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Original article

Methylenetetrahydrofolate Reductase Gene 677CT Polymorphism and Isolated Congenital Heart Disease in a Mexican Population

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ABSTRACT

Introduction and objectives: The frequency of the 677C>T mutation in the methylenetetrahydrofolate reductase gene in Mexico is one of the highest worldwide. Some studies have shown that both the homozygous state of this mutation and a high homocysteine concentration are associated with congenital heart disease. The aim of this study was to determine whether this association exists in the Mexican population.

Methods: Genotypes were analyzed in 60 patients with congenital heart disease and in their mothers, and the levels of homocysteine were determined in the latter group. The genotypes were compared with those of a control group (n=62) and of their mothers. All the possible mother-child genotype combinations were also compared.

Results: There were no significant differences in allele or genotype frequencies between the patients with congenital heart disease and the controls or their respective mothers ($P>.05$). Although no significant differences were observed when the homocysteine concentrations in the presence of the CC or the TT genotype were compared, a clear trend was observed ($P=.0621$). We found no significant differences in homocysteine concentrations in relation to folic acid intake. The study cases and controls did not differ in terms of the possible combinations of mother-child genotypes.

Conclusions: The frequencies obtained were consistent with those reported for Mexico. No significant differences were found between groups. Nor did we find any association between TT mutations in both the mother and child and hyperhomocysteinemia. There was no evidence of an association between any of the mother-child genotype combinations and congenital heart disease. Similar studies with larger numbers of patients are required to confirm or refute some of the trends observed in this report.

Polimorfismo 677CT del gen de la metilentetradihidrofolato reductasa y cardiopatías congénitas aisladas en población mexicana

Palabras clave:
Cardiopatía congénita
Mutación
Metilentetrahidrofolato reductasa
Hiperhomocisteinemia

RESUMEN

Introducción y objetivos: México tiene alta frecuencia de la mutación 677C>T del gen de la enzima metilentetrahidrofolato reductasa. Se ha demostrado que esta mutación en estado homocigoto y la hiperhomocisteinemia se asocian a cardiopatías congénitas. Nuestro objetivo es determinar si existe dicha asociación en la población mexicana.

Métodos: Se analizaron los genotipos de 60 pacientes con cardiopatías congénitas y sus madres, así como las concentraciones de homocisteína en estas, y se los comparó con los genotipos del grupo control (n = 62) y sus madres. También se compararon las combinaciones de los genotipos madre-hijo en ambos grupos.

Resultados: No se encontraron diferencias significativas de las frecuencias alélicas y genotípicas entre las pacientes con cardiopatía congénita y sus controles ni en sus madres ($p > 0,05$). Aunque no se encontraron diferencias entre la concentración de homocisteína y la presencia del genotipo CC o TT, la tendencia fue evidente ($p = 0,0621$). No se encontraron diferencias significativas en las concentraciones

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de homocisteína dependientes de la ingesta de ácido fólico. El análisis de las diferentes combinaciones genotípicas del binomio madre-hijo entre casos y controles no mostró diferencias significativas.
Conclusiones: Las frecuencias obtenidas concuerdan con las publicadas para nuestro país. No se encontraron diferencias significativas entre los grupos. Tampoco se encontró asociación de la mutación TT con hiperhomocisteinemia. No hay asociación entre las combinaciones genotípicas madre-hijo y las cardiopatías. Es necesario desarrollar estudios semejantes con un mayor número de pacientes para confirmar o descartar algunas tendencias observadas en este trabajo.

Abbreviations

CCHD: complex congenital heart disease
CHD: congenital heart disease
Hcy: homocysteine
MTHFR: methylenetetrahydrofolate reductase
OR: odds ratio
SCHD: simple congenital heart disease

INTRODUCTION

Congenital heart disease (CHD) affects 3 to 8 per 1000 live births all over the world.[1–3] In Mexico, it is the third most common cause of death among preschool children.[4] The etiology of CHD is complex and is characterized by genetic heterogeneity,[5,6] and multifactorial inheritance patterns are observed in the majority of cases. One of the risk factors of genetic CHD is the 677CT polymorphism of the gene encoding the enzyme methylenetetrahydrofolate reductase (MTHFR).[7,8] This enzyme is essential in folate metabolism, and participates in the homocysteine (Hcy) remethylation cycle and in the formation of methionine.

