Clinical and Applied Thrombosis/Hemostasis
Volume 29: 1-9
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DOI: 10.1177/10760296221151166
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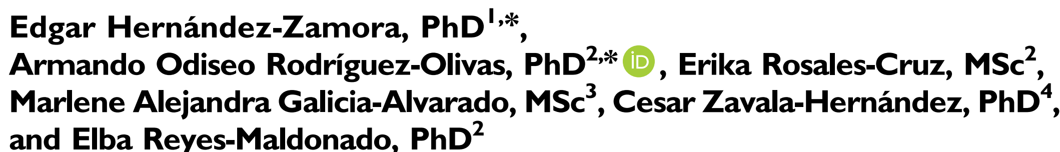
Prothrombin Time and Coagulation Factor IX as Hemostatic Risk Markers for Legg–Calvé–Perthes Disease


Edgar Hernández-Zamora, PhD[1,*], Armando Odiseo Rodríguez-Olivas, PhD[2,*], Erika Rosales-Cruz, MSc[2], Marlene Alejandra Galicia-Alvarado, MSc[3], Cesar Zavala-Hernández, PhD[4], and Elba Reyes-Maldonado, PhD[2]

Abstract

Background: Legg–Calvé–Perthes disease (LCPD) is a pediatric disorder that occurs due to the avascular necrosis of the femoral head and affects the range of motion of the hip in various degrees. Its etiology is still unknown, although it has been associated with coagulation abnormalities, however, the lack of reproducibility in the results in various studies has created a controversy as to whether hemostasis disorders are related to LCPD. On the other hand, there is little information on laboratory studies that could facilitate the diagnosis and treatment of LCPD.
Methods: Blood and plasma samples were tested from 25 patients with LCPD and 50 healthy controls, matched by sex and age. Cellular markers were evaluated through complete blood count, as well as coagulation times, coagulation factors activity, antithrombotic proteins, and homocysteine concentration.
Results: After assessing activity value frequencies in each group, the results showed more significant activity in some of the biological risk markers of thrombophilia, presenting a substantial difference in prothrombin time↘, FV↗, FVIII↗, FIX↗, and Hcy↗. These values imply that there may be hypercoagulable states in patients, which can cause thrombotic events.
Conclusions: Diminished prothrombin time and increase in FV activity, FVIII, FIX, and Hcy concentration support the hypothesis that microthrombi formation in small-caliber vessels could be causing avascularity and femoral necrosis, which are traits of LCPD. In addition, based on our results, we believe that the laboratory studies carried out are very useful in the diagnosis and treatment of LCPD.

Keywords
Legg–Calvé–Perthes disease, coagulation factors, antithrombotic proteins, homocysteine, laboratory

Date received: 8 October 2022; revised: 23 December 2022; accepted: 28 December 2022.

Background

Rare diseases (RDs) are characterized by low incidence, as well as difficulties obtaining a timely and accurate diagnosis.[1,2] Legg–Calvé–Perthes disease (LCPD) is an RD. This self-limited microvascular disorder causes an occlusion of the femoral head blood supply, resulting in idiopathic avascular osteonecrosis of the developing femoral head.[1,3,4] It occurs in children from 4 to 9 years old, mainly males at a 4:1 ratio to females. The incidence varies significantly within racial groups and is predominant in Caucasians. The etiology of LCPD is still unknown; it remains one of the most controversial conditions in pediatric orthopedics, and many aspects remain unclear, although it has been associated with hypercoagulable states (thrombophilia) and coagulation abnormalities.[1,5,6]

[1] Genomic Medicine, Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra (INR-LGII), México City, México
[2] Morphology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional (ENCB, IPN), México City, México
[3] Clinical Neurosciences, INR-LGII, México City, México
[4] Pathology Central Laboratory, INR-LGII, México City, México

*These authors have contributed equally.

Corresponding Authors:
Elba Reyes-Maldonado, Morphology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional (ENCB, IPN), Prolongación de Carpio y Plan de Ayala s/n, Col. Santo Tomás, Miguel Hidalgo, C.P. 11340, Mexico City, Mexico.
Email: elbareyesm@gmail.com

Armando Odiseo Rodríguez-Olivas, Morphology Department, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional (ENCB, IPN), Prolongación de Carpio y Plan de Ayala s/n, Col. Santo Tomás, Miguel Hidalgo, C.P. 11340, Mexico City, Mexico.
Email: orox3@hotmail.com

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The diagnosis of the disease is initially determined through physical exams and radiological studies.[7–10] Laboratory tests to support the clinical diagnosis and treatment of LCPD are scarce. However, little value is placed on routine clinical laboratory studies.

