4] years and 20 [2] years, $P = 0.069$), height (mean [SD], 178.2 [6.9]
9 cm and 176.0 [7.9] cm, $P = 0.790$) and body mass (mean [SD], 73.1 [12.3] kg and
10 74.1 [12.8] kg, $P = 0.583$).

11

12 In attempt to reduce heterogeneity female participants were not included in the
13 present study as the menstrual cycle is known to effect vasodilator capacity and
14 thermoregulation (Charkoudian and Stachenfeld, 2015; Hashimoto et al., 1995),
15 therefore the results of the present study should only be applied to young healthy
16 male participants.

17

18 *EXPERIMENTAL PROCEDURES AND MEASUREMENTS*

19 Participants attended the laboratory on one occasion where they received
20 iontophoresis of ACh. The technique of iontophoresis has been described previously
21 (Morris and Shore, 1996; Roustit et al., 2014). Briefly, iontophoresis is a non-invasive
22 method of transdermal drug delivery which transfers charged molecules using a low-
23 intensity electric current into and through the skin to a depth of approximately 2 mm
24 to 4 mm (Anderson et al., 2003). Iontophoresis was performed using both an anode
25 and cathode connected to a battery powered iontophoresis controller (MIC2, Moor

1 Instruments, UK). The iontophoresis chamber, which is a small Perspex ring (MIC-
2 ION1R-P1, Moor Instruments, UK) with an inner diameter of 9.5 mm, was filled with
3 approximately 0.2 mL of ACh (1 w/v % [55.05 mM], Sigma-Aldrich, UK), diluted in
4 water for injection. A laser Doppler probe (VP1T / 7, Moor Instruments, UK), utilised
5 to measure skin temperature and skin blood flow, was placed into the Perspex ring
6 and connected to a laser Doppler flowmetry monitor (moorVMS-LDF, Moor
7 Instruments, UK). Laser Doppler and iontophoresis data were recorded using a data
8 acquisition system and software (Powerlab and LabChart 7, AD Instruments, New
9 Zealand).

10

11 On the day of testing participants were asked to consume 150 mL of diluted orange
12 squash immediately prior to entering a temperature controlled chamber set at a dry
13 bulb temperature of 23.2 (0.8) °C. All participants rested for 30 minutes in a supine
14 position to allow skin temperature and skin blood flow to stabilise. Participants were
15 supine throughout the experiment and each skin site was cleaned with deionised
16 water prior to iontophoresis. Iontophoresis of ACh was delivered to either the right
17 medial or right lateral dorsal foot first using the anode, with the cathode placed
18 proximally within 5 cm to 10 cm. Secondly, iontophoresis was applied to the third or
19 fourth non-glabrous finger skin site (medial phalanx) on the right hand (Fig. 1).
20 Following this, participants were then asked to consume 150 mL of diluted orange
21 squash which contained dissolved aspirin tablets to the total of 600 mg of aspirin
22 (acetylsalicylic acid) (Boots Company, UK). Participants were blinded to the order of
23 placebo and aspirin. Aspirin irreversibly inhibits COX by acetylation of the active site
24 of COX (Vane and Botting, 2003; Vane, 1971) with this dose of aspirin shown to

1 inhibit 86 % of bradykinin-induced production of PGI₂ and 99 % inhibition of TXA₂
2 production by platelets at 30 minutes (Heavey et al., 1985).

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Placebo	Foot Site 1	Finger Site 1	Aspirin (600 mg)	Foot Site 2	Finger Site 2
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4 Fig. 1. Schematic of the experimental procedure

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6 Thirty minutes after aspirin treatment, iontophoresis began on the foot at a skin site
7 that had not been used (medial or lateral). Following this, iontophoresis was applied
8 to the second finger skin site (third or fourth). The reason for not using the same skin
9 site was that during pilot experiments the vasodilator response to iontophoresis of
10 ACh was much longer lasting than 30 minutes, thus using the same skin site would
11 influence subsequent skin blood flow results; this has been reported previously
12 (Brocx and Drummond, 2009). The order of participants' skin sites tested (lateral vs.
13 medial dorsal foot, third vs. fourth finger) was counter-balanced between
14 participants. Repeatability studies on six participants demonstrated that the
15 responses to ACh did not differ between sites (medial vs lateral foot; middle vs fourth
16 finger) and over time (two dose response curves to ACh following placebo).