The *MTHFR* gene, with locus on chromosome 1p36.3, consists of 11 exons.[9] Frosst et al. (1995)[10] identified a replacement of cytosine (C) by thymidine (T) at nucleotide 677, which generates a replacement of alanine by valine in the catalytic domain of the enzyme.[9,10] The homozygous T/T genotype causes a reduction in enzyme activity of up to 70%,[10] a circumstance that leads to an increase in Hcy levels when folic acid intake is insufficient.[9,10]

There is a strong ethnic component in the prevalence of the 677CT polymorphism; in the Italian population, the frequency of the T allele is 44% to 46%; in the Hispanic population living in the United States, it is 42%; in the French population, it is 36%; and in the Japanese population, it is 34%.[11–13] In the Mexican population, the 677CT polymorphism is even more frequent in mestizos, 44% to 58%, and in the indigenous Tarahumara population, it is 36%.[13–16] The frequencies of the T/T homozygous genotype in the Mexican mestizo population range between 19% and 34.8%.[14–16]

The 677CT polymorphism of the *MTHFR* gene has been found in a greater proportion in patients with CHD and their mothers than in the healthy population, indicating that the presence of the polymorphism is a risk factor for these conditions.[7,8,17–20] Moreover, the presence of the T/T genotype in patients with CHD is associated with a higher level of Hcy in the amniotic fluid during pregnancy.[8]

An insufficient intake of folic acid prior to conception is a risk factor for CHD. The periconceptional intake of vitamin supplements has been reported to reduce the risk of conotruncal defects by 59%.[17] The protective effect of the use of multivitamin supplements was evident in the mothers that took them during the periconceptional period, compared to those who took them after the second month of gestation.[17]

On the other hand, the risk of giving birth to a child with a conotruncal defect was 6.3 times higher for women with the T/T genotype who did not take folic acid supplements prior to conception.[20] However, this evidence tends to be inconsistent as some authors have found no risk of CHD associated with the T/T genotype in mothers of patients or in the patients themselves.[21–23]

Owing to the high frequency of the 677T/T polymorphism in the Mexican population and the high incidence of CHD, the objective of this study is to analyze the possible association between isolated CHD and the 677T/T genotype of the *MTHFR* gene.

METHODS

Patient Selection

This project was approved by the ethics and research committees of the *Instituto Nacional de Perinatología* and of the *Instituto Mexicano del Seguro Social*, in accordance with the guidelines of the Declaration of Helsinki. We included 60 mestizo pediatric patients with a diagnosis of isolated CHD and their mothers, after obtaining their informed consent. The patients were recruited between 1 March 2004 and 28 February 2005 in the *Servicio de Cardiopatías Congénitas* (Congenital Heart Disease Service), *Hospital de Cardiología, Centro Médico Nacional Siglo XXI*. The patients whose mothers had type 1 or type 2 diabetes mellitus or were taking antiseizure medication were excluded. Likewise, following informed consent of the mother-child pair, 62 healthy controls were recruited from the general mestizo population of the central region of Mexico.

Peripheral blood samples were collected in EDTA tubes and DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, United States). The amount and purity of the DNA were verified by spectrophotometry and by 1% agarose gel electrophoresis stained with ethidium bromide, respectively.

Determination of the 677CT Polymorphism

A 250-ng sample of genomic DNA was used for polymerase chain reaction (PCR) under the following conditions: 5 μL of 10X PCR Buffer (200 mM; Tris-HCl, pH 8.4, 500 mM KCl), 1 μL of 2.5 mM dNTP, 5% DMSO, 2 mM $MgCl_2$, 10 pmol of each oligonucleotide for the analysis of the 677CT polymorphism (sense: 5'GCAGGGAGCTTTGAGGCTGAC-3' and antisense: 5'AGGACGGTGCGGTGAGAGTG-3'), and 0.5 U of Taq polymerase (Invitrogene™), in a final volume of 50 μL. A 228-bp segment located in exon 4 was amplified; this segment contains a cut site for the restriction enzyme, *Hinf*I, (New England Biolabs™), the product of a substitution of C by T at position 677. The program employed consisted in: initial denaturation at 92 °C for 2 min and 35 cycles of denaturation at 92 °C for 2 min, annealing at 58 °C for 45 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. Then, a 15-μL aliquot of the PCR product was subjected to

digestion with 1 U of the restriction enzyme, *Hinf*I, in a total volume of 25 μL, and incubated for 3 h at 37 °C. The restriction fragments were analyzed by means of 4% agarose gel electrophoresis, followed by staining with ethidium bromide. When the 677CT polymorphism of the *MTHFR* gene was present, the digestion products were 172 bp and 56 bp in length.[24]