There are no prevalence data or reports in Mexico that evaluate the markers of hypercoagulability (thrombophilia) in patients with LCPD, which highlights the need for research in Latin American populations. Most of the studies in the literature reference the Caucasian population, and the reports have shown hemostatic alterations such as the presence of the factor V Leiden mutation, elevated levels of factor VIII, and resistance to activated protein C. Moreover, due to the ambiguity of the existing reports, it has not been possible to reach a consensus on whether or not hypercoagulable states (thrombophilia) and coagulation abnormalities trigger LCPD.[1,11–14]

This study aims to bring attention to this disease, as well as to help resolve the existing controversy about the etiology of LCPD and the hypercoagulable states by analyzing thrombophilia risk markers in a population of Mexican patients from the National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra (INR-LGII).

Methods

This was a retrospective case-control study. The cases included first-time or recurrent patients of all ages, both sexes, with a clinical and radiological diagnosis of LCPD, without other bone diseases or diseases related to coagulation abnormalities, and without any pharmacological treatment. There were also healthy controls, matched with the patients in a 2:1 proportion by age and sex, with radiological studies showing that they did not present any alteration in the femur or hip. In addition, they had no history of hematological or thrombotic pathologies and were not receiving any pharmacological treatment. All patients were recruited from the Orthopedic Service at INR-LGII in Mexico City. In addition, we obtained the weight (kg), height (m), and body mass index (BMI). Both groups were selected under the guidelines of the Norma Oficial Mexicana NOM-253-SSA1-2012 for blood banks.

A blood sample was taken from each participant in a tube with 3.8% sodium citrate. All hemolyzed or lipemic samples were discarded. The plasma was separated, by centrifugation at 2500 × g for 15 min. The samples were analyzed using commercial kits. The blood parameters were analyzed using Mindray Hematology Analyzer BC6800. The plasma was detached, and the samples were analyzed using commercial kits (HemosIL™) in a coagulation analyzer (coagulometer) IL ACL Elite / Pro. The coagulation times were: thrombin time, 0009758515; prothrombin time (PT), RecombiPlasTin, 2G-0020002950; and activated partial thromboplastin time (aPTT), liquid, 0020006300. The coagulation factors (CF) were: Factor I (Fibrinogen) HS PLUS 0008469810; Factor II (Prothrombin) 0008466050; Factor V (Proacelerin) 0020011500; Factor VIII (Antihemophilic A) 0020011800; Factor IX (Antihemophilic B) 0020011900; Factor X (Stuart–Prower Factor) 0020010000; Factor XI (Antihemophilic C) 0020011300; Factor XII (Hageman factor) 0020201200; Von Willebrand factor 0020002300 (VWF); Homocysteine 0020007800 (Hcy) (Instrumentation Laboratory SpA-V.le Monza 338 – 20128 Milano, Italy); Protein C, HemosiLTM 0020300500; and Antithrombin (AT) liquid, HemosiLTM 0020002500.

According to the International Federation of Clinical Chemistry (IFCC) and the Institute of Laboratory and Clinical Standards (CLSI), we established the suggested reference values (SRV) for a pediatric population, stratified by age ranges; we made determinations in 200 healthy minors.

Statistics

The Kolmogorov–Smirnov test was used to verify whether the data distribution was consistent with a normal distribution curve. A Mann–Whitney U test or Student's t-test was applied in the comparative analysis. A binary logistic regression analysis was performed using the Wald method (forward stepwise), to identify thrombophilia risk markers related to the presence of LCPD, based on the contribution of the following indicators: PT, FVIII, FIX, and Hcy, and the degree of fit was estimated using the classification table. The linear correlation values between the predictor variables of the model were verified in order to verify the aforementioned assumption of collinearity. The correlation matrix between the variables was −0.12.

Ethical Aspects

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

In total, 25 patients (23 males and 2 females) and two controls for each patient (46 males and 4 females) were recruited and matched by sex and age (Table 1). The patients had a mean age of 16.3 years and a mean age at diagnosis of 5.6 years, the mean age for the control group was 15.8 years (Table 1). No significant differences were found in the average weight and height. The average height of the patients was 1.48 ± 0.23 m and 1.49 ± 0.23 m in the controls, while the average

Table 1. Population Characteristics.

