The detection and measurement of interleukin-6 in venous and capillary blood samples, and in sweat collected at rest and during exercise.

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Abstract

Purpose: This study aimed to quantify the relationship between venous and capillary blood sampling methods for the measurement of plasma interleukin-6 (IL-6). A parallel study was conducted to determine the possibility of measuring IL-6 in sweat using an enzyme-linked immunosorbent assay (ELISA) and investigate the relationship between plasma and sweat derived measures of IL-6.**Methods:** Twelve male participants were recruited for the measurement of IL-6 at rest and during exercise (study 1).An additional group of five female participants were recruited for the measurement of IL-6 in venous blood versus sweat at rest and following exercise (study 2). In study 1, venous and capillary blood samples were collected at rest and in response to exercise. In study 2, venous and sweat samples were collected following exercise. **Results:** Mean plasma IL-6 concentration was not different between venous and capillary blood sampling methods either at rest (4.27 ± 5.40 vs. 4.14 ± 4.45 $pg.ml^{-1}$), during (5.40 ± 5.17 vs. 5.58 ± 6.34 $pg.ml^{-1}$), or in response to exercise (6.95 ± 6.37 vs. 6.99 ± 6.74 $pg.ml^{-1}$). There was no IL-6 detectable in sweat either at rest or following exercise. **Conclusion:** There are no differences in the measurement of plasma IL-6 using either venous or capillary blood sampling methods. Capillary measurement represents a minimally invasive way of measuring IL-6 and detecting changes in IL-6 which are linked to fatigue and overtraining.

Keywords: Interleukin-6, venous blood, capillary blood, sweat, exercise, biomarker

Abbreviations:

ANOVA: Analysis of variance

ELISA: enzyme-linked immunosorbent assay

IL-6: interleukin-6

MID: mid exercise

POST: post exercise

PRE: pre exercise

PRE (2): repeated pre exercise

rhIL-6: recombinant IL-6

$\dot{V}O_{2peak}$: peak oxygen consumption

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine which has been demonstrated to exert pro- and anti-inflammatory effects in response to a number of physiological perturbations including exercise (Fischer. 2006) and illness (Christiansen et al. 2013). In response to exercise, IL-6 undergoes the most dramatic increase of any cytokine, with the increase dependent on the duration and intensity of the exercise bout (Febbraio and Pedersen 2002; Fischer 2006), with high intensity exercise resulting in the most pronounced response (Leggate et al. 2010). Elevations in IL-6 are closely related to both increased feelings of fatigue at rest (Spath-Schwalbe et al. 1998) and impaired running performance (Robson-Ansley et al. 2004). Therefore, the measurement of IL-6 throughout the course of training may be beneficial to the management of training load and athletic performance in elite athletes. However, the measurement of IL-6 is inherently invasive, using venous blood sampling and requires strict patient control and lengthy laboratory preparation to gain reliable results. Therefore, it would be of benefit to develop a non- or minimally invasive measurement of IL-6, using capillary blood samples, similar to those already commercially available and used in the field to measure blood lactate and glucose from capillary samples. However, differences between cytokine concentrations in capillary and venous blood have been reported previously (Eriksson et al. 2007), but the relationship for IL-6 from venous and capillary blood plasma requires investigation.

Attempts have been made to detect cytokines in a variety of other body fluids using non-invasive methods including saliva (Cox et al. 2008; Minetto et al. 2005; Minetto et al. 2007) and sweat (Cizza et al. 2008; Marques-Deak et al. 2006) with varying degrees of success. Saliva has been shown to be a poor medium for cytokine measurement, as there is no correlation between IL-6 concentrations in blood versus saliva either at rest (Cox et al. 2008) or following strenuous exercise (Minetto et al. 2005). However, sweat may present a more promising conduit for non-invasive measurement of systemic IL-6. Marques-Deak et al., collected sweat from females over a 24 hour period and reported that IL-6 was present in measurable concentrations, above those of plasma IL-6 (Cizza et al. 2008; Marques-Deak et al. 2006). Importantly, the IL-6 concentrations detected in sweat and from venous plasma were correlated and the concentrations appeared comparable (Marques-Deak et al. 2006), suggesting that sweat may be a potential vehicle for biomarker measurement. However,

although some have been successful in measuring other cytokines including IL-1α, IL-1β and IL-8 in sweat in response to exercise (Dai et al. 2013), to our knowledge, no other groups have successfully reported the presence of IL-6 in sweat at rest or in response to exercise in healthy individuals.

