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Angiotensin converting enzyme gene polymorphism and respiratory muscle function in infants



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Title:

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ABSTRACT

Objective: Angiotensin converting enzyme (ACE) gene contains a polymorphism consisting of either the presence (I) or absence (D) of a 287 base pair fragment. Recent studies have suggested that the I-allele may be associated with superior exercise endurance; respiratory muscle function may be similarly influenced. The pressure-time index of inspiratory muscles (PTImus) is a measure of the load-capacity ratio of the inspiratory muscles. The objective of this study was to determine whether infants homozygous for the I-allele have lower PTImus compared to infants homozygous for the D-allele or heterozygous I/D.

Patients and Methods: One hundred thirty two infants were studied. ACE genotyping was performed by polymerase chain reaction amplification, using DNA from peripheral blood. PTImus was calculated as $(P_{i_{mean}}/P_{i_{max}}) \times (T_i/T_{tot})$, where $P_{i_{mean}}$ was the mean inspiratory pressure estimated from airway pressure, generated 100 milliseconds after an occlusion ($P_{0.1}$), $P_{i_{max}}$ was the maximum inspiratory pressure and T_i/T_{tot} was the ratio of the inspiratory time to the total respiratory cycle time. $P_{i_{max}}$ was the largest pressure generated during brief airway occlusions performed at the end of a spontaneous crying effort.

Results: Infants with I/I genotype had significantly lower PTImus than infants with either D/D or I/D genotypes ($p=0.000007$). ACE genotype was significantly related ($p=0.005$) to PTImus measurements, independent of other factors that may affect respiratory muscle function.

Conclusion: These results suggest that an association of ACE genotypes with PTImus measurements may exist in infants.

INTRODUCTION

Angiotensin I-converting enzyme (ACE) converts angiotensin I to vasoactive and aldosterone-stimulating peptide angiotensin II and it is an important component of the local renin-angiotensin systems (RASs).^{1,2} There is a significant variation in circulating ACE activity among individuals, which may be genetically determined. The human ACE gene is located on chromosome 17 (17q23) and contains a two allele polymorphism, consisting of either the presence (insertion, I) or absence (deletion, D) of a 287 base pair (bp) fragment.³ The deletion is associated with increased ACE activity in both tissue and circulation and accounts for about 44% of the intra-individual variation in plasma ACE activity in Caucasians.³ Recent studies have suggested that I-allele may be associated with superior exercise endurance,^{4,5} partly through improved skeletal muscle metabolic efficiency.⁶ Respiratory muscle function may be similarly influenced.

Several methods have been used to assess the endurance properties of respiratory muscles. Diaphragmatic pressure-time index (PTIdi) is a measure of the load-capacity ratio of the diaphragm.⁷ In adults, a PTIdi greater than 0.15-0.18 may indicate impending diaphragm fatigue.⁷ Determination of PTIdi, however, is invasive, requiring the use of an esophageal catheter. A non-invasive method of measuring pressure-time index of inspiratory muscles (PTImus) has been validated in both adults⁸ and infants.⁹

The aim of this study was to test the hypothesis that infants homozygous for the I-allele may have lower PTImus compared to infants homozygous for the D-allele or heterozygous I/D.

METHODS

Patients

Infants cared for at the Neonatal Intensive Care Unit- Pediatric Department of the University General Hospital of Patras, Greece, were eligible for the study. Infants were entered into the study if parents gave informed written consent. The study was approved by the local Research Ethics Committee. Part of the studied population was recruited from a study examining the association of ACE genotypes and circulating blood ACE on diaphragmatic function in infants. All infants were studied awake, before discharge, in supine position and at least one hour after a feed. Infants had no respiratory symptoms for at least 3 days before measurement. Furthermore, infants were on full oral feeds, had serum electrolytes, calcium, magnesium and phosphates within normal range and did not receive any methylxanthines.

ACE genotype determination

ACE genotyping was performed on DNA extracted from whole blood, collected from an indwelling catheter or via peripheral venipuncture during routine blood sampling. The method has been previously described.¹⁰ Briefly, DNA was extracted from 200 μ l blood using Qiam spin columns (Blood mini kit- Qiagen, QIAGEN Inc., Germantown, U.S.A). DNA was analyzed by electrophoresis on an agarose gel. DNA amplification of the 16th ACE intron was performed using two sets of primers flanking the polymorphic site, (outer and inner primers), because mistyping of the D/D genotype is reported to occur using the conventional amplification with insertion/deletion (I/D) flanking primer.¹¹

Measurement of PTImus

Airflow was measured using a pneumotachograph (Mercury F10L, GM Instruments, Kilwinning, Scotland) connected to a differential pressure transducer (DP45, range ± 2 cm H₂O, Validyne Corp, Northridge, CA, USA). Airway pressure (Paw) was measured from a side port on the pneumotachograph using a differential pressure transducer (DP45, range ± 100 cm H₂O, Validyne Corp, Northridge, CA, USA). The signals from the pressure transducers were amplified using a carrier amplifier (Validyne CD 280, Validyne Corp, Northridge, CA, USA). The pressure and flow signals were recorded and displayed in real time on a computer (Dell Optiplex GX620, Dell Inc., Texas, U.S.A) running LabviewTM software (National Instruments, Austin, Texas, U.S.A) with analog-to-digital sampling at 100 Hz (16-bit NI PCI-6036E, National Instruments, Austin, Texas, U.S.A).

P_{0.1} was calculated as the airway pressure generated 100 milliseconds after an occlusion, using a face mask and a unidirectional valve which allowed expiration but not inspiration, while the infant was quietly breathing. At least four airway occlusions were performed and the average P_{0.1} was calculated. Pressure-time index of the inspiratory muscles (PTImus), was calculated as: $PTImus = (P_{i_{mean}}/P_{i_{max}}) \times (T_i/T_{tot})$ where P_{i_{mean}} was the average airway pressure during inspiration, obtained from the formula $P_{i_{mean}} = 5 \times P_{0.1} \times T_i$.¹² P_{i_{max}} was the maximum inspiratory airway pressure, T_i was the inspiration time and T_{tot} was the total time for each breath calculated from the airway flow signal. To measure P_{i_{max}}, a facemask (total deadspace, 4.5 mL) was held firmly over the infant's nose and mouth and the airway was occluded at the end of a spontaneous crying effort using a unidirectional valve attached to the pneumotachograph, which allowed expiration, but not inspiration. The occlusion was

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3 maintained for at least four inspiratory efforts. At least three airway occlusions were
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5 performed and the maximum Pi_{max} achieved for individual, was recorded.
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8 **Statistical analysis**

