Hemostatic markers and polymorphisms in three Mexican families with Legg-Calvé-Perthes disease.

Armando Rodríguez (orox3@hotmail.com)
Instituto Politecnico Nacional Escuela Nacional de Ciencias Biologicas https://orcid.org/0000-0002-4447-8343
Edgar Hernández-Zamora
Instituto Nacional de Rehabilitacion Luis Guillermo Ibarra Ibarra
Leonora Casas-Ávila
Instituto Nacional de Rehabilitación: Instituto Nacional de Rehabilitacion Luis Guillermo Ibarra Ibarra
Margarita Valdés Flores
Instituto Nacional de Rehabilitacion Luis Guillermo Ibarra Ibarra
Ruth Angelica Lezama Palacios
Instituto Politecnico Nacional Escuela Nacional de Ciencias Biologicas
Elba Reyes Maldonado
Instituto Politecnico Nacional Escuela Nacional de Ciencias Biologicas

Research Article

Keywords: Etiology, Heritage, Familial cases, Hemostasis, LCPD.

Posted Date: July 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1785603/v1

Abstract

Background: Legg-Calvé-Perthes Disease (LCPD) is a condition caused by avascular necrosis of the femoral head. Although its etiology is still not fully understood, evidence suggests heritable thrombotic and inflammatory disorders and other factors may be implicated in its onset and progress. Our objective is to describe, in the three enrolled families, the genetic, biochemical, and environmental factors that may be associated with the etiology and development of LCPD.

Methods: We evaluated the following gene alterations: MTHFR, CBS, PT, FVL, FVIII, FIX, PAI-1, eNOS, IL-23R, and TNF-α, and their relationship with LCPD. Additionally, we assessed thrombophilia-associated markers (FI, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, FvW, PC, PS, AT, and homocysteine) using coagulometry methods.

Results: Seven patients with LCPD and 14 healthy volunteers were enrolled. Concentrations in hemoglobin ($p \leq 0.05$), fibrinogen ($p \leq 0.05$), homocysteine ($p =<0.05$), FVIII ($p \leq 0.05$), and factor IX activity percentage ($p \leq 0.05$) showed statistically significant differences. Our results show that all participants of this study present at least one mutated allele for the MTFHR (rs1801133) and IL-23R (rs1569922) polymorphisms, as well as isolated cases with other genetic variants.

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

Background

Legg-Calvé-Perthes disease (LCPD) is 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.

Between 1909 and 1910, Waldenström in Sweden, Calvé in France, Perthes in Germany, and Legg in the United States, presented several studies that classified and described LCPD as a new and uncharacterized pathology. LCPD has a very variable incidence, it ranges between 0.4/100000 and 29.0/100000, and its appearance is mainly in males. Unfortunately, our country has no prevalence data, but it is considered a low-incidence disease due to its occurrence and appearance. There are multiple theories about the etiology of LCPD; however, many remain controversial due to a lack of foundation or reproducibility. Nonetheless, the interruption of blood flow to the femoral head and subsequent ischemic necrosis seem to be critical events in developing LCPD since the pathological and structural changes characteristic of LCPD are perceptible after these. At the moment, there is considerable evidence of the absence of blood flow to the affected FH; histological studies have also shown changes consistent with ischemic necrosis of the deep portion of the articular cartilage [1, 2]. Research shows that at least two ischemic episodes might be necessary for LCPD to develop. However, studies in animal models have found that a single ischemic event produces changes like those found in LCPD [3, 4]. 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 [3, 5].

There is evidence that genetic mechanisms may be involved in the etiology of LCPD; the proposed mechanisms include inheritance patterns ranging from autosomal recessive to polygenic. While in families with a high rate of affected individuals, there appears to be an autosomal dominant mode of inheritance [6, 7], 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 [8]. Because of this, some authors describe the possibility of a more significant association of LCPD among relatives.

