Cardiovascular Fitness Is Negatively Associated With Homocysteine Levels in Female Adolescents

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Objective: To examine the association between cardiovascular fitness and homocysteine levels in adolescents.

Design: Cross-sectional study.

Setting: Madrid, Murcia, Granada, Santander, and Zaragoza, Spain.

Participants: One hundred fifty-six Spanish adolescents (76 boys and 80 girls) aged (mean ± SD) 14.8 ± 1.4 years.

Main Exposures: Cardiovascular fitness was measured by the 20-m shuttle run test. Pubertal stage, birth weight, smoking status, and socioeconomic status were determined, and the sum of 6 skinfold thickness measurements, and serum folic acid and vitamin B12 levels were measured. Methylenetetrahydrofolate reductase (MTHFR; 677C>T genotype) polymorphism was done by DNA sequencing.

Main Outcome Measure: Fasting homocysteine levels.

Results: Mean values of homocysteine were significantly higher in the MTHFR 677CT and TT genotype subgroups compared with the CC genotype subgroup in adolescent boys, whereas in adolescent girls, mean values of homocysteine were significantly higher in the MTHFR 677CT and TT genotype subgroup compared with the CC and CT genotype subgroups. Multiple regression analyses showed that cardiovascular fitness was significantly associated with homocysteine levels in female adolescents after controlling for potential confounders including the MTHFR 677C>T genotype (β = −0.40; semipartial correlation = −0.35; P = .007). No associations were found between cardiovascular fitness and homocysteine levels in male adolescents (β = 0.12; semipartial correlation = 0.08; P = .51).

Conclusion: The results suggest that cardiovascular fitness is negatively associated with homocysteine levels in female adolescents after controlling for potential confounders including MTHFR 677C>T genotype.

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Homocysteine has been suggested to be an independent risk factor for several multisytem diseases, including coronary heart disease, stroke, dementia, and Alzheimer disease, as well as for risk of hip fracture and pregnancy complications. Moreover, elevated homocysteine levels have been associated with increased oxidative stress and endothelial damage, although the mechanisms are not yet clarified. In children, elevated homocysteine levels are positively associated with cardiovascular disease in their parents, grandparents, and other relatives. Homocysteine levels are influenced by modifiable and nonmodifiable factors. Among the nonmodifiable factors, age and sex seem to have a specific role. Levels of homocysteine are higher in adolescent boys than in adolescent girls, and this sex effect seems to be enhanced during and after puberty. Genetic factors also seem to affect homocysteine levels. Elevated levels of homocysteine can be caused by mutations in enzymes involved in homocysteine metabolism, which give dysfunctional enzymes, for example, the single-nucleotide polymorphism at position 677 in the methylenetetrahydrofolate reductase (MTHFR) gene for MTHFR. Methylenetetrahydrofolate reductase is a key enzyme in homocysteine metabolism. The common polymorphism 677C>T gives a thermolabile form of the enzyme. Subjects homozygous for this mutation (or TT genotype) have higher levels of homocysteine compared with subjects with CC or CT genotypes.

Deficient serum levels of both folic acid and vitamin B12 have been associated with elevated homocysteine levels in children, adults, and elderly persons. Lifestyle factors such as smoking, lack of physical activity, excessive alcohol intake, and obesity have been associated with elevated levels of homocysteine in adults.

Poor cardiovascular fitness (CVF) is another important risk factor for cardiovascular disease and is a predictor of morbidity and all-cause mortality. Kuo et al have recently described a significant negative association between CVF and homocysteine levels in women.
Cardiovascular fitness has been negatively associated with features of metabolic syndrome in children and adolescents,
and with plasma lipid profile in both overweight and nonoverweight adolescents. However, studies examining the association between CVF and homocysteine levels in adolescents are lacking. We hypothesized that there would be a negative correlation between CVF and homocysteine levels in adolescents. For public health strategies and preventive purposes, it is of interest to understand the relative influence of modifiable factors on homocysteine levels from an early age.

