

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

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

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36 **Abstract**

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

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

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

## 63 1. Introduction

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

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

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

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

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

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

## 112 2. Materials and Methods

### 113 2.1 Study overview

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

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

136

### 137 2.2 Participants

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

140

### 141 2.3 Nutrition consultation

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

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

## 150 **2.4 Performance tests**

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

156 20km TT - During the first (familiarisation) and third visits (baseline test) participants  
157 performed a 20 km - TT on a Velotron<sup>®</sup> cycle ergometer (RacerMate Inc. Seattle, WA,  
158 USA).

159 GXT - During the second (familiarization), fourth and fifth visits participants conducted a  
160 GXT, to determine baseline LT and  $W_{peak}$ . These tests were performed on an electronically  
161 braked cycle ergometer (Lode-excalibur sport, Groningen, the Netherlands) and consisted of  
162 4-min stages separated by 30-s rest periods until exhaustion. Capillary blood samples were  
163 taken at rest, after each completed stage, and immediately following exhaustion, and were  
164 analysed by a YSI 2300 STAT Plus system (Yellow Springs, Ohio, USA). LT was calculated  
165 by the modified DMAX method, as previously reported (4, 5).

166 VO<sub>2peak</sub> test - After five min rest following the GXT, VO<sub>2peak</sub> was measured using a calibrated  
167 Quark CPET metabolic system (COSMED, Rome, Italy).

168

## 169 **2.5 Muscle biopsies and blood sampling**

170 Muscle biopsies: Muscle biopsies were performed on the *vastus lateralis* muscle of the  
171 participant's dominant leg. Following injection of a local anaesthetic (2 mL, 1% Xylocaine),  
172 incisions were made and the biopsy needle inserted. Muscle samples were collected with  
173 manual suction (13). To minimize acute changes induced by muscle biopsy procedures, a  
174 new incision was made for each muscle biopsy. Following collection, the samples (50-200  
175 mg) were immediately blotted on filter paper to remove excess blood, with a small portion  
176 (10-15 mg) immediately processed for the determination of mitochondrial respiration (15).  
177 The remaining portion of the muscle was snap-frozen in liquid nitrogen and stored at -80 °C  
178 for subsequent analysis.

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

185

## 186 **2.6 Muscle and blood analysis**

187 Genotyping

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

#### 196 Plasma ACE content analysis

197 For quantitation of ACE enzyme content in plasma, Abcam Human ELISA Kit (ab119577 –  
198 ACE (CD143)) was used (Abcam, Cambridge, United Kingdom). All samples were stored in  
199 -80 °C freezer before analysis. After thawing on ice, plasma samples were diluted 50 times  
200 with sample diluent buffer. 100µl of diluted samples were added to plate in duplicates, sealed  
201 and incubated at 37°C for 90 minutes. 100 µL of 1X Biotinylated Anti-Human ACE antibody  
202 was added into each well and the plate was incubated another 60 minutes at 37°C. The plate  
203 was then washed three times with 300 µL 0.01 M PBS (8.5 g NaCl, 1.4 g Na<sub>2</sub>HPO<sub>4</sub> and 0.2  
204 g NaH<sub>2</sub>PO<sub>4</sub> added to 1L distilled water, and pH adjusted to 7.2 - 7.6). 100 µL of 1X Avidin-  
205 Biotin-Peroxidase Complex working solution was added into each well and the plate was  
206 incubated at 37°C for 30 minutes. The plate was then washed five times with 0.01M PBS, 90  
207 µL of prepared TMB Colour Developing Agent were added into each well and the plate was  
208 incubated at 37°C avoiding light for 25 minutes. 100 µL of prepared TMB Stop Solution was  
209 added into each well. The O.D. absorbance at 450 nm was obtained with a microplate reader  
210 within 15 minutes after adding the stop solution.

#### 211 Western blots

212 Approximately 15 mg of frozen muscle samples were homogenized in ice-cold  
213 RadioImmunoPrecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM  
214 NaCl, 0.5% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA with  
215 protease/phosphatase inhibitors, 1 mM PMSF, 1 g/mL Aprotinin, 1 g/ml Leupeptin, 1mM  
216 Benzamidine, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM Na Pyrophosphate, 1 mM DTT, 1 mM NaF and  
217 proteinase/phosphatase inhibitor cocktail ) using a TissueLyser II (Qiagen, Hilden, Germany)  
218 for 2 × 1 minute at 30 Hz, and rotated for 1 h at 4°C. Muscle lysates were stored at -80°C  
219 until further analysis. Total protein content of muscle lysates was determined using the  
220 Bradford protein assay (Bio-Rad Laboratories, Hercules, United States).