Furthermore, hemostatic alterations such as hypofibrinolysis and 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 essential 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 [9–12].

Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of tissue plasminogen activator (t-PA) and urokinase (uPA), the activators of plasminogen and, thus, of fibrinolysis PAI-1. This inhibitor is produced mainly in the endothelium, although it is also secreted by other cell types, such as adipose tissue. In addition, PAI-1 is involved in angiogenesis, and some polymorphisms in the PAI-1 gene have been related to osteonecrosis femoral head (ONFH). [13, 14]

Nitric oxide (NO) participates in multiple physiological processes: angiogenesis, thrombosis, coagulation, and fibrinolysis, among others. Nitric oxide synthetase is the main enzyme in NO metabolism; it has three isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). There is evidence of the association between polymorphisms of eNOS and cardiovascular diseases (coronary artery disease, chronic heart failure, hypertension, atherosclerosis, stroke, renal diseases, and avascular necrosis of the femoral head). It has been proposed that LCPD could involve alterations in the vascularization of the femoral head. Therefore, sequence variations in the eNOS gene could alter nitric oxide synthesis and affect the progression of LCPD [15, 16].

Interleukin 23 (IL-23) is a proinflammatory cytokine recognized through the binding of the IL-12 receptor (IL-12R) with the IL-23 receptor (IL-23R). However, IL-23 promotes inflammation, primarily through recognition by the IL-23R. It has recently been reported that IL-23 deficient mice were resistant to collagen-induced arthritis. In addition, IL-23R has been related to different inflammatory disorders, and some of its variations have been associated with ONFH. [17, 18].

Finally, tumor necrosis factor α (TNF-α) is also a pro-inflammatory cytokine with a central role in the immune response, among other functions. In particular, it is related to bone remodeling since it stimulates osteoclastogenesis and simultaneously inhibits some functions of osteoblasts. Some variations in the TNF-α gene have been associated with osteonecrosis of the femoral head, although no relation was found between these and LCPD [12, 19]

In Mexico, there are no studies regarding familial cases of LCPD. Therefore, this study aims to describe, in three families with several members suffering from LCPD, some environmental factors, genetic polymorphisms, and biochemical hemostatic markers that could be involved in the etiology of LCPD.

Methods

This study is descriptive. It was conducted in three families and included seven patients with LCPD diagnosed through radiography and the study of the family's medical history. Furthermore, fourteen healthy donors were selected as a control group, 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 (Qiagen), per the manufacturer's protocol. Polymorphisms of CBS T833C (rs:115742905), MTHFR C677T (rs:1801133),PT G20210A (rs:1799963), FVL (rs:6025), FVIII (rs:598706), FIX (6048), PAI-1 (rs:1799889), eNOS (rs:17899983, rs:2070744), IL-23R (rs:1569922, rs:154655686, rs:7539625), and TNF-α (rs180062) 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 any significant difference between groups. [20].

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 INR-LGII Research and Ethics Committees reviewed and approved the study protocol.

Results

We studied three families, including ten individuals with LCPD. Not all patients participated in the study (Fig. 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 feet (P003, P004, and P005) and/or practiced high-impact sports such as taekwondo (P001). Interestingly, Family 2 (Fig. 1B) also had close relatives who suffered from osteoarthritis.

When studying hemoglobin (Hb) values and risk factors for thrombophilia, there were significant differences in the amount of Hb ($p \leq 0.05$), fibrinogen ($p \leq 0.05$), homocysteine (Hcy) ($p \leq 0.05$), and in the percentage of FVIII ($p \leq 0.05$) and FIX activity ($p \leq 0.05$) (Fig. 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 studied a group of Mexican children and adolescents (controls) with plasma from 191 participants, ages 0 to 18. There were differences between the reagent manufacturer's established parameters and the values found. Therefore, in this report, we set 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 [21] 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).

