**MEDICINE AND PHARMACY UNIVERSITY OF CRAIOVA**
**DOCTORAL SCHOOL**

# DOCTORAL DISSERTATION

## *Research on developing a molecular testing system to evaluate pregnancy morbidity*

**-Abstract-**

**PHD COORDINATOR**
**PhD. Dr. Francisc Mixich**

**PHD STUDENT**
**Andreea Gabriela Anghel**

**CRAIOVA**
**2015**

**CONTENTS**

**STAGE OF KNOWLEDGE**

**1.** DETERMINANTS OF BAD OBSTETRIC HISTORY OF PREGNANT WOMEN

Bad obstetric history involves unfavorable previous fetal development, two or more consecutive recurrent spontaneous abortions, intrauterine fetal death antecedents, retarded intrauterine growth, stillbirth, early neonatal death and/or congenital abnormalities. There can be genetic causes, hormonal causes, abnormal and immune maternal reactions or maternal infections.

1.1. Immunological causes of bad obstetric history pregnancy

Immunological factors of a pregnancy with bad obstetric history, or miscarriages, fall into autoimmune and alloimmune factors.

Autoimmune factors:

-Antiphospholipid antibodies

-Antinuclear antibodies

-Antithyroid antibodies

Alloimmune factors:

There is a debate over human leukocyte antigens (HLA) would be responsible for such alloimmune reactions.

1.2. Genetic causes of bad obstetric history pregnancy

Most miscarriages are caused by an abnormal embryo karyotype. At least 50% of first trimester abortions are cytogenetic abnormals. This does not include abnormalities caused by genetic diseases such as Mendeliene genetic disease or certain loci mutations ( hereditary thrombophilia etc). Other examples can be the polygenic or multifactorial diseases, that can not be detected by cytogenetic evaluations.

1.3. Infectious causes of bad obstetric history pregnancy

Viral infections

Microbial infections

Toxoplasma

## PERSONAL CONTRIBUTION

## INTRODUCTION

The research wishes to suggest the standardization and widespread introduction of molecular tests for monitoring pregnancy morbidity. The scientific rigor of molecular tests, clearly superior to serological tests, could improve both mother and fetus health by reducing the influence of risk factors that lead to premature birth, mortality or fetal morbidity. Current investigations are primarily based on immunological ELISA testing methods. The obtained data are still under discussion due to severe difficulties in interpreting the potential fetal exposure during pregnancy. These issues also raise the question of identifying more sensitive and specific methods. Today, molecular biology methods have become more adaptable and easy to use for clinical laboratory diagnosis, having superior technical performance.

## RESEARCH AIMS

1. The research was performed in order to obtain reduced pregnancy morbididty rate, which has not been noticed in the last decade In addition, the incidence of intrauterine growth restriction has not changed visibly over the last 20 years. The research wishes to demonstrate that maternal exposure to a number of risk factors (infectious and genetic) that significantly cause pregnancy morbidity, if investigated in a standardized way, can ensure objective pregnancy oversight to reduce side effects such as mortality and fetal morbidity.
2. The research wishes to issue a basal protocol for pregnant women with pregnancy morbidity, which consists in the standardization and widespread use of molecular tests that is economic and efficient to monitor morbidity in pregnancy, that will be used by competent

institutions and introduced in the minimum package of medical services offered to pregnant woman with risk factors.

