Lifestyle patterns and endocrine, metabolic and immunological biomarkers in European adolescents: The HELENA study

César Agostinis-Sobrinho *[1], PhD; Sonia Gómez-Martínez*[2], PhD; Esther Nova[2], PhD; Aurora Hernandez[2], PhD; Idoia Labayen [3], PhD; Antonios Kafatos[4], PhD; Frederic Gottand[5], PhD; Dénes Molnár[6], PhD; Marika Ferrari[7], PhD; Luis A. Moreno[8], PhD; Nathalie Michels[9], PhD; Azahara Ruperez [8] ,PhD; Jonatan R. Ruiz [10], PhD; Ascensión Marcos[2] PhD;
*Both authors contributed equally to this work

Corresponding Author

César Agostinis-Sobrinho.

Faculty of Health Science – Klaipeda University.

Herkaus Manto g. 84, 92294 Klaipėda.

Phone number: + 55 44 91809281.

Email: cesaragostinis@hotmail.com

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1- Faculty of Health Sciences - Klaipeda University – Lithuania

2- Department of Metabolism and Nutrition, Institute of Food Science and Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain

3- Department of Health Sciences, Public University of Navarra, Pamplona, Avda. 18 Barañáisn s/n., 31008, Spain

4- Faculty of Medicine, University of Crete, Crete, Greece.

5- Faculty of Medicine, University Lille, Lille, France.

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6- Department of Pediatrics, Medical School, University of Pécs, Pecs, Hungary

7- Council for Agricultural Research and Economics, Research Center for Food and Nutrition, Rome, Italy.

8 - Growth, Exercise, NUtrition and Development (GENUD) Research Group, University of Zaragoza, Food and Agriculture Institute of Aragón (IA2), Health Research Institute of Aragón (ISS Aragón), Zaragoza, Spain

9 - Department of Public Health, Faculty of Medicine and Health Sciences, Ghent University, De Pintelaan 185, Block K3, 4th Floor, 9000, Ghent, Belgium.

10- PROFITH "PROmoting FITness and Health through physical activity" Research Group, Department of Physical Education and Sport, Faculty of Sport Sciences, University of Granada, Granada, Spain.

Corresponding Author: César Agostinis-Sobrinho. Faculty of Health Science – Klaipeda University. Herkaus Manto g. 84, 92294 Klaipėda. Phone number: + 55 44 91809281. Email: cesaragostinis@hotmail.com

ABSTRACT

OBJECTIVE: To evaluate the association of lifestyle patterns related to physical activity (PA), sedentariness and sleep with endocrine, metabolic and immunological health biomarkers in European adolescents.

METHODS: The present cross-sectional study comprised 3528 adolescents (1845 girls) (12.5 –17.5 years) enrolled in the Healthy Lifestyle in Europe by Nutrition in Adolescence Study. Cluster analysis was performed by including body composition, PA by accelerometry, self-reported sedentary behaviors and sleep duration. We also measured endocrine, metabolic and immunological biomarkers.

RESULTS: Three-cluster solutions were identified: **1-** (light-PA time, moderate-vigorous-PA time and sedentary time); **2- (**light-PA time, moderate-vigorous-PA time, sedentary time and sleep time); **3-** (light-PA time, moderate-vigorous-PA time, sedentary time and body composition). In addition, each cluster solution was defined as: —healthy", —medium healthy" and —unhealthy" according to the presented rating. Analysis of variance showed that overall the healthiest groups from the three clusters analyzed presented a better metabolic profile. A decision tree analysis showed that leptin had a strong association with cluster 3 in both boys and girls, HDL-c had the strongest association with clusters 1 and 3 in boys. Cortisol had the strongest association with cluster 1. HOMA index (homeostatic model assessment) and C3 showed a strong association with cluster 3 in girls.

CONCLUSIONS: Our results support the existence of different interactions between metabolic health and lifestyle patterns related to PA, sedentariness and sleep, with some gender-specific findings. These results highlight the importance to consider multiple

lifestyle related health factors in the assessment of adolescents' health in order to plan favourable strategies.

Key words: Youth, cardiometabolic biomarkers, physical activity, sedentary behavior, sleep time.

INTRODUCTION

Adolescence is characterized linked to key changes in lifestyle habits, as well as metabolic and psychological functioning (1). Physical activity (PA), sedentary behaviors, sleep time, and body composition are critical yet modifiable, adolescent's health-related behaviors (2,3).

There is growing evidence indicating that PA, particularly moderate-to-vigorous PA (MVPA), seems to be independently associated with insulin resistance, blood lipid concentrations, blood pressure, inflammatory proteins, and cardiorespiratory fitness in children and adolescents (3,4). Moreover, it has been suggested that the promotion of PA and the reduction of excessive weight may reduce metabolic risk factors in adolescents (1). On the other hand, increased time spent in sedentary behaviors is associated with increased risk of all-cause and cardiovascular disease mortality (5).

