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Ricardo Jorge Rodrigues Ferreira

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UNIVERSIDADE DO PORTO

INSTITUTO CIÊNCIAS BIOMÉDICAS ABEL-SALAZAR

MESTRADO INTEGRADO EM MEDICINA

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Angiotensin-converting enzymes in adolescents and their association with blood pressure, obesity and gender: a cross-sectional study

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RESUMO

INTRODUÇÃO:
A hipertensão arterial é o principal fator de risco para mortalidade a nível mundial, afetando cerca de 5% de todas as crianças e adolescentes. O sistema renina-angiotensina-aldosterona tem um papel fundamental na regulação da pressão arterial, com as enzimas conversoras da angiotensina ECA e ECA2 a terem um papel essencial neste processo, que, no entanto, ainda não foi claramente demonstrado na adolescência. O objetivo deste estudo foi o de, numa amostra de adolescentes, estudar a atividade da ECA, ECA2 e a sua relação com o índice de massa corporal, com o sexo e com a pressão arterial.

MÉTODOS:
Foi realizada uma análise transversal de 172 adolescentes de 14-16 anos, incluídos na coorte Geração XXI (Portugal). A colheita de dados incluiu medidas antropométricas e pressão arterial ambulatória de 24 horas. A atividade das enzimas ECA e ECA2 foi determinada por via de ensaios fluorométricos. A atividade da ECA foi avaliada usando Hippuryl-His-Leu (h-HL) and Z-Phe-His-Leu (Z-FHL) como substratos. A atividade da ECA2 foi avaliada usando Mca-APK(Dnp), na presença e ausência de DX600 (inibidor da ECA2).

RESULTADOS:
Níveis mais elevados de atividade da ECA h-HL foram detetados em adolescentes com peso normal [mediana (P25-P75): (7.8 (4.4-15.0) vs 3.9 (2.6-14.0) vs 3.1 (1.8-5.4), nmol/ml/min, p for trend = 0.022; respetivamente em peso normal, excesso de peso e obesos]. Uma tendência linear crescente ao longo de classes de IMC foi observada para o rácio da atividade da ECA Z-FHL sobre a ECA h-HL, com um deteção de um rácio mais elevado nos obesos [3.2 (2.2-6.8) vs 4.5 (2.1-14.0) vs 7.5 (3.9-8.4), nmol/ml/min, p for trend = 0.026. respetivamente em peso normal, excesso de peso e obesos]. Nos rapazes, uma tendência linear decrescente verificou-se para a atividade da ECA h-HL [9.5 (4.8 – 17.9) vs 3.7 (3.0 – 7.7) vs 3.5 (2.1 – 5.9) nmol/ml/min, p for trend=0.020; respetivamente em peso normal, excesso de peso e obesos] e para o rácio da atividade da ECA2 sobre a ECA Z-FHL [0.6 (0.2 – 1.8) vs 1.5 (0.4 – 3.4) vs 1.0 (0.4 – 5.2), p for trend=0.022; respetivamente em peso normal, excesso de peso e obesos]. Em rapazes obesos, valores progressivamente mais elevados de z-score de pressão arterial diastólica foram observados ao longo de tercis de atividade da ECA2 (p = 0.003) e também ao longo de tercis de atividade do rácio da ECA2 sobre a ECA Z-FHL (p = 0.047). Em raparigas, valores progressivamente mais baixos de z-score da pressão arterial sistólica foram observados ao longo de tercis de atividade da ECA2 (p = 0.042).

CONCLUSÕES:
Na adolescência, as atividades da ECA e ECA2 são influenciadas pelo índice de massa corporal e pelo género. São necessários mais estudos para elucidar o papel das hormonas sexuais e da adiposidade no controlo da pressão arterial efetuado pelo sistema renina-angiotensina-aldosterona.

PALAVRAS-CHAVE: ECA, Enzima conversora de angiotensina; ECA2, Enzima conversora de angiotensina 2; SRAA, Sistema renina-angiotensina-aldosterona; Hipertensão arterial; Obesidade; Género; Adolescentes

ABSTRACT

BACKGROUND:
Hypertension is the number one risk factor for mortality worldwide, affecting around 5% of children and adolescents. The renin-angiotensin-aldosterone system is a key regulator of blood pressure (BP), with angiotensin-converting enzyme (ACE) and ACE2 having a fundamental role that, however, has not been clearly recognised in adolescence. We aimed to, in a sample of adolescents, study the activities of ACE and ACE2 across body mass index (BMI) classes and between sexes, and to evaluate the relationship between these enzymes' activity and BP.

METHODS:
We performed a cross-sectional study of 172 adolescents aged 14-16 years old, included in the Generation XXI cohort (Portugal). Data collection included anthropometric measurements and 24-hour ambulatory BP monitoring. ACE and ACE2 activities were determined through fluorometric assays. ACE activity was assessed using Hippuryl-His-Leu (h-HL) and Z-Phe-His-Leu (Z-FHL) as substrates. ACE2 activity was assessed using Mca-APK(Dnp) as a substrate, in the presence and absence of DX600 (ACE2 inhibitor).

RESULTS:
Higher activity of ACE h-HL was found in the normal weight adolescents [median (P25-P75): 7.8 (4.4-15.0) *vs* 3.9 (2.6-14.0) *vs* 3.1 (1.8-5.4), nmol/ml/min, p for trend = 0.022; in normal weight, overweight and obese groups, respectively]. A significant linear trend across BMI classes was also found for the ratio of ACE Z-FHL over ACE h-HL activity, with a higher ratio in the obese group [3.2 (2.2-6.8) *vs* 4.5 (2.1-14.0) *vs* 7.5 (3.9-8.4), p for trend = 0.026; in normal weight, overweight and obese groups, respectively]. In boys, a significant linear trend across BMI classes was found for the ACE h-HL activity [9.5 (4.8 – 17.9) *vs* 3.7 (3.0 – 7.7) *vs* 3.5 (2.1 – 5.9), nmol/ml/min, p for trend=0.020; in normal weight, overweight and obese groups, respectively] and for the ratio of ACE2 over ACE Z-FHL activity [0.6 (0.2 – 1.8) *vs* 1.5 (0.4 – 3.4) *vs* 1.0 (0.4 – 5.2), p for trend=0.022, in normal weight, overweight and obese groups, respectively]. Regarding obese boys, increasingly higher daytime diastolic BP z-score values were observed across tertiles of ACE2 activity (p = 0.003) and also across tertiles of the ratio of ACE2 over ACE Z-FHL activity (p = 0.047). In girls, lower systolic BP z-score values were observed across tertiles of ACE2 activity (daytime systolic BP p = 0.019; nighttime systolic BP p = 0.018).

CONCLUSIONS:
In adolescence ACE and ACE2 activities are influenced by BMI and gender. Further research is necessary to elucidate the role of sexual hormones and adiposity in the RAAS regulation of BP.

KEYWORDS: ACE, Angiotensin-converting enzyme; ACE2, Angiotensin-converting enzyme 2; RAAS, Renin-angiotensin-aldosterone system; Arterial hypertension; Obesity; Gender; Adolescents.

ABBREVIATIONS

ABPM, Ambulatory blood pressure monitoring

ACE, Angiotensin-converting-enzyme

ACE2, Angiotensin-converting-enzyme 2

AGT, Angiotensinogen

Ang I, Angiotensin I

Ang II, Angiotensin II

Ang-[1-5] Angiotensin-(1-5)

Ang-[1-7] Angiotensin-(1-7)

BMI, Body mass index

BP, Blood pressure

DBP, Diastolic blood pressure

h-HL, Hippuryl-His-Leu

MAP, Mean arterial pressure

RAAS, Renin-angiotensin-aldosterone system

SBP, Systolic blood pressure

SD, Standard deviation

WHO, World Health Organization

Z-FHL, Z-Phe-His-Leu

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INTRODUCTION

Hypertension is a major risk factor for various diseases, including cardiovascular, renal and neurocognitive diseases[1] and has been classified as the number one risk factor for mortality globally[2]. It is estimated to affect 5% of children and adolescents in the world, with recent global data showing a worldwide prevalence increase in this age group[3,4,5]. Moreover, there is evidence that hypertension in childhood, and mainly during adolescence, tracks into adulthood, increasing the cardiovascular risk of these individuals, especially in the presence of obesity[1].

