
Abd Elhadi Mohammed Agena Musa

(B.Sc. in Hematology and Immunohematology, University of Al-imam Almahdi 1998)

(M.Sc. in Hematology and Immunohematology, University of Gezira 2006)

A Thesis
Submitted to University of Gezira in Fulfillment of the Requirements for the Degree of PhD in Medical Laboratory Sciences
In
Hematology &Immunohematology
Department of Hematology &Immunohematology
Faculty of Medical Laboratory Sciences

2018
قال تعالى:
(ووالا سمون في العلم يقولون آمنا به كلامك عند ربا وما يذكر إلا أول الألباب) صدق الله العظيم
الآية (7) سورة آل عمران

Abd Elhadi Mohamed Agena Musa

Supervision Committee:

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Anwaar A.Y. Kordofani</td>
<td>Main Supervisor</td>
<td>............</td>
</tr>
<tr>
<td>Prof. Mohammed Alsanosi Ahmed</td>
<td>Co-Supervisor</td>
<td>............</td>
</tr>
<tr>
<td>Prof. BakriYoussif Mohamed Nour</td>
<td>Co-Supervisor</td>
<td>............</td>
</tr>
</tbody>
</table>

Date: 29/3/2018

Abd Elhadi Mohammed Agena Musa

Examination Committee:

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Anwaar A.Y. Kordofani</td>
<td>Chairperson</td>
<td>………………</td>
</tr>
<tr>
<td>Prof. Babiker Mohamed Ahmed</td>
<td>External Examiner</td>
<td>……………</td>
</tr>
<tr>
<td>Dr. Soad Fadul Allah Ali</td>
<td>Internal Examiner</td>
<td>……………</td>
</tr>
</tbody>
</table>

Date: 29/3 /2018
Declaration

I declare that this PhD thesis entitled was designed and submitted by me for the PhD degree in medical laboratory sciences under the guidance and supervision of Prof. Anwaar Ahmed Youssif Kordofani, Prof. Mohammed Al-Sanossi Ahmed, and Prof. Bakri Youssif Mohammed Nour.

The interpretations put forth are based on my reading and understanding of the original texts and they are not published anywhere in the form of books, monographs or articles and websites which I have made use of acknowledged at the respective place in the text.

For the present thesis that I am submitting to the University, no degree, diploma, or distinction has been conferred on me before, either in this or in any other University.

Date: 2018

Research Student

Abd Elhadi Mohamed Agena Musa
Dedication

To Sole of...
My father Mohammed...
To...
My mother Khadija...
Moreover, to...
My small Family
For their Continuous Encouragement and Support..

Abd Elhadi
Acknowledgement

I would like to thank:

- Prof Anwaar Kordofani (Department of Pathology. Faculty of Medicine. University of Khartoum), Prof. Bakri Youssif Mohamed Nour (Department of Medical Parasitology and Entomology. Blue Nile Institute For Endemic Diseases. University of Gezira) and Prof. Mohammed Al-Sanossi Ahmed (Department of Obstetrics and Gynecology. Faculty of Medicine. University of Gezira) for their supervision, kindness and great help to conduct this study.

- The staff of laboratories of Saudi, Omdurman Friendship, and Omdurman Maternal Hospital for their co-operation and providing great facilities to selection of patients, collection, storing of blood samples and doing the initial laboratory investigations.

- The staff of Rebat University research laboratory for providing facilities to do DNA extraction from blood.

- The staff of Sudan University for sciences and technology research laboratory for providing facilities for doing molecular investigations (PCR).

- The staff of Gezira private laboratory for providing great facilities for doing serological investigations (antiphospholipid antibodies (IgG/IgM) and anticardiolipin antibodies (IgG/IgM)) ELISA technique.

- Patients and healthy individuals for participating in this study.

Abd Elhadi Mohamed Agena Musa

Abstract

Background: Thrombophilia is a health problem, yet more studies are required to take this health problem and ensure. Objectives: The objective of this study is to determine the prevalence of undiscovered thrombophilic factors and their relation to recurrent pregnancy loss (RPL) in women who have had two or more previous pregnancy losses. This study is a prospective case control study, conducted from August 2013 to February 2018 in Mohammed Ali Alshaikh (Saudi) Hospital for Obstetrics and Gynecology, Friendship Hospital and Omdurman Maternal Hospital. Methods: A total of 100 women with history of two or more consecutive recurrent miscarriage as case group and 51 healthy women with no history of miscarriage as control group were screened for the presence of FVLeiden, prothrombin gene mutation, antiphospholipid antibodies APA (IgG/IgM), and anticardiolipin antibodies ACA (IgG/IgM). Also, prothrombin time PT, activated partial thromboplastin time APTT and Platelet counts were done for patients and controls. Results: The prevalence of FV Leiden G1691A gene mutation (homozygous and heterozygous) was found in 9(9%) and 11(11%) of patients with RPL and in 0(0%) and 2(3.9%) of controls. P value (0.000) and (0.000), respectively. The prevalence of prothrombin G20210A gene mutation (homozygous and heterozygous) was found in 11(11%) and 5(5%) of patients with RPL and in 3(5.8%) and 0(0%) of controls. P value (0.000) and (0.000), respectively. Anticardiolipin antibodies IgG/IgM were positive in 12(12%) and 7(7%) of patients with RPL, and in 3(5.8%) and 2(3.9%) controls. P value (0.000) and (0.000) respectively. Antiphospholipid antibodies IgG/IgM were positive in 10(10%) and 11(11%) of patients with RPL, and in 2(3.8%) and 1(1.9%) of controls. P value (0.000) and (0.007), respectively. PT, APTT, and platelet counts (thrombocytopenia) in women with RPL were affected significantly. P. value (0.000), (0.000), and (0.000), respectively. Conclusion: Based upon the results we concluded that the point mutation in factor V Leiden G1691A and prothrombin G20210A might play a role in RPL among Sudanese women. Anticardiolipin antibodies ACA (IgG/IgM) and antiphospholipid antibodies APA (IgG/IgM) have significant effect on RPL. PT, APTT, and platelet counts in women with RPL were affected significantly. Women with RPL should be screened for thrombophilia.
تخثر الدم كعامل مسبب لإسقاط الحمل المتكرر في النساء السودانيات، ولاية الخرطوم، السودان (2018)

عبد الهادي محمد عجينة موسي

ملخص الدراسة

مرض تخثر الدم يعتبر من المشاكل الصحية، وعلي يومنا هذا مجموعة من الدراسات الإضافية مطلوبة لأخذ وحل هذه المشكلة الصحية. الأهداف: تحديد معدل انتشار عوامل تخثر الدم الوراثية والمكتسبة لنة بالإسقاط المتكرر للحمل في النساء اللاتي تعرضن سابقا لإسقاط حمل مكرر مرتين أو أكثر. هذه دراسة مقارنة أجريت في الفترة من أغسطس 2013 إلى فبراير 2018م في مستشفى محمد علي الشيخ لأمراض النساء والتوليد (مستشفى السعودي) أم درمان، مستشفى الصداقة أم درمان ومستشفى الولادة أم درمان. 100 امرأة حملت تاريخ إسقاط متتابع للحمل مرتين أو أكثر (مرضي) و 51 امرأة لم ت تعرض لإسقاط حمل من قبل (أصحاء). تم اختيارهن لإجراء اختبارات مسحية للكشف عن تخثر الدم الوراثي (عامل 5 ليند و البروثرومبين جين) و تخثر الدم المكتسب (الأضداد من نوع ج و م للفوسفوليبيد و الكارديوليبين). كذلك تم قياس زمن البروثرومبين و زمن البروثرومبين الجزئي و تعداد الصفائح الدموية. درجة جينية متطابقة و متباينة اللاحلقة تعامل التجلط الخمس وجدت في (9%) و (11%) من المرضى المصابين بالإسقاط المتكرر للحمل. ووجدت في (9%) و (3.9%) من الأصحاء على التوالي. معدل b (0.000) على التوالي. كذلك الطفرة الجينية متطابقة و متباينة اللاحلقة للبروثرومبين وجدت في (11%) و (0%) على التوالي في النساء المصابات بالإسقاط المتكرر. ووجدت في (5.8%) و (0%) من الأصحاء معدل b (0.000) و (0.0000) على التوالي. الأضداد للكارديوليبين من نوع ج و م وجدت في (12%) و (7%) من النساء المصابات بالإسقاط المتكرر للحمل. ووجدت في (5.8%) و (3.9%) من الأصحاء معدل b (0.000) و (0.0000) على التوالي. الأضداد للفوسفوليبيد من نوع ج و م وجدت في (10%) و (11%) من النساء المصابات بالإسقاط المتكرر للحمل. ووجدت في (3.9%) و (1.9%) من الأصحاء معدل b (0.000) و (0.0007). على التوالي. زمن البروثرومبين و زمن البروثرومبين الجزئي و نقص الصفائح الدموية تأثرت بصورة واضحة في النساء المصابات بإسقاط الحمل المتكرر. معدل b (0.000) و (0.0000) على التوالي. خاتما أثبتت الدراسة وجود علاقة إحصائية بين الخلل الجيني لعامل التجلط 5 ليند و البروثرومبين والإسقاط المتكرر للحمل. ووجود علاقة إحصائية بين الأضداد للكارديوليبين والأضداد للفوسفوليبيد والإسقاط المتكرر للحمل. زمن البروثرومبين و زمن البروثرومبين الجزئي و تعداد الصفائح الدموية تأثرت بصورة واضحة في النساء المصابات بالإسقاط المتكرر للحمل. يجب إجراء اختبارات مسحية لتخثر الدم للنساء المصابات بالإسقاط المتكرر للحمل.
# Table of Contents

<table>
<thead>
<tr>
<th>Label</th>
<th>Content</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>آية كريمة</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Supervision Committee</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Examination Committee</td>
<td></td>
<td>Ii</td>
</tr>
<tr>
<td>Declaration</td>
<td></td>
<td>Iii</td>
</tr>
<tr>
<td>Dedication</td>
<td></td>
<td>Iv</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td></td>
<td>V</td>
</tr>
<tr>
<td>Abstract in English</td>
<td></td>
<td>Vi</td>
</tr>
<tr>
<td>Abstract in Arabic</td>
<td></td>
<td>Vi</td>
</tr>
<tr>
<td>Table of Contents</td>
<td></td>
<td>Vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td></td>
<td>Vi</td>
</tr>
<tr>
<td>List of figures</td>
<td></td>
<td>Ix</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

## Chapter One

1 Introduction | 1
1.1 Introduction | 1
1.2 Justification | 2
1.3 Objectives | 2
1.3.1 General Objective | 2
1.3.2 Specific Objectives | 2

## Chapter Two

2 Literature Review | 3
<table>
<thead>
<tr>
<th>Section</th>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Causes of Recurrent Miscarriage</td>
<td>3</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Anatomical conditions</td>
<td>3</td>
</tr>
<tr>
<td>2.1.1.1</td>
<td>Uterine conditions</td>
<td>3</td>
</tr>
<tr>
<td>2.1.1.2</td>
<td>Cervical conditions</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Chromosomal disorders</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2.1</td>
<td>Translocations</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2.2</td>
<td>Aneuploidy</td>
<td>4</td>
</tr>
<tr>
<td>2.1.3</td>
<td>Endocrine disorders</td>
<td>4</td>
</tr>
<tr>
<td>2.1.4</td>
<td>Thrombosis (Thrombophilia)</td>
<td>4</td>
</tr>
<tr>
<td>2.1.5</td>
<td>Immune factors</td>
<td>4</td>
</tr>
<tr>
<td>2.1.6</td>
<td>Antiphospholipid syndrome</td>
<td>4</td>
</tr>
<tr>
<td>2.1.7</td>
<td>Thyroid antibodies</td>
<td>5</td>
</tr>
<tr>
<td>2.1.8</td>
<td>Increased uterine NK cells</td>
<td>5</td>
</tr>
<tr>
<td>2.1.9</td>
<td>Parental HLA sharing</td>
<td>5</td>
</tr>
<tr>
<td>2.1.10</td>
<td>Male-specific minor histocompatibility</td>
<td>5</td>
</tr>
<tr>
<td>2.1.11</td>
<td>Ovarian factors</td>
<td>5</td>
</tr>
<tr>
<td>2.1.11.1</td>
<td>Luteal phase defect</td>
<td>5</td>
</tr>
<tr>
<td>2.1.12</td>
<td>Lifestyle factors</td>
<td>6</td>
</tr>
<tr>
<td>2.1.13</td>
<td>Infection</td>
<td>6</td>
</tr>
<tr>
<td>2.1.14</td>
<td>Chronic endometritis</td>
<td>6</td>
</tr>
<tr>
<td>2.1.15</td>
<td>Treatment</td>
<td>6</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>2.1.16</td>
<td>Psychological effects of miscarriage</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Thrombophilia</td>
<td>7</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Signs and symptoms of thrombophilia</td>
<td>9</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Types of thrombophilia</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2.1</td>
<td>Congenital thrombophilia</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2.2</td>
<td>Acquired thrombophilia</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2.3</td>
<td>Combined thrombophilia</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2.4</td>
<td>The ethnic effect of inherited thrombophilia</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.5</td>
<td>Unclear Causes</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.6</td>
<td>Diagnosis</td>
<td>15</td>
</tr>
<tr>
<td>2.2.2.6.1</td>
<td>Screening</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Factor V Leiden thrombophilia</td>
<td>17</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Pathophysiology of factor V Leiden</td>
<td>18</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Diagnosis of factor V Leiden</td>
<td>19</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Epidemiology of factor V Leiden</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>Prothrombin G20210A thrombophilia</td>
<td>20</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Signs and symptoms</td>
<td>21</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Terminology</td>
<td>22</td>
</tr>
<tr>
<td>2.5</td>
<td>Antiphospholipid Syndrome and Pregnancy</td>
<td>23</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Obstetric and non obstetric clinical features</td>
<td>24</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Pathophysiology</td>
<td>25</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Etiology</td>
<td>26</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Phospholipid release</td>
<td>26</td>
</tr>
<tr>
<td>2.5.5</td>
<td>Epitope mimicry in autoimmune disease</td>
<td>26</td>
</tr>
<tr>
<td>2.5.6</td>
<td>Epidemiology</td>
<td>27</td>
</tr>
<tr>
<td>2.5.6.1</td>
<td>Occurrence in the United States</td>
<td>27</td>
</tr>
<tr>
<td>2.5.6.2</td>
<td>Sex- and age-related demographics</td>
<td>27</td>
</tr>
<tr>
<td>2.5.7</td>
<td>Prognosis</td>
<td>27</td>
</tr>
<tr>
<td>2.5.8</td>
<td>Maternal morbidity</td>
<td>28</td>
</tr>
<tr>
<td>2.5.9</td>
<td>Maternal mortality</td>
<td>29</td>
</tr>
<tr>
<td>2.5.9.1</td>
<td>Prenatal morbidity</td>
<td>29</td>
</tr>
<tr>
<td>2.5.9.2</td>
<td>Prenatal mortality</td>
<td>29</td>
</tr>
<tr>
<td>2.5.10</td>
<td>Clinical and laboratory criteria</td>
<td>29</td>
</tr>
<tr>
<td>2.5.11</td>
<td>Laboratory criteria</td>
<td>30</td>
</tr>
<tr>
<td>2.5.12</td>
<td>Additional findings in APS</td>
<td>30</td>
</tr>
<tr>
<td>2.6</td>
<td>Previous studies</td>
<td>31</td>
</tr>
</tbody>
</table>

