Association of C677T Polymorphism in MTHFR Gene, High Homocysteine and Low HDL Cholesterol Plasma Values in Heterozygous Familial Hypercholesterolemia

Jose T Real1, 2, Sergio Martinez-Hervas1, 2, 3, Ana Barbara Garcia-Garcia2, 3, F Javier Chaves2, 3, Miguel Civera1, Juan F Ascaso1, 2, and Rafael Carmena1, 2

1Service of Endocrinology and Nutrition, Hospital Clínico Universitario, Department of Medicine, University of Valencia, Valencia, Spain
2CIBER de Diabetes y Enfermedades Metabolicas Asociadas (CIBERDEM)
3Unidad de Genotipado y Diagnostico Genetico, Fundación Investigacion, Hospital Clinico Universitario de Valencia, Valencia, Spain

Aim: to investigate the association of C677T polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene, homocysteine plasma values (Hcy), and plasma HDL cholesterol in heterozygous familial hypercholesterolemia (hFH).

Methods: One hundred and twenty-five hFH subjects were studied. Plasma lipid, lipoprotein, vitamin B12, folic acid and Hcy values were determined. C677T polymorphism in the MTHFR gene was detected by SSCP-PCR. Genetic diagnosis of FH was determined by a three-step protocol using SSCP-PCR, Southern blot, long PCR and automatic sequencing.

Results: We found significant differences in plasma HDL-C (CC 1.39 ± 0.34, CT 1.33 ± 0.39 and TT 1.14 ± 0.26 mmol/L, p = 0.028) between the C677T MTHFR genotypes, that were also found when gender, age, and BMI were included as covariables. In addition, Hcy values were significantly different between C/T MTHFR genotypes (CC 11.75 ± 2.9, CT 12.69 ± 2.88, TT 15.34 ± 2.1 µmol/L). The distribution of gender, smoking habit and LDLR gene mutations was similar among the three groups.

A significant correlation was found between Hcy plasma values and plasma HDL-C (r = -0.370, p = 0.003), but no correlations were found with age, BMI or other lipid and apo B plasma values.

Conclusion: In hFH subjects, the genotype TT and higher plasma Hcy levels were associated with lower HDL-C plasma values in FH subjects. More studies are needed to confirm our results and also to elucidate the exact mechanism of interaction between plasma homocysteine and lipid metabolism.


Key words: Total plasma homocysteine values, C677T MTHFR gene polymorphism, Familial hypercholesterolemia, HDL cholesterol

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disease defined at the molecular level by the presence of mutations in the LDL receptor (LDLR) gene and characterized by markedly elevated low density lipoprotein cholesterol (LDL-C) plasma levels, tendon xanthomata and increased risk of coronary heart disease (CHD)1). Despite the monogenic nature of the disease, FH shows great variability in phenotypic expression and risk of CHD. This expression and risk may be influenced by factors such as age, gender, diet, body mass index (BMI), low HDL-C, type of LDLR mutations or other genes2). New factors should be studied in order to explain this variability beyond LDL-C plasma values.
Hyperhomocysteinemia is a risk factor for cardiovascular disease. The prevalence of hyperhomocysteinemia (defined as plasma values ≥14 μmol/L) varies between 5% and 30% in the general population. The precise mechanism by which homocysteine promotes atherosclerosis is unknown, although there is strong evidence of its association. Homocysteine has a direct cytotoxic effect on endothelial cells and promotes in vivo endothelial dysfunction. Prothrombotic effects due to its interaction with procoagulant factors have also been shown.

Serum homocysteine (Hcy) levels are controlled and modulated by different factors. Higher Hcy values are found in males, older subjects, lower serum levels of folate and vitamin B12, mutations of methylene tetrahydrofolate reductase (MTHFR C677T polymorphism) and cystathionine-beta-synthase genes, several diseases (diabetes, renal insufficiency, cancer) and drugs (phenytoine, carbamacepine, ciclosporine, colestipol).

Westerbuck et al. have demonstrated in vitro homocysteine-induced endoplasmic reticulum stress that activates both unfolded protein responses and sterol regulatory element-binding proteins (SREBPs). The activation of SREBPs was associated with increased expression of the genes responsible for cholesterol/triglyceride biosynthesis and uptake, and with intraacellular accumulation of cholesterol. In addition, Mikael et al. have demonstrated that hyperhomocysteinemia decreased the expression of ApoAI in MTHFR (+/−) mice, and in patients with coronary heart disease (CHD), a negative correlation between plasma levels of Hcy and HDL-C was found.