The renin-angiotensin-aldosterone system (RAAS) has been known for many decades as a very important physiological regulator of blood pressure (BP). The cascade begins with angiotensinogen (AGT), which is cleaved by renin to form angiotensin I (Ang I). Subsequently occurs the conversion of Ang I to angiotensin II (Ang II) through the action of angiotensin-converting-enzyme (ACE). In parallel, angiotensin-converting-enzyme 2 (ACE2) mainly promotes the conversion of Ang II to angiotensin-(1-7) (Ang-[1-7]). Ang II is the main effector of the RAAS and exerts its effects through two receptors – AT1R and AT2R. The AT1R mediates the so-called classic effects, such as vasoconstriction, inflammation and fibrosis, as well as sodium and water retention through the release of aldosterone from the adrenal cortex. The AT2R mediates opposite effects, as does the Mas receptor when triggered by Ang-[1-7]; together, these two pathways represent a counter-regulatory arm opposing the Ang II detrimental cardiovascular outcomes[6,7]. This way, the balance between ACE and ACE2 activities is crucial for the balance between Ang II and Ang-[1-7] levels, which is essential for the overall effect of the RAAS. Both ACE and ACE2 are attached to the cell membrane but also exist as shedded soluble forms[8, 9, 10]. ACE shows two catalytic domains: The C-domain mainly metabolizes Ang I into Ang II; the N-domain mainly hydrolysis Ang-[1-7] into angiotensin-(1-5) (Ang-[1-5]), but also hydrolyses other bioactive peptides [11,12,13]. ACE2 shows a single catalytic domain [14,15].

In adults, primary hypertension is associated with overweight and obesity, namely with dysfunctional adipose tissue[16,17]. Adipose tissue is the second main endogenous source of AGT, with a positive and independent correlation existing between body weight and adipose tissue expression of AGT[16]. Also, the adipose tissue has all the necessary components for the production of Ang II, including AGT, renin and ACE. Plus, cell culture research has demonstrated that human adipocytes are able to produce Ang II[18]. *In vivo* experiments have demonstrated that mice with diet-induced obesity have higher levels of adipose tissue AGT that correlate with plasma AGT, Ang II and BP[19]. Interestingly, when feeding the same high-fat diet to mice knocked-out for adipocyte AGT and to wild-type mice, only the wild-type animals showed increases in

both plasma Ang II and BP[20]. This is in agreement with human studies where higher levels of renin, plasma AGT, ACE activity and aldosterone were observed in obese women compared to women with body mass index (BMI) within the normal range[21].

The high prevalence of obesity and overweight among children and adolescents is disturbing. Some studies have hypothesized on the effect that excess weight might have on the RAAS, and consequently on BP control and on the risk of hypertension[22] at young ages. In a previous study of our research group, we reported that BMI seems to play a key role in influencing the activities of ACE and ACE2 in prepubertal children, with significant differences existing between genders, in what concerns to RAAS regulation of BP[23]. Also, pediatric dyslipidemia was associated with increased urinary ACE activity, although no relation between urinary ACE activity and BP was established[24]. The link between the activities of ACE and ACE2 and BP regulation in adolescence remains to be explored.

In the present study, we aimed to compare the activities of ACE and ACE2 within different BMI classes in a sample of adolescents and to describe the relationship between the activity of these enzymes and ambulatory BP, taking into account the individuals' gender and BMI.

METHODS

Study design and sample collection

Adolescents aged 14-16 years old that have been followed since birth in a formerly established cohort research study (Generation XXI, Porto-Portugal)[25] were included in the present analysis. Of the original cohort (n=8647), 4590 children had an in-person follow-up visit at 7 years of age, therefore making them suitable for the ObiKid project, which aims to elucidate the effect of obesity in childhood and related comorbidities on kidney health. A subsample of 313 participants were assessed by the ObiKid project at 8-9 years of age[23]. For more details on screening and selection of individuals to the original ObiKid project previous publications can be searched[26]. In this work, our aim was to reevaluate all the ObiKid participants, formerly evaluated at 8-9 years of age. Tanner staging evaluation was performed for all participants, with all included being in a pubertal stage. Among the participants who were contacted to be involved in the ObiKid 2.0 reevaluation, 18 declined to participate due to personal restraints, 41 contacts could not be established, 77 were unable to plan visits during the recruitment period (more frequently due to the COVID-19 pandemic). Four ObiKid participants were excluded from the present reevaluation for chronic usage of medication (affecting either BP, lipidic metabolism or glucose). One additional participant was excluded for not having completed the evaluation (missing ABPM measurement). A total of 172 were finally included in the present study.

Data collection and variable definition

Study visits occurred at the Public Health and Forensic Sciences and Medical Education Department, Faculdade de Medicina da Universidade do Porto, Porto, Portugal. Anthropometric and general physical evaluations were completed following standard procedures and as formerly stated[25]. BMI was determined and BMI for-age values were classified according to the World Health Organization (WHO) growth reference data for BMI z-score into the following categories: normal weight (≤+1 standard deviation (SD), including only one child with thinness); overweight (>1 SD and ≤+2 SD), and obesity (>2 SD)[27]. As so, in our sample, 99 (57.6%) of adolescents were normal weight, 45 (26.2%) were overweight, and 28 (16.3%) were obese, according to the WHO reference. Classes of sex-specific adequate birthweight for gestational age were defined according to the 2013 revised Fenton growth reference curves[28].

Office BP assessment was performed with oscillometric validated sphygmomanometers (Elite 92125; Medel, Parma, Italy) comprising an adequately sized cuff in the right arm, by a nonphysician trained interviewer, three times with a 5-min interval, and the second and third evaluations were averaged for further analysis. Ambulatory BP monitoring (ABPM) was

measured for 24 hours with a portable non-invasive oscillometric BP recorder (Mobil-O-Graph ®, I.E.M. GmbH, Germany), in the non-dominant arm and with a cuff size adequately chosen in accordance to the circumference of the adolescent´s arm. BP evaluations were automatically set at intervals of 20-minute throughout daytime (from 08.00 AM to 08:00 PM) and at intervals of 30 minutes during the nighttime (from 00:00 PM to 06:00). A minimum monitoring period of 24 hours with gaps shorter than 2 hours was necessary in order to be accepted. The readings were used to determine mean 24 hours, day and night mean arterial pressure, systolic BP (SBP) and diastolic BP (DBP). SD scores for BP values were determined (least mean square method) according to the published reference values of the German Working Group on Pediatric Hypertension[29]. Sustained hypertension was defined according to the 2016 European Society of Hypertension guidelines[5]. For adolescents aged below 16 years old, sustained hypertension was defined as an average SBP and/or DBP at or above the 95th percentile for sex, age and height, either during the day, night or in the 24h on ABPM, according to reference values[5]. For adolescents aged 16 or older, sustained hypertension was defined as values above the adult cutoff levels for ABPM (daytime ≥ 135/85mmHg; nighttime ≥ 125/75mmHg; 24h: ≥ 130/80mmHg). It was considered as absence of dipping when a fall in the MAP during nighttime was <10% of the corresponding daytime MAP. Family history of hypertension was considered when at least one parent or sibling was reported to be affected.