The initial aim of the study was to quantify the relationship between venous and capillary blood sampling methods for the measurement of plasma IL-6 concentrations at rest and in response to exercise. It was hypothesised that venous and capillary IL-6 concentrations would be strongly correlated. A subsequent small scale proof of concept study aimed to determine whether IL-6 is present in sweat, and to examine the relationship between venous and sweat collection methods for IL-6 measurement at rest and in response to exercise. We hypothesised that IL-6 would be detectable in sweat and that this would be correlated to IL-6 concentrations found in venous blood plasma.

Methods

Participants

Twelve physically active healthy male participants volunteered to take part in the trials for the measurement of IL-6 in venous versus capillary blood plasma at rest and during exercise (mean ± SD); age 23.8 ± 3.5 years, height 177 ± 0.1 cm, body mass 72.9 ± 5.8 kg, body mass index 23.4 ± 1.3 $kg \cdot m^{-2}$; $\dot{V}O_{2peak}$ 3.6 ± 0.4 $L.min^{-1}$. Five healthy premenopausal active females age 22.7 ± 2.9 years height 164.8 ± 2.6 cm; body mass 60.5 ± 7.7 kg; body mass index 22.3 ± 2.5 $kg.m^{-2}$; $\dot{V}O_{2peak}$ 3.07 ± 0.1 $L.min^{-1}$ volunteered to participate in the study to determine the relationship between blood and sweat IL-6. All participants reported being recreationally active, taking part in at least three 30 min exercise sessions per week. Participants currently taking anti-inflammatory medication were excluded from the experiments. Participants were made aware of possible risks associated with participating, and provided written informed consent. All participants gave their informed consent prior to participation. Procedures were approved by the Local Ethics committee, and were carried out in accordance with the Declaration of Helsinki.

Study 1: Venous vs. capillary IL-6

Preliminary trials

Participants reported to the laboratory and measurements of body mass (Avery, Birmingham, UK) and height (Seca, Hamburg, Germany) were recorded. Peak oxygen uptake ($\dot{V}O_{2peak}$) was assessed using a continuous incremental exercise test until volitional exhaustion on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). The test protocol began at a workload of 100 W and was increased by 35 W every 3 min thereafter. Expired air was measured continuously using a calibrated online breath-by-breath gas analyser (Ultima CPX, MedGraphics, MN, USA), in addition to monitoring heart rate (RS200, Polar Electro, Kempele, Finland) and ratings of perceived exertion (Borg 1982) at the end of each incremental stage. Subsequent workloads equivalent to 60% $\dot{V}O_{2peak}$ were used in subsequent experimental trials.

Protocol

Participants reported to the laboratory at 0745 following an overnight fast, having remained inactive and refrained from caffeine and alcohol consumption in the previous 24 h. Body mass was recorded before participants were seated in a semi-supine position for resting venous and capillary blood sampling (PRE). Participants then cycled at a workload equivalent to 60% $\dot{V}O_{2peak}$ for 45 min. After 45 min, participants remained on the cycle ergometer while simultaneous venous and capillary blood samples were obtained (MID). Exercise was resumed for a further 30 min and immediately after, final venous and capillary samples were taken simultaneously (POST). Heart rate was monitored at 15-min intervals during the trials and participants consumed water *ad libitum* throughout. The exercise lasted for 75 minutes in total.

Blood sampling

Venous blood samples were collected by venepuncture from an antecubital vein in the right arm into a 10 ml vacutainer which had been pre-cooled and pre-treated with K^+EDTA (BD Biosciences, San Diego, USA). Capillary samples were obtained by fingerpick sampling from the left hand whereby 200 µl of blood was collected into two pre-cooled microvettes pre-treated with K^+EDTA (Sarstedt, Leicester, UK). Prior to collecting the resting capillary sample, the hand and forearm were warmed for 5 min using a warm-air hot box to arterialise the blood at the sample site (Lobley et al. 2013). Immediately after, the remaining samples

were centrifuged at 4,000g for 10 min at 4°C and the separated plasma was pipetted in to multiple eppendorfs and stored at -80°C for subsequent analysis.

Enzyme-linked immunosorbent assays

IL-6 was analysed using a sandwich enzyme-linked immunosorbent assay (ELISAs) as described in detail previously (Gray et al. 2008). All materials and chemical reagents were obtained from Sigma-Aldrich Ltd. (Poole, UK) unless otherwise specified. All incubation periods were at room temperature and the plate placed on a horizontal orbital plate shaker (Mini Orbital Shaker SOB, Stuart Scientific, UK) at 60 rpm unless otherwise stated. IL-6 concentrations were determined in relation to a four-parameter standard curve (GraphPad Prism, Version 4.00; San Diego California, USA). Plasma IL-6 concentrations were corrected for any changes in plasma volume during the exercise trials (Dill and Costill 1974). The inter-assay coefficient was 8.0% and the intra-assay coefficient 6.2%.