	Patients	Controls	*P*
Sex	Males: 23 Females: 2	Males: 46 Females: 4	N/A
Age	16.3 years	15.8 years	.80
Weight	45.3 kilos	52.3 kilos	.17
Height	1.5 m	1.5 m	.90
Body mass index	20.4	22.8	.90

Probability: $P \leq .05$.

weight was 46.21 ± 15.02 kg in the patients and 53.83 ± 24.06 kg in controls. In addition, the body mass index (BMI) is shown in Table 1.

In 17 patients, the left extremity was affected; five patients showed an affected right side, and three cases were bilateral. Most of the patients belonged to the blood group O (18 patients), followed to a lesser extent by group A (four patients), and only three patients belonged to group B. There were no cases of group AB. The entire population was type RH positive. Notably 80% of our patients referenced habitual exposure to tobacco smoke.

The blood groups, hemoglobin, and leukocytes presented a similar distribution to that reported in Latin American populations, and without significant differences between patients and controls. Regarding platelets and coagulation times, the thrombin time (TT) and activated partial thromboplastin time (aPTT) did not present significant differences between both groups nor did they exceed the reference values in any of the parameters analyzed; nevertheless, higher activity was found in the group of patients in terms of the TT, FI, FII, FXI, and FXII.

The PT was lower in patients and presented a significant difference without leaving the reference values (Table 2 and Figure 1), which has also been described by Chiari and Frank.[15]

As for the CF, VWF, and Hcy (Table 2), the mean, median, quartiles, percentages, and standard deviation were calculated. Both the mean and the median for the patients and the controls did not exceed the reference values in any of the parameters analyzed. However, when these parameters were evaluated with our SRV, significantly higher means were evident for the FV, FVIII, and FIX.[16,17]

According to the logistic regression model using the Wald method, the variables selected with statistical significance ($P < .05$) were the PT and FIX; so the resulting logistic function was: $P(Y) = 1/1 + \exp\ (5.362 - 1.05 \times 1 + 0.064 \times 2)$, where Y is the diagnosis (case/control), x1 represents the PT, and x2 represents the FIX.

The OR value of the PT variable was 0.348 (95% CI: 0.148–0.821) and the OR of the FIX variable was 1.067 (95% CI: 1.033–1.101); therefore, the presence of lower PT values and higher FIX levels were risk factors for LCPD disease (Figure 2).

Cox and Snell's R square and Nagelkerke's R square coefficients of determination indicated that 42.7% and 59.3% of the total residual variance was explained by the presence of the PT and FIX variables included in the model. The Hosmer–Lemeshow test for step 2 ($P = .557 > .05$) showed that the model fit the data correctly.

Likewise, the classification table showed the percentage of the correct classification for the positive cases was 69.6% (sensitivity of model) and 89.1% for the controls (specificity of model). In total, 57 cases were correctly classified by the model, which represented an 82.6% overall fit.

Discussion

Despite its low incidence, LCPD represents a worldwide health problem, so its study and understanding are crucial.[1,6] Unfortunately, it has not received the attention it needs in Latin American countries. LCPD research presents different challenges because its etiology is unknown. It is characterized by unilateral or bilateral idiopathic osteonecrosis of the

Table 2. Complete Blood Count, Coagulation Studies, Antithrombotic Proteins and Homocysteine.

Parameter (FRV, units) (SRV)	Patients ($N = 25$)	Controls ($N = 50$)	P
Leukocytes (N/O)	6.1 ± 1.3	6.6 ± 1.4	.1
Hemoglobin (N/O)	16.1 ± 1.9	15.4 ± 1.8	.1
PLTs (150–450 mm^3) (N/O)	264.5 ± 5	283.8 ± 72.7	.2
TT (15.8–24.9 s) (N/O)	17.5 ± 1.2	17.2 ± 1.8	.5
PT (11.8–13.7) (12.6–13.5)	**12.2 ± 0.8**	**13.0 ± 0.8**	**<.05***
aPTT (26–40.2 s) (28.1–31.9)	31.8 ± 2.7	30.1 ± 3.5	.4
Factor I (80–700 mg/dL) (248.8–360.0)	351.8 ± 87.2	328.4 ± 102.3	.2
Factor II (50%–150%) (97.9–136)	110 ± 14.6	108 ± 26.8	.1
Factor V (50%–150%) (39.2–115.1)	**134.4 ± 25.7**	**95.9 ± 38.6**	**<.05***
Factor VII (50%–129%) (87.0–132.0)	111.6 ± 21.8	115.2 ± 30.3	.6
Factor VIII (50%–150%) (43.5–81.5)	**102.6 ± 23.6**	**84.3 ± 37.6**	**<.05***
Factor IX (65%–150%) (72.6–94.8)	**130.1 ± 21.3**	**92.6 ± 21.8**	**<.05***
Factor X (77%–133%) (100.0–144.0)	117.2 ± 18.4	123.6 ± 26.7	.3
Factor XI (65%–150%) (65.1–142.7)	109.0 ± 19.5	106.1 ± 42.0	.7
Factor XII (50%–150%) (55.0–109.0)	94.8 ± 29.8	90.7 ± 38.7	.4
VWF Ag (66%–170%) (68.8–129.8)	86.7 ± 29.5	97.2 ± 35.3	.2
Hcy (5–15 µmol/L) (5.5–13.4)	**8.3 ± 1.3**	**6.9 ± 1.4**	**<.05***
PC (70%–140%) (83–128)	98.8 ± 16.1	104.3 ± 19.4	.2
AT (83%–128%) (68–130)	121.3 ± 16.8	118.8 ± 26.1	.7

