

Association of MTHFR rs1801133 and Homocysteine With Legg-Calvé-Perthes Disease in Mexican Patients

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Research

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Abstract

Background: Legg-Calvé-Perthes disease (LCPD) is an avascular osteonecrosis of the femoral epiphysis. It is a rare disease of unclear etiology in children. Alterations in coagulation, or the collagen gene have been described and could be associated with its etiology. Therefore, we set out to evaluate the following alterations: COL1A1 (rs1107946, rs2412298), COL2A1 (rs121912891 and rs387106558), MTHFR rs1801133, CBS rs115742905, and PT rs1799963 and their relationship with LCPD.

Methods: DNA was obtained and genotyped by real-time PCR with TaqMan probes. It was determined prothrombin and homocysteine (Hcy) by a coagulometric method. The variables were described as mean and standard deviation or percentages, and genotypic and allelic distributions were analyzed using the Student's t-test. In addition, the Hardy-Weinberg equilibrium, and OR.

Results: We studied 23 patients with LCPD and 46 controls. We did not find any association of the MTHFR, CBS, PT, COL1A1, and COL2A1 genetic variants with LCPD. However, when adjusting the data with the Hcy values for the MTHFR C677T polymorphism, the C/C genotypes showed an association with the recessive model ($p = 0.038$) with susceptibility to LCPD.

Conclusion: No association was found with the CBS, PT, COL1A1, and COL2A1 genes. Nevertheless, our results suggest a significant link between moderately elevated Hcy levels and the MTHFR C677T polymorphism in a cohort of Mexican children with LCPD.

Introduction

Due to its low prevalence, Legg-Calvé-Perthes disease (LCPD) is rare, particularly in Latin America. It is the avascular osteonecrosis of the femoral head that occurs in the pediatric population [1,2]. The interruption of the blood supply causes fragmentation and deformation of the femoral head, which leads to mechanical alterations in the hip [3]. Although there are several proposed causes, its etiology is still unknown. Thrombophilia is one of the most widely accepted since the tendency to develop states of hypercoagulability could lead to the development of necrosis [4-6]. On the other hand, described collagen gene alterations could also be associated with the etiology of the disease [7].

The methylenetetrahydrofolate reductase enzyme (MTHFR), together with the Cystathionine β -synthase (CBS), regulates the serum homocysteine (Hcy) level. MTHFR C677T and CBS T833C polymorphisms, Hcy levels, and other risk factors like polymorphisms of prothrombin G20210A (PT G20210A) have been linked to the development of prothrombotic states [8]. Various studies have suggested that these conditions could be related to the etiology of LCPD [9,10].

LCPD consists of sequential stages of involvement of the femoral epiphyses, including subchondral fracture, fragmentation, reossification, and healing with residual deformity. Genetic factors have been related to the etiology of LCPD, but the causal gene has not been identified. Nonetheless, COL2A1 gene mutations have been implicated in the etiology of LCPD because collagen is the essential matrix protein of all connective

tissues. Some of these alterations could cause structural defects of type II collagen related to the major clinical aspects [11,12].

On the other hand, the COL1A1 gene encodes the alpha-1 protein chain of type I collagen, the primary protein of bones. Some research focused on the COL1A1 polymorphisms associates them significantly with low bone mineral density, osteoporosis, increased fracture risk, and osteonecrosis [13,14]. Also, the deleterious effect of Hcy on bones may be mainly mediated by the binding of the molecule to collagen, which interferes with the formation of type I collagen cross-links [15]. Furthermore, several studies report a significant relationship between hypercoagulability and osteonecrosis [16]. Because of the above, our objective is to evaluate the following alterations involved with coagulation and collagen genes: MTHFR C677T rs1801133, CBS T833C rs115742905, and PT G20210A rs1799963; the following collagen-related polymorphisms: COL1A1 G1997T rs1107946, COL1A1 C1663T rs2412298, COL2A1 G2306A rs121912891, and COL2A1 G3665A rs387106558; and their relationship with LCPD.