**Chapter Three**

<p>| 3     | Materials and Methods     | 36 |
| 3.1   | Study Site                | 36 |
| 3.1.1 | Study Design              | 36 |
| 3.1.2 | Study Population and sample size | 36 |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Subsection</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>Materials and/or Methods</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Ethical clearance</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Samples Collection</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>3.2.2.1</td>
<td>Venous blood</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>3.2.2.2</td>
<td>Sample Storage</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Data Collection</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Data analysis</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Laboratory Tests</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1</td>
<td>Hematological Investigations</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1.1</td>
<td>Platelet counts (PC).</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1.2</td>
<td>Prothrombin Time (PT).</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1.3</td>
<td>Activated Partial Thromboplastin Time (APTT)</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.2</td>
<td>Serological investigations</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.2.1</td>
<td>Anti-cardiolipin antibodies (IgG/IgM) ELISA</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.2.2</td>
<td>Anti-phospholipids antibodies (IgG/IgM) ELISA</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.3</td>
<td>Molecular Investigations</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.3.1</td>
<td>DNA Extraction from Blood</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.3.2</td>
<td>Prothrombin Gene Mutation (FII) PCR</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.3.3</td>
<td>Factor V Leiden Gene Mutation (FV) PCR</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1</td>
<td>Initial Hematological Tests</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1.1</td>
<td>Sysmex</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>Section</td>
<td>Subsection</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>3.2.5.1.1.1</td>
<td>Principle</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>3.2.5.1.2</td>
<td>Platelets Analysis Flow in Whole Blood</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>3.2.5.1.3</td>
<td>Coagulation Tests</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>3.2.5.1.3.1</td>
<td>Dia Med - CD4</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>3.2.5.1.3.1.1</td>
<td>Principle</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>3.2.5.1.3.2</td>
<td>Prothrombin Time (PT)</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>3.2.5.1.3.2.1</td>
<td>Principle</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>3.2.5.1.3.2.2</td>
<td>Test procedure</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>3.2.5.1.3.3</td>
<td>Activated Partial Thromboplastin Time (APTT)</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>3.2.5.1.3.3.1</td>
<td>Principle</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>3.2.5.1.3.3.2</td>
<td>Procedure</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>3.2.5.2</td>
<td>Serological investigations</td>
<td></td>
<td>41</td>
</tr>
<tr>
<td>3.2.5.2.1</td>
<td>Anti Cardiolipin Antibodies IgG ELISA</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>3.2.5.2.1.1</td>
<td>Principle</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>3.2.5.2.1.2</td>
<td>Procedure</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>3.2.5.2.2</td>
<td>Anti Cardiolipin Antibodies IgM ELISA</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>3.2.5.2.2.1</td>
<td>Principle</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>3.2.5.2.2.2</td>
<td>Procedure</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>3.2.5.2.3</td>
<td>Anti Phosphatidylserine Antibodies IgG ELISA</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>3.2.5.2.3.1</td>
<td>Principle</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>3.2.5.2.3.2</td>
<td>Procedure</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>3.2.5.2.4</td>
<td>Anti-Phosphatidylserine antibodies IgM ELISA</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>3.2.5.2.4.1</td>
<td>Principle</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>3.2.5.2.4.2</td>
<td>Procedure</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3</td>
<td>Molecular Investigations</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3.1</td>
<td>DNA extraction from blood.</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3.1.1</td>
<td>Procedure</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3.2</td>
<td>Polymerase Chain Reaction (PCR)</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3.2.1</td>
<td>Description</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>3.2.5.3.2.2</td>
<td>Procedure</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter Four</strong></td>
<td></td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Results and Discussion</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Results</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>4.1.1</td>
<td>Demographical data</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>4.1.1.1</td>
<td>Age distribution of patients</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>4.1.2</td>
<td>Clinical data</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4.1.2.1</td>
<td>Times of recurrent abortion</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4.1.3</td>
<td>Laboratory results</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4.1.3.1</td>
<td>FV Leiden gene mutation</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4.1.3.2</td>
<td>Prothrombin gene mutation</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>4.1.3.3</td>
<td>Combined Thrombophilia</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>4.1.3.4</td>
<td>Antiphospholipid antibodies</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>4.1.3.5</td>
<td>Anticardiolipin antibodies</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>4.1.3.6</td>
<td>Basic coagulation screening</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>4.1.3.7</td>
<td>Platelet counts</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Discussion</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

**Chapter Five**

| 5 | Conclusion and Recommendations | 70 |
| 5.1 | Conclusion | 70 |
| 5.2 | Recommendations | 71 |
| References | 72 |
| Appendices | 83 |
| Appendix 1 | 83 |
| Appendix 2 | 85 |
| Appendix 3 | 86 |
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distribution of factor V Leiden in Women with recurrent miscarriage and control Women.</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>Distribution of Prothrombin G20210A in Women with recurrent miscarriage and control Women.</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Distribution of Antiphospholipid Antibodies (IgG) in Women with recurrent miscarriage and control Women</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>Distribution of Antiphospholipid Antibodies (IgM) in Women with recurrent miscarriage and control Women</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>Distribution of Anticardiolipin Antibodies (IgG) in Women with recurrent miscarriage and control Women</td>
<td>57</td>
</tr>
<tr>
<td>6</td>
<td>Distribution of Anticardiolipin Antibodies (IgM) in Women with recurrent miscarriage and control Women</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>Distribution of PT, APTT and Platelet counts in Women with recurrent miscarriage and control Women</td>
<td>57</td>
</tr>
<tr>
<td>8</td>
<td>Distribution of abortion by trimester in Women with recurrent miscarriage.</td>
<td>58</td>
</tr>
<tr>
<td>9</td>
<td>PCR primers used in genotyping the polymorphisms.</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>Combined factor V Leiden and prothrombin gene mutation in Women with recurrent miscarriage.</td>
<td>59</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The coagulation system, often described as a &quot;cascade&quot;,</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>consists of a group of proteins that interact in the</td>
<td></td>
</tr>
<tr>
<td></td>
<td>formation of a fibrin-rich clot</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Consanguinity of patients and their couples</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>Number of recurrent abortion in patients</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>Consanguinity of patients and their couples</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>Times of recurrent abortion in patients</td>
<td>80</td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>Anti Cardiolipin Antibodies</td>
</tr>
<tr>
<td>ADH</td>
<td>Anti Diuretic Hormones</td>
</tr>
<tr>
<td>APA</td>
<td>Anti Phospholipids Antibodies</td>
</tr>
<tr>
<td>APTT</td>
<td>Active Partial Tissue Thromboplastin</td>
</tr>
<tr>
<td>BT</td>
<td>Bleeding Time</td>
</tr>
<tr>
<td>btw</td>
<td>Between</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous Ambulatory Protenium Dialysis</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Conc</td>
<td>Concentration</td>
</tr>
<tr>
<td>D.W</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>Fig</td>
<td>Figure</td>
</tr>
<tr>
<td>FII</td>
<td>Factor II</td>
</tr>
<tr>
<td>FVL</td>
<td>Factor Five Leiden</td>
</tr>
<tr>
<td>g/dl</td>
<td>Gram per Deciliter</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>IVD</td>
<td>In vitro Diagnosis</td>
</tr>
<tr>
<td>mEq</td>
<td>Mill equivalent</td>
</tr>
<tr>
<td>mg/dl</td>
<td>Milligram per Deciliter</td>
</tr>
<tr>
<td>mmol/l</td>
<td>Mill mole/Liter</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PC</td>
<td>Para Thyroid Hormones</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelet</td>
</tr>
<tr>
<td>PLT/IgG</td>
<td>Platelet Associated IgG</td>
</tr>
<tr>
<td>PC</td>
<td>Platelet Counts</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin Time</td>
</tr>
<tr>
<td>PTH</td>
<td>Para Thyroid Hormones</td>
</tr>
<tr>
<td>R 1</td>
<td>Reagent 1</td>
</tr>
<tr>
<td>R 2</td>
<td>Reagent 2</td>
</tr>
<tr>
<td>R 3</td>
<td>Reagent 3</td>
</tr>
<tr>
<td>RcoF</td>
<td>Ristocetin co-Factor</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restricted Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RPL</td>
<td>Recurrent pregnancy loss</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Sig</td>
<td>Significant</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Science</td>
</tr>
<tr>
<td>STD</td>
<td>Standard</td>
</tr>
<tr>
<td>TC</td>
<td>Treated conservatively</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>ACL</td>
<td>Anticardiolipin</td>
</tr>
<tr>
<td>APL</td>
<td>Antiphospholipid</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid Syndrome</td>
</tr>
<tr>
<td>LAC</td>
<td>Lupus Anticoagulants</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous Thromboembolism</td>
</tr>
</tbody>
</table>
Chapter One

1. Introduction

1.1. Introduction:

Recurrent pregnancy loss (RPL), either early or late in the gestational period is a serious problem and has both psychological and social impacts on the women who suffer from it. In some cases, it may lead to divorce or other social problems. Miscarriage is common, with most studies showing the incidence of this complication occurring before 20 weeks’ gestation varies between 8–20%, with 80% of these occurring in the first 12 weeks of pregnancy. (Regan L, et al, 2000) The real rate of miscarriage may be much higher than reported, since many women miscarry before realizing that they are pregnant. In one study, human chorionic gonadotrophin was checked daily from the expected time of ovulation until the next menstrual period in order to detect a pregnancy as early as possible; this yielded a miscarriage rate of 31%. (Wilcox AJ, et al, 1988) Recurrent pregnancy loss is defined as 2 or more spontaneous abortions before the 20th week of gestation, which affects approximately 5% of women of reproductive age (Regan L, et al, 2000), Habitual or recurrent miscarriage (RM) is defined as the loss of three or more consecutive and clinically-recognized pregnancies before 20 weeks’ gestation; this affects 1–2% of women. (Saravelos SH, et al, 2012) This incidence increases to 5% when it is defined as a loss of two or more clinically recognized pregnancies before 20 weeks’ gestation. (Hogge WA, et al, 2003) RPL may be classified as early (losses at or before 20 weeks’ gestation) or late (losses after 20 weeks’ gestation). Patients may be classified as suffering from primary RPL when they have never had a live birth or from secondary RPL when they have had recurrent losses following a successful pregnancy. RPL has now been deemed a major cause of female infertility. (Sarig G, et al, 2002) Thrombophilia is a common cause of RPL and may be seen in 40–50% of cases. (Brenner B, et al, 1999) (Visser J, et al, 2011) Pregnancy is a hypercoagulable state and if the pregnancy is affected by thrombophilia, the hypercoagulable state becomes worse and may impair blood flow through the maternal veins, leading to deep vein thrombosis, and clots in the placental blood vessels, leading to fetal growth restriction and/or fetal demise. (Colman-Brochu S, et al, 2004) (Regan L, et al, 2000) Due to this fact, anticoagulants have become very popular for treating
RPL. The aim of this review is to find the strength of the association between thrombophilia and RPL. Thrombophilia may be hereditary or acquired.

1.2 Justification

RPL is a very common event in obstetric practice. Thrombophilia in its acquired and hereditary forms is considered one of the most important causes of such losses. In Sudan, no much attention is given to this point and the majority of the affected women are not properly investigated to rule out thrombophilia. Detection of acquired or inherited factors as causes of pregnancy loss is extremely important since with proper management the risk of pregnancy loss can be eliminated.

1.3 Objectives

1.3.1 General Objective

To study the effect of acquired and hereditary thrombophilia on recurrent pregnancy loss in Sudanese women.

1.3.2 Specific Objectives:

- To determine the polymorphism of factor V Leiden G1691A gene mutation in Sudanese women with recurrent pregnancy loss.

- To determine the polymorphism of prothrombin G20210A gene mutation in Sudanese women with recurrent pregnancy loss.

- To evaluate the platelet counts and hypercoagulability state in Sudanese women with recurrent pregnancy loss.

- To determine the frequency of anticardiolipin antibodies in Sudanese women with recurrent pregnancy loss.

- To determine the frequency of anti-phosphatidyl serine antibodies in Sudanese women with recurrent pregnancy loss.
Chapter Two

2. Literature Review

2.1. Causes of Recurrent Miscarriage:

There are various causes for recurrent miscarriage, and some are treatable. Some couples never have a cause identified, often after extensive investigations. (Van D B, et al, 2011) About 50-75% of cases of Recurrent Miscarriage are unexplained. (Rodger MA, et al, 2008)

2.1.1. Anatomical conditions:

The anatomical conditions associated with recurrent miscarriage include

2.1.1.1. Uterine conditions:

A uterine malformation is considered to cause about 15% of recurrent miscarriages. The most common abnormality is a uterine septum, a partition of the uterine cavity. MRI or a combined laparoscopy hysteroscopy of the uterus makes the diagnosis. In addition, uterine leiomyomata could result in pregnancy loss.

2.1.1.2. Cervical conditions:

In the second trimester, a weak cervix can become a recurrent problem. Such cervical incompetence leads to premature pregnancy loss resulting in miscarriages or preterm deliveries.

2.1.2. Chromosomal disorders:

Chromosomal disorders divided into

2.1.2.1. Translocations:

A balanced translocation or Robertsonian translocation in one of the partners leads to unviable fetuses that are miscarried. This explains why a karyogram is often performed in both partners if a woman has experienced repeated miscarriages. About 3% of the time a chromosomal problem of one or both partners can lead to recurrent pregnancy loss. Although patients with such a chromosomal problem are more likely to miscarry, they may also deliver normal or abnormal babies.
2.1.2.2. Aneuploidy:

Aneuploidy may be a cause of a random spontaneous as well as recurrent pregnancy loss. (Christiansen O, et al, 2014) Aneuploidy is more common with advanced reproductive age reflecting decreased germ cell quality.