## RESEARCH METODOLOGY

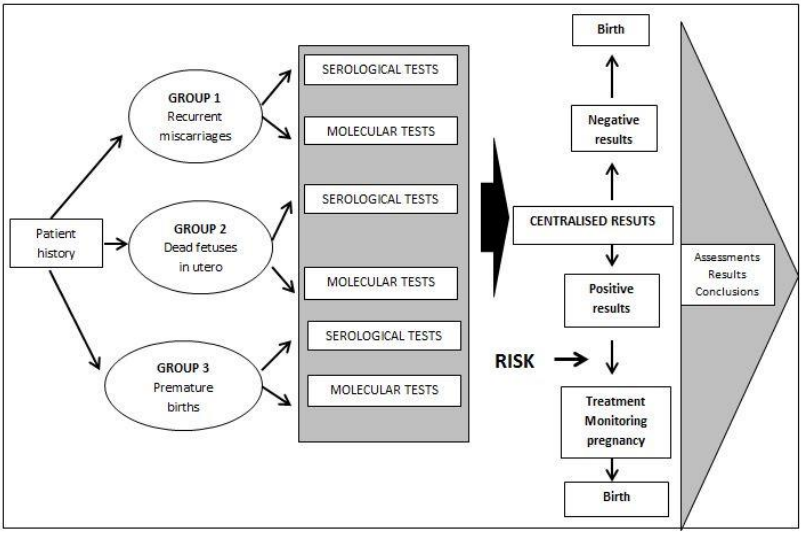
### Study materials

The studied cases come from multiple gynecological cabinets in major cities of Oltenia (Craiova, Slatina, Târgu Jiu, Târgu Cărbunești, Balș, Băilești), in 2010-2014. The study group, selected from a number of 1914 pregnant women with bad obstetric history that include reccurent spontaneous abortion, misscarriages, stillbirths, retarded intrauterine growth, premature birth or congenital abnormalities. The control group, comprising multiparous pregnant women without negative history belong to the same source. Out of the 1914 pregnant women, 108 were identified having bad history pregnancies. Therefore, 108 pregnant women with risk pregnancies history and 108 multiparous pregnant women with normal risk were included in this study, a total of 216 patients.

### Data collection

All the 1914 pregnant women were identified within specialised offices. A database was established based on data sheets drawn up with patients agreement. They were dynamically monitored trhoughout pregnancy, and the results were introduced into the database, which allowed corresponding correlations to be made. The only inclusion criteria was the presence of pregnant women at first antenatal consultation, which established through anamnesis, the bad history of pacient`s previous pregnancies.

**Studied groups**




**Serological tests**

ELISA test for *Toxoplasma Gondi*
ELISA test for *Rubella*
ELISA test for *Cytomegalovirus (CMV)*
ELISA test for *Herpes simplex virus type 2.*
ELISA test for *Parvovirus B19.*
ELISA test for *Varicela zoster*
ELISA test for Antiphospholipid antibodies

**Molecular tests**

Real time quantitative PCR for Toxoplasma
Real time quantitative PCR for CMV
Real time quantitative RT PCR for Rubella
Real time quantitative PCR for Herpes simplex type 2
Real time quantitative PCR for Parvovirus B19
Real time quantitative PCR for Varicella Zoster virus

PCR-RFLP for G1691A mutation analysis (V Leiden factor) and G20210A prothrombin gene mutation.

**Data analysis**

Obtained data were complied in Microsoft Excel calculation sheets. To identify any close association between definitely close data,we used the Chi-square test using an Epi Info software (version 3.5.1). Data were expressed as percentage or as allele frequency. The allele frequency was calculated using gene-counting methods. Statistical analysis was preformed using a SPSS software, version 11.5. The genotype distribution for each mutation, the frequency of heterozygotes and homozygotes were compared between patients and control with Pearson`s Chi-square test. The value of $p<0.05$ was considered significant.

**RESULTS**

Serological and molecular investigations for Toxoplasma

About 42.59% of the cases from the study group are seropositive and 13.88% from the control group. IgG seropositivity reaches 30.55%, respectively 10.18%. For IgG and IgM antibodies, seropositivity reaches 12.03% of the study group and 3.70% of the control group. The difference between seropositivities is significant (p=0.00). *Toxoplasma* DNA determinations have identified only 8 cases from the risk group (7,40%) and 3 cases from the control group (2,77%).

Serological and molecular investigations for *Rubella*

Serological IgM positive cases, which mean the presence of an acute infection, have an absolutely insignificant number and no statistical significance between the control and study groups. Furthermore, the

3, respectively 4 cases of IgM positivity, have not been confirmed by molecular tests.

Serological and molecular investigations for *Cytomegalovirus* (CMV)

Serological IgM positive cases, which mean the presence of an acute infection, have an absolutely insignificant number and no statistical significance between the control and study groups. Amongst the 2 cases, respectively 1 case of IgM seropositivity, molecular tests have confirmed the presence of ADN CMV in one case from the control group.

Serological and molecular investigations for *Herpes simplex* type 2

There is not a significant difference between the control group and the group of women with bad obstetric history. Moreover, only the presence of IgM antibodies is important for the fetal risk, because this means the presence of an acute infection, so the number of positive cases for IgM (6, respectively 7) means that there is a difference between the two groups, also insignificant. This IgM positive cases were have been confirmed by molecular tests only up to a third.

Serological and molecular investigations for *Parvovirus B19*

Serological IgM positive cases, which means the present of an acute infection, have not been identified neither in the study group nor in the control group. Molecular tests have confirmed serological results.

Serological and molecular investigations for *Varicela zoster*

Serological IgM positive cases, which means the present of an acute infection, have not been identified neither in the study group nor in the control group. Molecular tests have confirmed serological results.

Serological investigations for Antiphospholipid antibodies

IgG seropositivity is only 27,77%, respectively 8,33%. For IgG and IgM antibodies, seropositivity reaches 5,55% in the study group and 0% at the control group. The difference between the seropositivities is significant (p=0.03).

