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TITLE PAGE

Title: Iron and vitamin status biomarkers and its association with physical fitness in adolescents. The HELENA study.

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Running head: Micronutrient biomarkers and fitness in adolescents

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ABSTRACT

There is a lack of studies that analyse the association between micronutrient-related biomarker status and physical fitness in adolescents. In the present study, biochemical parameters for iron and vitamin status were studied along with objective measures of physical fitness in healthy male and female European adolescents. 1089 adolescents (580 girls, 12.5-17.5 years) from the HELENA cross-sectional study were included. Hierarchical linear models were performed to determine the associations between micronutrient biomarkers and physical fitness. Age, seasonality, latitude, body mass index, menarche (in girls) and physical activity were used as covariates. For cardiorespiratory fitness, concentrations of hemoglobin, retinol and vitamin C in male adolescents and, β-carotene and 25(OH)D in female adolescents were associated with VO_{2max}. For muscular fitness, concentrations of hemoglobin, β-carotene, retinol and α-tocopherol in male adolescents and β-carotene and 25(OH)D in female adolescents were associated with better performance of the standing long jump test. In summary, concentrations of hemoglobin and most antioxidant vitamins in male adolescents and β-carotene and 25(OH)D in female adolescents were positively associated with cardiorespiratory and muscular fitness, after controlling for relevant confounders. The associations between physical fitness and iron or vitamin status observed in this cross-sectional study in adolescents should be followed up by a study specifically designed to evaluate causal relationships.

Keywords: iron status, vitamins, 25-hydroxyvitamin D, physical performance, adolescence.

INTRODUCTION

Adolescence is a critical period of growth, development and for the acquisition of healthy behaviors (31). Appropriate nutrition during this period is a basic requirement to express genetic potential that together with physical activity (PA) will influence later adult and elderly health outcomes. Physical fitness is a set of attributes related to a person´s ability to perform physical activities that require aerobic capacity, endurance, strength or flexibility and is mainly determined by a combination of regular PA and genetically inherited ability (9). Physical fitness is frequently evaluated in adolescents and it is known to be a a powerful marker for present (youth) and future (adult) health (39). Scientific evidence shows that the adolescents´ performance in these tests has declined in the last three decades (49, 50). For some micronutrients an at least marginally deficient nutritional status has been also identified (3, 52). It has been stated in the literature that physical fitness interacts with the nutritional status of the individuals (29), which at the same time can differ according to gender, age, latitude, etnicity, climate, seasonality, genetic background, adiposity and lifestyle factors (4, 8, 16, 23, 41, 47, 51).

It is known that hemoglobin is the oxygen carrier to the muscles. Some studies have shown that iron status is associated with physical fitness, mainly in its cardiorespiratory dimension (54). Some vitamins, such as vitamin C, have biological effects which are associated with sports performance and recovery from intense training, such as antioxidant function, inmunocompetence, collagen metabolism and carnitine biosynthesis (29). 25-hydroxyvitamin D [(25(OH)D] has been shown to affect skeletal muscle strength and function, acting on calcium mechanisms, cell proliferation and differentiation, preventing against insulin resistance and arachidonic acid mobilization, and other mechanisms (7, 15), being also related to fitness performance (16, 21). Nevertheless, the association between status of some micronutrients and physical performance remain still controversial. In

addition, limited information is available concerning this association in the adolescent population, taking into account the important effect of the above-mentioned confounders. In this study, we hypothesized that iron and vitamin status status is directly associated with cardiorespiratory (CRF) and muscular fitness in adolescents.

MATERIAL AND METHODS

Study design

The HELENA-CSS is a European Union-funded project 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) (33). Detailed descriptions of the HELENA sampling and recruitment approaches, standardization and harmonization processes, data collection, analysis strategies, quality control activities and inclusion criteria have been described in detail elsewhere (32). An extended and detailed manual of operations was designed for and thoroughly read by every researcher involved in the field work before the data collection started. Parents and adolescents signed an informed consent, the protocol was approved by Research Ethics Committees of each city involved and the study has been performed following the ethical guidelines of the Declaration of Helsinki 1964 (revision of Edinburgh 2000), Convention of Oviedo (1997), the Good Clinical Practice, and the legislation about clinical research in humans in each of the participating countries.