2.1.3. Endocrine disorders:

Women with hypothyroidism are at increased risk for pregnancy losses. Unrecognized or poorly treated diabetes mellitus leads to increased miscarriages. Women with polycystic ovary syndrome also have higher loss rates possibly related to hyperactive insulinemia or excess androgens. Inadequate production of progesterone in the luteal phase may set the stage for RPL.

2.1.4. Thrombophilia:

An important example is the possible increased risk of miscarriage in women with thrombophilia (propensity for blood clots). The most common problem is the factor V Leiden and prothrombin G20210A mutation. (Christiansen O, et al, 2014) Some preliminary studies suggest that anticoagulant medication may improve the chances of carrying pregnancy to term but these studies need to be confirmed before they are adopted in clinical practice. (Nielsen, H S, et al, 2011) Note that many women with thrombophilia go through one or more pregnancies with no difficulties, while others may have pregnancy complications. Thrombophilia may explain up to 15% of recurrent miscarriages.

2.1.5. Immune factors:

A common feature of immune factors in causing recurrent pregnancy loss appears to be a decreased maternal immune tolerance towards the fetus. (Cicinelli E, et al, 2014)

2.1.6. Antiphospholipid syndrome:

The antiphospholipid syndrome is an autoimmune disease that is a common cause of recurrent pregnancy loss. (Empson, M, et al, 2005) (Christiansen O, et al, 2014) Around 15% of the women who have recurrent miscarriages have high levels of antiphospholipid antibodies. Women who have had more than one miscarriage in the first trimester, or a miscarriage in the second trimester, may have their blood tested for antibodies, to determine if they have antiphospholipid syndrome. (Empson, M, et al, 2005) Women diagnosed with antiphospholid syndrome generally take aspirin or
heparin in subsequent pregnancies, but questions remain due to the lack of high quality trials. (De Jong, P. G, et al, 2013)

2.1.7. Thyroid antibodies:  
Anti-thyroid autoantibodies are associated with an increased risk of recurrent miscarriage with an odds ratio of 2.3 with a 95% confidence interval of 1.5–3.5. (Regan L, et al, 2000)

2.1.8. Increased uterine NK cells:  
Natural Killer Cells, a type of white blood cell, are present in uterine tissue. High levels of these cells may be linked to RPL but high numbers or the presence of these cells is not a predictor of pregnancy loss in women who have not have had a miscarriage. (Oliver-Williams, C. T, et al, 2013)

2.1.9. Parental HLA sharing:  
Earlier studies that perhaps paternal sharing of HLA genes would be associated with increased pregnancy loss have not been confirmed.

2.1.10. Male-specific minor histocompatibility:  
Immunization of mothers against male-specific minor histocompatibility (H-Y) antigens has a pathogenic role in many cases of secondary recurrent miscarriage, that is, recurrent miscarriage in pregnancies succeeding a previous live birth. An example of this effect is that the male: female ratio of children born prior and subsequent to secondary recurrent miscarriage is 1.49 and 0.76 respectively. (Trogstad, L, et al, 2009)

2.1.11. Ovarian factors:  
These include

2.1.11.1. Luteal phase defect:  
The issue of a luteal phase defect is complex. The theory behind the concept suggests that an inadequate amount of progesterone is produced by the corpus luteum to maintain the early pregnancy. Assessment of this situation was traditionally carried out by an endometrial biopsy, however recent studies have not confirmed that such assessment is valid. (Christiansen O, et al, 2014) Studies about the value of progesterone supplementation remain deficient; however, such supplementation is commonly carried out on an empirical basis.
2.1.12. Lifestyle factors:

While lifestyle factors have been associated with increased risk for miscarriage in general, and are usually not listed as specific causes for RPL, every effort should be made to address these issues in patients with RPL. Of specific concern are chronic exposures to toxins including smoking, alcohol, and drugs. (Christiansen O, et al, 2014)

2.1.13. Infection:

A number of maternal infections can lead to a single pregnancy loss, including listeriosis, toxoplasmosis, and certain viral infections (rubella, herpes simplex, measles, cytomegalovirus, coxsackie virus). However, there are no confirmed studies to suggest that specific infections will lead to recurrent pregnancy loss in humans. Malaria, syphilis, and brucellosis can also cause recurrent miscarriage. (Christiansen O, et al, 2014)

2.1.14. Chronic endometritis:

Chronic Endometritis (CE) due to common bacteria has been found to be prevalent in some women with a history of recurrent miscarriage. One study found that 71 percent of women who tested positive for this condition were successfully treated by an antibiogram-based antibiotic treatment. 78.4 percent of these women subsequently became pregnant in the year following treatment. The study concludes that "CE is frequent in women with recurrent miscarriages," and that "antibiotic treatment seems to be associated with an improved reproductive outcome." The authors also conclude, "that hysteroscopy should be a part of the diagnostic workup of infertile women complaining of unexplained recurrent miscarriage." (Wong, LF, et al, 2014)

2.1.15. Treatment:

If the likely cause of recurrent pregnancy loss can be, determined treatment is to be directed accordingly. In pregnant women with a history of recurrent miscarriage, anticoagulants seem to increase the live birth rate among those with antiphospholipid syndrome and perhaps those with congenital thrombophilia but not in those with unexplained recurrent miscarriage. (Oliver-Williams, et al, 2013) One study found that in many women with chronic endometritis, "fertility was restored after appropriate antibiotic treatment."
There are currently no treatments for women with unexplained recurrent pregnancy loss. The majority of patients are counseled to try to conceive again, and chances are about 60% that the next pregnancy is successful without treatment. However, each additional loss worsens the prognostic for a successful pregnancy and increases the psychological and physical risks to the mother. Aspirin has no effect in preventing recurrent miscarriage in women with unexplained recurrent pregnancy loss. (Baumann K, et al., 2013) Immunotherapy has not been found to help. There is currently one drug in development, NT100, which is in clinical trials for the treatment of unexplained recurrent miscarriage. The study investigates the role of NT100 in improving maternal-fetal tolerance for women with unexplained recurrent miscarriage. (Trogstad, L, et al., 2009)

In certain chromosomal situations, while treatment may not be available, in vitro fertilization with preimplantation genetic diagnosis may be able to identify embryos with a reduced risk of another pregnancy loss, which then would be transferred. However, in vitro fertilization does not improve maternal-fetal tolerance imbalances.

Close surveillance during pregnancy is generally recommended for pregnant patients with a history of recurrent pregnancy loss. Even with appropriate and correct treatment, another pregnancy loss may occur as each pregnancy develops its own risks and problems.

2.1.16. Psychological effects of miscarriage:

There is significant, and often unrecognized, psychological and psychiatric trauma for the mother – for many, miscarriage represents the loss of a future child, of motherhood, and engenders doubts regarding her ability to procreate. (Borissoff JI, et al., 2011) Studies have shown that a significant percentage of women experience grief, depression, and anxiety, and that there is an increased risk of major depressive disorder following a miscarriage. The psychological effects can persist for 6 months to 3 years and tend to deepen with additional miscarriages.

2.2. Thrombophilia:

An increased tendency to form internal blood clots because of a genetic or immune system abnormality. The clots might occur in veins or arteries, posing a risk to the person's health. A person can have a thrombophilia-related disorder with no outward
symptoms. Many doctors test for these disorders as a part of a recurrent miscarriage testing workup. Thrombophilia is associated with miscarriage and later pregnancy loss, because women with these disorders may form small blood clots that cause problems with the placenta or the umbilical cord. Antiphospholipid Syndrome is a common cause of recurrent miscarriages. Genetic, or hereditary thrombophilias, are associated with greater risk of stillbirth. The most common of these conditions is Factor V Leiden.

Thrombophilia is a term, which describes the increased tendency of excessive blood clotting. It is a normal phenomenon during pregnancy, where there is an increase in most clotting factors, such as factor VIII, Von Willebrand factor, platelets, fibrinogen, and factor VII. During pregnancy, there is also an increase in prothrombin fragment 1 + 2 and D-dimer. (Hathaway, WE, et al, 1993)(De Boer K, et al, 1989)

When investigating patients with RPL, it is very important to exclude other possible causes of the losses, such as uterine malformation; diabetes mellitus; connective tissue diseases such as systemic lupus erythematosus (SLE); chromosomal abnormalities, and thyroid dysfunction. (Castori M, et al, 2013)(Brenner B, et al, 1999)

Thrombophilia is associated with the high risk of fetal loss in early and late pregnancy. It is either inherited, acquired or a combination of both.

Inherited thrombophilia is the leading cause of maternal thromboembolism and are associated with an increased risk of certain adverse recurrent miscarriage including second- and third-trimester fetal loss, abruptions, and severe intrauterine growth restriction, and early onset, severe preeclampsia. (Kyrle PA, et al, 2010) Inherited thrombophilia are the leading cause of maternal thrombo embolism and are associated with an increased risk of certain adverse recurrent miscarriage including second and third trimester fetal loss, abruptions, severe intrauterine growth restriction, and early-onset, severe preeclampsia. (Bertina RM, et al, 1994) Current information suggests that all patients with a history of prior venous thrombotic events and those with these characteristic adverse pregnancy events should be evaluated for thrombophilia Current information suggests that all patients with a history of prior venous thrombotic events and those with these characteristic adverse pregnancy events should be evaluated for thrombophilia. The most common inherited thrombophilic disorders are deficiencies of antithrombin III, protein C and protein S, Factor V Leiden mutation, methylene
tetrahydrofolate reductase (MTHFR) and prothrombin gene mutation (G20210A). (Kyrle PA, et al, 2010)

2.2.1. Signs and symptoms of thrombophilia:

The most common conditions associated with thrombophilia are deep vein thrombosis (DVT) and pulmonary embolism (PE), which are referred to collectively as venous thromboembolism (VTE). DVT usually occurs in the legs, and is characterized by pain, swelling, and redness of the limb. It may lead to long-term swelling and heaviness due to damage to valves in the veins.(Scarvelis D, et al, 2006) The clot may also break off and migrate (embolize) to arteries in the lungs. Depending on the size and the location of the clot, this may lead to sudden-onset shortness of breath, chest pain, palpitations and may be complicated by collapse, shock and cardiac arrest.(Heit JA, et al, 2007) (Agnelli G, et al, 2010)

Venous thrombosis may also occur in more unusual places: in the veins of the brain, liver (portal vein thrombosis and hepatic vein thrombosis), mesenteric vein, kidney (renal vein thrombosis) and the veins of the arms.(Heit JA, et al, 2007) Whether thrombophilia also increases the risk of arterial thrombosis (which is the underlying cause of heart attacks and strokes) is less well established. (Heit JA, et al, 2007) (Middeldorp S, et al, 2008) (De Moerloose P, et al, 2007)

Thrombophilia has been linked to recurrent miscarriage, (Rai R, et al, 2006) and possibly various complications of pregnancy such as intrauterine growth restriction, stillbirth, severe pre-eclampsia and abruptio placenta. (Heit JA, et al, 2007)

Protein C deficiency may cause purpura fulminans, a severe clotting disorder in the newborn that leads to both tissue death and bleeding into the skin and other organs. The condition has also been described in adults. Protein C and protein S deficiency have also been associated with an increased risk of skin necrosis on commencing anticoagulant treatment with warfarin or related drugs.(Heit JA, et al, 2007) (Baglin T, et al, 2010)
2.2.2. Types of thrombophilia:

Thrombophilia can be congenital or acquired. Congenital thrombophilia refers to inborn conditions (and usually hereditary, in which case "hereditary thrombophilia" may be used) that increase the tendency to develop thrombosis, while, on the other hand, acquired thrombophilia refers to conditions that arise later in life.

2.2.2.1. Congenital thrombophilia:

The most common types of congenital thrombophilia are those that arise because of over activity of coagulation factors. They are relatively mild, and are therefore classified as "type II" defects. (Crowther MA, et al, 2003) The most common ones are factor V Leiden (a mutation in the F5 gene at position 1691) and prothrombin G20210A, a mutation in prothrombin (at position 20210 in the 3' untranslated region of the gene). (Finan RR, et al, 2002) (Azem F, et al, 2004)


It is unclear whether congenital disorders of fibrinolysis (the system that destroys clots) are major contributors to thrombosis risk. (Crowther MA, et al, 2003) Congenital deficiency of plasminogen, for instance, mainly causes eye symptoms and sometimes problems in other organs, but the link with thrombosis has been more uncertain. (Mehta R, et al, 2008)

Blood group determines thrombosis risk to a significant extent. Those with blood groups other than type O are at a two to four-fold relative risk. O blood group is associated with reduced levels of Von Willebrand factor because of increased clearance and factor VIII, which is related to thrombotic risk. (Rosendaal FR, et al, 2009)

2.2.2.2. Acquired thrombophilia:

A number of acquired conditions augment the risk of thrombosis. A prominent example is antiphospholipid syndrome. (Mitchell RS, et al, 2007) (Rosendaal FR, et al, 2005) which is caused by antibodies against constituents of the cell membrane,
particularly lupus anticoagulant (first found in people with the disease systemic lupus erythrematosus but often detected in people without the disease), anti-cardiolipin antibodies, and anti-β2-glycoprotein-1 antibodies; it is therefore regarded as an autoimmune disease. In some cases, antiphospholipid syndrome can cause arterial as well as venous thrombosis. It is also more strongly associated with miscarriage, and can cause a number of other symptoms (such as live do reticularis of the skin and migraine). (Ruiz-Irastorza G, et al, 2010)

Heparin-induced thrombocytopenia (HIT) is due to an immune system reaction against the anticoagulant drug heparin (or its derivatives). (Mitchell RS, et al, 2007) Though it is named for associated low platelet counts, HIT is strongly associated with risk of venous and arterial thrombosis. (Keeling D, et al, 2006) Paroxysmal nocturnal hemoglobinuria (PNH) is a rare condition resulting from acquired alterations in the PIGA gene, which plays a role in the protection of blood cells from the complement system. PNH increases the risk of venous thrombosis but is also associated with hemolytic anemia (anemia resulting from destruction of red blood cells). (Regan L, et al, 2000) Both HIT and PNH require particular treatment. (Keeling D, et al, 2006) (Brodsky RA, et al, 2008)

Hematologic conditions associated with sluggish blood flow can increase risk for thrombosis. For example, sickle-cell disease (caused by mutations of hemoglobin) is regarded as a mild prothrombotic state induced by impaired flow. (Mitchell RS, et al, 2007) Similarly, myeloproliferative disorders, in which the bone marrow produces too many blood cells, predispose to thrombosis, particularly in polycythemia vera (excess red blood cells) and essential thrombocytosis (excess platelets). Again, these conditions usually warrant specific treatment when identified. (Papadakis E, et al, 2010)