Determination of Homocysteine Concentrations

The quantification of L-homocysteine was carried out by competitive immunoassay in serum of case mother-child pairs (children with CHD and their mothers), using an IMMULITE® 2000 analyzer (Siemens Medical Solutions Diagnostics, Malvern, Pennsylvania). The samples were processed in accordance with manufacturer specifications. The sample volume required was 15 μL and the sensitivity of the assay for Hcy was 0.5 μmol/L.

Prenatal Folic Acid Intake by the Mothers in the Study Cases

All the mothers in the case pairs had been asked directly in the questionnaire whether they had received folic acid supplementation alone or as part of a multivitamin preparation, at doses of 400 μg. The intake was classified as pregestational or gestational; in the latter cases, the mothers were asked when the supplementation had begun and how long it continued.

Statistical Analysis

Both the allele frequencies and the observed and expected heterozygosity were estimated using a gene-counting method. We determined whether the genotype distribution complied with Hardy-Weinberg equilibrium by applying the Monte Carlo method (Guo and Thompson, 1992). The differences between groups/populations were assessed by means of exact tests using the TFPGA (Tools For Population Genetics Analysis) program, version 3.1 (Miller, 1998). The odds ratio (OR) for each comparison was estimated with the Epi Info™ 2000 software package. The level of significance for the exact tests was assessed carrying out 5000 simulations with a 95% confidence interval (95%CI). To establish an association between the genotypes of the mother-child pairs and the presence of CHD, we applied the χ^2 test with a confidence level of 95%. In the mothers of patients with CHD, we determined the relationship between Hcy concentrations, genotypes, and folic acid intake, and compared the Hcy concentrations between the different genotypes of the patients with CHD using ANOVA. In addition, Pearson correlation coefficients were applied to the Hcy level of each patient with CHD with respect to the presence of the risk allele in the genotype, converted to a continuous variable as follows: CC=1, CT=2, TT=3.

RESULTS

We analyzed the findings in 60 pediatric patients with isolated CHD and their mothers, as well as 62 controls and their mothers. Study participants came from the Federal District and 9 Mexican states (State of Mexico, Guanajuato, Guerrero, Queretaro, Morelos, Nayarit, Oaxaca, Puebla, and Veracruz), whereas the 62 controls were from the Federal District and the State of Mexico. The patient group, recruited in a children's hospital, included newborn infants and individuals up to the age of 17 years, with a mean age of 5.6 years. The controls ranged in age between 18 and 26 years, with a mean age of 20 years. The patient group consisted of 33 girls (55%) and 27 boys (45%); among the controls there were 24 women (38.7%) and 38 men (61.3%). The mean age of the mothers of the patients at the time of their pregnancy was 26 years (24 years for the mothers of the controls). The cardiac anomalies in the cases studied are listed in Table 1.

The CHD population in this study came from a wide range of geographic regions, from northern, central, and southern Mexico; despite this fact, the allele and genotype frequencies were found to be homogeneous (Table 2). The absence of significant differences in the allele and genotype frequencies of the patients as compared to the controls and of the mothers of the patients vs those of the control group ($P>.05$) was a noteworthy finding. The genotypes in the study cases and in the controls deviated from Hardy-Weinberg equilibrium ($P<.05$), a circumstance that could be attributed in both groups to the higher than expected number of heterozygotes, although these results are compatible with the so-called "hybrid vigor" for the 677CT polymorphism; our study was not designed to

Table 1
Types of Isolated Congenital Heart Disease in the Patients Recruited in the *Servicio de Cardiopatías Congénitas* of *Hospital de Cardiología* del *Centro Médico Siglo XXI* of the *Instituto Mexicano del Seguro Social* (Mexico DF)