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10 Data was tested for normality using the Shapiro-Wilk and D'Agostino skewness
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12 tests. Differences between ACE genotype groups were assessed for statistical
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14 significance using the Kruskal-Wallis and Dunn's post-hoc non-parametric tests and
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16 Cramer's V test, as appropriate. Stepwise multiple regression analysis was
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18 performed to determine whether the polymorphism of the ACE gene is related to
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20 PTImus measurements, independently to gestational (GA) and postnatal (PNA) age,
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22 birthweight (BW), gender and support from mechanical ventilation.
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27 Statistical analysis was performed using StatView 5.0 (SAS Institute, Inc., NC,
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29 USA), NCSS 2007 (NCSS, Utah, USA) and SPSS version 17 (SPSS Inc, Chicago,
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31 IL, USA).
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36 **RESULTS**

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41 Between January 2007 and August 2008 from the 532 infants admitted in the
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43 Neonatal Intensive Care Unit of the University of Patras, one hundred thirty two
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45 infants were included in the study. Fifty eight infants required ventilation in the initial
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47 stage of their illness. The characteristics of the study population are presented in
48
49 table 1.
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53 Eighteen infants (13.6%) were homozygous for the I-allele (I/I), 41 (31.1%)
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55 homozygous for the D-allele (D/D) and 73 infants (55.3%) were heterozygous I/D. A
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57 Hardy-Weinberg equilibrium (HWE) calculation was performed to examine whether
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3 genotype frequencies remain constant in the studied population. Participants were in
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5 HWE (Chi square for HWE 2.613, $p=0.106$).
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8 Overall, there were no significant differences in the characteristics of the infants
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10 with I/I, D/D and I/D ACE genotypes (table 2). Only four infants were oxygen
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12 dependent at 36 weeks postmenstrual age (1 infant with I/I, 2 with I/D and 1 with D/D
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14 ACE genotypes). Infants with I/I genotype had lower PTImus than infants with either
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16 D/D or I/D genotypes (Kruskal-Wallis, $p=0.000007$; Dunn's test, z -value= 3.58,
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18 $p<0.01$ and z -value= 4.88, $p<0.01$, respectively) (table 2), (figure). No difference in
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20 regards to PTImus values was found between infants with D/D and I/D genotypes
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22 (Dunn's test, z -value=1.39, n.s). Analysis of the components of the PTImus has
23
24 shown that only the ratio of Pi_{mean} to Pi_{max} was lower in infants with the I/I genotype,
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26 compared to infants with either the D/D or I/D genotypes (Kruskal-Wallis, $p=$
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28 0.000005; Dunn's test, z -value= 3.33, $p<0.01$ and z -value= 4.91, $p<0.01$,
29
30 respectively). Lower values of Pi_{mean} in infants with I/I genotype compared to infants
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32 with either D/D or I/D genotypes, were also observed (Kruskal-Wallis, $p= 0.0009$;
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34 Dunn's test, z -value= 2.61, $p<0.05$ and z -value= 3.73, $p<0.01$, respectively) (table 2).
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36 Pi_{max} measurements were not statistically different between the three groups (table
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38 2). Stepwise regression analysis revealed that ACE genotypes were significantly
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40 related to the PTImus measurements after logarithmic transformation, independent
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42 of GA, BW, PNA, gender and support from mechanical ventilation ($p=0.005$) (table
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53 In the subgroup of infants that never required any form of ventilatory support, 10
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55 infants (13.5%) were homozygous I/I, 20 (27.0%) homozygous D/D and 44 infants
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57 (59.5%) were heterozygous I/D. Infants in this subgroup were in HWE (Chi square
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59 for HWE 3.304, $p=0.069$). No significant difference in the characteristics of the
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3 infants with I/I, D/D and I/D ACE genotypes, was found (table 4). Infants with I/I
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5 genotype had lower PTImus than infants with either D/D or I/D genotypes (Kruskal-
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7 Wallis, $p= 0.0022$; Dunn's test, $z\text{-value}= 3.07$, $p<0.01$ and $z\text{-value}= 3.39$, $p<0.01$,
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9 respectively), (table 4). No difference regarding PTImus values was found between
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11 infants with D/D and I/D genotypes (Dunn's test, $z\text{-value}= 0.0008$, n.s). The ratio of
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13 Pi_{mean} to Pi_{max} was lower in infants with the I/I genotype, compared to infants with
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15 either the D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0015$; Dunn's test, $z\text{-value}=$
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17 2.95 , $p<0.01$ and $z\text{-value}= 3.58$, $p<0.01$, respectively). Pi_{max} measurements were not
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19 statistically different between the three groups (table 4), but Pi_{mean} was lower in
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21 infants with I/I genotype, compared to infants with either D/D or I/D genotypes
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23 (Kruskal-Wallis, $p= 0.003$; Dunn's test, $z\text{-value}= 3.31$, $p<0.01$ and $z\text{-value}= 3.00$,
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25 $p<0.01$, respectively). In this subgroup, forty six infants never required any oxygen
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27 supplementation; 6 infants (13.0%) were homozygous I/I, 13 (28.3%) homozygous
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29 D/D and 27 infants (58.7%) were heterozygous I/D. Infants in the subgroup that
30
31 never required any oxygen supplementation were in HWE (Chi square for HWE
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33 1.872 , $p=0.171$). The median (range) of PTImus of the whole subgroup was 0.078
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35 (0.028-0.119). Infants with I/I ACE genotype had lower PTImus than infants with
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37 either D/D or I/D genotypes [median (range), 0.040 (0.028-0.083) versus 0.073
38
39 (0.052-119) and 0.080 (0.040-117), respectively; Kruskal-Wallis, $p= 0.0075$; Dunn's
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41 test, $z\text{-value}= 2.55$, $p<0.05$ and $z\text{-value}= 3.11$, $p<0.01$, respectively]. The ratio of
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43 Pi_{mean} to Pi_{max} and Pi_{mean} were lower in infants with the I/I ACE genotype, compared
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45 to infants with either D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0047$; Dunn's test, $z\text{-}$
46
47 $value= 2.42$, $p<0.05$ and $z\text{-value}= 3.27$, $p<0.01$, respectively and Kruskal-Wallis, $p=$
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49 0.019 ; Dunn's test, $z\text{-value}= 2.46$, $p<0.05$ and $z\text{-value}= 2.74$, $p<0.05$, respectively).
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6 In the subgroup of the infants who required ventilation in the initial stage of their
7 illness, 8 infants (13.8%) were homozygous I/I, 21 (36.2%) homozygous D/D and 29
8 infants (50%) were heterozygous I/D. Participants were in HWE (Chi square for HWE
9 0.1622, $p=0.687$). Infants with I/I ACE genotype had lower PTImus than infants with
10 either D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0024$; Dunn's test, $z\text{-value}= 1.98$,
11 $p<0.05$ and $z\text{-value}= 3.37$, $p<0.01$, respectively).
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22 DISCUSSION