The parameters in bold text are difference significative, for which the mean and the standard deviation are presented [X (± SD)]. *$P \leq .05$.
Abbreviations: FRV, fabricant reference values; SRV, suggest references values; PLTs, platelets; TT, thrombin time; PT, prothrombin time; aPTT, activated partial thromboplastin time; VWF, von Willebrand Factor; PC, protein C; PS, protein S; AT, antithrombin; Hcy, homocysteine; N/O, data no obtained.

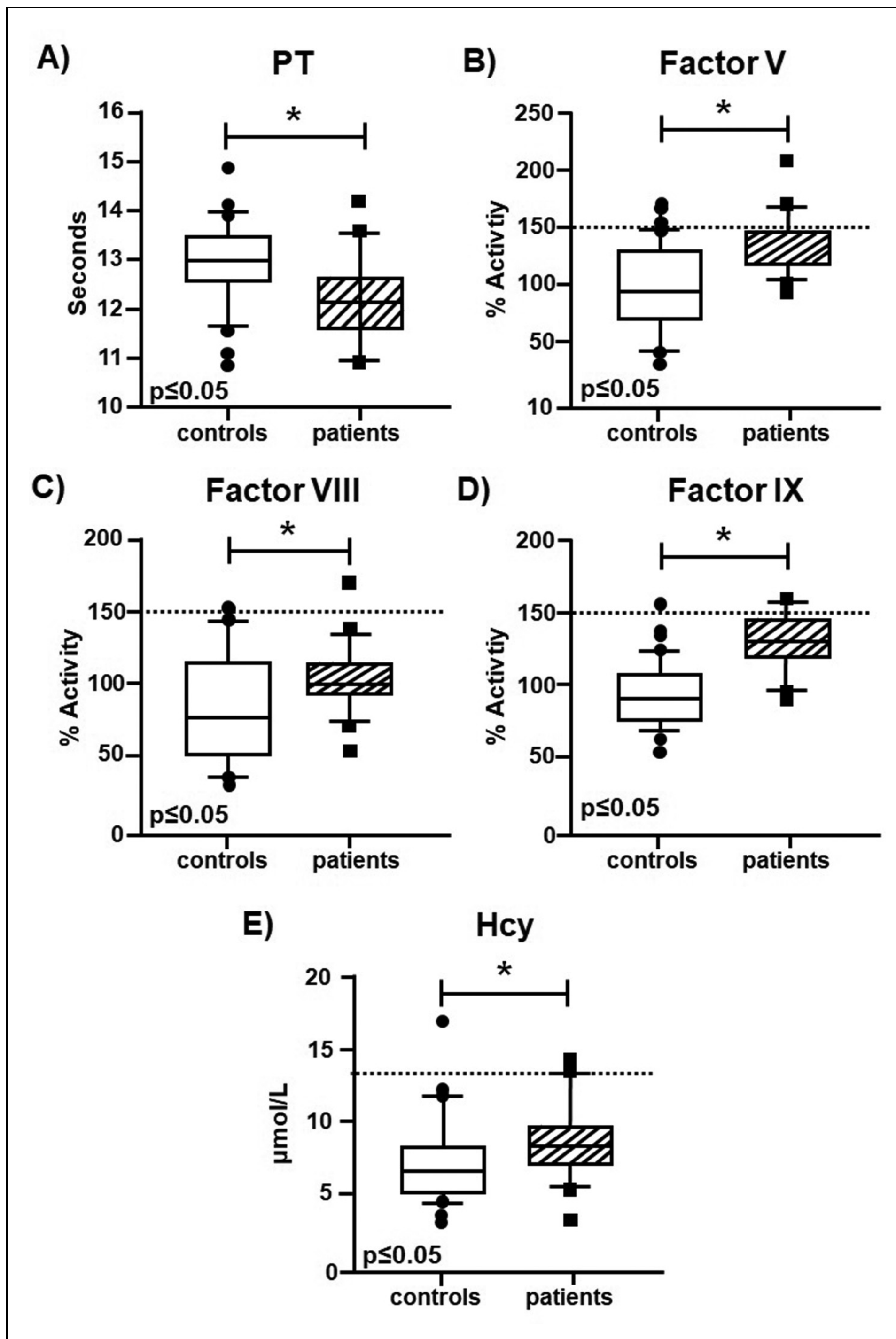

Figure 1. Comparison of patients ($n = 25$) versus controls ($n = 50$). Tests: (A) Prothrombin time; (B) Factor V; (C) Factor VIII; (D) Factor IX; (E) Homocysteine. Mustache graphs showing the median as well as the 10-90 percentile and out-of-range values. (•, • •, ■, ■ ■) Outliers are represented with circles outside the mentioned percentiles. The *P*-value comparing both groups (patients and controls) is also