Functional Polymorphism rs13306560 of the MTHFR Gene Is Associated with Essential Hypertension in a Mexican-Mestizo Population

Running title: Pérez-Razo et al.; MTHFR gene polymorphism and essential hypertension

Juan Carlos Pérez-Razo, BSc1*; Luis Javier Cano-Martinez, MSc2*;
Gilberto Vargas Alarcón, PhD3; Samuel Canizales-Quinteros, PhD4;
Nancy Martínez-Rodríguez, PhD3; Patricia Canto, MD, PhD5; Bladimir Roque-Ramírez, PhD2;
Carlos Palma-Flores, PhD2; Rosa Esteban-Martínez, MSc6; Berenice López-Hernández, PhD2;
David Rojano-Mejía1; Ramón M. Coral-Vazquez, PhD6,8

1División de Medicina Genómica, 2División de Investigación Biomédica, 3Subdirección de Enseñanza e Investigación, CMN 20 de Noviembre–ISSSTE; 4Departamento de Fisiología y Grupo de Estudio en Genómica y Proteómica de Enfermedades Cardiovasculares, Instituto Nacional de Cardiología “Ignacio Chávez”; 5Unidad de Genómica de Poblaciones Aplicada a la Salud, Facultad de Química–UNAM, Instituto Nacional de Medicina Genómica; 6Unidad de Investigación en Obesidad, Facultad de Medicina–UNAM, Clínica de Obesidad, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán; 7Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, Instituto Politécnico Nacional; 8Unidad de Medicina Física y Rehabilitación Centro, UMAE, Hospital de Traumatología y Ortopedia “Lomas Verdes”, Instituto Mexicano del Seguro Social, México, D.F., México

*contributed equally

Correspondence:
Ramón M. Coral-Vázquez,
Sección de Estudios de Posgrado e Investigación
Escuela Superior de Medicina, Plan de San Luis y Díaz Mirón s/n,
Col. Casco de Santo Tomas, Del. Miguel Hidalgo
C.P. 11340, México, D.F., Mexico
Tel.: +5255 57296300, x62820
Fax: +5255 57296300x62820
E-mail: rmcoralv@gmail.com / rcoral@ipn.mx

Journal Subject Codes: [20] Other etiology
Abstract:

**Background** - Polymorphisms of *MTHFR* have been associated with diastolic blood pressure, hypertension and other cardiovascular diseases; however, results of these studies are still controversial. In this study we sought to determine whether two functional variants (rs1801133 and rs13306560) within the *MTHFR* gene are associated with hypertension in Mexican-Mestizos.

**Methods and Results** - We performed a case-control study with 1214 subjects including adults and children in order to test for the association of both SNPs with essential hypertension (EH). The adult group included 764 participants (372 patients and 391 controls) and the group of children included 418 participants (209 patients and 209 controls). rs13306560 was associated with EH in adults (OR = 4.281, 95% CI: 1.841–9.955, P = 0.0003) with a statistical power above of 0.8. In children, none of the polymorphisms was associated with EH. Additionally, we assessed the effect of the rs13306560 polymorphism on the *MTHFR* promoter region by means of luciferase reporter gene assays using human umbilical vein endothelial cells HUVECs. Cells transfected with the pMTHFRaLUC construct showed an ~25% reduction in luciferase activity (P = 0.003). Furthermore, the promoter activity was reduced considerably by *in vitro* methylation of CpG sequences.

**Conclusions** - Our data suggest that the rs13306560 polymorphism of the *MTHFR* gene may be part of the observed hypertension process in Mexican-Mestizo populations, but further studies are warranted. In addition, the allele A of the rs133065 polymorphism as well as the *in vitro* methylation of CpGs reduced the promoter activity of the *MTHFR* regulatory region.