Study sample

The geographical distribution of the 10 cities (>100,000 inhabitants) was not random and not represented by the strata, but it was decided according to the following criteria: representation of territorial units (countries) of Europe according to geographical location

(North/South/East/West), cultural reference and socioeconomic situation; and selection of a territorial unit (city) in the country, which had an experienced research group to perform the study. The age range considered valid for the HELENA study was 12.5-17.5 y. All the analyses conducted on the HELENA data are adjusted by a weighing factor to balance the sample according to the theoretical age distribution foreseen. A total of 3528 adolescents, 1683 males and 1845 females, were considered eligible for the HELENA analyses.

In the HELENA-CSS protocol, it was established that blood samples were obtained randomly in one third of the population sample. Therefore, a total of 1089 adolescents (509 males and 580 females) were included in this report. To make maximum use of the data, all valid data on physical fitness components were included in this report. Consequently, sample sizes vary for the different physical fitness tests and biomarkers.

Anthropometric measurements

International guidelines for anthropometry in adolescents were used in the HELENA study (35). Barefoot and in light indoor clothing, body weight (Kg) and height (cm) were measured with an electronic scale (Type SECA 861), precision 100 g, range 0 – 150 Kg and a stadiometer (Type Seca 225), precision 0,1 cm, range 70 – 200 cm, respectively. Body mass index (BMI) was calculated as body weight (Kg) divided by height (m) squared.

Physical fitness tests

The physical fitness components, *i.e.* muscular fitness and aerobic capacity, were assessed by the physical fitness tests previously described in detail (37). The scientific rationale for the selection of all of these tests, as well as their reliability in young people, was previously published (38). Lower-body muscular strength was assessed with the *standing long jump* test (SLJ), which has shown to be a good indicator of overall muscular fitness in youth (10).

156 This test was performed twice and the best score was retained. CRF was assessed with the
157 *20m shuttle run* test (stage). A stage is the period of time in which the speed maintains
158 constant. In this test, the initial speed is 8.5km·h^{-1}, which is increased by 0.5km·h^{-1}·min^{-1} (1
159 min equals one stage) (24). Léger equation was used to estimate the maximal oxygen
160 consumption (VO_{2max}) from 20m shuttle run test (28). This test was performed only once.
161
162 *Specimen collection and biochemical analyses*
163 The blood sampling procedure and sample logistics have been described in detail elsewhere
164 (22). Briefly, fasting blood samples (24.3 ml) were collected by venipuncture at school
165 between 8 and 10 o'clock in the morning after a 10-hour overnight fast. Samples for the
166 different analyses were manipulated *in situ* as described below and transported according to
167 the protocol to the central laboratory in Bonn, Department of Nutrition and Food Sciences,
168 for further manipulation. Whole-blood samples for the red blood parameters, including
169 hemoglobin, were sent directly to the local laboratory of each country to be analysed.
170 A specific handling, transport and traceability system for biological samples was developed
171 for the HELENA study and was already described by González-Gross et al. (22). Blood
172 samples were obtained between October 2006 and June 2007 and in October 2007. Blood
173 sampling date was dependent from local field work planning, agreement of the school and
174 availability and capacity of the central laboratory.
175
176 *Iron status assessment*
177 Blood sampling procedure and laboratory measures for iron status indicators [STfR and
178 serum ferritin] have been described elsewhere (20, 22). Briefly, sTfR and serum ferritin
179 were measured using ELISA (enyme-lynked immunosorbent assay) (19) in the Human
180 Nutrition Laboratory of the National Research Institute on Food and Nutrition (Rome, Italy).

A commercially available control sample from Bio-Rad Liquichek Immunology Control Level 3 (Bio-Rad, Milan, Italy) was used to obtain a calibration curve on each plate.

Provitamin A (ß-carotene), vitamin A (retinol) and vitamin E (α-tocopherol) measurements

ß-carotene, retinol and α-tocopherol were analysed by reversed phase high-performance liquid chromatography using UV detection (RP-HPLC) (Sykam Gilching Germany) in serum. The vacutainer was centrifuged for 15 min at 3,500 r.p.m. at 4°C. Standards (ß-carotene, retinol, α-tocopherol), hexane and isopropanol were obtained from Sigma Aldrich (Germany) and had all HPLC-grade. The variation of the method is below 3% for all the vitamins. The samples were stable over 24 hours at room temperature (coefficient of variation (CV) vitamin E = 4.6%; vitamin A = 3.2%).

Vitamin C measurement

For vitamin C measurements the heparin tubes were put immediately on ice and centrifuged within 30 min (3,500 r.p.m. for 15 min.). For stabilization, heparin plasma was precipitated with a 6% (w/w) perchloric acid solution spiked with metaphosphoric acid (1:1). The precipitated samples were transported at a stable temperature of 4-7°C within 24h to the central laboratory and stored at -80°C until analysis. Plasma Vitamin C was analysed by RP-HPLC using UV-detection (Sykam Gilching Germany). The CV of the Method was 1.7%.

