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The *ACE* I/D gene variant predicts ACE enzyme content in blood but not the ACE, UCP2 and UCP3 protein content in human skeletal muscle in the Gene SMART study

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Running head: *ACE* I/D gene variant and ACE, UCP2 and UCP3 protein content

Abstract

Angiotensin Converting Enzyme (ACE) is expressed in human skeletal muscle. The *ACE* I/D polymorphism has been associated with athletic performance in some studies. Studies suggested that the *ACE* I/D gene variant is associated with ACE enzyme content in the serum, and there is an interaction between ACE and Uncoupling Proteins 2 and 3 (UCP2, and UCP3). However, no studies have explored the effect of *ACE* I/D on ACE, UCP2 and UCP3 protein content in human skeletal muscle. Utilising the Gene SMART cohort (n=81), we investigated whether the *ACE* I/D gene variant is associated with ACE enzyme content in blood, and ACE, UCP2, and UCP3 protein content in skeletal muscle at baseline, and following a session of High-Intensity Interval Exercise (HIIE). Using a stringent and robust statistical analyses, we found that the *ACE* I/D gene variant was associated with ACE enzyme content in blood ($p<0.005$) at baseline, but not the ACE, UCP2, and UCP3 protein content in muscle at baseline. A single session of HIIE tended ($0.005 < p < 0.05$) to increase blood ACE content immediately post exercise, while muscle ACE protein content was lower 3 hours post a single session of HIIE ($p<0.005$). Muscle UCP3 protein content decreased immediately post a single session of HIIE ($p<0.005$), and remained low 3 hours post exercise. However, those changes in the muscle were not genotype-dependent. In conclusion, The *ACE* I/D gene variant predicts ACE enzyme content in blood but not the ACE, UCP2 and UCP3 protein content of human skeletal muscle.

Key words: Exercise, ACE, gene variant, uncoupling proteins

New & Noteworthy: This paper described the association between *ACE* I/D gene variant and ACE protein content in blood and, ACE, UCP2 and UCP3 protein content in skeletal muscle at baseline and after exercise, in a large cohorts of healthy males. Our data suggest that *ACE* I/D is strong predictor of blood ACE content but not muscle ACE content.

1. Introduction

Angiotensin Converting Enzyme (ACE) is a key enzyme of the renin–angiotensin systems (RAS). ACE is cleaved from an anchoring stalk of endothelial cells that line blood vessels and is released into the blood circulation (10). ACE then cleaves angiotensin I (Ang I), a weak vasoconstrictor, into angiotensin II (Ang II), a much stronger one that triggers the release of aldosterone (26). Ang II exerts its agonist effect on angiotensin II type 1 receptor (AT1R), and leads to an increased blood pressure (27). ACE is also expressed in skeletal muscle (18), and is associated with increased blood pressure and glucose homeostasis (11).

ACE has been shown to influence exercise capacity (18). ACE inhibition increases peak aerobic capacity in patients with congestive heart failure (CHF) (12). ACE inhibitors were reported to improve peak oxygen capacity (VO_2) in CHF by reducing the limitation from peripheral muscle factors (17). The *ACE I/D* gene variant, insertion (the I allele) or deletion (the D allele) of an Alu sequence of 287 base pairs in intron 16 of the *ACE* gene, has been identified three decades ago (33). The I allele was reported to be associated with endurance performance in high-altitude mountaineers (24). This association between I allele and endurance capacity was subsequently replicated in elite athletes (25, 27). However, conflicting results exist in literature (30, 38), suggesting that this association requires biological/physiological confirmation. Indeed, Rigat *et al* (33) reported that people who harbour the DD genotype has ~50% higher level of the ACE enzyme content in blood, compared with their II counterparts. Danser *et al*. also showed that carriers of the D allele have higher ACE enzyme activity in the heart (8). It is, however, unclear if the *ACE I/D* gene polymorphism influences the ACE protein content in human skeletal muscle.

Studies suggested that there is an interaction between ACE and the mitochondrial Uncoupling Proteins 2 and 3 (UCP2, and UCP3). UCP2 and UCP3 are expressed in skeletal muscle and are involved in the regulation of muscle metabolism (34). An animal study reported that AT1R antagonist treatment downregulated UCP2 expression in mouse pancreas (6). Deletion of the angiotensin II type 2 receptor (AT2R) was reported to induce gene expression of UCP2 and UCP3 in mouse skeletal muscle (42). A recent study further demonstrated that UCP2 regulates ACE gene expression directly (10). RNA interference against UCP2 in human umbilical vein endothelial cells resulted in a higher ACE mRNA expression (10). Yet, no studies have explored the effect of *ACE I/D* gene polymorphism on UCP2 and UCP3 protein content in human skeletal muscle.

When skeletal muscle is engaged in endurance work, there is a need to maintain blood pressure control and glucose homeostasis (11). The ACE enzyme is critical for optimal regulation of muscle bioenergetics and the maintenance of blood and glucose homeostasis (11). Importantly, twenty minutes of bicycle exercise at 70% VO_{2max} resulted in elevated serum ACE activity (39). Yet, it is unknown how exercise influences ACE protein content in skeletal muscle. UCP2 and UCP3, on the other hand, have a rapid turnover (2). Acute exercise tends to decrease muscle UCP2 gene expression (36), and induces the mRNA expression of UCP3, but not UCP3 protein content (35). However, it is still unclear whether the *ACE* I/D polymorphism is associated with decreased/increased ACE, UCP2 and UCP3 protein content in human skeletal muscle in response to acute exercise.

Therefore, the aims of this study were to investigate whether: 1) the *ACE I/D* gene variant is associated with physiological characteristics (such as Peak Oxygen Uptake (VO_{2peak}), Lactate

Threshold (LT), Power Peak (W_{peak})) at baseline (i.e.; pre-exercise); 2) the *ACE I/D* gene variant is associated with ACE enzyme content in blood, and ACE, UCP2, and UCP3 protein content in skeletal muscle at baseline; and 3) the *ACE I/D* gene variant is associated with ACE enzyme content changes in blood, and ACE, UCP2, and UCP3 protein content changes in muscle following a single session of High-Intensity Interval Exercise (HIIE).

2. Materials and Methods

2.1 Study overview

This study is part of a large multi-centre study, the Gene SMART study (Gene and Skeletal Muscle Adaptive Response to Training), which has been approved by Victoria University Human Ethics Committee (HRE13-223). The study methods were previously published elsewhere (40). Briefly, participants provided medical clearance to satisfy the predetermined study criteria prior to starting the study. Details regarding the study structure and protocol were then provided. Diet habits were assessed by questionnaire, and physical activity was monitored by accelerometers. Baseline exercise testings were conducted to determine baseline physical level. Baseline exercise testing comprised of two 20 km Time Trials (20 km TT) and three Graded Exercise Test to exhaustion (GXTs).