17

18 The iontophoresis protocol employed in the present study is the same as previously
19 used (Maley et al., 2015) which consisted of six pulses of 25 μ A (0.5 mC) followed by
20 one pulse of 50 μ A (1mC) and one of 100 μ A (2 mC) applied for 20 seconds
21 separated by 60 second intervals in which no current was applied. On completion of
22 the protocol, and after an interval of five minutes, the protocol was repeated on the
23 next skin site. Blood pressure from the contralateral arm was recorded pre- and post-

1 iontophoresis application and measured using an automated monitor (Minimon 7137
2 Plus, Kontron Instruments, UK) for calculation of mean arterial pressure (MAP).

3

4 *DATA ANALYSES*

5 Due to high skin resistance, it was not possible to deliver all the current pulses in
6 each skin site for all participants; this occurred more in the AFD participants.
7 Therefore, only those who could receive the first pulse of iontophoresis were
8 included in analyses (see results). As skin resistance during iontophoresis charges
9 of 100 μ A have been reported to influence the vasodilator response to ACh (Pienaar
10 et al., 2014; Puissant et al., 2014) we investigated whether this was true for lower
11 iontophoresis charges. Following placebo treatment, the skin blood flow responses
12 (average over the six pulses of 25 μ A) were correlated with electrical skin resistance
13 (average over the six pulses of 25 μ A) and were plotted for CAU and AFD separately
14 and R^2 calculated. Skin resistance was calculated by monitoring the applied voltage
15 and dividing this by the current application, displayed in kilohms.

16

17 Blood pressure remained constant throughout the iontophoresis protocol (see
18 results) therefore skin blood flow at baseline was expressed as laser Doppler units
19 (LDU) rather than cutaneous vascular conductance. Average skin blood flow in
20 response to iontophoresis of ACh was calculated over the final 20 seconds of the
21 interval between successive pulses and between 40 to 60 seconds after the final
22 pulse. These responses were expressed as percentage change from that prior to
23 iontophoresis (averaged over 20 seconds and set at 0 %). ED50, expressed as 95 %
24 confidence intervals was calculated using GraphPad (Version 5, USA). Maximum
25 skin blood flow and area under the curve (AUC) were calculated for each participant.

1 The point at which the skin blood flow was at a maximum point was not always
2 identified following the final pulse, therefore maximum skin blood flow was taken
3 from wherever it was highest.

4

5 Statistical analyses were conducted using IBM SPSS for Windows version 20 (IBM
6 SPSS Statistics, USA). Normality of data was assessed using Shapiro-Wilks
7 statistical analysis. An α value of 0.05 was used to determine statistical significance.
8 Baseline skin blood flow, skin temperature and MAP between- and within-groups
9 were compared using an independent and paired samples *t*-test, respectively. ED50,
10 maximal percentage change, AUC between-groups was analysed using an
11 independent samples *t*-test or a Mann-Whitney U test, respectively (statistical test
12 utilised determined by normality testing). ED50, maximal percentage change, AUC
13 within-groups was analysed using a paired samples *t*-test or a Wilcoxon signed rank
14 test. Non-parametric analysis was utilised to assess skin blood flow over time. Effect
15 sizes were calculated using Cohen's *d* for parametric data (denoted by *d* in text) and
16 Rosenthal's *r* for non-parametric data (denoted by *r* in text). Data within figures are
17 presented as mean (SD)

18

19 **RESULTS**

20 *MEAN ARTERIAL PRESSURE*

21 MAP at baseline for CAU and AFD following placebo (mean [SD], 83 [8] mmHg and
22 87 [8] mmHg, respectively, $P = 0.627$) and COX inhibition (mean [SD], 84 [5] mmHg
23 and 88 [9] mmHg, respectively, $P = 0.064$) did not differ between- or within-groups
24 (CAU $P = 0.748$, AFD $P = 0.805$).

25

1 **BASELINE SKIN BLOOD FLOW AND SKIN TEMPERATURE**

2 There were no differences in baseline skin blood flow or skin temperature between-
 3 or within-groups for either the foot or finger skin sites following treatment of either
 4 placebo or COX inhibition (Table 1).

5

6 Table 1. Mean (SD) baseline skin blood flow (LDU) and skin temperature (°C) for the
 7 foot and finger skin sites following placebo or COX inhibition

