

1 **TITLE PAGE**

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

4

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45 J.V. and F.R.P.L. wrote the paper; L.G.M., G.V.R., M.G.G. and L.M.A. had primary  
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49 **Running head:** Micronutrient biomarkers and fitness in adolescents

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59

60 **ABSTRACT**

61 There is a lack of studies that analyse the association between micronutrient-related  
62 biomarker status and physical fitness in adolescents. In the present study, biochemical  
63 parameters for iron and vitamin status were studied along with objective measures of  
64 physical fitness in healthy male and female European adolescents. 1089 adolescents (580  
65 girls, 12.5-17.5 years) from the HELENA cross-sectional study were included. Hierarchical  
66 linear models were performed to determine the associations between micronutrient  
67 biomarkers and physical fitness. Age, seasonality, latitude, body mass index, menarche (in  
68 girls) and physical activity were used as covariates. For cardiorespiratory fitness,  
69 concentrations of hemoglobin, retinol and vitamin C in male adolescents and,  $\beta$ -carotene and  
70 25(OH)D in female adolescents were associated with  $VO_{2max}$ . For muscular fitness,  
71 concentrations of hemoglobin,  $\beta$ -carotene, retinol and  $\alpha$ -tocopherol in male adolescents and  
72  $\beta$ -carotene and 25(OH)D in female adolescents were associated with better performance of  
73 the standing long jump test. In summary, concentrations of hemoglobin and most antioxidant  
74 vitamins in male adolescents and  $\beta$ -carotene and 25(OH)D in female adolescents were  
75 positively associated with cardiorespiratory and muscular fitness, after controlling for  
76 relevant confounders. The associations between physical fitness and iron or vitamin status  
77 observed in this cross-sectional study in adolescents should be followed up by a study  
78 specifically designed to evaluate causal relationships.

79

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

## 81 INTRODUCTION

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

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

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

110

## 111 **MATERIAL AND METHODS**

### 112 *Study design*

113 The HELENA-CSS is a European Union-funded project conducted on adolescents from 10  
114 European cities: Stockholm (Sweden), Athens and Heraklion (Greece), Rome (Italy),  
115 Zaragoza (Spain), Pecs (Hungary), Ghent (Belgium), Lille (France), Dortmund (Germany)  
116 and Vienna (Austria) (33). Detailed descriptions of the HELENA sampling and recruitment  
117 approaches, standardization and harmonization processes, data collection, analysis  
118 strategies, quality control activities and inclusion criteria have been described in detail  
119 elsewhere (32). An extended and detailed manual of operations was designed for and  
120 thoroughly read by every researcher involved in the field work before the data collection  
121 started. Parents and adolescents signed an informed consent, the protocol was approved by  
122 Research Ethics Committees of each city involved and the study has been performed  
123 following the ethical guidelines of the Declaration of Helsinki 1964 (revision of Edinburgh  
124 2000), Convention of Oviedo (1997), the Good Clinical Practice, and the legislation about  
125 clinical research in humans in each of the participating countries.

126

### 127 *Study sample*

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

131 (North/South/East/West), cultural reference and socioeconomic situation; and selection of a  
132 territorial unit (city) in the country, which had an experienced research group to perform the  
133 study. The age range considered valid for the HELENA study was 12.5-17.5 y. All the  
134 analyses conducted on the HELENA data are adjusted by a weighing factor to balance the  
135 sample according to the theoretical age distribution foreseen. A total of 3528 adolescents,  
136 1683 males and 1845 females, were considered eligible for the HELENA analyses.  
137 In the HELENA-CSS protocol, it was established that blood samples were obtained  
138 randomly in one third of the population sample. Therefore, a total of 1089 adolescents (509  
139 males and 580 females) were included in this report. To make maximum use of the data, all  
140 valid data on physical fitness components were included in this report. Consequently,  
141 sample sizes vary for the different physical fitness tests and biomarkers.