Participants underwent a 48-h control diet prior to muscle biopsies to reduce confounding effects from diet. An experienced medical doctor collected a muscle biopsy from the *vastus lateralis* muscle, along with a blood sample from participants after 12h fasting. Immediately after the baseline resting biopsy, participants underwent a session of HIIE tailored to their baseline fitness on an electronically braked cycle ergometer (Velotron®, Racer Mate Inc, Seattle, USA). The exercise session comprised a 5-min warm-up at 60W and 8 high-intensity intervals of 2 min each, interspaced by 1-min rest periods at 60W (work:rest ratio = 2:1). For each participant, the intensity was calculated as LT + 40% of the difference between the participants' individually determined Wpeak and the LT (LT + 40% (Wpeak-LT)). Immediately after the completion of the HIIE session, the second muscle biopsy and blood sample were collected. Three hours after the completion of the HIIE session, the third muscle biopsy and blood sample were collected. Participants remained fasted during the whole trial. The study flow is outlined in Figure 1.

2.2 **Participants**

Eighty-one unrelated moderately-trained males (Age: 31.0 ± 8.3, VO_{2peak}: 46.3 ± 7.0 mL/min/kg), Caucasian (for > 3 generations), aged 18-45, participated in the study.

2.3 Nutrition consultation

Each participant was provided with individualised, pre-packaged meals for the 48h prior to the biopsy day. The energy content of the provided meals were calculated using the Mifflin St-Jeor equation, which takes into account body mass, height, age and physical activity level (23). The macronutrient profile of the diet was based on the current Australian National Health and Medical Research Council (NHMRC) guidelines (i.e. 15-20% protein, 50-55% carbohydrates, < 30% fat and < 10% saturated fat). Participants were also required to refrain

from strenuous exercise, alcohol and caffeine consumption for the 48 h prior to the biopsy day.

2.4 *Performance tests*

Baseline performance tests were conducted as reported previously (22, 40). Briefly, all participants completed a familiarisation and baseline testing. All visits were separated by a minimum of 48 h. In addition, participants were required to refrain from exercise, alcohol and caffeine consumption for 24 h before all tests. The familiarisation and baseline testing consisted of the following:

20km TT - During the first (familiarisation) and third visits (baseline test) participants performed a 20 km - TT on a Velotron® cycle eogometer (RacerMate Inc. Seattle, WA, USA).

GXT - During the second (familiarization), fourth and fifth visits participants conducted a GXT, to determine baseline LT and W_{peak}. These tests were performed on an electronically braked cycle ergometer (Lode-excalibur sport, Groningen, the Netherlands) and consisted of 4-min stages separated by 30-s rest periods until exhaustion. Capillary blood samples were taken at rest, after each completed stage, and immediately following exhaustion, and were analysed by a YSI 2300 STAT Plus system (Yellow Springs, Ohio, USA). LT was calculated by the modified DMAX method, as previously reported (4, 5).

VO_{2peak} test - After five min rest following the GXT, VO_{2peak} was measured using a calibrated Quark CPET metabolic system (COSMED, Rome, Italy).

2.5 Muscle biopsies and blood sampling

Muscle biopsies: Muscle biopsies were performed on the *vastus lateralis* muscle of the participant's dominant leg. Following injection of a local anaesthetic (2 mL, 1% Xylocaine), incisions were made and the biopsy needle inserted. Muscle samples were collected with manual suction (13). To minimalize acute changes induced by muscle biopsy procedures, a new incision was made for each muscle biopsy. Following collection, the samples (50-200 mg) were immediately blotted on filter paper to remove excess blood, with a small portion (10-15 mg) immediately processed for the determination of mitochondrial respiration (15). The remaining portion of the muscle was snap-frozen in liquid nitrogen and stored at −80 ˚C for subsequent analysis.

Blood sampling: Venous blood samples were collected through cannulation immediately after each muscle biopsy (22). Five millilitres of venous blood were collected with BD Vacutainer EDTA blood collection tubes (Becton, Dickinson and Company, USA); the tubes were then inverted 6-10 times, centrifuged at 3,500 rpm for 10 minutes at 4˚C, and the resulted supernatant plasma samples were collected and aliquoted into Eppendorf tubes. The residual blood was saved for DNA extraction.

2.6 Muscle and blood analysis

Genotyping

Genomic DNA was extracted from residual blood samples using the GeneJET Genomic Whole Blood DNA Purification Kit (#K0781 Thermo Scientific, MA, USA), as well as using the MagSep Blood gDNA Kit (Eppendorf, Hamburg, Germany). *ACE I/D* genotypes were determined using the TaqMan SNP assay (rs4343, Applied Biosystems, Foster City, California, United States) by Mastercycler® ep realplex2 (Eppendorf, Hamburg, Germany), and QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, California, United States). Genotyping was validated by another researcher with a new set of DNA samples (41).

Plasma ACE content analysis

For quantitation of ACE enzyme content in plasma, Abcam Human ELISA Kit (ab119577 – ACE (CD143)) was used (Abcam, Cambridge, United Kingdom). All samples were stored in -80 °C freezer before analysis. After thawing on ice, plasma samples were diluted 50 times with sample diluent buffer. 100µl of diluted samples were added to plate in duplicates, sealed and incubated at 37°C for 90 minutes. 100 µL of 1X Biotinylated Anti-Human ACE antibody was added into each well and the plate was incubated another 60 minutes at 37°C. The plate was then washed three times with 300 µL 0.01 M PBS (8.5 g NaCl, 1.4 g Na2HPO4 and 0.2 g NaH2PO4 added to 1L distilled water, and pH adjusted to 7.2 - 7.6). 100 µL of 1X Avidin-Biotin-Peroxidase Complex working solution was added into each well and the plate was incubated at 37°C for 30 minutes. The plate was then washed five times with 0.01M PBS, 90 µL of prepared TMB Colour Developing Agent were added into each well and the plate was incubated at 37°C avoiding light for 25 minutes. 100 µL of prepared TMB Stop Solution was added into each well. The O.D. absorbance at 450 nm was obtained with a microplate reader within 15 minutes after adding the stop solution.

