ORIGINAL ARTICLE

Homocysteine Concentrations and Molecular Analysis in Patients with Congenital Heart Defects

Luciano C. Galdieri, Santiago R. Arrieta, Célia M.C. Silva, Carlos A.C. Pedra, and Vânia D’Almeida

Department of Pediatrics, Department of Medicine, and Department of Health Sciences, Universidade Federal de São Paulo UNIFESP/EPM, São Paulo, Brazil

Instituto Dante Pazzanese de Cardiologia, São Paulo, Brazil

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Background. Congenital heart defects are the result of incomplete heart development and, like many diseases, have been associated with high homocysteine concentration.

Methods. We evaluated homocysteine, folic acid and vitamin B12 concentrations, and the mutations 677C>T and 1298A>C in MTHFR, 844ins68 in CBS and 2756A>G in MTR genes in 58 patients with congenital heart defects, 38 control subjects, and mothers of 49 patients and 26 controls.

Results. Control and patients presented normal range concentrations for homocysteine (7.66 ± 3.16 μM and 6.95 ± 3.12 μM, respectively), folic acid (8.31 ± 3.00 ng/mL and 11.84 ± 10.74 ng/mL) and vitamin B12, (613.56 ± 307.57 pg/mL and 623.37 ± 303.12 pg/mL), which did not differ among groups. For the mothers studied, homocysteine and vitamin B12 concentrations also did not differ between groups. However, folic acid concentrations of mothers showed significant difference, the highest values being in the group of patients. No difference was found in allele frequencies among all groups studied.

Conclusions. In the studied groups, high homocysteine seems not to be correlated with congenital heart defects, as well as folic acid and vitamin B12. The mutations studied, in isolation, were not related to congenital heart defects, but high concentration of maternal homocysteine is associated with the presence of three or four mutated alleles. © 2007 IMSS. Published by Elsevier Inc.

Key Words: Homocysteine, Folic acid, Methylenetetrahydrofolate reductase, Methionine synthase, Cystathionine-β-synthase, Congenital heart defects.

Introduction

Many diseases have been associated with high plasma homocysteine concentration; however, the situation seems to be more evident in cases of neural tube defects and cardiovascular diseases (1–3). Other malformations related to neural crest are also influenced by high homocysteine concentrations, such as cleft palate and congenital heart defects (4,5).

Congenital heart defects are mainly the result of incomplete development of the heart during the first 6 weeks of pregnancy (5). It can be associated with a genetic syndrome (e.g., Down Syndrome) or be isolated (multifactorial origin), meaning that there is a presumed genetic basis that is susceptible to or modified by environmental factors (6).

Goldmuntz et al. (7) mentioned that congenital heart defects are the most common major malformations, occurring in 4–8/1000 live births in the U.S., being the major cause of infant mortality due to congenital abnormalities (8). The incidence of congenital heart defects does not vary...
by race; however, the frequency of certain lesions varies according to gender.

Data from the Latin American Collaborative Study of Congenital Malformations (ECLAMC) from 1967–1997 showed 845 congenital heart defects from a total of 34,102 children with congenital abnormalities (total of 2,027,270 births) (9). A Brazilian study showed that the prevalence of congenital heart defects is 5.49/1000 live births (10).

The association between folic acid deficiency and congenital malformations is well known, in animal (11) and epidemiological studies (12–15) alike. Folic acid deficiency can cause neural tube defects (16) as well as congenital heart defects (6).

Several studies have demonstrated that the supplementation of multivitamins with folic acid reduces the occurrence of neural tube defects (11–15).

Several studies have suggested that neural crest cells might be particularly susceptible to the teratogenic effects of homocysteine (5,6,11), a biomarker of folate metabolism. Data reported by Tierney et al. (16) show that elevated homocysteine directly disrupts normal neural crest cell formation in vivo and that homocysteine treatment decreased the number of these cells and increased the number of neural tube cells.

The genetic background of individuals is another factor to consider when evaluating homocysteine concentrations. In this respect, the enzymes 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine-β-synthase (CBS), methionine synthase (MTR), and methionine synthase reductase (MTRR) can also interfere in its concentration (17,18). These factors had already been related to neural tube defects and congenital heart defects, as the mutations present in these patients (2,19) were the same in their respective mothers (6,20).