**Key words:** hypertension, MTHFR, polymorphism, gene, essential, hypertension, MTHFR, gene, glycoprotein VI polymorphism genetics
Introduction

Hypertension is a common condition that according to a global study will affect 1.56 billion adults by the year 2025.\(^1\) Despite this, essential hypertension (EH) remains as a complex phenotype in which genetic determinants remain largely undefined.\(^2,3\) A recent large study of Sardinian families estimated a broad-sense heritability of ~65% and ~45% for systolic and diastolic blood pressure, respectively\(^4\). In general, large scale epidemiologic studies suggest that the heritability of blood pressure exceeds 50%, justifying a search for genetic variants influencing susceptibility to EH\(^5,6\). Indeed, the human genome contains several genetic variants that may influence the blood pressure and have been associated to hypertension.\(^7,8\) In this regard, the AGTRAP-MTHFR-CLCN6-NPPA-NPPB gene cluster at 1p36 has been associated with cardiac dysfunction, blood pressure (BP), and renal disease.\(^2,9-11\) MTHFR is a key element within this gene cluster that codifies for the enzyme methylenetetrahydrofolate reductase, which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5 methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine.\(^12\) The T allele of the rs1801133 (formerly C677T or A222V) generates an MTHFR thermolabile enzyme with reduced activity, and the homozygous state of this allele has been associated with a moderately elevated plasma homocysteine level.\(^13,14\) It has been proposed that hyperhomocysteinemia induces oxidative injury of vascular endothelial cells, arteriolar constriction, renal dysfunction and increased sodium reabsorption.\(^15-17\) Results of two meta-analyses suggest that the MTHFR C677T polymorphism is associated with an increased risk of hypertension, at least in Asian and Caucasian populations.\(^18,19\)

Our group previously found that the 677TT genotype is associated with a reduced risk of preeclampsia in Maya-Mestizo women.\(^20\) However, no study has examined the possible
association of this polymorphism with EH in Mexican-Mestizo populations.

Moreover, Tomaszewski et al.\textsuperscript{10} described another polymorphism (rs13306560) located in the promoter region of \textit{MTHFR/CLNC6} that has a strong association with diastolic blood pressure and proposed by \textit{in silico} analysis the potential functionality of this variant. Furthermore, the research group suggested that this polymorphism is associated with blood pressure independently of the thermolabile form of MTHFR generated by the 677T allele of the rs1801133. This is supported by the observations that the rs13306560 is not in linkage disequilibrium with rs1801133 and that this has a minor association with diastolic blood pressure.\textsuperscript{10} Despite this, there are no published studies seeking the association of the rs13306560 with EH.

Therefore, the aim of this study was to gain insight on the potential role of rs1801133 and rs13306560 in EH in Mexican-Mestizo adults and children as well as to determine by \textit{in vitro} studies the functionality of the rs13306560.

\textbf{Methods}

\textbf{Subjects}

The study was approved by the Institute’s Human Research and Ethics Committees. All participants provided written informed consent prior to inclusion in the study. A case-control association study was performed. There were 1181 individuals (763 adults and 418 children) admitted into the study. Mexican ancestry was considered as Mexican-mestizo individuals born in Mexico and who have a Spanish-derived last name and can be traced back to the third generation.\textsuperscript{21}

The adult group included 372 patients with EH referred to the National Institute of Cardiology (INCICH) in Mexico City and 391 normotensive individuals recruited among blood
donors at the INCICH with systolic and diastolic blood pressures <120 and <80 mmHg, respectively, and not taking any antihypertensive medications. Hypertension was defined as systolic blood pressure (BP) ≥140 mmHg, diastolic BP ≥90 mmHg or the use of at least one class of antihypertensive drugs. Secondary hypertension was ruled out using a detailed health questionnaire and clinical evaluation. None of the patients had evidence of cardiac or renal failure. Furthermore, all individuals were evaluated for various clinical characteristics as previously reported.22

In regard to the group of children, we included 418 unrelated school-age Mexican-Mestizo children of whom 209 were hypertensive and 209 were non-hypertensive. In this case, each hypertensive child was age- and gender-matched with a non-hypertensive child. The definition of hypertension in children and adolescents was in concordance with the criteria previously reported with a basis on the normative distribution of BP in healthy children.23 Children with prehypertension and stage 1 and 2 hypertension were included in the case group (Table 1). Additionally, several anthropometric and biochemical parameters were measured in all individuals as described in a previous study.24

**Genotyping and Quality Control**

Genomic DNA was isolated from peripheral blood using a commercial kit based on the salt fractionation method (QIAmp 96 DNA Blood Kit, Qiagen, Hilden, Germany). The rs1801133 and rs13306560 single nucleotide polymorphisms (SNPs) were genotyped using 5’ TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA, assay ID: C__1202883_20 and C__30914969_10) on a LightCycler® 480 Instrument (Roche Diagnostics Ltd., Basel, Switzerland) according to the manufacturer’s instructions. Genotyping call rate surpassed 95% for all SNPs tested, with no discordant genotypes in 10% of duplicate samples.
Constructs and Plasmids