included. Stressed with an asterisk (*) are samples whose comparisons presented a significant difference during the comparative analysis when applying a Mann–Whitney U test or a student's t-test, depending on the case. ($*P \leq 0.05$). Activity (%) = percentage of activity, μmol/L = micromoles per liter.

proximal femoral epiphysis, in which the vascular system is highly compromised. It has been proposed that its etiology involves successive vascular occlusions, in which hypercoagulable states would play a central role. The proposed mechanism is that microthrombi lodge in the vessels that nourish the femur, blocking the supply of blood, which

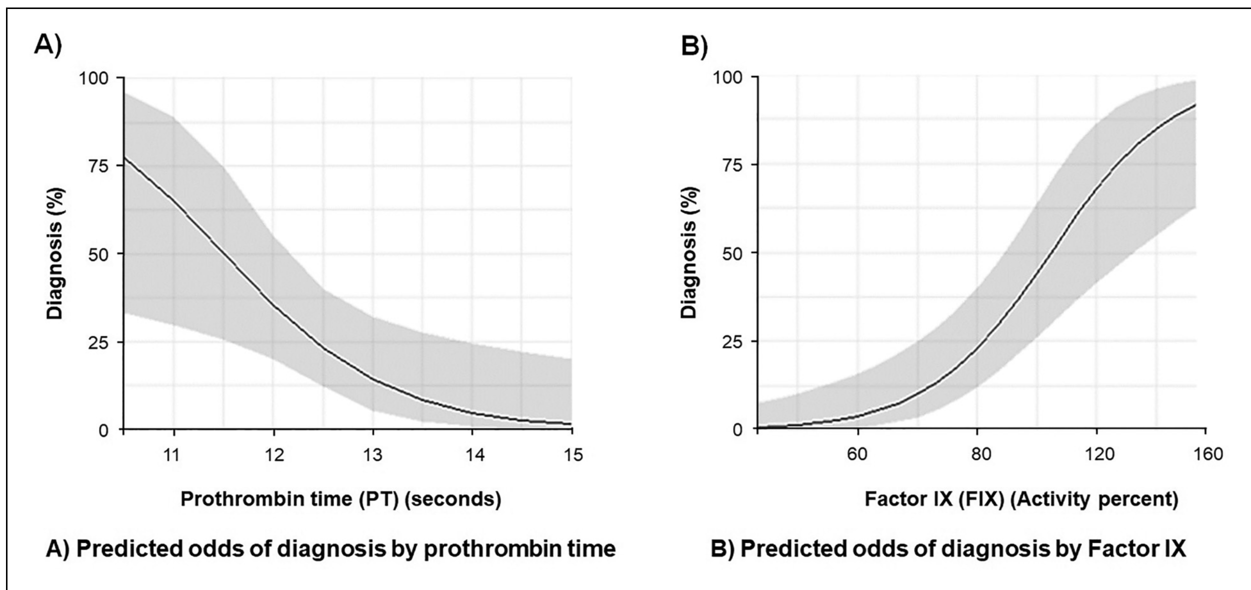

Figure 2. Predicted odds of diagnosis by (A) prothrombin time and (B) Factor IX. In this model, the PT showed sensitivity and specificity, as well as the activity of factor IX, routine tests in hematology laboratories.

