Familial cases of Mexican patients with Legg-Calvé-Perthes disease

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Research

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Abstract

Background

Legg-Calvé-Perthes Disease (LCPD) is described as an avascular necrosis of the femoral head. Although its etiology is still not fully understood, evidences suggest heritable thrombotic disorders and other factors may be implicated in its onset and progress. Our objective is to describe, in three enrolled families, the genetic, biochemical and environmental factors that may be associated with the etiology and development of LCPD.

Methods

Therefore, we set out to evaluate the following alterations of collagen genes: MTHFR rs1801133, CBS rs115742905, and PT rs1799963 and their relationship with LCPD. Thrombophilia associated markers (FI, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, FvW, PC, PS, AT, and homocysteine) were evaluated using coagulometry methods. Results: Seven LCPD patients and 14 healthy volunteers were enrolled. Concentrations in hemoglobin ($p \leq 0.0001$), fibrinogen ($P \leq 0.0001$), homocysteine ($p = 0.0414$), factor IX activity percentage ($p \leq 0.0001$), and protein S ($p = 0.0478$) showed statistically significant differences. None of the evaluated polymorphisms showed statistically significant differences. However, all patients presented the mutated MTHFR C677T polymorphism in a homozygous (T/T) or heterozygous manner (C/T).

Conclusions

Our results show environmental elements from every family and hemostatic disorders may be involved in suffering and developing LCPD. Also, heritable factors could contribute to the onset of the disease. Clearly, environmental, genetic, and prothrombotic factors are involved in this pathology.

Background

Legg-Calvé-Perthes disease (LCPD) is considered a rare disease due to its low incidence and unknown etiology. LCPD presents as uni- or bilateral avascular necrosis of the femoral head (FH), which affects the range of motion of the hip to varying degrees and causes pain in the affected limb that intensifies during and after physical activity [1,2]. Between 1909 and 1910, Waldenström in Sweden, Calvé in France, Perthes in Germany, and Legg in the United States, presented several studies where they classified and described LCPD as a new and uncharacterized pathology [3,4]. LCPD has a very variable incidence, it ranges between 0.4/100000 and 29.0/100000, and its appearance is mainly in males. Unfortunately, in our country, there is no prevalence data, but, due to its occurrence and appearance, it is considered as a low incidence disease [5-7]. There are multiple theories about the etiology of LCPD; however, many of them remain controversial due to lack of foundation and/or reproducibility. Nonetheless, the interruption of blood flow to the femoral head and subsequent ischemic necrosis seem to be key events in the development of LCPD, since, after these, the pathological and structural changes characteristic of LCPD are perceptible. At the moment, there is multiple evidence of absence of blood flow to the affected FH; in addition, histological studies have shown changes consistent with ischemic necrosis of the deep portion of the articular cartilage [8]. Research shows that it appears that at least two ischemic episodes are necessary for LCPD to develop, although studies in animal models have found that a single ischemic event produces changes like those found in LCPD [9-11]. Necrosis will lead to the decay of the mechanical and support properties of the bone and articular cartilage, resulting in the deformation of the FH due to the mechanical forces applied to it [9,12].

There is evidence that genetic mechanisms may be involved in the etiology of LCPD, and inheritance patterns ranging from autosomal recessive to polygenic have been proposed. While in families with a high rate of affected individuals there appears to be an autosomal dominant mode of inheritance [13-15], Gray et al. found that the rate of occurrence of LCPD in first-, second- and third-degree relatives combined was 1:39, and 1:26 among siblings, i.e. 35 and 50 times greater than in the general population [16]. Because of this, some authors describe the possibility of a greater association of LCPD among relatives.

Furthermore, hemostatic alterations such as hypofibrinolysis and/or hypercoagulable states are proposed as triggering factors of LCPD. Studies present high levels of fibrinogen and factor VIII (FVIII), in addition to polymorphisms such as the factor V Leiden mutation and the prothrombin 20210 (G/A), polymorphism (PT G20210A), as possible causal factors. C667T polymorphisms of methylenetetrahydrofolate reductase (MTHFR C667T) and T833C polymorphism of cystathionine beta synthase (CBS T833C), which are characterized by increased levels of homocysteine (Hcy) in the blood, are proposed as causative agents of LCPD. It is important to mention that, although no apparent relationship of these polymorphisms to LCPD was found, it was reported that MTHFR polymorphisms are implicated in a wide variety of thromboembolic diseases and that elevated Hcy levels have been related to osteonecrosis [17-20].