Baseline Skin Blood Flow (LDU)						
	Foot			Finger		
	Placebo	COX ib	Within	Placebo	COX ib	Within
CAU	12 (6) <i>n</i> = 12	11 (4) <i>n</i> = 12	<i>P</i> = 0.165	54 (19) <i>n</i> = 11	47 (23) <i>n</i> = 11	<i>P</i> = 0.111
AFD	10 (7) <i>n</i> = 12	8 (3) <i>n</i> = 12	<i>P</i> = 0.312	52 (25) <i>n</i> = 10	48 (23) <i>n</i> = 8	<i>P</i> = 0.089
Between	<i>P</i> = 0.571	<i>P</i> = 0.081		<i>P</i> = 0.890	<i>P</i> = 0.950	
Skin Temperature (°C)						
CAU	27.1 (1.3) <i>n</i> = 12	26.8 (1.3) <i>n</i> = 12	<i>P</i> = 0.079	29.4 (0.8) <i>n</i> = 11	28.9 (1.1) <i>n</i> = 11	<i>P</i> = 0.172
AFD	27.0 (1.1) <i>n</i> = 12	26.6 (1.3) <i>n</i> = 12	<i>P</i> = 0.121	28.8 (0.6) <i>n</i> = 10	28.5 (0.7) <i>n</i> = 8	<i>P</i> = 0.167
Between	<i>P</i> = 0.848	<i>P</i> = 0.998		<i>P</i> = 0.084	<i>P</i> = 0.355	

8 Note: COX ib stands for COX inhibition

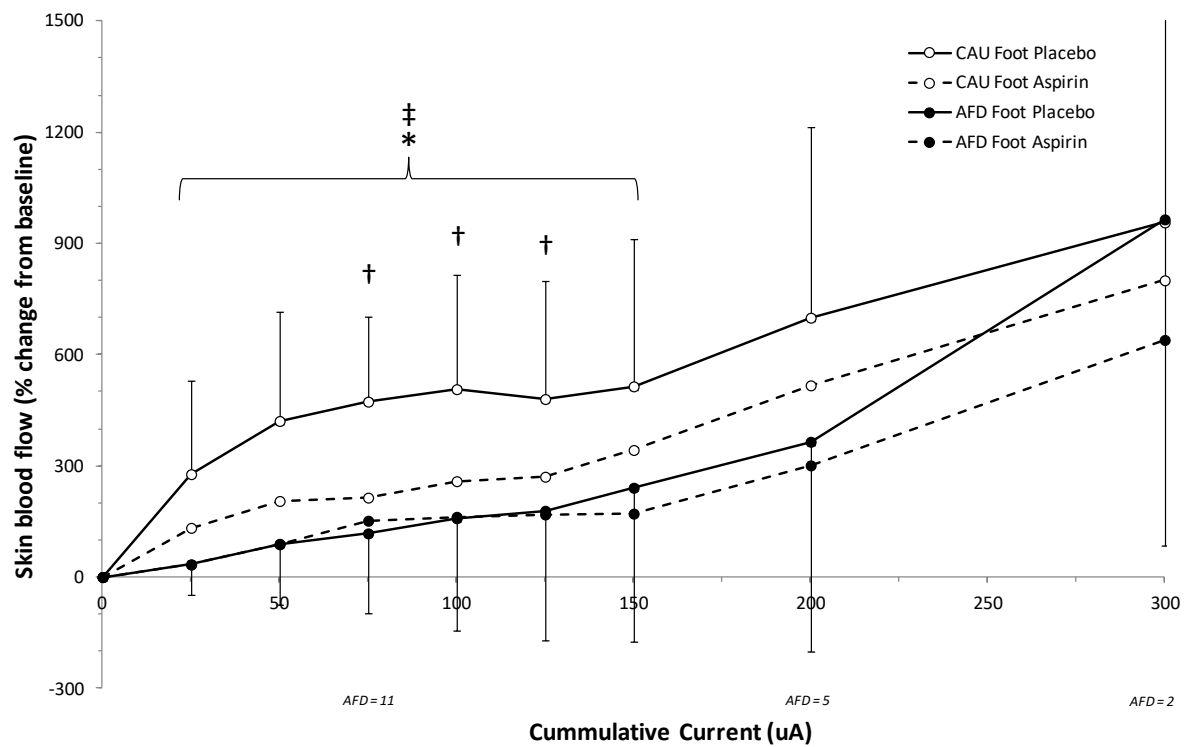
9 **RESPONSES TO ACETYLCHOLINE**

10 **FOOT SKIN SITE**

11 **WITHIN-GROUPS**

12 Fig. 2 shows the skin blood flow responses to ACh for the foot skin site in CAU and
 13 AFD. CAU experienced a reduced vasodilator response to ACh following COX
 14 inhibition (Fig. 2). Additionally, in CAU following COX inhibition ED50 occurred at a
 15 greater cumulative current (Table 2, *P* = 0.005), AUC was smaller (*P* = 0.031, *d* =
 16 0.80) but maximal vasodilatation did not differ. COX inhibition did not affect the
 17 vasodilator response to ACh in AFD.

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1

2 Fig. 2. Mean (SD) skin blood flow responses in the foot skin site for both placebo and
 3 COX inhibition trials. * Significant difference between CAU and AFD for placebo trial
 4 ($P < 0.05$). ‡ Significant difference between CAU and AFD for COX inhibition trial (P
 5 < 0.05). † Significant difference between placebo and COX inhibition trial for CAU (P
 6 < 0.05).