Methods

We carried out a case-control study. All patients were recruited from the Orthopedic Service at Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra (INR-LGII) in Mexico City. This study included a total of 69 subjects: 23 cases of less than 18 years of age with a clinical and radiological diagnosis of LCPD and 46 healthy controls, matched with the patients 2:1 according to age and gender. In addition, we obtained weight, height, and body mass index (BMI). Patients diagnosed with other bone diseases, diseases related to coagulation abnormalities, and those under pharmacological treatment were excluded to avoid modifying bone metabolism and anticoagulant therapy.

Genotyping

DNA was obtained from peripheral blood leukocytes. Genotyping was performed by real-time PCR with TaqMan probes in the conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA). Briefly, reactions were performed in 25 ml volume, containing TaqMan PCR master mix (1X), TaqMan probe (100 nm), primers (900nm of each), and DNA (25 ng). Samples were run in a StepOne Real-Time PCR System (Applied Biosystems). Cycling conditions: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and a final cycle at 60°C for one min.

Determination of coagulation factor FII and Hcy

Plasma was obtained from peripheral blood in tubes with 3.8% sodium citrate. The HemosiTTM 0020012200 chromogenic kit and the HemosiTTM 0020007800 kit for Hcy were used in ACL-7000 elite pro equipment to determine the prothrombin coagulation factor II (FII). The results were expressed as a percentage of activity for FII and in $\mu\text{mol} / \text{L}$ for Hcy.

Statistical analysis

The variables are described as mean \pm standard deviation or as percentages (%) where appropriate. Differences in genotypic and allelic distribution between case and control groups were analyzed using

Fisher's exact test or Student's t-test. The Hardy-Weinberg equilibrium (HWE) was calculated for the genotype frequencies in cases and controls using a Chi-square goodness of fit test in SNPStats. The odds ratio (OR) and the confidence interval (95% CI) were calculated to assess the relative risk of having the disease according to the relative frequency of different genotypes between cases and controls with PAST 4.03. Furthermore, the inheritance patterns for SNPs and associations were estimated using SNPStats (<http://www.snpstats.net/start.htm>).

Ethical aspects

The cases were patients with LCPD. Controls were selected under the official Mexican standard NOM-253-SSA1-2012 guidelines, all participants received oral and written information, and parents or guardians signed a letter of consent. This study was approved by the INR-LGII research and ethics committees.

Results

23 patients (21 men and 2 women) and 46 controls (42 men and 2 women) were recruited. The average height in patients was 1.48 ± 0.23 m compared to 1.49 ± 0.23 m in controls. The average weight was 46.21 ± 15.02 kg in patients, compared to 53.83 ± 24.06 Kg in controls, without significant difference between patients and controls. Similarly, BMI presented no significant differences between patients and controls.

Prothrombin activity did not show a significant difference between the mean of the patients compared to the controls. However, regarding the levels of Hcy, there were significant differences in the mean of the patients in comparison to the controls (Figure 1).

The genotype frequencies of the MTHFR rs1801133 polymorphism were in HWE in controls and patients ($p = 0.76$ and $p = 0.40$, respectively). The CBS rs115742905 and PT rs1799963 variants are not found in HWE because the mutant allele is absent in either case. The wild genotype resulted in 100% in both populations (Table 1).

The genotype frequency of the COL1A1 rs1107946 variant was not found in HWE with respect to the controls. No significant differences were observed for the genotype and allelic distribution for the polymorphism between the patients with LCPD and the controls. The COL1A1 variant rs24122948 was found in HWE, but no differences were observed. In the case of COL2A1 rs24122948 and rs106558, we did not find the presence of the mutant alleles (Table 1).

Table 1 presents the link of MTHFR to age, which showed a significant association with the recessive inheritance model.

Discussion

The prevalence of LCPD is highly variable in different populations. Since it is a rare disease, there are very few reports in Mexico and Latin America that describe it. Children with LCPD begin with pain in one or both legs and a slight limp, usually attributed to falls or poor posture. The pain sometimes goes away temporarily, but the lameness continues. Thus, a long journey of visits to various medical services begins, and it usually

does not end with a specific diagnosis nor adequate treatment. The correct diagnosis is only given when patients finally go to specialized pediatric orthopedic institutions, such as the INR-LGII. Nevertheless, the number of cases reported in recent years in the INR-LGII is still six to eight cases per year, and each year the number decreases, for the lack of information does not allow an early and specific diagnosis.