**METHODS**

**PARTICIPANTS**

The study participants were a subsample of the AVENA (Alimentación y Valoración del Estado Nutricional de los Adolescentes Españoles [Food and Assessment of the Nutritional Status of Spanish Adolescents]) study, which was designed to assess the health and nutritional status of adolescents. The AVENA study design has been reported in detail elsewhere. Data were collected from November 6, 2000, to June 28, 2002, in 5 Spanish cities: Madrid, Murcia, Granada, Santander, and Zaragoza. Data in the present article are from adolescents in whom both homocysteine levels and MTHFR genotypes were measured (n = 156; 76 boys and 80 girls).

A comprehensive verbal description of the nature and purpose of the study was given to both the adolescents and their teachers. Written consent to participate was requested from parents and adolescents. Adolescents with a personal history of cardiovascular disease, who were taking medication at the time of the study, or who were pregnant were excluded. The study protocol was performed in accord with the ethical standards established in the 1961 Declaration of Helsinki (as revised in Hong Kong in 1989 and in Edinburgh, Scotland, in 2000) and was approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla, Santander.

Before any testing was performed, the parents completed a questionnaire, part of which addressed the adolescent’s previous and current health status. Socioeconomic status was also assessed in the questionnaire and was defined by the educational achievement and occupation of the father. According to this information and following the recommendation of the Spanish Society for Epidemiology, the adolescents were classified into 5 socioeconomic categories: low, medium low, medium, medium high, and high. Smoking status at the time of the study was reported via questionnaire completed by the adolescents, and they were categorized as smoker, nonsmoker, and occasional smoker (ie, once a week).

**PHYSICAL EXAMINATION**

Anthropometric measurements were obtained as described elsewhere. In brief, skinfold thickness was measured to the nearest 0.2 mm at the biceps, triceps, subscapular, suprailliac, thigh, and calf on the left side of the body. The sum of the 6 skinfold thicknesses was used as an indicator of body fat. These measurements correlate highly with measured body fat percentage in adolescents of similar ages as measured with dual-energy x-ray absorptiometry.

Identification of pubertal stage was assessed according to the method of Tanner and Whitehouse. Self-reported breast development in adolescent girls and genital development in adolescent boys was used for pubertal stage classification.

**MEASUREMENT OF CVF**

Cardiovascular fitness was assessed by the 20-m shuttle run test as previously described. In brief, participants were required to run between 2 lines 20 m apart while keeping pace. Running pace was determined by audio signals emitted from a prerecorded cassette tape. The initial speed was 8.5 km/h, which was increased by 0.5 km/h per minute (1 minute equal to 1 stage). The tape used was calibrated over 1 minute. Subjects were instructed to run in a straight line, to pivot on completing a shuttle, and to pace themselves in accord with the audio signals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on 2 consecutive occasions or when the subject stopped because of fatigue. All measurements were carried out under standardized conditions on an indoor rubber-floored gymnasium. Constant vocal encouragement was given to participants throughout the test. All participants were familiar with the test because the 20-m shuttle run test is one of the fitness tests included in the physical education curriculum in Spain. Adolescents were instructed to abstain from strenuous exercise in the 48 hours preceding the test.

Cardiovascular fitness was considered as the number of stages completed (precision of 0.5 steps) for being the most direct measurement obtained. Moreover, for the purpose of comparing the results with those of previous publications, maximal oxygen consumption (V\text{O}_\text{max}, milliliters per kilogram per minute) was estimated by the Leger equation, where

\[
V\text{O}_\text{max} = 31.025 + [3.2385 - 20.65+1.3638A] + 0.1366S, \text{ where } A \text{ is age and } S \text{ is final speed} (S = 8 + 0.5 \times \text{number of stages completed} ).
\]

The reliability and validity of this test has been shown in young persons.