Study 2: Venous vs. sweat IL-6

Preliminary Trial

Participants reported to the laboratory at 0800 and measurements of body mass and height were recorded. $\dot{V}O_{2peak}$ was assessed using a continuous incremental exercise test until volitional exhaustion on an electromagnetically braked cycle ergometer. Following a 3 min warm-up period of cycling at 50 W, workload was increased by 1 W every 3 seconds until volitional exhaustion. Expired air was measured using a continuous, breath-by-breath gas analyser, and heart rate was monitored throughout.

Sweat collection

The previously published disposable sweat collection method was used to obtain sweat samples (Brisson et al. 1991). Briefly, on a clean working surface using gloved hands, a sweat patch consisting of laboratory film paper (Parafilm, American Can Co.) was attached to the adhesive side of a sterile wound dressing (Opsite, Smith & Nephew) to form a collection chamber once applied to the skin surface. The skin was cleaned with an alcohol swab and collection patches were attached to the left and right scapular and further secured with tape (Transpore, 3M, UK). The sweat collection area was 12 cm^2, with sweat aspirated using a blunt fill needle (BD, Oxford, UK) and 5ml syringe (BD, Oxford, UK) following a 20

minute washout period and on exercise completion. Collected sweat was aliquotted into pre-weighed eppendorfs. Eppendorfs were then re-weighed, centrifuged at 13,000g for 20 minutes and stored on ice before freezing at -80°Cprior to further analysis.

Exercise trial

Participants reported to the laboratory at 0800 following an overnight fast, having avoided strenuous activity and refrained from caffeine and alcohol consumption in the previous 24 h. A cannula was inserted into an antecubital vein to allow venous blood sampling at baseline, following 20 min of the trial, and immediately at the end of the trial (60 minutes).

Participants entered an environmental chamber set at 32°C and 60% relative humidity, where they performed 60 min cycle ergometer exercise at a workload corresponding to 60% $\dot{V}O_{2peak}$. Following 20 min of exercise, the first sweat and venous blood samples were collected (washout period) and the sweat patches replaced for the remaining 40 min of the exercise, after which final sweat and blood samples were collected. Regional sweat rates were determined according to the change in mass of the aspirating syringe and sweat rates calculated as follows:

$$Sweat\ rate\ (ml.cm^{-2}.min^{-1}) = \frac{sweat\ volume\ (ml) \div area(cm^2)}{time\ (min^{-1})}$$

Resting trial

Participants reported to the laboratory at 0800as described previously, and a cannula inserted into an antecubital vein. The skin of the scapula was cleaned, and sweat patches applied. Participants then entered an environmental chamber set at 45°C, 50% relative humidity, for 60 min. Paired venous blood and sweat samples were taken at the same time points as in the exercise trial. Regional sweat production was determined as above.

Protein assay

The protein concentration of sweat samples was assessed using the Quant-IT assay kit (Invitrogen, Paisley, UK)and carried out according to the manufacturer's instructions. 5 µL of sweat was added to each well of a 96 well plate along with 100 µL fluorescent reagent diluted

1:200 in dilution buffer. Fluorescence was determined with excitation wavelength 470 nm and emission wavelength 570 nm.

Interleukin-6assay

Venous IL-6 was assayed via ELISA as in study 1. Sweat samples were made to 10% FCS to eliminate non-specific binding of IL-6 to the plastic ware used during the analysis. This ensures an equivalent final protein concentration as used in the plasma IL-6 assays. Sweat samples were added to the wells without dilution as previous experiments (not reported here) had indicated that there was little of no IL-6 present. The IL-6 standard curves, carried out in the presence of sweat, indicate that there is no matrix effect. All other procedures remained the same. Samples were analysed in triplicate, with an intra-assay coefficients of variation of <5%.

IL-6 detection in artificial and real sweat

To assess the capability of an ELISA to detect IL-6 in sweat, and to clarify if there was any interference of the sweat matrix on the IL-6 antibodies used in the ELISA, a set of dilution series was prepared in TBS/FCS, artificial sweat and pooled human sweat samples. Artificial sweat was prepared according to the standard reported previously (Midander et al. 2007). Eight-point standard curves were set up for TBS/FCS and artificial sweat, while a three-point series spike with 10 $pg.ml^{-1}$ rhIL-6 was prepared in real sweat. Absorbance was read at 490 nm and 650 nm.