Laboratory procedures

A venous blood sample was collected from all participants after an overnight fast of at least 8 hours and examined for ACE and ACE2 activities by fluorimetric assays according to previous studies[30,31] at the Department of Biomedicine – Unit of Pharmacology and Therapeutics of the Faculty of Medicine of Porto, Portugal. For the ACE activity assay we used Hippuryl-His-Leu (h-HL) and Z-Phe-His-Leu (Z-FHL) as substrates; h-HL is more rapidly hydrolyzed by the C-domain than by the N-domain[12]. For the ACE2 activity assay we used Mca-APK(Dnp), as the substrate and the assay was performed in the presence of 10 µM captopril to inhibit ACE and in the presence or absence of the selective ACE2 inhibitor DX600. Besides each enzymatic activities' ratios were calculated to infer on the contribution between the two catalytic domains of ACE (ACE Z-FHL/h-HL activity ratio) and of ACE2 in relation to ACE (ACE2/ACE Z-FHL activity ratio). Concerning the ACE Z-FHL/ACE h-HL activity ratio, it has been suggested that it is approximately 1 when ACE Z-FHL and ACE h-HL are equally active in the sample; is close to or lower that 0.7 if the ACE h-HL is more active than the ACE Z-FHL; and is close to or higher than 4.5 if the ACE Z-FHL is more active than the ACE h-HL[12].

Creatinne and sodium were assessed in a 24-h urine sample collected from all individuals, in the Clinical Pathology Department of Centro Hospitalar Universitário São João, Porto, Portugal. The 24-h urine samples were validated if urinary creatinine was within the range of 11.3–28.0 mg/kg per day (according to age- and sex specific reference values[32]) and if urinary volume was >300 mL. The parents and the adolescents were informed on the adequate methods for collecting the 24-h urine sample, and upon sample delivery compliance was checked once again with a short inquiry form. Based on these criteria, all urine samples of the enrolled adolescents were considered suitable for analysis. An ion-selective electrode method was used to analyze sodium in urine, and creatinine was evaluated by a standard automated clinical chemistry analyzer (Olympus AU 5400 automated analyzer; Beckman–Coulter, Brea, California, USA). The estimated salt intake was projected from 24-h urinary sodium excretion as 1 mEq of sodium being equal to 0.0588 g of salt.

Ethics

All phases of the study complied with the Ethical Principles for Medical Research Involving Human Subjects expressed in the Declaration of Helsinki. The baseline and follow-up evaluations were approved by the University of Porto Medical School/ S. João Hospital Centre Ethics Committee, except the 13-year follow-up that was approved by the ISPUP Ethics Committee. At baseline and follow-up evaluations, all procedures were explained to participants, and an informed consent was signed by one of the parents or legal guardians (at 13 years, it was also signed by the participants). The baseline evaluation was approved by the Data Protection National Commission and the study follows the EU General Data Protection Regulation under close supervision of the Data Protection Office of ISPUP.

Statistical analysis

Statistical analysis was accomplished using IBM SPSS Statistics 29.0. Data are presented as mean and SD or as the median and 25th and 75th percentiles (P25– P75), as appropriate. Differences between groups in continuous variables were calculated with Student's t tests or Mann–Whitney tests, as adequate. Relationships between categorical variables were evaluated using chi-square tests. The distribution of ACE activity (evaluated by Z-FHL and h-HL), ACE Z-FHL / ACE h-HL activity ratio, ACE2 activity, and ACE2 / ACE Z-FHL activity ratio by BMI classes and the distribution of ambulatory BP z-score values by tertiles of ACE and ACE2 activities and ratios, separately in girls and boys, and in accordance to BMI classes, are presented in boxplot graphs. The p values for linear trend across the groups characterized in the boxplots were calculated by linear regression, adjusted for age (in years). Multivariate linear regression models were

employed in order to perform a quantification of the effect of ACE activity (evaluated by Z-FHL and h-HL), ACE Z-FHL/ACE h-HL activity ratio, ACE2 activity, ACE2/ACE Z-FHL activity ratio, on various ABPM z-score variables, taking into account the influence of age (years), family history of hypertension (at least one parent or sibling reported to be affected), class of birthweight for gestational age, estimated salt intake (g/24 h) and additionally for BMI z-score when appropriately signed, in the whole sample and in adolescents with normal weight and overweight or obesity, in girls and boys separately. The p values were considered statistically significant if <0.05.

RESULTS

A total of 172 adolescents (55.8% boys) were included in the present analysis, with a median (P25-P75) age of 14 (14-14) years. Demographic and anthropometric characteristics and BP data are presented in Table I, for the study population and separately for adolescents with normal weight (n=99), overweight (n=45) and obesity (n=28). Adolescents in the overweight and obese groups presented higher median (P25-P75) age than those in the normal weight group [14 (14-16) vs 14 (14-16) vs 14 (14-14); $p < 0.001$)]. Regarding office BP z-score values, both values for SBP and DBP [median (P25-P75)] increased across BMI classes [SBP: -0.09 (-0.61 – 0.49) vs 0.15 (-0.40 – 0.85) vs 0.85 (0.24 – 1.59), $p < 0.001$; DBP: 0.72 (0.17 – 1.02) vs 0.89 (0.23 – 1.36) vs 1.16 (0.63 – 1.64), $p = 0.006$; in normal weight, overweight and obese groups, respectively]. Concerning ambulatory BP values, no statistically significant differences were observed for daytime or nighttime SBP and DBP z-score values across BMI classes. The median estimated salt intake was higher in overweight and obese groups than in the normal weight group [8.00 (5.29 – 10.94) vs 10.18 (7.59 – 11.65) vs 9.41 (7.59 – 14.00), $p = 0.049$, in normal weight, overweight and obese groups, respectively].

Within our population of adolescents, the following medians (P25-P75) were obtained: 22.19 nmol/mL/min (14.25-40.34) for ACE Z-FHL activity; 5.81 nmol/mL/min (3.04-13.05) for ACE h-HL activity; 4.11 (2.41-7.44) for the ACE Z-FHL/ACE h-HL activity ratio; 25.81 nmol/mL/min (6.70-59.78) for ACE2 activity; 1.00 (0.28-3.11) for ACE2/ACE Z-FHL activity ratio. The distribution of ACE and ACE2 activities and of ACE Z-FHL/ACE h-HL and ACE2/ACE Z-FHL activity ratios according to BMI classes is depicted in Figure 1. When comparing BMI classes, the higher levels of activity for ACE h-HL were found amongst the normal weight adolescents, with a significant linear trend across BMI groups [7.8 nmol/mL/min (4.4-15.0) vs 3.9 nmol/mL/min (2.6-14.0) vs 3.1 nmol/mL/min (1.8-5.4), p for trend = 0.022, in normal weight, overweight and obese groups, respectively], which reflected in a significant linear trend across BMI classes for the ACE Z-FHL/ACE h-HL activity ratio, with a higher ratio in the obese group [3.2 (2.2-6.8) *vs* 4.5 (2.1-14.0) *vs* 7.5 (3.9-8.4), p for trend = 0.026, in normal weight, overweight and obese groups, respectively]. As for ACE Z-FHL and ACE2 activities and for ACE2/ACE Z-FHL activity ratio, no statistically significant differences were found. When each sex was considered separately (Figure 2), a significant linear trend across BMI classes was found in boys for the ACE h-HL activity [9.5 nmol/mL/min (4.8 – 17.9) vs 3.7 nmol/mL/min (3.0 – 7.7) vs 3.5 nmol/mL/min (2.1 – 5.9), p for trend = 0.020, in normal weight, overweight and obese groups, respectively] and for the ACE2/ACE Z-FHL activity ratio [0.6 (0.2 – 1.8) vs 1.5 (0.4 – 3.4) vs 1.0 (0.4 – 5.2), p for trend=0.022,

in normal weight, overweight and obese, respectively]. No statistically significant differences were found neither in boys for the other parameters nor in any of them in girls.