Vitamin B_6, B_{12} (cobalamin and holo-transcobalamin) and folate (plasma and red blood cell) measurements

For the measurement of vitamin B_6 (pyridoxal 5'-phosphate), aliquots of EDTA whole blood were sent by cooled transport to the central laboratory and stored at -80°C until bunched analysis. Pyridoxal 5'-phosphate was measured by HPLC (Varian Deutschland GmbH,

206 Darmstadt, Germany; CV = 1%) with a modified method of Kimura et al. (26). For vitamin
207 B_{12} status, cobalamin and holo-transcobalamin were determined. For folate status, plasma
208 folate and red blood cell (RBC) folate were determined. For the measurement of cobalamin
209 and plasma folate, blood was collected in heparinized tubes, immediately placed on ice, and
210 centrifuged within 30 min (3500 rpm for 15 min). The supernatant fluid was transported at a
211 stable temperature of 4-7°C to the central laboratory and stored there at -80°C until assayed.
212 After measuring the hematocrit in situ, EDTA whole blood was sent to the central laboratory
213 for the RBC folate analysis. EDTA whole blood was diluted 1:5 with freshly prepared 0.1%
214 ascorbic acid for cell lysis incubated for 60 min in the dark before storage at -80°C.
215 Cobalamin, plasma and RBC folate were measured by competitive immunoassay (Immulite
216 2000, DPC Biermann GmbH, Bad Nauheim, Germany); (CV for plasma folate = 5.4%, RBC
217 folate = 10.7%, Cobalamin = 5.0%). Sera for measuring holo-transcobalamin were obtained
218 by centrifuging blood collected in evacuated tubes without anticoagulant at 3,500 r.p.m for
219 15 min within one hour. Once sent to the central laboratory, the sera were aliquoted and
220 stored at -80°C until transport in dry ice to the biochemical lab at the Universidad
221 Politécnica de Madrid for analysis (Laboratorio número 242 de la Red de Laboratorios de la
222 Comunidad de Madrid). Holo-transcobalamin was measured by microparticle enzyme
223 immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK; CV = 5.1%) with the use
224 of AxSym (Abbot Diagnostics, Abbott Park, IL, USA).
225
226 *25(OH)D measurement*
227 For vitamin D status, plasma 25(OH)D was measured. Blood was collected in EDTA tubes
228 transported at room temperature to the central laboratory at IEL within 24 h. There it was
229 centrifuged at 3,500 rpm for 15 min at 4ºC and the supernatant stored at -80°C till analysis.

Plasma 25(OH)D was analysed by ELISA using a kit (OCTEIA 25-Hydroxy Vitamin D) from Immunodiagnostic System (Germany) and measured with a SunriseTM Photometer by TECAN (Germany). The IDS OCTEIA 25(OH)D kit is an enzyme immunoassay intended for the quantitative determination of 25(OH)D and other hydroxylated metabolites in human serum or plasma. The sensitivity of this method is 5 nmol/L 25(OH)D and the variation is below 6 %. The mean recovery of 25(OH)D is 101%. The coefficient of variation for the method was below 1%.

Seasonality

A variable was computed by re-coding the original variable "Blood drawing date" into "Seasonality", as follows: winter (from 21st December to 20st March, coded as 1), autumn (from 21st September to 20th December, coded as 2), spring (from 21st March to 20th June, coded as 3) and summer (from 21st June to 20th September, coded as 4), as it was performed in previous studies (16). As the HELENA study was performed during the academic year, few adolescents (N=25) were assessed in the first days of summer, and they were included along with those assessed during spring. Therefore, the final variable was composed by 3 groups: winter (coded as 1), autumn (coded as 2) and spring (coded as 3).

Latitude of residence

The latitude of the study centers was also taken into account as a confouder in the analyses. The latitude of each city was obtained from http://maps.google.es/. Latitudes of the involved cities were: Stockholm (59º33´ N), Dortmund (51º51´ N), Ghent (51º06´ N), Lille (50º63´ N), Vienna (48º21´ N), Pecs (46º07´ N), Rome (41º89´ N), Zaragoza (41º66´ N), Athens (37º98´ N) and Heraklion (35º33´ N). To make use of this data, latitudes were added to the database as numeric variables with two decimals (i.e. Stockholm = 59.55).