The approach to the disease is complicated because its etiology is not yet clear. Genes involved in bone remodeling and architecture have been studied. Furthermore, elevated levels of Hcy have been linked to osteonecrosis [17]. For this reason, we decided to explore variants in the COL1A1 gene that have been studied in relation to osteoporosis and fractures [18,19]. However, none of these variants was informative in the studied sample of Mexican patients with LCPD. It has also been suggested that COL2A1, involved in osteonecrosis of the femoral head, is related to skeletal dysplasia due to failure of cartilage development and growth [20]. However, none of these alterations had significant differences between the patients and the controls studied. Additionally, COL2A1 is suggested to be one of the representative causal genes, as Li et al. found a mutation in COL2A1 G1888A in a Chinese family affected by LCPD and osteonecrosis of the femoral head [12,21], and Miyamoto et al. identified a mutation in COL2A1 G3508A in a Japanese family with LCPD. These findings differ from our results since we found no relationship with LCPD [22].

The PT G20210A mutation increases the risk of thrombosis [23]. López et al. studied 90 children with LCPD, and no patient had a family or personal history of early thrombotic events. However, four children with LCPD (4.4%) were heterozygous for the G20210A polymorphism compared to controls but without any significant association [24]. Vosmaer et al. found an increase in the incidence of LCPD in the presence of the prothrombin mutation when the FVL polymorphism was also present [25]. Our study did not find significant differences that relate this polymorphism, PT rs1799963, with LCPD.

Common alterations in some genes related to Hcy metabolism, such as MTHFR C677T and CBS T833C, have been shown to cause increased plasma homocysteine levels, thus bringing about a predisposition to thrombosis [26]. Our study did not find these variants, CBS T833C rs5742905, neither in controls nor in patients. Azarpira et al. showed that the MTHFR C677T polymorphism was not associated with the risk of LCPD in Iranian children [27]. When we analyzed the results for the MTHFR C677T rs1801133 polymorphism, it did not present a significant association with the risk of susceptibility to LCPD. However, the frequency of the mutant allele was higher than in the Iranian population, even though several epidemiological case-control studies have found that MTHFR polymorphisms could not play a significant role in the susceptibility to the development of osteonecrosis of the femoral head [28,29]. In this study, we also found that the levels of Hcy between cases and controls present a significant difference ($p < 0.05$). In addition, when adjusting the value of the MTHFR C677T polymorphism with age, we found a significant association in the recessive model (OR 5.62 (1.02-30.93), $p = 0.038$).

In general, hyperhomocysteinemia is a risk factor for various diseases: vascular, neurological, diabetes, psoriasis, cancers, including osteoporosis, among others [30]. In addition, Hcy is thought to alter bone remodeling [31]. Furthermore, it has been described that short-term moderate hyperhomocysteinemia affects bone and cartilage characteristics. Trabecular bone microarchitecture is especially sensitive to hyperhomocysteinemia. It shows clearly negative bone balance, mainly due to a decrease in trabecular

numbers and markedly reduced trabecular connections, which indicates multiple alterations in collagen due to homocysteine accumulation in bones, indicative of broken collagenous cross-links [32].

Conclusion

This is the first study to evaluate genetic aspects of coagulation mediators and type I and II collagen in Mexican patients with LCPD. We did not find a relationship of collagen alterations (COL1A1 rs1107946, COL1A1 rs2412298, COL2A1 rs121912891, and COL2A1 rs387106558) or coagulation alterations (CBS T833C rs115742905 and PT G20210A rs1799963) with LCPD. However, we found an association of the MTHFR rs1801133 polymorphism in a recessive model adjusted by age and elevated levels of Hcy in a population of Mexican children with LCPD.

Abbreviations

LCPD: Legg-Calvé-Perthes disease.

COL1A1: Collagen type I alpha 1 chain.

COL2A1: Collagen type II alpha 1 chain.