142

#### 143 *Anthropometric measurements*

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

149

#### 150 *Physical fitness tests*

151 The physical fitness components, *i.e.* muscular fitness and aerobic capacity, were assessed  
152 by the physical fitness tests previously described in detail (37). The scientific rationale for  
153 the selection of all of these tests, as well as their reliability in young people, was previously  
154 published (38). Lower-body muscular strength was assessed with the *standing long jump* test  
155 (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  $8.5\text{km}\cdot\text{h}^{-1}$ , which is increased by  $0.5\text{km}\cdot\text{h}^{-1}\cdot\text{min}^{-1}$  (1  
159 min equals one stage) (24). Léger equation was used to estimate the maximal oxygen  
160 consumption ( $\text{VO}_{2\text{max}}$ ) 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 (enzyme-linked immunosorbent assay) (19) in the Human  
180 Nutrition Laboratory of the National Research Institute on Food and Nutrition (Rome, Italy).



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

183

184 *Provitamin A ( $\beta$ -carotene), vitamin A (retinol) and vitamin E ( $\alpha$ -tocopherol) measurements*

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

192

193 *Vitamin C measurement*

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

200

201 *Vitamin B<sub>6</sub>, B<sub>12</sub> (cobalamin and holo-transcobalamin) and folate (plasma and red blood  
202 cell) measurements*

203 For the measurement of vitamin B<sub>6</sub> (pyridoxal 5'-phosphate), aliquots of EDTA whole blood  
204 were sent by cooled transport to the central laboratory and stored at -80°C until bunched  
205 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<sub>12</sub> 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<sub>12</sub> 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 4°C and the supernatant stored at -80°C till analysis.

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

237

### 238 *Seasonality*

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

247

### 248 *Latitude of residence*

249 The latitude of the study centers was also taken into account as a confounder in the analyses.  
250 The latitude of each city was obtained from <http://maps.google.es/>. Latitudes of the involved  
251 cities were: Stockholm (59°33′ N), Dortmund (51°51′ N), Ghent (51°06′ N), Lille (50°63′  
252 N), Vienna (48°21′ N), Pecs (46°07′ N), Rome (41°89′ N), Zaragoza (41°66′ N), Athens  
253 (37°98′ N) and Heraklion (35°33′ N). To make use of this data, latitudes were added to the  
254 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<sub>6</sub>, 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

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

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

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

295

## 296 **RESULTS**

297 Table 1 shows descriptive characteristics of the sample by sex.

### 298 **Micronutrient status and CRF in adolescents**

299 Results from the hierarchical linear models investigating the relationship between the  
300 adolescent CRF (assessed by  $VO_{2max}$ ), and their micronutrient-related biomarker status are  
301 presented in tables 2 and 3. In male adolescents, adjusted results showed that hemoglobin  
302 ( $\beta = 0.192$ ), retinol and vitamin C ( $\beta = 0.148$  and  $\beta = 0.186$ , respectively) were associated with  
303  $VO_{2max}$  (table 2). In female adolescents, adjusted results showed that  $\beta$ -carotene ( $\beta = 0.101$ )  
304 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 ( $\beta= 0.203$ ),  $\beta$ -carotene ( $\beta= 0.160$ ), retinol ( $\beta= 0.128$ ) and  $\alpha$ -tocopherol ( $\beta= -$   
311  $0.135$ ) were associated with SLJ (table 4). In female adolescents, adjusted results showed  
312 that  $\beta$ -carotene ( $\beta= 0.177$ ) and 25(OH)D ( $\beta= 0.125$ ) 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,  $<0.75$ mg/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  $\beta$ -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 immunocompetence 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  $\beta$ -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  $\beta$ -carotene on performance remains to be determined (29). Regarding muscular  
346 fitness (SLJ), we found positive associations with  $\beta$ -carotene (in both sexes) and retinol in  
347 male adolescents, after controlling for relevant confounders. In addition,  $\alpha$ -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  $\alpha$ -tocopherol supplementation. . However, there are no studies  
352 analyzing the relationship between  $\alpha$ -tocopherol and the lower limbs strength and therefore,  
353 the physiological factors behind cannot be elucidated, suggesting the need of more studies.