A 563-bp DNA fragment of the *MTHFR* regulatory sequence (-441/+122) was PCR amplified from DNA of a heterozygous patient for the rs13306560 variant. Oligonucleotides used to amplify the promoter sequence were forward: KpnI/MTHFR F GCGC GGTACC

```
  gtaggggtatgagaaaagacc
```

and reverse: HindIII/MTHFR R GCGC AAGCTT

```
  gtcaggttgctggagagg
```

PCR conditions were as follows: one cycle at 95 °C for 3 min, 31 cycles at 95 °C for 45 s, 62 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 7 min.

Amplified DNA was cloned in the pGEM-T Easy (Promega, Madison, WI, USA) and sequenced employing a dye terminator cycle sequencing reaction kit (ABI PRISM 310, Applied Biosystems, Foster City, CA, USA). Clones containing allele G or A of the rs13306560 were subcloned into the KpnI/HindIII sites of pGL3-basic (Promega) containing luciferase reporter gene and sequenced using a dye terminator cycle sequencing reaction kit (ABI PRISM 310, Applied Biosystems).

**In vitro Methylation**

Ten micrograms of the assessed vectors were methylated *in vitro* with the CpG methyltransferase (M. SssI, New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s instructions and the protocol described by Mamruth et al.25 The effectiveness of methylation was examined by digestion of an aliquot with methylation-sensitive SsII restriction enzyme.

**Cell Culture, Transfection, and Reporter Gene Activity Analysis**

HUVECs were isolated from human umbilical cord segments and cultured as previously reported.26 Two hundred thousand cells corresponding to 90% confluence were seeded in 9.5 cm² culture wells; cells were transfected 48 h later using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. One microgram
of each MTHFR promoter construct was transiently cotransfected with 250 ng of pRL/CMV (Promega) plasmid used to normalize luciferase activities. Luciferase activity in transfected HUVECs was measured 48 h post-transfection using the DualLuciferase Reporter Assay System (Promega) according to the manufacturer’s instructions in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Luciferase activity was then normalized to the protein concentration of each cell lysate.

**Statistical Analysis**

Demographic and clinical variables between hypertensive and non-hypertensive groups were analyzed using SPSS v.16.0 (SPSS Inc., Chicago, IL, USA). Numeric variables not normally distributed are presented as median and range and were compared using the Mann–Whitney U test. Comparisons between categorical variables and deviations from Hardy–Weinberg equilibrium (HWE) were carried out using $\chi^2$ test. Normally distributed numeric variables are presented as mean ± standard deviation and compared using the Student t test.

A multivariable logistic regression analysis was used to test the associations between genotype and hypertension under additive, dominant and recessive inheritance models, reporting the most significant. Odds ratio and P-value were adjusted for Z score BMI, triglycerides and cholesterol in children; glucose, triglycerides, type 2 diabetes and family history in adults. In the case of rs13306560 with low genotype counts, Fisher’s exact test was also used to evaluate the association between genotype and hypertension. A two-way non-parametric ANOVA was used to evaluate the effect of the rs13306560 alleles and the *in vitro* CpG methylation on the promoter activity of regulatory cloned sequence of the MTHFR gene.

Statistical power to detect an association of rs13306560 and rs1801133 with hypertension at an alpha of 0.05 was calculated taking into account the frequencies of the polymorphisms and
the prevalence of hypertension in the childhood and adult populations\textsuperscript{27, 28}, in addition to other parameters (Table 2), under an additive model using QUANTO software (http://hydra.usc.edu/GxE/).

**Results**

**Characteristics of the Studied Populations**

Clinical and demographic characteristics of patients and controls of both studied populations are shown in Table 3. With regard to children, Z-score of BMI, triglycerides and cholesterol were significantly higher in the group with EH than in non-hypertensive individuals. The other characteristics were not significantly different between groups.

In the adult groups, individuals with EH showed significantly higher levels of triglycerides and glucose as compared with the levels observed in non-hypertensive individuals. They also showed significant differences in family history and diabetes mellitus. The other characteristics were not significantly different between groups (Table 3).