A= Adenine, T=Thymine, G=Guanine, C=Cytosine, MTHFR= methylenetetrahydrofolate reductase, PT= prothrombin, CBS= cystathionine beta-synthase, COL1A=, type I collagen COL2A1= type II collagen, FVL= Factor V Leiden, FVIII= Factor VIII, FIX= Factor IX, PAI-1= Plasminogen activator inhibitor-1, eNOS=Endothelial nitric oxide synthase, IL-23R= interleukin-23 receptor, TNF-α= tumor necrosis factor Alpha, *: adult, = bilateral cases.

In addition, all the members of this study have at least one mutated allele for the MTFHR (1801133) and IL-23R (1569922) polymorphisms, and there are isolated cases with other genetic variants (Table 2).

Finally, we didn't find association between polymorphisms of CBS T833C (rs:115742905), PT G20210A (rs:1799963), FVL (rs:6025), FVIII (rs:598706), FIX (6048), PAI-1 (rs:1799889), eNOS (rs:17899983, rs:2070744), IL-23R (rs:154655686, rs:7539625), and TNF-α (rs180062).

Discussion

In each of the families studied, we observed particular characteristics. For example, some members suffered from osteoarthritis in Family 2, the one with the highest number of patients with LCPD (Fig. 1B). This pathology has been previously related to LCPD [22], so it would be of interest to continue studying 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, and some of them are present in our population, like socioeconomic deprivation, which is an important factor to consider since a higher incidence of the disease has been observed in the population with lower socioeconomic levels. The socioeconomic level of our population ranged between middle, lower-middle, and lower classes, so it can be considered an environmental factor and could be related to the predisposition to LCPD due to poor nutrition, urbanization, and other variables [23, 24].

Another factor in our group of patients is exposure to wood and tobacco smoke, which several studies have considered to be a relevant 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 [25–27].

Moreover, the population shows mechanical load distribution disturbances, such as overdue arches or the practice of high-impact sports like taekwondo and gymnastics, 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 [28, 29].

A family study was performed with the presence of related patients, so genetically, it is proposed that inherited factors could cause LCPD. Although no evidence was found that could relate the polymorphisms studied to LCPD, we cannot rule out that the development of this disease could involve other genetic alterations [6, 7].

Regarding laboratory studies, all our patients presented high hemoglobin levels, a factor linked to increased blood viscosity and, probably, to subsequent thrombotic events [30–32]. In addition, our results show different hemostatic alterations in every individual analyzed, presenting out-of-range values in one or more parameters.

When comparing patients with controls, significant differences were found in some parameters, such as fibrinogen. Interestingly, 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 [33]. This is an example of how the relationship between 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 [34].

In some autoimmune diseases, proinflammatory interleukins have been linked to the development of osteonecrosis. Therefore, it is assumed that these interleukins are related to the appearance or development of LCPD. IL23 is a proinflammatory cytokine that is recognized after the binding of IL-23R and IL-12R [17]. The IL-23R signal translation pathway has been extensively studied for its relationship with proinflammatory diseases and different pathologies that cause bone deterioration. In addition, the signaling pathway that occurs after the activity of IL-23R has plethoric effects on osteoblast and osteoclast differentiation since it can inhibit or stimulate both processes [35]. Razawy et al. showed that 7-week-old IL-23R-/- mice have a bone mass similar to age-matched littermate control mice. In contrast, 12-week-old IL-23R-/- mice have significantly lower trabecular and cortical bone mass, shorter femurs, and more fragile bones [35]. Our results suggest a relationship between inflammation, alterations in the IL-23R, and the etiology of LCPD.

Homocysteine disturbances have emerged as risk factors for multiple pathological conditions, such as osteoporosis, venous thrombosis, osteonecrosis, and LCPD. In contrast, elevated Hcy levels have been associated with increased oxidative stress in the bone microenvironment, which 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 reduced bone blood flow and possibly affect angiogenesis. It has been described that osteoblast activity is affected by Hcy concentration, and Hcy concentration itself can be altered by factors such as diet and lifestyle.