Sleep duration is another critical factor for adolescent's health and health-related behaviors (2). It has previously reported that the sleep time is inversely associated with C-reactive protein levels (6) and a healthier immune profile in adolescents (8). Moreover, adolescents who sleep less than 8 h/day have higher adiposity markers (9).

The presence of metabolic risk factors and obesity during childhood and adolescence seems to track and predict the development of metabolic disorders later in life (9). Adipose tissue is a well-known source of inflammation, considered as a complex and highly active metabolic endocrine organ that can secrete various molecules, such as leptin, C3 and C4 complement factors, and cortisol among others (10). Leptin is an adipokine that has been recognized to have a major role on energy balance and to appear in high serum concentrations in adolescents with obesity (11). Leptin concentrations in adolescents are positively associated with body mass index (BMI), body fat (BF %) and homeostasis model assessment-insulin resistance (HOMA-

IR) (12) and with a high cardiometabolic risk in adolescents (11). In addition, leptin has also been associated with sleep deprivation (13), and PA levels (14). C3 and C4 complement factors are acute-phase proteins, important components of the immune system that are associated with increased levels of cardiovascular disease risk factors with pro-inflammatory effects (15) and have been proposed as a potential biomarkers of CVD risk in adolescents (11) . Cortisol has a main role in regulating immune responses, particularly inflammation (16). In addition, it is considered to be a reliable marker of stress with an influence on adipose tissue and energy balance (17).

Although the individual effect of different lifestyle patterns such as PA, sedentary behaviors, sleep time, and body composition on immunological and inflammatory biomarkers has been already described in the scientific literature, the combined influence, such as in cluster analysis of healthy PA and sleep lifestyle patterns (including anthropometric factor) on endocrine, metabolic, and inflammatory biomarkers is still unknown. Therefore, identify subpopulations with similar patterns thought the cluster analysis to better understand the underlying linking of the combination of several lifestyle patterns' determinants on the metabolic health in adolescents seems pertinent.

The current study was aimed at evaluating the association between lifestyle patterns (including PA, sedentary behavior, sleep time, and anthropometric factor) and endocrine, metabolic and immunological health markers in European adolescents.

METHODS

Study design and sample selection

The Healthy Lifestyle in Europe by Nutrition in Adolescence-Cross-sectional Study (HELENA-CSS) is a multi-center European Union-funded project aiming to obtain reliable data from European adolescents aged 12.5-17.5 years about how dietary habits and patterns, body composition, levels of PA and fitness as well as different biomarkers are related to nutritional status (18,19). This study was conducted on adolescents from 10 European cities: Stockholm (Sweden), Athens and Heraklion (Greece), Rome (Italy), Zaragoza (Spain), Pecs (Hungary), Ghent (Belgium), Lille (France), Dortmund (Germany), and Vienna (Austria) (18). The methodology used in this study has been published elsewhere (19).The total eligible HELENA-CSS population consisted of 3528 adolescents. Blood samples were obtained for a third of the HELENA-CSS participants, resulting in a representative sub-population of 1089 adolescents (approximately 100 boys and girls per city). The size of this population was previously calculated as sufficient to account for the expected variability in blood measurements. For the purposes of the present study, adolescents with valid data for each selected variable were finally included in the analysis. The HELENA study was performed following the ethical guidelines of the Declaration of Helsinki 1964 (as revised in 2000). Written informed consent was obtained from both the adolescents and their parents or guardians.

Assessment of physical activity

The ActiGraph® monitor (ActiGraph® GT1M®, Pensacola, Florida, USA) was used to assess objectively the physical activity in free-living conditions (20). Adolescents were instructed to wear the accelerometer against the lower back for 7 consecutive days using an elastic waistband. The accelerometer was worn during awake time and removed

during water-based activities. The interval of time (epoch) was set at 15 sec in accordance with consensus recommendations for children and adolescents. To be included in the analysis, participants had to wear the accelerometer for a minimum of 8 h/day for at least 3 days a week. Inactive, light, moderate and vigorous PA was defined as 100, 101-1999, 2000-3999 and 4000 counts/min, respectively. The assessment of time spent in moderate-to-vigorous physical activity was based on a cut-off of 2000 counts·min^{-1} (21). The data were analyzed as time spent in physical activity (min·day^{-1}) and as mean counts/day.

Assessment of sedentary behaviors

A self-report sedentary behavior questionnaire (designed *ad hoc*) was administered during the school hours as described elsewhere (22). Adolescents reported the time spent viewing TV, playing with computer games, playing with console games, surfing by internet for reasons other than studying, surfing by internet due to study reasons, and studying (non-school time) for both week and weekend days. The sedentary questionnaire is valid and reliable (23)

Sleep time assessment

Habitual sleep time was estimated through a questionnaire with the following questions: ―Howmany hours (and minutes) do you usually sleep during week days?" and ―How many hours (and minutes) do you usually sleep during weekend days? A total weekly sleep score was calculated as: [(min weekday × 5) + (min weekend day × 2)]/7 (7).