255

256 *Age at menarche*

257 A quantitative variable was computed as a measure of time (months) as follows: menarche
258 (months) = age (months) at the moment of blood drawing – age (months) at the moment of
259 menarche. Those girls (n=104) who had no menarche before blood drawing were considered
260 as "0" for the analyses.

261

262 *Physical activity assessment*

263 A uni-axial accelerometer (Actigraph GT1M, Manufacturing Technology Inc. Pensacola,
264 FL, USA) was used to assess physical activity (PA) as described previously (44). In this
265 study, the interval of time (epoch) was set at 15 seconds. The time spent (minutes/day) at
266 moderate PA (MPA) [4-6 metabolic equivalents (METs)] was calculated based upon a cut-
267 off of 2000-3999 counts per minute (cpm). The time spent (minutes/day) at vigorous PA (>6
268 METs) was calculated based upon a cut-off of 4000 cpm. Further, moderate to vigorous PA
269 (MVPA) (>4 METs) was calculated as the sum of moderate and vigorous PA. The cut-offs
270 to define the intensity categories are similar to those used in previous studies (17).
271 Subjects were classified as non-active adolescents (<60 minutes/day of MVPA) and active
272 adolescents (≥60 minutes/day of MVPA) according to the recent guidelines launched by the
273 U.S. Department of Health and Human Services and other medical institutions (1).

274

275 *Statistical methods*

276 After square root transformation of serum ferritin and natural logarithm transformation of
277 sTfR, β-carotene, retinol, vitamin B_6, cobalamin, holo-transcobalamin, plasma folate and
278 RBC folate, all the residuals showed a satisfactory pattern (normal distribution). Since
279 interactions between sex and the studied variables were observed ($p<0.001$), results are

given separately by gender. Descriptive data were assessed by one-way analysis of variance (ANOVA) for normally distributed variables and by *U* Mann Whitney for non-normally distributed variables.

The relationships among micronutrient biomarkers, i.e. iron, hidrosoluble and liposoluble vitamins and the performance in the physical fitness tests, i.e. 20m shuttle run and SLJ, were assessed using hierarchical linear models. The adjusted analysis was conducted according to a previously formulated hierarchical model, including different models: 1) age, seasonality and latitude 2) nutritional status and menarche (in females) 3) MVPA and 4-7) micronutrient biomarkers. At each model of micronutrient biomarkers, the variables were controlled for those at the same level and the levels above (models 1-3) (53). For a variable to remain in the model, a significance level of $P < 0.20$ was required.

All the analyses were performed using the Statistical Package for Social Sciences software (SPSS, v. 15.0 for WINDOWS; SPSS Inc., Chicago, IL, USA), and values of $p<0.05$ were considered statistically significant. Figures were performed using Sigmaplot (v. 10.0 for WINDOWS; Systat Software Inc., San José, California).

RESULTS

Table 1 shows descriptive characteristics of the sample by sex.

Micronutrient status and CRF in adolescents

Results from the hierarchical linear models investigating the relationship between the adolescent CRF (assessed by VO_{2max}), and their micronutrient-related biomarker status are presented in tables 2 and 3. In male adolescents, adjusted results showed that hemoglobin ($\beta= 0.192$), retinol and vitamin C ($\beta= 0.148$ and $\beta= 0.186$, respectively) were associated with VO_{2max} (table 2). In female adolescents, adjusted results showed that β-carotene ($\beta= 0.101$) and 25(OH)D ($\beta= 0.091$) were associated with VO_{2max} (table 3).

305

306 **Micronutrients and muscular fitness in adolescents**

307 Results from the hierarchical linear models investigating the relationship between the
308 adolescent muscular fitness (assessed by SLJ), and their micronutrient-related biomarker
309 status are presented in tables 4 and 5. In male adolescents, adjusted results showed that
310 hemoglobin (β= 0.203), β-carotene (β= 0.160), retinol (β= 0.128) and α-tocopherol (β= -
311 0.135) were associated with SLJ (table 4). In female adolescents, adjusted results showed
312 that β-carotene (β= 0.177) and 25(OH)D (β= 0.125) were associated with SLJ (table 5).

313

314 **DISCUSSION**

315 In the present study we examined cross-sectional associations of micronutrient-related
316 biomarker status with the performance in physical fitness tests in a large sample of European
317 adolescents from ten cities. The studied cities were equivalent and comparable among
318 countries but the samples were representative for the cities and not for the countries (32). To
319 the best of our knowledge, there is limited literature concerning the association among
320 micronutrient status and physical fitness in adolescents.