Western blots

Approximately 15 mg of frozen muscle samples were homogenized in ice-cold RadioImmunoPrecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA with protease/phosphatase inhibitors, 1 mM PMSF, 1 g/mL Aprotinin, 1 g/ml Leupeptin, 1mM Benzamidine, 1 mM Na3VO4, 5 mM Na Pyrophosphate, 1 mM DTT, 1 mM NaF and proteinase/phosphatase inhibitor cocktail) using a TissueLyser II (Qiagen, Hilden, Germany) for 2 × 1 minute at 30 Hz, and rotated for 1 h at 4°C. Muscle lysates were stored at -80°C until further analysis. Total protein content of muscle lysates was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, United States).

Protein extracts were loaded on TGX Stain-Free™ Precast gels (Bio-Rad Laboratories, Hercules, United States), separated for 120 minutes at 100V and subsequently transferred to PolyVinyl DiFluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, United States) using a Trans-Blot ® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, United States). Thereafter, blots were blocked for 60 minutes in 5% milk in tris-buffered saline (TBS) and washed with TBS plus 0.1% Tween at room temperature, followed by incubation with ACE, UCP2 and UCP3 primary antibodies (1:1000 dilution) overnight at 4˚C. After washing, the membranes were incubated with the appropriate secondary antibodies for 60 minutes at room temperature and revealed using a chemiluminescent substrate (Bio-Rad Laboratories, Hercules, United States). Light emission was recorded using ChemiDocTM MP System (Bio-Rad Laboratories, Hercules, United States) and quantified by image analysis

software (Image Lab, Bio-Rad Laboratories, Hercules, United States). Protein content was then normalized to total protein analysis by TGX Stain-Free™ gel (Bio-Rad Laboratories, Hercules, United States) (Eaton et al., 2013).

2.7 Data analysis

We used robust linear models adjusted for age to test the effect of the *ACE I/D* polymorphism on outcomes at baseline, using the *MASS* package in the R statistical software. We used linear mixed models (with the *lme4* package) adjusted for age to test the effect of a single session of HIIE, and to test for a possible interaction between the *ACE I/D* polymorphism and a single session of HIIE, on the changes of measured outcomes immediately after and 3h post exercise. UCP3 protein levels were not normally distributed and were log transformed before running the analyses. We treated DD, ID and II genotypes as separate groups. p-values were adjusted for multiple comparisons using the Benjamini and Hochberg method, and all reported p-values are adjusted p-values. An adjusted p value < 0.005 was considered significant (3).

3. Results

3.1. *ACE I/D* gene variant is not associated with baseline fitness levels

The *ACE I/D* genotype distribution in our sample was 27 DD, 39 ID, and 15 II individuals, which is similar to the general population. There was a trend toward higher W_{peak} and LT in the DD participants compared to their ID and II counterparts. However, this trend was abolished after using a robust multiple comparison statistical approach (p = 0.072 for Wpeak and LT) (Table 1).

Table 1. Physiological characteristics among different ACE I/D genotypes

	DD (n=27)	**ID (n=39)**	**II (n=15)**	**Raw p-value**	**Adjusted p-value***
W_{peak} (W)	311.6 (79.8)	285.6 (52.0)	256.5 (36.7)	0.024	0.072
LT (W)	229.6 (74.3)	206.0 (43.8)	180.9 (33.0)	0.029	0.072
VO_{2peak} (mL/min/kg)	48.5 (7.3)	46.1 (6.9)	42.6 (6.6)	0.37	0.37

Data are presented as Mean ± SD. *p-value after BH correction, from a robust linear model.

3.2. *ACE I/D* gene variant is associated with ACE content in blood, but not ACE, UCP2 or UCP3 protein content in muscle at baseline

We then tested whether there were any differences in ACE content in blood, and any differences in ACE, UCP3 and UCP2 protein content in muscle at baseline between the different genotypes. II individuals had only about half the amount of ACE content in blood, compared with ID and DD individuals (p = 0.000015, Table 2). However, there were no differences in ACE protein content in skeletal muscle between genotypes at baseline (p =

0.46, Table 2). Similarly, there were no differences in UCP2 or UCP3 protein content in muscle between genotypes at baseline (p = 0.084 and p = 0.46, Table 2).

We next tested whether ACE content in blood is correlated with ACE content in skeletal muscle at baseline, and found no significant correlation between the two (p = 0.81, r = 0.028). We further tested the correlation between ACE blood and muscle content according to different *ACE I/D* genotypes. There was no correlation among DD individuals (p = 0.96, r = 0.012), ID individuals (p = 0.52, r = 0.11), or II individuals (p = 0.78, r = 0.088) at baseline.

Table 2. ACE content in blood, ACE, UCP2 and UCP3 content in muscle at baseline

	DD (n=27)	**ID (n=39)**	**II (n=15)**	**Raw p-value**	**Adjusted p-value***
ACE content in blood at baseline (pg/mL)	4281 (1731)	4676 (1729)	2233 (607.0)	0.000015	NA
ACE protein content in muscle at baseline (arbitrary unit, AU)	1.86 (0.77)	1.58 (0.58)	1.63 (0.41)	0.23	0.46
UCP2 protein content in muscle at baseline (AU)	1.3 (0.76)	1.5 (1.0)	1.1 (0.73)	0.32	0.46
UCP3 protein content in muscle at baseline (AU)†	0.91 (0.32)	1.4 (1.4)	0.88 (0.28)	0.028	0.084

Data are presented as Mean ± SD. *p-value after BH correction, from a robust linear model. †Data were not normally distributed and were log-transformed for the statistical test

3.3. A single session of HIIE reduced UCP3 protein content in muscle but did not affect ACE content in blood, or ACE and UCP2 protein content in muscle.

Using a stringent adjusted p-value threshold of 0.005, there was a trend for an increase in ACE blood content immediately after exercise (mean fold change = 0.13, p = 0.0053), but no changes 3h after HIIE (p = 0.87) (Table 3 and Figure 2a). We noted a small decrease of muscle UCP3 protein content immediately after exercise (mean fold change = 0.06, p = 0.0035, Table 3 and Figure 2d). There were no changes in muscle ACE or UCP2 protein content immediately or 3h post HIIE (Table 3 and Figure 2b,c).

We next tested whether the changes of ACE blood content are correlated with changes of ACE content in skeletal muscle after HIIE. There was no significant correlation immediately after HIIE (p = 0.083, r = 0.20) or 3h post HIIE (p = 0.43, r = 0.094).