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 [36–39]. All the patient members of the studied families presented the mutated polymorphism in a homozygous or heterozygous manner. In addition, homocysteinemia has important effects on bone density and its relationship with collagen since it interrupts the crosslinking of collagen molecules. [39, 40].

Conclusions

Our findings in this studied population of familial cases with LCPD agree 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 and inflammation disorders may be involved in the development of LCPD. In addition, we presume that, since we found familial cases, and there are cases with genetic variants, 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 studying different polymorphisms, such as that of MTHFR and IL-23R, to know if these polymorphisms are related to LCPD. This study aimed to highlight the multifactorial picture of this pathology that involves diverse environmental, genetic, inflammatory and prothrombotic factors.

Abbreviations

LCPD
Legg-Calvé-Perthes disease.
FH
femoral head.
FVIII
factor VIII.
PT
Prothrombin.
FVL
Factor V Leiden mutation.
PT G20210A
Prothrombin gene mutation (or Factor II mutation)
MTHFR
methylenetetrahydrofolate reductase.
CBS
cystathionine beta-synthase.
Hcy
Homocysteine.
ONFH
Osteonecrosis femoral head.
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.
NO
Nitric oxide.

NOS
Nitric oxide synthetase.
eNOS
Endothelial isoform.
iNOS
Inducible isoform.
nNOS
Neuronal isoform.
IL
Interleukin.
IL-12R
IL-12 receptor.
IL-23R
IL-23 receptor.
TNF-α
Tumor necrosis factor α.

Declarations

Acknowledgments

Thanks to reviewer Alejandra Tapia Alcazar for her assistance in preparing this manuscript.

In loving memory of M.D. Antonio Redón Tavera, head of the Pediatric Hip Service and Ph.D. M.D. Margarita Valdés Flores, Genomic Medicine, INR-LGII.

Funding

This study had no specific funding.

Contributions

EHZ, AORO, MVF and ERM conceived and designed the experiments. ERC, LCA, 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.