Anthropometry

The anthropometric methods followed in the HELENA-CSS have been already described in detail by Nagy *et al*. (2008) (24). Skinfold thickness were measured to the nearest 0.2 mm in triplicate in the left side at triceps and subscapular using a Holtain

Caliper (Crymmych, UK), and percentage body fat was estimated (Slaughter et al, 1988). Thereafter, fat mass index was calculated as body fat (kg) divided by the height in squared meters (kg/m^2). BMI Z-score and waist circumference Z-score were transformed into age-and sex-specific Z-scores. A clustered anthropometry factor was created from the following variables: BMI Z-score, fat mass index and waist circumference Z-score. To create the anthropometry factor, these variable values were summed, where the lowest values were indicative of a healthier body composition profile. Pubertal status was assessed during a medical examination by a physician/pediatrician according to the methodology described by Tanner and Whitehouse (24).

Blood sampling and measurements of biomarkers

Blood samples were collected by venipuncture at school between 8:00 and 10:00 in the morning after a 10-hour overnight fast. The methodology for collection, preparation and shipping of the blood samples was standardized amongst all participating cities. Cortisol and leptin were measured by fluorescence polarization enzymoassay (AxSYM analyser; Abbott Diagnostics, Illinois, U.S.A.). C3 and C4 complement factors were analyzed by nephelometry (Behring Diagnostics, Spanish National Research Council, CSIC, Madrid). High density lipoprotein cholesterol (HDL-c) was measured using enzymatic methods (Dade Behring, Schwalbach, Germany). Insulin concentrations were measured using an Immulite 200 analyser (DPC Bierman GmbH, Bad Nauheim, Germany). Glucose was measured using enzymatic methods (Dade Behring, Schwalbach, Germany). Homeostasis model assessment (HOMA) calculation was used as a measure of insulin resistance (glucose x insulin/22.5).

Statistical analyses

The analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 24.0 for WINDOWS; SPSS INC, Chicago) and the level of significance was set at $p<0.05$. Study sample characteristics are presented as mean (SD), unless otherwise stated. The normality of distribution for the variables was investigated by the Kolmogorov-Smirnov test. Differences between genders were analyzed by t-test. All subsequent analyses were performed separately for boys and girls.

To identify subpopulations with similar patterns of light physical activity (LPA), MVPA, sedentariness, sleep and anthropometry factor a K-means cluster analysis was used. Because PA variables, sedentariness, sleep and anthropometry factor had different arithmetic scales, Z-scores of all variables were calculated to standardize the data set before clustering. In a first step, several possible cluster solutions were identified and compared to provide information necessary for the following procedure.

Based on the behavior patterns, a three-clusters solution seemed the most informative of the study population as well as resulting in adequately sized emerging groups. All clusters had both PA (LPA and MVPA) and sedentary behaviors as common characteristics essential for growth, development and health in youth.

Cluster 1 (LPA time, MVPA time and sedentary time) resulted in three groups, which were labeled from most to least healthy considering LPA and MVPA as health promoters and sedentary time as negative for health. **Cluster 2** (LPA time, MVPA time, sedentary time and sleep time) resulting groups were labeled from most to least healthy bearing in mind the same definition as in Cluster 1 plus sleep time as a positively or negatively health situation depending on the sleep time. **Cluster 3** (LPA time, MVPA time, sedentary time and anthropometry factor) resulting groups were labeled from most to least healthy, bearing in mind the same definition as in Cluster 1 plus the anthropometry factor as positive or negative for health depending on the calculated

value obtained. In each of the three clusters the healthiest group was defined as that showing the highest rating (group H); the medium rating was defined as group MH and the less healthy group was defined as group UH showing the lowest rating. Means of lifestyle patterns and patterns of the cluster model within all groups tested are summarized in table 1 and table 1S respectively.

Values of the selected variables (cortisol, leptin, C3, C4, HDL-c and HOMA index) were compared between the three groups by General Linear Model, GLM (ANOVA with Bonferroni and Tamhane post-hoc criterion used to assess statistical significance regarding group pairs) and non-parametric analysis (Kruskal-Wallis; with Mann-Whitney post-hoc criterion to assess statistical significance regarding group pairs). Tanner stages were included like fixed effect to the initial simple GLM. Due to the small numbers of individuals found within Tanner stages I and II, these two groups were combined to allow the statistical analysis.

Decision tree (DT) analysis was used to evaluate relationships between several dependent variables (cortisol, leptin, HDL-c, HOMA index, C3 and C4) and the independent variables Cluster 1, Cluster 2 y Cluster 3. Chi-squared Automatic Interaction Detection (CHAID) algorithm was chosen.