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

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

## 235 **2.7 Data analysis**

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

246

## 247 **3. Results**

248

### 249 **3.1. *ACE I/D* gene variant is not associated with baseline fitness levels**

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

255

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

	<b>DD (n=27)</b>	<b>ID (n=39)</b>	<b>II (n=15)</b>	<b>Raw p-value</b>	<b>Adjusted p-value*</b>
$W_{peak}$ (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

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

258

### 259 **3.2. *ACE I/D* gene variant is associated with ACE content in blood, but not ACE, UCP2 260 or UCP3 protein content in muscle at baseline**

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

266 0.46, Table 2). Similarly, there were no differences in UCP2 or UCP3 protein content in  
267 muscle between genotypes at baseline ( $p = 0.084$  and  $p = 0.46$ , Table 2).

268 We next tested whether ACE content in blood is correlated with ACE content in skeletal  
269 muscle at baseline, and found no significant correlation between the two ( $p = 0.81$ ,  $r = 0.028$ ).  
270 We further tested the correlation between ACE blood and muscle content according to  
271 different *ACE I/D* genotypes. There was no correlation among DD individuals ( $p = 0.96$ ,  $r =$   
272  $0.012$ ), ID individuals ( $p = 0.52$ ,  $r = 0.11$ ), or II individuals ( $p = 0.78$ ,  $r = 0.088$ ) at baseline.

273

274 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

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

276 †Data were not normally distributed and were log-transformed for the statistical test

277

### 278 3.3. A single session of HIIE reduced UCP3 protein content in muscle but did not affect 279 ACE content in blood, or ACE and UCP2 protein content in muscle.

280 Using a stringent adjusted p-value threshold of 0.005, there was a trend for an increase in  
281 ACE blood content immediately after exercise (mean fold change = 0.13,  $p = 0.0053$ ), but no  
282 changes 3h after HIIE ( $p = 0.87$ ) (Table 3 and Figure 2a). We noted a small decrease of  
283 muscle UCP3 protein content immediately after exercise (mean fold change = 0.06,  $p =$   
284  $0.0035$ , Table 3 and Figure 2d). There were no changes in muscle ACE or UCP2 protein  
285 content immediately or 3h post HIIE (Table 3 and Figure 2b,c).

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

289

290 Table 3. ACE content in blood, ACE, UCP2 and UCP3 protein content in muscle following  
291 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
	<b>Pre</b>	<b>3HP</b>		<b>Adjusted p-value*</b>
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

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

293 †Data were not normally distributed and were log-transformed for the statistical test

294

#### 295 **3.4. ACE I/D gene variant did not modulate the effect of HIIE on ACE, UCP2 or UCP3** 296 **in blood or muscle.**

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

300 We further tested the correlation between ACE blood content and skeletal muscle content  
301 according to different *ACE I/D* genotypes after HIIE. There was no correlation among DD  
302 individuals ( $p = 0.053$ ,  $r = 0.41$ ), ID individuals ( $p = 0.97$ ,  $r = 0.0054$ ), or II individuals ( $p =$   
303  $0.19$ ,  $r = 0.41$ ) immediately after HIIE. However, three hours post HIIE, there was a tendency  
304 for correlation among II individuals ( $p = 0.039$ ,  $r = 0.60$ ), but not DD ( $p = 0.65$ ,  $r = 0.099$ ) or  
305 ID ( $p = 0.44$ ,  $r = -0.13$ ) individuals.

306

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

	<b>DD</b>		<b>ID</b>		<b>II</b>		<b>Raw p-value</b>	<b>Adjusted p-value</b>
	Pre	Post	Pre	Post	Pre	Post		
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	<b>Raw p-value</b>	<b>Adjusted p-value</b>
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

309

310

311 **4. Discussion**

312

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

324 *The association between ACE genotype and baseline fitness levels*

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

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

334 In line with the literature (33), *ACE* DD carriers had 68% higher ACE levels in the serum  
 335 compared with *ACE* II carriers at baseline. Elite endurance athletes have higher frequency of  
 336 the *ACE* II genotype, and lower frequency of the DD genotype, in some studies (31), and this  
 337 has been associated with low ACE content in the blood. 20 minutes of bicycle exercise at  
 338 70%  $VO_{2\text{max}}$  also increased serum ACE enzyme activity (39). Therefore, it may be intuitive  
 339 to think that an acute session of HIIE would lead to higher blood ACE level. However, we  
 340 did not observe significant changes of plasma ACE content after an acute HIIE session.

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

343 as the ACE enzyme activity in plasma (37). Plasma Ang II level increased following acute  
344 exercise (39). The regulation of ACE by Ang II is mediated, at least partly by Mitogen-  
345 Activated Protein Kinase (MAPK) pathway (p38 and p42/44) (9, 20), and one session of  
346 exercise (60 min of cycling at 70% of VO<sub>2</sub> max) activated MAPK the p42/44 MAPK  
347 signalling pathway in human skeletal muscle (1). The other possible mediator of Ang II  
348 induced downregulation of ACE is possibly UCP2, Ang II has been shown to upregulate  
349 UCP2 (42), while UCP2 has been reported to inhibit ACE expression (10). Based on our  
350 data, we hypothesis that one session of HIIE leads to elevated ACE content and Ang II in the  
351 blood (showed here in the results section), while more Ang II enters to skeletal muscle and  
352 results in lower ACE expression in skeletal muscle.