Molecular investigations for G1691A mutations (V Leiden Factor)

Factor V Leiden mutation is significantly associated with venous thromboembolic disease in the group of pregnant women with bad obstetric history with an odds ratio (95% CI) = 6,41(1,81-22,76); p = 0,004.

Molecular investigations for prothrombin gene G20210A mutations

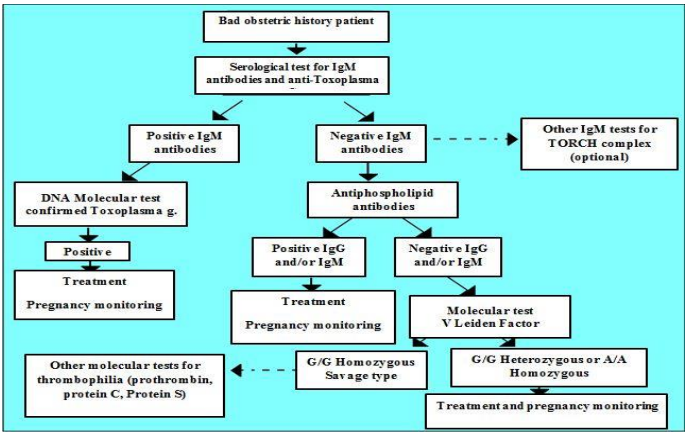
G20210A prothrombin gene mutation frequency has not a significant difference between the study group and the control group.

**DISCUSSIONS**

Analyzing the results from the table, we can notice that a significant association between bad obstetric history and risk during pregnancy, if we compare the results with the ones obtained by the study group, exists in the cases of *Toxoplasma* infections, antiphospholipid antibodies and V Leiden factor. For any other determinations, our results present an insignificant association, or show no associations. In the light of these data, a drastic reconsideration over the set of tests that a pregnant patient with pregnancy failures history has to make is required.

| Tabel 11. Summary of results obtained in study group and control group | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Infectious causes** | IgG Seropositivity | | IgM Seropositivity | | Molecular tests | | Statistics Association degree |
| | Study group | Control | Study group | Control | Study group | Control | |
| Toxoplasma | 33(30.55) | 11(10.18) | 13(12.03) | 4(3.70) | 8(7.40) | 3(2.77) | **Significant** |
| Rubella | 105(97.22) | 104(96.29) | 3(2.77) | 3(2.77) | 0(0) | 0(0) | **Insignificant** |
| CMV | 106(98,14) | 107(99,07) | 2(1,85) | 1(0,92) | 1(0,92) | 0(0) | **Insignificant** |
| Herpes 2 | 33(30,55) | 41 (37,9) | 6 (5,55) | 7(6,48) | 2(1,85) | 1(0,92) | **Insignificant** |
| Parvovirus B19 | 6(5,55) | 4(3,70) | 0(0) | 0(0) | 0(0) | 0(0) | **Insignificant** |
| Varicella zoster | 8(5,55) | 8(5,55) | 0(0) | 0(0) | 0(0) | 0(0) | 7(6,49) |
| **Immunological causes** | IgG Seropositivity | | IgM Seropositivity | | - | | Statistics Association degree |
| | Study group | Control | Study group | Control | | | |
| Antiphospholipid antibodies | 30(27.77) | 9(8,33) | 9(8,33) | 1(0,92) | | | **Significant** |
| **Genetic causes Hereditary throbophilia** | Savage type G/G | | G/A Heterozygous | | A/A Homozygous | | Statistics |
| | Study group | Control | Study group | Control | Study group | Control | |
| V Leiden G1691A Factor | 91(84,25) | 105(97,22) | 17(15,75) | 3 (2,78) | 0 | 0 | **Significant** |
| Prothrombin G20210A | 103(95,37) | 101(93,51) | 5(4,63) | 7(6,49) | 0 | 0 | **Insignificant** |

Regarding the context of the data form our study, we propose the following investigations algorithm for pregnant women with bad obstetrical history:



**CONCLUSIONS**

1. A significant association between the bad obstetric history and pregnancy risk, if we compare the results obtained by the control group, exists only in the cases of Toxoplasma infections, antiphospholipid syndrome and V Leiden factor. In all the other determinations, our results show either no significant associations or no associations.
2. Having obtained these results, a drastic reconsideration over the set of tests that a pregnant patient with pregnancy failures history has to make is required.
3. The infectious causality of these pregnancy failures greatly narrows. From our results, we can conclude that the most important test of the TORCH complex, is the test for Toxoplasma g. and, even in this case, is sufficient only the IgM antibodies testing.
4. Based on scientific literature data, and on our results we can say that testing TORCH complex for IgG antibodies is useless. It would be enough only the IgM antibodies testing, and if positive, the results can be confirmed by molecular tests of highlighting the nucleic acid of the pathogenic agent. Serological tests cost would thus reduce to half, and the difference could be invested in molecular testing which is more accurate and provide more precise data.
5. The investigation of antispermatic antibodies however, has a particular value in finding causes of a bad obstetric history, and the results that we obtained confirme that the degree of associating risk to women with bad obstetric history, compared to the control group is statistically significant.
6. Among genetic tests regarding risk for trombophilia , V Leiden factor mutation has a significant value, as stated in most data from literature. G20210A prothrombin gene mutation investigation, which is rare, has a low value, and we obtained insignificant risk values.