**Association of rs1801133 and rs13306560 with EH**

Hardy–Weinberg equilibrium test was performed for the polymorphisms under study. The distribution of the observed genotypes did not differ from what was expected in either the patient or control groups ($P >0.05$).

Allele and genotype frequencies of the rs1801133 and rs13306560 polymorphisms are presented in Table 4. In children there was no association of the polymorphism rs1801133 (C\textsubscript{677}T\textrightarrow\text{A222}V) with EH under additive model (OR = 1.111, 95\% CI: 0.822–1.503, $P = 0.4925$) and any other model used. In contrast, in the adult population, under the additive model, the MTHFR 677TT + TC genotypes conferred with low significance a decreased risk of hypertension (OR=0.768, 95\% CI:0.624-0.947, $P=0.0133$) after adjusting by glucose,
triglycerides, type 2 diabetes and family history, with a statistical power of 0.763 according to the OR obtained.

Regarding polymorphism rs13306560, in children it was not significantly associated with hypertension (OR = 2.706, 95% CI: 0.947–7.732, P = 0.0891). In adults, the presence of the allele MTHFR -23A (at -23 bp of the transcription start site, sequence accession number: NG_008766) (Fig. 1A) was associated, under the additive model, with an increased risk of hypertension (OR = 4.281, 95% CI: 1.841–9.955, P = 0.0003).

**Bioinformatic Analysis**

In order to gain insight into the possible implications of the rs13306560 polymorphism in the MTHFR promoter, we used the MatInspector software that utilizes a wide library of matrix descriptions for transcription factor binding sites in DNA sequences. The analysis showed that the polymorphism is adjacent to a site for Kruppel-like transcription factors (Fig. 1A) whose activity may be differently affected depending on the allele present.

**In vitro Functionality of rs13306560**

Because the rs13306560 is located in the putative core promoter of the MTHFR gene (Fig. 1A), we decided to examine the effect of either allele G or A on the promoter activity. To this end, we transfected HUVECs with a luciferase reporter gene construct harboring the MTHFR promoter sequence with the G or A allele of the rs13306560. Cells transfected with the construct pMTHFRaLUC showed an ~25% reduction in luciferase activity (P = 0.003) compared to those transfected with pMTHFRgLUC (Fig. 1B). A similar result was obtained when the experiment was carried out in human umbilical vein smooth muscle cells (data not shown). On the other hand, a computational-based analysis by Tomaszewski et al. and by our group showed the presence of a CpG island in the regulatory sequence of the MTHFR/CLCN6, opening the
possibility of DNA methylation as a mechanism for gene expression regulation. In order to explore this, we methylated in vitro the two clones containing the promoter of MTHFR with each allele of the rs13306560 polymorphism (Fig. 1). In both cases the expression level of the reporter gene was diminished almost to the level of the negative control.

Discussion

In this study we analyze the presence of the association of rs13306560 and rs1801133, located in the promoter region and exon 4 of the MTHFR gene, respectively, in children and adults with and without EH. With regard to the childhood population, we found no association of the rs1801133 (C677T) polymorphism with EH. A similar effect has been reported in a study conducted with Chinese hypertensive children. On the other hand, in the adult population there was a low significant protective effect of allele 677T, which contrasts with previous reports associating the 677TT genotype with an increased risk of hypertension in Asian, Caucasian and Chinese populations.

In a previous study we observed a protective effect of the 677TT genotype with preeclampsia in Maya-Mestizo women. The presence of the 677T allele of the MTHFR gene produces a thermolabile enzyme due to the presence of a valine residue instead of an alanine residue in the amino acid sequence of the protein, which has been associated with a high level of total homocysteine (tHcy) in plasma. However, a study performed in subjects from Mexico City reported one of the highest frequencies of the 677T allele and no correlation of the TT homozygous genotype with tHcy levels. In addition, these investigators proposed that high folic acid intake due to the Mexican diet may exert a positive selection on the TT genotype. Consistent with this hypothesis, a previous study by our group showed that several Mexican Amerindian populations have the highest reported frequency of the 677T allele.
Regarding the rs13306560 polymorphism, an extensive study with European nuclear families showed that this variant, located in the MTHFR-CLCN6 locus, is strongly associated with diastolic blood pressure. In this study we found a risk association trend between the -23T allele and hypertension in the childhood population. Concerning the adult populations, the -23T allele was significantly associated with an increased risk of EH under an additive model. The associated differences observed between the two populations may be ascribed to the exposure time of the individuals to different factors that modified BP, such as physical activity, dietary salt intake, alcohol use, smoking habit, age, and BMI. Based on this premise, individuals with the risk allele who are exposed to several environmental risk factors may have an increased possibility of developing EH. Furthermore, it is important to underline that the selection criteria were different between adults and children. In the first case, individuals were selected from patients who arrived at the hospital for treatment; in the second case, the samples were obtained from a group recruited from a summer camp for children of employees of the Mexican Health Ministry and from a public junior high school in Mexico City.