MTHFR: methylenetetrahydrofolate reductase.

CBS: cystathionine beta synthase.

PT: Prothrombin.

DNA: Deoxyribonucleic acid.

Hcy: Homocysteine.

INR-LGII: Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra Ibarra".

BMI: Body mass index.

FII: Coagulation factor II.

HWE: Hardy-Weinberg equilibrium.

OR: Odds ratio.

CI: Confidence interval.

SNPs: Single nucleotide polymorphisms.

NOM: Norma Oficial Mexicana.

Declarations

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Contributions

EHZ, AORO, and ERM conceived and designed the experiments. JGBP, LCA, MVF, AORO and EHZ collected blood samples and clinical data. JGBP and AORO performed the experiments. EHZ, AORO, JGBP and ERM wrote the paper. All authors read and approved the final manuscript.

Ethics declarations

All participants received oral and written information about the study and signed a letter of consent. The study protocol was reviewed and approved by the INR-LGII Research and Ethics Committees.

Consent for publication

All authors consent to publish.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All relevant data used in this study have been included in the manuscript. The corresponding author can be contacted if any further information is needed.

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Tables

Table 1. Association of MTHFR rs1801133, CBS rs115742905, PT rs1799963, COL1A1 rs1107946 and rs2412298, COL2A1 rs121912891 and rs387906558 with Legg-Calvé-Perthes Disease

SNP and Model	Genotype	Controls n (%)	Cases n (%)	OR (95% CI)	p-value #	OR (95% CI) *	p-value *
MTHFR rs1801133							
	T/T	15 (32.6%)	6 (26.1%)	1.00		1.00	
Codominant	C/T	21 (45.6%)	9 (39.1%)	1.07 (0.31-3.66)		0.43 (0.08-2.30)	
	C/C	10 (21.7%)	8 (34.8%)	2.00 (0.53-7.54)	0.51	3.69 (0.56-24.56)	0.07
Dominant	T/T	15 (32.6%)	6 (26.1%)	1.00		1.00	
	C/T-C/C	31 (67.4%)	17 (73.9%)	1.37 (0.45-4.19)	0.58	0.97 (0.23-3.99)	0.96
Recessive	T/T-C/T	36 (78.3%)	15 (65.2%)	1.00		1.00	
	C/C	10 (21.7%)	8 (34.8%)	1.92 (0.63-5.81)	0.25	5.62 (1.02-30.93)	0.038
CBS rs115742905							
	T/T	46 (100.0%)	23 (100.0%)	Monomorphic SNP		Monomorphic SNP	
—————	T/C	0 (0.0%)	0 (0.0%)				
	C/C	0 (0.0%)	0 (0.0%)				
PT rs1799963							
	G/G	46 (100.0%)	23 (100.0%)				
—————	G/A	0 (0.0%)	0 (0.0%)	Monomorphic SNP		Monomorphic SNP	
	A/A	0 (0.0%)	0 (0.0%)				
COL1A1 rs1107946							
	G/G	21	13	1.00		1.00	

		(45.6%)	(56.5%)				
Codominant	G/T	13 (28.3%)	6 (26.1%)	0.75 (0.23- 2.45)	0.63	0.73 (0.22- 2.42)	
	T/T	12 (26.1%)	4 (17.4%)	0.54 (0.14- 2.03)		0.53 (0.14- 2.00)	0.62
Dominant	G/G	21 (45.6%)	13 (56.51%)	1.00		1.00	
	G/T-T/T	25 (54.4%)	10 (43.5%)	0.60 (0.17- 2.11)	0.41	0.63 (0.23- 1.75)	0.38
Recessive	G/G-G/T	34 (73.9%)	19 (82.6%)	1.00		1.00	
	T/T	12 (26.1%)	4 (17.4%)	0.60 (0.17- 2.11)	0.41	0.59 (0.17- 2.10)	0.41
COL1A1 rs2412298							
	C/C	39 (84.8%)	19 (82.6%)	1.00		1.00	
—————	C/T	7 (15.2%)	4 (17.4%)	1.17 (0.31- 4.50)	0.82	1.18 (0.31- 4.55)	0.81
COL2A1 rs121912891							
	G/G	46 (100.0%)	23 (100.0%)	Monomorphic SNP		Monomorphic SNP	
—————	G/A	0 (0.0%)	0 (0.0%)				
	A/A	0 (0.0%)	0 (0.0%)				
COL2A1 rs387906558							
	G/G	46 (100.0%)	23 (100.0%)	Monomorphic SNP		Monomorphic SNP	
—————	G/A	0 (0.0%)	0 (0.0%)				
	A/A	0 (0.0%)	0 (0.0%)				