7 Error bars included for CAU and AFD placebo only for reader clarity.

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1 Table 2. Maximum, ED50 and area under the curve (AUC) skin blood flow response
 2 to ACh in the foot skin site following placebo or COX inhibition treatment

		Variable			
			ED50 (µA)	Max (%)	AUC
Within	Foot CAU	PLACEBO <i>n</i> = 12	54 to 116	^943 (490)	4808 (2678)
		COX IB <i>n</i> = 12	116 to 174 †	^775 (784)	2998 (1761) †
			<i>P</i> = 0.005	<i>P</i> = 0.308	<i>P</i> = 0.031
	Foot AFD	PLACEBO <i>n</i> = 12	150 to 271	^81 (370)	^190 (1329)
COX IB <i>n</i> = 12		118 to 418	^50 (148)	^95 (894)	
		<i>P</i> = 0.757	<i>P</i> = 0.117	<i>P</i> = 1.000	
Between	Foot placebo	CAU <i>n</i> = 12	54 to 116	^943 (490)	^4516 (2601)
		AFD <i>n</i> = 12	153 to 302 *	^81 (370) *	^190 (1329) *
			<i>P</i> < 0.001	<i>P</i> = 0.003	<i>P</i> = 0.001
	Foot COX ib	CAU <i>n</i> = 12	116 to 174	^775 (784)	^3120 (3170)
AFD <i>n</i> = 12		97 to 424	^50 (148) *	^95 (894) *	
		<i>P</i> = 0.159	<i>P</i> < 0.001	<i>P</i> = 0.002	

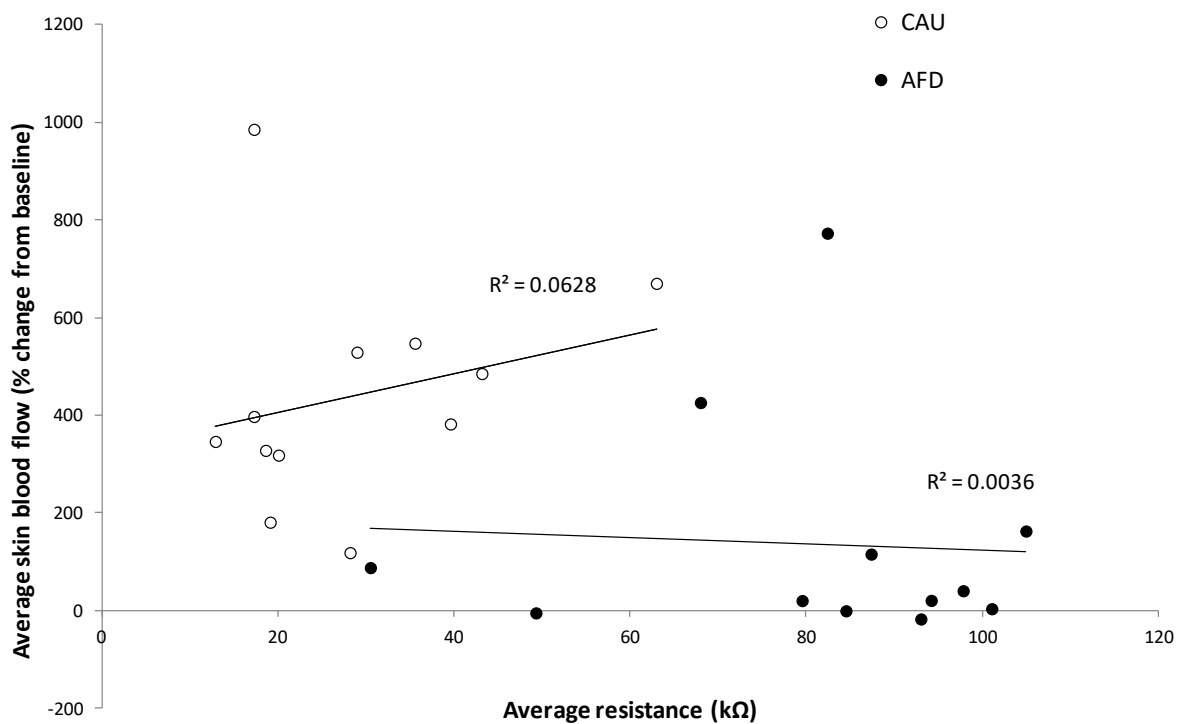
3 Max given as median (IQR) percentage change from baseline, ED50 given as 95 % confidence
 4 intervals (microamps) and AUC given as mean (SD) or median (IQR). Note: as pairwise analyses
 5 were conducted within-groups, the values reported do not always match the between-groups
 6 analyses which included all participants or until a participant did not receive all applied current. †
 7 Significant difference between placebo and COX inhibition (*P* < 0.05). * Significant difference between
 8 CAU and AFD (*P* < 0.05). ^ Median (IQR).