Few studies so far have investigated the effect of Hcy and C677T polymorphism of the MTHFR gene on cardiovascular risk and phenotypic expression in FH subjects. Kawashiri et al. have demonstrated that mutation of the MTHFR gene in FH male subjects was associated with progression of CHD through elevations of plasma Hcy levels. In children with FH, Tonstad et al. have demonstrated that C677T polymorphism of the MTHFR gene was associated with higher plasma Hcy values and that these values were associated with a parenteral history of CHD. Pinciotta et al. demonstrated in 249 FH adult patients that C/T MTHFR polymorphism was the major determinant of plasma Hcy values, and that such values were the most significant predictor of CHD in these patients.

In summary, elevated plasma levels of Hcy may cause endoplasmic reticulum stress and dysregulation of cholesterol and triglyceride biosynthetic pathways, with decreased expression of ApoAI. As a consequence, we hypothesize that the TT genotype of the MTHFR gene associated with higher levels of Hcy may be related with different lipoprotein phenotypes in a genetic model of hypercholesterolemia.

The present study was undertaken to investigate the association of C677T polymorphism with the MTHFR gene and Hcy plasma values with the lipid phenotype in heterozygous familial hypercholesterolemia.

Subjects and Methods

Subjects

The study population consisted of 125 hFH subjects who had been consecutively referred to our Lipid Clinic. All subjects were Caucasian and lived in the Valencia region. The institutional ethics committee approved the protocol and all subjects gave written informed consent to enter the study.

Diagnostic criteria for hFH included: plasma levels of total and LDL-C higher than the 95th percentile corrected for both age and sex, presence of tendon xanthomata, coronary artery disease in the proband or in a first-degree relative, and bimodal distribution of total and LDL-C levels in the family, indicating an autosomal dominant pattern of phenotype IIa.

Study Design

The study was conducted on an outpatient basis under the supervision of a physician and a clinical dietician at the Lipid Clinic. All potentially FH subjects were individually screened for 6 weeks (visit −1) before the study, to verify inclusion and exclusion criteria, explain different study phases and obtain the informed consent. This pre-study visit included a complete medical history, physical examination and a laboratory test. All potentially FH subjects were included after 6 weeks before the beginning of the study. A complete medical history and physical examination were obtained in all participants. BMI was calculated as weight divided by height squared (kg/m²). Blood pressure was measured at a sitting position and after a 5-minute rest. The mean value of three measurements was considered.

Inclusion criteria were: heterozygous FH, age 18–65 years. Exclusion criteria were: homozygous FH, presence of metabolic, hepatic, renal, or endocrine disease, history of coronary events, treatment with drugs that could affect lipid metabolism and ethanol consumption > 30 g/d. Coronary events included episodes of angina with a positive exercise test or positive coronaryography or documented history of myocardial infarction (MI).
A total of 125 hFH subjects were included in the study protocol at visit 0. This visit included a physical examination, dietary advice on a standard cholesterol lowering diet (NCEP-1), and blood sampling. Caloric intake was controlled in order to keep body weight stable throughout the study. Subjects were instructed to maintain their regular level of physical activity and lifestyle. After 4 weeks, visit 1 included a physical examination verifying the objectives of weight, control of dietary advice on the NCEP-1 diet, and blood sampling after a 10–12-hour fast. Laboratory tests included plasma lipids, lipoproteins, vitamin B12, folic acid and Hcy plasma values, DNA extraction and genetic analysis (see laboratory methods).

**Laboratory Methods**

At visit 1, blood samples were obtained after a 10–12-hour fast on the same morning (8.00 h–9.00 h) in the Metabolic Unit with a standard venipuncture technique after sitting for 10–15 min.

Plasma total cholesterol and triglyceride levels were measured by standard enzymatic techniques\(^{(16)}\). HDL-C was measured after precipitation of apoB-containing lipoproteins with polyanions\(^{(17)}\) and very low density lipoprotein cholesterol (VLDL-C) after separation of VLDL (d<1.006 g/mL) by ultracentrifugation\(^{(18)}\). LDL-C was calculated by subtracting VLDL and HDL-C from total cholesterol. Total plasma apoB and ApoAI were measured by immunoturbimetry. Fasting plasma total homocysteine values were determined by immunoassay (CMX; Abbot). Fasting folic acid and vitamin B12 were measured by the standard method.

The coefficients of variation for biochemical parameters were <7\%.

DNA extraction was performed by the standard procedure. Genetic diagnosis of FH was established using a three step protocol as previously described\(^{(15)}\).

In 102 clinically diagnosed hFH subjects, a mutation of the LDLR gene was detected. The distribution of LDLR gene mutations according to the type of mutation (null/no null mutation) is shown in Table 1. The description and definition of the type of LDLR mutation have been previously defined by our group\(^{(15)}\).

C677T polymorphism on the MTHFR gene was detected by the SSCP-PCR procedure based on the description by Frosst et al\(^{(19)}\) with minor modifications.