Cancer, particularly when metastatic (spread to other places in the body), is a recognized risk factor for thrombosis. (Heit JA, et al, 2007) (Rosendaal FR, et al, 2005) A number of mechanisms have been proposed, such as activation of the coagulation system by cancer cells or secretion of procoagulant substances. Furthermore, particular cancer treatments (such as the use of central venous catheters for chemotherapy) may increase the risk of thrombosis further. (Prandoni P, et al, 2005)
Nephrotic syndrome, in which protein from the bloodstream is released into the urine due to kidney diseases, can predispose to thrombosis; (Mitchell RS, et al, 2007) this is particularly the case in more severe cases (as indicated by blood levels of albumin below 25 g/l) and if the syndrome is caused by the condition membranous nephropathy. (Hull RP, et al, 2008) Inflammatory bowel disease (ulcerative colitis and Crohn's disease) predispose to thrombosis, particularly when the disease is active. Various mechanisms have been proposed. (Heit JA, et al, 2007) (Preston FE, et al, 1996)

Pregnancy is associated with an increased risk of thrombosis. This probably results from a physiological hypercoagulability in pregnancy that protects against postpartum hemorrhage. (Bourjeily G, et al, 2010)

The female hormone estrogen, when used in the combined oral contraceptive pill and in premenopausal hormone replacement therapy, has been associated with a two to six fold increased risk of venous thrombosis. The risk depends on the type of hormones used, the dose of estrogen, and the presence of other thrombophilic risk factors. (Crowther MA, et al, 2003) Various mechanisms, such as deficiency of protein S and tissue factor pathway inhibitor, are said to be responsible. (Trogstad, L, et al, 2009)

Obesity has long been regarded as a risk factor for venous thrombosis. It more than doubles the risk in numerous studies, particularly in combination with the use of oral contraceptives or in the period after surgery. Various coagulation abnormalities have been described in the obese. Plasminogen activator inhibitor-1, an inhibitor of fibrinolysis, is present in higher levels in people with obesity. Obese people also have larger numbers of circulating micro vesicles (fragments of damaged cells) that bear tissue factor. Platelet aggregation may be increased, and there are higher levels of coagulation proteins such as von Willebrand factor, fibrinogen, factor VII and factor VIII. Obesity also increases the risk of recurrence after an initial episode of thrombosis. (Stein PD, et al, 2009)

2.2.2.3. Combined Thrombophilia:

Combined thrombophilia (which is either a combination of acquired and IT, or a combination of more than one inherited thrombophilic gene defect) has been identified by several researchers as a cause of both early and late RPL; however, the frequency of combined thrombophilia is not clear. (Papadakis E, et al, 2010) (Ivanov P, et al, 2011)
2.2.1.4. The ethnic effect of inherited thrombophilia:

In a study of the effect of ethnicity on RM and IT, Baumann et al. found that in a uniform ethnic group the prevalence of various congenital thrombophilic markers did not differ. Thus, when investigating a multi-ethnic cohort of women, the prevalence of hereditary thrombophilia may differ because the basic prevalence in different ethnic groups varies. (Baumann K, et al, 2013) The prevalence of thrombophilia in the general population varies from 1.1% in Lebanon to 2.5% in India. (Hoteit R, et al, 2012)

2.2.2.5. Unclear Causes:

A number of conditions that have been linked with venous thrombosis are possibly genetic and possibly acquired. (Rosendaal FR, et al, 2005) These include: elevated levels of factor VIII, factor IX, factor XI, fibrinogen and thrombin-activatable fibrinolysis inhibitor, and decreased levels of tissue factor pathway inhibitor. Activated protein C resistance that is not attributable to factor V mutations is probably caused by other factors and remains a risk factor for thrombosis. (Rosendaal FR, et al, 2005)

There is an association between the blood levels of homocysteine and thrombosis, (Rodger MA, et al, 2008) although this has not been reported consistently in all studies. (Rosendaal FR, et al, 2009) Homocysteine levels are determined by mutations in the MTHFR and CBS genes, but also by levels of folic acid, vitamin B6 and vitamin B12, which depend on diet. (Crowther MA, et al, 2003)

Fig. 1:
The coagulation system, often described as a "cascade", consists of a group of proteins that interact in the formation of a fibrin-rich clot.
Thrombosis is a multifactorial problem because there are often multiple reasons why a person might develop thrombosis. These risk factors may include any combination of abnormalities in the blood vessel wall, abnormalities in the blood flow (as in immobilization), and abnormalities in the consistency of the blood. Thrombophilia is caused by abnormalities in blood consistency, which is determined by the levels of coagulation factors and other circulating blood proteins that participate in the "coagulation cascade". (Rosendaal FR, et al, 2005)

Normal coagulation is initiated by the release of tissue factor from damaged tissue. Tissue factor binds to circulating factor VIIa. The combination activates factor X to factor Xa and factor IX to factor IXa. Factor Xa (in the presence of factor V) activates prothrombin into thrombin. Thrombin is a central enzyme in the coagulation process: it generates fibrin from fibrinogen, and activates a number of other enzymes and cofactors (factor XIII, factor XI, factor V and factor VIII, TAFI) that enhance the fibrin clot. (Baglin T, et al, 2012) The process is inhibited by TFPI (which inactivates the first step catalyzed by factor VIIa/tissue factor), antithrombin (which inactivates thrombin, factor IXa, Xa and XIa), protein C (which inhibits factors Va and VIIIa in the presence of protein S), and protein Z (which inhibits factor Xa). (Crowther MA, et al, 2003)

In thrombophilia, the balance between "procoagulant" and "anticoagulant" activity is disturbed. The severity of the imbalance determines the likelihood that someone develops thrombosis. Even small perturbances of proteins, such as the reduction of antithrombin to only 70–80% of the normal level, can increase the thrombosis risk; this is in contrast with hemophilia, which only arises if levels of coagulation factors are markedly decreased. (Crowther MA, et al, 2003)

In addition to its effects on thrombosis, hypercoagulable states may accelerate the development of atherosclerosis, the arterial disease that underlies myocardial infarction and other forms of cardiovascular disease. (Borissoff JI, et al, 2009) (Borissoff JI, et al, 2011)
2.2.2.6. Diagnosis:

Tests for thrombophilia include complete blood count (with examination of the blood film), prothrombin time, partial thromboplastin time, thrombodynamics test, thrombin time and reptilase time, lupus anticoagulant, anti-cardiolipin antibody, anti-β2 glycoprotein 1 antibody, activated protein C resistance, fibrinogen tests, factor V Leiden and prothrombin mutation, and basal homocysteine levels. (Heit JA, et al, 2007) Testing may be more or less extensive depending on clinical judgment and abnormalities detected on initial evaluation. (Heit JA, et al, 2007)

2.2.2.6.1. Screening:

There are divergent views as to whether everyone with an unprovoked episode of thrombosis should be investigated for thrombophilia. Even those with a form of thrombophilia may not necessarily be at risk of further thrombosis, while recurrent thrombosis is more likely in those who have had previous thrombosis even in those who have no detectable thrombophilic abnormalities. (Middeldorp S, et al, 2008) (Baglin T, et al, 2010) Recurrent thromboembolism, or thrombosis in unusual sites (e.g. the hepatic vein in Budd-Chiari syndrome), is a generally accepted indication for screening. It is more likely to be cost-effective in people with a strong personal or family history of thrombosis. (Wu O, et al, 2005) In contrast, the combination of thrombophilia with other risk factors may provide an indication for preventative treatment, which is why thrombophilia testing may be performed even in those who would not meet the strict criteria for these tests. (Dalen JE, et al, 2008) Searching for a coagulation abnormality is not normally undertaken in patients in whom thrombosis has an obvious trigger. For example, if the thrombosis is due to immobilization after recent orthopedic surgery, it is regarded as "provoked" by the immobilization and the surgery and it is less likely that investigations will yield clinically important results. (Mitchell RS, et al, 2007) (Dalen JE, et al, 2008)

When venous thromboembolism occurs when a patient is experiencing transient major risk factors such as prolonged immobility, surgery, or trauma, testing for thrombophilia is not appropriate because the outcome of the test would not change a patient's indicated treatment. (Chong, L, et al, 2013) (Baglin, T, et al, 2010) In 2013, the American Society of Hematology, as part of recommendations in the Choosing Wisely campaign, cautioned against overuse of thrombophilia screening; false
positive results of testing would lead to people inappropriately being labeled as having thrombophilia, and being treated with anticoagulants without clinical need (Chong, L., et al., 2013)

In the United Kingdom, professional guidelines give specific indications for thrombophilia testing. It is recommended that testing be done only after appropriate counseling, and hence the investigations are usually not performed at the time when thrombosis is diagnosed but later. (Baglin T, et al, 2010) In particular situations, such as retinal vein thrombosis, testing is discouraged altogether because thrombophilia is not regarded as a major risk factor. In other rare conditions generally linked with hypercoagulability, such as cerebral venous thrombosis and portal vein thrombosis, there is insufficient data to state for certain whether thrombophilia screening is helpful, and decisions on thrombophilia screening in these conditions are therefore not regarded as evidence-based. (Baglin T, et al, 2010) Recurrent miscarriage is an indication for thrombophilia screening, particularly antiphospholipid antibodies (anti-cardiolipin IgG and IgM, as well as lupus anticoagulant), factor V Leiden and prothrombin mutation, activated protein C resistance and a general assessment of coagulation through an investigation known as thromboelastography. (Rai R, et al, 2006)

Women who are planning to use oral contraceptives do not benefit from routine screening for thrombophilies, as the absolute risk of thrombotic events is low. If either the woman or a first-degree relative has suffered from thrombosis, the risk of developing thrombosis is increased. Screening this selected group may be beneficial, (Tchaikovski SN, et al, 2010) but even when negative may still indicate residual risk. (Baglin T, et al, 2010) Professional guidelines therefore suggest that alternative forms of contraception be used rather than relying on screening. (Baglin T, et al, 2010)

Thrombophilia screening in people with arterial thrombosis is generally regarded unrewarding and is generally discouraged, (Begin T, et al, 2010) except possibly for unusually young patients (especially when precipitated by smoking or use of estrogen-containing hormonal contraceptives) and those in whom revascularization, such as coronary arterial bypass, fails because of rapid occlusion of the graft. (De Moerloose P, et al, 2007)
2.3. Factor V Leiden thrombophilia:

When there is a mutation of the *FVL* gene (arginine amino acid is substituted by glutamine amino acid at position number 506 of factor V), this may result in the formation of a protein which is resistant to the action of activated protein C, called anti-protein C (aPC). The aPC removes the inhibitory effect of protein C on the clotting mechanism and enhances the conversion of prothrombin to thrombin, subsequently enhancing the formation of clots. (Sehirali S, *et al.*, 2005) This absent or reduced activity of antithrombin leads to increased levels of thrombin and clot formation.

Factor V Leiden thrombophilia. (De Stefano V, *et al.*, 1995) is a genetic disorder of blood clotting. Factor V Leiden is a variant (mutated form) of human factor V (one of several substances that help blood clot) that causes an increase in blood clotting (hypercoagulability). With this mutation, the anticoagulant protein secreted (that stops factor V from causing inappropriate clotting) is inhibited, leading to an increased tendency to form dangerous, abnormal blood clots. (Ridker PM, *et al.*, 1997) Factor V Leiden is the most common hereditary hypercoagulability (prone to clotting) disorder amongst ethnic Europeans. (Gregg JP, *et al.*, 1997). (De Stefano V, *et al.*, 1998). It is named after the Dutch city Leiden, where it was first identified in 1994 by Prof. Bertina *et al.* (Bertina RM, *et al.*, 1994).

Factor V is one of the essential clotting factors in the coagulation cascade. Its active form, factor Va, acts as a cofactor allowing factor X to stimulate the conversion of prothrombin to thrombin. Thrombin is then able to cleave fibrinogen to fibrin and a fibrin clot is formed.

Activated protein C is a natural anticoagulant it limits the extent of clotting by destroying factor V and reducing further thrombin formation. Factor V Leiden (FVL) mutation (named after the Dutch university where it was discovered) is a point mutation in the gene for clotting factor V. It has autosomal dominant inheritance and is the most common cause of inherited thrombophilia the mutation of Factor V Leiden causes acquired protein C resistance, resulting in thrombophilia both in veins and spiral arteries of the placenta. (Azem F, *et al.*, 2004) The association between the FVL mutation and RPL seems stronger for non-recurrent second-trimester pregnancy loss compared with recurrent early pregnancy loss. (Aznar J, *et al.*, 2005) Women with factor V Leiden have a substantially increased risk of clotting in pregnancy (and on
estrogen-containing birth control pills or hormone replacement) in the form of deep vein thrombosis and pulmonary embolism. They also may have a small increased risk of preeclampsia, may have a small increased risk of low birth weight babies, may have a small increased risk of miscarriage and stillbirth due to either clotting in the placenta, umbilical cord, or the fetus (fetal clotting may depend on whether the baby has inherited the gene) or influences the clotting system may have on placental development. (Baglin T, et al, 2012)

2.3.1. Pathophysiology of factor V Leiden:

In the normal person, factor V functions as a cofactor to allow factor Xa to activate the enzyme thrombin. Thrombin in turn cleaves fibrinogen to form fibrin, which polymerizes to form the dense meshwork that makes up the majority of a clot. Activated protein C (aPC) is a natural anticoagulant that acts to limit the extent of clotting by cleaving and degrading factor V.

Factor V Leiden is an autosomal dominant genetic condition that exhibits incomplete penetrance, i.e. not every person who has the mutation develops the disease. The condition results in a factor V variant that cannot be as easily degraded by aPC (activated Protein C). The gene that codes the protein is referred to as F5. Mutation of this gene—a single nucleotide polymorphism (SNP) is located in exon 10. (Jennifer Bushwitz, et al, 2006) As a missense, substitution of base G to base A, it changes the protein’s amino acid from arginine to glutamine. Depending on the chosen start, the position of the nucleotide variant is either at position 1691 or 1746. It also affects the amino acid position for the variant, which is either 506 or 534. (Together with the general lack of nomenclature standard, this variance means that the SNP can be referred to in several ways, such as G1691A, c.1691G>A, 1691G>A, c.1746G>A, p.Arg534Gln, Arg506Gln, R506Q or rs6025.) Since this amino acid is normally the cleavage site for aPC, the mutation prevents efficient inactivation of factor V. When factor V remains active, it facilitates overproduction of thrombin leading to generation of excess fibrin and excess clotting. (Jennifer Bushwitz, et al, 2006)

The excessive clotting that occurs in this disorder is almost always restricted to the veins, where the clotting may cause a deep vein thrombosis (DVT). If the venous clots break off, these clots can travel through the right side of the heart to the lung where they block a pulmonary blood vessel and cause a pulmonary embolism. It is
extremely rare for this disorder to cause the formation of clots in arteries that can lead to stroke or heart attack, though a "mini-stroke", known as a transient ischemic attack, is more common. Given that this disease displays incomplete dominance, those who are homozygous for the mutated allele are at a heightened risk for the events detailed above versus those that are heterozygous for the mutation. (Jennifer Bushwitz, *et al.*, 2006)

### 2.3.2. Diagnosis of factor V Leiden:

Suspicion of factor V Leiden being the cause for any thrombotic event should be considered in any Caucasian patient below the age of 45, or in any person with a family history of venous thrombosis.

