

UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA,

DOCTORAL SCHOOL

PHD THESIS

**CLINICAL, HISTOPATHOLOGICAL, IMMUNOHISTOCHEMICAL AND
ULTRASONOGRAPHY ASPECTS OF PREGNANT WOMEN WITH AND
WITHOUT HEREDITARY THROMBOPHILIA**

- ABSTRACT-

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2019

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KEY WORDS: thrombophilia, histopathology, immunohistochemistry, heparine, preeclampsia.

I. KNOWLEDGE STAGE

I.1. Introduction

Thrombophilia (TPh) is defined as inadequate predisposition to form clots. The prevalence of this condition is constantly increasing. Over 50% of people with a thrombotic episode of unknown cause have changes in coagulation factors [Varga EA, 2012]. Hemostasis classically presents an intrinsic and an extrinsic pathway, with a common moment being the generation of factor X and an enzyme complex (prothrombinase) leading to the activation of prothrombin with thrombin formation [Handin RI 2004; Cucuianu M, 1994]. During pregnancy, a pregnant woman undergoes significant physiological and anatomical changes that help to accommodate and feed the fetus, changes in all maternal systems and organs, reversible shortly after birth [Soma-Pillay P, 2016]. Hypercoagulability, hemodilution, increased plasma volume, decreased haemoglobin, haematocrit and erythrocytes, platelet count (below 150,000 / mm³), appears especially in the second and third trimester of pregnancy [Barini R, 2013].

I.2. Hereditary thrombophilia

People with hereditary thrombophilia (TPh) either have an inability to produce adequate amounts of normal proteins, or have the ability to produce abnormal proteins. The most common hereditary thrombophilia are [Kupfermanc MJ, 1999]: PII gene mutation, FVL gene mutation, C677T or A1298T MTHFR gene mutation, PAI-1 gene mutation, PC, PS and AT III deficiency. In both acquired and hereditary TPh, the balance between procoagulant and anticoagulant activity of plasma factors is disturbed [Armstrong EM, 2014]. The risk of thrombosis during pregnancy may be increased in women with inherited or acquired thrombophilia [Folkeringa N, 2007], being higher in those with multiple defects of coagulation factors [Varga EA, 2008]. Not all thrombophilia confer a risk of thrombosis during pregnancy.

Severe preeclampsia (PE), placental abruption, intrauterine growth restriction (IUGR) and intrauterine fetal death are the most feared complications during pregnancy, with an additional risk of maternal and fetal morbidity and mortality. Their causes often remain incompletely elucidated. All of these conditions may be associated with abnormal placental vascularization or haemostasis disorders that may lead to inadequate placental circulation [Sohlberg S, 2014; Ariel I, 2004].

I.3. Placental histopathological and immunohistochemical characteristics

Physiologically, the microscopic aspect of the mature placenta is represented by a mass rich in chorionic villi of different sizes, containing stroma and multiple blood vessels. In pathological conditions, such as IUGR or PE, the placental lesions described in the histological examination are: single or multiple placental infarctions, fibrinoid necrosis, syncytial knots, avascular villi, dilated stem villi accompanied by stasis, trophoblastic degeneration, and calcifications. Abnormal development of the placenta may occur, which may influence fetal-maternal circulation through hypoperfusion, reducing the blood flow to the uterine arteries, with major repercussions on the fetus [Dekker GA, 1998].

Immunohistochemical testing has undergone a global development, representing one of the important tests used to detect a more specific clinical or histological features. The most important placental changes are related to vascularization. In highlighting the placental perfusion disorders we use anti-CD34 monoclonal antibody. Once the ischemia was installed, the oxidative stress appears, and was specifically identified with the help of anti-hypoxia-inducing factor-1 (HIF-1) α and anti-endothelial nitric oxide synthase (eNOS) monoclonal antibodies. [Siemerink MJ, 2012; Zimna A, 2015; Förstermann U, 2011].

I.4. Doppler ultrasonography

Non-invasive method most often used to obtain important information during pregnancy. It is a useful tool for monitoring and detecting perinatal complications such as PE, IUGR, intrauterine fetal death, but also for fetal malformations [Solomon LJ, 2010] and for detection of uteroplacental insufficiency [Khong SL, 2015]. Indications for using this type of examination are [Bhide A, 2013]: reduced fetal movements, history of IUGR or prematurity, intrauterine fetal death, PE, maternal smoking or illicit drug use, gestational diabetes, placental abruption. Doppler indices, pulsatility and resistance indices (PI, RI) at the uterine arteries are thus calculated to detect early any obstetric complications.