RESULTS

Descriptive characteristics of the participants are presented in **Table 2**. Boys spent more time in LPA, MVPA, sedentary behavior and sleep times, and presented higher waist circumference than girls. In contrast, girls presented higher fat mass index than boys. After stratifying the total sample by gender, the selected dependent variables showed few correlations among them (data not shown). Table 3 shows those variables with significant values in each cluster both in boys and girls. When the analyses were repeated including Tanner stages the results remained unmodified (data not shown).

To better understand the relationship between the above mentioned clusters (1, 2 and 3) and the dependent variables, we used the DT method. Regarding cluster 1, the healthiest group showed the lowest cortisol values [healthy (10.8±4.4; 11.5±7.0 µg/dL) *vs* medium healthy/unhealthy (12.3±5.2; 13.2±7.1 µg/dL, in boys and girls, respectively)]. Similar results were also observed in cluster 2 [healthy (10.5±4.4; 11.3±6.6 µg/dL) *vs* medium healthy/unhealthy (12.4±5.2; 13.3±7.2 µg/dL, in boys and girls, respectively)]. However, in cluster 3, this difference in cortisol levels was only significant in boys [healthy (10.5±4.3 µg/dL) *vs* medium healthy/unhealthy (12.5±5.3 µg/dL, respectively)]. (Supplemented figure 1S).

When it was evaluated whether cortisol had a strong interaction with any of the clusters, we only found significant results in boys. Firstly, cortisol had the strongest interaction with cluster 1. Cortisol from healthy group had the strongest interaction with cluster 3, and cortisol from medium healthy and unhealthy groups had the strongest interaction with cluster 2 (figure 1A).

In addition, leptin had strong interactions with cluster 3 in boys (healthy/medium healthy (7.2±10.2) ng/mL *vs* unhealthy *(*13.1±18.0) ng/mL) and in girls (healthy (19.1±15.0) ng/mL *vs* medium healthy/unhealthy (36.6±27.3) ng/mL). When it was evaluated whether leptin had the strongest interaction with any of the clusters, we found that leptin had the first strong interaction with cluster 3 in both boys and girls. In boys, leptin from both unhealthy and healthy/medium groups had a strong interaction with cluster 2 (figure 2A). On the other hand, leptin in girls from the medium healthy/unhealthy group had a strong interaction with cluster 1 (figure 2B).

On the other hand, HDL-c showed that healthy groups (boys and girls) from cluster 3 presented the highest values [healthy (55.1±9.7 and 58.4±10.3 for boys and girls, respectively) mg/dL *vs* medium healthy/unhealthy (51.9±10.1 and 55.7±11.2 for

boys and girls respectively) mg/dL, respectively] (figure 2C). When it was evaluated whether HDL-c had the strongest interaction with any of the clusters, we found that HDL-c had the strongest interaction with clusters 1 and 3 in boys (figure 1D).

HOMA index could confirm the results that we observed by ANOVA in girls and we found that healthy girls group from cluster 3 presented the lowest mean (2.1±1.2) *vs* medium healthy/unhealthy (2.5±1.9) (figure 1C).

Moreover, for C3 and C4 complement factors DTP confirmed the results found by ANOVA test; C3 mean was higher in unhealthy boys group from cluster 3 (1.17±0.20) g/L *vs* healthy/medium healthy (1.11±0.16) g/L); the C4 mean was lower in healthy girls group (0.20±0.07) g/L *vs* medium healthy/unhealthy (0.22±0.07) g/L. In addition, when it was evaluated whether C3 had strong interaction with any of the clusters, we found that C3 had the strongest interaction with cluster 3. Subsequently, healthy/medium healthy groups had the strongest interaction with cluster 1 (figure 1B)

DISCUSSION

Our results support the existence of different associations on metabolic health depending on the behavioral pattern considered, and with some differences between genders. Overall the healthiest groups from the three analyzed clusters presented a healthier metabolic profile in boys and girls. However, despite some gender-specific differences, cluster 3 (the one including all studies lifestyle patterns) showed more associations with the studied endocrine, metabolic and immunological markers. Collectively, these findings suggest the existence of a close biological relationship between the combination of lifestyle patterns' and metabolic health.

Adolescence is a period of life that has been associated with several metabolic changes and a decrease in PA levels (1). Although there is evidence supporting that PA (25), sedentary behavior (5), sleep time (26), and body composition (9) can influence metabolic health, we are not aware of previous studies examining the combination of these variables on endocrine, metabolic and immunological markers in adolescents.