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

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

370

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

372 Uncoupling proteins are mitochondrial transporters which regulate mitochondrial function  
373 and cellular metabolism (10). UCP2 is critical in maintaining fatty acid oxidation (21), while  
374 UCP3 is highly expressed in skeletal muscle and has been previously reported to involve in  
375 the process of mitochondrial biogenesis (19). UCP2 and 3 have a rapid turnover (2), and we  
376 therefore investigated the effect of acute HIIE on their protein content in skeletal muscle. In  
377 the present study, a single session of HIIE did not change UCP2 protein content. No studies  
378 have reported the protein content of UCP2 after acute exercise, and there is discrepancy  
379 regarding the effect of acute exercise on UCP2 gene expression. One study reported that  
380 acute exercise tended to decrease muscle UCP2 gene expression in humans (36). However, a  
381 different study reported on higher UCP2 gene expression after acute exercise in mice (7). On  
382 the other hand, UCP3 protein content significantly decreased immediately post HIIE, and  
383 remained at low-levels three hours post HIIE. This finding is different from previous studies  
384 showing no changes in UCP3 protein levels following acute exercise (14, 35). The  
385 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 than 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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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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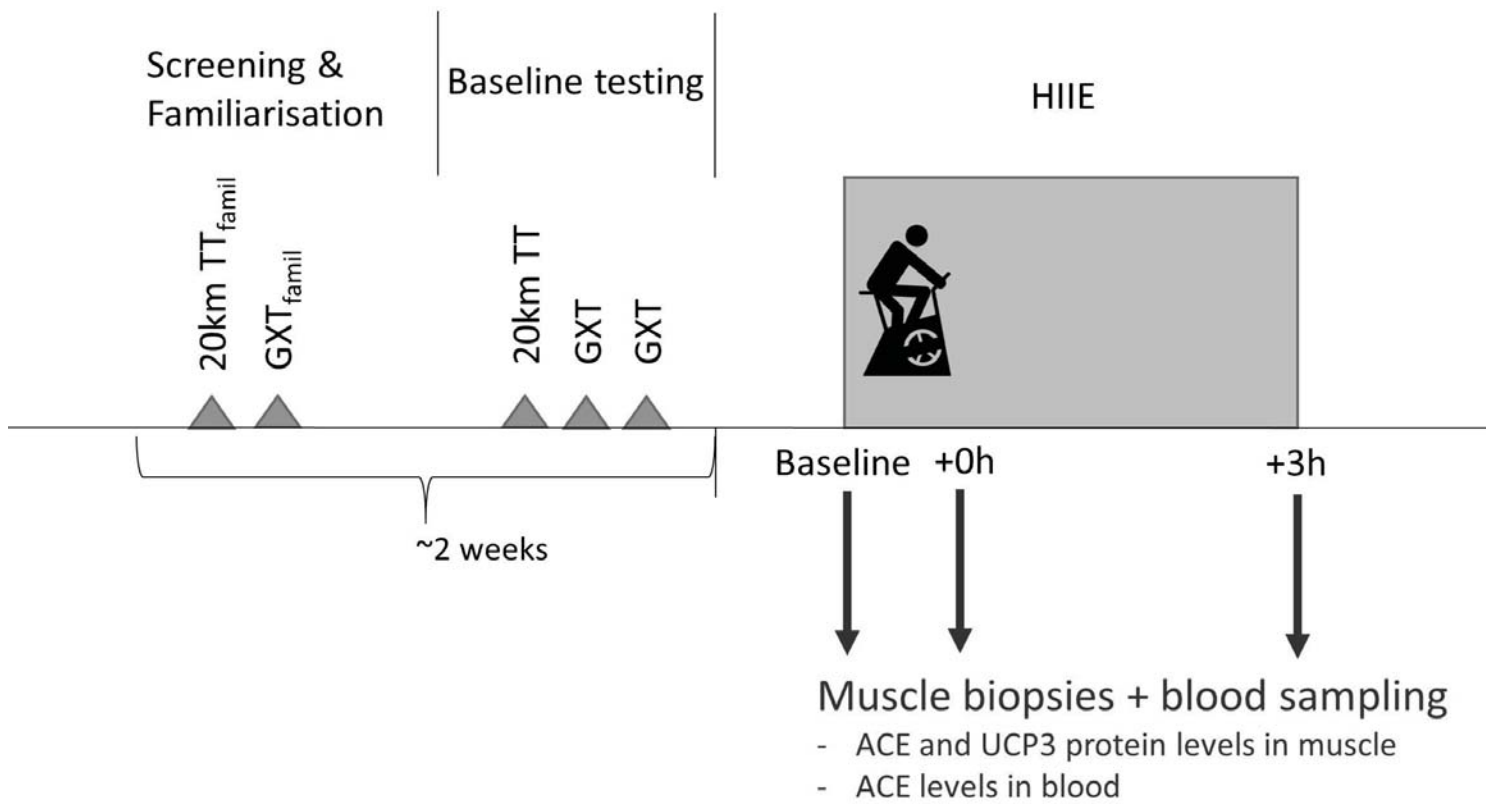
584 **Figure 2.** The fold change of ACE content in blood, ACE, UCP2 and UCP3 protein content  
585 in muscle following HIIE. A. Fold change of blood ACE content after HIIE. B. Fold change  
586 of muscle ACE protein content after HIIE. C. Fold change of muscle UCP2 protein content  
587 after HIIE. D. Fold change of muscle UCP3 protein content after HIIE.