References

1. Rodríguez-Olivas AO, Hernández-Zamora E, Reyes-Maldonado E. Legg-Calvé-Perthes disease overview. Orphanet J Rare Dis. 2022;17(1):125.
2. Kim HK. Pathophysiology and new strategies for the treatment of Legg-Calvé-Perthes disease. J Bone Joint Surg Am. 2012;94(7):659–69.
3. Joseph B. Natural history of early-onset and late-onset Legg-Calve-Perthes disease. J Pediatr Orthop. 2011;31(2 Suppl):152–5.
4. Sanchis M, Zahir A, Freeman MA. The experimental simulation of Perthes disease by consecutive interruptions of the blood supply to the capital femoral epiphysis in the puppy. J Bone Joint Surg Am. 1973 Mar;55(2):335–42.
5. Kim HK, Herring JA. Pathophysiology, classifications, and natural history of Perthes disease. Orthop Clin North Am. 2011;42(3):285–95.
6. Al-Omran AK, Sadat-Ali M. Legg-Calve-Perthes disease in two generations of male family members: a case report. J Orthop Surg (Hong Kong). 2012;21(2):258–61.
7. Miyamoto Y, Matsuda T, Kitoh H, Haga N, Ohashi H, Nishimura G, Ikegawa S. A recurrent mutation in type II collagen gene causes Legg-Calvé-Perthes disease in a Japanese family. Hum Genet. 2007;121(5):625–9.
8. Gray IM, Lowry RB, Renwick DH. Incidence and genetics of Legg-Perthes disease (osteochondritis deformans) in British Columbia: evidence of polygenic determination. J Med Genet. 1972;9(2):197–202.
9. Balasa VV, Gruppo RA, Glueck CJ, Wang P, Roy DR, Wall EJ, Mehlman CT, Crawford AH. Legg-Calve-Perthes disease and thrombophilia. J Bone Joint Surg Am. 2004;86(12):2642–7.
10. Vosmaer A, Pereira RR, Koenderman JS, Rosendaal FR, Cannegieter SC. Coagulation abnormalities in Legg-Calvé-Perthes disease. J Bone Joint Surg Am. 2010;92(1):121–8.
11. Woratanarat P, Thaveeratitharm C, Woratanarat T, Angsanuntsukh C, Attia J, Thakkinstian A. Meta-analysis of hypercoagulability genetic polymorphisms in Perthes disease. J Orthop Res. 2014;32(1):1–7.
12. Azarpira MR, Ghilian MM, Sobhan MR, Mehdinezhad-Yazdi M, Aghili K, Miresmaeili SM, Neamatzadeh H. Association of MTHFR and TNF-α genes polymorphisms with susceptibility to Legg-Calve-Perthes disease in Iranian children: A case-control study. J Orthop. 2018;15(4):984–7.
13. Liang XN, Xie L, Cheng JW, Tan Z, Yao J, Liu Q, Su W, Qin X, Zhao JM. Association between PAI-1 4G/5G Polymorphisms and osteonecrosis of the femoral head: a meta-analysis. Thromb Res. 2013;132(2):158–63.
14. Zeng Z, Wang B, Pan H. Relation between osteonecrosis of the femoral head and PAI-1 4G/5G gene polymorphism: a meta-analysis. Int J Clin Exp Med. 2015;15(11):20337–42. 8(.
15. Zhao Y, Liao S, Lu R, Dang H, Zhao J, Ding X. Endothelial nitric oxide synthase gene polymorphism is associated with Legg-Calvé-Perthes disease. Experimental and therapeutic medicine. 2016;11(5):1913–7.
16. Azarpira MR, Ghilian MM, Sobhan MR, Mahdinezhad-Yazdi M, Aghili K, Ahrar H, Neamatzadeh H. Association of eNOS 27-bp VNTR, 894G > T and 786T > C polymorphisms with susceptibility to Legg-Calve-Perthes Disease in Iranian children. J Orthop. 2019;16(2):137–40.
17. Kim TH, Hong JM, Oh B, Cho YS, Lee JY, Kim HL, Lee JE, Ha MH, Park EK, Kim SY. Association of polymorphisms in the Interleukin 23 receptor gene with osteonecrosis of femoral head in Korean population. Exp Mol Med. 2008;40(4):418–26.
18. Srzentić S, Nikčević G, Spasovski D, Baščarević Z, Živković Z, Terzic-Šupić Z, Matanović D, Djordjević V, Pavlović S, Spasovski V. Predictive genetic markers of coagulation, inflammation and apoptosis in Perthes disease—Serbian experience. Eur J Pediatr. 2015;174(8):1085–92.
19. Peng Y, Liu Y, Huang D, Huang W, Shao Z. Association of TNF-α-308(G/A) and – 238(G/A) polymorphisms with non-traumatic osteonecrosis of the femoral head risks: a meta-analysis. Int Orthop. 2018;42(7):1711–21.
20. Swofford D, Selander R. BIOSYS-1 – A Fortran program for the comprehensive analysis of electrophoresis data in population genetics and systematics. J Heredity. 1981;2(4):281–3.
21. Kenneth K, Marshall L, Josef P, Marcel M, Oliver P, Linda B, Michael C. Williams hematology. New York: McGraw-Hill Education; 2016.
22. Hsu JE, Baldwin KD, Tannast M, Hosalkar H. What is the evidence supporting the prevention of osteoarthritis and improved femoral coverage after shelf procedure for Legg-Calvé-Perthes disease? Clin Orthop Relat Res. 2012;470(9):2421–30.