Table 3. ACE content in blood, ACE, UCP2 and UCP3 protein content in muscle following HIIE

	Pre	**Post**	**Raw p-value**	**Adjusted p-value***
ACE level in blood (pg/mL)	4115 (1810)	4430 (1818)	0.0053	NA
ACE protein level in muscle (AU)	1.7 (0.63)	1.6 (0.64)	0.037	0.074

UCP2 protein level in muscle (AU)	1.4 (0.89)	1.5 (0.94)	0.11	0.11
UCP3 protein level in muscle (AU)†	1.1 (1.0)	0.97 (0.48)	0.0012	0.0035
	Pre	**3HP**		**Adjusted p-value***
ACE level in blood (pg/mL)	4115 (1810)	4138 (1731)	0.87	NA
ACE protein level in muscle (AU)	1.7 (0.63)	1.5 (0.71)	0.0057	0.017
UCP2 protein level in muscle (AU)	1.4 (0.89)	1.6 (1.0)	0.018	0.018
UCP3 protein level in muscle (AU)†	1.1 (1.0)	0.99 (0.65)	0.0084	0.017

Data are presented as Mean ± SD. *p-value after BH correction, from a robust linear model. †Data were not normally distributed and were log-transformed for the statistical test

3.4. *ACE I/D* gene variant did not modulate the effect of HIIE on ACE, UCP2 or UCP3 in blood or muscle.

We then tested whether the *ACE I/D* polymorphism is associated with ACE level in blood, as well as ACE, UCP2 and UCP3 protein levels in muscle, following HIIE. There were no differences in response to a single bout of HIIE between the different genotypes (Table 4).

We further tested the correlation between ACE blood content and skeletal muscle content according to different *ACE I/D* genotypes after HIIE. There was no correlation among DD individuals ($p = 0.053$, $r = 0.41$), ID individuals ($p = 0.97$, $r = 0.0054$), or II individuals ($p = 0.19$, $r = 0.41$) immediately after HIIE. However, three hours post HIIE, there was a tendency for correlation among II individuals ($p = 0.039$, $r = 0.60$), but not DD ($p = 0.65$, $r = 0.099$) or ID ($p = 0.44$, $r = -0.13$) individuals.

Table 4. ACE content in blood, ACE, UCP2 and UCP3 protein content in muscle after HIIE according to *ACE I/D* genotypes

	DD		**ID**		**II**			
	Pre	Post	Pre	Post	Pre	Post	**Raw p-value**	**Adjusted p-value**
ACE level in blood (pg/mL)	4281 (1731)	4488 (1486)	4676 (1729)	4947 (1969)	2233 (607.0)	2878 (968.8)	0.37	NA
ACE protein content in muscle (AU)	1.9 (0.77)	1.7 (0.74)	1.6 (0.58)	1.5 (0.62)	1.6 (0.41)	1.6 (0.51)	0.79	0.84
UCP2 protein content in muscle (AU)	1.3 (0.76)	1.5 (0.94)	1.5 (1.0)	1.5 (0.87)	1.1 (0.73)	1.2 (1.2)	0.42	0.84
UCP3 protein content in muscle (AU)	0.91 (0.32)	0.83 (0.23)	1.4 (1.4)	1.1 (0.62)	0.88 (0.28)	0.86 (0.18)	0.079	0.24
	Pre	3HP	Pre	3HP	Pre	3HP	**Raw p-value**	**Adjusted p-value**
ACE level in blood (pg/mL)	4281 (1731)	4272 (1511)	4676 (1729)	4635 (1799)	2233 (607.0)	2498 (667.2)	0.66	NA

ACE protein content in muscle (arbitrary unit, AU)	1.9 (0.77)	1.6 (0.70)	1.6 (0.58)	1.4 (0.70)	1.6 (0.41)	1.6 (0.76)	0.72	0.72
UCP2 protein content in muscle (AU)	1.3 (0.76)	1.4 (0.78)	1.5 (1.0)	1.9 (1.2)	1.1 (0.73)	1.0 (0.53)	0.098	0.29
UCP3 protein content in muscle (AU)	0.91 (0.32)	0.85 (0.27)	1.4 (1.4)	1.2 (0.88)	0.88 (0.28)	0.84 (0.19)	0.18	0.37

4. Discussion

We explored the influence of the *ACE I/D* gene variant on ACE enzyme content in blood, as well as ACE, UCP2 and UCP3 protein content in human skeletal muscle, pre-and-post HIIE. We also analyzed the influence of *ACE I/D* polymorphism on fitness levels (W_{peak}, LT, VO_{2peak}) at baseline. In all cases, we utilized a robust statistical approach with a stringent p-value (< 0.005). The *ACE I/D* genotype was associated with plasma ACE content; DD individuals had significantly (68%) higher ACE content at baseline compared with II individuals. A single session of HIIE tended to increase blood ACE levels immediately post exercise, while muscle ACE protein content tended to be lower 3 hours post a single session of HIIE ($0.005 < p < 0.05$). Moreover, muscle UCP3 protein content decreased immediately post a single session of HIIE, and remain low 3 hours post exercise. Those changes in muscle were not *ACE* I/D genotype-dependent.

The association between ACE genotype and baseline fitness levels

The *ACE I/D* gene variant has previously been associated with athletic performance (24). We investigated a possible association between the *ACE I/D* gene variant and physiological parameters at baseline. However, we did not observe differences in W_{peak}, VO_{2peak} or LT (three strong markers of exercise performance) between DD and II participants. Our findings are consistent with previous studies in trained Polish male, and female athletes (29) and indicated that the participants were well-matched for fitness levels at baseline, regardless of their genotype. This prompted us to further investigate any *ACE I/D* genotype effects in the molecular level.

The influence of the ACE I/D gene variant on ACE content in blood and muscle

In line with the literature (33), *ACE* DD carriers had 68% higher ACE levels in the serum compared with *ACE* II carriers at baseline. Elite endurance athletes have higher frequency of the *ACE* II genotype, and lower frequency of the DD genotype, in some studies (31), and this has been associated with low ACE content in the blood. 20 minutes of bicycle exercise at 70% VO_{2max} also increased serum ACE enzyme activity (39). Therefore, it may be intuitive to think that an acute session of HIIE would lead to higher blood ACE level. However, we did not observe significant changes of plasma ACE content after an acute HIIE session.

The ACE mRNA expression and enzyme activity are regulated by angiotensin II (Ang II). Ang II infusion significantly reduced ACE mRNA levels in the lung and in the testis, as well

as the ACE enzyme activity in plasma (37). Plasma Ang II level increased following acute exercise (39). The regulation of ACE by Ang II is mediated, at least partly by Mitogen-Activated Protein Kinase (MAPK) pathway (p38 and p42/44) (9, 20), and one session of exercise (60 min of cycling at 70% of VO2 max) activated MAPK the p42/44 MAPK signalling pathway in human skeletal muscle (1). The other possible mediator of Ang II induced downregulation of ACE is possibly UCP2, Ang II has been shown to upregulate UCP2 (42), while UCP2 has been reported to inhibit ACE expression (10). Based on our data, we hypothesis that one session of HIIE leads to elevated ACE content and Ang II in the blood (showed here in the results section), while more Ang II enters to skeletal muscle and results in lower ACE expression in skeletal muscle.