Concerning leptin the unhealthy adolescent groups had the highest concentrations. These results concur with those found by other authors (11,27,28). Leptin directly regulates the production of several cytokines, although it displays both pro- and anti-inflammatory properties (29). High levels of leptin are associated with pro-inflammatory risk, impaired vascular function, insulin resistance, and obesity (30). We also studied leptin across all clusters and DT analyses showed the strongest interaction between leptin and cluster 3 in both boys and girls. Interestingly, cluster 2 had a stronger interaction in boys while cluster 1 only in girls. These results might be explained by the gender-differences of behavioral patterns and leptin concentrations. Indeed, leptin is mainly produced by adipose tissue and its circulating concentrations are positively associated with fatness (31). However, leptin action is not limited to

adipose tissue, occurring also in the skeletal muscle, liver and intestinal function (31). In addition, leptin has also a circadian rhythm and sleep deprivation is associated with changes in the leptin concentrations, which could lead to an increase in appetite and consequently in weight gain (13). However, the association between sleep time and leptin may be modified by gender (13), which may explain the strong interaction with cluster 2 only in boys.

Cortisol, a glucocorticoid hormone synthesized by the adrenal cortex, is considered the stress hormone and can be influenced by several biological conditions, including increased body weight (17). When cortisol concentration was assessed in each cluster, the lowest values belonged to the healthiest groups, both in boys and girls, except in cluster 3, where this outcome is valid only for boys. This outcome is in agreement with that found by other authors (7,32). Surprisingly, although medium healthy girls group from cluster 3 met MVPA recommendations, they presented the worst anthropometry factor (table 1). Sex-differences have been found in the psychosocial behaviors during youth (33); in fact, this result may be partially caused by the different socio-cultural rules and expectations applied to girls, especially focusing on weight gain (34). In addition, depression and other psychological alterations have been pointed out to lead to obesity, especially in girls (35). We could speculate that these characteristics may explain to some extent our results taking into account that depressed people (especially, girls) have been shown to have the highest risk of obesity (35). These findings could explain the absence of any subsequent interaction with the other clusters in girls in our DT analyses. Indeed, no direct significant associations have been shown between cortisol and body composition by other authors (36), although the current results suggest the interest to include also the anthropometry factor, due to the significant interaction with clusters 1 and 3 in boys. Particularly, we observed this

interaction within the healthy group and the lowest cortisol levels found in this group. We found the lowest cortisol levels in healthy/unhealthy groups which could imply that adolescents who spent a lot of time in sedentary behaviors and sleep less than 8 (h/d) had increased levels of stress. These findings were in line with Pérez de Heredia et al., (2014) who showed that sleep duration in European adolescents was inversely associated with cortisol concentrations (7).

C3 and C4 complement factors have been associated with a high cardiovascular risk in adolescents (11). In our population, we found that unhealthy boys from cluster 3 presented the highest C3 concentration and healthy girls group from cluster 3 presented the lowest C4 concentration. These findings are consistent with Martinez-Gomez *et al*. (2011) (37) who observed that PA has an indirect role in inflammation through fitness and body fat in adolescents. Besides, Andersen *et al*., (2015) (38) did not find any association between PA and C4. However, when all the patterns were analyzed together, C3 showed the strongest association with cluster 3 and (from healthy/medium healthy groups with cluster 1), highlighting the sedentary behavior and physical activity interactions with this biomarker. These findings emphasize, again, the importance of including these lifestyle habit patterns together with body composition in relation to immunological and inflammatory biomarkers.

In our population, when we studied the HDL-c levels and HOMA index across the clusters, we found that HDL-c had the strongest association with clusters 1 and 3 in boys. In addition, the healthy girls group from cluster 3 had the lowest HOMA index level. These findings concur with other authors (39,40). Consistently, Hardy et al. (2010) showed that adolescent boys spending two or more hours per day of screen time on weekdays have twice the risk of abnormal levels of insulin and HOMA index

compared with those peers spending less than two hours per day of screen time on weekdays (41).

In summary, the present study investigating apparently healthy European adolescents has public health and clinical implications, since, adolescence is a period in which many risky behaviors start entailing a major impact on adults health (1). In addition, the present results emphasize the importance to analyze these lifestyle habit patterns together with body composition in relation to the metabolic health in this period of life. This approach seems to be useful to promote healthy habits related to physical activity, sedentariness and sleep in adolescents, as at this age they become more independent and acquire more responsibility in taking care of themselves.

Several limitations in this study should be considered and caution should be taken in the interpretation of the findings. Firstly, our cross-sectional design does not allow us to establish causality. Secondly, the use of self-reported behavioral measures in our analyses, yet this questionnaire is valid and reliable to be used with adolescents(23). Thirdly, a single fasting baseline measurement of biomarkers was made, which could limit its accurateness. However, the big size of the sample may counteract this limitation. It would have been desirable to have objective data on sleep habits, but unfortunately, polysomnography is not feasible in population based studies, and the adolescents did not wear the accelerometer during sleep time. Further research using emerging endocrine, metabolic and immunological health markers is needed to contrast our results and provide new insights regarding the influence of PA, sedentary behavior, sleep time duration and body composition at early ages.

In conclusion, we reported the existence of different interactions on metabolic health depending on the behavioral patterns established, with some differences according to gender. Our analysis emphasizes that not only an active lifestyle and less

sedentary time, but also an adequate sleep pattern and anthropometry, may reduce the likelihood to be engaged in a health-risk behavior.