23. Kealey WD, Moore AJ, Cook S, Cosgrove AP. Deprivation, urbanisation and Perthes' disease in Northern Ireland. J Bone Joint Surg Br. 2000;82(2):167–71.
24. Gordon JE, Schoenecker PL, Osland JD, Dobbs MB, Szymanski DA, Luhmann SJ. Smoking and socio-economic status in the etiology and severity of Legg-Calvé-Perthes' disease. J Pediatr Orthop B. 2004;13(6):367–70.
25. Perry DC, Thomson C, Pope D, Bruce CE, Platt MJ. A case-control study to determine the association between Perthes' disease and the recalled use of tobacco during pregnancy, and biological markers of current tobacco smoke exposure. Bone Joint J. 2018;99-B(8):1102–8.
26. Daniel AB, Shah H, Kamath A, Guddettu V, Joseph B. Environmental tobacco and wood smoke increase the risk of Legg-Calvé-Perthes disease. Clin Orthop Relat Res. 2012;470(9):2369–75.
27. DiGiacomo SI, Jazayeri MA, Barua RS, Ambrose JA. Environmental Tobacco Smoke and Cardiovascular Disease. Int J Environ Res Public Health. 2018;16(1):96.
28. Larson AN, Kim HK, Herring JA. Female patients with late-onset Legg-Calvé-Perthes disease are frequently gymnasts: is there a mechanical etiology for this subset of patients? J Pediatr Orthop. 2012;33(8):811–5.
29. Ponce de León Samper MC, Herrera Ortiz G, Castellanos Mendoza C. Relationship between flexible flat foot and developmental hip dysplasia. Rev Esp Cir Ortop Traumatol. 2015;59(5):295–8.
30. Ibrahim NA, Hassan FM, Elgari MM, Abdalla SE. Risk factors for deep vein thrombosis of lower extremities in Sudanese women. Vasc Health Risk Manag. 2018;14:157–64.
31. Folsom AR, Wang W, Parikh R, Lutsey PL, Beckman JD, Cushman M. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Hematocrit and incidence of venous thromboembolism. Res Pract Thromb Haemost. 2020;4(3):422–8.
32. Hassan WU, Syed MJ, Alamgir W, Awan S, Bell SM, Majid A, Wasay M. Cerebral Venous Thrombosis at High Altitude: Analysis of 28 Cases. Cerebrovasc Dis. 2019;48(3–6):184–92.
33. Dilley A, Hooper WC, Austin H, Jamil M, Miller C, Stokes M, Evatt B, Eldridge J. The beta fibrinogen gene G-455-A polymorphism is a risk factor for Legg-Perthes disease. J Thromb Haemost. 2003;1(11):2317–21.
34. Lechner D, Wiener C, Weltermann A, Eischer L, Eichinger S, Kyrle PA. Comparison between idiopathic deep vein thrombosis of the upper and lower extremity regarding risk factors and recurrence. J Thromb Haemost. 2008;6(8):1269–74.
35. Razawy W, Alves CH, Koedam M, Asmawidjaja PS, Mus A, Oukka M, Leenen P, Visser JA, van der Eerden B, Lubberts E. IL-23 receptor deficiency results in lower bone mass via indirect regulation of bone formation. Sci Rep. 2021;11(1):10244.
36. Behera J, Bala J, Nuru M, Tyagi SC, Tyagi N. Homocysteine as a Pathological Biomarker for Bone Disease. J Cell Physiol. 2017;232(10):2704–9.
37. Quéré I, Gris JC, Dauzat M. Homocysteine and venous thrombosis. Semin Vasc Med. 2005;5(2):183–9.
38. Liew SC, Gupta ED. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: epidemiology, metabolism and the associated diseases. Eur J Med Genet. 2015;58(1):1–10.
39. Buendía-Pazarán JG, Hernández-Zamora E, Rodríguez-Olivas AO, Casas-Ávila L, Valdés-Flores M, Reyes-Maldonado E. Association of MTHFR rs1801133 and homocysteine with Legg-Calvé-Perthes disease in Mexican patients. Orphanet J Rare Dis. 2022;17(1):123.
40. Saito M, Marumo K. The Effects of Homocysteine on the Skeleton. Curr Osteoporos Rep. 2018;16(5):554–60.