354 In recent years, vitamin D has been the most widely studied micronutrient in relation with  
355 physical fitness. The concentrations of 25(OH)D in adolescents participating in the  
356 HELENA study were recently described and published (23). In our study, we found positive  
357 associations between 25(OH)D, cardiorespiratory and muscular fitness only in female  
358 adolescents, after controlling for relevant confounders. Our results support previous  
359 evidence that shows a positive association between circulating 25(OH)D and CRF ( $VO_{2max}$ )  
360 (16, 34, 42). In addition, our results support previous studies that showed positive  
361 associations between 25(OH)D and muscular fitness (assessed through handgrip test and/or  
362 pliometric tests) in females (21, 42, 55). It has been shown that vitamin D affects skeletal  
363 muscle strength and function, acting on calcium mechanisms, cell proliferation and  
364 differentiation, preventing against insulin resistance and arachidonic acid mobilization, or  
365 other mechanisms (7, 15). However, with this study it is not possible to disentangle the  
366 mechanisms by which 25(OH)D affects cardiovascular and muscle function in our  
367 population.

368

### 369 *Limitations and strengths*

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



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

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

392

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596

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

Variables	Males				Females				
	n	Mean	SD	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 <sup>2</sup> )	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 <sub>2max</sub> (ml/Kg/min)	404	44.2	± 7.9	428	36.0	± 5.4			
<b>Iron status</b>									
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)				
<b>Antioxidant vitamins</b>									
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			
<b>Hidrosoluble vitamins</b>									
Vitamin B <sub>6</sub> (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)				
<b>Vitamin D</b>									
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<sub>2max</sub>**, 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 - $VO_{2max}$							
Model	Variables	Unadjusted analysis			Adjusted analysis		
		$\beta$	P	n	$\beta$	P*	n
1 <sup>a</sup>	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 <sup>a</sup>	BMI	-0.445	<0.001	404	-0.374	<0.001	404
3 <sup>a</sup>	MVPA	0.329	<0.001	264	0.205	<0.001	264
4 <sup>b</sup>	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 <sup>c</sup>	$\beta$ -carotene †	0.210	<0.001	355	-0.048	0.481	
	Retinol †	-0.080	0.133	349	0.148	0.031	221
	$\alpha$ -tocopherol	0.007	0.895	349	-0.050	0.417	
	Vitamin C	0.173	0.001	400	0.186	0.005	
6 <sup>d</sup>	Vitamin B <sub>6</sub> †	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 <sup>e</sup>	25(OH)D	0.113	0.029	372	0.088	0.127	250

$\beta$  is the standardized regression coefficient.

<sup>a</sup> The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

<sup>b</sup> Significant variables shown in models 1-3 + model 4 were included

<sup>c</sup> Significant variables shown in models 1-3 + model 5 were included

<sup>d</sup> Significant variables shown in models 1-3 + model 6 were included

<sup>e</sup> 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 transferrin receptor;  **$VO_{2max}$** , maximal oxygen consumption.

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



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

		Females - $\text{VO}_{2\text{max}}$					
		Unadjusted analysis			Adjusted analysis		
Model	Variables	$\beta$	P	n	$\beta$	P*	n
<b>1<sup>a</sup></b>	Age	-0.445	< <b>0.001</b>	427	-0.492	< <b>0.001</b>	
	Seasonality	0.213	< <b>0.001</b>	424	0.224	< <b>0.001</b>	424
	Latitude	0.178	< <b>0.001</b>	428	0.233	< <b>0.001</b>	
<b>2<sup>a</sup></b>	BMI	-0.321	< <b>0.001</b>	428	-0.226	< <b>0.001</b>	419
	Menarche	-0.380	< <b>0.001</b>	425	-0.101	<b>0.039</b>	
<b>3<sup>a</sup></b>	MVPA	0.100	<b>0.039</b>	296	0.039	0.413	290
<b>4<sup>b</sup></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	
<b>5<sup>c</sup></b>	$\beta$ -carotene †	0.189	< <b>0.001</b>	371	0.101	<b>0.042</b>	354
	Retinol †	-0.004	0.934	364	0.064	0.214	
	$\alpha$ -tocopherol	0.041	0.432	364	-0.029	0.540	
	Vitamin C	0.069	0.161	417	0.013	0.779	
<b>6<sup>d</sup></b>	Vitamin B <sub>6</sub> †	0.130	<b>0.010</b>	388	0.026	0.572	366
	Cobalamin †	0.203	< <b>0.001</b>	403	0.016	0.785	
	Holo-Transcobalamin †	0.102	<b>0.040</b>	409	0.067	0.231	
	Plasma folate †	0.108	<b>0.030</b>	402	-0.051	0.387	
	RBC folate †	0.109	<b>0.030</b>	398	0.084	0.156	
<b>7<sup>e</sup></b>	25(OH)D	0.027	0.593	396	0.091	<b>0.030</b>	391