I.5. Thrombophilia treatment

There are until recently many recommendations regarding the use of anticoagulants during pregnancy, but not all are correct and documented. Heparin (enoxaparin, dalteparine, tinzaparin, or anti-factor Xa - danaparoid molecules) are the most commonly used anticoagulants because of the inability to cross the placenta, starting with unfractionated heparin, which is the one that produces the most complications [Christiansen OB, 2005].

Antivitamins k, are not recommended during pregnancy due to placental passage and teratogenicity.

II. PERSONAL CONTRIBUTIONS

II.1. Objective

The main objective of this research was to establish a correlation between hereditary TPh (high or low risk of thrombosis) and the histopathological and / or immunohistochemical changes found in the placental tissue, through clinical, genetic, histological, immunohistochemical and ultrasound studies.

II.2. Methodology

We initially carried out a retrospective study, for choosing the study group, by analysing the observation sheets, birth registers and electronic databases of the two clinics, between January 2013 and December 2016. We then proposed a prospective study on this group of patients, between January 2017 - December 2018, which included patients from the Obstetrics and Gynaecology Clinics of the Philanthropy Hospital and of the County Emergency Hospital in Craiova. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova.

The research was divided into 4 studies: the clinical-demographic study, which aims to identify the risk factors for each patient; Genetic study, useful to identifying the mutations of the coagulation factors specific to each patient and their correlation with pre-existing risk factors; Histopathological and immunohistochemical study, the most important study based on the detection of placental lesions specific to each type of TPh; Ultrasonographic study, which consists in monitoring each patient and evaluating the response to anticoagulant therapy and in early identification of obstetric complications.

The study group consists of 90 non-smoking Caucasian women with a history of obstetric or thrombotic complications and who did not benefit from testing for genetic mutations of coagulation factors and whose placental tissue from first pregnancy is available. Out of 18,000 births, 1,562 did not meet the selection criteria, a number of 1,128 patients having already been genetically tested to detect coagulation factor mutations. 120 patients obtained a new pregnancy between January 2017 and December 2018. Finally, after proper

counselling, 30 patients refused to participate in the study. Therefore, the final study group included 90 patients.

After birth, the obtained placental tissue was immediately fixed in 10% formalin solution for a period of 24 hours, processed and included in paraffin at the Clinical of Pathological Anatomy of the Philanthropy Hospital and of the County Emergency Hospital in Craiova. These were subsequently processed in the Center for Microscopic and Immunological Studies of the UMF, Craiova, and additional staining techniques were used, after the informed consent of all the women included in the study was obtained. They were used: classical morphological staining Hematoxylin eosin (HE), trichrome Goldner - Szeckeli staining, to identify collagen fibres, coloured in green and Periodic acid Schiff hematoxylin (PAS-hematoxylin) to identify glycosaminoglycans, coloured in pink.

Further, placental tissues were investigated by IHC analysis: the anti-CD34 monoclonal antibody that allowed us to identify the blood vessels in the placental tissue. Endothelial nitric oxide antibody (eNOS), and anti-hypoxia inducing factor (HIF-1) α , which play an important role in hypoxia.

Pregnant women were than investigated dynamically using Doppler ultrasonography in TI, TII and TIII until birth, or more often depending on the changes that have occurred. As reference values were taken into account: IP, IR, protodiastolic NOTCH and specific percentiles for weight and gestational age.

III. RESULTS

Clinical-demographic study

All 90 patients had a personal history of at least one obstetric or thrombotic complication: PE, IUGR, recurrent early or late pregnancy loss, intrauterine fetal death, placental abruption, and/or personal or family history of thrombosis such as myocardial infarction, venous thromboembolism (VTE) or stroke. As results there was a relatively high incidence of spontaneous abortions in the first trimester, both in patients with TPh (65%) and in those without a coagulation factor mutation (70%), with no statistical significance ($P = 0.709$), respectively ($P = 0.299$).

Genetic study

In terms of genetic characteristics, the study group was divided into group A, consisting of 5 patients with high risk thrombophilia (5.5%) and 35 patients with low risk TPh (39%) and in group B, made up of 50 pregnant women whose thrombophilia tests were negative (55.5%). A relatively low prevalence of high-risk TPh is observed, 5.5% (5 of 90 cases) of patients had homozygous FVL or double heterozygous FVL / PII gene mutation. In the low-risk TPh group, 23 patients had heterozygous MTHFR and PAI-1 gene mutations, homozygous, or heterozygous / homozygous status. Another 10 patients were with heterozygous FVL gene mutation and 2 cases out of 90 with heterozygous PII gene mutation.

Histopathological study

We were able to highlight, important placental differences between patients with high-risk TPh and those with low-risk or TPh-. In all patients with high-risk TPh, the predominant placental lesions were multiple placental infarctions (Figure I) and syncytial knots (+++). In patients with low risk TPh, these pathological placental changes could be detected in 40% of cases, with lower intensity or were strictly focal. Fibrinoid necrosis (+) was also found in this group, single placental infarction, syncytial knots (+++) with localized character. These lesions were described in less than 30% of women with TPh- (group B). In 60% of cases, the conventional HP examination revealed aspects compatible with normal placenta.