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Table 1. Means of lifestyle patterns within all the groups tested in European adolescents

	Boys			Girls		
	H	MH	UH	H	MH	UH
Cluster 1	***n=547***	***n=736***	***n=279***	***n=569***	***n=840***	***n=365***
LPA (min/d)	209±39	146±28	191±38	203±35	143±23	161±29
MVPA (min/d)	89±23	53 ±16	64±22	68±20	43±14	46±15
Sedentariness (min/d)	256±109	367±116	724±198	318±107	249±96	629±161
Cluster 2	***n=536***	***n=777***	***n=313***	***n=563***	***n=881***	***n=363***
LPA (min/d)	208±40	146±29	183±40	197±36	143±25	168±33
MVPA (min/d)	86±24	55±18	62±22	62±21	44±16	52±20
Sedentariness (min/d)	289±126	335±118	711±197	289±127	276±101	625±168
Sleep time (min/d)	532±63	455±58	485±68	537±56	456±54	452±67
Cluster 3	***n=601***	***n=342***	***n=696***	***n=895***	***n=538***	***n=392***
LPA (min/d)	206±41	174±39	147±31	152±29	199±38	151±31
MVPA (min/d)	87±23	64±21	51±15	49±17	61±23	44±16
Sedentariness (min/d)	274±116	689±200	348±117	268±101	295±118	620±167
Anthropometry factor	-032±077	-0,35±089	0,48±1,04	-0,64±0,59	0,93±087	0,18±0,80

Data are presented as mean ±SD. LPA, Light Physical Activity; MVPA, Moderate-to-Vigorous Physical Activity; min, minutes; d. day.
H, healthy; MH, medium healthy; UH, unhealthy
Cluster 1: LPA, MVPA and sedentariness; *Cluster 2:* LPA, MVPA, sedentariness and sleep time; *Cluster 3:* LPA, MVPA, sedentariness and anthropometry factor

Table 2. Characteristics of the subjects

	Whole group	Boys	Girls	T-Test
	n =3528	**n =1683**	**n =1845**	**(p=values)**
Age (years)	14.7±1.2	14.8±1.2	14.7±1.2	0.085
BMI (kg/m^2)	21.4±3.7	21.5±4.0	21.3±3.5	0.097
Waist circumference (cm)	72.2±8.9	74.4±9.5	70.2±7.9	**<0.001**
Fat mass index (Kg/m^2)	5.3±3.0	4.7±3.5	5.8±2.5	**<0.001**
LPA (min/d)	169±42	174±45	165±39	**<0.001**
MVPA (min/d)	58±24	67±25	51±10	**<0.001**
Sedentariness (min/d)	367±196	389±207	347±184	**<0.001**
Sleep time (min/d)	483±70	486±70	481±69	**0.033**

Data are presented as mean ±SD. LPA, Light Physical Activity; MVPA, Moderate-to-Vigorous Physical Activity; min, minutes; d. day.

Table 3. Selected variables (means) in European adolescents according to cluster groups and stratified for sex

		Boys							Girls					
		n	H	**n**	MH	**n**	UH		**n**	H	**n**	MH	**n**	UH
Cluster 1														
Cortisol (µg/dL)	*	166	10.8±4.4[a]	171	12.6±5.1[b]	77	11.8±5.4[ab]	*	185	11.5±7.0[a]	245	13.3±7.0[b]	83	13.1±7.2[ab]
HDL-c (mg/dL)	#	187	54.5±9.9[a]	196	52.5 ±9.9[B]	97	52.0±10.3[B]		197	56.7±11.5	262	57.2±10.9	93	57.1±9.41
Leptin (ng/mL)	#	168	8.8±12.1[AB]	182	9.2±14.7[B]	83	11.1±15.8[a]	#	191	27.8±24.7[B]	251	27.5±24.5[B]	88	31.5±21.7[a]
Cluster 2														
Cortisol (µg/dL)	*	168	10.5±4.4[a]	179	12.7±5.1[b]	83	11.7±5.2[ab]	*	169	11.3±6.6[a]	266	13.2±7.2[b]	88	13.4±7.2[a]
Leptin (ng/mL)	#	173	10.1±13.5[a]	187	8.3±13.4[B]	89	11.1±17.0[A]	#	174	26.1±22.0[A]	275	28.1±25.6[a]	90	32.5±22.3[B]
Cluster 3														
Cortisol (µg/dL)	*	167	10.5±4.3[a]	92	12.2±5.2[b]	172	12.7±5.3[b]		249	12.4±6.5	169	12.3±7.6	107	13.5±7.3
HDL-c (mg/dL)	*	186	55.1±9.7[a]	112	52.9±10.3[ab]	201	51.4±9.9[b]	*	265	58.4±10.3[a]	180	55.0±12.0[b]	119	56.7±9.8[ab]
Leptin (ng/mL)	*	169	7.5±11.1[a]	101	6.6±8.7[a]	179	13.0±18.0[b]	*	255	19.0±15.0[a]	174	37.9±28.7[b]	112	34.6±25.0[b]
C3 (g/L)	*	178	1.12±0.15[a]	106	1.10±0.17[a]	182	1.17±0.19[b]	#	254	1.13±0.19[A]	167	1.16±0.18[B]	113	1.14±0.14[AB]
C4 (g/L)		176	0.210±0.071	105	0.205±0.061	182	0.212±0.066	*	251	0.203±0.065[a]	164	0.219±0.065[b]	113	0.224±0.066[b]
HOMA index	#	185	2.2±1.7[A]	107	2.1±1.8[a]	198	2.7±2.8[B]	*	263	2.1±1.2[a]	171	2.6±2.2[b]	117	2.4±1.4[ab]