$\beta$  is the standardized regression coefficient

<sup>a</sup> The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

<sup>b</sup> Significant variables shown in models 1-3 + model 4 were included

<sup>c</sup> Significant variables shown in models 1-3 + model 5 were included

<sup>d</sup> Significant variables shown in models 1-3 + model 6 were included

<sup>e</sup> 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 transferrin receptor; **VO<sub>2max</sub>**, maximal oxygen consumption.

**Categorical variables:** Seasonality (1= winter, 2= autumn, 3= spring); MVPA (0= <60 min/day, 1=  $\geq$  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							
Model	Variables	Unadjusted analysis			Adjusted analysis		
		$\beta$	P	n	$\beta$	P*	n
1 <sup>a</sup>	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 <sup>a</sup>	BMI	-0.256	<0.001	463	-0.291	<0.001	462
3 <sup>a</sup>	MVPA	0.142	0.014	300	0.132	0.010	300
4 <sup>b</sup>	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 <sup>c</sup>	$\beta$ -carotene †	0.227	<0.001	412	0.160	0.013	
	Retinol †	0.241	<0.001	404	0.128	0.039	254
	$\alpha$ -tocopherol	-0.063	0.204	404	-0.135	0.017	
	Vitamin C	0.109	0.019	458	-0.003	0.965	
6 <sup>d</sup>	Vitamin B <sub>6</sub> †	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 <sup>e</sup>	25(OH)D	0.078	0.105	430	0.085	0.120	285

$\beta$  is the standardized regression coefficient

<sup>a</sup> The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

<sup>b</sup> Significant variables shown in models 1-3 + model 4 were included

<sup>c</sup> Significant variables shown in models 1-3 + model 5 were included

<sup>d</sup> Significant variables shown in models 1-3 + model 6 were included

<sup>e</sup> 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 transferrin receptor.

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

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

		<b>Females - SLJ</b>					
		Unadjusted analysis			Adjusted analysis		
<i>Model</i>	<i>Variables</i>	$\beta$	<i>P</i>	<i>n</i>	$\beta$	<i>P</i> *	<i>n</i>
<b>1<sup>a</sup></b>	Age	0.177	<b>&lt;0.001</b>	536	0.142	<b>0.001</b>	
	Seasonality	0.052	0.231	531	0.000	0.998	531
	Latitude	0.299	<b>&lt;0.001</b>	537	0.284	<b>&lt;0.001</b>	
<b>2<sup>a</sup></b>	BMI	-0.336	<b>&lt;0.001</b>	537	-0.313	<b>&lt;0.001</b>	532
	Menarche	-0.037	0.400	533	-0.082	0.090	
<b>3<sup>a</sup></b>	MVPA	0.143	<b>0.006</b>	373	0.108	<b>0.021</b>	369
<b>4<sup>b</sup></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	<b>0.036</b>	489	-0.020	0.709	
<b>5<sup>c</sup></b>	$\beta$ -carotene †	0.257	<b>&lt;0.001</b>	470	0.177	<b>0.003</b>	305
	Retinol †	-0.048	0.298	464	-0.080	0.182	
	$\alpha$ -tocopherol	0.029	0.534	465	0.003	0.954	
	Vitamin C	0.114	<b>0.009</b>	512	-0.019	0.727	
<b>6<sup>d</sup></b>	Vitamin B <sub>6</sub> †	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	
<b>7<sup>e</sup></b>	25(OH)D	0.081	0.070	501	0.125	<b>0.010</b>	350

$\beta$  is the standardized regression coefficient

<sup>a</sup> The effect of each variable on the outcome was adjusted for other variables in the same model or above in the hierarchical model

<sup>b</sup> Significant variables shown in models 1-3 + model 4 were included

<sup>c</sup> Significant variables shown in models 1-3 + model 5 were included

<sup>d</sup> Significant variables shown in models 1-3 + model 6 were included

<sup>e</sup> 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 transferrin receptor.

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