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27 In this study, an association between ACE genotypes and PTImus measurements
28 in infants, was demonstrated. Infants homozygous for the I-allele had lower PTImus
29 values than infants homozygous for the D-allele and heterozygous I/D. No difference
30 in regards to PTImus values was found between infants with D/D and I/D genotypes.
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32 Furthermore, the ACE polymorphism was related to the PTImus measurements,
33 independent of other factors, which could affect respiratory muscle performance. The
34 association between ACE genotypes and PTImus measurements was replicated on
35 the subgroup of infants that required ventilation in the initial stage of their illness and
36 on the subgroup of infants that never required any form of ventilatory support.
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48 One limitation of our study was the fact that measurements in an age-matched
49 control group, consisting of infants in the postnatal ward, were not performed. The
50 recruitment of such babies is extremely difficult. To overcome this issue, we
51 measured infants in the neonatal unit that are free of factors that could possibly
52 interfere with respiratory muscle function such as requirement of respiratory support.
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3 One part of the whole population consisted of infants that never required oxygen
4 supplementation, indeed, and a separate analysis was performed in this subgroup.
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10 Fatigue of respiratory muscles may result in an inability to maintain adequate
11 alveolar ventilation and respiratory failure. A simple method of studying the
12 endurance properties of inspiratory muscles, is the measurement of the duration that
13 a specific submaximal task (maximum sustainable ventilation, external mechanical
14 load) can be maintained (time limit or endurance time, TLim), referred to as the point
15 where the inspiratory muscles failed to maintain the task, despite maximal effort.⁷
16
17 However, this kind of test is difficult to be applied to infants. Diaphragmatic pressure-
18 time index (PTIdi), the product of the ratio of mean inspiratory transdiaphragmatic
19 pressure per breath ($P_{di_{mean}}$) to maximum transdiaphragmatic pressure ($P_{di_{max}}$) and
20 the ratio of the inspiratory time (T_i) to the total respiratory cycle time (T_{tot}), is closely
21 related to the TLim.¹³ The determination of PTIdi, however, is rather invasive, since
22 it requires the placement of an esophageal catheter. Analysis of inspiratory muscle
23 function by measurement of PTImus was first described by Gaultier et al.¹² As
24 measurement of P_{O_1} is non-invasive, measurement of PTImus has the advantage of
25 being relatively easy to be performed. This index was validated in adults.⁸ Studies in
26 healthy children, demonstrated that PTImus ranged between 0.036 and 0.090.^{14,15} In
27 spontaneously breathing infants, PTImus was compared to PTIdi and an agreement
28 between the two methods, using Bland and Altman analysis, was found.⁹ In this
29 study, PTImus measurements in infants that never required any oxygen
30 supplementation ranged between 0.028 and 0.119, something that is close to the
31 values observed in healthy children.
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Several studies have suggested that there is an association of the ACE I/D polymorphism with exercise performance. British Army recruits were examined before and after an identical 10-week general physical training program.⁵ Although, pre-training performance was independent of genotype, the improvement in performance was 11-fold greater among those of I/I, compared with D/D genotype.⁵ Consistently, a significant excess of the I/I genotype was found in Australian national rowers attending their pre-Olympics selection trial, compared to matched healthy controls.¹⁶ The frequency of I-allele was higher in British Olympic-standard distance runners.⁴ Furthermore, genotype distribution and I-allele frequency differed significantly between elite mountaineers, who had ascended beyond 7000 m without the use of supplemental oxygen and controls.⁵ All such studies are prone to difficulties in comparison, as the definition of the “elite athlete” and the heterogeneity of the elite athlete phenotype may be variable. The polymorphism of the ACE gene has been examined in infants, but not in association with muscle endurance.^{17,18}

The association of the ACE genotype and respiratory muscle function, has not been widely examined yet. In a study of COPD (Chronic Obstructive Pulmonary Disease) patients and healthy control subjects, the ACE genotype was related to the regulation of skeletal muscle aerobic work efficiency, but it was not associated with the ventilatory responses to exercise.¹⁹ The relationship between circulating ACE activity, ACE I/D genotype, mechanical efficiency and the maximal rate of oxygen uptake has been assessed in untrained women.²⁰ Neither circulating ACE activity nor ACE genotype showed a statistically significant association with any of the endurance phenotypes measured. However, ACE inhibition in heart failure increases respiratory muscle strength²¹ and exercise tolerance.^{22,23}