Data are presented as mean ±SD. H, healthy; MH, medium healthy; UH, unhealthy

Significant differences in boys and girls for each cluster, as assessed by the One-way ANOVA test* and post hoc Bonferroni and Tamhone a,b,c p≤0.05

Non-parametric analysis, Kruskal-Wallis# and post hoc Mann-Whitney U test (exact p-value of Montecarmelo) A,B,C p≤0.05

Figure 1. DTP: Cortisol, C3, HDL-C levels and HOMA index according to the three clusters in European adolescents

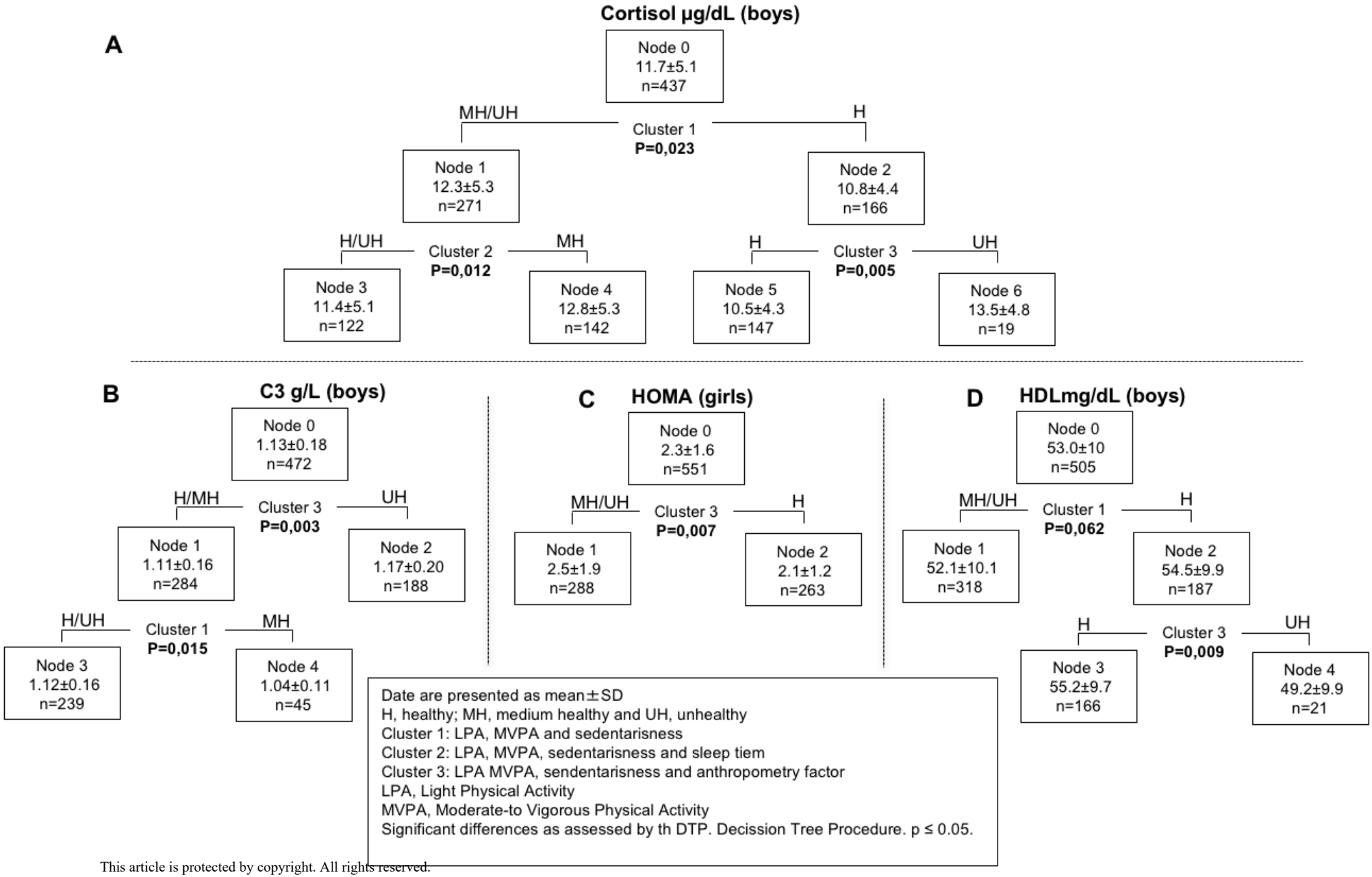
