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 (VO₂) 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% VO₂max 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 (VO₂peak), 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 W_{peak} and the LT (LT + 40% (W_{peak}-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\textsubscript{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\textsubscript{2peak} test - After five min rest following the GXT, VO\textsubscript{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 W_peak and LT) (Table 1).

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

<table>
<thead>
<tr>
<th></th>
<th>DD (n=27)</th>
<th>ID (n=39)</th>
<th>II (n=15)</th>
<th>Raw p-value</th>
<th>Adjusted p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W_peak (W)</td>
<td>311.6 (79.8)</td>
<td>285.6 (52.0)</td>
<td>256.5 (36.7)</td>
<td>0.024</td>
<td>0.072</td>
</tr>
<tr>
<td>LT (W)</td>
<td>229.6 (74.3)</td>
<td>206.0 (43.8)</td>
<td>180.9 (33.0)</td>
<td>0.029</td>
<td>0.072</td>
</tr>
<tr>
<td>VO_2peak (mL/min/kg)</td>
<td>48.5 (7.3)</td>
<td>46.1 (6.9)</td>
<td>42.6 (6.6)</td>
<td>0.37</td>
<td>0.37</td>
</tr>
</tbody>
</table>

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 =
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

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

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

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Raw p-value</th>
<th>Adjusted p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE level in blood (pg/mL)</td>
<td>4115 (1810)</td>
<td>4430 (1818)</td>
<td>0.0053</td>
<td>NA</td>
</tr>
<tr>
<td>ACE protein level in muscle (AU)</td>
<td>1.7 (0.63)</td>
<td>1.6 (0.64)</td>
<td>0.037</td>
<td>0.074</td>
</tr>
</tbody>
</table>
### ACE content in blood, ACE, UCP2 and UCP3 protein content in muscle after HIIE according to ACE I/D genotypes

<table>
<thead>
<tr>
<th></th>
<th>DD</th>
<th>ID</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>ACE level in blood (pg/mL)</td>
<td>4281 (1731)</td>
<td>4488 (1486)</td>
<td>4676 (1729)</td>
</tr>
<tr>
<td>ACE protein content in muscle (AU)</td>
<td>1.9 (0.77)</td>
<td>1.7 (0.74)</td>
<td>1.6 (0.58)</td>
</tr>
<tr>
<td>UCP2 protein content in muscle (AU)</td>
<td>1.3 (0.76)</td>
<td>1.5 (0.94)</td>
<td>1.5 (1.0)</td>
</tr>
<tr>
<td>UCP3 protein content in muscle (AU)</td>
<td>0.91 (0.32)</td>
<td>0.83 (0.23)</td>
<td>1.4 (1.4)</td>
</tr>
<tr>
<td>ACE level in blood (pg/mL)</td>
<td>4281 (1731)</td>
<td>4272 (1511)</td>
<td>4676 (1729)</td>
</tr>
</tbody>
</table>

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
| 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) | 1.7 (1.0) | 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_\text{peak}, LT, VO_2\text{peak}* 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_\text{peak}, VO_2\text{peak}*, 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_2\text{max}* 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
moderately intensity continuous exercise, we utilised high-intensity interval exercise in the present study. This decrease, however, was not ACE I/D variant-dependent.

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

Study limitations

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

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

Conclusions and future directions

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


Figure legend

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

**Figure 2.** The fold change of ACE content in blood, ACE, UCP2 and UCP3 protein content in muscle following HIIE. A. Fold change of blood ACE content after HIIE. B. Fold change of muscle ACE protein content after HIIE. C. Fold change of muscle UCP2 protein content after HIIE. D. Fold change of muscle UCP3 protein content after HIIE.
Screening & Familiarisation

20km TT<sub>Famil</sub>
GXT<sub>Famil</sub>

Baseline testing

20km TT
GXT
GXT

HIIE

Baseline +0h +3h

~2 weeks

Muscle biopsies + blood sampling
- ACE and UCP3 protein levels in muscle
- ACE levels in blood