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10 **BETWEEN-GROUPS**

11 AFD demonstrated lower vasodilatation compared with CAU in response to ACh
 12 following both placebo and COX inhibition (Fig. 2). Following placebo treatment
 13 ED50 occurred at a greater cumulative current for AFD compared with CAU (Table
 14 2, *P* < 0.001), and maximal vasodilatation (*P* = 0.003, *r* = 0.59) as well as AUC (*P* =
 15 0.001, *r* = 0.62) were lower in AFD than CAU. Following COX inhibition, ED50 did

1 not differ between groups, however maximal vasodilatation ($P < 0.001$, $r = 0.67$) as
 2 well as AUC ($P = 0.002$, $r = 0.60$) were lower in AFD compared with CAU.
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 4 No relationship was observed between electrical skin resistance and skin blood flow
 5 responses in the foot skin site for either CAU or AFD (Fig. 3).



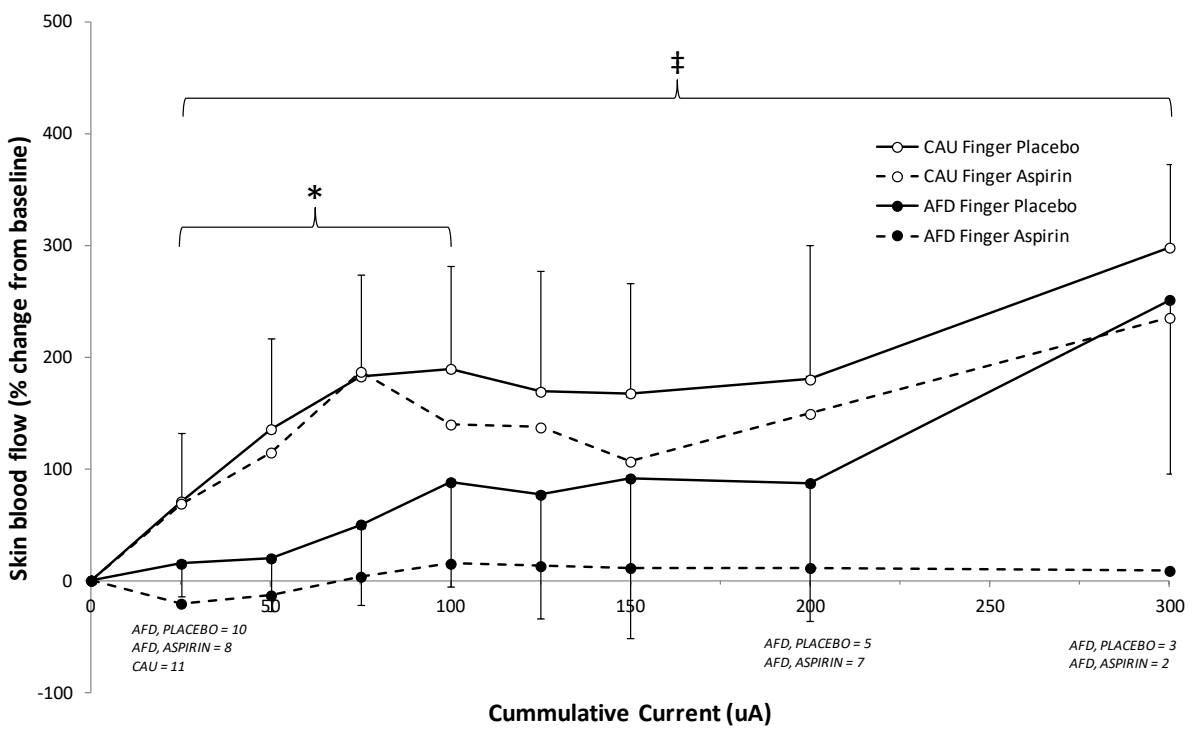
6
 7 Fig. 3. Relationship between average skin blood flow (%) and average electrical skin
 8 resistance (kΩ) for CAU and AFD in the foot skin site during 25 μ A iontophoresis
 9 pulses of ACh following placebo treatment

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 11 *FINGER SKIN SITE*
 12 *WITHIN-GROUPS*

13 Fig. 4 shows the skin blood flow responses to ACh for the finger skin site in CAU and
 14 AFD. For CAU, COX inhibition did not affect the vasodilator response to ACh. This
 15 was confirmed with no difference in ED50, maximal vasodilatation or AUC (Table 3).