The aim of this work was to evaluate concentrations of plasma homocysteine, folic acid, and vitamin B12 as well as mutations in the genes of the enzymes MTHFR, MTR, and CBS, involved in homocysteine metabolism and to relate them with the etiology of congenital heart defects.

Materials and Methods

Patients

The study was performed using samples of patients and control subjects from the São Paulo Hospital, and Federal University of São Paulo (UNIFESP/EPM) and from the Institute of Cardiology “Dante Pazzanese”.

The 58 selected patients, aged between 0 and 11 years, presented isolated cardiopathies (not associated with genetic syndromes or other malformations). All patients had their diagnoses confirmed by echocardiogram or cardiac catheterization.

All patients’ mothers were invited to participate and complete a clinical questionnaire on gestation, childbirth, education, occupation and clinical history, as well as diet and folic acid supplementation during pregnancy. Race was assigned based on morphological phenotype and family origin. Latin (mainly Portugal, Italy, and Spain) or Europeans (Germany, Holland, Hungary, Poland, Lebanon) were classified as whites. Non-whites include pure black people, pure Amerindians or biracial children. This ethnic characterization is the same used by the 1993 World Health Organization Latin America Study of Congenital Malformations.

The control group was composed of 38 healthy children, observing the following exclusion criteria: presence of congenital malformations (cardiopathies, defects of the neural tube, cleft lip, obstructive uropathies, and alterations of limbs). Mothers who presented diabetes, hypertension, specific hypertension disease of pregnancy, cardiopathies, systemic lupus erythematosus, renal insufficiency, and HIV were excluded from both groups.

The protocol was approved by the ethics committees of UNIFESP/EPM (CEP n˚1303/01) and Institute of Cardiology “Dante Pazzanese” (CEP no. 3120/2002), and written informed consent was obtained from all mothers before participation.

Methods

Blood samples were collected into vacuum tubes without anticoagulant and containing EDTA and immediately chilled on ice. Aliquots of the serum and plasma were stored at −80°C until determinations.

Total plasma homocysteine concentrations were determined by high performance liquid chromatography (HPLC) with fluorimetric detection and isocratic elution as reported by Pfeiffer et al. (21).

Serum folic acid and vitamin B12 measurements were performed in the Laboratory of Clinical Analysis Vitae, using HPLC and radioimmunoassay methods, respectively.

Genomic DNA extraction was performed using peripheral blood collected into tubes containing EDTA from all children and mothers, according to the methodology described by Miller et al. (22).

Genotyping for the mutations 677C>T and 1298A>C of MTHFR and 2756A>G of MTR were performed by polymerase chain reaction (PCR) followed by digestion by the enzymes HinfI (23), MboII (24), and HaeIII (25), respectively. The diagnosis of the mutation 844ins68 of CBS was performed by PCR as described by Tsai et al. (26).

All statistical analyses were carried out using statistical analysis software (SAS-Statistical Analysis System for Windows, version 6.12, SAS Institute Inc., 1989–1996, Cary, NC), with the level of significance set at p <0.05. The statistical analysis was performed using chi-square test or Fisher’s exact test (expected values <5), Mann-Whitney
test, and Kolmogorov-Smirnov test. To assess between-group differences we performed a stepwise multiple logistic regression.

Results

Characteristics of the Population

Blood samples of 58 patients and 38 control subjects, coming from the São Paulo Hospital and Institute of Cardiology “Dante Pazzanese”, which assists people with the same socioeconomic and demographic backgrounds, were collected. Also, 49 samples of patients’ mothers and 26 samples of control mothers were obtained. The mean age (years ± SD) of the children was 4.44 ± 3.31 for the control group and 3.27 ± 3.14 for the patients. The mean (years ± SD) age of the mothers was 29.27 ± 6.64 for the control group and 29.18 ± 7.03 for the patients.

The racial composition of the children was as follows: the control group was comprised of 52.63% white and 47.37% non-white subjects; the patients’ group was comprised of 22.41% white and 77.59% non-white subjects. We found significant differences for the racial component between the two groups. The highest prevalence of non-white was in the group of patients ($\chi^2 = 9.29; DF = 1; p = 0.0023$).