We also explored, for the first time, the association between ACE blood content and ACE muscle content in healthy, moderately-trained individuals, and found no significant correlation both at baseline and after HIIE. The study by Reneland et al., has reported no correlation between ACE enzyme activity in blood and ACE activity in skeletal muscle among hypertensive patients (32), while an early study has also reported a discrepancy between plasma and lung angiotensin-converting enzyme activity in a rat model (16).

We found no association between *ACE I/D* polymorphism and ACE protein content in muscle, at baseline or post exercise. Extensive literature exist on the association between *ACE I/D* polymorphism and athletic performance, and physiological parameters (28). However, we are not aware of any study looking at the association between ACE content in muscle and ACE content in blood according to *ACE I/D* polymorphism, and the possible biological mechanism(s) involved. We therefore suggest that although the RAS exists in skeletal muscle, and the ACE is expressed in muscle, it might not be affected by an acute session of HIIE; alternatively, the exercise effects on ACE muscle content may require a longer exercise intervention. We therefore suggest that future work will focus on looking at the influence of the *ACE I/D* polymorphism on ACE protein content after a chronic exercise intervention rather than acute one.

The influence of the ACE I/D gene variant on UCP2 and UCP3 protein content in muscle

Uncoupling proteins are mitochondrial transporters which regulate mitochondrial function and cellular metabolism (10). UCP2 is critical in maintaining fatty acid oxidation (21), while UCP3 is highly expressed in skeletal muscle and has been previously reported to involve in the process of mitochondrial biogenesis (19). UCP2 and 3 have a rapid turnover (2), and we therefore investigated the effect of acute HIIE on their protein content in skeletal muscle. In the present study, a single session of HIIE did not change UCP2 protein content. No studies have reported the protein content of UCP2 after acute exercise, and there is discrepancy regarding the effect of acute exercise on UCP2 gene expression. One study reported that acute exercise tended to decrease muscle UCP2 gene expression in humans (36). However, a different study reported on higher UCP2 gene expression after acute exercise in mice (7). On the other hand, UCP3 protein content significantly decreased immediately post HIIE, and remained at low-levels three hours post HIIE. This finding is different from previous studies showing no changes in UCP3 protein levels following acute exercise (14, 35). The discrepancy could be due to the different format of exercise, while previous studies employed

386 moderately intensity continuous exercise, we utilised high-intensity interval exercise in the
387 present study. This decrease, however, was not *ACE I/D* variant-dependent.

388 There are several possible explanations as to why our current results are different from the
389 previously reported. Our exercise intervention consisted of short, high-intensity intervals (one
390 session, eight bouts of 2 min exercise), which could have triggered different molecular
391 pathways then the traditional continuous endurance exercise. Another possible explanation is
392 that the II genotype is associated with endurance athletes only at the elite level, and not
393 necessarily with exercise responses in moderately-trained participants.

394 *Study limitations*

395 Compared to traditional exercise studies we have assessed a relatively large number of
396 participants (n=81 and muscle biopsies (n=81 X 3 time points). However, when divided by
397 genotypes the numbers are still insufficient to identify a strong genotype effect. This
398 speculation is supported by our observation that there was a tendency of changes in blood
399 ACE content and muscle ACE, UCP2 and UCP3 protein content after acute exercise, while
400 there was no difference after dividing participants according to *ACE I/D* genotypes.
401 Furthermore, muscle biopsies, by nature, may result in damaged muscle tissue. We performed
402 three muscle biopsies in a very short period of time, which may have resulted in repetitive
403 tissue damage possibly led to up/down regulations of tissue repair molecular pathways.

404 The association between the *ACE I/D* genotype and endurance performance has mostly been
405 found at the high end of the performance spectrum, and our study population was
406 recreationally active males. Becoming an elite athlete requires intensive and chronic exercise
407 training leading to massive adaptations and extreme muscle phenotypes. It is possible that the
408 training stimulus we utilised (a session of HIIE) was insufficient to observe influence of the
409 *ACE I/D* genotype on ACE, UCP2 or UCP3 muscle content.

410 *Conclusions and future directions*

411 In conclusion, the results of the present study provide evidence of the *ACE I/D* genotype as a
412 strong predictor for ACE enzyme content in the blood. However, the *ACE I/D* did not predict
413 skeletal muscle ACE, UCP2 or UCP3 protein content at baseline or post HIIE. These results,
414 combined with the absence of significant differences in baseline endurance characteristics,
415 add to the growing body of literature suggesting that there might be other muscle targets that
416 can explain if and why the *ACE I/D* influences muscle performance and adaptations to
417 exercise training. Therefore, future studies, utilising longer periods of exercise, should focus
418 on discovering the molecular pathways by which the *ACE I/D* influences exercise
419 adaptations. Understanding both genetic/environmental contributions and how they differ
420 between individuals could be beneficial in understanding elite performance and adaptation to
421 training and muscle function in both healthy and diseased populations. Finally, because the
422 *ACE* genotype showed strong association with ACE enzyme level in the blood, other markers
423 in the RAS system, such as Ang II, may be worth to be measured in both blood and skeletal
424 muscle.

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431 References:

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433 1. **Aronson D, Violan MA, Dufresne SD, Zangen D, Fielding RA, and Goodyear LJ**. Exercise
434 stimulates the mitogen-activated protein kinase pathway in human skeletal muscle. *The Journal of*
435 *clinical investigation* 99: 1251-1257, 1997.
436 2. **Azzu V, Mookerjee SA, and Brand MD**. Rapid turnover of mitochondrial uncoupling protein
437 3. *The Biochemical journal* 426: 13-17, 2010.
438 3. **Benjamin DJ, Berger JO, Johannesson M, Nosek BA, Wagenmakers E-J, Berk R, Bollen KA,**
439 **Brembs B, Brown L, and Camerer C**. Redefine statistical significance. *Nature Human Behaviour* 2: 6,
440 2018.
441 4. **Bishop D, Jenkins DG, and Mackinnon LT**. The relationship between plasma lactate
442 parameters, Wpeak and 1-h cycling performance in women. *Medicine and science in sports and*
443 *exercise* 30: 1270-1275, 1998.
444 5. **Bishop D, Jenkins DG, McEniery M, and Carey MF**. Relationship between plasma lactate
445 parameters and muscle characteristics in female cyclists. *Medicine and science in sports and exercise*
446 32: 1088-1093, 2000.
447 6. **Chu KY, and Leung PS**. Angiotensin II Type 1 receptor antagonism mediates uncoupling
448 protein 2-driven oxidative stress and ameliorates pancreatic islet beta-cell function in young Type 2
449 diabetic mice. *Antioxid Redox Signal* 9: 869-878, 2007.
450 7. **Cortright RN, Zheng D, Jones JP, Fluckey JD, DiCarlo SE, Grujic D, Lowell BB, and Dohm GL**.
451 Regulation of skeletal muscle UCP-2 and UCP-3 gene expression by exercise and denervation. *The*
452 *American journal of physiology* 276: E217-221, 1999.
453 8. **Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, and**
454 **Schunkert H**. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion
455 polymorphism. *Circulation* 92: 1387-1388, 1995.
456 9. **Day RM, Yang Y, Suzuki YJ, Stevens J, Pathi R, Perlmutter A, Fanburg BL, and Lanzillo JJ**.
457 Bleomycin upregulates gene expression of angiotensin-converting enzyme via mitogen-activated
458 protein kinase and early growth response 1 transcription factor. *Am J Respir Cell Mol Biol* 25: 613-
459 619, 2001.
460 10. **Dhamrait SS, Maubaret C, Pedersen-Bjergaard U, Brull DJ, Gohlke P, Payne JR, World M,**
461 **Thorsteinsson B, Humphries SE, and Montgomery HE**. Mitochondrial uncoupling proteins regulate
462 angiotensin-converting enzyme expression: crosstalk between cellular and endocrine metabolic
463 regulators suggested by RNA interference and genetic studies. *BioEssays : news and reviews in*
464 *molecular, cellular and developmental biology* 38 Suppl 1: S107-118, 2016.
465 11. **Dietze GJ, and Henriksen EJ**. Angiotensin-converting enzyme in skeletal muscle: sentinel of
466 blood pressure control and glucose homeostasis. *J Renin Angiotensin Aldosterone Syst* 9: 75-88,
467 2008.
468 12. **Drexler H, Banhardt U, Meinertz T, Wollschlager H, Lehmann M, and Just H**. Contrasting
469 peripheral short-term and long-term effects of converting enzyme inhibition in patients with
470 congestive heart failure. A double-blind, placebo-controlled trial. *Circulation* 79: 491-502, 1989.
471 13. **Edge J, Eynon N, McKenna MJ, Goodman CA, Harris RC, and Bishop DJ**. Altering the rest
472 interval during high-intensity interval training does not affect muscle or performance adaptations.
473 *Experimental physiology* 98: 481-490, 2013.

14. **Fernstrom M, Tonkonogi M, and Sahlin K**. Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *The Journal of physiology* 554: 755-763, 2004.
15. **Granata C, Oliveira RS, Little JP, Renner K, and Bishop DJ**. Training intensity modulates changes in PGC-1alpha and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 30: 959-970, 2016.
16. **Huang H, Arnal JF, Llorens-Cortes C, Challah M, Alhenc-Gelas F, Corvol P, and Michel JB**. Discrepancy between plasma and lung angiotensin-converting enzyme activity in experimental congestive heart failure. A novel aspect of endothelium dysfunction. *Circ Res* 75: 454-461, 1994.
17. **Jondeau G, Dib JC, Dubourg O, and Bourdarias JP**. Relation of functional improvement in congestive heart failure after quinapril therapy to peripheral limitation. *Am J Cardiol* 79: 635-638, 1997.
18. **Jones A, and Woods DR**. Skeletal muscle RAS and exercise performance. *Int J Biochem Cell Biol* 35: 855-866, 2003.
19. **Jones TE, Baar K, Ojuka E, Chen M, and Holloszy JO**. Exercise induces an increase in muscle UCP3 as a component of the increase in mitochondrial biogenesis. *American journal of physiology Endocrinology and metabolism* 284: E96-101, 2003.
20. **Koka V, Huang XR, Chung AC, Wang W, Truong LD, and Lan HY**. Angiotensin II up-regulates angiotensin I-converting enzyme (ACE), but down-regulates ACE2 via the AT1-ERK/p38 MAP kinase pathway. *Am J Pathol* 172: 1174-1183, 2008.
21. **Kukat A, Dogan SA, Edgar D, Mourier A, Jacoby C, Maiti P, Mauer J, Becker C, Senft K, Wibom R, Kudin AP, Hultenby K, Flogel U, Rosenkranz S, Ricquier D, Kunz WS, and Trifunovic A**. Loss of UCP2 attenuates mitochondrial dysfunction without altering ROS production and uncoupling activity. *PLoS genetics* 10: e1004385, 2014.
22. **Levinger I, Yan X, Bishop D, Houweling PJ, Papadimitriou I, Munson F, Byrnes E, Vicari D, Brennan-Speranza TC, and Eynon N**. The influence of alpha-actinin-3 deficiency on bone remodelling markers in young men. *Bone* 98: 26-30, 2017.
23. **Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, and Koh YO**. A new predictive equation for resting energy expenditure in healthy individuals. *The American journal of clinical nutrition* 51: 241-247, 1990.
24. **Montgomery HE, Marshall R, Hemingway H, Myerson S, Clarkson P, Dollery C, Hayward M, Holliman DE, Jubb M, World M, Thomas EL, Brynes AE, Saeed N, Barnard M, Bell JD, Prasad K, Rayson M, Talmud PJ, and Humphries SE**. Human gene for physical performance. *Nature* 393: 221-222, 1998.
25. **Myerson S, Hemingway H, Budget R, Martin J, Humphries S, and Montgomery H**. Human angiotensin I-converting enzyme gene and endurance performance. *J Appl Physiol (1985)* 87: 1313-1316, 1999.
26. **Navar LG**. Physiology: hemodynamics, endothelial function, renin-angiotensin-aldosterone system, sympathetic nervous system. *J Am Soc Hypertens* 8: 519-524, 2014.
27. **Nazarov IB, Woods DR, Montgomery HE, Shneider OV, Kazakov VI, Tomilin NV, and Rogozkin VA**. The angiotensin converting enzyme I/D polymorphism in Russian athletes. *European journal of human genetics : EJHG* 9: 797-801, 2001.
28. **Orysiak J, Mazur-Rozycka J, Busko K, Gajewski J, Szczepanska B, and Malczewska-Lenczowska J**. Individual and combined influence of ACE and ACTN3 genes on muscle phenotypes in Polish athletes. *Journal of strength and conditioning research / National Strength & Conditioning Association* 2017.
29. **Orysiak J, Zmijewski P, Klusiewicz A, Kaliszewski P, Malczewska-Lenczowska J, Gajewski J, and Pokrywka A**. The association between ace gene variation and aerobic capacity in winter endurance disciplines. *Biol Sport* 30: 249-253, 2013.