**Table 1. Mutations of the LDL receptor gene and C677T genotype distribution in 102 FH subjects**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Null mutations (n=32)</th>
<th>No null mutations (n=70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>9 (28.1%)</td>
<td>29 (41.4%)</td>
</tr>
<tr>
<td>CT</td>
<td>13 (40.6%)</td>
<td>31 (44.3%)</td>
</tr>
<tr>
<td>TT</td>
<td>10 (31.3%)</td>
<td>10 (14.3%)</td>
</tr>
</tbody>
</table>

Mean values of quantitative variables were compared with one-way ANOVA and ANCOVA. Proportions were compared with contingency tables and the chi-square (\(x^2\)) test or Fischer’s exact test (\(n<5\)). Multiple regression analysis was used to estimate the independent contributions of age, gender, BMI to mean baseline lipid and apo B plasma values.

**Results**

In the 125 hFH subjects, the distribution of C677T polymorphism was 43 (34\%) CC, 55 (44\%) CT and 27 (22\%) TT. The distribution of gender, smokers and the type of LDLR gene mutations was similar between groups (Table 1, 2).

Using one-way ANOVA (Table 2) we found significant differences in plasma HDL-C (CC 1.39±0.34, CT 1.33±0.39 and TT 1.14±0.26 mmol/L, \(p=0.028\)) and ApoAI (CC 1.33±0.30, CT 1.32±3.25, TT 1.14±2.02 g/L, \(p=0.04\)) between C677T MTHFR genotypes. No significant differences were observed for age, BMI, TC, LDL-C, TG, VLDL-C, folic acid and vitamin B12. Hcy plasma values were significantly higher in TT group compared to CC and CT groups (CC 11.75±2.9, CT 12.69±2.88, TT 15.34±2.1 μmol/L) (Table 2).

In multivariate analysis (ANCOVA), using plasma lipid values as dependent variables, gender and MTHFR genotypes as independent variables and age as covariables we found significant differences in HDL-C plasma values (\(p=0.033\)) between MTHFR genotypes. No interaction was observed between gender and these genotypes. These differences in plasma HDL-C values were still found when age and BMI were included as covariables.

Other lipid values were significantly related to age: TC (Beta 0.931, \(p=0.005\)), TG (Beta 0.838, \(p=0.001\)) and LDL-C (Beta 0.697, \(p=0.034\)), but not to MTHFR genotypes.

As expected, Hcy plasma values were significantly higher in males than females (11.8±4.75 μmol/L vs 9.75±5.07 μmol/L, respectively, \(p=0.015\)). A signifi-
cant negative correlation was found between Hcy plasma values and plasma HDL-C ($\rho = 0.370$, $p = 0.003$), but no correlations were found with age, BMI and other lipid and apo B plasma values. In linear regression analysis using plasma Hcy values as dependent variables and age, gender and HDL-C as independent variables, only the latter reached significance in the model ($\rho = 0.350$, $p = 0.015$).

**Discussion**

This is the first study demonstrating an association between the TT genotype of the MTHFR gene and plasma Hcy values with the lipoprotein phenotype in FH subjects. FH subjects with the unfavorable genotype (TT) of the MTHFR gene and FH with higher plasma Hcy values showed significantly lower values of plasma HDL-C, even in the presence of variables (age, smoking habit, gender, BMI and type of LDLR gene mutations) known to modulate plasma HDL-C values.

It is well known that FH subjects express great variability in CHD risk, even among carriers of the same LDL receptor mutation. Thus, it is important to search for determinants of high cardiovascular risk in FH subjects beyond the LDL plasma concentrations. Our results indicate an association between three CHD risk markers: the C677T genotype of MTHFR, and high Hcy and low HDL-C plasma values. This should be verified by other studies in different FH populations.

The mechanism responsible for the association among the C677T genotype, and Hcy and HDL-C plasma values is still unknown. Recently, Westerbuck et al.\(^9\) have demonstrated in vitro a homocysteine-induced endoplasmic reticulum stress that activates the sterol regulatory element binding proteins (SREBPs). SREBP1a and SREBP2 are important regulatory proteins of cholesterol and triglyceride synthesis and uptake. The activation of SREBPs was associated with increased expression of the genes responsible for cholesterol/triglyceride biosynthesis and uptake, and with intracellular accumulation of cholesterol. Moreover, Westerbuck et al.\(^9\) observed hepatic steatosis in hyperhomocysteinemic mice that could be related to homocysteine-induced alteration in the regulatory uptake of cholesterol. These results suggest that plasma Hcy could influence cholesterol uptake in mamalian cells.