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3 The enhanced endurance performance in the ACE I/I genotype may be due to
4 higher oxygen delivery, conversion of fast-twitch, type II, to slow-contracting, type I,
5 muscle fibers, increased mitochondrial numbers and density, and raised myoglobin
6 content of the muscle.²⁴ ACE I-allele compared to D-allele is associated with an
7 increased percentage of fatigue-resistant type I skeletal muscle fibres²⁵ and hence
8 with enhanced mechanical⁶ and metabolic²⁶ efficiency of the trained muscle. The
9 effect of ACE genotype on energy balance, might be mediated through several
10 different pathways, such as systemic endocrine or nutritional mechanisms.²⁶ Local
11 RASs exist in several tissues. The adipose RAS may alter mobilization of substrate
12 from fat stores and affect metabolism.²⁶ Finally, the local skeletal muscle RAS may
13 modify the use of substrate and enhance the metabolic efficiency of the muscles.²⁶
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30 Several factors may affect respiratory muscle endurance such as nutrition,²⁷
31 prolonged ventilatory support,²⁸ drugs,^{29,30,31} as well as phosphate,³² calcium³³ and
32 magnesium³⁴ blood levels. All infants were measured prior to discharge, being on full
33 enteral feeds, they did not receive any medication and their biochemistry blood tests
34 were within the normal range during measurements.
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45 PTImus integrates all components that may affect inspiratory muscles while
46 breathing at rest, i.e, timing of breathing cycle, inspiratory demand at rest and
47 maximal inspiratory force reserve. When we considered these components, the
48 timing of the breath cycle (T_i/T_{tot}) and the maximal inspiratory force reserve ($P_{i_{max}}$)
49 were not statistically different between infants with I/I, D/D and I/D ACE genotype.
50 However, the $P_{i_{mean}}/P_{i_{max}}$ ratio and the $P_{i_{mean}}$ (inspiratory demand at rest), were
51 lower in infants with the I/I compared with infants with D/D and I/D genotypes. A
52 higher inspiratory demand at rest ($P_{i_{mean}}$) in infants with D/D and I/D, compared with
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3 infants with I/I ACE genotypes, could suggest that a higher elastic and/or resistive
4 load (existing lung disease) imposed on the respiratory muscles of infants with I/D
5 and D/D genotypes compared with infants with I/I ACE genotypes, may exist.
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8 Indeed, infants with non-I/I ACE genotypes have been suggested to be more likely to
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10 develop bronchopulmonary dysplasia, perhaps as a consequence of the role of ACE
11 activity in the inflammatory cascade.¹⁷ However, in this study, no difference in
12 regards to bronchopulmonary dysplasia rate between infants with I/I, I/D and D/D
13 ACE genotypes was noted. In addition, the same association between ACE
14 genotypes and Pi_{mean} was replicated in a subgroup of infants that never required any
15 form of respiratory support and in infants that never required any oxygen
16 supplementation.
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32 Fatigue threshold of inspiratory muscles has not yet been established in infants.
33 All infants measured, were spontaneously breathing on air. Therefore, the PTImus
34 results of all infants would be expected to lie far from the fatigue threshold. These
35 results, however, indicate an increased effectiveness of inspiratory muscles of
36 infants with the I/I ACE genotype, due to a lower energy demand and thus to a lower
37 risk of respiratory muscle fatigue, under conditions that increase inspiratory load.^{35,36}
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39 The findings of this study suggest that in clinical practice, infants with I/I ACE
40 genotype, if supported by mechanical ventilation, may succeed extubation more
41 often than infants with I/D and D/D ACE genotypes or may have a lower risk of
42 respiratory failure under conditions that increase inspiratory load, such as respiratory
43 infections.
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In conclusion, these results suggest that an association of ACE genotypes with

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3 PTImus measurements may exist in infants, independent of other factors, which
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5 could affect respiratory muscle function.
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10 11 12 **ACKNOWLEDGMENTS**

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19 20 **COMPETING INTERESTS**

21
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23
24 to a conflict of interest.
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32 None
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36 37 **ETHICS COMMITTEE APPROVAL**

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39 The study was approved by the Ethics Committee of the University Hospital of
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41 Patras, Patras, Greece
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Figure legend

Comparison of PTImus results of infants with I/I (n=18), I/D (n=73) and D/D (n=41) ACE genotypes. Box and whisker plot: fine horizontal lines represent 10th, 25th, 50th, 75th and 90th centiles of PTImus. Outliers are plotted as discrete data points.

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Table 1. Characteristics of the study population. Data are demonstrated as n (%) or median (range).

Number of studied infants	132	
Gestational age (weeks)	35.5	(27-40)
Birth weight (g)	2680	(900-4540)
Gender (male)	76	(57.6)
Preterm	80	(60.6)
Duration of mechanical ventilation (days)	0	(0-59)
Diagnoses on admission		
Respiratory distress syndrome (RDS)	45	(34.1)
Transient tachypnea of the newborn (TTN)	23	(17.4)
Meconium aspiration syndrome	5	(3.8)
Infection	17	(12.9)
Congenital pneumonia	6	(4.5)
Prematurity (low birth weight)	26	(19.7)
Birth depression	7	(5.3)
Intrauterine growth retardation	3	(2.3)
<u>At the time of measurement</u>		
Postnatal age (days)	9	(1-107)
Postmenstrual age (PMA) (weeks)	37.1	(32.4–46.7)
Preterm (PMA<37 weeks)	61	(46.2)
Weight (g)	2550	(1850-4610)

Table 2. Characteristics and PTImus results of infants with I/I, I/D and D/D ACE genotypes. Data are demonstrated as n (%) or median (range in parentheses).

ACE genotypes (n)	II (18)	ID (73)	DD (41)	p value
Gestational age				
(weeks)	36 (27-40)	35 (28-40)	36(29-40)	0.648 ^a
Birth weight (g)				
	2780 (960-4150)	2700 (900-4080)	2580 (1070-4540)	0.598 ^a
Male gender	8 (44.4%)	42 (57.5%)	26 (63.4%)	0.118 ^b
Duration of mechanical ventilation (days)				
	0 (0-59)	0 (0-21)	1 (0-8)	0.857 ^a
<u>At the time of measurement</u>				
Postnatal age (days)	8.5 (1-107)	9 (1-62)	9 (1-56)	0.692 ^a
Weight (kg)				
	2660 (1850-4050)	2560 (1850-4140)	2450 (1880-4610)	0.471 ^a
PTImus				
	0.046 (0.023-0.085)	0.077 (0.040-0.170)	0.071 (0.036-0.119)	0.000007 ^a
Ti/Tot	0.39 (0.27-0.58)	0.43(0.31-0.55)	0.45(0.35-0.51)	0.081 ^a
Pi_{mean}/Pi_{max}				
	0.118 (0.052-0.171)	0.182 (0.096-0.373)	0.160 (0.086-0.322)	0.000005 ^a
Pi_{mean} (cmH₂O)				
	7.77 (3.71-13.48)	10.18 (5.86-19.21)	9.98 (4.48-17.64)	0.0009 ^a
Pi_{max} (cmH₂O)				
	66.9 (46.8-88.7)	58.6 (32.3-97.4)	62.8 (28.0-91.8)	0.271 ^a

^a Kruskal-Wallis test ^b Cramer's V test

Table 3. Results of stepwise regression analysis

	Partial Correlation		
	Coefficient	t Value	Significance
Dependent variable: PTImus			
ACE gene polymorphism	0.245	2.870	0.005
Birth weight	-0.176	-2.035	0.044
Gestational age	0.011	0.125	0.901
Postnatal age	0.020	0.222	0.825
Gender	-0.033	-0.376	0.708
Duration of mechanical ventilation	-0.061	-0.694	0.489

Table 4. Characteristics and PTImus results of infants with I/I, I/D and D/D ACE genotypes that never required respiratory support. Data are demonstrated as n (%) or median (range in parentheses).