Biochemical Measurements

All the collected samples were quantified for homocysteine values. Folic acid and vitamin B$_{12}$ were quantified in part of the samples because, in some cases, the amount of sample collected was insufficient for the quantification of both vitamins (Table 1).

We did not find significant differences in the average values of homocysteine (Kruskal-Wallis $p = 0.284$), folic acid (Kruskal-Wallis $p = 0.638$) and vitamin B$_{12}$ (Kruskal-Wallis $p = 0.887$) among the groups of children. We also did not find significant differences in the values of homocysteine (Kruskal-Wallis $p = 0.138$) and vitamin B$_{12}$ (Kruskal-Wallis $p = 0.2693$) among the groups of mothers. We observed, however, significant differences in folic acid concentrations between the groups of mothers (Kruskal-Wallis $p = 0.00052$; Kolmogorov-Smirnov test $p < 0.001$); the highest values were found in the group of patients’ mothers.

Stepwise multiple logistic regression analysis including the biochemical parameters revealed that maternal folic acid presented a significant association ($p = 0.009$, OR = 1.34; 95% CI = 1.07—1.67).

Molecular Analysis

The distribution of the mutated alleles in children and mothers is shown in Table 2. There were no significant differences ($\chi^2$ and Fisher’s exact test) in the frequencies between patients and control subjects or in the frequencies between groups of mothers.

The Hardy-Weinberg equation showed that all mutations were in equilibrium in the patients’ and control groups as well as in the mothers’ groups (data not shown).

Biochemical and Molecular Relationships

We compared the biochemistry parameters (homocysteine, folic acid and vitamin B$_{12}$ concentrations) and the different genotypes for the studied mutations using Kruskal-Wallis test for three categories (wild-type, homozygote mutated and heterozygote) and Mann-Whitney test for two categories (wild-type vs. heterozygote and homozygote mutated). However, we found a reduction trend in folic acid concentration in the control group with genotype TT (TT: 5.51 ± 2.07; CT: 8.76 ± 3.04; CC: 8.96 ± 2.85; Kruskal-Wallis $p = 0.0586$) when compared with the other genotypes (CT, CC) of the MTHFR gene. We also found statistical difference in the concentration of vitamin B$_{12}$ (AG: 353.60 ± 251.29; GG: 461.00; AA: 734.58 ± 272.81; Mann-Whitney $p = 0.0130$), as the concentration of children with genotypes AG and GG was lower than those with genotype AA of the MTR gene.

We also performed a comparison of the biochemical variables (homocysteine, folic acid and vitamin B$_{12}$) for children and mothers considering the number of mutated alleles. In some cases, to perform the analysis some categories were grouped due to their small frequency (e.g.,

<table>
<thead>
<tr>
<th>Table 1. Homocysteine, folic acid and vitamin B$_{12}$ concentrations in the groups of children and their mothers</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Children</td>
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<tr>
<td>Control subjects</td>
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<tr>
<td>Patients</td>
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<tr>
<td>Mothers</td>
</tr>
<tr>
<td>Control subjects</td>
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<tr>
<td>Patients</td>
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</tbody>
</table>

Data are mean ± SD.
Hcy, homocysteine.
$p < 0.05$ (Kruskal-Wallis test).
heterozygote and homozygote mutated subjects, or three and four mutated alleles). Significant difference was found in the concentration of homocysteine depending on the number of mutated alleles in patients (Table 3), as well as in their mothers (Table 4).

### Discussion

Control children were selected in accordance with exclusion criteria, avoiding children and respective mothers with possible factors that could modify homocysteine concentration. We studied patients up to 11 years old, an age at which values of homocysteine do not vary significantly. Increases occur only after the age of 11 (27).

Mothers did not mention the use of substances that could intervene with the biochemical parameters either in the critical period of neural crest formation (28) or in the period of blood collection. No medical incident occurred during pregnancies that could justify the presence of cardiopathies.