There are a few different methods by which this condition can be diagnosed. Most laboratories screen 'at risk' patients with either snake venom (e.g. dilute Russell's viper venom time) based test or an APTT based test. In both methods, the time it takes for blood to clot is decreased in the presence of the factor V Leiden mutation. This is done by running two tests simultaneously; one test is run in the presence of activated protein C (APC) and the other, in the absence. A ratio is determined based on the two tests and the results signify to the laboratory whether APC is working or not. These are quick, three-minute, automated tests that most hospital laboratories can easily perform.

There is also a genetic test that can be done for this disorder. The mutation (a 1691G→A substitution) removes a cleavage site of the restriction endonuclease MnlI, so PCR, treatment with MnlI, and then DNA electrophoresis will give a diagnosis.

### 2.3.3. Epidemiology of factor V Leiden:

Studies have found that about 5% of Caucasians in North America have factor V Leiden. The condition is less common in Latin Americans and African-Americans and is extremely rare in people of Asian descent.

Up to 30 percent of patients who present with deep vein thrombosis (DVT) or pulmonary embolism have this condition. The risk of developing a clot in a blood vessel depends on whether a person inherits one or two copies of the factor V Leiden mutation. Inheriting one copy of the mutation from a parent (heterozygous) increases by fourfold to eightfold the chance of developing a clot. People who inherit two copies of the mutation (homozygous), one from each parent, may have up to 80 times the
usual risk of developing this type of blood clot.(Rochat RW, et al, 2005) Considering that the risk of developing an abnormal blood clot averages about 1 in 1,000 per year in the general population, the presence of one copy of the factor V Leiden mutation increases that risk to between 4 in 1,000 to 8 in 1,000. Having two copies of the mutation may raise the risk as high as 80 in 1,000. It is unclear whether these individuals are at increased risk for recurrent venous thrombosis. While only 1 percent of people with factor V Leiden have two copies of the defective gene, these homozygous individuals have a more severe clinical condition. The presence of acquired risk factors for venous thrombosis—including smoking, use of estrogen-containing (combined) forms of hormonal contraception, and recent surgery—further increase the chance that an individual with the factor V Leiden mutation will develop DVT.

Women with factor V Leiden have a substantially increased risk of clotting in pregnancy (and on estrogen-containing birth control pills or hormone replacement) in the form of deep vein thrombosis and pulmonary embolism. They also may have a small increased risk of preeclampsia, may have a small increased risk of low birth weight babies, may have a small increased risk of miscarriage and stillbirth due to either clotting in the placenta, umbilical cord, or the fetus (fetal clotting may depend on whether the baby has inherited the gene) or influences the clotting system may have on placental development.(Rodger MA, et al, 2008) Note that many of these women go through one or more pregnancies with no difficulties, while others may repeatedly have pregnancy complications, and still others may develop clots within weeks of becoming pregnant. (Rodger MA, et al, 2008)

2.4. Prothrombin G20210A thrombophilia:

Prothrombin G20210A refers to a human gene mutation that increases the risk of blood clots. The "G20210A" refers to the fact that the mutation is a guanine (G) to adenine (A) substitution at position 20210 of the DNA of the prothrombin gene. This mutation (or more accurately, single-nucleotide polymorphism or variant), is commonly associated with increased risk of occurrence and recurrence of the disease venous thromboembolism (VTE), including both deep vein thrombosis (DVT) and pulmonary embolism (PE). As of 2005, it was believed that most carriers of the mutation never develop VTE in their lifetimes. (Rai R, et al, 2006) Other blood clotting pathway mutations that increase the risk of clots include factor V Leiden.
Prothrombin G20210A was identified in the 1990s is almost exclusively present in Caucasians. It is estimated to have originated in that population slightly over 20,000 years ago. (Kniffin, Cassandra L, et al, 2012) About 2 to 3% of Caucasians carry the variant. (Rosendaal FR, et al, 2005)

A mutation of the prothrombin gene (G20210A) will facilitate the formation of thrombin and clot formation in heterozygous individuals, who have a two-fold higher risk of clotting in comparison to non-carriers. Women with hyper homocysteinaemia show a folic acid deficiency, also resulting in a two-fold increase in clotting within homozygous women. (Cosmi B, et al, 2013) (Yildiz G, et al, 2012) The exact mechanism by which IT causes implantation failure and subsequent RPL is unclear. It has been suggested that thrombophilia may lead to a syncytiotrophoblast invasion of the maternal blood vessels, which in turn leads to the formation of microthrombosis at the site of implantation, resulting in implantation failure and RPL. (Nair RR, et al, 2012)

2.4.1. Signs and symptoms:


It confers a 2- to 3-fold higher risk of VTE. Deficiencies in the anticoagulants Protein C and Protein S give a higher risk (5- to 10-fold). (Rosendaal FR, et al, 2009) Behind non-O blood type. (Regan L, et al, 2000) and factor V Leiden, prothrombin G20210A is one of the most common genetic risk factors for VTE. (Martinelli I, et al, 2010) It was realized in 1996 that a particular change in the genetic code produces the body to make too much of the prothrombin protein. By having too much prothrombin, it increases the chances the blood clotting. Individuals who carry the condition have the prothrombin mutation, which can be inherited by offspring. (Velayuthaprabhu S, et al, 2005)
Having the prothrombin mutation increases the risk of developing a DVT (Deep vein thrombosis), known as a blood clot in the deep veins, often but not always in the legs. DVTs are threatening as they can damage the veins throughout the body, causing pain and swelling, and sometimes leading to disability. Most variety of people who have this prothrombin gene mutation do not require any treatment but need to be cautious throughout periods when the possibility of getting a blood clot may be enlarged (e.g. after surgery, during long flights etc.); occasionally people with the mutation may need to go on blood thinning medication to decrease the risk of developing blood clots. As there is no cure for the mutation, studies throughout the world are becoming conversant, emitting various medications in order to decrease risk factors.

Heterozygous carriers who take combined birth control pills are at a 15-fold increased risk of VTE,( Rosendaal FR, et al, 2005) while carriers also heterozygous with factor V Leiden have an approximate 20-fold higher risk. ( Rosendaal FR, et al, 2009) In a recommendation statement on VTE, genetic testing for G20210A in adults that developed unprovoked VTE was disadvised, as was testing in asymptomatic family members related to G20210A carriers who developed VTE. Baglin T, et al, 2012) In those who develop VTE, the results of thrombophilia tests (wherein the variant can be detected) rarely play a role in the length of treatment.( Degen SJ, et al, 1987) More recent studies have shown that G20210A mutation is associated with RM and other studies found that the incidence of G20210A mutation was rare in women with RM. (Baglin T, et al, 2012)

The polymorphism is located in a noncoding region of the prothrombin gene (3' untranslated region nucleotide 20210. (Ye Z, et al, 2006) replacing guanine with adenine. The position is at or near where the pre-mRNA will have the poly-A tail attached. (Poort SR, et al, 1996)

2.4.2. Terminology:

Because prothrombin is also known as factor II, the mutation is also sometimes referred to as the factor II mutation or simply the prothrombin mutation; in either case, the names may appear with or without the accompanying G20210A location specifies (unhelpfully, since prothrombin mutations other than G20210A are known).
2.5. Antiphospholipid Syndrome and Pregnancy:

Antiphospholipid syndrome, or APS, is an autoimmune disorder in which the body's immune system reacts against certain normal substances present in blood. A person with APS will have a greater than average tendency to form blood clots. APS is associated with greater odds of pregnancy loss. An estimated 10% to 25% of women with recurrent miscarriages have APS, and a person with APS will often have no other symptoms of the disorder until she experiences recurrent miscarriages. Some controversy exists over whether APS causes late or early pregnancy losses. Most doctors agree that APS can cause late losses but the evidence is unclear on whether APS causes early losses. As part of the normal recurrent miscarriage workup, doctors’ test for specific antibodies called anticardiolipin antibodies or lupus anticoagulant antibodies. Testing positive for these antibodies may indicate APS. In APS, the body’s immune system recognizes the phospholipids, which are a part of the cell membrane, as a foreign substance and thus produces antibodies against them. However, other studies have shown that antiphospholipid antibodies (aPL) often act against a protein cofactor called β2-glycoprotein-1. This protein cofactor helps the aPL to adhere to the phospholipids in the cell membrane. The aPL consist of 20 antibodies, but only the lupus anticoagulant and anticardiolipin antibodies (immunoglobulin G and immunoglobulin M, but not immunoglobulin A [IgA]) have been shown to be of clinical significance. In women with SLE, adverse live-birth outcomes were significantly associated with positive anticardiolipin IgA and anti-beta 2 glycoproteins. (Molad Y, et al, 2005) The mechanism by which APS causes implantation failure and subsequent RPL is unclear. It has been suggested that, as in thrombophilia, APS may lead to a syncytiotrophoblast invasion of the maternal blood vessels, leading to the formation of microthrombosis at the site of implantation, resulting in implantation failure and RPL.

Antiphospholipid syndrome (APS) is associated with pregnancy complications, including preeclampsia, thrombosis, autoimmune thrombocytopenia, fetal growth restriction, and fetal loss.

APS is classified as primary or secondary, depending on its association with other autoimmune disorders. Primary APS is diagnosed in patients demonstrating the clinical and laboratory criteria for the disease without other recognized autoimmune disease.
Secondary APS is diagnosed in patients with other autoimmune disorders, such as systemic lupus erythromatosus (SLE). Women with the clinical features of APS should be tested for three antiphospholipid antibodies that have proven association with the diagnosis of APS: lupus anticoagulant (LAC), anticardiolipin (aCL) antibody, and anti-beta-2-glycoprotein I antibody.

These antibodies predispose to clotting in vivo, predominantly by interfering with the antithrombotic role of PLs. The antiphospholipid (aPL) auto-antibodies bind moieties on negatively charged PLs or moieties formed by the interaction of negatively charged PLs with other lipids, PLs, or proteins.

2.5.1. Obstetric and non-obstetric clinical features:

Obstetric features of APS are as follows:
- Unexplained fetal death or stillbirth
- Recurrent pregnancy loss - 3 or more spontaneous abortions with no more than 1 live birth
- Unexplained second or third trimester fetal death
- Severe preeclampsia at less than 34 weeks’ gestation
- Unexplained severe fetal growth restriction
- Chorea gravidarum

Non-obstetric features of APS are as follows:
- Non traumatic thrombosis or thromboembolism (venous or arterial)
- Stroke, especially in individuals aged 24-50 years
- Unexplained amaurosis fugax
- Autoimmune thrombocytopenia
- Autoimmune hemolytic anemia
- Prolongation of a clotting assay
- Livedoreticularis
- SLE or other connective tissue disorder
- False-positive serologic test result for syphilis
2.5.2. Pathophysiology:

Biologic effects mediated by the human aPL antibodies include the following:

- Reactivity with endothelial structures that disturbs the balance of prostaglandin E2/thromboxane production
- Interaction with platelet PLs, with consequent up regulation of platelet aggregation
- Dysregulation of complement activation

Interaction of aPL with phosphatidylserine exposed during trophoblast syncytium formation, which raises the possibility of a more direct effect of these autoantibodies on placental structures. In patients with primary APS, the presence of the 3 aCL isotypes plus LAC has been associated with a higher number of recurrent spontaneous abortions, compared with other possible combinations of aCL isotypes. (Human aCL antibodies cause placental necrosis in BALB/c mice).

The association between aPL antibodies and particular human leukocyte antigen (HLA) alleles and HLA-linked epitopes has been reported in studies of patients with lupus erythromatosus (eg, HLA-DR7, HLA-DR4). The HLA-DR3 phenotypes seem to predispose to the formation of aCL antibodies and antinuclear antibodies (ANAs), but this has not been confirmed in patients, and particular HLA alleles associated with recurrent miscarriage have not been reported.

Animals immunized with aCL or with the cofactor beta-2 glycoprotein I (b2GPI) develop clinical manifestations of APS, including fetal loss, thrombocytopenia, and neurologic and behavioral dysfunction, along with elevated levels of aPL antibodies.

The aCL antibodies bind to b2GPI, or a complex formed by this b2GPI is a platelet adhesion glycoprotein and cardiolipin. Exposure of endothelial cells to anti-b2GPI antibodies and their corresponding peptides leads to the inhibition of endothelial cell activation, as shown by decreased expression of the adhesion molecules E-selectin, intercellular adhesion molecule, and vascular cell adhesion molecule and of monocyte adhesion.

In vivo infusion of each of the anti-b2GPI antibodies into BALB/c mice followed by administration of the corresponding specific peptides prevents the peptide-treated mice from developing experimental APS. These fascinating results suggest that the use of synthetic peptides that focus on neutralization of pathogenic anti-b2GPI antibodies represents a possible new therapeutic approach to APS.
Passive transfer into naive mice of inherently heterogeneous aPL antibody populations—from humans with APS or from autoimmune mice—either affinity-purified or as part of whole immunoglobulin fractions, has been shown to induce growth retardation and fetal loss.

2.5.3. Etiology:

Like other autoimmune disorders, APS does not have a known etiology, although it is known that the passive transfer of maternal antibodies mediates autoimmune disorders in the fetus and newborn. The mechanism of excess autoantibody production and immune complex formation is not well understood.

Certain genetic factors may be important, as indicated by a number of family and twin studies for SLE and the demonstration of an increased frequency of HLA-DR2, HLA-DR3, and HLA-DR4 null alleles in patients with SLE. As with other autoimmune disorders, women have a higher incidence than men and the diagnosis is more likely to be made in women of reproductive age.

2.5.4. Phospholipid release:

PL molecules are ubiquitous in nature and are present in the inner surface of the cell (ie, on the inner or outer surface of the cell membrane or intracellular organelles) and in microorganisms. Therefore, during infectious disease processes, including viral (eg, HIV, Epstein-Barr virus [EBV], cytomegalovirus [CMV], adenoviruses), bacterial (eg, bacterial endocarditis, tuberculosis, Mycoplasma pneumonia), spirochetal (eg, syphilis, leptospirosis, Lyme disease), and parasitic (eg, malaria infection) infections, the disruption of cellular membranes may occur during cell damage. PLs are consequently released, stimulating aPL antibodies.