321 In our study, hemoglobin concentration was positively associated with CRF (assessed
322 through VO_{2max}) in male adolescents after controlling for relevant confounders. The
323 influence of iron status (i.e. hemoglobin) on physical fitness has been studied and
324 demonstrated during decades (12, 36). A recent study showed that severe iron deficiency
325 (males, <0.75mg/L and females, <0.73 mg/L) impaired aerobic capacity, assessed through
326 VO_{2max} (54). Our finding of a positive association between blood hemoglobin and muscular
327 fitness (assessed through SLJ) in male adolescents is novel, but previous studies found
328 positive associations between serum ferritin and the performance in SLJ in males (5),

329 enhancing the importance of iron status not only in relation to CRF but also muscular
330 fitness.
331 The concentrations of antioxidant vitamins in adolescents participating in the HELENA
332 study were recently described and published (8). In our study, we found that concentrations
333 of retinol and vitamin C in male adolescents and β-carotene in female adolescents were
334 positively associated with CRF, after controlling for relevant confounders. Our results agree
335 with those shown in the study of Suboticanec-Buzina et al. (48) who reported that vitamin C
336 supplementation resulted in a significant increase in CRF (VO_{2max}) in adolescents with
337 initially lower values. In contrast, studies performed in young adults showed that vitamin C
338 was not associated with CRF (13, 56). Therefore, there is no a clear evidence of a benefit of
339 vitamin C supplementation on physical performance, suggesting further research. However,
340 vitamin C may exert permissive effects on physiologic functions, such as antioxidant,
341 inmunocompetence and collagen repair, which are associated with recovery from intense
342 training, and as consequence, promoting performance (29). Our results support the positive
343 correlations found in female adolescents between β-carotene concentrations and CRF
344 (VO_{2max}) in the study of Lloyd et al. (30); however, a later review showed that any positive
345 effects of β-carotene on performance remains to be determined (29). Regarding muscular
346 fitness (SLJ), we found positive associations with β-carotene (in both sexes) and retinol in
347 male adolescents, after controlling for relevant confounders. In addition, α-tocopherol was
348 negatively associated with muscular fitness (SLJ) in male adolescents. Some studies showed
349 that the performance in standard exercise tests (45), CRF (VO_{2max}) (45), muscular fitness
350 (handgrip test) (46), swimming endurance (27, 45), or blood lactate concentrations (27) was
351 not affected by long-term α-tocopherol supplementation. . However, there are no studies
352 analyzing the relationship between α-tocopherol and the lower limbs strength and therefore,
353 the physiological factors behind cannot be elucidated, suggesting the need of more studies.

In recent yearts, vitamin D has been the most widely studied micronutrient in relation with physical fitness. The concentrations of 25(OH)D in adolescents participating in the HELENA study were recently described and published (23). In our study, we found positive associations between 25(OH)D, cardiorespiratory and muscular fitness only in female adolescents, after controlling for relevant confounders. Our results support previous evidence that shows a positive association between circulating 25(OH)D and CRF (VO_{2max}) (16, 34, 42). In addition, our results support previous studies that showed positive associations between 25(OH)D and muscular fitness (assessed through handgrip test and/or pliometric tests) in females (21, 42, 55). It has been shown that vitamin D affects skeletal muscle strength and function, acting on calcium mechanisms, cell proliferation and differentiation, preventing against insulin resistance and arachidonic acid mobilization, or other mechanisms (7, 15). However, with this study it is not possible to disentangle the mechanisms by which 25(OH)D affects cardiovascular and muscle function in our population.

Limitations and strengths

Although we controlled for several potential confounders, we cannot be certain that other unmeasured confounders have not influenced our observations. Cross-sectional studies provide evidence of associations. However, in this specific case, it seems reasonable to think that micronutrient status can influence physical fitness, whereas it is not so clear the mechanisms by which physical fitness could determine higher or lower micronutrient status. It should also be considered that sexual maturation was not included as a covariate in our analyses, due to it was not available on the whole sample. However, the age of the adolescents was used instead, which showed a slight and stronger association with the dependent variables.

Despite the aforementioned, this is the first study reporting the association between different physical fitness components (i.e. CRF and muscular fitness) and a large number of micronutrient biomarkers in a large sample of European adolescents. The fitness tests used in the present report showed a good criterion-related validity in adolescents (43). In addition, this study includes important sets of confounders, i.e. age, seasonality, latitude, BMI, menarche (in females) and MVPA, which is crucial to analyse the association among micronutrient-related biomarker status and physical fitness.