Cardiac diagnosis	Genotype			No. of patients
	CC	CT	TT	
VSD		4	3	7
ASD	2	8	1	11
AC		3		3
Tricuspid atresia	1	2		3
Aortopulmonary window		1		1
Mitral stenosis	1			1
Aortic stenosis		1		1
Pulmonary valve stenosis		1		1
Vascular ring		1		1
TF		1	1	2
PA + VSD	1	1		2
CTD		1		1
TGV		1	1	2
Single ventricle and single atrium			1	1
Single ventricle and single atrium + PA		1		1
Single ventricle		2		2
Single ventricle + PS		1	1	2
Single ventricle + PA	1			1
DORV	1	2		3
DORV + VSD		1		1
DORV + PS		1		1
DORV + PS + dextrocardia		1		1
TAPVC + ASD		1	1	2
TAPVC		1		1
Ebstein anomaly		2	1	3
ASD + aortic valve stenosis			1	1
ASD + pulmonary valve stenosis		1		1
PA + PDA			1	1
Mitral stenosis + tricuspid regurgitation		1		1
AC + VSD + PDA		1		1
Total	7 (11.7)	41 (68.3)	12 (20)	60 (100)

AC, aortic coarctation; ASD, atrial septal defect; CTD, conotruncal defect; DORV, double outlet right ventricle; PA, pulmonary atresia; PDA, patent ductus arteriosus; PS, pulmonary stenosis; TAPVC, total anomalous pulmonary venous connection; TF, tetralogy of Fallot; TGV, transposition of the great vessels; VSD, ventricular septal defect.
Data are expressed as no. (%).

Table 2
Comparison of the Allele and Genotype Distribution of the 677CT Polymorphism of the *MTHFR* Gene in Patients, in Controls, and in Their Respective Mothers

	Children					Mothers				
MTHFR	Genotype			Allele		Genotype			Allele	
	C/C	C/T	T/T	C	T	C/C	C/T	T/T	C	T
Patients	7 (11.7)	41 (68.3)	12 (20)	55 (45.8)	65 (54.2)	8 (13.3)	38 (63.3)	14 (23.3)	54 (45)	66 (55)
Controls	9 (14.5)	46 (74.2)	7 (11.3)	64 (51.6)	60 (48.4)	13 (21)	37 (59.7)	12 (19.3)	63 (50.8)	61 (49.2)
Comparison[a]		$P \geq .788$[b]		$P = .844$			$P \geq .264$[b]		$P = .364$	

Data are expressed as no. (%).
[a] Fisher exact test to compare patients and controls.
[b] Lower probability value when comparing genotypes grouped to test different models of inheritance for the T risk allele: *a)* recessive ([C/C + C/T] and T/T), and *b)* dominant (C/C and [C/T + T/T]).

Table 3
Comparison of the Allele and Genotype Distribution of the 677CT Polymorphism of the *MTHFR* Gene in Patients With Complex Congenital Heart Disease and Those With Simple Congenital Heart Disease

	Genotype			Allele	
	C/C	C/T	T/T	C	T
CCHD	5 (14.3)	23 (65.7)	7 (20)	33 (47.14)	37 (52.8)
SCHD	2 (8)	18 (72)	5 (20)	22 (44)	28 (56)
Comparison[a]		$P = .4546$[b]		$P = .7334$	

CCHD, complex congenital heart disease; SCHD, simple congenital heart disease.
Data are expressed as no. (%).
[a] Fisher exact test to compare patients and controls.
[b] Lower probability value when comparing genotypes grouped to test different models of inheritance for the T risk allele: *a)* recessive ([C/C + C/T] and T/T), and *b)* dominant (C/C and [C/T + T/T]).

demonstrated this phenomenon, and the finding does not warrant a more in-depth discussion in this respect. We should point out that genotyping errors were ruled out by evaluating each sample in triplicate and by the use of positive and negative controls for PCR and enzymatic digestion, in addition to having the results (genotypes) read by 2 independent observers.