ACE genotypes (n)	II (10)	ID (44)	DD (20)	p value
Gestational age				
(weeks)	36(32-39)	35.5(30-40)	36.5(30-40)	0.946 ^a
Birth weight (g)				
	2780 (1350-3650)	2725 (1130-4080)	2740 (1070-4540)	0.982 ^a
Male gender				
	5 (50%)	26 (59.1%)	12 (60%)	0.065 ^b
<u>At the time of measurement</u>				
Postnatal age (days)				
	4(1-26)	6(1-39)	8.5(1-41)	0.146 ^a
Weight (kg)				
	2675 (1950-3500)	2620 (1890-4140)	2685 (1880-4610)	0.889 ^a
PTImus				
	0.043 (0.028-0.085)	0.076 (0.040-0.117)	0.074 (0.052-0.119)	0.0022 ^a
Ti/Tot				
	0.39 (0.27-0.51)	0.43 (0.34-0.55)	0.46 (0.35-0.51)	0.058 ^a
Pi_{mean}/Pi_{max}				
	0.118 (0.092-0.171)	0.177 (0.096-0.336)	0.163 (0.116-0.306)	0.0015 ^a
Pi_{mean} (cmH₂O)				
	7.77 (4.68-10.13)	9.70 (6.20-19.21)	10.25 (7.39-16.07)	0.003 ^a
Pi_{max} (cmH₂O)				
	61.4 (46.8-88.7)	58.6 (36.9-97.4)	63.3 (32.7-91.8)	0.637 ^a

^a Kruskal-Wallis test ^b Cramer's V test

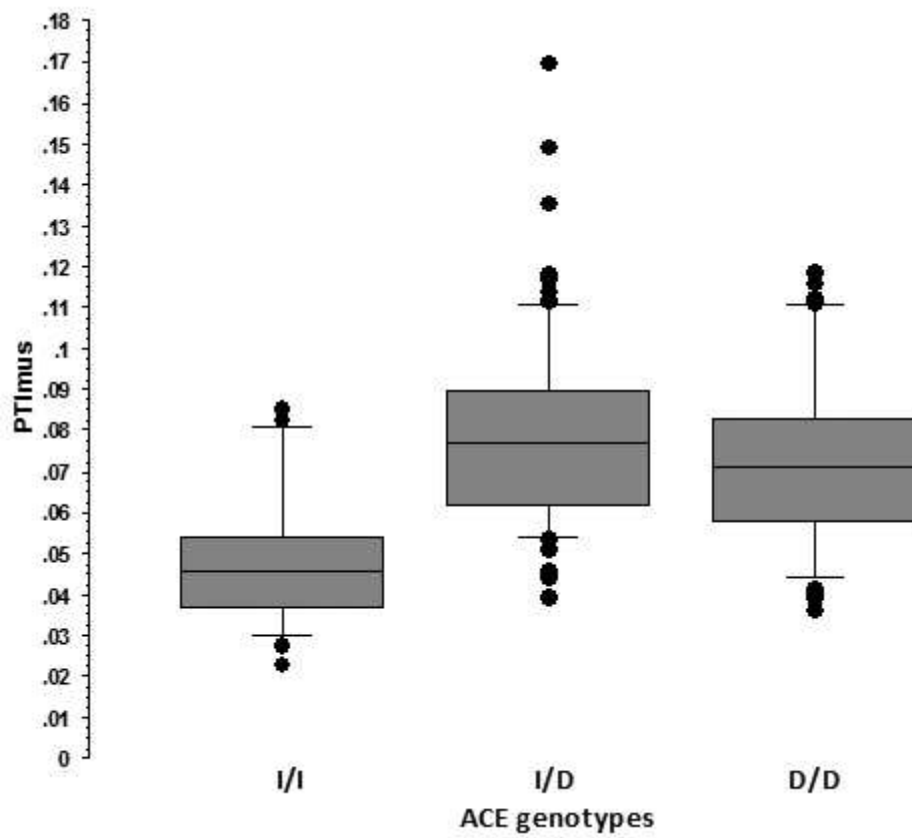


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Title:

Angiotensin converting enzyme gene polymorphism and respiratory muscle function in infants

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Running Head

ACE gene and respiratory muscle function

Word count

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ABSTRACT

Objective: Angiotensin converting enzyme (ACE) gene contains a polymorphism consisting of either the presence (I) or absence (D) of a 287 base pair fragment. Recent studies have suggested that the I-allele may be associated with superior exercise endurance; respiratory muscle function may be similarly influenced. The pressure-time index of inspiratory muscles (PTImus) is a measure of the load-capacity ratio of the inspiratory muscles. The objective of this study was to determine whether infants homozygous for the I-allele have lower PTImus compared to infants homozygous for the D-allele or heterozygous I/D.

Patients and Methods: One hundred thirty two infants were studied. ACE genotyping was performed by polymerase chain reaction amplification, using DNA from peripheral blood. PTImus was calculated as $(P_{i_{mean}}/P_{i_{max}}) \times (T_i/T_{tot})$, where $P_{i_{mean}}$ was the mean inspiratory pressure estimated from airway pressure, generated 100 milliseconds after an occlusion ($P_{0.1}$), $P_{i_{max}}$ was the maximum inspiratory pressure and T_i/T_{tot} was the ratio of the inspiratory time to the total respiratory cycle time. $P_{i_{max}}$ was the largest pressure generated during brief airway occlusions performed at the end of a spontaneous crying effort.

Results: Infants with I/I genotype had significantly lower PTImus than infants with either D/D or I/D genotypes ($p=0.000007$). ACE genotype was significantly related ($p=0.005$) to PTImus measurements, independent of other factors that may affect respiratory muscle function.

Conclusion: These results suggest that an association of ACE genotypes with PTImus measurements may exist in infants.

INTRODUCTION

Angiotensin I-converting enzyme (ACE) converts angiotensin I to vasoactive and aldosterone-stimulating peptide angiotensin II and it is an important component of the local renin-angiotensin systems (RASs).^{1,2} There is a significant variation in circulating ACE activity among individuals, which may be genetically determined. The human ACE gene is located on chromosome 17 (17q23) and contains a two allele polymorphism, consisting of either the presence (insertion, I) or absence (deletion, D) of a 287 base pair (bp) fragment.³ The deletion is associated with increased ACE activity in both tissue and circulation and accounts for about 44% of the intra-individual variation in plasma ACE activity in Caucasians.³ Recent studies have suggested that I-allele may be associated with superior exercise endurance,^{4,5} partly through improved skeletal muscle metabolic efficiency.⁶ Respiratory muscle function may be similarly influenced.