In summary, concentrations of hemoglobin and most antioxidant vitamins in males and β-carotene and 25(OH)D in females were positively associated with cardiorespiratory and muscular fitness in a large sample of European adolescents, after controlling for relevant confounders. The associations between physical fitness and iron or vitamin status observed in this cross-sectional study in adolescents should be followed up by a study specifically designed to evaluate causal relationships.

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411

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Table 1. Descriptive characteristics of the studied adolescents by sex.

	Males			Females		
Variables	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**
Age (y)	509	15.02 ±	1.23	580	15.04 ±	1.18
Body mass (kg)	509	62.8 ±	14.4	580	56.3 ±	10.1
Height (cm)	509	170.2 (163.8-177)		580	162 (157.5-166.6)	
BMI (kg/m^2)	509	21.5 ±	4.0	580	21.4 ±	3.4
Seasonality (Winter/autumn/spring) (%)	509	(28/31/41)		580	(24/33/43)	
MVPA (min/day)	318	69.0 ±	25.1	395	51.5 ±	20.5
SLJ (cm)	463	184.7 ±	32.9	537	144.8 ±	25.6
$VO_{2\ max}$ (ml/Kg/min)	404	44.2 ±	7.9	428	36.0 ±	5.4
Iron status						
Hemoglobin (g/dL)	508	14.7 ±	1.2	569	13.4 ±	1.1
sTfR (mg/L)	469	5.9 (4.7-7.0)		536	5.7 (4.5-7.1)	
Serum ferritin (µg/L)	477	32.8 (18.2-50.0)		532	24.4 (14.1-38.7)	
Antioxidant vitamins						
β-carotene (ng/ml)	452	192.5 (130.8-279.2)		506	219.9 (148.6-311.3)	
Retinol (ng/ml)	445	349.7 (282.7-411.5)		501	325.5 (277.0-402.2)	
α-tocopherol (µg/ml)	447	9.5 ±	1.9	507	10.3 ±	2.1
Vitamin C (mg/L)	503	10.03 ±	3.28	554	10.64 ±	3.31
Hidrosoluble vitamins						
Vitamin B_6 (pmol/L)	453	53.7 (37.9-79.3)		528	46.5 (32.1-70.1)	
Cobalamin (pmol/L)	501	328.39 ±	125.26	554	368.28 ±	155.31
Holo-Transcobalamin (pmol/L)	469	58.7 (44.2-72.1)		551	57.7 (45.3-74.5)	
Plasma folate (nmol/L)	497	16.0 (11.8-22.1)		552	16.2 (12.2-22.8)	
RBC folate (nmol/L)	495	728.5 (568.5-952.9)		545	707.9 (544.4-931.6)	
Vitamin D						
25(OH)D (nmol/L)	471	57.38 ±	22.63	538	60.20 ±	23.41

25(OH)D, 25-hydroxyvitamin D; **MVPA**, moderate to vigorous physical activity; **RBC**, red blood cells; **SD**, standard deviation; **SLJ**, standing long jump; **sTfR**, soluble transferrin receptor; **VO_{2max}**, maximal oxygen consumption

ANOVA was performed for normally distributed variables (mean ± SD) and *U* Mann Whitney for non-normally distributed variables (median and interquartil intervals)

Table 2. Association of micronutrient-related biomarker status and potential confounders with cardiorespiratory fitness (VO_{2max}, ml/kg/min) in male European adolescents.

		Males - VO_{2max}					
		Unadjusted analysis			Adjusted analysis		
Model	*Variables*	*β*	*P*	*n*	*β*	*P**	*n*
1 [a]	Age	-0.186	**<0.001**	404	-0.217	**<0.001**	
	Seasonality	0.139	**0.005**	404	0.135	**0.003**	404
	Latitude	0.311	**<0.001**	404	0.324	**<0.001**	
2 [a]	BMI	-0.445	**<0.001**	404	-0.374	**<0.001**	404
3 [a]	MVPA	0.329	**<0.001**	264	0.205	**<0.001**	264
4 [b]	Hemoglobin	-0.010	0.836	400	0.192	**0.002**	
	sTfR †	0.000	0.995	376	-0.010	0.861	246
	Serum ferritin ‡	-0.149	**0.003**	384	-0.022	0.700	
5 [c]	β-carotene †	0.210	**<0.001**	355	-0.048	0.481	
	Retinol †	-0.080	0.133	349	0.148	**0.031**	221
	α-tocopherol	0.007	0.895	349	-0.050	0.417	
	Vitamin C	0.173	**0.001**	400	0.186	**0.005**	
6 [d]	Vitamin B_6 †	0.042	0.425	358	0.058	0.363	
	Cobalamin †	0.162	**0.001**	396	0.084	0.261	
	Holo-Transcobalamin †	0.090	0.086	369	-0.031	0.689	222
	Plasma folate †	0.019	0.713	394	-0.004	0.964	
	RBC folate †	0.049	0.329	392	-0.008	0.925	
7 [e]	25(OH)D	0.113	**0.029**	372	0.088	0.127	250