When analysing the distribution of ABPM z-score values by tertiles of ACE activity, according to BMI classes, in either sex, no statistically significant association was found either for ACE Z-FHL activity or ACE h-HL activity (data not shown). But, in overweight boys, higher daytime SBP z-score values were observed across tertiles of ACE Z-FHL/ACE h-HL activity ratio (ß-coefficient (CI 95%): 0.387 (0.003 to 0.770), p = 0.048). The distribution of ABPM z-score values by tertiles of ACE2 activity and of ACE2/ACE Z-FHL activity ratio, according to BMI classes and separately in girls and boys is presented in Figures 3 – 4 respectively. In normal weight girls, lower daytime SBP z-score values were observed across tertiles of ACE2 activity (ß-coefficient (CI 95%): 0.308 (-0.604 to -0.012), p = 0.042). In obese boys, increasingly higher daytime DBP z-score values were observed across tertiles of ACE2 activity (ß-coefficient (CI 95%): 0.810 (0.361 to 1.259), p = 0.003) and also across tertiles of ACE2/ACE Z-FHL activity ratio (ß-coefficient (CI 95%): 0.687 (0.012 to 1.363), p = 0.047).

When using multivariate linear regression analysis, daytime SBP z-score decreased by 0.28 [(95% CI -0.51 to -0.05), p = 0.019] and nighttime SBP z-score decreased by 0.36 [(CI 95%): -0.36 (-0.66 to -0.06), p = 0.018] per tertile of ACE2 activity in girls. An effect was also observed after adjustment for BMI z-score, but no longer after stratification by BMI groups. In obese boys, daytime DBP z-score increased (ß-coefficient (CI 95%): 0.82 (0.34 to 1.31), p = 0.006). No other statistically significant effects were found in either sex for ACE2 activity or for the ratio of ACE2 over ACE Z-FHL (Tables II-III), nor for ACE activity (ACE Z-FHL or ACE h-HL) for the ratio of ACE Z-FHL over ACE h-HL activity or for the ratio of ACE2 over ACE h-HL activity (data not shown).

DISCUSSION

Our study is the first to show a predominance of ACE N-domain activity over ACE C-domain activity in obese adolescents and a higher ACE2 activity over that of ACE in overweight/obese boys. Also, our multivariate linear regression analysis shows that in obese boys, tertiles of ACE2 activity are positively associated with daytime DBP z-score values, while in girls, tertiles of ACE2 are negatively associated with both daytime and nighttime SBP z-score values. In girls, ACE2 activity was negatively associated with daytime and nighttime SBP z-scores even after adjusting for BMI.

An association between obesity and activation of the RAAS has classically been described[33]. The adipose tissue is recognised to produce AGT and Ang II[16]. Plus, it has been reported that ACE2 mRNA levels in visceral fat, but not subcutaneous fat, correlate with BMI[34]. Moreover, a study in lung epithelial cells revealed higher ACE2 expression in cells from obese individuals[35]. Besides, obesity is also associated with insulin resistance, kidney damage and is itself a chronic inflammatory state. All these factors contribute to a higher degree of activation of the RAAS in obesity. We, for the first time, report that in the group of obese adolescents included in our study, there is a higher ratio of ACE Z-FHL over ACE h-HL activity, thus reflecting a higher activity of ACE N-domain over its C-domain. Indeed, the ACE Z-FHL / ACE h-HL activity ratio was higher than 1 in the all BMI classes, but it was 4.5 and 7.5 in the overweight and the obese groups, respectively, which suggests that the ACE N-domain is more active than the ACE C-domain in obese adolescents[12]. Moreover, particularly in boys with overweight/obesity, ACE2 is more active than ACE, since we found a higher ACE2 / ACE Z-FHL activity ratio in this group (being < 1.0 in normal weight boys and > 1.0 in overweight / obese). Together, these results suggest that in adolescent boys, obesity triggers ACE2 activity eventually to counteract the deleterious effects of Ang II. However, since the conversion of Ang-[1-7] into Ang-[1-5] by the ACE N-domain seems to overcome the conversion of Ang I into Ang II by the ACE C-domain, the putative protective ACE2 activation might be blunted. Also, high ACE N-domain activity will cleave the anti-inflammatory, anti-fibrotic peptide AcSDKP, which might also account for the blunted protection mechanism[36]. Increased ACE2 levels have been detected in hypertensive, obese and type 2 diabetic patients[37,38], which might be associated with higher enzyme activity, and thus be a biomarker of (response to) disease[39], as might be the case in our sample of overweight/obese boys. Differently, in adolescent girls, we observed no association with BMI neither concerning ACE nor ACE2 activities, which suggests that these enzymes are not a part of the pathophysiology of obesity in the female sex.

As the RAAS is mainly associated with BP regulation, and ACE and ACE2 are crucial for the RAAS balance, we also looked for the association of ACE and ACE2 with BP, according to BMI and, due to the gender differences found, separately in boys and girls. Our data in boys are in line with the analysis of the impact of BMI on ACE and ACE2 activities since in obese boys, daytime DBP z-score values increased across tertiles of ACE2 activity. This was quite unexpected since higher ACE2 activity should increase the levels of Ang-[1-7], which is a known vasodilator, thus would be expected to decrease BP. As so, one might speculate that in adolescent boys, obesity prompts a non-effective protective mechanism that expands to a positive deleterious effect on DBP regulation. Differently, we observed that in girls, SBP z-score values decrease across tertiles of ACE2 activity, with no effect across tertiles of ACE activity. As referred, this might be explained by increased conversion of Ang II into Ang-[1-7], which through its vasodilatory effects would lower BP. Interestingly, a study by Harp JB et al showed that weight loss can promote a decrease in ACE activity that is, however, not accompanied by changes in BP [40]. A similar effect was verified in our results for ACE C-domain activity, as even though this enzyme's activity decreased across BMI classes, this did not translate into changes in BP values. Similarly, obese adolescents presented with a higher relevance of ACE N-domain over ACE C-domain but this too did not translate into alterations in BP. As our results reveal that increased ACE2 activity levels have contrasting outcomes in obese boys opposite to all girls, it is reasonable to speculate that RAAS enzymes others than ACE and ACE2 or other regulatory systems, play a role. Most probably endocrine hormone systems, as those related to the BMI and hormonal effects of oestrogen and testosterone.

It is well-known that BP values increase during growth, specifically during puberty, and this effect is more noticeable in males than in females [41]. The pubertal decrease in insulin sensitivity, the effects of growth hormone and insulin-like growth factor 1, but also the impact of the changing levels of reproductive hormones are some of the factors believed to be involved in these changes [42]. Androgens are described to have a dual effect on BP but several studies tend to associate higher testosterone levels with higher SBP values, and not the contrary, with a similar effect on BP being described in children with hyperandrogenism [42, 43]. While in adolescent girls estradiol can promote the synthesis of hepatic AGT, the BP-lowering effect of estradiol prevails, thus providing cardiovascular protection through various mechanisms, including increased nitric oxide synthesis resulting in vasodilation, promotion of angiogenesis, antioxidative activity, improvement of lipid profile and cardiac remodelling[44,45]. This is particularly noticeable in individuals with estrogen deficiency, whereas patients with polycystic ovary syndrome and Turner's syndrome display higher BP values [46, 47]. As for progesterone, this hormone is also reported to contribute to lower BP levels [40].

The findings of the present study contrast with our previous study in prepubertal children (8-9 years old)[23], where ACE and ACE2 activities increased with BMI and the associations with BP were positive, restricted to tertiles of ACE activity (not ACE2 activity) and only observed in girls. Now, in adolescent girls, we observe a negative association between tertiles of ACE2 and BP, which might reflect the protective effect of estrogens on CV disease. In contrast, in prepubertal boys, there was no association between tertiles of ACE or ACE2 activity and BP, but in adolescent boys, obesity seems to negatively affect BP indirectly through an inefficient ACE2-mediated mechanism.