In Mexico, there are no studies related to LCPD. Therefore, the aim of this study is to describe, in three families with several members suffering from LCPD, some environmental, genetic, and biochemical factors that could be involved in the etiology of LCPD.

Methods

This study was conducted in three families that include of a total of seven patients with LCPD diagnosed on the basis of radiography and the study of the family medical history. Furthermore, fourteen healthy donors were selected as control group; they were matched by age, gender, size, and weight. Both groups were recruited in the Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra Ibarra" (INR-LGII).

A blood sample was taken from each participant and collected in a tube whit EDTA K2, and a tube with 3.8% sodium citrate. All hemolyzed or lipemic samples were discarded. Hematic biometry was performed in a Coulter LH 780 hematology automated analyzer. Citrated plasma was separated, and the samples were analyzed using commercial kits (HemosIL™) in a coagulation analyzer IL ACL Elite / Pro for each determination: TP – HemosiLTM PT RecombiPlasTin 2G 0020002950. The International Normalized Ratio (INR) was calculated automatically from the PT values. TTPa – HemosiLTM APTT-SP (liquid) 0020006300. Factor I – HemosiLTM 0008469810. Factor II – HemosiLTM 0008466050. Factor V - HemosiLTM 0020011500. Factor VII – HemosiLTM 0020011700. Factor VIII – HemosiLTM 0020011800. Factor IX - HemosiLTM 0020011900. Factor X - HemosiLTM 0020010000. Factor XI - HemosiLTM 0020011300. Factor XII – HemosiLTM 0020201200. Antigenic von Willebrand factor – HemosiLTM 0020002300. Protein C – HemosiLTM 0020300500. Free S protein – HemosiLTM 0020002700. Liquid antitrombin – HemosiLTM 0020002500.

DNA was extracted from whole blood using a commercial kit Puregene (Quiagen), in accordance with the manufacturer's protocol. Polymorphisms of CBS T833C (rs:115742905), MTHFR C677T (rs:1801133), and PT G20210A (rs:1799963) genes were genotyped by real-time PCR, with Taqman® probes labeled with FAM or VIC (Applied Biosystems, Foster city, CA, USA), in a Real Time Step One PCR System (Applied Biosystems).

Statistics

A database in GraphPad Prism version 8.0.0 for Windows was designed for the data obtained; a comparative analysis (Mann–Whitney *U* test, *t* Student) was performed to determine if there was any significant difference between groups. Gene frequencies, summary of genetic variation parameters, and deviations from Hardy-Weinberg equilibrium were determined by using the BIOSYS-1 program [21].

Ethical Aspects

The patients were people diagnosed with LCPD through clinical and radiological assessments. The controls were people without radiological alterations in the femur and hip and with no history of thrombophilia or any other ailing. Both groups were selected under the guidelines of the Norma Oficial Mexicana NOM-253-SSA1-2012 for blood banks. 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.

Results

Three families, including ten individuals with LCPD were studied. Not all patients were included in the study (Figure 1). According to the family medical history, four children were included: P002, P004, P006, and P007, with an average age of 11 ± 6.3 years and 77.9 ± 89.3 cm in height; and three adults: P001, P003, and P005 with an average age of 43.6 ± 5.1 years and 165 ± 3 cm in height, all of them male. The adults indicated that they were smokers, and all patients had habitual exposure to wood and tobacco smoke. In Mexico, six socioeconomic levels have been described, each with different incomes and consumption habits. Our population ranged between levels C and D, corresponding to the middle, lower middle and lower classes (https://www.amai.org/).

There were two bilateral cases (P001 and P004), and it was common to find patients who had flat foot (P003, P004, and P005) and/or practiced high-impact sports such as taekwondo (P001). An interesting fact is that Family 2 (Figure 1B) also had close relatives who suffered from osteoarthritis.

When studying hemoglobin (Hb) values and risk factors for thrombophilia, significant differences were found in the amount of Hb ($p \leq 0.0001$), fibrinogen ($p \leq 0.0001$), homocysteine (Hcy) ($p = 0.0414$), and in the percentage of FIX activity ($p \leq 0.0001$) and protein S ($p = 0.0478$) (Figure 2).