30. **Papadimitriou ID, Papadopoulos C, Kouvatsi A, and Triantaphyllidis C**. The ACE I/D polymorphism in elite Greek track and field athletes. *J Sports Med Phys Fitness* 49: 459-463, 2009.
31. **Puthucheary Z, Skipworth JR, Rawal J, Loosemore M, Van Someren K, and Montgomery HE**. The ACE gene and human performance: 12 years on. *Sports Med* 41: 433-448, 2011.
32. **Reneland R, Haenni A, Andersson PE, Andren B, and Lithell H**. Skeletal muscle angiotensin-converting enzyme and its relationship to blood pressure in primary hypertension and healthy elderly men. *Blood Press* 8: 16-22, 1999.
33. **Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, and Soubrier F**. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *The Journal of clinical investigation* 86: 1343-1346, 1990.
34. **Schrauwen P, and Hesselink M**. UCP2 and UCP3 in muscle controlling body metabolism. *J Exp Biol* 205: 2275-2285, 2002.
35. **Schrauwen P, Hesselink MK, Vaartjes I, Kornips E, Saris WH, Giacobino JP, and Russell A**. Effect of acute exercise on uncoupling protein 3 is a fat metabolism-mediated effect. *American journal of physiology Endocrinology and metabolism* 282: E11-17, 2002.
36. **Schrauwen P, Troost FJ, Xia J, Ravussin E, and Saris WH**. Skeletal muscle UCP2 and UCP3 expression in trained and untrained male subjects. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 23: 966-972, 1999.
37. **Schunkert H, Ingelfinger JR, Hirsch AT, Pinto Y, Remme WJ, Jacob H, and Dzau VJ**. Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circ Res* 72: 312-318, 1993.
38. **Scott RA, Irving R, Irwin L, Morrison E, Charlton V, Austin K, Tladi D, Deason M, Headley SA, Kolkhorst FW, Yang N, North K, and Pitsiladis YP**. ACTN3 and ACE genotypes in elite Jamaican and US sprinters. *Medicine and science in sports and exercise* 42: 107-112, 2010.
39. **Woods D, Sanders J, Jones A, Hawe E, Gohlke P, Humphries SE, Payne J, and Montgomery H**. The serum angiotensin-converting enzyme and angiotensin II response to altered posture and acute exercise, and the influence of ACE genotype. *European journal of applied physiology* 91: 342-348, 2004.
40. **Yan X, Eynon N, Papadimitriou ID, Kuang J, Munson F, Tirosh O, O'Keefe L, Griffiths LR, Ashton KJ, Byrne N, Pitsiladis YP, and Bishop DJ**. The gene SMART study: method, study design, and preliminary findings. *BMC genomics* 18: 821, 2017.
41. **Yang R, Shen X, Wang Y, Voisin S, Cai G, Fu Y, Xu W, Eynon N, Bishop DJ, and Yan X**. ACTN3 R577X Gene Variant Is Associated With Muscle-Related Phenotypes in Elite Chinese Sprint/Power Athletes. *Journal of strength and conditioning research / National Strength & Conditioning Association* 31: 1107-1115, 2017.
42. **Yvan-Charvet L, Even P, Bloch-Faure M, Guerre-Millo M, Moustaid-Moussa N, Ferre P, and Quignard-Boulange A**. Deletion of the angiotensin type 2 receptor (AT2R) reduces adipose cell size and protects from diet-induced obesity and insulin resistance. *Diabetes* 54: 991-999, 2005.

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574 **Figure legends**

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576 **Figure 1**. Study Design. Each participant underwent baseline exercise testings including two
577 20 km Time Trials (20 km TT) and three Graded Exercise Test to exhaustion (GXTs). After a
578 resting biopsy and blood sampling after an overnight fasting, each participant performed a single
579 session of high-intensity interval exercise (HIIE), the second muscle biopsy and blood sample
580 were collected immediately after HIIE. The third muscle biopsy and blood sample were
581 collected three hours after the completion of the HIIE.

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584 **Figure 2**. The fold change of ACE content in blood, ACE, UCP2 and UCP3 protein content
585 in muscle following HIIE. A. Fold change of blood ACE content after HIIE. B. Fold change
586 of muscle ACE protein content after HIIE. C. Fold change of muscle UCP2 protein content
587 after HIIE. D. Fold change of muscle UCP3 protein content after HIIE.