**HOMOCYSTEINE, SERUM FOLIC ACID, AND VITAMIN B\textsubscript{12} ASSAYS**

With the subject in the supine position, blood samples were obtained by venipuncture after an overnight fast, using vacuum tubes (Vacutainer; Becton, Dickinson and Co, Franklin Lakes, NJ), and placed on ice immediately. The fasting state was verbally confirmed by the subject before blood sampling. All samples were processed within 1 hour by centrifugation, divided into aliquots, and the portions stored at −80°C until withdrawn for analysis. Homocysteine in acidified citrated plasma was assayed using a fluorescence polarization immunoassay on an IMX unit (Abbott Laboratories, Abbott Park, Ill). Serum folic acid and vitamin B\textsubscript{12} levels were measured using the fluorometric method with an IMX automatic analyzer (Abbott Laboratories).

**MTHFR GENOTYPING**

Total blood DNA was extracted and purified from 500 µL of whole blood anticoagulated with EDTA using the Quiagen procedure described by Higuchi. Genotyping of the 677C>T variant in the human MTHFR gene was performed by means of polymerase chain reaction and allele-specific restriction digestion of the amplified products with the restriction enzyme Hinf1 (GE Healthcare, Buckinghamshire, England), as previously described by Froost et al.

**STATISTICAL ANALYSIS**

Data are given as mean ± SD unless otherwise indicated. After serum folic acid and vitamin B\textsubscript{12} concentrations were normalized by natural logarithm transformation, all of the residuals showed a satisfactory pattern.

The effect on homocysteine levels of sex and MTHFR 677C>T were analyzed by 1-way analysis of variance because there was a significant interaction between sex and MTHFR
The subgroup means were compared using the Tukey test. After bivariate correlation analysis, multiple regression analyses were used to study the relation between homocysteine levels and CVF after controlling for potential confounders. We used an extended-model approach: Model 1 examined the influence of CVF on homocysteine levels after controlling for age, pubertal stage, birth weight, smoking status, socioeconomic status, and the sum of 6 skinfold measurements. Model 2 examined the influence of CVF on homocysteine levels after controlling for the confounders included in model 1 plus serum folic acid and vitamin B12 levels. Model 3 examined the influence of CVF on homocysteine levels after controlling for the cofounders included in model 1 and model 2 plus the MTHFR 677C>T genotype. Semipartial correlation was used as a measure of the relationship between CVF and homocysteine levels after controlling for the effect that 1 or more additional variables (eg, age or birth weight) had on one of those variables. The analyses were performed using Statistical Package for Social Sciences software (version 14.0 for Windows; SPSS Inc, Chicago, Ill), and the level of significance was set at $P = .05$.

### RESULTS

#### DATA COMPLETENESS AND BASELINE CHARACTERISTICS

Both homocysteine levels and the MTHFR genotype were measured in 156 adolescents (76 boys and 80 girls). Of these, 23% of the adolescents refused to continue the 20-m shuttle run test because of discomfort or distress, and their results are not included in the final data sample. The observed power for the sample size was 0.40. Pubertal stage was obtained from 96% of the subjects, and skinfold thickness data from 94%. Birth weight, socioeconomic status, and smoking status were available for 93%, 87%, and 71% of the subjects, respectively.

The descriptive characteristics of the study sample are given in Table 1. Adolescent boys were significantly heavier and taller than adolescent girls, and girls had significantly higher skinfold thicknesses. Adolescent boys had significantly...
higher levels of homocysteine, lower levels of serum vitamin B₁₂, and significantly higher CVF levels (Table 1). The results of the regression models using the homocysteine level as the outcome variable are given in Table 2. The results of the regression models using the homocysteine level as the outcome variable are given in Table 2. The results of the regression models using the homocysteine level as the outcome variable are given in Table 2. The results of the regression models using the homocysteine level as the outcome variable are given in Table 2. The results of the regression models using the homocysteine level as the outcome variable are given in Table 2.
found only in female adolescents. Our findings support those of a previous study that examined the relationship between homocysteine levels and CVF in adults.\textsuperscript{27} Kuo et al\textsuperscript{27} showed that high homocysteine levels were negatively associated with estimated CVF in women. However, they did not find any association in men, which is in accord with our results. These results suggest that sex hormones may have a role in mediating the CVF-homocysteine association, exerting different effects in female and male subjects; however, further studies to determine whether this is the case are needed. One longitudinal study observed 499 independent community-dwelling elderly persons for 3 years and found that those with elevated homocysteine levels were at increased risk of decline in physical function.\textsuperscript{48} However, CVF data were not provided and a comparison by sex was not performed.