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For AFD, COX inhibition tended to reduce maximal vasodilatation ($P = 0.064$, $d = 1.28$) and AUC ($P = 0.053$, $d = 1.32$). Calculation of ED50 was not possible for AFD following COX inhibition as no distinctive dose-response curve could be fitted to the data.



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Fig. 4. Mean (SD) skin blood flow responses in the finger skin site for both placebo and COX inhibition trials. * Significant difference between CAU and AFD for placebo trial ($P < 0.05$). ‡ Significant difference between CAU and AFD for COX inhibition trial ($P < 0.05$). Error bars included for CAU and AFD placebo only for reader clarity.

1 Table 3. Maximum, ED50 and area under the curve (AUC) skin blood flow response
 2 to ACh in the finger skin site following placebo or COX inhibition treatment

			Variable		
			ED50 (µA)	Max (%)	AUC
Within	Finger CAU	Placebo <i>n</i> = 11	49 to 98	301 (76)	1542 (597)
		COX IB <i>n</i> = 11	24 to 137	311 (222)	1255 (872)
			<i>P</i> = 0.646	<i>P</i> = 0.902	<i>P</i> = 0.273
	Finger AFD	Placebo <i>n</i> = 8	105 to 187	188 (139)	642 (632)
COX IB <i>n</i> = 8		-	57 (43)	22 (202)	
		Unable to calculate	<i>P</i> = 0.064	<i>P</i> = 0.053	
Between	Finger placebo	CAU <i>n</i> = 11	49 to 98	301 (76)	1542 (597)
		AFD <i>n</i> = 10	125 to 282 *	160 (139) *	539 (660) *
			<i>P</i> < 0.001	<i>P</i> = 0.013	<i>P</i> = 0.002
	Finger COX ib	CAU <i>n</i> = 11	24 to 137	^287 (162)	1255 (872)
AFD <i>n</i> = 8		-	^53 (88) *	35 (218) *	
		Unable to calculate	<i>P</i> = 0.001	<i>P</i> = 0.001	

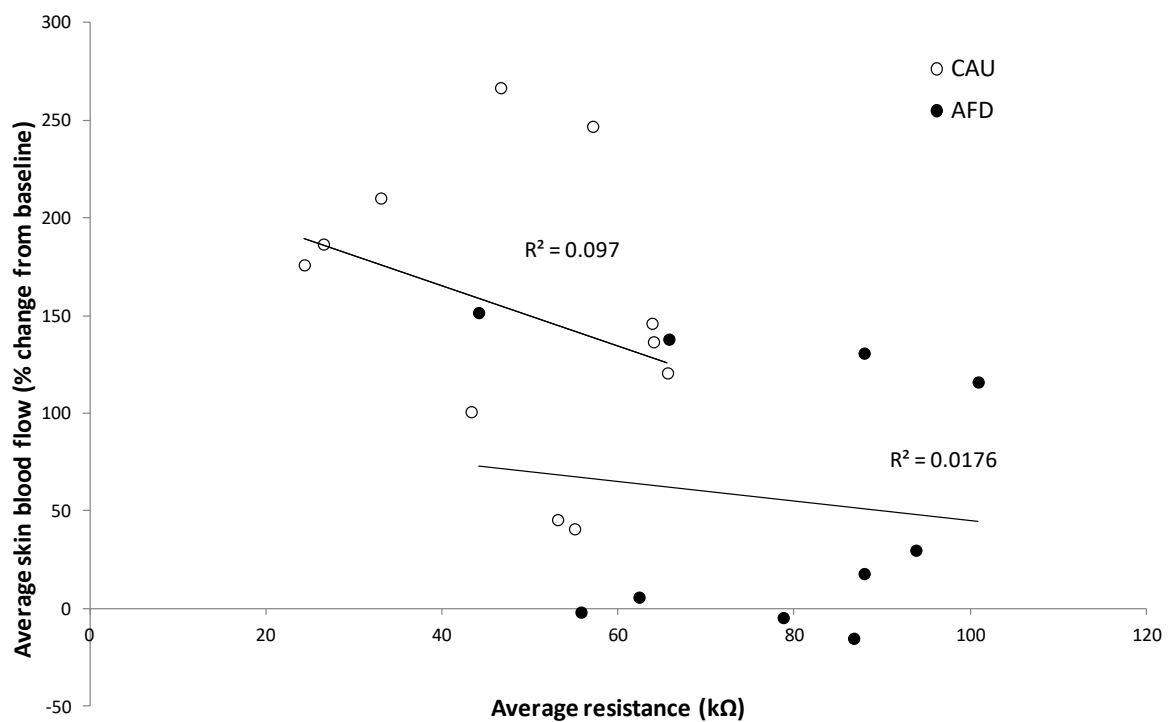
3 Max given as mean (SD) or median (IQR) percentage change from baseline, ED50 given as 95 %
 4 confidence intervals (microamps) and AUC given as mean (SD). Note: as pairwise analyses were
 5 conducted within-groups, the values reported do not always match the between-groups analyses
 6 which included all participants or until a participant did not receive all applied current. * Significant
 7 difference between CAU and AFD (*P* < 0.05). ^ Median (IQR).