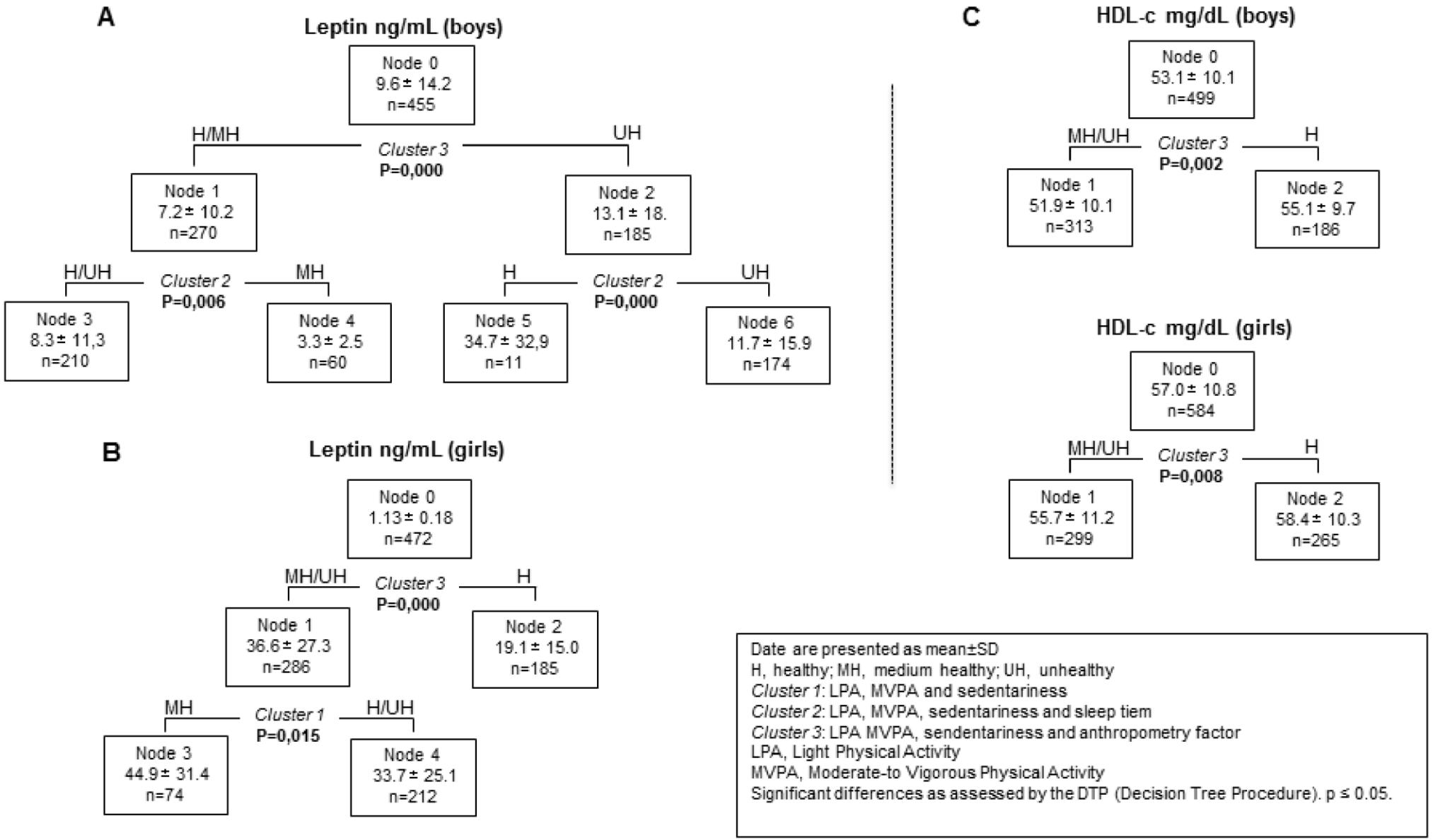


Figure 2. DTP: Leptin and HDL-c levels according to the three clusters in European adolescents



Date are presented as mean±SD
H, healthy; MH, medium healthy; UH, unhealthy
Cluster 1: LPA, MVPA and sedentariness
Cluster 2: LPA, MVPA, sedentariness and sleep tiem
Cluster 3: LPA MVPA, sendentariness and anthropometry factor
LPA, Light Physical Activity
MVPA, Moderate-to Vigorous Physical Activity
Significant differences as assessed by the DTP (Decision Tree Procedure). $p \leq 0.05$.