None of the previous studies included the MTHFR 677C>T genotype, which affects homocysteine levels.\textsuperscript{16,19} Balasa et al\textsuperscript{16} found that the MTHFR 677C>T polymorphism was an independent determinant of homocysteine levels in 197 healthy US children aged 6 months to 16 years. Similarly, Papoutsakis et al\textsuperscript{19} reported in a sample of healthy Greek children that the TT genotype was associated with homocysteine concentrations. Homocysteine levels in our study sample were significantly higher in the MTHFR 6777C>T and TT genotype subgroups compared with the CC subgroup in adolescent boys, whereas in adolescent girls, mean values of homocysteine were significantly higher in the TT genotype subgroup compared with the CC and CT genotype subgroups.

In the present study, CVF was objectively measured by the 20-m shuttle run test. We did not have a direct measurement of VO\textsubscript{2max}, the most valid method of measuring CVF. However, from a practical point of view, field tests may be a better option than laboratory testing, especially in epidemiologic studies, because a large number of subjects can be tested at the same time, which enhances the motivation of the participants, and the tests are simple, safe, and often the only feasible choice, especially in school settings. The 20-m shuttle run test meets these criteria. Cardiovascular fitness was considered as the number of stages completed in the 20-m shuttle run test. However, CVF estimated from the Leger equation (VO\textsubscript{2max}, milliliters per kilogram per minute) was also provided for the purpose of making comparisons with other studies possible. When the analyses were performed using VO\textsubscript{2max} or speed (kilometers per hour) rather than the number of stages as the measurement of CVF, similar results were obtained.

Results from cross-sectional studies have shown associations between homocysteine levels and lifestyle-related factors.\textsuperscript{20,22-28} However, findings are different when analyzed prospectively.\textsuperscript{49,50} Duncan et al\textsuperscript{49} found that 6 months of exercise increased homocysteine levels in sedentary adults, whereas Randeva et al\textsuperscript{51} showed that 6 months of sustained brisk walking for 20 to 60 minutes 3 days a week significantly decreased homocysteine levels and increased CVF in young overweight and obese women with polycystic ovary syndrome, a group at increased risk of premature atherosclerosis. Similarly, a weight-reduction program that included physical activity had a positive effect on the homocysteine levels in obese children.\textsuperscript{52} Together, these results suggest that modifications in lifestyle-related factors may influence homocysteine levels in a different manner in children and adolescents than in adults.

The results from the present study should be interpreted with caution because of the limitations of the cross-sectional design; that is, direction of causality cannot be determined. Elevated homocysteine levels may be simply a marker of an unhealthy lifestyle that is associated with poor exercise capacity. The relationship between homocysteine levels and CVF should be studied prospectively. It must be borne in mind that the subjects in this study were healthy adolescents with no previously diagnosed cardiovascular disorders. Also, our study included a moderate number of participants. The observed power for the sample size was low (0.40), which may have masked the association between CVF and homocysteine levels in the adolescent boys. This warrants further investigation. However, we believe that covariates that may confound the measures of association in our study were appropriately considered and controlled for.

The results of this study suggest that CVF is negatively associated with homocysteine levels in adolescent girls after controlling for potential confounders including the MTHFR 677C>T genotype. These results should stimulate a debate on whether the metabolism of homocysteine could be one way in which the benefits of high CVF levels are exerted.

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REFERENCES

Why Is the Game Called Cat’s Cradle?

The term was originally cratch-cradle, and cratch is from Middle English crêche, meaning a rack in which hay is put for cattle. The first figure created with the string in cat’s cradle looks like a cratch.

—From Why Do We Say It? Castle Books, 1985