An important strength in our study is the fact that we analysed a large sample of adolescents and had access to a broad set of data regarding cardiovascular and metabolic risk factors, including 24h-ABPM values, which is a major strength since most studies rely solely on office BP values. Besides, it also strengthens the study the fact that activity of both ACE catalytic domains, and ACE2 activity were evaluated, thus allowing us to determine which domain of ACE activity prevails and to ascertain the balance between ACE and ACE2 activities. The sexual dimorphism pointed out by our results was only possible to ascertain by the Tanner staging. Although, hormonal data could have added important support to the interpretation of our results. Also, the cross-sectional design of the present analysis imposes that any causal inference from our results should be cautiously drawn. In the future, we intend to promote a longitudinal analysis of the available data and this cohort is expected to be followed until adulthood, therefore allowing for future longitudinal analysis that might be able to allow us to draw more solid conclusions. Moreover, we should also acknowledge the limitation of solely relying in BMI. Other forms of evaluating total body fat, including bioimpedance and imaging techniques, could have improved our ability of adequately accounting for the effect of visceral fat on these enzymes.

In brief, our study showed that that both ACE and ACE2 are affected by BMI during adolescence, and that during this developmental period, the RAAS control of BP seems to be greatly influenced not only by BMI but also by gender. Further research is necessary to clarify the role of sexual dimorphism and adiposity in the RAAS regulation of BP and we hope to contribute to this scientific field with further cross-sectional and longitudinal studies with this cohort until adulthood.

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TABLES AND FIGURES

TABLE I. Characterization of the study population, according to body mass index classification.

Data presented as median (P25-P75) or as number of individuals (% of total). p values obtained by Kruskall-Wallis test or Chi-square test.

[a] Birthweight for gestational age according to the Fenton curves [28].

[b] The classification of overweight and obesity is according to the World Health Organization classification for BMI z-scores [27].

[c] Non-dipping status was defined as a fall in the MAP during nighttime of <10 % of the corresponding daytime BP.

[d] Sustained hypertension was defined according to the 2016 European Society of Hypertension guidelines[5].

[e] Adolescents were considered to have a family history of hypertension when at least one parent or sibling was reported to be affected.

BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

	All n = 172	**Normal weight n = 99**	**Overweight n = 45**	**Obese n = 28**	**p**
Demographic and anthropometric data					
Age (years)	14.00 (14.00-14.00)	14.00 (14.00-14.00)	14.00 (14.00-16.00)	14.00 (14.00-16.00)	< 0.001
Male sex	96 (55.8%)	56 (56.6%)	28 (62.2%)	12 (42.9%)	0.262
Gestational age (weeks)	39.0 (38-40)	39.0 (38-40)	39.0 (38-40)	39.5 (38-40)	0.312
Birthweight (g)	3300 (3020 – 3498)	3200 (2990 – 3470)	3300 (2995 – 3537)	3404 (3162 – 3597)	0.100
Birthweight for gestational age[a]					0.479
Small (<10th percentile)	11 (6.4%)	9 (9.2%)	2 (4.4%)	0 (0%)	
Adequate (10th–90th percentile)	148 (86.1%)	82 (83.7%)	40 (88.9%)	2 (7.1%)	
Large (≥90th percentile)	12 (7.0%)	7 (7.1%)	3 (6.7%)	26 (92.9%)	
Birth lenght (cm)	49.5 (48.0-50.8)	49.0 (48.0-51.0)	49.5 (48.0-50.3)	50.0 (48.0-50.9)	0.926
Weight (kg)	59.1 (53.0-70.9)	54.0 (49.7-58.6)	67.7 (62.3-73.8)	84.6 (77.2-91.1)	< 0.001
Height (cm)	165.3 (160.5-170.8)	164.9 (160.5-169.6)	163.9 (160.2-170.3)	167.3 (160.9-172.8)	0.529
BMI (kg/m^2)[b]	21.9 (19.6-25.7)	20.0 (18.1-21.3)	24.8 (23.6-25.9)	30.3 (28.7-31.9)	< 0.001
z-score	0.9 (-0.3-1.7)	0.2 (-0.6-0.7)	1.5 (1.3-1.7)	2.4 (2.3-2.6)	< 0.001
Blood pressure data					
Office blood pressure SBP (mmHg)	112.0 (106.0-120.0)	110.0 (104.0-116.0)	114.0 (107.5-122.0)	120.0 (111.8-131.5)	< 0.001
z-score	0.1 (-0.5-0.8)	-0.1 (-0.6-0.5)	0.2 (-0.4-0.9)	0.9 (0.2-1.6)	< 0.001
DPB (mmHg)	74.0 (68.0-78.0)	72.0 (67.0-76.0)	74.0 (68.5-80.0)	78.5 (73.0-84.0)	0.001
z-score	0.8 (0.3-1.2)	0.7 (0.2-1.0)	0.9 (0.2-1.4)	1.2 (0.6-1.6)	0.006
24-h ambulatory blood pressure					
Daytime SBP (mmHg)	114.0 (109.0-118.5)	113.0 (108.3-117.0)	114.0 (110.0-119.0)	117.0 (111.8-122.8)	0.026
z-score	-0.9 [(-1.4)-(-0.3)]	-1.0 [(-1.4)-(0.4)]	-1.0 [(-1.3)-(0.4)]	-0.6 (-0.9-0.1)	0.074
Daytime DBP (mmHg)	67.0 (63.0-71.0)	67.0 (63.00-70.75)	67.0 (64.0-72.0)	68.5 (65.0-72.0)	0.265
z-score	-0.9 [(-1.5)-(-0.3)]	-0.9 [(-1.6)-(-0.3)]	-0.9 [(-1.5)-(-0.2)]	-0.8 [(-1.2)-(-0.1)]	0.486
Nightime SBP (mmHg)	106.0 (101.0-112.0)	105.0 (100.3-111.0)	107.0 (101.0-111.0)	109.0 (103.0-115.3)	0.063
z-score	0.2 (-0.3-0.8)	0.2 (-0.4-0.8)	0.1 (-0.3-0.6)	0.5 (0.0-1.3)	0.091
Nightime DBP (mmHg)	58.0 (55.0-62.5)	57.0 (54.0-62.0)	59.0 (55.0-62.0)	60.5 (57.0-65.0)	0.035
z-score	0.4 (-0.3-1.2)	0.2 (-0.3-1.1)	0.5 (-0.1-1.2)	0.9 (0.1-1.6)	0.055
Nondipping status[c]	80 (48.5%)	41 (42.7%)	25 (58.1%)	14 (53.8%)	0.175
Sustained hypertension[d]	22 (12.8%)	15 (15.2%)	4 (8.9%)	3 (10.7%)	0.544
Family history of hypertension[e]	26 (15,1%)	15 (15.2%)	6 (13.3%)	5 (17.9%)	0.869
Analytical parameters					
Estimated salt intake (g/24h)	9.1 (5.9-11.3)	8.0 (5.3-10.9)	10.2 (7.6-11.7)	9.4 (7.6-14.0)	0.038

TABLE II. Mean change in ambulatory blood pressure levels by tertile of ACE2 activity levels (in nmol/mL/min), in girls and boys separately

The values presented are regression coefficients (β) and 95% confidence intervals of BP z-score data for ACE2 activity levels (nmol/mL/min), estimated by linear regression models, with ABPM values as the dependent variables. Tertiles (T1, ≤ 2.0588; T2, 2.0588–7.6493; T3, >7.6493) were based on data from all enrolled participants. Model 1 adjusted for age (years), family history of hypertension (at least one parent or sibling reported to be affected), class of birthweight for gestational age, estimated salt intake (g/24h) and additionally for BMI z-score when duly signed.
BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure

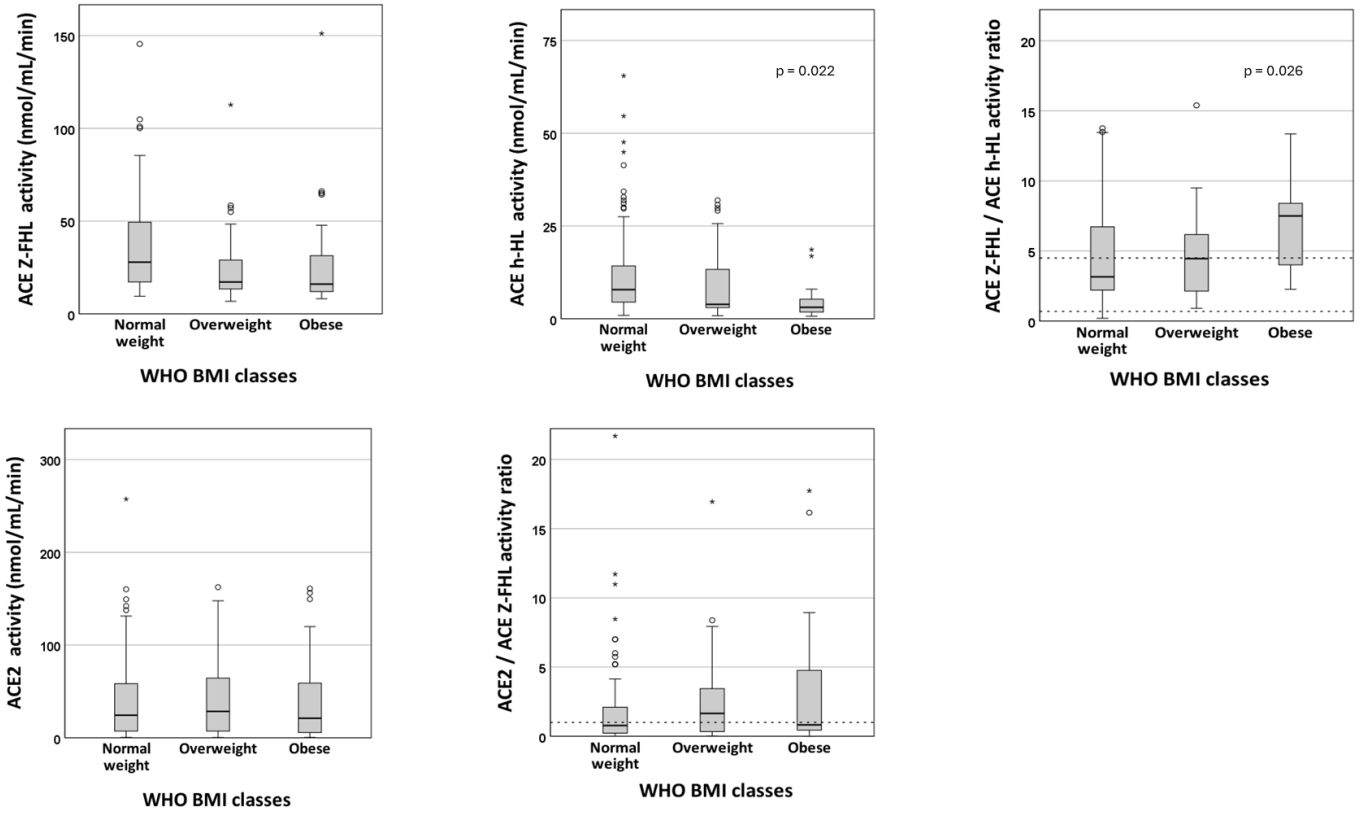
	Daytime SBP				Nighttime SBP			
	Girls		Boys		Girls		Boys	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
All								
Model 1	-0.28 [(-0.51)-(-0.05)]	0.019	0.12 (-0.11-0.33)	0.303	-0.36 [(-0.66)-(-0.06)]	0.018	0.16 (-0.07-0.39)	0.161
Model 1 + BMI z-score	-0.25 [(-0.49)-(-0.02)]	0.037	0.10 (-0.12-0.32)	0.365	-0.35 [(-0.66)-(-0.04)]	0.026	0.15 (-0.08-0.38)	0.190
Normal weight								
Model 1	-0.27 (-0.59-0.05)	0.100	0.11 (-0.23-0.45)	0.512	-0.35 (-0.75-0.06)	0.090	0.24 (-0.08-0.56)	0.133
Overweight								
Model 1	-0.39 (-1.17-0.39)	0.284	0.09 (-0.24-0.43)	0.562	-0.42 (-1.46-0.63)	0.381	0.21 (-0.26-0.67)	0.366
Obese								
Model 1	-0.16 (-0.70-0.39)	0.532	0.26 (-0.53-1.05)	0.447	-0.45 (-1.23-0.33)	0.226	-0.18 (-0.50-0.14)	0.214
	Daytime DBP				**Nighttime DBP**			
	Girls		Boys		Girls		Boys	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
All								
Model 1	-0.16 (-0.40-0.07)	0.166	0.15 (-0.09-0.38)	0.216	-0.11 (-0.41-0.19)	0.466	0.18 (-0.07-0.44)	0.159
Model 1 + BMI z-score	-0.14 (-0.37-0.10)	0.256	0.15 (-0.09-0.38)	0.227	-0.09 (-0.39-0.22)	0.580	0.18 (-0.08-0.44)	0.180
Normal weight								
Model 1	-0.15 (-0.49-0.19)	0.362	0.13 (-0.21-0.47)	0.448	0.06 (-0.34-0.45)	0.777	0.33 (-0.03-0.69)	0.073
Overweight								
Model 1	-0.24 (-1.21-0.72)	0.575	-0.17 (-0.64-0.30)	0.461	-0.23 (-1.46-1.01)	0.686	0.06 (-0.49-0.61)	0.828
Obese								
Model 1	-0.08 (-0.52-0.35)	0.669	0.82 (0.34-1.31)	0.006	-0.50 (-1.24-0.23)	0.153	-0.26 (-0.91-0.39)	0.364

TABLE III. Mean change in ambulatory blood pressure levels by tertile of the ACE2/ACE Z-FHL activity ratio, in girls and boys separately

The values presented are regression coefficients (β) and 95% confidence intervals of BP z-score data for the ratio of ACE2 over ACE N-domain activity levels, estimated by linear regression models, with ABPM values as the dependent variables. Tertiles (T1, ≤ 0.4713; T2, 0.4713–2.3075; T3, >2.3075) were based on data from all enrolled participants. Model 1 adjusted for age (years), family history of hypertension (at least one parent or sibling reported to be affected), class of birthweight for gestational age, estimated salt intake (g/24h) and additionally for BMI z-score when duly signed.
BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure

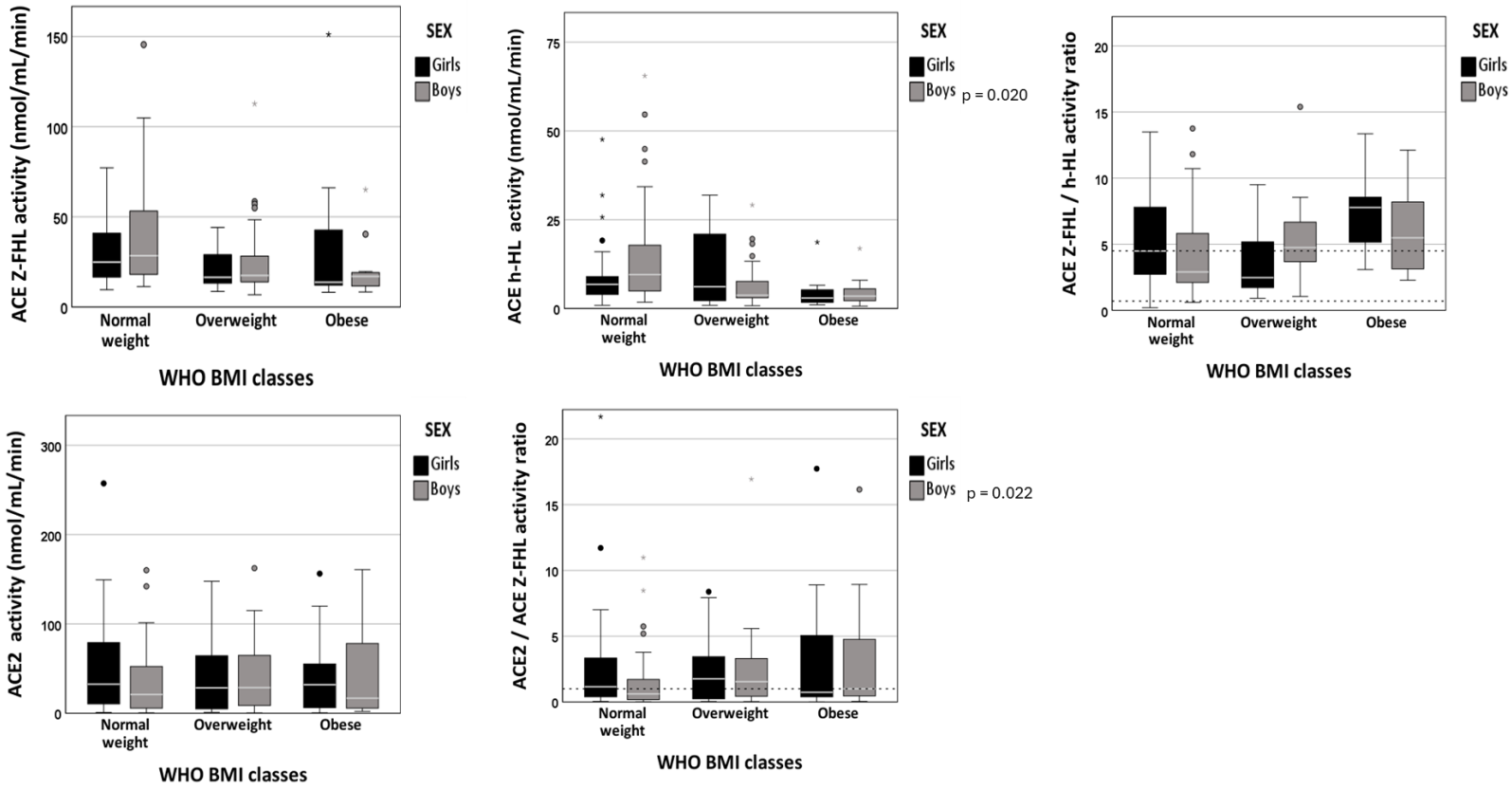
	Daytime SBP				Nighttime SBP			
	Girls		Boys		Girls		Boys	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
All								
Model 1	-0.15 (-0.41-0.10)	0.235	0.11 (-0.13-0.34)	0.373	-0.17 (-0.50-0.16)	0.297	0.14 (-0.10-0.38)	0.256
Model 1 + BMI z-score	-0.14 (-0.39-0.12)	0.289	0.08 (-0.15-0.32)	0.488	-0.16 (-0.50-0.17)	0.331	0.12 (-0.12-0.37)	0.320
Normal weight								
Model 1	-0.06 (-0.40-0.28)	0.731	0.12 (-0.28-0.52)	0.553	-0.13 (-0.55-0.30)	0..555	0.15 (-0.24-0.53)	0.447
Overweight								
Model 1	-0.35 (-1.33-0.64)	0.444	0.05 (-0.28-0.38)	0.752	-0.22 (-1.55-1.11)	0.714	0.16 (-0.29-0.62)	0.463
Obese								
Model 1	-0.17 (-0.83-0.50)	0.578	-0.15 (-1.13-0.82)	0.713	-0.46 (-1.44-0.52)	0.314	-0.11 (-0.53-0.31)	0.531
	Daytime DBP				**Nighttime DBP**			
	Girls		Boys		Girls		Boys	
	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
All								
Model 1	-0.02 (-0.27-0.23)	0.878	0.16 (-0.09-0.41)	0.203	0.04 (-0.28-0.36)	0.802	0.25 (-0.02-0.52)	0.071
Model 1 + BMI z-score	-0.004 (-0.26-0.25)	0.977	0.16 (-0.10-0.41)	0.216	0.05 (-0.27-0.37)	0.737	0.24 (-0.04-0.52)	0.087
Normal weight								
Model 1	0.08 (-0.27-0.43)	0.661	0.16 (-0.25-0.56)	0.433	0.24 (-0.16-0.63)	0.230	0.29 (-0.15-0.72)	0.189
Overweight								
Model 1	-0.30 (-1.48-0.88)	0.573	-0.07 (-0.53-0.39)	0.760	-0.16 (-1.69-1.36)	0.813	0.17 (-0.36-0.70)	0.509
Obese								
Model 1	-0.11 (-0.63-0.42)	0.661	0.56 (-0.42-1.55)	0.211	-0.61 (-1.50-0.29)	0.161	0.00 (-0.83-0.83)	1.000

FIGURE 1 - Distribution of ACE and ACE2 activities and activity ratios according to body mass index classes.