The study of the population in the U.S. (14) showed greater prevalence of whites among patients with congenital heart defects—the opposite of what we observed in our sample. However, it must be considered that, even with the adopted criteria, the lack of information about the ethnic origin of our population makes the division of the sample difficult in accordance with race. Data of Alves-Silva et al. (29) showed that the Brazilian population is highly heterogeneous and even the Brazilian Caucasian population has great Amerindian and African contributions by maternal ancestry and Portuguese contributions for paternal ancestry. However, many of the participants of this study were told to ignore any European, African or Amerindian component in their families.

Many other studies involving congenital cardiopathies investigated homocysteine only in the mothers, either in the plasma or in the amniotic fluid. We understand that it is also important to study the patients because our group had previously demonstrated high concentration of plasma homocysteine in children with spina bifida, suggesting homocysteine to be considered a risk factor for this injury (1).

Studying the amniotic fluid, Steegers-Theunissen et al. (30), as well as Wenstrom et al. (2), concluded that high concentrations of homocysteine must have an important role in the etiology of neural tube defects. Malinow et al. (31) showed a reduction of homocysteine concentrations between the umbilical vein and umbilical artery, suggesting a consumption of this amino acid by the embryo. Therefore, maternal homocysteine, either in the blood or amniotic fluid, can have implications in fetal development.

When the group of mothers was studied, we did not find an increase in homocysteine concentration. These data are not in accordance with the results reported by Kapusta et al. (5) who found an increase of 26.6% in mothers’ homocysteine concentration of 27 patients with congenital heart defects.

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>MTR</th>
<th>CBS</th>
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<tbody>
<tr>
<td>677C&gt;T</td>
<td>2756A&gt;G</td>
<td>844A&gt;G</td>
</tr>
<tr>
<td>Children</td>
<td>Mothers</td>
<td>Children</td>
</tr>
<tr>
<td>Subjects</td>
<td>Patients</td>
<td>Subjects</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>CC</td>
<td>18 (47.37%)</td>
<td>10 (38.46%)</td>
</tr>
<tr>
<td>CT</td>
<td>14 (36.84%)</td>
<td>15 (57.70%)</td>
</tr>
<tr>
<td>TT</td>
<td>6 (15.79%)</td>
<td>1 (3.84%)</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>CC</td>
<td>19 (50.00%)</td>
<td>15 (57.70%)</td>
</tr>
<tr>
<td>CT</td>
<td>16 (42.11%)</td>
<td>15 (57.70%)</td>
</tr>
<tr>
<td>TT</td>
<td>1 (2.80%)</td>
<td>1 (3.84%)</td>
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| MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; CBS, cystathionine-\(\beta\)-synthase.
Table 3. Comparison of the biochemical variables for patients considering the number of mutated alleles

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<th>3–4</th>
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<tbody>
<tr>
<td>Hcy (µM)</td>
<td>5.00 ± 1.64* (n = 7)</td>
<td>7.44 ± 3.16 (n = 18)</td>
<td>7.72 ± 3.38 (n = 23)</td>
<td>5.50 ± 2.39 (n = 9)</td>
</tr>
<tr>
<td>Folic acid (ng/mL)</td>
<td>6.35 ± 3.54 (n = 6)</td>
<td>7.47 ± 5.82 (n = 13)</td>
<td>12.00 ± 9.82 (n = 20)</td>
<td>22.65 ± 16.34 (n = 8)</td>
</tr>
<tr>
<td>Vitamin B12 (pg/mL)</td>
<td>618.40 ± 20.76 (n = 5)</td>
<td>607.08 ± 352.22 (n = 13)</td>
<td>668.30 ± 336.23 (n = 20)</td>
<td>540.63 ± 178.58 (n = 8)</td>
</tr>
</tbody>
</table>

*p = 0.0405 (Kruskal-Wallis).

heart defects, compared with 56 control women. Hobbs et al. (32) also showed increased levels of homocysteine in 224 patients with congenital heart defect, when compared with 90 controls.

The high concentrations of folic acid in the groups of patients’ mothers, compared to the group of control mothers, differ from our expectations because folic acid is considered a prevention factor for congenital malformations. Shaw et al. (33), studying the folate carrier polymorphism 80A>G that raises the concentrations of plasma folic acid, found a high risk of conotruncal heart defects in children with mutated alleles. Thus, even suitable concentrations of plasma folic acid may not enter the cells, being unavailable to act in the diverse biochemical reactions leading to normal development of the heart.