Statistical analysis

Data were analysed using SPSS 19.0 (Statistical Package for Social Sciences Inc.; Chicago, IL, USA). These data were initially tested for distribution using the Kolmogorov-Smirnov test and subsequently log-transformed on occasions where data were not normally distributed, specifically for capillary and venous concentrations of IL-6 obtained in study 1. All other data were confirmed to be normally distributed. Venous and capillary blood IL-6 concentrations were compared using repeated measures two-factor ANOVA. The strength of the relationship between sampling methods was assessed using the Pearson-product-moment correlation coefficient. As the Pearson-product-moment correlation coefficient solely provides information on the strength of the relationship between measurements, Bland-Altman analysis (Bland et al. 1986) was performed to give an indication of the agreement

between variables. Plasma and sweat IL-6 data were compared with independent factorial ANOVA. Effect sizes were calculated using Cohen's *d*, with effect sizes of <0.2 classified as small, 0.4-0.6 as medium and > 0.8 as large (Cohen 1988). Results are expressed as mean ± SEM. Statistical significance was accepted at $P< 0.05$.

Results

Study 1 – Venous vs. capillary IL-6 concentration

At all time points there were significant correlations between IL-6 concentrations taken from venous and capillary blood. At rest the correlation coefficient between venous and capillary plasma IL-6 was strong ($r = 0.942$, $R^2 = 0.887$, $P< 0.01$), and was maintained with the onset of exercise ($r = 0.930$, $R^2 = 0.865$, $P< 0.01$) and following exercise ($r = 0.947$, $R^2 = 0.897$, $P< 0.01$, figure 1).

Figure 1 near here

Mean plasma IL-6 concentration was not different between venous and capillary blood sampling methods during ($d = 0.04$, $P = 0.751$) or in response to exercise ($d = 0.02$, $P = 0.882$). There was however a significant difference in venous compared to capillary derived IL-6 at rest ($d = 0.3$, $P < 0.05$; figure 2). In order to investigate this disparity further and to ensure that the result reflected a true baseline, the resting measure was repeated in all 12 participants under the same conditions at least 7 days later. No difference between resting IL-6 derived from either venous or capillary sampling was found in these repeated measurements ($d = 0.07$, $P = 0.803$, figure 2) and there was a strong correlation between measures ($r = 0.912$, $R^2 = 0.832$, $P<0.01$, figure 1A). These data were then used in all subsequent analysis. There was a significant main effect of time on IL-6 concentration ($P<0.05$). The concentration of IL-6 increased following exercise in both venous (42%) and capillary (66%) samples (both $P< 0.05$). There was no main effect of sample site ($P = 0.365$), nor an interaction effect (time *x* sample site, $P= 0.494$). Correspondingly, effect sizes at each time point were trivial (PRE $d = 0.06$, MID $d = 0.04$, POST $d = 0.02$).

Figure 2 near here

Bland-Altman plots (figure 3) provide a schematic illustration of the agreement between venous and capillary sampling methods for the detection of IL-6.

Figure 3 near here

Study 2 – Plasma vs. sweat IL-6

Sweat rate

During the resting trial, there was no detectable sweat collected during the initial 20 minute washout period. In the exercise condition, following the washout period, sweat rate was 0.6 ± 0.4 $ml.cm^2.min^{-1}$. During the main collection period, sweat rate in the exercise trial, was significantly higher than during the resting trial (2.6 ± 1.4 $ml.cm^{-2}.min^{-1}$ and 0.4 ± 0.1 $ml.cm^{-2}.min^{-1}$ respectively, $d = 2.2$, $P<0.05$).

Detection of IL-6 in real and artificial sweat

The TBS/FCS and artificial sweat dilution series were assayed in quadruplicate, and the real sweat series was assayed in duplicate. Both real and artificial sweat series closely overlay on a single standard curve, with a mean assay coefficient of variation of 3.6% (figure 4A).

Figure 4 near here

IL-6 detection in venous blood plasma

IL-6 was detected in venous plasma samples from all participants in both conditions. In the exercise trial, IL-6 concentration showed a tendency to increase over time, although this was not significant ($P = 0.269$). During the resting trial, IL-6 remained the same

throughout the course of the trial (figure 4B). There were no differences in IL-6 concentration at any time point between conditions (figure 4B).

IL-6 detection in sweat

None of the sweat samples contained any detectable IL-6. When comparing absorbance between sweat samples and the blank standard (TBS + 10% FCS), there was no discernible difference.

Discussion

The present series of studies demonstrate that, when plasma IL-6 concentrations from either venous or capillary blood are compared using ANOVA, which considers the group mean and associated variance, there is no difference between the concentrations of plasma IL-6 measured in venous and capillary blood samples during, or in response to exercise. In addition, we have shown that IL-6 does not appear in sweat in concentrations similar to, or even above that of plasma, counter to previous reports (Cizza et al. 2008; Marques-Deak et al. 2006). Therefore, in agreement with previous proteomic analysis of sweat (Raiszadeh et al. 2012), it is unlikely that sweat acts as a plasma transudate, at least in the case of IL-6.