causes the characteristic necrosis of LCPD. The results in multiple studies have supported this theory; however, the lack of the reproducibility of these results has given rise to controversies as to whether hemostasis is involved in the development and progression of LCPD.[11,12,18–21]

Physiologically, the hemostatic balance in children is different from that of adults. The development of the hemostasis system matures and changes over time, from fetal life to old age. Because of these changes, it is necessary to determine the SRV for each stage of life, including childhood. Therefore, the reference values (RV) must be calculated for each specific population and clinical laboratory.[16,17]

Both active and passive (environmental) cigarette smoke exposure predisposes to cardiovascular events, since smoking increases fibrinogen levels, platelet counts, and prothrombin activity, and reduces PT. On the other hand, smoke influences all phases of atherosclerosis from endothelial dysfunction to acute clinical events, the latter being largely thrombotic. Smoke exposure has been related to LCPD previously by other studies.[1,22,23] This was an environmental factor with hemostatic repercussions present in our population.

The model of coagulation cascade (MCC) is explained as successive proteolytic steps. It was useful in the understanding of how the coagulation enzymatic process occurs in plasma-based in vitro coagulation. The MCC suggests that the extrinsic and intrinsic pathways work as semi-independent pathways, while the clinical manifestations of individual factor deficiencies oppose this concept. The cell-based model of coagulation shows that hemostasis works as a complex, dynamic, and comprehensive system, where the hyperactivity of one or more factors affects the entire system.[24,25] When we observed higher average values in several of the CFs in the patient group, a hemostatic imbalance was present. We assume this imbalance activated mechanisms, which altered the results in one or more routine tests, such as the prothrombin time.

The PT is a routine test in the clinical laboratory, with high sensitivity and specificity, as well as low cost and easy access. The PT measures the time required for coagulation to occur after the addition of tissue factor (TF) to citrated plasma. In this way, the formation of a clot is simulated, and its formation time can be measured. The PT depends on the concentrations of factors in the extrinsic (FVII-TF) and common pathways (FX, FV, FII, and fibrinogen) and is shortened in the presence of traces of thrombin or other activated factors that may be produced in hypercoagulable states.[23,26,27] The normal range of the PT according to the manufacturer's RV is 11.8–13.7 s; however, our SRV was 12.6–13.5 s. Values above the RV are related to hemorrhagic pathologies, while values lower than 10 s are linked to prothrombotic states. The results obtained in this work show less time was required for the formation of a clot in patients, evidencing greater activity in hemostasis (Table 2).

Alterations in coagulation factors have been described in different populations of patients with LCPD, including factor V, and its mutation Factor V Leiden (FVL). Factor V, also called the proaccelerin or labile factor, is the plasma cofactor for the prothrombinase complex that activates prothrombin to thrombin. It is synthesized primarily by the liver. Plasma FV circulates as a 330-kDa single-chain polypeptide that is the inactive procoagulant. Although most FV is present in plasma, approximately 20% of the circulating FV is found within platelet granules. It plays a key role in the etiology of venous thromboembolism (VTE) and atherothrombotic cardiovascular events. The Leiden mutation of factor V (FLV) is the most studied thrombotic disorder. FVL is a mutation that is inherited in an autosomal dominant manner, with incomplete dominance, expressing a variant of factor V that cannot be