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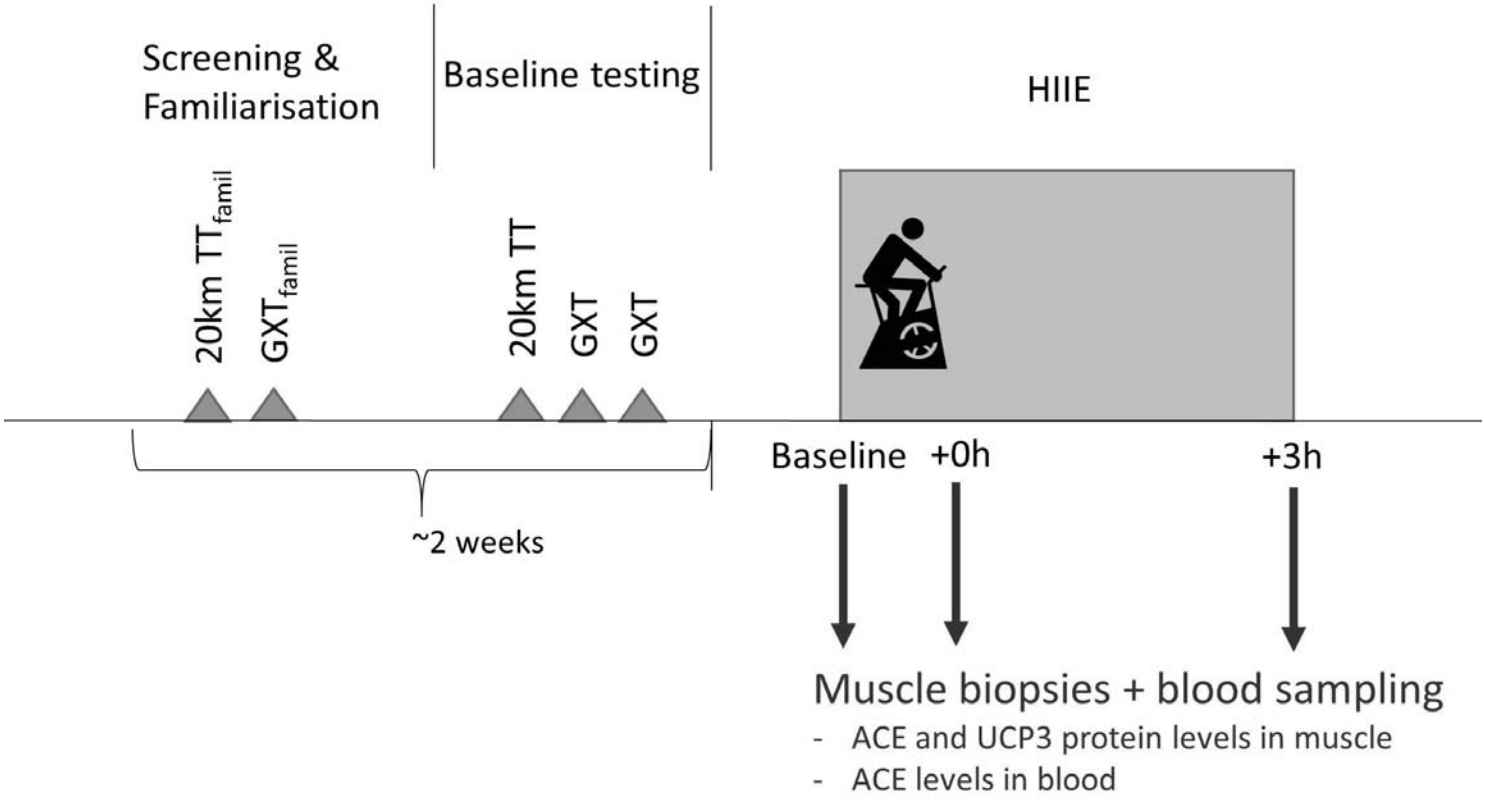
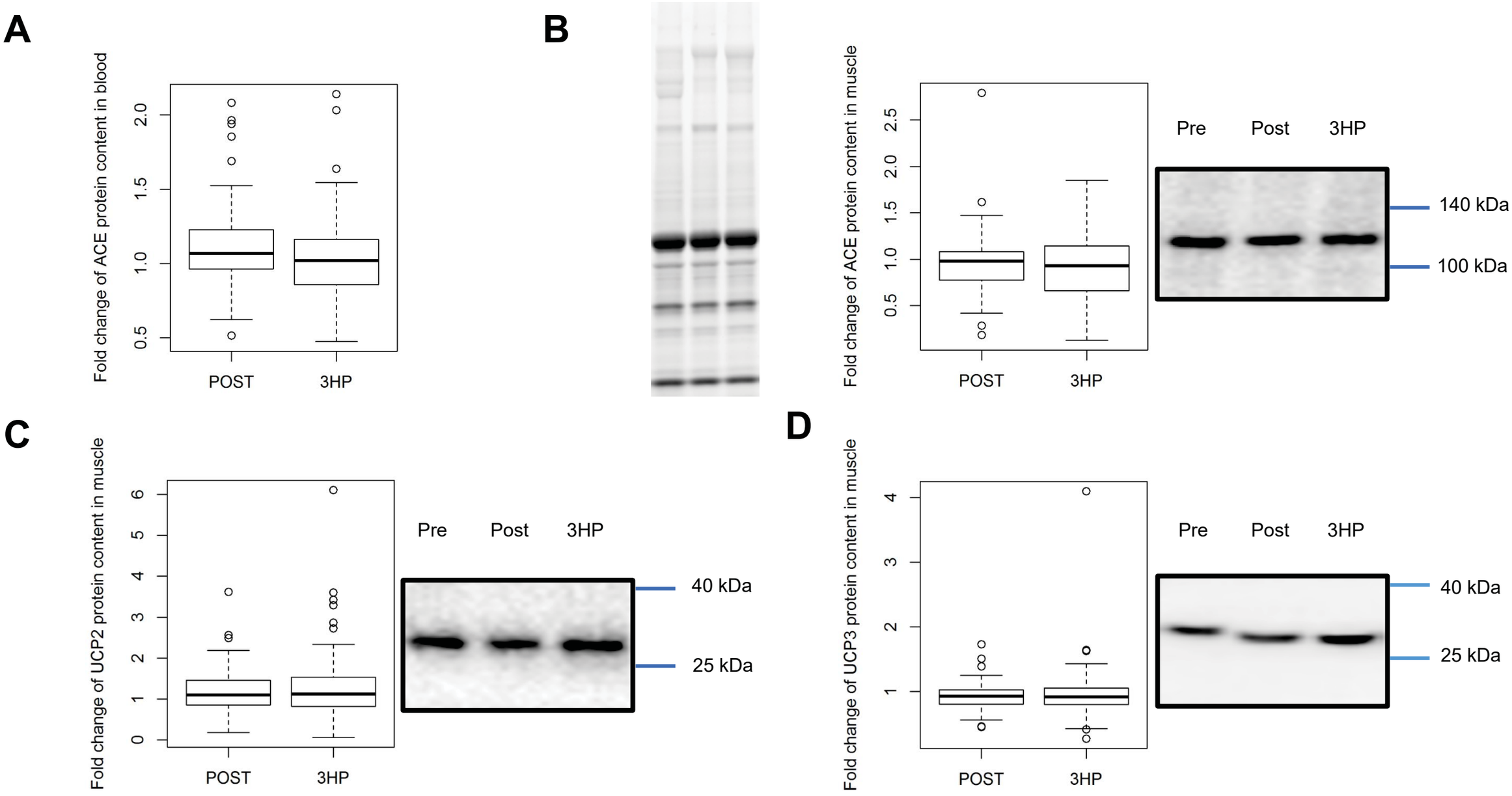

Screening &
Familiarisation
Baseline testing
HIIE
20km TT famil
GXT famil
20km TT
GXT
GXT
~2 weeks
Baseline
+0h
+3h
Muscle biopsies + blood sampling
- ACE and UCP3 protein levels in muscle
- ACE levels in blood


A
Fold change of ACE protein content in blood
0.5
1.0
1.5
2.0
POST
3HP
B
C
Fold change of UCP2 protein content in muscle
0
1
2
3
4
5
6
POST
3HP
Pre
Post
3HP
40 kDa
25 kDa
D
Fold change of ACE protein content in muscle
0.5
1.0
1.5
2.0
2.5
POST
3HP
Pre
Post
3HP
140 kDa
100 kDa
Fold change of UCP3 protein content in muscle
1
2
3
4
POST
3HP
Pre
Post
3HP
40 kDa
25 kDa