Several methods have been used to assess the endurance properties of respiratory muscles. Diaphragmatic pressure-time index (PTIdi) is a measure of the load-capacity ratio of the diaphragm.⁷ In adults, a PTIdi greater than 0.15-0.18 may indicate impending diaphragm fatigue.⁷ Determination of PTIdi, however, is invasive, requiring the use of an esophageal catheter. A non-invasive method of measuring pressure-time index of inspiratory muscles (PTImus) has been validated in both adults⁸ and infants.⁹

The aim of this study was to test the hypothesis that infants homozygous for the I-allele may have lower PTImus compared to infants homozygous for the D-allele or heterozygous I/D.

METHODS

Patients

Infants cared for at the Neonatal Intensive Care Unit- Pediatric Department of the University General Hospital of Patras, Greece, were eligible for the study. Infants were entered into the study if parents gave informed written consent. The study was approved by the local Research Ethics Committee. Part of the studied population was recruited from a study examining the association of ACE genotypes and circulating blood ACE on diaphragmatic function in infants. All infants were studied awake, before discharge, in supine position and at least one hour after a feed. Infants had no respiratory symptoms for at least 3 days before measurement. Furthermore, infants were on full oral feeds, had serum electrolytes, calcium, magnesium and phosphates within normal range and did not receive any methylxanthines.

ACE genotype determination

ACE genotyping was performed on DNA extracted from whole blood, collected from an indwelling catheter or via peripheral venipuncture during routine blood sampling. The method has been previously described.¹⁰ Briefly, DNA was extracted from 200 μ l blood using Qiam spin columns (Blood mini kit- Qiagen, QIAGEN Inc., Germantown, U.S.A). DNA was analyzed by electrophoresis on an agarose gel. DNA amplification of the 16th ACE intron was performed using two sets of primers flanking the polymorphic site, (outer and inner primers), because mistyping of the D/D genotype is reported to occur using the conventional amplification with insertion/deletion (I/D) flanking primer.¹¹

Measurement of PTImus

Airflow was measured using a pneumotachograph (Mercury F10L, GM Instruments, Kilwinning, Scotland) connected to a differential pressure transducer (DP45, range ± 2 cm H₂O, Validyne Corp, Northridge, CA, USA). Airway pressure (Paw) was measured from a side port on the pneumotachograph using a differential pressure transducer (DP45, range ± 100 cm H₂O, Validyne Corp, Northridge, CA, USA). The signals from the pressure transducers were amplified using a carrier amplifier (Validyne CD 280, Validyne Corp, Northridge, CA, USA). The pressure and flow signals were recorded and displayed in real time on a computer (Dell Optiplex GX620, Dell Inc., Texas, U.S.A) running LabviewTM software (National Instruments, Austin, Texas, U.S.A) with analog-to-digital sampling at 100 Hz (16-bit NI PCI-6036E, National Instruments, Austin, Texas, U.S.A).

P_{0.1} was calculated as the airway pressure generated 100 milliseconds after an occlusion, using a face mask and a unidirectional valve which allowed expiration but not inspiration, while the infant was quietly breathing. At least four airway occlusions were performed and the average P_{0.1} was calculated. Pressure-time index of the inspiratory muscles (PTImus), was calculated as: $PTImus = (P_{i_{mean}}/P_{i_{max}}) \times (T_i/T_{tot})$ where P_{i_{mean}} was the average airway pressure during inspiration, obtained from the formula $P_{i_{mean}} = 5 \times P_{0.1} \times T_i$.¹² P_{i_{max}} was the maximum inspiratory airway pressure, T_i was the inspiration time and T_{tot} was the total time for each breath calculated from the airway flow signal. To measure P_{i_{max}}, a facemask (total deadspace, 4.5 mL) was held firmly over the infant's nose and mouth and the airway was occluded at the end of a spontaneous crying effort using a unidirectional valve attached to the pneumotachograph, which allowed expiration, but not inspiration. The occlusion was

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3 maintained for at least four inspiratory efforts. At least three airway occlusions were
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5 performed and the maximum $P_{i_{max}}$ achieved for individual, was recorded.
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8 **Statistical analysis**

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10 Data was tested for normality using the Shapiro-Wilk and D'Agostino skewness
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12 tests. Differences between ACE genotype groups were assessed for statistical
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14 significance using the Kruskal-Wallis and Dunn's post-hoc non-parametric tests and
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16 Cramer's V test, as appropriate. Stepwise multiple regression analysis was
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18 performed to determine whether the polymorphism of the ACE gene is related to
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20 PTImus measurements, independently to gestational (GA) and postnatal (PNA) age,
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22 birthweight (BW), gender and support from mechanical ventilation.
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27 Statistical analysis was performed using StatView 5.0 (SAS Institute, Inc., NC,
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29 USA), NCSS 2007 (NCSS, Utah, USA) and SPSS version 17 (SPSS Inc, Chicago,
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36 **RESULTS**