It is worth mentioning that the diagnosis of hemorrhagic alterations in the Clinical Chemistry Laboratory requires specific hemostasis tests. In parallel to this work, we conducted a study in a group of Mexican children and adolescents (controls), with plasma from 191 participants of ages from 0 to 18. There were differences between the reagent's manufacturers established parameters and the values found; therefore, in this report, we established reference values for coagulation times, coagulation factors, von Willebrand factor (WVF), and antithrombotic proteins (proteins C and protein S) according to the International Federation of Clinical Chemistry (IFCC) and the Institute of Laboratory and Clinical Standards (CLSI). For Hb, the range described for children aged 11 to 18 was taken [22] to assess the group of children in this study, finding values outside the range, including some above the reference value for adults. The group of adults also presented values above the normal range (Table 1).

Finally, when determining the prevalence of the polymorphisms MTHFR C677T (rs1801133), PT G20210A (rs1799963), and CBS T833C (rs115742905) in both groups, there were no significant differences.

Table 1. Patient's hemoglobin, coagulation factors, natural anticoagulants, homocysteine values AND MTHFR C677T polymorphism.

	Hb	FI	FII	FV	FVII	FVIII	FIX	FX	FXI	FXII	WVF	PC	PS	AT	Hcy	MTHFR
	g/dL	mg/dL	%	%	%	%	%	%	%	%	%	%	%	%	µmol/L	C677T
RV	12-18	80-700	50-150	50-150	50-129	50-150	65-150	77-133	65-150	50-150	66-170	70-140	63-135	83-128	4-11.2	Ala222Val
PRVC	13.5-17.5	249-360	98-136	39-115	87-132	44-82	73-94	100-144	65-142	55-109	69-130	83-128	85-112	108-144	DNS	rs1801133
P01*^	**18.3**	414	138	**208**	**173**	104	144	**159**	106	90.8	81.7	110	**135**	108	**13.1**	**T/T**
P02	**18.6**	**405**	130	**125**	107	**94.8**	**130**	118	109	**104**	48.4	102	**128**	121	7.2	C/**T**
P03*	**18.2**	453	100	146	126	112	**157**	121	113	108	104	85	**133**	81	**14.2**	C/**T**
P04^	**19.1**	**545**	94.4	**134**	122	**117**	**147**	116	124	80.7	124	87	**132**	122	9.7	C/**T**
P05*	**18.1**	395	118	146	110	110	132	121	122	80.7	72.4	**147**	127	**147**	6.5	C/**T**
P06	**17.9**	**452**	130	**117**	90.4	**98**	**157**	111	103	104	50.2	**130**	**117**	**130**	9.4	C/**T**
P07	**17.5**	**496**	118	**133**	92.4	**99.1**	**125**	107	130	93.1	53.7	**135**	87	**135**	7.6	**T/T**

P: Patient. Hb: hemoglobin. F: Coagulation factor. VWF: Von Willebrand factor. PC: Protein C. PS: Protein S, AT: Antithrombin. Hcy: Homocysteine.

MTHFR C677T: Methylenetetrahydrofolate reductase C677T polymorphism. C: cysteine nucleotide. T: thymine nucleotide. RV: Reference value, PRVC: Proposed reference value for children, black values are all those which were out of range. DNS: Data not shown. *: adult, ^: bilateral disease.

g/dL= grams per deciliter, mg/dL= milligrams per deciliter, µmol/L= micromoles per liter, %= percentage of activity.

Discussion

In each of the families studied, we observed particular characteristics. For example, in Family 2, the one with the highest number of patients with LCPD (Figure 1B), there were also members who suffered from osteoarthritis. This pathology has been previously related to LCPD [23], so it would be of interest to continue studying in order to know if, in our population, there could be alterations related to bone development possibly associated with LCPD.

LCPD is a complex disease; the lack of knowledge regarding its etiology is considered the main obstacle to its study. Different etiological factors have been described as the causative agents of LCPD, some of them present in our population, such as socioeconomic deprivation, which is an important factor to consider, since it has been observed that, in the population with lower socioeconomic levels, there is a higher incidence of the disease. The socioeconomic level of our population ranged between middle, lower middle, and lower classes, which can be considered as an environmental factor and could be related to the predisposition to LCPD due to poor nutrition, urbanization and other variables [24,25].

Another factor present in our group of patients is exposure to wood and tobacco smoke, which has been proposed in several studies as an important factor in the appearance of LCPD. According to what has been described, wood or tobacco smoke may be related to alterations in hemostasis by various mechanisms [26-28].

Moreover, in our population, there are disturbances in the distribution of mechanical loads, such as overdue arches and/or the practice of high-impact sports like taekwondo, which could lead to the development of LCPD due to the discrepancy of the forces applied on the hip and femur, as well as venous occlusion [29,30].