Tables

Table 1
Patient's hemoglobin, coagulation factors, natural anticoagulants and homocysteine values.

	Hb	FI	FII	FV	FVII	FVIII	FIX	FX	FXI	FXII	WVF	PC	PS	AT	Hcy
	g/dL	mg/dL	%	%	%	%	%	%	%	%	%	%	%	%	µmol/L
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
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
P01*^	**18.3**	414	138	**208**	**173**	104	144	**159**	106	90.8	81.7	110	**135**	108	**13.1**
P02	**18.6**	**405**	130	**125**	107	**94.8**	**130**	118	109	**104**	48.4	102	**128**	121	7.2
P03*	**18.2**	453	100	146	126	112	**157**	121	113	108	104	85	**133**	81	**14.2**
P04^	**19.1**	**545**	94.4	**134**	122	**117**	**147**	116	124	80.7	124	87	**132**	122	9.7
P05*	**18.1**	395	118	146	110	110	132	121	122	80.7	72.4	**147**	127	**147**	6.5
P06	**17.9**	**452**	130	**117**	90.4	**98**	**157**	111	103	104	50.2	**130**	**117**	**130**	9.4
P07	**17.5**	**496**	118	**133**	92.4	**99.1**	**125**	107	130	93.1	53.7	**135**	87	**135**	7.6

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

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.

Table 2 is available in the Supplementary Files section.

Figures

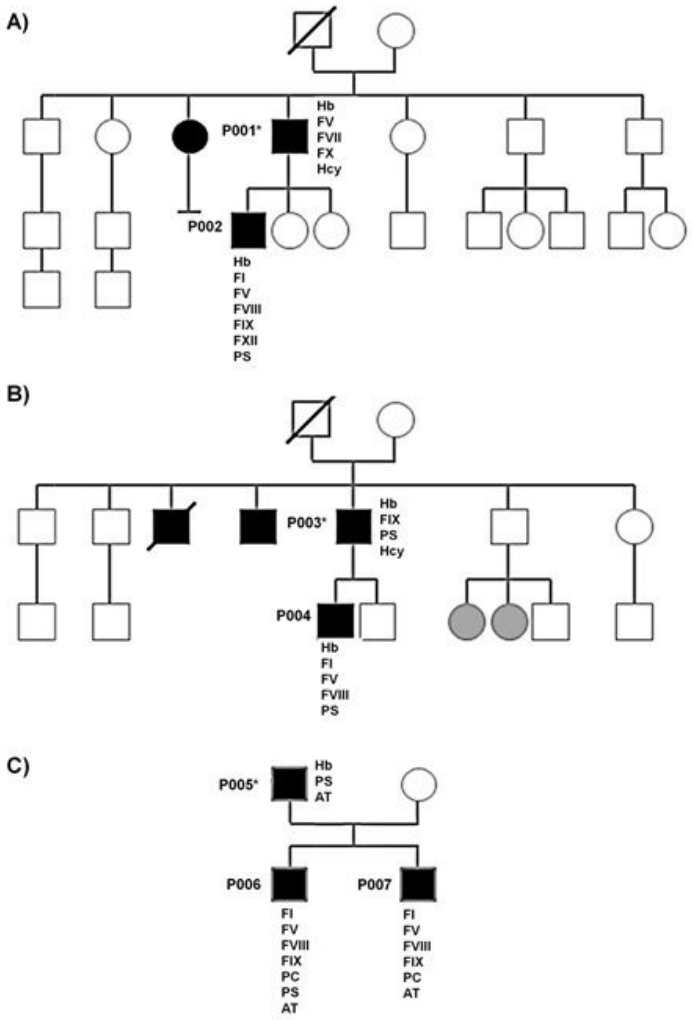

Figure 1

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, FC = coagulation factor, PC = protein C, PS = protein S, AT = Antithrombin, Hcy = homocysteine, Asterisk = adult (*), mentioned values are all those which were out of range.