β is the standardized regression coefficient.
[a] The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model
[b] Significant variables shown in models 1-3 + model 4 were included
[c] Significant variables shown in models 1-3 + model 5 were included
[d] Significant variables shown in models 1-3 + model 6 were included
[e] Significant variables shown in models 1-3 + model 7 were included
† Log-transformed data; ‡ Square-root transformed data
* Variables with P > 0.2 were not included in the subsequent adjustment models
BMI, body mass index; **25(OH)D**, 25-hydroxyvitamin D; **MVPA,** moderate to vigorous physical activity; **RBC,** red blood cells; **sTfR,** soluble transferring receptor; **VO_2max,** maximal oxygen consumption.
Categorical variables: Seasonality (1= winter, 2= autumn, 3= spring); MVPA (0= <60 min/day, 1= ≥ 60 min/day)

Table 3. Association of micronutrient-related biomarker status and potential confounders with cardiorespiratory fitness (VO_{2max}, ml/kg/min) in female European adolescents.

		Females - VO_{2max}					
		Unadjusted analysis			Adjusted analysis		
Model	*Variables*	*β*	*P*	*n*	*β*	*P**	*n*
1 [a]	Age	-0.445	**<0.001**	427	-0.492	**<0.001**	
	Seasonality	0.213	**<0.001**	424	0.224	**<0.001**	424
	Latitude	0.178	**<0.001**	428	0.233	**<0.001**	
2 [a]	BMI	-0.321	**<0.001**	428	-0.226	**<0.001**	419
	Menarche	-0.380	**<0.001**	425	-0.101	**0.039**	
3 [a]	MVPA	0.100	**0.039**	296	0.039	0.413	290
4 [b]	Hemoglobin	0.041	0.401	418	0.038	0.390	
	sTfR †	0.022	0.661	390	-0.039	0.374	377
	Serum ferritin ‡	0.002	0.973	389	0.006	0.887	
5 [c]	β-carotene †	0.189	**<0.001**	371	0.101	**0.042**	
	Retinol †	-0.004	0.934	364	0.064	0.214	354
	α-tocopherol	0.041	0.432	364	-0.029	0.540	
	Vitamin C	0.069	0.161	417	0.013	0.779	
6 [d]	Vitamin B_6 †	0.130	**0.010**	388	0.026	0.572	
	Cobalamin †	0.203	**<0.001**	403	0.016	0.785	
	Holo-Transcobalamin †	0.102	**0.040**	409	0.067	0.231	366
	Plasma folate †	0.108	**0.030**	402	-0.051	0.387	
	RBC folate †	0.109	**0.030**	398	0.084	0.156	
7 [e]	25(OH)D	0.027	0.593	396	0.091	**0.030**	391

β is the standardized regression coefficient

[a] The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

[b] Significant variables shown in models 1-3 + model 4 were included

[c] Significant variables shown in models 1-3 + model 5 were included

[d] Significant variables shown in models 1-3 + model 6 were included

[e] Significant variables shown in models 1-3 + model 7 were included

† Log-transformed data; ‡ Square-root transformed data

* Variables with P > 0.2 were not included in the subsequent adjustment models

BMI, body mass index; **25(OH)D**, 25-hydroxyvitamin D; **MVPA,** moderate to vigorous physical activity; **RBC,** red blood cells; **sTfR,** soluble transferring receptor; **VO_2max,** maximal oxygen consumption.

Categorical variables: Seasonality (1= winter, 2= autumn, 3= spring); MVPA (0= <60 min/day, 1= ≥ 60 min/day)

Table 4. Association of micronutrient-related biomarker status and potential confounders with muscular fitness (SLJ, cm) in male European adolescents.