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41 Between January 2007 and August 2008 from the 532 infants admitted in the
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43 Neonatal Intensive Care Unit of the University of Patras, one hundred thirty two
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45 infants were included in the study. Fifty eight infants required ventilation in the initial
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47 stage of their illness. The characteristics of the study population are presented in
48
49 table 1.
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53 Eighteen infants (13.6%) were homozygous for the I-allele (I/I), 41 (31.1%)
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55 homozygous for the D-allele (D/D) and 73 infants (55.3%) were heterozygous I/D. A
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57 Hardy-Weinberg equilibrium (HWE) calculation was performed to examine whether
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3 genotype frequencies remain constant in the studied population. Participants were in
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5 HWE (Chi square for HWE 2.613, $p=0.106$).
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8 Overall, there were no significant differences in the characteristics of the infants
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10 with I/I, D/D and I/D ACE genotypes (table 2). Only four infants were oxygen
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12 dependent at 36 weeks postmenstrual age (1 infant with I/I, 2 with I/D and 1 with D/D
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14 ACE genotypes). Infants with I/I genotype had lower PTImus than infants with either
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16 D/D or I/D genotypes (Kruskal-Wallis, $p=0.000007$; Dunn's test, z -value= 3.58,
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18 $p<0.01$ and z -value= 4.88, $p<0.01$, respectively) (table 2), (figure). No difference in
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20 regards to PTImus values was found between infants with D/D and I/D genotypes
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22 (Dunn's test, z -value=1.39, n.s). Analysis of the components of the PTImus has
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24 shown that only the ratio of Pi_{mean} to Pi_{max} was lower in infants with the I/I genotype,
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26 compared to infants with either the D/D or I/D genotypes (Kruskal-Wallis, $p=$
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28 0.000005; Dunn's test, z -value= 3.33, $p<0.01$ and z -value= 4.91, $p<0.01$,
29
30 respectively). Lower values of Pi_{mean} in infants with I/I genotype compared to infants
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32 with either D/D or I/D genotypes, were also observed (Kruskal-Wallis, $p= 0.0009$;
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34 Dunn's test, z -value= 2.61, $p<0.05$ and z -value= 3.73, $p<0.01$, respectively) (table 2).
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36 Pi_{max} measurements were not statistically different between the three groups (table
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38 2). Stepwise regression analysis revealed that ACE genotypes were significantly
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40 related to the PTImus measurements after logarithmic transformation, independent
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42 of GA, BW, PNA, gender and support from mechanical ventilation ($p=0.005$) (table
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53 In the subgroup of infants that never required any form of ventilatory support, 10
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55 infants (13.5%) were homozygous I/I, 20 (27.0%) homozygous D/D and 44 infants
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57 (59.5%) were heterozygous I/D. Infants in this subgroup were in HWE (Chi square
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59 for HWE 3.304, $p=0.069$). No significant difference in the characteristics of the
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3 infants with I/I, D/D and I/D ACE genotypes, was found (table 4). Infants with I/I
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5 genotype had lower PTImus than infants with either D/D or I/D genotypes (Kruskal-
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7 Wallis, $p= 0.0022$; Dunn's test, $z\text{-value}= 3.07$, $p<0.01$ and $z\text{-value}= 3.39$, $p<0.01$,
8
9 respectively), (table 4). No difference regarding PTImus values was found between
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11 infants with D/D and I/D genotypes (Dunn's test, $z\text{-value}= 0.0008$, n.s). The ratio of
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13 Pi_{mean} to Pi_{max} was lower in infants with the I/I genotype, compared to infants with
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15 either the D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0015$; Dunn's test, $z\text{-value}=$
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17 2.95 , $p<0.01$ and $z\text{-value}= 3.58$, $p<0.01$, respectively). Pi_{max} measurements were not
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19 statistically different between the three groups (table 4), but Pi_{mean} was lower in
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21 infants with I/I genotype, compared to infants with either D/D or I/D genotypes
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23 (Kruskal-Wallis, $p= 0.003$; Dunn's test, $z\text{-value}= 3.31$, $p<0.01$ and $z\text{-value}= 3.00$,
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25 $p<0.01$, respectively). In this subgroup, forty six infants never required any oxygen
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27 supplementation; 6 infants (13.0%) were homozygous I/I, 13 (28.3%) homozygous
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29 D/D and 27 infants (58.7%) were heterozygous I/D. Infants in the subgroup that
30
31 never required any oxygen supplementation were in HWE (Chi square for HWE
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33 1.872 , $p=0.171$). The median (range) of PTImus of the whole subgroup was 0.078
34
35 (0.028-0.119). Infants with I/I ACE genotype had lower PTImus than infants with
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37 either D/D or I/D genotypes [median (range), 0.040 (0.028-0.083) versus 0.073
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39 (0.052-119) and 0.080 (0.040-117), respectively; Kruskal-Wallis, $p= 0.0075$; Dunn's
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41 test, $z\text{-value}= 2.55$, $p<0.05$ and $z\text{-value}= 3.11$, $p<0.01$, respectively]. The ratio of
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43 Pi_{mean} to Pi_{max} and Pi_{mean} were lower in infants with the I/I ACE genotype, compared
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45 to infants with either D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0047$; Dunn's test, $z\text{-}$
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47 $value= 2.42$, $p<0.05$ and $z\text{-value}= 3.27$, $p<0.01$, respectively and Kruskal-Wallis, $p=$
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49 0.019 ; Dunn's test, $z\text{-value}= 2.46$, $p<0.05$ and $z\text{-value}= 2.74$, $p<0.05$, respectively).
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6 In the subgroup of the infants who required ventilation in the initial stage of their
7 illness, 8 infants (13.8%) were homozygous I/I, 21 (36.2%) homozygous D/D and 29
8 infants (50%) were heterozygous I/D. Participants were in HWE (Chi square for HWE
9 0.1622, $p=0.687$). Infants with I/I ACE genotype had lower PTImus than infants with
10 either D/D or I/D genotypes (Kruskal-Wallis, $p= 0.0024$; Dunn's test, $z\text{-value}= 1.98$,
11 $p<0.05$ and $z\text{-value}= 3.37$, $p<0.01$, respectively).
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22 DISCUSSION

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27 In this study, an association between ACE genotypes and PTImus measurements
28 in infants, was demonstrated. Infants homozygous for the I-allele had lower PTImus
29 values than infants homozygous for the D-allele and heterozygous I/D. No difference
30 in regards to PTImus values was found between infants with D/D and I/D genotypes.
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32 Furthermore, the ACE polymorphism was related to the PTImus measurements,
33 independent of other factors, which could affect respiratory muscle performance. The
34 association between ACE genotypes and PTImus measurements was replicated on
35 the subgroup of infants that required ventilation in the initial stage of their illness and
36 on the subgroup of infants that never required any form of ventilatory support.
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48 One limitation of our study was the fact that measurements in an age-matched
49 control group, consisting of infants in the postnatal ward, were not performed. The
50 recruitment of such babies is extremely difficult. To overcome this issue, we
51 measured infants in the neonatal unit that are free of factors that could possibly
52 interfere with respiratory muscle function such as requirement of respiratory support.
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3 One part of the whole population consisted of infants that never required oxygen
4 supplementation, indeed, and a separate analysis was performed in this subgroup.
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10 Fatigue of respiratory muscles may result in an inability to maintain adequate
11 alveolar ventilation and respiratory failure. A simple method of studying the
12 endurance properties of inspiratory muscles, is the measurement of the duration that
13 a specific submaximal task (maximum sustainable ventilation, external mechanical
14 load) can be maintained (time limit or endurance time, T_{Lim}), referred to as the point
15 where the inspiratory muscles failed to maintain the task, despite maximal effort.⁷
16
17 However, this kind of test is difficult to be applied to infants. Diaphragmatic pressure-
18 time index (PTIdi), the product of the ratio of mean inspiratory transdiaphragmatic
19 pressure per breath ($P_{di_{mean}}$) to maximum transdiaphragmatic pressure ($P_{di_{max}}$) and
20 the ratio of the inspiratory time (T_i) to the total respiratory cycle time (T_{tot}), is closely
21 related to the T_{Lim}.¹³ The determination of PTIdi, however, is rather invasive, since
22 it requires the placement of an esophageal catheter. Analysis of inspiratory muscle
23 function by measurement of PTImus was first described by Gaultier et al.¹² As
24 measurement of P_{O_1} is non-invasive, measurement of PTImus has the advantage of
25 being relatively easy to be performed. This index was validated in adults.⁸ Studies in
26 healthy children, demonstrated that PTImus ranged between 0.036 and 0.090.^{14,15} In
27 spontaneously breathing infants, PTImus was compared to PTIdi and an agreement
28 between the two methods, using Bland and Altman analysis, was found.⁹ In this
29 study, PTImus measurements in infants that never required any oxygen
30 supplementation ranged between 0.028 and 0.119, something that is close to the
31 values observed in healthy children.
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Several studies have suggested that there is an association of the ACE I/D polymorphism with exercise performance. British Army recruits were examined before and after an identical 10-week general physical training program.⁵ Although, pre-training performance was independent of genotype, the improvement in performance was 11-fold greater among those of I/I, compared with D/D genotype.⁵ Consistently, a significant excess of the I/I genotype was found in Australian national rowers attending their pre-Olympics selection trial, compared to matched healthy controls.¹⁶ The frequency of I-allele was higher in British Olympic-standard distance runners.⁴ Furthermore, genotype distribution and I-allele frequency differed significantly between elite mountaineers, who had ascended beyond 7000 m without the use of supplemental oxygen and controls.⁵ All such studies are prone to difficulties in comparison, as the definition of the “elite athlete” and the heterogeneity of the elite athlete phenotype may be variable. The polymorphism of the ACE gene has been examined in infants, but not in association with muscle endurance.^{17,18}