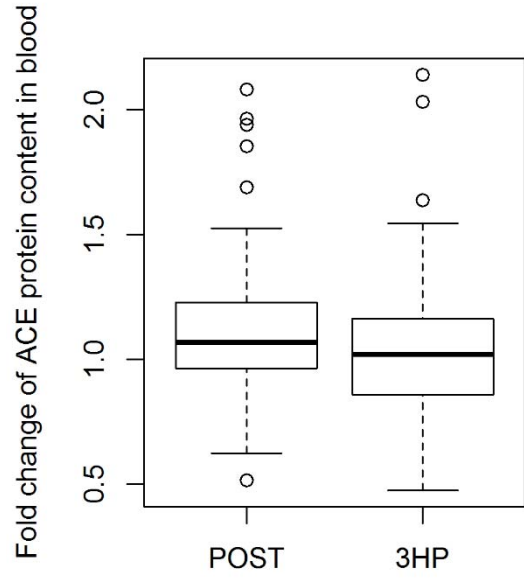
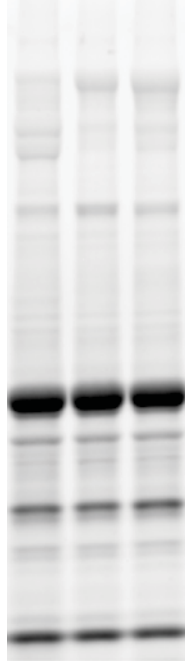
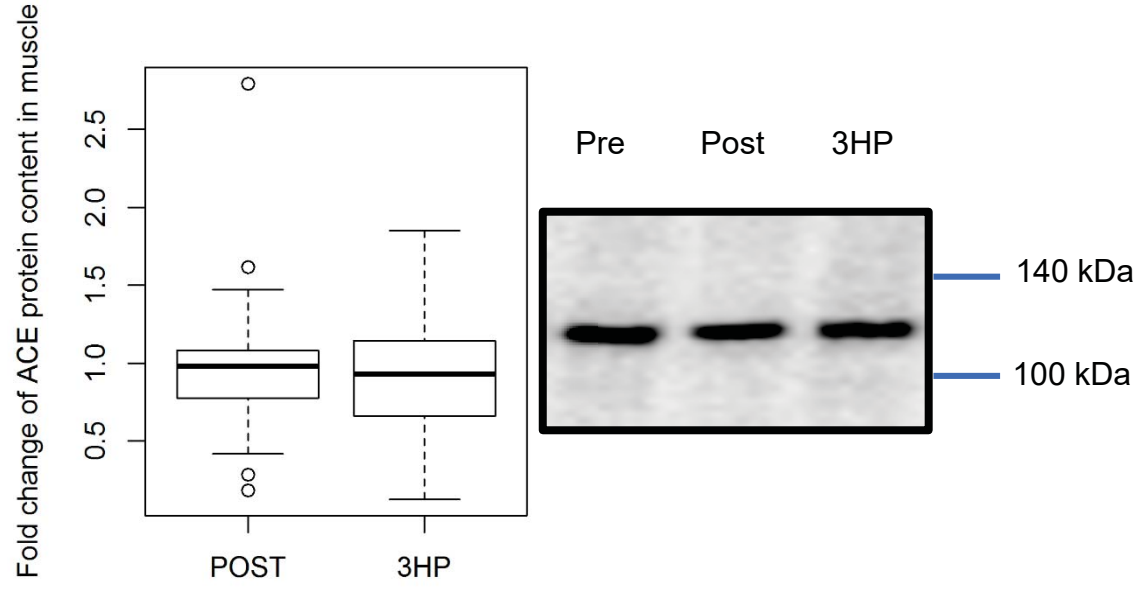
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