One unexpected finding was that despite high correlation, pre-exercise measurements of IL-6 differed between venous and capillary derived samples. One possible explanation for this is that the arterialisation protocol of the resting sample was not totally effective in fully arterialising the venous blood, especially as a 5 minute hot box exposure has been implemented elsewhere for peripheral arterialization of the blood (Lobley et al. 2013). Furthermore it is possible that if the peripheral blood vessels were not sufficiently vasodilated in response to heating, then it would be expected that the venous to capillary delivery would be restricted (Gallen and Macdonald. 1990), resulting in a discrepancy and potential time lag between venous and capillary appearance of IL-6. Although many report arterialization protocols of 5-10 minutes heat exposure, some advocate 20 minutes of heating to arterialize samples (Stephens et al. 2008). Given that we found no difference in IL-6 concentrations measured in either venous or capillary blood in response to exercise where peripheral blood flow would be elevated, it suggests that this is the likely cause. In order to validate this speculation, we repeated measures of resting IL-6 for both venous and capillary samples in all twelve participants following the same pre-experimental protocol, ensuring that the

arterialisation protocol was tightly adhered to. In this repeated measure we were able to demonstrate that there was no difference between plasma IL-6 when sampled from either venous or capillary sites following effective arterialisation. We are confident that this repeated measure is a true reflection of the baseline IL-6 taken from both venous and capillary sites, since the bias of 0.13 $pg.ml^{-1}$ was similar to that observed in response to, and after exercise. Therefore, this highlights the importance of ensuring full arterialisation, with a minimum of 5 minutes of heating, when taking resting measurements using capillary blood sampling at rest. However, despite the statistical difference at rest in the initial resting sample, the bias between sampling sites was only 0.67 $pg.ml^{-1}$, which is unlikely to be of clinical relevance when classifying inflammation status, given that chronic low level inflammation is defined as a two- to fourfold increase in circulating pro- and anti-inflammatory cytokines (Bruunsgaard. 2005).

In view of the strong positive correlations between venous and capillary IL-6, it appears that the capillary sampling may offer an alternative to venous sampling of IL-6 to quantify plasma IL-6 concentrations. Therefore, an important consideration is that a consistent sample site should be used for repeated measurements on the same individual over time, particularly since Bland-Altman analysis illustrated that the sampling sites were not consistently in complete agreement, most notably when the IL-6 concentration was above 10 $pg.ml^{-1}$. Given that a number of cytokines, and in particular IL-6, can be reliably measured in capillary blood plasma, it is of importance to develop a real-time, point of care analysis tool for the measurement of such biomarkers, which may speed up the diagnosis and treatment of a number of clinical conditions.

In view of previous reports showing that IL-6 was present in sweat at concentrations comparable to that of plasma (Cizza et al. 2008; Marques-Deak et al. 2006) a secondary proof of concept study was conducted. Only trace amounts of IL-6 were detected in any of the sweat samples. A trace amount of IL-6 was detected following exercise in one participant, which was just below the detection range of our assay and well below the normal plasma physiological range. It is possible that the IL-6 ELISA assay is inhibited by components in the sweat. Therefore, to determine the potential interference of the sweat matrix on the IL-6 ELISA, we tested both artificial and real sweat spiked with known concentrations of rhIL-6. When carried out in the presence of 10% FCS, this experiment showed that there was no difference in the detection of rhIL-6 between phosphate buffered saline solution, artificial and

real sweat, demonstrating that the ELISA protocol was not sensitive to any of the components of the sweat matrix. The assay was sensitive enough to detect IL-6 in human sweat and plasma at concentrations greater than 0.31 $pg.ml^{-1}$, which is well below the normal physiological plasma range of this cytokine. Therefore, it is somewhat surprising that we were unable to replicate the findings of others that IL-6 is present in sweat at concentrations above that of blood plasma (Cizza et al. 2008; Marques-Deak et al. 2006).This is especially so given that female participants were used for this part of the study, in order to directly replicate the subject group of the previous work and that plasma IL-6 was detected at all time points within the expected physiological range.