The association of the ACE genotype and respiratory muscle function, has not been widely examined yet. In a study of COPD (Chronic Obstructive Pulmonary Disease) patients and healthy control subjects, the ACE genotype was related to the regulation of skeletal muscle aerobic work efficiency, but it was not associated with the ventilatory responses to exercise.¹⁹ The relationship between circulating ACE activity, ACE I/D genotype, mechanical efficiency and the maximal rate of oxygen uptake has been assessed in untrained women.²⁰ Neither circulating ACE activity nor ACE genotype showed a statistically significant association with any of the endurance phenotypes measured. However, ACE inhibition in heart failure increases respiratory muscle strength²¹ and exercise tolerance.^{22,23}

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3 The enhanced endurance performance in the ACE I/I genotype may be due to
4 higher oxygen delivery, conversion of fast-twitch, type II, to slow-contracting, type I,
5 muscle fibers, increased mitochondrial numbers and density, and raised myoglobin
6 content of the muscle.²⁴ ACE I-allele compared to D-allele is associated with an
7 increased percentage of fatigue-resistant type I skeletal muscle fibres²⁵ and hence
8 with enhanced mechanical⁶ and metabolic²⁶ efficiency of the trained muscle. The
9 effect of ACE genotype on energy balance, might be mediated through several
10 different pathways, such as systemic endocrine or nutritional mechanisms.²⁶ Local
11 RASs exist in several tissues. The adipose RAS may alter mobilization of substrate
12 from fat stores and affect metabolism.²⁶ Finally, the local skeletal muscle RAS may
13 modify the use of substrate and enhance the metabolic efficiency of the muscles.²⁶
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30 Several factors may affect respiratory muscle endurance such as nutrition,²⁷
31 prolonged ventilatory support,²⁸ drugs,^{29,30,31} as well as phosphate,³² calcium³³ and
32 magnesium³⁴ blood levels. All infants were measured prior to discharge, being on full
33 enteral feeds, they did not receive any medication and their biochemistry blood tests
34 were within the normal range during measurements.
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45 PTImus integrates all components that may affect inspiratory muscles while
46 breathing at rest, i.e, timing of breathing cycle, inspiratory demand at rest and
47 maximal inspiratory force reserve. When we considered these components, the
48 timing of the breath cycle (T_i/T_{tot}) and the maximal inspiratory force reserve ($P_{i_{max}}$)
49 were not statistically different between infants with I/I, D/D and I/D ACE genotype.
50 However, the $P_{i_{mean}}/P_{i_{max}}$ ratio and the $P_{i_{mean}}$ (inspiratory demand at rest), were
51 lower in infants with the I/I compared with infants with D/D and I/D genotypes. A
52 higher inspiratory demand at rest ($P_{i_{mean}}$) in infants with D/D and I/D, compared with
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3 infants with I/I ACE genotypes, could suggest that a higher elastic and/or resistive
4 load (existing lung disease) imposed on the respiratory muscles of infants with I/D
5 and D/D genotypes compared with infants with I/I ACE genotypes, may exist.
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10 Indeed, infants with non-I/I ACE genotypes have been suggested to be more likely to
11 develop bronchopulmonary dysplasia, perhaps as a consequence of the role of ACE
12 activity in the inflammatory cascade.¹⁷ However, in this study, no difference in
13 regards to bronchopulmonary dysplasia rate between infants with I/I, I/D and D/D
14 ACE genotypes was noted. In addition, the same association between ACE
15 genotypes and Pi_{mean} was replicated in a subgroup of infants that never required any
16 form of respiratory support and in infants that never required any oxygen
17 supplementation.
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32 Fatigue threshold of inspiratory muscles has not yet been established in infants.
33 All infants measured, were spontaneously breathing on air. Therefore, the PTImus
34 results of all infants would be expected to lie far from the fatigue threshold. These
35 results, however, indicate an increased effectiveness of inspiratory muscles of
36 infants with the I/I ACE genotype, due to a lower energy demand and thus to a lower
37 risk of respiratory muscle fatigue, under conditions that increase inspiratory load.^{35,36}
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39 The findings of this study suggest that in clinical practice, infants with I/I ACE
40 genotype, if supported by mechanical ventilation, may succeed extubation more
41 often than infants with I/D and D/D ACE genotypes or may have a lower risk of
42 respiratory failure under conditions that increase inspiratory load, such as respiratory
43 infections.
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In conclusion, these results suggest that an association of ACE genotypes with

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3 PTImus measurements may exist in infants, independent of other factors, which
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5 could affect respiratory muscle function.
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10 11 12 **ACKNOWLEDGMENTS**

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19 20 **COMPETING INTERESTS**

21
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23
24 to a conflict of interest.
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31
32 None
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36 37 **ETHICS COMMITTEE APPROVAL**

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39 The study was approved by the Ethics Committee of the University Hospital of
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41 Patras, Patras, Greece
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Figure legend

Comparison of PTImus results of infants with I/I (n=18), I/D (n=73) and D/D (n=41) ACE genotypes. Box and whisker plot: fine horizontal lines represent 10th, 25th, 50th, 75th and 90th centiles of PTImus. Outliers are plotted as discrete data points.

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