The concentrations of vitamin B12 did not differ significantly between the studied groups of mothers in accordance with data presented by Hobbs et al. (32). van Roooji et al. (34) found lower concentrations of vitamin B12 in patients with cleft palate, when compared with the control group. In our study, vitamin B12 does not seem to be responsible for the etiology of congenital heart defects.

The analysis of all genotypes studied (i.e., MTHFR 677C>T and 1298A>C; MTR 2756A>G; CBS ins68pb) did not show significant differences among the children or mothers. Perez et al. (1), studying children with neural tube defects in a sample of the Brazilian population, did not find association of mutations 677C>T and 1298A>C in the children or in their respective mothers.

Arruda et al. (35) found a prevalence of 10% of white people with mutated allele 677T and only 1.45% in blacks and 1.2% in native Brazilians (Indians) with the same mutation. Considering the greater prevalence of non-whites in our study, we believe that this could interfere in the amount of mutated alleles 677T in the patients and control subjects studied. It could be one of the reasons for the absence of correlation between mutated genotypes and congenital heart defects.

Analyzing the biochemical parameters in relation to genotype MTR 2756A>G, we did not find correlations with homocysteine and folic acid concentrations either, but we found low concentrations of vitamin B12 in the group of control heterozygote children and mutated homozygote when compared to wild-type homozygote genotype (AG: 353.60 ± 251.29 GG: 461.00 vs. AA: 734.58 ± 272.81; Mann-Whitney p = 0.0130). This fact may be explained by the low concentrations of vitamin B12 found in our population, as suggested by Guerra-Shinohara et al. (36). In fact, we believe that these alterations, concerning folic acid as well as vitamin B12 concentrations, are related with reduced intake of these vitamins by the control group and are not due to the genetic background of our sample.

Differences between biochemical parameters and the presence of the genotype CBS ins68bp were not observed in all groups. Although this mutation is considered one of the causes of hyperhomocysteinemia (37), in our sample this did not happen.

Evaluating the amount of mutated alleles and biochemical parameters, we did not find significant differences in the control group and their respective mothers. We can conclude that, in these groups, the amount of mutated alleles in the same person did not influence homocysteine, folic acid, and vitamin B12 concentrations. However, when evaluating the groups of patients and their respective mothers, we observed that children who have one or two mutations also have higher homocysteine concentrations. The same occurs in the group of mothers, however, with the increase of homocysteine in the presence of three or four mutations. A similar fact was demonstrated by van der Put et al. (24) when they found high concentrations of homocysteine in heterozygotes in both mutations: 677C>T and 1298A>C. Nevertheless, we did not observe preferential composition between the mutated alleles for increased homocysteine concentrations (data not shown).

Our data could not indicate that increase in homocysteine concentration or the presence of the polymorphisms

Table 4. Comparison of the biochemical variables for patients’ mothers considering the number of mutated alleles

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<tbody>
<tr>
<td>Hcy (µM)</td>
<td>8.87 ± 2.04* (n = 7)</td>
<td>11.23 ± 8.29 (n = 13)</td>
<td>11.05 ± 4.72 (n = 21)</td>
<td>14.81 ± 3.61 (n = 8)</td>
</tr>
<tr>
<td>Folic acid (ng/mL)</td>
<td>7.33 ± 5.94 (n = 6)</td>
<td>8.64 ± 5.26 (n = 13)</td>
<td>9.19 ± 4.81 (n = 18)</td>
<td>9.40 ± 2.39 (n = 7)</td>
</tr>
<tr>
<td>Vitamin B12 (pg/mL)</td>
<td>363.29 ± 145.67 (n = 7)</td>
<td>361.85 ± 107.43 (n = 13)</td>
<td>363.42 ± 147.25 (n = 19)</td>
<td>481.43 ± 207.33 (n = 7)</td>
</tr>
</tbody>
</table>

*p = 0.0096 (Kruskal-Wallis).
studied in the genes MTHFR, MTR and CBS are correlated with congenital heart defects, which supports the hypothesis that the development of congenital heart defects has a multifactorial etiology. The accumulation of mutations in the genes of enzymes of homocysteine metabolism may cause an increase in homocysteine levels.

Acknowledgments
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