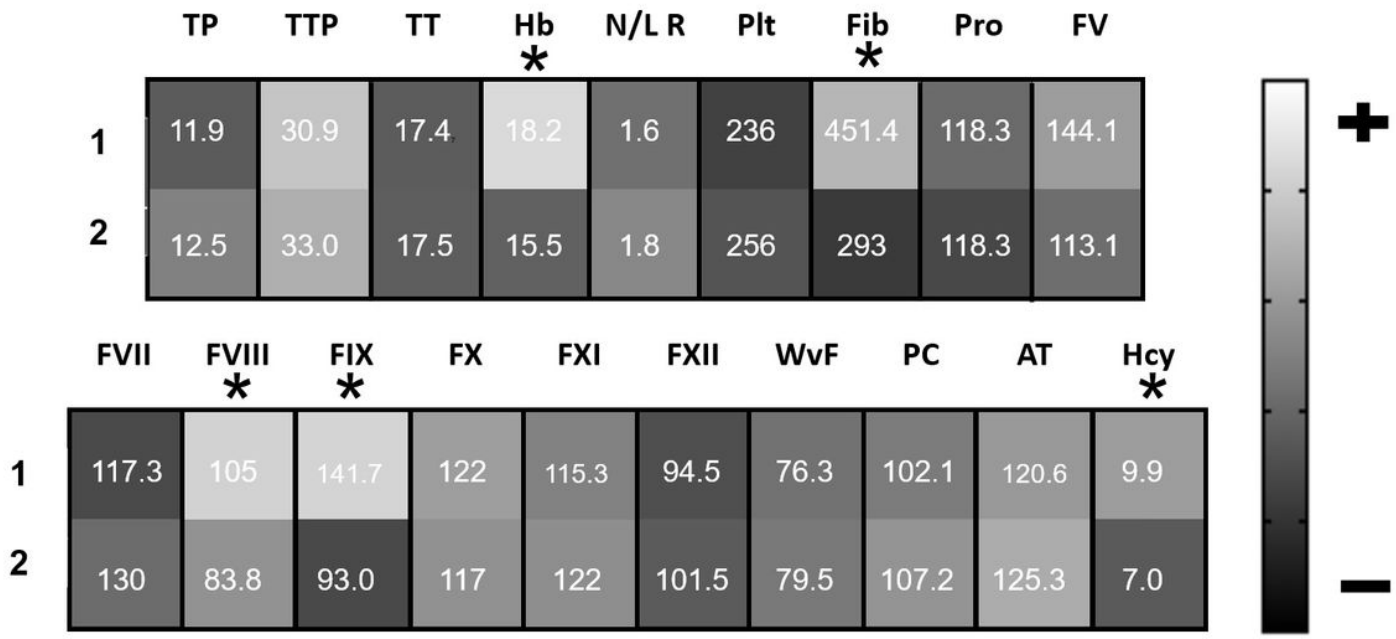

Figure 2

Concentration and activity of coagulation associated analytes in LCPD patients and healthy volunteers. Means are shown inside each box. 1 = Patients, 2 = controls, TP = prothrombin time, TTP = partial thromboplastin time, TT = thrombin time, Hb = hemoglobin, N/L R = neutrophil lymphocyte ratio, Plt = platelets, Fib = fibrinogen, Pro = prothrombin, FV = factor V, FVII = factor FVII, FVIII = factor VIII, FIX = factor IX, FX = factor X, FXI factor XI, FXII = factor XII; PC = protein C, PS = protein S, AT = antithrombin, Hcy = homocysteine, Asterisk = significative difference (*).

Supplementary Files

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

- Table2genotype.docx