8

9 **BETWEEN-GROUPS**

10 AFD demonstrated lower vasodilatation compared with CAU in response to ACh
 11 following both placebo and COX inhibition (Fig. 4). Following placebo in AFD, ED50
 12 occurred at a greater cumulative current than CAU (Table 3, *P* < 0.001). Additionally,
 13 maximal vasodilatation was lower (*P* = 0.013, *r* = 1.27) and AUC was smaller (*P* =
 14 0.002, *r* = 1.78) in AFD than CAU. Following COX inhibition, AFD demonstrated

1 lower maximal vasodilatation ($P = 0.001$, $r = 0.64$) and a smaller AUC ($P = 0.001$, $d =$
2 1.96) compared with CAU.
3
4 No relationship was observed between electrical skin resistance and skin blood flow
5 responses in the finger skin site in either CAU or AFD (Fig. 5).



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7 Fig. 5. Relationship between average skin blood flow (%) and average electrical skin
8 resistance (kΩ) for CAU and AFD in the finger skin site during 25 μ A iontophoresis
9 pulses of ACh following placebo treatment

10

11 DISCUSSION

12 The present study demonstrated that the vasodilator response to local application of
13 ACh in the foot and finger non-glabrous skin sites is lower in AFD compared with
14 CAU irrespective of COX inhibition. This data supports previous observations in the
15 hands and feet (Maley et al., 2015) and forearm (Cardillo et al., 1999; Jones et al.,

1 1999; Ozkor et al., 2014; Stein et al., 1997) where an attenuated vasodilator
2 response to ACh or methacholine was observed in AFD compared with CAU. The
3 effect of COX inhibition on the responses to ACh appeared to be site and ethnicity
4 dependant. CAU, but not AFD, experienced a lower vasodilator response to ACh
5 following COX inhibition in the foot skin site indicating the role of vasodilator
6 prostanoids, supporting previous findings in the forearm (Holowatz et al., 2005;
7 Kellogg et al., 2005; Noon et al., 1998); however, in the finger skin site, COX
8 inhibition did not affect CAU but tended to affect AFD vasodilatation.

9

10 It has been previously reported (Pienaar et al., 2014) that the higher skin resistance
11 in AFD individuals at iontophoresis currents of 100 μ A may be a possible cause of
12 the reduced response to ACh in AFD compared with CAU. However, no correlation
13 between electrical skin resistance and skin blood flow responses was observed in
14 the present study during the 25 μ A applied currents (Fig. 3 and Fig. 5). The obvious
15 differences in applied iontophoresis currents between studies could be a major factor
16 influencing results as previous investigations in healthy individuals have also
17 reported that electrical skin resistance influences the ACh-induced vasodilator
18 response to applied currents of 100 μ A (Puissant et al., 2014). Additionally, Pienaar
19 et al., (2014) correlated skin blood flow responses with electrical skin resistance but
20 did not separate CAU and AFD data. Therefore, the conclusion from Pienaar et al.,
21 (2014) that iontophoresis in AFD is limited by resistance more so in comparison to
22 CAU may be flawed as this ethnic group is known for higher skin resistance
23 (Johnson and Corah, 1963) and decreased endothelial reactivity (Cardillo et al.,
24 1999; Jones et al., 1999; Ozkor et al., 2014; Stein et al., 1997). Different skin sites
25 (i.e. forearm vs. foot) and amount of iontophoresis charge may also have influenced

1 the correlation between electrical skin resistance and skin blood flow responses.
2 Based on our observations we suggest during 25 μ A iontophoresis charges the
3 depressed ACh-induced vasodilator response in AFD is not due to high electrical
4 skin resistance in these individuals but due to another mechanism yet to be
5 identified.