The difference in reported results may be due to differing ways of stimulating sweat (exercise vs exogenous sweat gland stimulation), sweat collection methods (pouch vs pad) , the duration of collection and subject differences (healthy vs clinical) . In the studies by Marques-Deak et al. (Marques-Deak et al. 2006) and Cizza et al. (Cizza et al. 2008) participants underwent a 24 h collection period wearing an absorbent pad whilst carrying out their normal daily activity. This collection method involved the sweat and its constituent parts being absorbed by a pad in contact with the skin surface. To extract the sweat, the patches were then centrifuged and the spun and sweat was collected in a sterile tube (Marques-Deak et al. 2006; Sarno et al. 2001). It is possible that the resultant sweat available for analysis contains some contamination from the absorbent pad and may give rise to false positive or false negative results (Brisson et al. 1991). In the present study, this issue was circumvented by allowing the sweat to pool during the collection period before aspiration, thus negating any potential contamination from collection pads (Boysen et al. 1984). Furthermore, we employed a 20 minute washout sweat collection period to allow for any epidermal or sweat gland derived protein debris to be removed prior to collecting the main sweat sample for analysis. The possibility of epidermal contamination in collected sweat samples was also ruled out by Marques-Deak et al., who demonstrated that tape-sampling of the epidermis failed to detect any cytokines at a lower detection limit of 0.1 $pg.ml^{-1}$(Marques-Deak et al. 2006).

In addition, the participants used in the POWER studies were females in remission from depression (Cizza et al. 2008; Marques-Deak et al. 2006). As depression has on occasion been linked to alterations in cytokine levels, it is possible that the elevations reported by previously represent a pathological cytokine elevation. However, the data

regarding cyctokine elevation in depression is equivocal (Marques-Deak et al. 2006; Raison et al. 2006).

Finally, sweat is known to contain a number of protease enzymes (Baechle et al. 2006; Horie et al. 1986) and protease inhibitors (Park et al. 2011; Raiszadeh et al. 2012). It is possible that during the 40 minute main collection period, these enzymes may be responsible for degrading IL-6, rendering it undetectable by ELISA. However, the addition of rhIL-6 to both real and artificial sweat samples allowed for full recovery of IL-6 with no loss of signal strength. Therefore, it is unlikely that degradation is occurring during the 40 minute collection period, particularly in light of the fact that the incubation period in the ELISA was 2 hours in length. During this incubation period, there is potential exposure of spiked IL-6 to the protease enzymes in real sweat. As there is close replication of the concentration of IL-6 between both real and artificial sweat (lacking protease enzymes) it is unlikely that proteolytic degradation of IL-6 is occurring during the 40 minute collection period and is not the reason we are unable to report IL-6 in thermogenically induced sweat.

Clearly, the present data indicate that sweat is not merely a plasma transudate as previously suggested (Cizza et al. 2008; Marques-Deak et al. 2006). The fact that sweat IL-6 appears at concentrations above that of plasma (Cizza et al. 2008; Marques-Deak et al. 2006) suggests that the source of sweat IL-6 may arise from within the sweat gland itself (Grellner. 2002; Tian and Stacey. 2003) rather than from the epidermis (Marques-Deak et al. 2006) or skeletal muscle in response to exercise (Febbraio and Pedersen. 2002). Indeed, recent data suggests that the amino acid and proteomic profiles of sweat are very different to that of plasma (Mark and Harding. 2013). However, in agreement with Raiszadeh et al., the present data do not support the notion that IL-6 is present in sweat in detectable levels and we suggest that measurement of IL-6 in sweat is not a valid or reliable measurement of systemic IL-6. Moreover, of 180 proteins identified in sweat, only albumin, zinc α2 glycoprotein, gelsolin, apolipoprotein D and clusterin 1 were present in both sweat and plasma (Raiszadeh et al. 2012), indicating that future work aimed at using sweat as a potential vehicle for biomarkers or disease should focus on these proteins, as they have already been linked to a number of diseases and pathological conditions (Ceperuelo-Mallafré et al. 2009; Dubois et al. 2010; Hassan et al. 2008; Mracek et al. 2011; Rydén et al. 2012; Yang et al. 2013).

In summary, we have successfully demonstrated that there are no differences in the measurement of plasma IL-6 at rest, during, and in response to exercise using either venous or capillary blood sampling methods, and that the two measurement sites are strongly correlated. Thus, if one is aware of the potential pitfalls of capillary sampling, then it may represent as useful and minimally invasive measure for IL-6. We have successfully demonstrated that ELISA measurement is capable of detecting IL-6 inartificial sweat at concentrations well below the normal plasma physiological concentration. Despite this, we were unsuccessful in detecting IL-6 in sweat in all participants either at rest in a thermally challenging environment, or following exercise. As such, it appears that sweat is not a simple plasma transudate as previously suggested and is therefore not a reliable medium for the non-invasive measurement of IL-6 in healthy or athletic populations.