2.5.5. Epitope mimicry in autoimmune disease:

The SWISS PROT protein database revealed high homology between the hexapeptides that bind to ILA-1, ILA-3, and H-3 mAbs and the membrane particles of different bacteria and viruses. The sequence LKTPRV showed homology to 8 different bacteria (eg, Pseudomonas aeruginosa) and homologies to 5 types of viruses (ie, polyoma virus, human CMV, adenovirus).
The sequence TL-RVYK also shows homology to 8 different bacteria, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Shigella dysenteriae*, and to viruses such as EBV and HIV. Therefore, data may support the theory predicting that epitope mimicry is involved in the propagation of the autoimmune status.

2.5.6. Epidemiology:

The epidemiology of the disease include

2.5.6.1. Occurrence in the United States:

Women have been reported to account for approximately 80% of patients with APS. The aPL antibodies account for 65-70% of cases of venous thrombosis in women with venous thrombosis in unusual sites (eg, cerebral portal, splenic, subclavian and mesenteric veins). The aPL antibodies are detected in approximately 2% of all patients with non traumatic venous thrombosis.

Approximately 22% of women with APS have had venous thrombosis and 6.9% have had a cerebrovascular incident (over a median follow-up period of 60 mo); 24% of thrombotic events have been found to occur during pregnancy or the postpartum period. The rate for thrombosis or stroke is 5-12%. These observations suggest that women with documented APS should not take estrogen-progestin combination oral contraceptives.

2.5.6.2. Sex and age-related demographics:

Most cases of APS (80%) are in women. APS is predominantly diagnosed in reproductive-aged women (i.e., 15-55 y). This is similar to other autoimmune states.

2.5.7. Prognosis:

APS is one of the major causes of thrombosis and its complications in women, with arterial thrombosis, coronary artery occlusions, and venous thrombosis being reported in patients with this syndrome. Previous thrombosis in the face of a diagnosis of APS has been documented to have a recurrence rate of 25% per year in untreated patients.

A 2015 retrospective analysis by the European Registry on Obstetric Antiphospholipid Syndrome (EUROAPS) found very good maternal-fetal outcomes in women whose obstetric APS (OAPS) was treated. (Alijotas-Reig J *et al*, 2015)
Previous fetal loss appears to be a risk factor for fetal loss, preeclampsia, premature birth, and placenta-mediated complications in women with pure OAPS, according to a 2014 report from the Nimes Obstetricians and Hematologists Antiphospholipid Syndrome (NOH-APS) study. In addition, the incidence of such late-pregnancy complications were greater in the treated women with pure OAPS compared to non treated women negative for antiphospholipid antibodies.

The investigators defined pure OAPS as pregnancy morbidity in women with no previous history of thrombosis (i.e., repeated unexplained abortion < 10th gestational wk, unexplained fetal loss ≥ 10 gestational week, or premature birth < 34th gestational wk due to preeclampsia). (Bouvier S, et al, 2014) Treatment included low molecular weight heparin (LMWH) and low-dose aspirin (LDA).

### 2.5.8. Maternal morbidity:

Thrombosis, especially in patients with APS and a history of thrombosis, is a major concern. Morbidity may also be associated with anticoagulation in patients treated with heparin or low–molecular-weight heparins in pregnancy. Moreover, women with APS have an increased incidence of preeclampsia, which, when it occurs, frequently develops prior to 34 weeks’ gestation. The incidence of severe preeclampsia requiring premature delivery is also increased.

APS is also associated with infertility and pregnancy complications, such as spontaneous abortions, prematurity, and stillbirths.

**Landry-Guillain-Barré-Strohl syndrome**

Landry-Guillain-Barré-Strohl syndrome (LGBSS) of acute inflammatory demyelinating polyradiculoneuropathy, although exceedingly rare in pregnancy, can occur in patients with APS and lupus.

Patients usually present with progressive bilateral and symmetrical muscle weakness accompanied by mild sensory symptoms, including paresthesia, numbness, and tingling. The disease can progress to involve the respiratory muscles, resulting in respiratory failure. Two thirds of the patients have a history of viral-like infections 1-3 weeks prior to the onset of symptoms.

CMV infection has been incriminated as a potential etiologic agent in some pregnant patients presenting with LGBSS.
Acute inflammatory demyelinating polyradiculoneuropathy is a rare disease with an incidence of approximately 1-1.5 cases per 100,000 LGBSS cases per year.

2.5.9. Maternal mortality:

Mortality rates during pregnancy are not well characterized. Multiorgan failure has been described during pregnancy by Asherson, (Asherson RA, et al, 1989) and during postpartum by Kochenour. (Kochenour NK, et al, 1987)

2.5.9.1. Prenatal morbidity:

The aPL antibodies are found in 10-15% of women at high risk for fetal growth restriction. Neonatal morbidity and mortality may be influenced by indicated preterm delivery for maternal severe preeclampsia or fetal growth restriction.

Neonatal lupus dermatitis, a variety of systemic and hematologic abnormalities, and isolated congenital heart block has been associated with APS and SLE.

2.5.9.2. Prenatal mortality:

Fetal deaths at or beyond 20 weeks' gestation may be attributable to APS involvement. The rate of fetal loss may exceed 90% in untreated patients with APS. Therapy (including aspirin and heparin) can reduce the rate of fetal loss to 25%, as described by Cowchock et al. (Cowchock FS, et al, 1992)

History

The “international consensus statement for the diagnosis of antiphospholipid syndrome,” published in 1999 by Wilson et al, serves as a set of criteria similar to that for the diagnosis of other autoimmune disorders. The criteria were updated in 2006 to reflect new insights into APS. The diagnosis requires that the patient have at least 1 clinical and 1 laboratory criterion. (Wilson WA, et al, 1999) (Miyakis S, et al, 2006)

2.5.10. Clinical and laboratory criteria:

The clinical criteria for APS include the following:

- One or more clinical episodes of arterial, venous, or small-vessel thrombosis, occurring within any tissue or organ

- One or more unexplained deaths of morphologically normal fetuses at or after 10 weeks’ gestation
- One or more premature births of morphologically normal fetuses at or before 34 weeks’ gestation because of eclampsia or severe preeclampsia or features consistent with placental insufficiency

- Three or more consecutive, unexplained spontaneous abortions before 10 weeks’ gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded

2.5.1.1. Laboratory criteria:

Criteria for laboratory testing, which are consistent with current clinical management guidelines from the American Congress of Obstetricians and Gynecologists, include the following: (Asherson RA, et al, 1989)

Anticardiolipin antibodies - Anticardiolipin IgG or IgM antibodies present at moderate or high levels (ie, >40 GPL or MPL or >99th percentile) in the blood on 2 or more occasions at least 12 weeks apart

Lupus anticoagulant - LAC detected in the blood on 2 or more occasions at least 12 weeks apart, according to the guidelines of the International Society on Thrombosis and Hemostasis. (Miyakis S, et al, 2006)

Anti-beta_2-glycoprotein I antibodies IgG or IgM - In titers above the 99th percentile for normal as defined by the laboratory performing the test, on 2 or more occasions at least 12 weeks apart

2.5.12. Additional findings in APS:

Findings in APS can also include the following:


. Positive result from the Coombs test

. Hemolytic anemia

. Chorea

. Chorea gravid arum

Physical Examination
The diagnosis of APS is based primarily on clinical history and laboratory data. Patients with secondary APS are more likely to have findings on physical examination, although some physical findings may be associated with primary APS. Thrombosis and stroke are possible residual neurologic findings in APS.

Cutaneous manifestations of APS can include the following:

- Digital cyanosis
- Lived oreticularis
- Digital gangrene
- Leg ulcers
- Discoid rash - i.e., a raised, erythematous patch with keratotic scaling and follicular plugging; older lesions may be atrophic Photosensitivity.

**2.6. Previous studies:**

A study done by Majid (2013), among 80 Iranian women with recurrent pregnancy loss was reported that no prothrombin G20210A gene mutations in cases and controls was found and concluded these data did not confirm that prothrombin gene G20210 mutation might play a role in recurrent pregnancy loss in Iranian women. This result was disagreement with findings from the several large meta-analysis studies that have explored to determine any significant association between the prothrombin gene G20210 and the presence of recurrent pregnancy loss. (Majid A, *et al*, 2013)

Yıldız Gazi (2012) has conducted a study about inherited thrombophilia with recurrent pregnancy loss in Turkish women. He was reported that no statistically significant difference was found between the study and the control groups in terms of the prevalence of Factor V Leiden (G1691A), prothrombin G20210A and MTHFR C677T gene mutations. P. value (0.534), (0.452), and (0.656), respectively. Furthermore, the prevalence of multiple gene mutations was not statistically different between the groups. Routine screening for Factor V Leiden (G1691A), prothrombin G20210A and MTHFR C677T gene mutations in patients with a history of recurrent pregnancy loss is not recommended in Turkish women (Yildiz G, *et al*, 2012).

Gihan and Osama, (2013) reported that when studies causes of RPL among Saudi women. The study including 142 females, 72 had a history of 2 or more events of fetal
loss in any of the 3 trimesters of pregnancy. The other 70 were clinically healthy women with a good obstetric history taken as a control group. Detection of FV Leiden (G1691A) and Prothrombin G20210A gene mutations were done. The results indicated that the total mutation carriage rate (AA and AG) among cases for Prothrombin G20210A gene was higher in frequency than FVL. Both were significantly higher than controls. For FV Leiden P. value (0.0001, while for prothrombin gene P. value (0.0001). (Gihan EH, et al, 2013)

Robert M, (2010), has studied a total of 157 women with recurrent pregnancy loss RPL and 60 healthy controls. The prothrombin gene mutation was seen in 3.8% of them and concluded that there was no association between the prothrombin gene G20210A mutation and pregnancy loss. (Robert M, et al, 2010)

Asaad M A. (2016) has conducted a study on point mutation in factor V Leiden G1691A and factor II G20210A and effect on coagulation profile and frequency of recurrent spontaneous abortions among Sudanese women. He found that the prevalence of heterozygous FVL gene mutation was found to be 8 % in women with RPL but in control group, it found to be 6.4%. P. value (0.66). The prevalence of heterozygous prothrombin gene was 3% among cases with RPL, but no mutant gene was, detected among control group P. Value (0.091). Normal Prothrombin time (PT) and activated partial thromboplastin time (APTT) was found in women with recurrent pregnancy loss (RPL), and they were not affected significantly. P. value (0.93) and (0.69), respectively). (Asaad M A. et al, 2016)

Also in Sudan, study done by Ahmed Bolad (2013), to determine the frequency of the primary and secondary antiphospholipid syndrome and the haemostatic abnormalities in women with history of RPL. He investigates APA, ACA, PT, and APTT as well as platelet counts. He found that the frequencies of both APA and ACA were 20%. He reported that significant correlation between the presence of APA and ACA/IgG and RPL. P value (0.03) and (0.04) respectively. The correlation between the presence of thrombocytopenia and recurrent miscarriage was significant. P. value (0.001). 5% of patients had prolonged PT. 8% of women with RPL had prolonged APTT and significant correlation obtained when compared with controls. P. value (0.018). (Ahmed Bolad, et al, 2013)
In Sudan, Safinaz A (2016) studied eighty-three pregnant Sudanese women with history of recurrent miscarriage, who attended to hospital and eighty healthy controls. They were tested for anticardiolipin IgG antibodies. She found that the frequency of positive anticardiolipin antibody among Sudanese women with RPL was 5 (6%) while it was 0.00% in control group. There was significant increase in means of anticardiolipin (2± 0.239) for patient with RPL versus (1.94± 0.00) for control, P. value (0.000). (Safinaz. A, et al, 2016)

In Russia, Kovalevsky G (2013) reported the frequency of thrombocytopenia (2-10%) in 540 women Russian with recurrent pregnancy loss. Thrombocytopenia in these women was presumably due to anti platelet autoantibodies directed against platelet-bound B2 GPI. The combined odds ratios for the association between RPL and FVL gene mutation and between RPL and prothrombin G20210A gene mutation were 2.0 (95% confidence interval, 1.5-2.7; P. value (0.001) and 2.0 (95% confidence interval, 1.0 4.0; P. value (0.03), respectively. (Kovalevsky G, et al, 2013)

In France, a study report by Cauchi and others in 145 French showed that only 7% of women with recurrent pregnancy loss had prolonged APTT. P. value (0.019) and that was due to the presence of antiphospholipid antibodies and lupus anticoagulant (LA). (Cauchi, MN, et al, 2003)

In Nigeria, a study reported by O. A Awodu (2003) among 100 Nigerian women with recurrent pregnancy loss to assess hemostasis by using activated partial thromboplastin time APTT. He mentioned that Prolonged APTT was seen in 3% of women with RPL P. value (0.018). (O A Awodu, et.al. 2003)

In Egypt, Omneya M. Osman (2015) conducts a research on inherited thrombophilia and early pregnancy loss among Egyptian women. 100 patients of unexplained RPL, and 43 healthy controls were investigated for inherited thrombophilic factors (FV Leaden G1691A, prothrombin G20210A and MTHFR gene mutation). She found that heterozygous FVL was found in 58% of patients and in 41.9% of controls (p. value = 0.105) and heterozygous prothrombin gene was found in 11% of patients and in 4.7% of controls. P. value (0.34). (Omneya M. Osman, et al, 2015)

In Brazil, R. O. Goncalves (2016) conducts a research on Association between thrombophilia polymorphism MTHFR C677T, FV leaden and prothrombin G20210A
and recurrent miscarriage in Brazilian women. 137 women with two or more consecutive first trimester miscarriage and 100 healthy women with no history of pregnancy loss as controls. The relevant gene was amplified by PCR. He found that heterozygous FVL gene mutation was found in 4(2.9%) of patients and in 2(2%) of controls (p. value > 0.05) and heterozygous prothrombin gene mutation was found in 4(2.9%) of patients and in 0% of controls (p. value > 0.05). (R. O. Goncalves, et al, 2016)

In Iran, a study was done by Alireza. Parand (2013) on inherited thrombophilia and recurrent pregnancy loss, among 90 randomly selected Iranian women with three or more consecutive miscarriage and 44 healthy women with no history of pregnancy loss as controls, to determine the frequency of FV Leaden, prothrombin gene mutation as well as protein C ans[d protein S deficiency. He found that heterozygous FVL was found in 15(13.5%) of patients and in 6(2.6%) of controls. P. value (0.081) and heterozygous prothrombin gene was found in 2(0.9%) of patients and in 1(0.4%) of controls. P. value (0.58). (Alireza. Parand, et al, 2013)

Vora SA (2008) study 430 Indian women with RPL and 100 normal control women with no history of RPL or other obstetric complications. They screened for the presence of antiphospholipid antibodies (APA), anticardiolipin antibodies (ACA), and annexin V. APA were seen in 12.2% of patients with RPL. P. value (˂0.05). ACA IgG/IgM antibodies were found in 27.9% of patients with RPL. P. value (˂0.05).