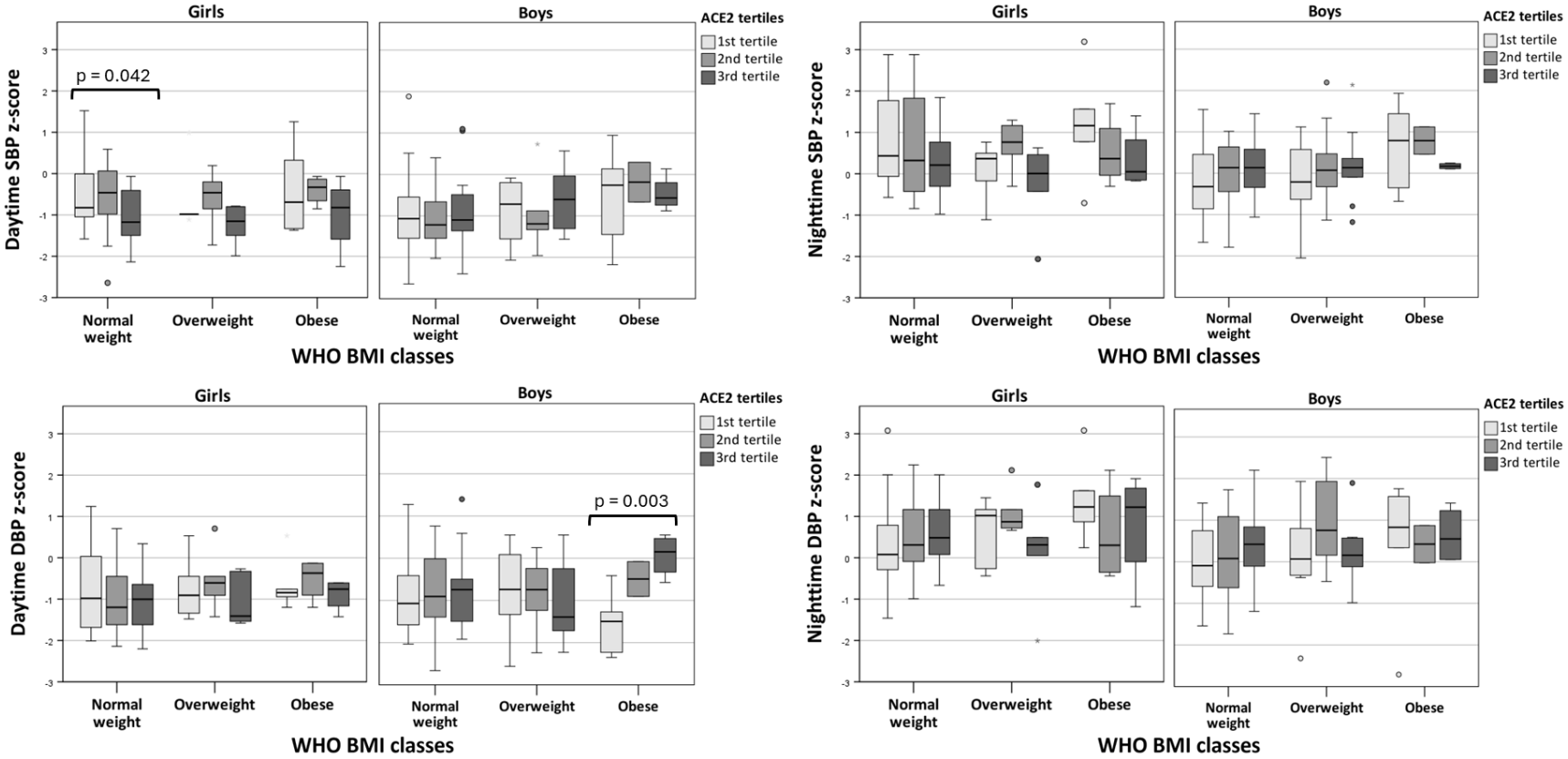
In all graphics a reference Y axis line is drawn at the corresponding median value. In the ACE Z-FHL / ACE h-HL activity ratio graphic, dashed Y axis reference lines are drawn at values 0,7 and 4,5. In the ACE2 / ACE Z-FHL activity ratio graphic, a dashed Y axis reference line is drawn at value 1,0. The classification of overweight and obesity is according to the World Health Organization classification for BMI z-scores [27]. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; WHO, World Health Organization. Only significant p values for linear trend across groups are displayed (calculated by linear regression and adjusted for age).

FIGURE 2 - Distribution of ACE and ACE2 activities and activity ratios according to body mass index classes, separated by sexes.



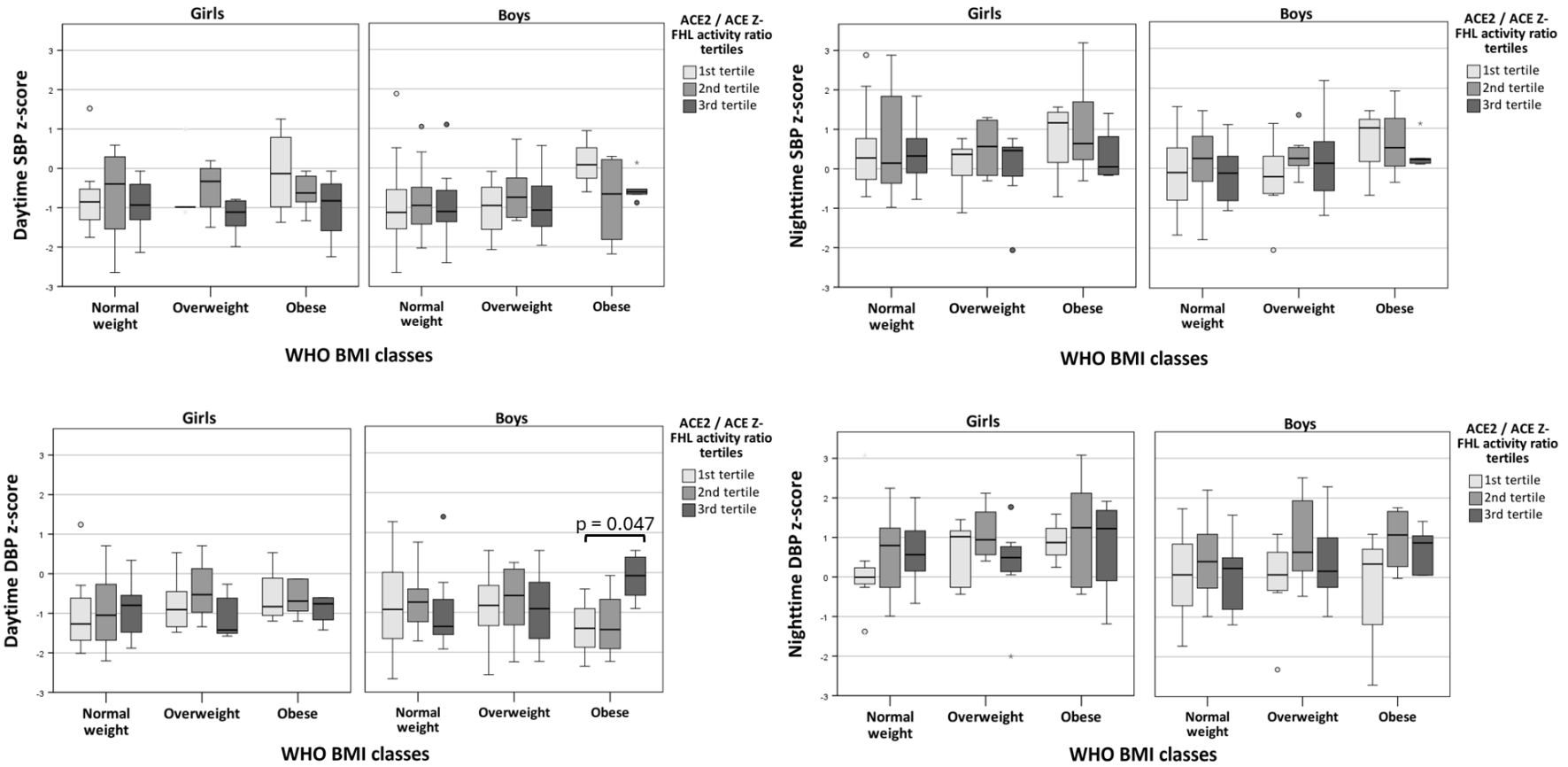
In all graphics a reference Y axis line is drawn at the corresponding median value. In the ACE Z-FHL / ACE h-HL activity ratio graphic, dashed Y axis reference lines are drawn at values 0,7 and 4,5. In the ACE2 / ACE Z-FHL activity ratio graphic, a dashes Y axis reference line is drawn at value 1,0. The classification of overweight and obesity is according to the World Health Organization classification for BMI z-scores [27]. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; WHO, World Health Organization. Only significant p values for linear trend across groups are displayed (calculated by linear regression and adjusted for age).

FIGURE 3 - Distribution of ambulatory blood pressure z-score values by tertiles of ACE2 activity according to body mass index classes, separately in boys and girls



The body mass index groups were defined according to the WHO classification for body mass index z-score values [27]. Tertiles (T1, ≤ 2.0588; T2, 2.0588–7.6493; T3, >7.6493) were based on data from all enrolled participants. The BP z-score values are expressed as medians (horizontal line in box). Only significant p values for linear trend across groups are shown (calculated by linear regression).

FIGURE 4. Distribution of ambulatory blood pressure z-score values by tertiles of ACE2/ACE Z-FHL activity ratio according to body mass index classes, separately in boys and girls.



The body mass index groups were defined according to the WHO classification for body mass index z-score values [27]. Tertiles (T1, ≤ 0.4713; T2, 0.4713–2.3075; T3, >2.3075) were based on data from all enrolled participants. The BP z-score values are expressed as medians (horizontal line in box). Only significant p values for linear trend across groups are shown (calculated by linear regression).

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