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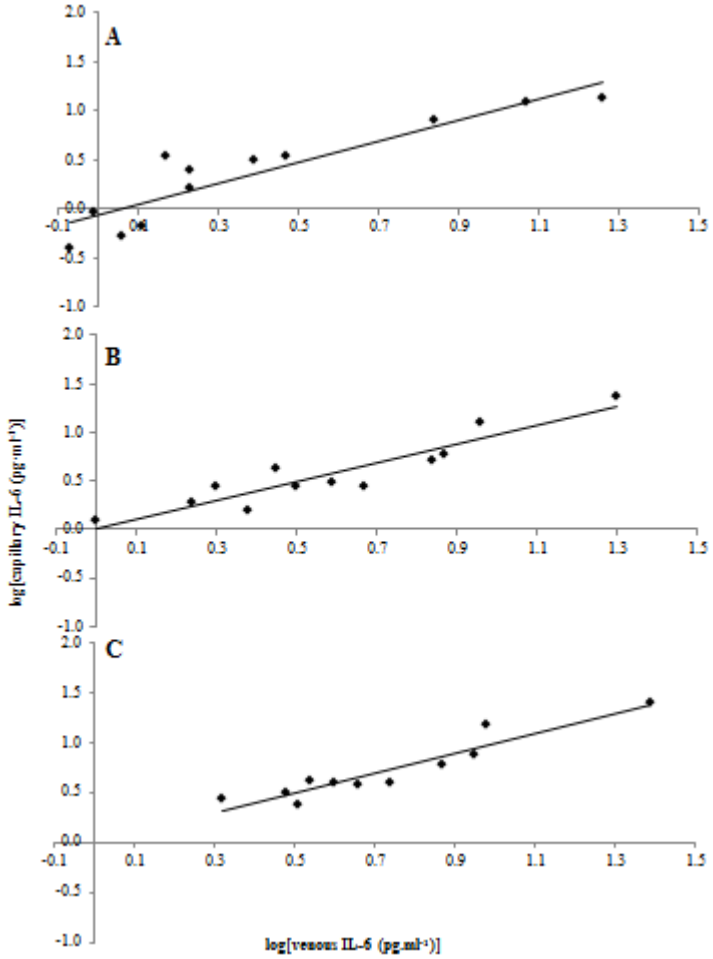

Figure 1: Correlation between venous and capillary blood sampling methods for the measurement of plasma IL-6 concentration during exercise. A) IL-6 at 0 minutes (P <0.01; B) IL-6 correlation at 45 minutes (P <0.01; C) IL-6 correlation at 75 minutes (P <0.01)

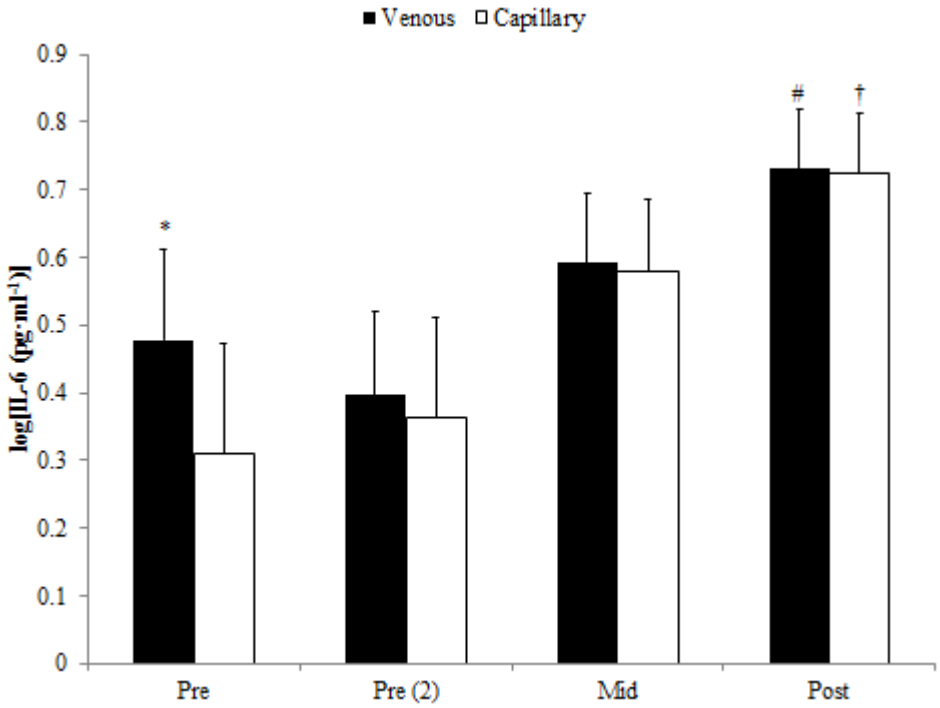

Figure 2: Plasma IL-6 concentration measured from venous and capillary blood sampling at rest and in response to exercise. There was an effect of time on IL-6 concentration (P< 0.05) with elevations for both capillary and venous concentrations post exercise (Post) versus pre exercise (Pre2) (both P < 0.05). On verification of the initial baseline measurement (Pre1), there was no difference in IL-6 concentration. There was no difference between sampling methods in response to 45 min (MID) and immediately after 75 min (POST) of exercise. = venous higher than capillary (P <0.05), # = venous different to pre (P < 0.05), † = capillary different to pre (P < 0.05). Data shown as mean ± SEM*