Sater MS (2013), conducted a study involving 277 women with RPL and 288 healthy control women. Antiphospholipid IgG antibodies, anticardiolipin IgG/IgM antibodies and anti prothrombin IgM antibodies were measured. Anticardiolipin IgG antibodies were positive in 36.5% of patients with RPL and in 4.5% of controls. P. value (0,001). Antiphospholipid IgG antibodies were found in 33.5% of patients with RPL, and in 1.5% of controls. P. value (0.001).
Chapter Three

3. Materials and Methods

3.1. Study Site

3.1.1. Study Design:

This study is an observational prospective comparative case control study.

3.1.2. Study Population and sample size:

Hundred Sudanese women who experienced two or more of the adverse pregnancy outcomes during their reproductive age, and who came for the out patients clinics at (Mohammed Ali Alshaikh Hospital (Saudi Hospital), Friendship Hospital, and Omdurman Maternal Hospital, during the period between 2013 – 2018 was included in this study, and 51 healthy women who attended the same medical facilities with at least more than 2 normal pregnancies and without any history of recurrent miscarriages as controls.

3.2. Materials and/or Methods:

3.2.1. Ethical clearance:

The ethics committee (Ministry of health, Khartoum state) was approved the study. Written informed consent was obtained from participants.

3.2.2. Samples Collection:

Venous blood was collected in 3.8% tri-sodium citrate and EDTA.

3.2.2.1. Venous blood:

The area of collection was cleaned using 70% alcohol, left to dry and then 1.8 ml of blood was transferred to 0.2 ml of 3.8% tri-sodium citrate container keeping 1: 9 ratios. In addition, 2.5 ml of blood was transferred into two EDTA blood containers. Then the blood was mixed thoroughly with the anticoagulant without frothing and labeled. (Regan L, et al, 2000)

3.2.2.2. Sample Storage:

EDTA anticoagulated blood samples and plasma samples were stored at -20c for molecular and serological analysis.
3.2.3. Data Collection:
A questionnaire was designed to collect demographical and clinical data from patients and controls. (See appendices)

3.2.4. Data analysis:
Data were entered and analyzed by SPSS programme (version: 21).

3.2.5. Laboratory Tests:
Included the following:

3.2.5.1. Hematological investigations:
3.2.5.1.1. Platelet counts (PC).
3.2.5.1.2. Prothrombin Time (PT).
3.2.5.1.3. Activated Partial Thromboplastin Time (APTT).

3.2.5.2. Serological investigations:
3.2.5.2.1. Anti-cardiolipin antibodies (IgG/IgM) ELISA.
3.2.5.2.2. Anti-phospholipids antibodies (IgG/IgM) ELISA.

3.2.5.3. Molecular Investigations:
3.2.5.3.1. DNA Extraction from Blood.
3.2.5.3.2. Prothrombin Gene Mutation (FII) PCR.
3.2.5.3.3. Factor V Leiden Gene Mutation (FV) PCR

3.2.5.1. Initial Hematological Tests:
Hematological tests were done to confirm the diagnosis of hereditary and acquired thrombophilic patients.
These tests included:

3.2.5.1.1. Sysmex: KXN-21
Is an automated machine used for determination of some hematological parameters.

3.2.5.1.1. Principle
DC Detection Method:
Blood sample is aspirated, measured to a predetermined volume, diluted at the specified ratio, and then fed into each transducer. The transducer chamber has a minute
hole called the aperture. On both side of the aperture, there are the electrodes between which flows direct current. Blood cells suspended in the diluted sample pass through the aperture, causing direct current resistance to change between the electrodes. As direct current resistance changes, the blood cells size is detected as electric pulses. Blood cells count is calculated by counting the pulses, and a histogram of blood cells size is plotted by determining the pulse size. Also analyzing a histogram makes it possible to obtain various analysis data.

**3.2.5.1.2. RBC/PLT Analysis Flow:**

**RBC/PLT Analysis Flow in Whole Blood Mode:**

1. Blood was aspirated from the sample probe into the sample rotor value.
2. 4.0 ml (0.004 ml) of blood was measured by the sample rotor value is diluted into 1:500 with 1.996 ml of diluents and brought to the mixing chamber as diluted sample. (First step dilution).
3. Out of the 1:500 dilution sample, 0.04 ml was measured by the sample rotor value, diluted into 1:25000 with 1.960 ml of diluents, then transferred RBC\PLT transducer chamber. (Second step dilution).
4. 0.25 ml of the sample in the RBC/PLT transducer chamber was aspirated through an aperture. At this time RBCs and platelets were counted by the DC detection method. Normal value 150000 – 400000 c/cu mm.

**3.2.5.1.3. Coagulation Tests:**

For detecting the intrinsic and extrinsic coagulation factors.

**3.2.5. 1.3.1. Dia Med - CD4:**

Coagulometer which uses a turbidity method (Clotting Method) was used.

**3.2.5.1.3.1.1. Principle:**

The thrombin catalyzed conversion of fibrinogen to fibrin is the final reaction in the coagulation cascade. Fibrin formation results in an increase in sample turbidity which is detected by the photometer. Photometric detection is started manually by pressing the "OPTIC" key with simultaneous addition of the test reagent. Alternatively, the reaction is started by the addition of the test reagent using the auto pipette. The time between the start of the photometric detection and the turning point of the reaction curve is the result. The result is displayed in seconds on the liquid crystal display (and printed automatically to the thermal printer).
3.2.5. 1.3.2. Prothrombin Time (PT):

For determination of the efficiency of the extrinsic pathway of coagulation cascade.

3.2.5.1.3.2.1. Principle:

Thromboplastin activates the coagulation factors of the extrinsic system in plasma in the presence of calcium ions. The clotting time depends on the concentration of factors 11, V, VII and X. A prolonged clotting time indicates deficiency of one or several of these factors.

Reagent:

diaplastin, (calcium-thromboplastin rabbit brain) liquid, ready to use.

Stability:

Diaplastin: approx. 1 year at 2 – 8 C°

1 week at 18 – 25 C°

2 days at 37 C°

Sample preparation:

Mix 9 parts of freshly collected blood with 1 part of tri-sodium citrate 0.11 mol/L. Centrifuge immediately for 5 minutes at RCF 1500 - 2000 g (approx. 3000 rpm) and transfer plasma into a clean test tube. Plasma should be tested within 3 hours of blood collection.

System preparation:

1. Turn on instrument and wait for green LED light to come on.
2. Check setup system if necessary.
3. Check setup test.
4. Return to main menu and enter "Analysis" by pressing #1.

Select PT with the UP/down arrow keys or enter the numeric test code, #01.

3.2.5.1.3.2.2. Test procedure:

1. Pre-warm Diaplastin reagent for 15-20 minutes.
2. Pipette 0.05 ml Plasma to Cuvettes (s).
3. Pre-warm Plasma for 2-5 minutes. Press the "TIMER 1" key to start stopwatch.
4. Transfer Cuvettes to measuring position.
5. While incubating, press "OPTIC 1". If selected, enter pat. I dent. with numeric keys of UP/down keys. Confirm by pressing "OPTIC 1" again. The message "ACTIVE" is displayed and channel 1 is ready to start the reaction. Repeat for the remaining
channels.
6. Add 0.1 ml pre-warmedDiaPlastin reagent. The test will automatically start using the auto pipette.

CATION:
(When the test procedure is running, pressing the "OPTIC 1" and the "Enter" keys will interrupt the test).
Repeat for remaining channels.
7. The instrument will read for 300 seconds. If no clot is detected, the display will read "+++ +".
8. The result is displayed in seconds.
Reference values:
12 - 14 seconds

3.2.5.1.3.3. Activated Partial Thromboplastin Time (APTT):
For determination of the efficiency of the intrinsic pathway of coagulation cascade.

3.2.5.1.3.3.1. Principle:
Cephaloplastin active the coagulation factors of the intrinsic system in Plasma in the presence of calcium ions. The clotting time depends on the activity of factors VIII, IX, and XII as well as of I, II, V and X.
The contact activation by complexes kaolin reduces the clotting time considerably and avoids turbidity, to be used also for optical measurements. Due to its high sensitivity to heparin, DiaCelin can be used for heparin determination.

Reagent:
1. DiaCelin - (Cephaloplastin, rabbit brain, with complexes kaolin), Liquid
2. Calcium chloride, 0.02 mol".

Stability:
Dia Colin. Approx. 1 year at 2-8 °C.
1 week at 18-25 °C.
2 days at 37 °C.

Sample preparation:
- Mix 9 parts of freshly collected blood with 1 part of sodium citrate (38 g/l "Hydrated" or 32 g/l "dehydrated") or (0.11 mol/l).
- Centrifuge immediately for 5 minutes at RCF 1500-2000 g (approx. 3000 rpm) and
transfer Plasma into a clean test tube.
- Plasma should be tested within 3 hours of blood collection.
- Blood collection preferably in plastic tubes.

System preparation:
1. Turn on instrument and wait for green LED light to come on.
2. Check system setup if necessary.
3. Check test setup.
4. Return to main menu and enter "Analysis" by pressing #1. Select APTT with the UP/down arrow keys or enter the numeric test code, #03.

3.2.5.1.3.3.2. Test procedure:
1. Pre-warmDiaCelin and CaCL2 reagent for 15 - 20 minutes.
2. Pipette 0.05 ml of plasma into Cuvette.
3. Add 0.05 ml pre-warmedDiaCelin reagent. Incubate for exactly 3 minutes.
4. Press the "TIMER 1" key to start stop-watch 1.
5. Transfer Cuvette to measuring position.
6. While incubating, press "OPTIC 1". If selected, enter pat. Indent. With numeric key or UPDown key. Confirm by pressing "OPTIC 1" again. The message "ACTIVE" is displayed and channel 1 is ready to start the reaction repeat for the remaining channels.
7. Add 0. G5 ml pre-warmed CaCl2 reagent. The test will. Automatically start using the auto pipette. (CATION: When the test procedure is running, pressing the "OPTIC 1" and the "Enter" keys will interrupt the test).
8. The instrument will read for 300 seconds. If no clot is detected, the de Splay will read "+++.
9. The result is displayed in seconds.
Normal values:
26 - 35 seconds.

3.2.5.2. Serological investigations:
The serological investigations include anti cardiolipin and anti-phospholipid antibodies.
3.2.5.2.1. Anti-Cardiolipin Antibodies IgG ELISA:

ELISA for quantitative determination of anti-cardiolipin antibodies (IgG).

3.2.5.2.1.1. Principle:

The test is based on the immobilization and purified human-beta-glycoprotein1to the solid phase of micro titer strips and subsequent binding of anti-cardiolipin antibodies from patient serum.

The bound antibodies are detected with a peroxidase labeled secondary antibody that is directed against human IgG. After addition of substrate solution, a color appears which intensity is proportional to the concentration and the avidity of the detected antibodies. Following the addition of stop solution, the color switches from blue to yellow.

Contents:

Micro titer Strips (MTP) in one strip holder:
8-well snap-off strips ready for use coated with cardiolipin and purified human beta-2-glycoprotein 1.

Calibrator IgG (CAL) white cap:
Human serum, inked according to concentration, ready for use.
Anti-cardiolipin level: 31.25U/ml (1), 62.5 U/ml (2), 125 U/ml (3), 250 U/ml (4), 500 U/ml (5).

Negative Control serum (NC) green cap:
Human, ready for use.

Positive Control Serum (PC) red cap:
Human, ready for use. Concentrations are stated on the labels.

Washing Buffer (WASH) black cap:
Concentrate (20X) for 1
TRIS buffer PH 6.9±0.2

Dilution Buffer (DIL) blue cap:
Ready for use
Phosphate buffer PH 7.3±0.2

Conjugate Solution (CON) white cap:
Anti-human-IgG HRP conjugate, ready for use.

TMB Solution (SUB) black cap:
Sulphuric acid, ready for use 0.5 mol/l

Adhesive strip.
Stability:
The reagent is stable up to the stated expiry dates on the individual labels when stored at 2 – 8 c˚.

Reagent Preparation:
- Allow the test kit and all its components to reach room temperature before use.
- Used bottled should be closed carefully and stored at 2 – 8 c˚.
- Store (SUB) protected from light.
- Don’t use polystyrene vessels for handling of (CON).
- To avoid potential microbial and/or chemical contamination, unused reagent should never be transferred into the original vials.
- Any crystallized salt inside the bottle of (WASH) must be resolved before use. Dilute 1 part of (WASH 20X) with 19-part DW. WASH is stable for 6 weeks stored at 2 – 8 c˚.

Specimen:
Patient serum or plasma.
- Use samples freshly collected or freeze samples at –20 c˚. Freeze and thaw once only.
- Do not use serum inactivated by heat treatment at 56 c˚.
- Allow the samples to reach room temperature (30 min.).
- Dilute samples 1: 101 with DIL (add 10mL sample to 1 ml DIL)

3.2.5.2.1.2. Procedure:
- Pipette 100µL of diluted patient sample, CAL, PC and NC into MTP, for blank use DIL instead of sample dilution, seal MTP with adhesive strip.
- Incubate for 1 hour at RT.
- Discard the solution from MTP. Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper and cloth.
- Pipette 100 µL CON and seal MTP with adhesive strip.
- incubate for 30 min. at RT.
- Discard the solution from MTP.
- Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper or cloth.
- Pipette 100 µL SUB and incubate for 10 min. at room temperatures. Above 25 c˚ the substrate incubation could be shortened, but should never fall short of 5 min.
- Add 100 µL STOP per well.
Read absorbance value at 540 nm within the next 100 min. after stopping. Bichromatic measurement with a reference wavelength at 620 – 690 nm is recommended.

3.2.5.2.2. Anti-Cardiolipin Antibodies IgM ELISA:

ELISA for quantitative determination of anti-cardiolipin antibodies (IgM).

3.2.5.2.2.1. Principle:
The test is based on the immobilization and purified human-beta-glycoprotein1 to the solid phase of micro titer strips and subsequent binding of anticardiolipin antibodies from patient serum.
The bound antibodies are detected with a peroxidase - labeled secondary antibody that is directed against human IgM. After addition of substrate solution, a color appears which intensity is proportional to the concentration and the avidity of the detected antibodies. Following the addition of stop solution, the color switches from blue to yellow.