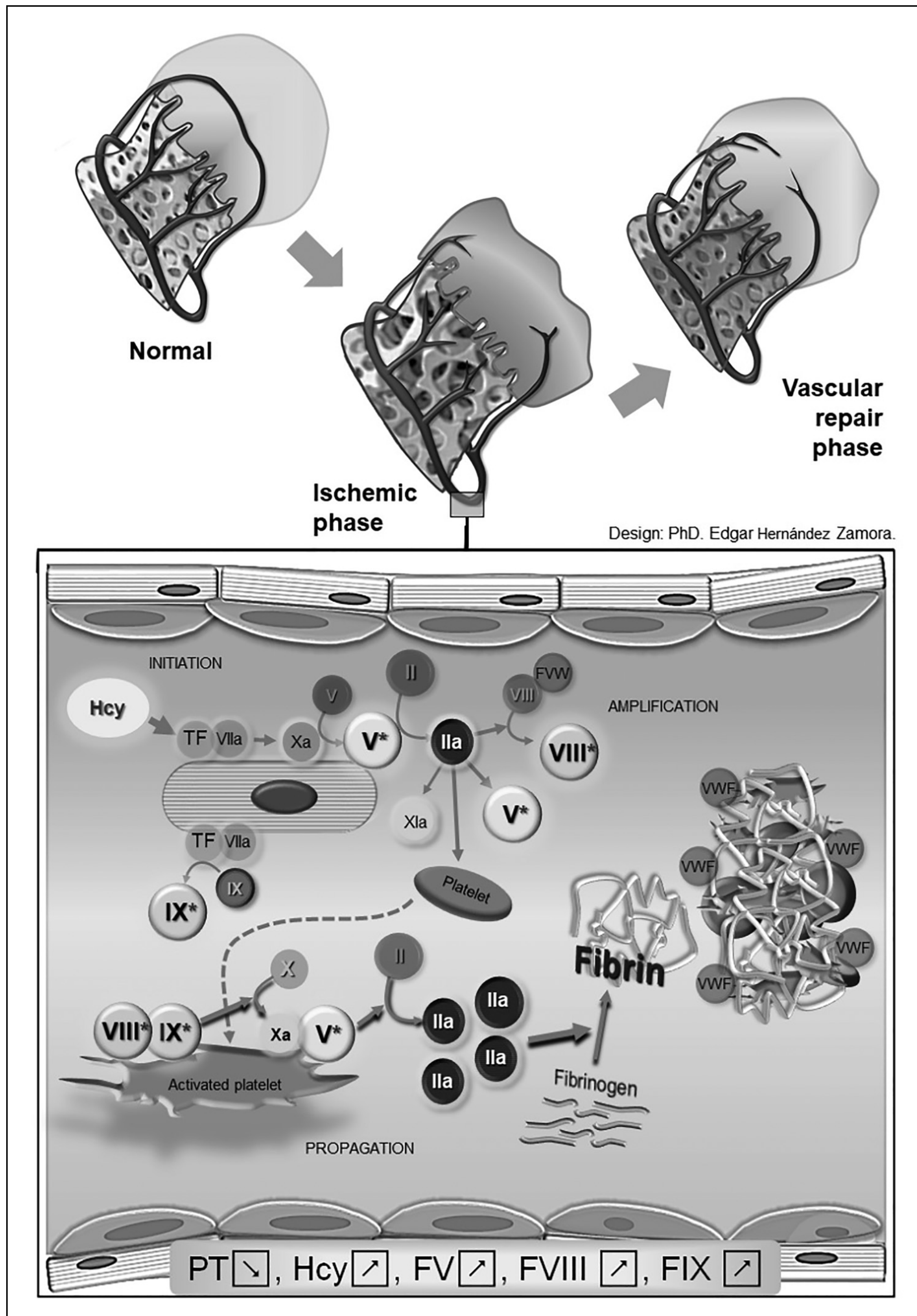

Figure 3. The top represents the evolution of femoral head deformity following ischemic necrosis in LCPD. On the bottom is the cell-based model of coagulation and fibrin formation. In this model, thrombin generation occurs in three phases: Initiation, amplification, and propagation. In this model, the increase of Hcy, and of the factors V, VIII, and IX are hemostatic alterations that favor hypercoagulable states (thrombophilia) that may play a central role in the etiology of LCPD. Hcy: Homocysteine. Tissue Factor: TF. Coagulation factors: II, V, VII, VIII, IX y X. VWF: von Willebrand factor. PT: Prothrombin time.

inactivated by protein C, resulting in an increased activity of the circulating FV. The evaluation of the activity of the FV could provide important information in addition to having a lower cost and being more accessible in routine laboratories.[28–32]

FVIII is synthesized in hepatocytes, lymphoid tissue, kidney, and endothelial cells. It is a protein of 2332 amino acids, with an approximate weight of 293 kilodaltons.[33] The main function of factor VIIIa, together with factor IXa and phospholipids in the presence of $Ca+2$, consists of activating factor X, thus participating as a complex in clot formation. In patients with venous thromboembolism and LCPD, FVIII has been shown to have high values. Different studies have

shown that the risk of recurrent venous thrombosis is also significantly increased in patients with high FVIII levels. Diverse evidence supports the hypothesis that elevated FVIII levels constitute a risk of important thrombophilia. In addition, Kristoffersen et al showed the PT decreased while the FVIII% increased, and the PT levels normalized as the FVIII% decreased.[12,34–38]

Coagulation factor IX is a vitamin K-dependent blood protein, with a molecular weight of 65 000 KDa. It is produced mainly by the liver, and it plays a key role in the intrinsic pathway of coagulation. Diverse studies suggest that it may have a critical role in hemostasis and deep venous thrombosis. Studies relate higher levels and activities of FIX to diseases such as idiopathic venous thromboembolism, coronary heart disease, and myocardial infarction.[39,40] It is important to note that this is the first time FIX has been associated with LCPD, a topic that should be further studied.