		Males - SLJ					
		Unadjusted analysis			Adjusted analysis		
Model	*Variables*	*β*	*P*	*n*	*β*	*P**	*n*
1 [a]	Age	0.382	**<0.001**	462	0.372	**<0.001**	
	Seasonality	0.001	0.983	463	-0.044	0.294	462
	Latitude	0.288	**<0.001**	463	0.273	**<0.001**	
2 [a]	BMI	-0.256	**<0.001**	463	-0.291	**<0.001**	462
3 [a]	MVPA	0.142	**0.014**	300	0.132	**0.010**	300
4 [b]	Hemoglobin	0.286	**<0.001**	459	0.203	**0.001**	
	sTfR [†]	-0.109	**0.025**	426	-0.007	0.897	273
	Serum ferritin [‡]	0.105	**0.029**	433	0.080	0.156	
5 [c]	β-carotene [†]	0.227	**<0.001**	412	0.160	**0.013**	
	Retinol [†]	0.241	**<0.001**	404	0.128	**0.039**	254
	α-tocopherol	-0.063	0.204	404	-0.135	**0.017**	
	Vitamin C	0.109	**0.019**	458	-0.003	0.965	
6 [d]	Vitamin B_6 [†]	0.024	0.631	414	0.030	0.619	
	Cobalamin [†]	0.035	0.461	454	0.014	0.846	
	Holo-Transcobalamin [†]	-0.005	0.910	424	0.091	0.213	252
	Plasma folate [†]	-0.113	**0.016**	453	0.028	0.721	
	RBC folate [†]	0.005	0.912	451	-0.088	0.267	
7 [e]	25(OH)D	0.078	0.105	430	0.085	0.120	285

β is the standardized regression coefficient
[a] The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model
[b] Significant variables shown in models 1-3 + model 4 were included
[c] Significant variables shown in models 1-3 + model 5 were included
[d] Significant variables shown in models 1-3 + model 6 were included
[e] Significant variables shown in models 1-3 + model 7 were included
[†] Log-transformed data; [‡] Square-root transformed data
* Variables with P > 0.2 were not included in the subsequent adjustment models
BMI, body mass index; **25(OH)D**, 25-hydroxyvitamin D; **MVPA,** moderate to vigorous physical activity; **RBC,** red blood cells; **SLJ,** standing long jump; **sTfR,** soluble transferring receptor.
Categorical variables: Seasonality (1= winter, 2= autumn, 3= spring); MVPA (0= <60 min/day, 1= ≥ 60 min/day)

Table 5. Association of micronutrient-related biomarker status and potential confounders with muscular fitness (SLJ, cm) in female European adolescents.

		Females - SLJ					
		Unadjusted analysis			Adjusted analysis		
Model	*Variables*	*β*	*P*	*n*	*β*	*P**	*n*
1 [a]	Age	0.177	**<0.001**	536	0.142	**0.001**	
	Seasonality	0.052	0.231	531	0.000	0.998	531
	Latitude	0.299	**<0.001**	537	0.284	**<0.001**	
2 [a]	BMI	-0.336	**<0.001**	537	-0.313	**<0.001**	532
	Menarche	-0.037	0.400	533	-0.082	0.090	
3 [a]	MVPA	0.143	**0.006**	373	0.108	**0.021**	369
4 [b]	Hemoglobin	-0.032	0.463	526	0.008	0.878	
	sTfR †	0.045	0.319	491	0.058	0.280	329
	Serum ferritin ‡	-0.095	**0.036**	489	-0.020	0.709	
5 [c]	β-carotene †	0.257	**<0.001**	470	0.177	**0.003**	
	Retinol †	-0.048	0.298	464	-0.080	0.182	305
	α-tocopherol	0.029	0.534	465	0.003	0.954	
	Vitamin C	0.114	**0.009**	512	-0.019	0.727	
6 [d]	Vitamin B_6 †	0.056	0.218	492	0.087	0.099	
	Cobalamin †	0.060	0.179	511	0.046	0.488	
	Holo-Transcobalamin †	0.035	0.426	512	0.036	0.581	326
	Plasma folate †	-0.004	0.931	510	-0.058	0.383	
	RBC folate †	0.030	0.508	505	0.084	0.223	
7 [e]	25(OH)D	0.081	0.070	501	0.125	**0.010**	350

β is the standardized regression coefficient

[a] The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

[b] Significant variables shown in models 1-3 + model 4 were included

[c] Significant variables shown in models 1-3 + model 5 were included

[d] Significant variables shown in models 1-3 + model 6 were included

[e] Significant variables shown in models 1-3 + model 7 were included

† Log-transformed data; ‡ Square-root transformed data

* Variables with P > 0.2 were not included in the subsequent adjustment models

BMI, body mass index; **25(OH)D**, 25-hydroxyvitamin D; **MVPA,** moderate to vigorous physical activity; **RBC,** red blood cells; **SLJ,** standing long jump; **sTfR,** soluble transferring receptor.

Categorical variables: Seasonality (1= winter, 2= autumn, 3= spring); MVPA (0= <60 min/day, 1= ≥ 60 min/day)