**SELECTIVE REFERENCES**

**Surpam RB, Kamlakar UP, Khadse RK, Qazi MS, Jalgaonkar SV**. Serological study for TORCH infections in women with bad obstetric history. J Gynec Obstet India 2006;56:41-43.

**Meka A, Reddy BM.** Recurrent spontaneous abortion: an overview of genetic and non-genetic background. Int J Hum Gen 2006;6:109-117.

**Singh G, Sidhu K**, Bad obstetric history: A prospective study. Med J Armed Forces India 2010; 66, 2: 117–120.

**Koppad C, Lakshmi KS,** Immunological Causes of Bad Obstetric History. Journal of Evidence based Medicine and Healthcare 2014; 1(16): 2086-2099.

**Djelmis J, Radoncic E, Ivanisevic M**. Antiphospholipid antibody syndrome and fetal outcome. Acta Med Croatica 2001; 55 (3): 123-30.

**Giannakopoulos B, Passam F, Rahgozar S, Krilis SA.** Current concepts on the pathogenesis of the antiphospholipid syndrome. Blood 2007; 109 (2): 422-30.

**Greer IA**. Thrombophilia: implications for pregnancy outcome. Thromb Res 2003; 109:73-81.

**Zonouzi AP, Chaparzadeh N, Ghorbian S, Sadaghiani MM, Farzadi L, Ghasemzadeh A, Kafshdooz T, Sakhinia M, Sakhinia E.** The association between thrombophilic gene mutations and recurrent pregnancy loss. Journal of Assisted Reproduction and Genetics 2013; 30(10).

**Robertson L, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GD, Walker ID.**. Thrombophilia in pregnancy: a systematic review. Br J Haematol 2006;132(2):171–196.

**Montoya JG, Liesenfeld O.** "Toxoplasmosis". Lancet 2004; 363 (9425): 1965–76.

**Trifa A, Popp R.** Thrombophilia – It's time to stick to the international Guidelines. Rom. J. Rare Dis 2015; Suppl 1: 18-19.

**CURRICULUM VITAE**

**Personal information:**

Name / Surname: ***Anghel Andreea Gabriela***
Address: Unirii st., Craiova (Romania)
Mobile number: 0745363512
E-mail: andeea_anghel@yahoo.com
Nationality: Romanian
Date of birth: 23/08/1985
Gender: Female

**Professional experience:**

Period: 01/10/2010–Present
Occupation or position held: Dentist
Business or sector: Medicine/ Health

**Education and Training:**

2000-2004 – National College "Fratii Buzesti", Craiova
2004-2010 – Faculty of Dentistry, University of Medicine and Pharmacy of Craiova
2010-2011 – Master "Prosthetic Rehabilitation with implant support", University of Medicine and Pharmacy of Craiova
2011-Present – PhD at Molecular and Cellular Biology Department, University of Medicine and Pharmacy of Craiova

**Personal skills :**
Mother tongue(s) – Romanian
Other language(s):

(*) Common European Framework of reference for Languages

| | Understanding | | | | Speaking | | | | Writing | |
|---|---|---|---|---|---|---|---|---|---|---|
| European level | Listening | | Reading | | Spoken interaction | | Spoken production | | | |
| English | C2 | Proficient user | C2 | Proficient user | C2 | Proficient user | C2 | Proficient user | C2 | Proficient user |
| French | C1 | Proficient user | C1 | Proficient user | B2 | Independent user | B2 | Independent user | B2 | Independent user |
| Spanish | B2 | Independent user | B2 | Independent user | B2 | Independent user | B2 | Independent user | B2 | Independent user |

**Computer skills:**

- Good command of Microsoft Office Word
- Microsoft Office Excel
- Microsoft Office Power Point

**Driving licence** : Category B

**BDI Personal Publications (International Database):**

- -Original Paper - Serological Screening for Toxoplasma G in women with bad obstetric history in Oltenia Region, Romania. (Current Health Science Journal, Volume 40, 2014 Supplement 7)
- -Original Paper- The predictive value of A1298C and C677T MTHFR gene mutations for the recurrent spontaneous abortions risk (Current Health Science Journal, Volume 41, 2014 Supplement 1)