The population with CHD (n=60) was stratified into 2 groups: patients with complex congenital heart disease (CCHD), consisting of 35 patients (58.33%); and those with simple congenital heart disease (SCHD), with 25 patients (41.67%). The evaluation of the T/T, C/T, and C/C genotypes and the T and C alleles according to type of heart disease in the patients with CCHD and those with SCHD revealed no significant differences in the genotype or allele frequencies ($P > .05$) (Table 3).

The Hcy concentrations in the mothers did not differ significantly when they were grouped by genotypes, alleles, or folic acid intake ($P = .7465$). The differences in the Hcy levels were determined according to genotype (Table 4). The comparison of the distribution of Hcy concentrations in the C/C and T/T groups revealed differences that nearly reached statistical significance ($P = .0621$). The correlation between the Hcy levels and the presence of the T allele was not statistically significant ($P = .2497$; $r^2 = 0.42$). In all, 41.7% of the mothers of children with CHD had not received folic acid therapy. The remaining 58.3% of the mothers who took supplements was divided between those who received them prior to conception (8.57%) and those who took them during their pregnancy (91.43%), after week 6 of gestation. We examined the possible relationship between the folic acid intake and Hcy levels by comparing them in women who received supplements and in those who did not, finding values of 8.20 (2.39) μg/L and 8.0164 (3.4) μg/L, respectively, in the mothers of patients with CHD, with no significant differences ($P > .05$).

The analysis of the genotypes in the mother-child pairs resulted in 7 combinations and, although no significant differences were found between the case pairs and the control pairs in terms of any of these combinations, we observed a trend toward a greater frequency of the CT/TT combination among the case pairs as compared to the controls (OR=2.9; 95%CI, 0.98-10.87) (Table 5).

Table 4
Homocysteine Concentrations in Patients With Congenital Heart Disease According to the Genotype of the 677CT Polymorphism of the *MTHFR* gene

	Patients, no.	Homocysteine (μg/L), mean (SD)	SE
C/C	7	6.8 (2.4)	0.9
T/C	41	9.3 (5.5)	0.8
T/T	12	11.6 (5.6)	1.6

SD, standard deviation; SE, standard error.
ANOVA test. 95% confidence interval.

DISCUSSION

Congenital heart disease is one of the major causes of death in children both in Mexico and in other countries. Due to the high frequency of the 677CT polymorphism of the *MTHFR* gene in the Mexican population, we were mainly interested in determining

Table 5
Genotype Combinations Found in the Case Mother-Child Pairs and Control Mother-Child Pairs

Genotype	Study cases, no. (%)	Controls, no. (%)
TT/TT	3 (5)	3 (4.8)
TT/CT	11 (18.3)	9 (14.5)
CT/TT	9 (15)	4 (6.4)
CT/CT	24 (40)	28 (45.2)
CT/CC	5 (8.3)	5 (8.1)
CC/CT	6 (10)	9 (14.5)
CC/CC	2 (3.3)	4 (6.5)
Total	60 (100)	62 (100)

Study cases vs controls (χ^2), $P = .7465$; standard error=0.014.

what genetic factor could be associated with the alterations leading to the development of CHD in the embryo.

The allele frequencies observed in the groups studied here were along the lines of those reported previously in the mestizo population in northwestern, central and southeastern Mexico.[15–17] The majority of our patient population (85%) was from central Mexico (Federal District, State of Mexico, Guanajuato, Queretaro, Morelos and Puebla). The remainder of the mestizo population (15%) came from western and southern Mexico (the states of Nayarit, Guerrero, Oaxaca and Veracruz), where we found a frequency of 22% for the T/T genotype and of 56% for the T allele; these incidences also correspond to those reported previously.[15–17] The initial evaluation of the genotype and allele frequencies in the two groups revealed the predominance of the T allele in the patients and their mothers, whereas in the controls we found a prevalence of the C allele.

The statistical analysis applied to the frequencies of the C/C, C/T, and T/T genotypes in the patient group vs the control group, and in the mothers of the subjects in the two groups, demonstrated that there were no significant differences ($P>.05$). In the light of this finding, we can say that it is not possible to associate any CHD with the presence of the 677CT polymorphism of the *MTHFR* gene in the patient group with respect to the controls and their respective mothers in our population. The types of CHD studied here were heterogeneous in accordance with their embryonic origin, and there were both SCHD and CCHD. Despite the fact that the most serious CCHD (thus defined on the basis of their high rates of mortality) are not found in a great proportion of the older patients, the differences in the frequencies observed for each type of heart disease enable us to affirm that, for the purpose of this study, the type of CHD does not introduce a bias in the modification of these frequencies.