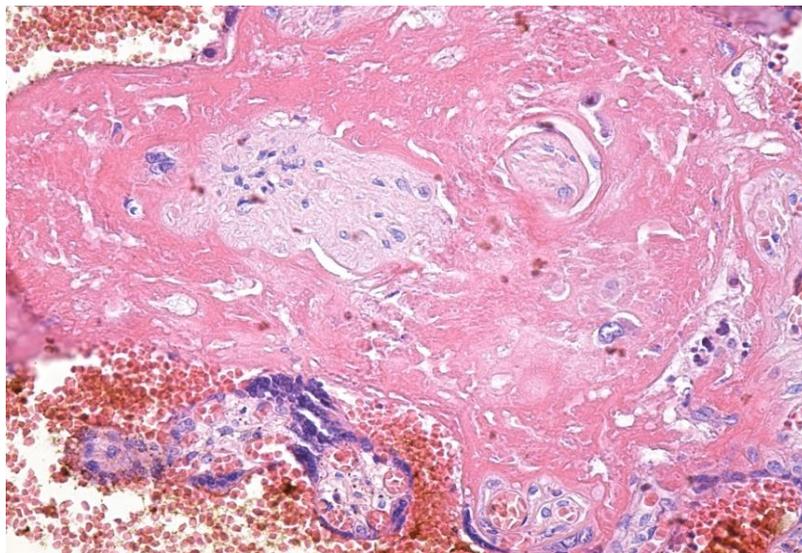


Figure I. Histopathological examination; Multiple placental infarctions in a patient with high-risk thrombophilia.

Immunohistochemical study

Immunohistochemistry was used to highlight more specifically the lesions appeared at histological examination and to establish a pattern between them and thrombophilia tests.

An expression of CD34 (+) and even CD34 (-) with large areas of fibrinoid necrosis (+++) was observed in 100% of women with high risk TPh, in under 40% of women with low risk TPh and in 34% with TPh-, showing an important statistical significance, $p = 0.01$. CD34 (+++) immunoreactivity was noted in 3 patients (60%) with high risk TPh, in 6 patients (17.1%) with low risk TPh and in 7 patients (14%) with TPh-, suggesting increased angiogenesis, but without statistical significance, $p = 0.05$. An increased immunoexpression of hypoxia-induced factor 1 (HIF-1) α (+++) (Figure II) was found in 100% of cases with high-risk TPh, in 14% with low-risk TPh, and in 12% with TPh-.

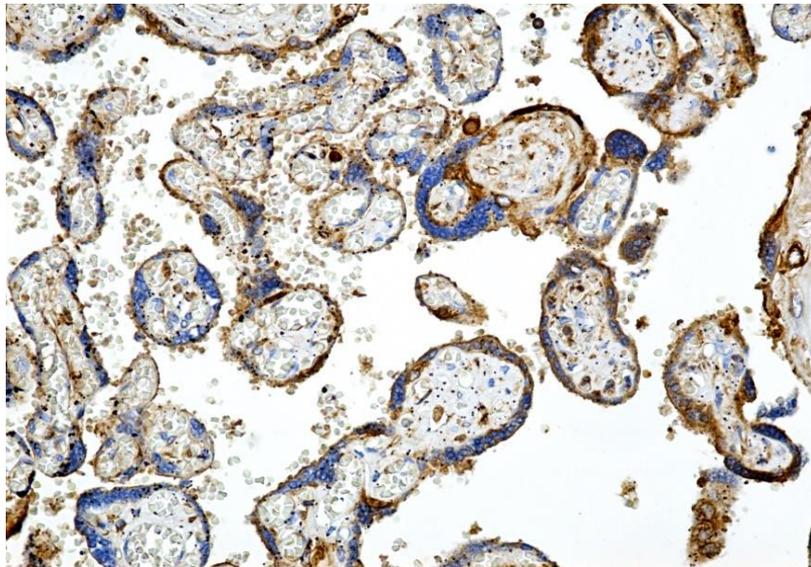


Figure II. Immunohistochemical examination; Increased HIF-1 α immunoexpression in a patient with high-risk thrombophilia.

ENOS (+++) immunoexpression was observed in the placental tissue of all 5 women with high-risk TPh, with a significant statistical significance $P < 0.001$. ENOS (+) immunoexpression was observed in both high-risk and low-risk TPh patients (<35%). By correlating the expression of immunohistochemical markers and genetic tests, a statistical significance can be observed in the case of HIF-1 α (+++), eNOS (+++) but also CD34 (-) / (+) immunoexpression with $p < 0.05$. Could not reveal a statistical significance between genetic tests and the immunoexpression of HIF (-), eNOS (-) but also CD 34 (+++) markers with $p > 0.05$.