Bioinformatic analysis has suggested the functionality of the rs13306560 polymorphism and that the ancestral allele G is conserved in higher vertebrates. Additional analysis conducted by our group showed that this polymorphism is located at -23 bp from the transcription start site, a segment that may be within the basal promoter of the MTHFR. Interestingly, presence of the -23T allele reduced, in ~25%, the promoter activity of the regulatory sequence cloned to direct the expression of the luciferase gene as compared with the clone that harbored the -24G allele. Patients with EH presented a higher frequency of the allele A. Consequently, individuals with this allele may have a reduced expression of the MTHFR transcript and the concomitant diminution of the protein level. It is possible that due to this effect the -23A allele is mediating
its association with EH. According to other computational analyses, the rs13306560 polymorphism is adjacent to a consensus site for Krupel-like factors (KLFs). Because of this, it may be hypothesized that the presence of every allelic variant affects differentially the activity of this site. In this regard, KLFs regulate the transcription of some genes by recruiting other transcription factors, chromatin remodelers, and transcription machinery to specific promoters. Likewise, the rs13306560 polymorphism is located within a region containing a CpG island found in the intergenic union of MTHFR and CLCN6. Considering this, we observed that the in vitro methylation of the MTHFR regulatory sequence drastically reduced the expression of the luciferase reported gene regardless of the rs13066560 allele present in the promoter. In this sense, the degree of methylation of the regulatory sequence could be another factor that determines the level of expression of the MTHFR. Kerkel et al. proposed that methylation of some regulatory sequences may be determined according to some dimensions by specific SNPs. In addition, epigenetic mechanisms have been associated with the development of hypertension. Epigenesis is another factor that may explain the observed differences in genetic association between children and adults with polymorphism rs13066560 because a shorter exposure to environmental factors may be associated with fewer epigenetic changes. Therefore, it may be relevant to test in future investigations if the rs13066560 polymorphism has influence over the methylation of the CpG islands located in the MTHFR/CLCN6 regulatory sequence. Interestingly, a study by Crider et al. showed that the CC genotype of rs1801133 variant was associated with hypomethylation under folate depletion. In view of this, a possible effect of both rs13066560 and rs1801133 in MTHFR/CLCN6 RNA and protein level via genetic or epigenetic mechanisms remains to be proven.

In relation to CLCN6, the polymorphism is located in the 5’ UTR region at +31 bp of the
transcription start site, using as a reference the reported sequences of the CLCN6 (accession number: NG_008766). Flister et al., using a genetically modified murine model, showed that the Clcn6 gene plays a relevant role in blood pressure modulation. Accordingly, the rs13306560 variant could mediate its association with EH, modifying the expression of both MTHFR and CLN6. Further studies are needed to confirm this hypothesis.

In summary, our results propose that rs13306560 may play a role in the development of EH in the Mexican-Mestizo population because the polymorphism was associated with an increased risk of the disease. In addition, we determined that this polymorphism affects the promoter activity of the regulatory sequence of the MTHFR and that this promoter capacity is drastically reduced by in vitro CpG methylation. Additional studies are needed to evaluate whether the polymorphisms also affect the expression level of the CLCN6 and whether epigenetic mechanisms on the MTHFR/CLCN6 regulatory sequences may be involved in the development of EH.

Acknowledgments: Luis Javier Cano Martínez was a M.Sc. candidate supported by Consejo Nacional de Ciencia y Tecnología (CONACyT, México). Rosa Esteban-Martínez is a Ph.D candidate supported by Consejo Nacional de Ciencia y Tecnología (CONACyT, México). Sharon Morey, Executive Editor, Scientific Communications, assisted in the English review of the manuscript.