Raw data; * Adjusted by age; Significant values are shown in bold.

Figures

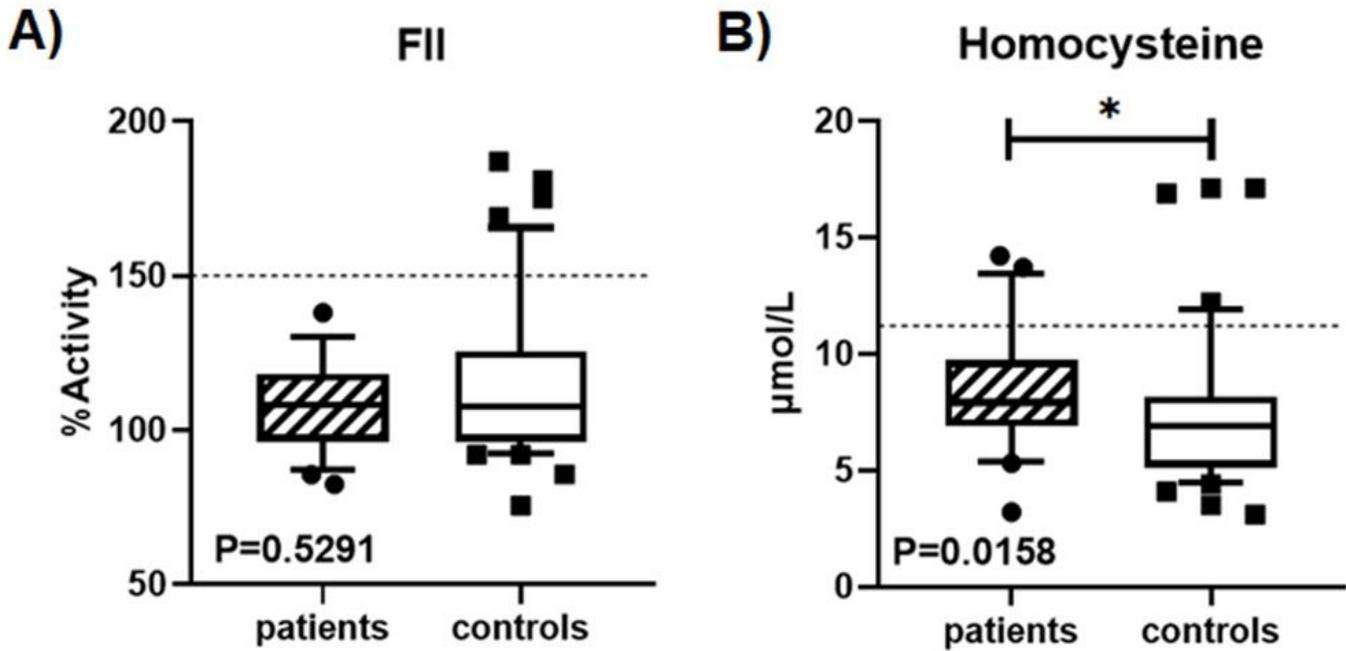


Figure 1

Comparison of A) Factor II (prothrombin) and B) Homocysteine. Mustache graphs showing the median and the 10-90 percentile. Out-of-range values (•, ••, ■, ■■, outliers) are represented with dots outside the mentioned percentiles. The p-value from the comparison between patients and controls is also shown. Compared samples that showed a significant difference according to the Mann-Whitney U-test are highlighted with an asterisk (*), dotted line= maximum proposed reference value for children (···).