Ultrasonographic study

The ultrasonographic aspects of women with high risk TPh and in treatment with LMWH were normal, without changes during pregnancy, at birth or postpartum. Only one patient in this group had mild PE. In 5 patients with low-risk TPh, Doppler ultrasonography show in TIII an increased value of RI, PI and the presence of protodiastolic incision (notch). Doppler changes were reported in specific nomograms, showing a percentile below 10% in a patient with heterozygous FVL gene mutation and below 5% in a patient with heterozygous PII gene mutation. The remaining 3 patients developed severe preeclampsia TA 160 / 90mmhg respectively 170/100mmhg, 1 patient with heterozygous FVL gene mutation, and 2 double heterozygotes MTHFR / PAI-1 gene mutation. Among patients without TPh, 6 had obstetric complications, of which 4 IUGR, with percentages below 10% for gestational age and 2 PE with systolic blood pressure values between 160-200mmhg and diastolic between 90-120mmhg.

Pregnancy outcome according to LMWH therapy.

In patients with increased risk of thrombosis, in the presence of the anticoagulant treatment, the risk of developing PE is 0.29 (OR 4; 95% CI 0.374-42,801), much lower than patients with TPh- where the risk is 1.5 times higher (OR 0.64; 95% CI 0.123-3,400) and 0.66 (OR 1.545; 95% CI 0.294-8.121) in patients with low risk TPh (Table I).

	Relative risk		Confidence interval 95%	
	OR	min	Max	
High risk TPh	,294	4,000	,374	42,801
Low risk TPh	,667	1,545	,294	8,121
TPh-	1,500	,647	,123	3,400
Nr of cases	90			

Table I: Relative risk and likelihood of developing preeclampsia (PE) in a new pregnancy; OR- odds ratio / odds ratio; TPh - thrombophilia.

The risk of developing IUGR is 1.33 times higher (OR 0.735; 95% CI 0.127-4,241) in patients with low-risk thrombophilia and 0.75 (OR 1.360; 95% CI 0.236-7,845) in those without TPh. Analysing the pregnancies outcome, it appears that there are no major differences between patients with low-risk thrombophilia and patients without TPh, so the probability to develop obstetric complications was similar between these two groups of

patients. The pregnancy outcome is favorable, out of all patients included in the study, only 6 developed IUGR with a birth weight below 2500 grams, compared to the rest of the cases whose fetus had a birth weight over 2500 grams.

IV. DISCUSSIONS

The results of this study may partially explain the pathophysiology of thrombophilia associated with placental lesions. Thus, a severe genetic mutation of the coagulation factors produces a thrombogenic effect in the placental vessels, with a high incidence of multiple infarction and fibrinoid necrosis. Moreover, there are also placental perfusion disorders with the installation of oxidative stress. This stimulates the production of the antiangiogenic factors, with local vasoconstriction and hypoxia, which could produce a disorder of normal fetal development. After hypoxia and vasoconstriction are installed, a nitric oxide release occurs in the affected vessel. After vasodilation the relaxation of smooth muscle cells occurs. These mechanisms have also recently been cited in patients with obstetric complications [Aouache R, 2018; Förstermann U, 2011; Kay HH, 2006; Sultana Z, 2017; Marinoni E, 1997]. There have been no publications in the present or in the past that attempt to demonstrate an association between thrombophilia and IHC test at the placental level, which is why further studies are needed on larger groups of patients.

V. CONCLUSIONS

The objectives of the study were achieved by analysing the clinical demographic, genetic, HP, IHC data and by careful ultrasonographic monitoring of pregnancy progression in the presence or absence of anticoagulant therapy. According to our preliminary results, low-risk TPh shows no clinical changes, HP or IHC in addition to those seen in patients without TPh, most likely because they occur due to other causes and not due to thrombophilia. As strengths we mention, a strictly standardized protocol in placental tissue analysis and pregnancy management. In our opinion, in patients with previous obstetric and / or thrombotic complications, placenta should be investigated initially using HP and IHC techniques. We can speculate that this approach would be more cost effective and not only in comparison to the extended TPh testing for large populations. The next step would be to test the patients selectively, for the mutations of the coagulation factors, only when the HP and IHC examinations were encountered: multiple placental infarcts, fibrinoid necrosis (+++), syncytial knots (+++) in association with an immunoexpression of CD34 (-) / (+), HIF-1

(+++)) and eNOS (+++)). In this way a much larger proportion of patients will be found to have high risk TPh and the anticoagulant treatment would be administered only when needed.

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