Hcy is an intermediate product of methionine and cysteine metabolism. In plasma, approximately 1% circulates as free, 70% to 80% is bound to proteins, and 20% to 30% circulates as Hcy dimers. The plasma-free Hcy values are 5.0 to 15.0 μmol/L in adults.[41] In children, they are 5.5–13.4 μmol/L for males and 4.9–11.9 μmol/L for females.[42] Hcy and especially hyperhomocysteinemia (HHcy) have significant effects on bone remodeling, blood flow, and hemostasis (Figure 3), systems that are altered in LCPD. In addition, HHcy has been linked to osteoporosis and ischemic heart disease, among others. Hcy at a concentration of 10 μmol/L or greater is considered a risk factor in the development of cardiovascular diseases and ischemic heart disease. Furthermore, an association has been found between an increased Hcy plasma level and a lower PT. Hcy induces the expression of TF, which when transmitted through blood can contribute to thrombosis but not to hemostasis.[41–46] We assume that since we did not find a difference between the FVII activity of the patients and controls, other factors were activated by the FVII–TF complex. This could be related to a local increase in the TF in patients, due to a moderately elevated Hcy concentration, resulting in a lower PT. These results in patients with LCPD coincided with those that have been described in pregnant women and patients with HIV.[23,46–48] In this study, several patients exceeded those high values of Hcy, and some polymorphisms in the Methylenetetrahydrofolate reductase (MTHFR) were present in patients with LCPD.[32,49,50]

Tests can be integrated into a model and be considered acceptable, if both the specificity and the sensitivity have a high level, of at least 75%. Thus, the PT and FIX were considered markers of hemostatic risk for Legg–Calvé–Perthes disease.

This work had some limitations because it was studying a rare disease; among these were the following: The sample size of the patients was small, which caused difficulties in investigation due to the low number of cases. Moreover, the patients presented diagnostic and follow-up difficulties, which did not allow a greater number test to be carried out on each patient.

Conclusion

Our results showed a decrease in the PT, although it is not clear why it was lower, as well as an increase in the activity of factors V and VIII, and for the first time, an increase in the activity of factor IX in LCPD patients, which are related to greater activity in hemostasis. On the other hand, we also found high Hcy concentrations, related to LCPD, thrombosis, and multiple bone diseases. It is likely that this plays a significant part in the etiology of LCPD, affecting the bone, vascular system, or hemostasis by various mechanisms. The increase in the activity of some CFs and the concentration of the Hcy supports the theory that hemostatic alterations such as thrombophilia may play a central role in the etiology of LCDP (Figure 3).

Despite great advances in the study of coagulation, there is still much to investigate. The hemostatic system is dynamic, with multiple interactions between cells and plasma proteins, as well as with inflammatory mechanisms and environmental factors. Thrombotic events have been reported to have an impact on the occurrence and severity of LCPD. This is why knowing the state of a patient's hemostasis would provide important information in clinic. The laboratory tests described in this work allow us to recognize a general panorama of hemostasis, and they are affordable and can be performed in any health institution. In particular, the PT and FIX support the diagnosis of LCPD, in conjunction with the imaging tests used.

Abbreviations

LCPD	Legg-Calvé-Perthes disease
INR-LGII	Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra Ibarra"
VWF	Von Willebrand factor
Hcy	Homocysteine
NOM	Norma Oficial Mexicana
BMI	Body mass index
TT	Thrombin Time
aPTT	activated Partial Thromboplastin Time
PT	Prothrombin Time
CF	Coagulation factors
PLTs	platelets
MTHFR	methylenetetrahydrofolate reductase

Acknowledgments

Thanks to reviewer Alejandra Tapia Alcazar for her assistance in the preparation of this manuscript. In loving memory of M.D. Antonio Redón Tavera, head of the Pediatric Hip Service; and Ph. D. Margarita Valdes Flores, Genomic Medicine, both from INR-LGII.

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.

Contributions

EHZ, AORO, and ERM conceived and designed the experiments. ERC, AORO, CZH, and EHZ collected blood samples and clinical data. AORO and ERC performed the experiments. EHZ, AORO, MAGA, and ERM carried out the analysis and wrote the paper. All authors read and approved the final manuscript.

Consent for Publication

All authors consent to publish.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article

ORCID iD

Armando Odiseo Rodríguez-Olivas https://orcid.org/0000-0002-4447-8343

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