6
7 In elderly and / or hypertensive individuals, COX inhibition restores the vasodilator
8 response to ACh through an increase in nitric oxide bioavailability (Taddei et al.,
9 1997a, 1997b). In comparison, COX inhibition attenuates the vasodilator response to
10 ACh in young normotensive individuals (Holowatz et al., 2005; Kellogg et al., 2005).
11 Thus, COX products appear to facilitate vasodilatation in young normotensive
12 individuals, but elicit vasoconstriction in older / hypertensive individuals. In the
13 present study it was hypothesised that COX inhibition in AFD may have augmented
14 the vasodilator response to ACh by inhibiting the COX associated oxidative stress
15 (Kukreja et al., 1986; Taddei et al., 1998; Viridis et al., 2013) and vasoconstrictor
16 prostanoid contribution; however, this was not observed. Therefore, it appears either,
17 (1) the COX pathway is not (or as) active in young healthy AFD males, or (2) the
18 lower vasodilator response to ACh in AFD is not due to the COX pathway. Given that
19 finger skin blood flow tended to decrease with COX inhibition (Table 3) we cannot
20 provide evidence for an inactive COX pathway in AFD.

21
22 In contrast to our results and the studies mentioned above (Holowatz et al., 2005;
23 Kellogg et al., 2005), Hendry and Marshall (2004) reported COX inhibition
24 augmented the response to ACh in the fingers of young healthy individuals. It is not
25 clear why the present study observed different responses but a direct comparison

1 between studies is not possible as methodological differences exist (e.g. 100 μ A vs.
2 25 μ A, respectively).

3

4 Given that AFD did not experience an augmented vasodilator response to ACh with
5 COX inhibition, the present study suggests other mechanisms are accountable for
6 the lower vasodilator response compared with CAU. It is well documented that both
7 nitric oxide and prostanoids are involved in the ACh-induced vasodilatation
8 (Holowatz et al., 2005; Kellogg et al., 2005; Noon et al., 1998). Another mechanism
9 by which vasodilatation occurs in response to ACh is through endothelial-dependent
10 hyperpolarising factors (EDHFs) (Brunt et al., 2015). Given that prostanoids
11 production would be negligible upon COX inhibition, it is assumed that the ACh-
12 induced vasodilatation would be mainly mediated through nitric oxide or EDHFs.
13 EDHFs are unlikely to be compromised in AFD as a recent study demonstrated that
14 EDHFs provide a compensatory mechanism eliciting vasodilatation in response to
15 intra-arterial infusion of ACh in AFD, but not CAU (Ozkor et al., 2014). It is known
16 that nitric oxide bioavailability is often lower in AFD compared with CAU due, in part,
17 to an increased oxidative stress (Kalinowski et al., 2004). It is possible oxidative
18 stress sources other than COX, such as superoxide produced from the enzyme
19 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Paravicini and
20 Touyz, 2008), may react with nitric oxide forming peroxynitrite resulting in less
21 bioavailability of nitric oxide and lower vasodilatation (Münzel et al., 2010).

22

23 Whilst prostanoids appear to play a role in the vasodilator response to ACh (Fig. 2)
24 and in other settings such as whole-body heating (McCord et al., 2006), they are not
25 involved in the vasodilator response to local heating (Dahmus et al., 2013; Golay et

1 al., 2004; McCord et al., 2006). This demonstrates that pharmacological protocols
2 such as those used to deliver ACh may not always reflect what occurs in an applied
3 setting. Recently, Belvins et al., (2014) provided preliminary evidence that COX
4 inhibition may reduce cold-induced vasoconstriction for CAU during local cooling of
5 the foot. While in the present study COX was not responsible for the lower
6 vasodilator response to ACh in AFD, COX may play a role during local cooling as
7 this enzyme releases TXA₂ (Sernerl et al., 1990, 1981) and reactive oxygen species
8 (Kukreja et al., 1986) which potentiate vasoconstriction (Bailey et al., 2005; Hamberg
9 et al., 1975). Based on this information it is hypothesised that COX may play some
10 role in the exaggerated vasoconstrictor response in AFD during cooling, thereby
11 contributing to the increased risk of NFCI. Future research should investigate the
12 role of prostanoids during local cooling to elucidate the reasons for the skin blood
13 flow and skin temperature differences between CAU and AFD during local cooling of
14 the extremities.

15

16 It is concluded that the attenuated endothelial reactivity to locally delivered ACh in
17 AFD compared with CAU in foot and finger skin sites is not due to an altered function
18 of COX in AFD; therefore, other pathways appear to be responsible.

19

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22

23 **AUTHORS CONTRIBUTIONS**

24 All authors contributed to the design of the research protocol; M J Maley collected
25 and analysed data; all authors interpreted results of experiments; M J Maley

1 prepared tables, figures and drafted manuscript; all authors edited and revised
2 manuscript; all authors approved final version of manuscript.

3

4 **STATEMENT OF CONFLICTS OF INTEREST**

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7

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