In addition, Mikael et al.\(^10\), have demonstrated that hyperhomocysteinemia decreased the expression of ApoAI and increased the expression of CYP7A1 in MTHFR (+/−) mice. The same authors have also demonstrated a significant negative correlation between ApoAI plasma values and Hcy levels ($r = -0.33$) in 60 males with CHD and a significant negative correlation between Hcy plasma values and HDL-C levels.

### Table 2. General characteristics, plasma lipid, apo B, folic acid, vitamin B12 and Hcy plasma values in FH subjects divided according to the C677T MTHFR genotype

<table>
<thead>
<tr>
<th></th>
<th>CC genotype ($n = 43$)</th>
<th>CT genotype ($n = 55$)</th>
<th>TT genotype ($n = 27$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.52 (19.46)</td>
<td>38.39 (18.27)</td>
<td>40.63 (17.9)</td>
</tr>
<tr>
<td>Males %</td>
<td>38%</td>
<td>48%</td>
<td>44%</td>
</tr>
<tr>
<td>No smokers %</td>
<td>81%</td>
<td>83%</td>
<td>85%</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25.77 (4.26)</td>
<td>25.8 (4.3)</td>
<td>26.48 (5.0)</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>8.73 (1.64)</td>
<td>8.89 (1.74)</td>
<td>8.61 (1.77)</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.28 (0.63)</td>
<td>1.35 (1.01)</td>
<td>1.25 (0.63)</td>
</tr>
<tr>
<td>VLDL-C (mmol/L)</td>
<td>0.59 (0.35)</td>
<td>0.56 (0.35)</td>
<td>0.54 (0.27)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.39 (0.35)</td>
<td>1.33 (0.35)</td>
<td>1.14 (0.26)*, **</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>6.77 (1.60)</td>
<td>6.98 (1.78)</td>
<td>6.73 (1.74)</td>
</tr>
<tr>
<td>Apo B (g/L)</td>
<td>1.54 (0.40)</td>
<td>1.55 (0.42)</td>
<td>1.57 (0.35)</td>
</tr>
<tr>
<td>Apo AI (g/L)</td>
<td>1.33 (3.03)</td>
<td>1.32 (3.25)</td>
<td>1.14 (2.02)*, **</td>
</tr>
<tr>
<td>Vitamin B12 (pg/mL)</td>
<td>384.17 (49.59)</td>
<td>377.27 (44.67)</td>
<td>354.87 (59.14)</td>
</tr>
<tr>
<td>Folic acid (ng/mL)</td>
<td>11.03 (5.64)</td>
<td>11.61 (4.69)</td>
<td>11.70 (5.93)</td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>11.75 (2.9)</td>
<td>12.69 (2.88)</td>
<td>15.34 (2.1)*, **</td>
</tr>
</tbody>
</table>

*p ≤ 0.04 (ANOVA)  **p < 0.05 (corrected by gender, age, BMI and tHcy)

Abbreviations: TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, apo B: apolipoprotein B, ApoAI: apolipoprotein AI, Hcy: plasma homocysteine.
(r = -0.24), similar to our results in hFH subjects free of clinical CHD.

The negative correlation between Hcy and HDL-C and ApoA-I has been confirmed in mice with targeted deletions of the genes for apoE and cystathionine beta-synthase (CBS), an animal model of dyslipidemia and hypercholesterolemia. High plasma Hcy accelerates spontaneous atherosclerosis in CBS (-/-) / apoE (-/-) mice, reduces the concentration of circulating HDL, apoA-I and large HDL particles, inhibits HDL function and enhances HDL-C clearance\textsuperscript{20, 21}.

In other studies, high plasma Hcy values activated NF-kB and through inhibition of PPAR alpha downregulated the expression of ApoAI and HDL-C plasma values\textsuperscript{22, 23}.

Our results, carried out in a human genetic model of hypercholesterolemia and high coronary risk, are in agreement with the commented results of other studies that link high Hcy plasma values with low HDL-C plasma values. FH subjects with higher plasma Hcy values and FH carriers of the TT genotype of the MTHFR gene showed significantly lower plasma HDL-C values, independently of gender, age, BMI, the type of LDLR gene mutation, folic acid and vitamin B 12 values. In addition, different studies have linked higher Hcy values in FH patients with CHD\textsuperscript{11-14}. Case-control studies have also demonstrated that Hcy negatively correlated with HDL-C levels in patients with CHD\textsuperscript{24}.

In conclusion, the unfavorable TT genotype and higher plasma Hcy levels were associated with lower HDL-C plasma values in FH subjects. The TT genotype in FH subjects may confer a higher cardiovascular risk by negatively affecting HDL-C and Hcy levels. More studies are needed to confirm our results and to elucidate the exact mechanism of interaction between plasma homocysteine and lipid metabolism in FH subjects.

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