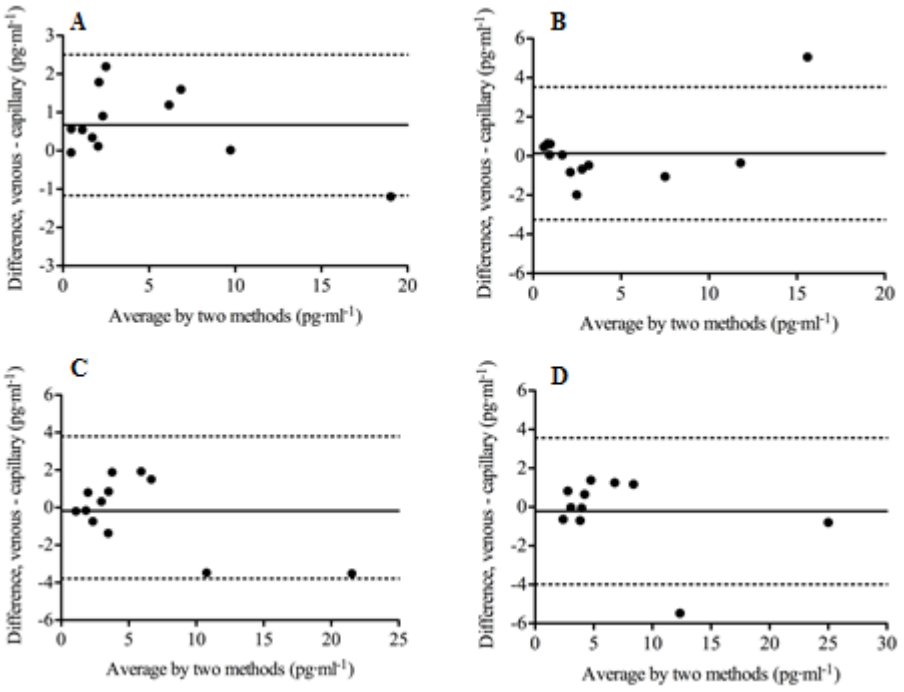

Figure 3: The 95% limits of agreement and mean differences between venous and capillary blood sampling for the measurement of plasma IL-6 at: A) rest; B) repeated resting sample, C) 45 minutesof cycling at 60% $\dot{V}O_{2peak}$ *andD) 75 minutes of cycling at 60%* $\dot{V}O_{2peak}$

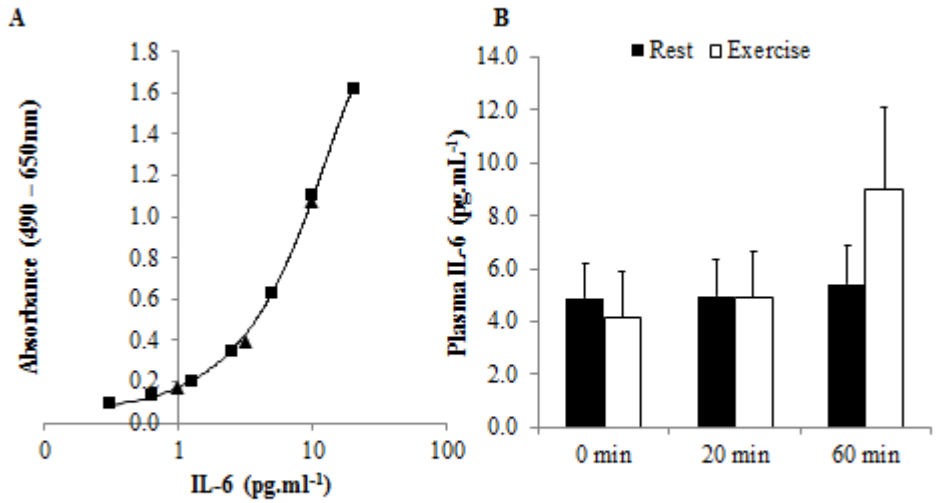

Figure 4: The detection of spiked rhIL-6 in real and artificial sweat in study two. A) The standard curve for spiked recovery of IL-6 in artificially made sweat (■) and real sweat (▲) indicates that there is no interference of the sweat matrix on the detection of IL-6 across a physiological range. B) Plasma IL-6 concentration did not differ between resting and exercise trials or in response to exercise. All data presented as mean ± SEM.