Funding Sources: This work was supported by Consejo Nacional de Ciencia y Tecnología, México (grant #: 2011-C01-161909).

Conflict of Interest Disclosures: None.

References:


Table 1: Blood pressure percentiles of the childhood population

<table>
<thead>
<tr>
<th>Percentile</th>
<th>SBP</th>
<th>DBP</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Normal</td>
<td>5th–89th</td>
<td>10th–89th</td>
<td>110</td>
<td>99</td>
</tr>
<tr>
<td>Prehypertension</td>
<td>90th–94th</td>
<td>90th–94th</td>
<td>53</td>
<td>39</td>
</tr>
<tr>
<td>Cases Stage 1 Hptn</td>
<td>95th–99th</td>
<td>95th–99th</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>Cases Stage 2 Hptn</td>
<td>99th–100th</td>
<td>99th–100th</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>

SBP: systolic blood pressure; DBP: diastolic blood pressure.

The percentiles were calculated for each group as described in Methods and only the highest and lowest percentiles of each group are indicated.
<table>
<thead>
<tr>
<th>SNP</th>
<th>*Sample size per group</th>
<th>Model of inheritance</th>
<th>Allele frequency</th>
<th>Population risk</th>
<th>Range effect assumed (OR)</th>
<th>Statistical power</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13306560</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>209</td>
<td>Additive</td>
<td>0.04</td>
<td>0.05</td>
<td>2.5-4</td>
<td>0.86-0.99</td>
</tr>
<tr>
<td>Adults</td>
<td>372</td>
<td>Additive</td>
<td>0.04</td>
<td>0.3</td>
<td>2.5-4</td>
<td>0.94-0.99</td>
</tr>
<tr>
<td>rs1801133</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>209</td>
<td>Additive</td>
<td>0.54</td>
<td>0.05</td>
<td>0.822-1.5</td>
<td>0.36-0.66</td>
</tr>
<tr>
<td>Adults</td>
<td>372</td>
<td>Additive</td>
<td>0.49</td>
<td>0.3</td>
<td>0.625-0.947</td>
<td>0.99-0.45</td>
</tr>
</tbody>
</table>

*In the case of children the sample size corresponded to a matched case-control design and in the adults was an unmatched case-control design (1:1).
Table 3: Clinical and biochemical parameters of the study groups

<table>
<thead>
<tr>
<th></th>
<th>Nonhypertensive</th>
<th>Hypertensive</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Children</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>209</td>
<td>209</td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>99 (47%)</td>
<td>99 (47%)</td>
<td>0.922</td>
</tr>
<tr>
<td>Female (%)</td>
<td>110 (53%)</td>
<td>110 (53%)</td>
<td>0.922</td>
</tr>
<tr>
<td>Age (years)</td>
<td>8.86 ± 1.99</td>
<td>8.86 ± 1.99</td>
<td>0.921</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>102.5 ± 8.2</td>
<td>118.5 ± 8.9</td>
<td>2 x 10^{-16}</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>61.1 ± 6.4</td>
<td>70.9 ± 10.2</td>
<td>2 x 10^{-16}</td>
</tr>
<tr>
<td>Z-score, BMI</td>
<td>0.84 ± 0.97</td>
<td>1.35 ± 0.97</td>
<td>2 x 10^{-5}</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>88 (53–128)</td>
<td>90 (60–144)</td>
<td>0.581</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>92 (16–373)</td>
<td>118 (18–380)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>173 (38–254)</td>
<td>179 (101–317)</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>102 (40–158)</td>
<td>109 (52–257)</td>
<td>0.054</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>46.5 (25–92)</td>
<td>44 (23–89)</td>
<td>0.073</td>
</tr>
<tr>
<td>WHR</td>
<td>0.89 (0.74–1.07)</td>
<td>0.90 (0.62–2.26)</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>391</td>
<td>372</td>
<td></td>
</tr>
<tr>
<td>Male [n (%)]</td>
<td>166 (41)</td>
<td>210 (53.5)</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>108 ± 10.3</td>
<td>133 ± 19.1</td>
<td>2 x 10^{-15}</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>67 ± 7.1</td>
<td>80 ± 10.3</td>
<td>2 x 10^{-12}</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 (29–75)</td>
<td>58 (29–81)</td>
<td>0.998</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>27.3 ± 3.4</td>
<td>29.5 ± 3.9</td>
<td>0.251</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>89 (58–391)</td>
<td>94 (71–269)</td>
<td>3 x 10^{-4}</td>
</tr>
<tr>
<td>*Glucose NDM (mg/dL)</td>
<td>88 ± 7.9</td>
<td>91 ± 8.2</td>
<td>0.322</td>
</tr>
<tr>
<td>†Glucose DM (mg/dL)</td>
<td>127 (79–391)</td>
<td>131 (77–281)</td>
<td>0.483</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>141 (43–420)</td>
<td>157 (50–652)</td>
<td>0.005</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>190.2 ± 33.5</td>
<td>192.4 ± 36.1</td>
<td>0.386</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>116 ± 29.0</td>
<td>117 ± 32.1</td>
<td>0.736</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>47.1 ± 12</td>
<td>44.2 ± 11</td>
<td>0.070</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus, n (%)</td>
<td>62 (15.8)</td>
<td>93 (24.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>Family history of hypertension, n (%)</td>
<td>147 (37.5)</td>
<td>204 (54.6)</td>
<td>6 x 10^{-6}</td>
</tr>
<tr>
<td>Tobacco smoking, n (%)</td>
<td>97 (24)</td>
<td>71 (19.0)</td>
<td>0.055</td>
</tr>
<tr>
<td>Use of alcohol, n (%)</td>
<td>284 (72.6)</td>
<td>276 (73.9)</td>
<td>0.358</td>
</tr>
</tbody>
</table>