These findings show that the pathogenic processes involved in the development of the heart in the patient group differ and involve simultaneous cellular events that follow a chronological order over short periods of time, a circumstance that makes it more difficult to detect an association in this group of conditions.

Hyperhomocysteinemia in the mother is a factor related to the presence of CHD, an observation that is supported by studies in which it was found that up to 46.2% of the mothers of children with these defects had hyperhomocysteinemia under fasting conditions and when they had no multivitamin supplementation, vs only 14.3% of the mothers of the healthy controls.[25] Moreover, a study in mothers of these patients demonstrated that maternal hyperhomocysteinemia increases the risk of giving birth to a child with CHD by 2.9 to 4.4 times compared to women who do not have this condition.[26] One of the elements that causes the elevation in plasma Hcy is the presence of the 677CT polymorphism in the homozygous state.[27]

In our study, we determined the Hcy concentrations in the patients and in their mothers, and found no differences between the patients with the C/C and the T/T genotype. This indicates that, at least in the population studied here, either the homozygous genotype for this mutation is not a determining factor in establishing the Hcy concentrations or folic acid supplementation has a direct effect on these concentrations, a circumstance that, ultimately, does not protect against the cardiac condition. These results have been observed in other populations[23] in which it has not been possible to relate the hyperhomocysteinemia associated with the 677CT polymorphism with the presence of CHD. However, the difference in the Hcy levels between the patients with CC and TT genotypes was close to the limit of significance ($P=.06$) and therefore this finding, in particular, should be confirmed or refuted.

Although the CT/TT genotype combination in the mother-child pair in patients and controls did not differ significantly, the OR (2.9; 95%CI, 0.975-10.871) indicates that, in the long run, an association might be found if the sample size were increased. It is noteworthy that no differences in the OR were found for the TT/TT genotypes in the case mother-child pairs, probably because: *a)* this genotype combination is not associated with the types of heart disease studied in this report, and *b)* the number of cases analyzed for each type of cardiac condition was insufficient to enable the detection of this association in the analysis of the mother-child pair genotype.

On the other hand, the CT/TT genotype combination (OR=2.9) could confer a higher risk of CHD and confirms studies in which the T/T genotype has been found in patients with this alteration.[7,8,18,19] According to the results obtained in this study, the risk of CHD may be the consequence of the genotype combination in the mother-child pair, which could increase the Hcy concentrations[27] during embryonic development, with the resulting deficiency in nucleic acid synthesis during gestation. This could indicate that the combination of the heterozygous state in the mother and T/T homozygosity in the fetus is a risk factor for CHD. This is significant due to the fact that the frequency of the T allele in the Mexican mestizo population is as high as 58.5%.[14–16]

Among the limitations of the study, we can point to the small size of the sample population analyzed, considering that: *a)* the type I error produced by the small sample size calls for the confirmation of these results in a larger study population; *b)* the confidence intervals found in the analysis of the genotype combinations are wide, and *c)* the reported frequency of the 677CT polymorphism in the Mexican population as a whole is the highest in the world. It is important to mention that the marginal OR (2.9) and its 95%CI (0.975-10.871) indicate that increasing the size of the study population would make it possible to detect the association with the phenotype being analyzed. On the other hand, the Hcy concentrations found in the patients with CHD and the CC genotype do not differ significantly from those recorded in patients with the TT genotype, probably due to prenatal folic acid supplementation. However, this effect is inevitable when the cross-sectional design of the study is taken into consideration.

CONCLUSIONS

According to the findings of the present study, it is not possible to establish an association between the 677CT polymorphism of the *MTHFR* gene and the presence of heart disease in the Mexican mestizo population. There were no significant differences in the Hcy concentrations in the patients and their mothers related to the presence of the C/C or T/T genotype, or to the maternal intake of folic acid. Similar studies with larger numbers of patients need to be carried out.

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CONFLICTS OF INTERESTS

None declared.

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