A family study was performed with the presence of related patients, so genetically it is proposed that LCPD could be caused by inherited factors. Although no evidence was found that could relate the polymorphisms studied to LCPD, the fact that other genetic alterations could be involved in the development of this disease cannot be ruled out [31,32].

Regarding laboratory studies, all our patients presented high hemoglobin levels, a factor linked to increased blood viscosity and, probably, to subsequent thrombotic events [33-35]. In addition, our results show that there are different hemostatic alterations in every individual analyzed, presenting out-of-range values in one or more parameters.

When patients were compared with controls, significant differences were found in some parameters, such as fibrinogen. It is interesting to note that the circulating amount of fibrinogen, the G455A polymorphism in the β-chain of fibrinogen, and its interaction with exposure to tobacco smoke have been described as risk factors for LCPD [36]. This is an example of how the relationship of environmental, genetic, and metabolic factors may be related to the development of LCPD.

Because the elevated levels of FIX have been established as risk factors for lower limb venous thrombosis, and given that we found higher FIX activity in the patient group, it is presumable that FIX could play a role in the development of LCPD [37].

Homocysteine disturbances have emerged as risk factors for multiple pathological conditions, such as osteoporosis, venous thrombosis, osteonecrosis, and LCPD [38,39], while elevated Hcy levels have been associated with increased oxidative stress in the bone microenvironment. This could lead to increased osteoclast differentiation and activity. Additionally, oxidative stress decreases the viability of nitric oxide through the production of superoxide anions, which would result in decreased bone blood flow and possibly affect angiogenesis. It has been described that osteoblast activity is affected by Hcy concentration [38]. Hcy concentration, on the other hand, can be altered by factors such as diet and lifestyle [39].

The C677T polymorphism is a point mutation in position 677 on the methylenetetrahydrofolate reductase (MTHFR) gene with the substitution of cysteine to thymine nucleotide. This point mutation causes the substitution of alanine to valine in the MTHFR enzyme. The single nucleotide polymorphism of this gene reduces the ability of the MTHFR enzyme to catalyze the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and leads to the rise of plasma homocysteine levels in the homozygous mutated subjects, while the heterozygous mutated subjects have mildly raised Hcy levels compared to the normal, non-mutated controls [40]. All the patient's members of the families studied, presented the mutated polymorphism in a homozygous or heterozygous manner.

Based on the study of clotting factors and Hcy in a group of patients with LCPD, we decided to study only familial cases and began with the study of MTHFR. Our findings in this studied population of familial cases with LCPD agrees with previous studies where the same factors have been related to LCPD onset and development. We also observed that some environmental factors specific to each family, together with hemostatic disorders, may be involved in the development of LCPD. In addition, we presume that, since we found familial cases, it is very likely that there is an important contribution of inherited genetic factors. Although with this sample we cannot establish a specific type of inheritance. We intend to continue with the study of different polymorphisms, such as that of MTHFR. To establish if these polymorphisms are related to the LCPD. In this study, we wish to highlight the presence of a multifactorial picture, in which diverse environmental, genetic, and prothrombotic factors are involved in this pathology.

Abbreviations

LCPD: Legg-Calvé-Perthes disease.

FH: femoral head.

FVIII: factor VIII.

PT: Prothrombin.

MTHFR: methylenetetrahydrofolate reductase.

CBS: cystathionine beta synthase.

Hcy: Homocysteine.

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

EDTA K2: Ethylenediaminetetraacetic acid disodium salt.

INR: International Normalized Ratio.

DNA: Deoxyribonucleic acid.

FAM: Fluorescein amidite.

NOM: Norma Oficial Mexicana.

Hb: Hemoglobin.

IFCC: International Federation of Clinical Chemistry.

CLSI: Institute of Laboratory and Clinical Standards.

Declarations

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Contributions

EHZ, AORO, and ERM conceived and designed the experiments. ERC, LCA, MVF, AORO and EHZ collected blood samples and clinical data. ERC and AORO performed the experiments. EHZ, AORO, 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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Figures