Data are mean ± SD or median (range).
*Glucose NDM: non-diabetes mellitus.
†Glucose DM: diabetes mellitus.
SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; WHR: waist/hip ratio; BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
Table 4: Association of rs13306560 and rs1801133 of the MTHFR gene with essential hypertension in children and adults

<table>
<thead>
<tr>
<th></th>
<th>*rs13306560</th>
<th>rs1801133</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype (%)</td>
<td>Allele frequency (%)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-hypertensive</td>
<td>204 (97.61)</td>
<td>5 (2.38)</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>196 (93.77)</td>
<td>13 (6.22)</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-hypertensive</td>
<td>384 (98.2)</td>
<td>7 (1.8)</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>346 (92.7)</td>
<td>27 (7.3)</td>
</tr>
</tbody>
</table>

*Association was determined by Fisher’s exact test. When the association was analyzed by multivariable logistic regression analysis adjusting odds ratio and P-value for Z score BMI, triglycerides and cholesterol in children, and for glucose, triglycerides, type 2 diabetes and family history in adults, the values were similar to those obtained with the Fisher’s exact test (Children: OR=2.57, CI [0.857-7.705], P=0.0919; Adults: 4.83, CI [2.041-10.43], P=0.0003).

†Association was evaluated by a multivariable logistic regression analysis. Odds ratio and P-value were adjusted for Z score BMI, triglycerides and cholesterol in children; glucose, triglycerides, type 2 diabetes and family history in adults.
Figure Legend:

Figure 1: rs13306560 affects the promoter activity of MTHFR regulatory sequence. A) Schematic diagram of the MTHFR promoter indicating the presence of both allelic variants (-24, G/C) and the consensus sequence for Kruppel-like factor KLF. The promoter sequence with each allele was cloned in pGL3 vector to direct the expression of the luciferase gene as described in Materials and Methods. B) The presence of allele A (pMTHFRaLUC) reduces the promoter activity of the sequence in ~25% as compared with the promoter activity observed in cells transfected with the MTHFR regulatory sequence harboring the allele G (pMTHFRgLUC) (\( *P = 0.0007 \)). Methylation of both clones drastically reduced the activity of the promoter independently of the allele present (\( \dagger P < 10^{-4} \)). n=5 independent duplicate experiments; means were compared by two-way nonparametric ANOVA.
A

G/A (CACCC)
-23 bp KLF LUCIFERASE

B

Fold activation

Unmethylated Methylated Unmethylated Methylated

pMTHRFgLUC pMTHRFaLUC
Functional Polymorphism rs13306560 of the MTHFR Gene Is Associated with Essential Hypertension in a Mexican-Mestizo Population


Circ Cardiovasc Genet. published online May 28, 2015;
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/early/2015/05/28/CIRCGENETICS.114.000942