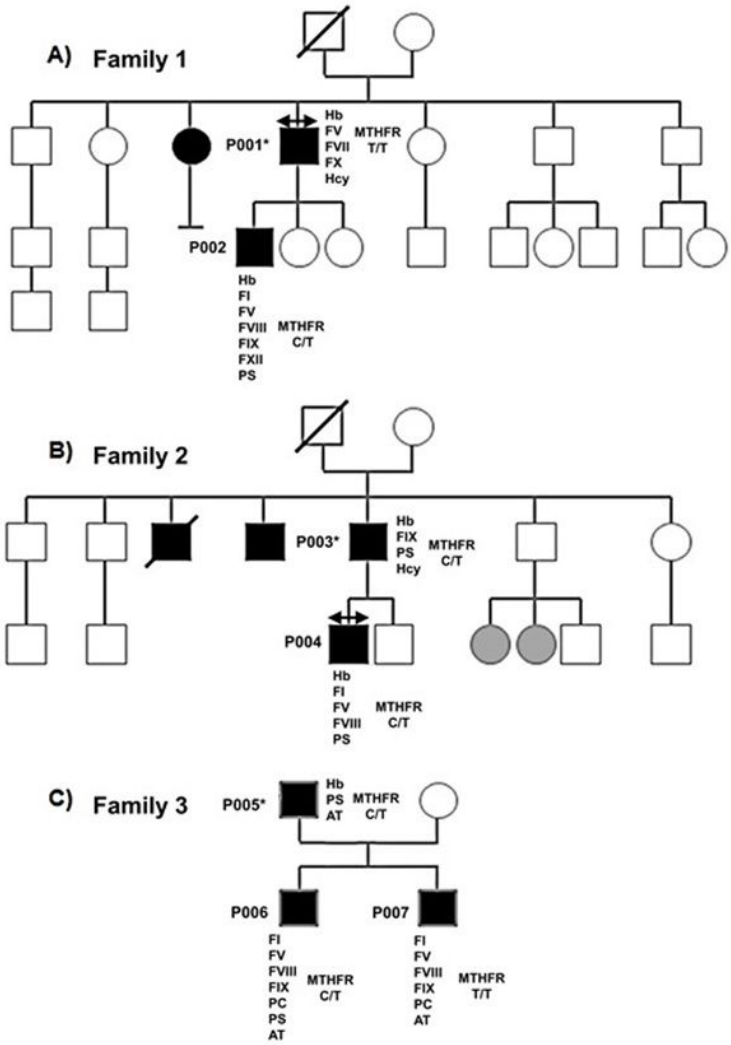

Figure 1

Family tree of LCPD studied individuals. A) Family 1. B) Family 2. C) Family 3. Square = males, circle= females, slash= deceased, black= LCPD, grey= osteoarthritis, P= patient, Hb= hemoglobin, F= coagulation factor, PC=protein C, PS= Protein S, AT= antithrombin, Hcy= Homocysteine, asterisk =Adult (*), mentioned values are all those which were out of range, double arrow = bilateral cases (↔).

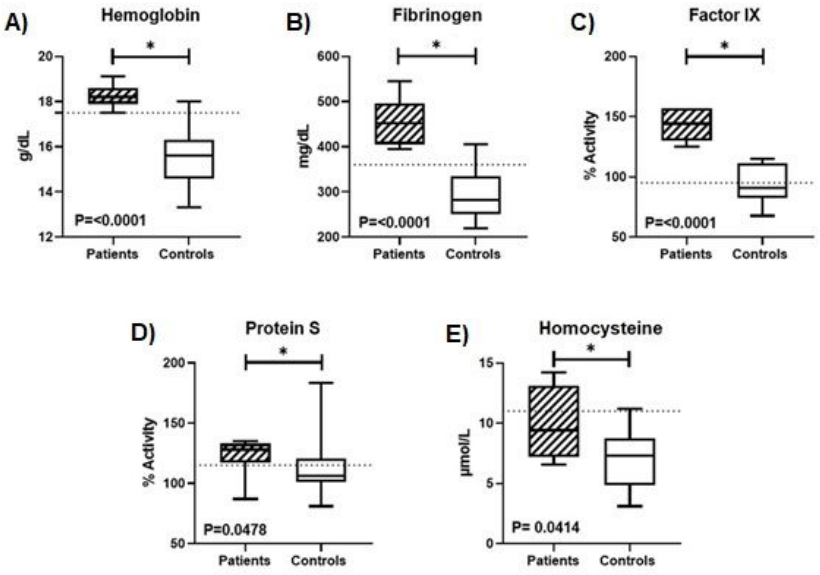

Figure 2

Concentration and activity of coagulation associated analytes in LCPD patients and healthy volunteers. A) Hemoglobin; B) Fibrinogen; C) Factor IX; D) Protein S; E) Homocysteine. %= percentage, g/dL= grams per deciliter, mg/dL =milligrams per deciliter, µmol/L= micromoles per liter, dotted line= maximum proposed reference value for children (····), asterisk= significant difference (*), P= P value.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DR.EDGARHERNANDEZ.pdf