Contents:
Micro titer Strips (MTP) in one strip holder:
8-well snap-off strips ready for use coated with cardiolipin and purified human beta-2-glycoprotein 1.
Calibrator IgG (CAL) white cap:
Human serum, inked according to concentration, ready for use.
Anti-cardiolipin level: 31.25U/ml (1), 62.5 U/ml (2), 125 U/ml (3), 250 U/ml (4), 500 U/ml (5).
Negative Control serum (NC) green cap:
Human, ready for use.
Positive Control Serum (PC) red cap:
Human, ready for use. Concentrations are stated on the labels.
Washing Buffer (WASH) black cap:
Concentrate (20X) for 1
TRIS buffer PH 6.9±0.2
Dilution Buffer (DIL) blue cap:
Ready for use
Phosphate buffer PH 7.3±0.2
Conjugate Solution (CON) white cap:
Anti-human-IgG HRP conjugate, ready for use.
TMB Solution (SUB) black cap:
Sulphuric acid, ready for use 0.5 mol/l
Adhesive strip.

Stability:
The reagent is stable up to the stated expiry dates on the individual labels when stored at 2 – 8 c˚.

Reagent Preparation:
-Allow the test kit and all its components to reach room temperature before use.
- Used bottled should be closed carefully and stored at 2 – 8 c˚.
- Store (SUB) protected from light.
- Don’t use polystyrene vessels for handling of (CON).
- To avoid potential microbial and/or chemical contamination, unused reagent should never be transferred into the original vials.
- Any crystallized salt inside the bottle of (WASH) must be resolved before use. Dilute 1 part of (WASH 20X) with 19-part DW. WASH is stable for 6 weeks stored at 2 – 8 c˚.

Specimen:
Patient serum or plasma.
-Use samples freshly collected or freeze samples at – 20 c˚. Freeze and thaw once only.
Do not use serum inactivated by heat treatment at 56 c˚.
- Allow the samples to reach room temperature (30 min.).
-Dilute samples 1: 101 with DIL (add 10mL sample to 1 ml DIL)

3.2.5.2.2.2. Procedure:
- Pipette 100µL of diluted patient sample, CAL, PC and NC into MTP, for blank use DIL instead of sample dilution, seal MTP with adhesive strip.
- Incubate for 1 hour at RT.
- Discard the solution from MTP. Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper and cloth.
- Pipette 100 µL CON and seal MTP with adhesive strip.
- incubate for 30 min. at RT.
- Discard the solution from MTP.
- Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper or cloth.
- Pipette 100 µL SUB and incubate for 10 min. at room temperatures. Above 25°C the substrate incubation could be shortened, but should never fall short of 5 min.
- Add 100 µL STOP per well.
- Read absorbance value at 540 nm within the next 100 min. after stopping. Bi-chromatic measurement with a reference wavelength at 620 – 690 nm is recommended.

3.2.5.2.3. Anti-phosphatidyl serine Antibodies IgG ELISA:

ELISA for quantitative determination of anti-Phosphatidylserine antibodies (IgG).

3.2.5.2.3.1. Principle:
The test is based on the immobilization and purified human-beta-glycoprotein1 to the solid phase of micro titer strips and subsequent binding of anti-phosphatidylserine antibodies from patient serum.
The bound antibodies are detected with a peroxidase labeled secondary antibody that is directed against human IgG. After addition of substrate solution, a color appears which intensity is proportional to the concentration and the avidity of the detected antibodies. Following the addition of stop solution, the color switches from blue to yellow.

Contents:
Micro titer Strips (MTP) in one strip holder:
8-well snap-off strips ready for use coated with phosphatidylserine and purified human beta-2-glycoprotein 1.
Calibrator IgG (CAL) white cap:
Human serum, inked according to concentration, ready for use.
Anti-phosphatidylserine level: 6.25/ml (1), 12.5 U/ml (2), 25 U/ml (3), 50 U/ml (4), 100 U/ml (5).

Negative Control serum (NC) green cap:
Human, ready for use.

Positive Control Serum (PC) red cap:
Human, ready for use. Concentrations are stated on the labels.

Washing Buffer (WASH) black cap:
Concentrate (20X) for 1
TRIS buffer PH 6.9±0.2
Dilution Buffer (DIL) blue cap:
Ready for use
Phosphate buffer PH 7.3±0.2
Conjugate Solution (CON) white cap:
Anti-human-IgG HRP conjugate, ready for use.
TMB Solution (SUB) black cap:
Sulphuric acid, ready for use 0.5 mol/l
Adhesive strip.
Stability:
The reagent is stable up to the stated expiry dates on the individual labels when stored at 2 – 8 c˚.
Reagent Preparation:
- Allow the test kit and all its components to reach room temperature before use.
- Used bottled should be closed carefully and stored at 2 – 8 c˚.
- Store (SUB) protected from light.
- Don’t use polystyrene vessels for handling of (CON).
- To avoid potential microbial and/or chemical contamination, unused reagent should never be transferred into the original vials.
- Any crystallized salt inside the bottle of (WASH) must be resolved before use. Dilute 1 part of (WASH 20X) with 19 part DW. WASH is stable for 6 weeks stored at 2 – 8 c˚.
Specimen:
Patient serum or plasma.
- Use samples freshly collected or freeze samples at – 20 c˚. Freeze and thaw once only. Do not use serum inactivated by heat treatment at 56 c˚.
- Allow the samples to reach room temperature (30 min.).
- Dilute samples 1: 101 with DIL (add 10mL sample to 1 ml DIL)

3.2.5.2.3.2. Procedure:

- Pipette 100 µL of diluted patient sample, CAL, PC and NC into MTP, for blank use DIL instead of sample dilution, seal MTP with adhesive strip.
- Incubate for 1 hour at RT.
- Discard the solution from MTP. Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper and cloth.
- Pipette 100 µL CON and seal MTP with adhesive strip.
- Incubate for 30 min. at RT.
- Discard the solution from MTP.
- Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper or cloth.
- Pipette 100 µL SUB and incubate for 10 min. at room temperatures. Above 25 c˚ the substrate incubation could be shortened, but should never fall short of 5 min.
- Add 100 µL STOP per well.
- Read absorbance value at 540 nm within the next 100 min. after stopping. Bidichromatic measurement with a reference wavelength at 620 – 690 nm is recommended.

3.2.5.2.4. Anti-Phosphatidylserine Antibodies IgM ELISA:

ELISA for quantitative determination of anti-phosphatidylserine antibodies (IgM).

3.2.5.2.4.1. Principle:

The test is based on the immobilization and purified human-beta-glycoprotein1 to the solid phase of micro titer strips and subsequent binding of anti-phosphatidylserine antibodies from patient serum.

The bound antibodies were detected with a peroxidase labeled secondary antibody that is directed against human IgM. After addition of substrate solution, a color appears which intensity is proportional to the concentration and the avidity of the detected antibodies. Following the addition of stop solution, the color switches from blue to yellow.
Contents:

Micro titer Strips (MTP) in one strip holder:
8-well snap-off strips ready for use coated with phosphatidylserine and purified human beta-2-glycoprotein 1.

Calibrator IgM (CAL) white cap:
Human serum, inked according to concentration, ready for use.
Anti- phosphatidylserine level: 6.25U/ml (1), 12.5 U/ml (2), 25 U/ml (3), 50 U/ml (4), 100 U/ml (5).

Negative Control serum (NC) green cap:
Human, ready for use.

Positive Control Serum (PC) red cap:
Human, ready for use. Concentrations are stated on the labels.

Washing Buffer (WASH) black cap:
Concentrate (20X) for 1
TRIS buffer PH 6.9±0.2

Dilution Buffer (DIL) blue cap:
Ready for use
Phosphate buffer PH 7.3±0.2

Conjugate Solution (CON) white cap:
Anti-human-IgG HRP conjugate, ready for use.

TMB Solution (SUB) black cap:
Sulphuric acid, ready for use 0.5 mol/l
Adhesive strip.

Stability:
The reagent is stable up to the stated expiry dates on the individual labels when stored at 2 – 8 c˚.

Reagent Preparation:
-Allow the test kit and all its components to reach room temperature before use.
- Used bottled should be closed carefully and stored at 2 – 8 °C.
- Store (SUB) protected from light.
- Don’t use polystyrene vessels for handling of (CON).
- To avoid potential microbial and/or chemical contamination, unused reagent should never be transferred into the original vials.
- Any crystallized salt inside the bottle of (WASH) must be resolved before use. Dilute 1 part of (WASH 20X) with 19-part DW. WASH is stable for 6 weeks stored at 2 – 8 °C.

Specimen:
Patient serum or plasma.
- Use samples freshly collected or freeze samples at – 20 °C. Freeze and thaw once only. Do not use serum inactivated by heat treatment at 56 °C.
- Allow the samples to reach room temperature (30 min.).
- Dilute samples 1: 101 with DIL (add 10mL sample to 1 ml DIL)

3.2.5.2.4.2. Procedure:
- Pipette 100 µL of diluted patient sample, CAL, PC and NC into MTP, for blank use DIL instead of sample dilution, seal MTP with adhesive strip.
- Incubate for 1 hour at RT.
- Discard the solution from MTP. Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper and cloth.
- Pipette 100 µL CON and seal MTP with adhesive strip.
- incubate for 30 min. at RT.
- Discard the solution from MTP.
- Wash MTP 3 times using 300 µL WASH per well.
- Discard WASH and knock out residues on an absorbent paper or cloth.
- Pipette 100 µL SUB and incubate for 10 min. at room temperatures. Above 25 °C the substrate incubation could be shortened, but should never fall short of 5 min.
- Add 100 µL STOP per well.
- Read absorbance value at 540 nm within the next 100 min. after stopping. Bi-chromatic measurement with a reference wavelength at 620 – 690 nm is recommended.
3.2.5.3. Molecular Investigations:

Molecular analysis:

The detection of Prothrombin and factor V Leiden gene were analyzed by Polymerase chain reaction (PCR) method.

3.2.5.3.1. DNA Extraction from Blood.


Isolation of Genomic DNA from Whole Blood Wizard® Genomic DNA Purification Kit:

Instructions for use of product A1120, A1123, A1125 AND A1620.

ORDERING / TECHNICAL INFORMATION: www.promega.com • Phone 608-274-4330 or 800-356-9526 • Fax 608-277-2601 ©1999–2010 Promega Corporation. All Rights Reserved.

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Lyses Solution</th>
<th>Protein Precipitation Solution</th>
<th>Isopropanol</th>
<th>DNA Rehydration Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell</td>
<td>Nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300µl</td>
<td>900µl</td>
<td>300µl</td>
<td>100µl</td>
<td>300µl</td>
</tr>
<tr>
<td>1ml</td>
<td>3ml</td>
<td>1ml</td>
<td>330µl</td>
<td>1ml</td>
</tr>
<tr>
<td>3ml</td>
<td>9ml</td>
<td>3ml</td>
<td>1ml</td>
<td>3ml</td>
</tr>
<tr>
<td>10ml</td>
<td>30ml</td>
<td>10ml</td>
<td>3.3ml</td>
<td>10ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>800µl</td>
<td></td>
</tr>
</tbody>
</table>

As little as 20µl can be processed with this system.

Please see Technical Manual #TM050, Section 3.C.
3.2.5.3.1.1. Procedure:

Red Blood Cell Lyses

1. Using volumes from the table above combine the appropriate volumes of Cell Lyses Solution and blood. Mix by inversion.

2. Incubate for 10 minutes at room temperature.

3. Centrifuge: ≤ 300µl sample 13,000–16,000 × g*; 20 seconds 1–10 ml sample 2,000 × g; 10 minutes


Nuclei Lyses and Protein Precipitation

5. Using volumes from the table above, add Nuclei Lysis Solution and mix by inversion.

6. Add Protein Precipitation Solution; vortex for 20 seconds.

7. Centrifuge: ≤ 300µl sample 13,000–16,000 × g*; 3 minutes 1–10 ml sample 2,000 × g; 10 minutes DNA Precipitation and Rehydration

8. Transfer supernatant to a new tube containing isopropanol (using volumes from table above). Mix.

9. Centrifuge: ≤ 300 µl sample 13,000 –16,000 × g*; 1 minute 1–10 ml sample 2,000 × g/ 1 minute


12. Aspirate the ethanol and air-dry the pellet (10–15 minutes).

13. Rehydrate the DNA in the appropriate volume of DNA Rehydration Solution for 1 hour at 65°C or overnight at 4°C. *Maximum speed on a micro centrifuge.

*Additional protocol information is available in Technical Manual #TM050, available online at www.promega
3.2.5.3.2. Polymerase Chain Reaction (PCR):

Maxime PCR PreMix Kit (i-Taq):

3.2.5.3.2.1. Description:
Maxime PCR PreMix Kit (i-Taq) is the product what is mixed every component i-Taq DNA polymerase, dNTPs mixture, reaction buffer and so on in one tube for 1 rxn PCR. You can do PCR just add a template DNA, primer set and DW.

Storage:
Store at -20 °C; under this condition, it is stable for at least a year.

Contents:
Maxime PCR PreMix (i-Taq, for 20 µL rxn).
Component in (20 µL reaction):
i-Taq DNA polymerase (5U/µL) 2.5 U
dNTPs 2.5 U
Reaction buffer (10X) 1X
Gel loading buffer 1X

3.2.5.3.2.2. Procedure:
1. Add template DNA and primers into Maxime PCR PreMix tubes (i-Taq).

Note 1: Recommended volume of template and primer: 3 µL - 9 µL (3 µL).

Appropriate amount of DNA template samples:
cDNA: 0.5 – 10 of first RT reaction volume.
Genomic DNA: 0.1 – 1 ug for single copy.

Note 2: Appropriate amount of primers:
Primer: 5 – 20 pmol/µL each (sense and anti-sense).

267- base pair (bp) segment of the factor V gene was amplified used specific forward primer (5’TCA GGC AGG AAC AAC ACC AT-3’) and reverse primer 5’GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT 3 wild type and mutant.
345-bp genomic DNA fragment encompassing a part of the prothrombin gene that contains the mutation was amplified by PCR using specific primers Forward (5’TCT AGA AAC AGT TGC CTG GC-3’) and reverse primer (5’ATA GCA CTG GGA GCA TTG AAG C-3) wild type and mutant.

2. Add distilled water into the tube to a total volume of 20 µL (do not calculate the dried components)

   Example: Total 20 µL reaction volume.

<table>
<thead>
<tr>
<th>PCR reaction Mixture</th>
<th>Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>1 – 2 µL</td>
</tr>
<tr>
<td>Primer (F: 10 pmol/ µL)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Primer (R: 10 pmol/ µL)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16 -17 µL</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

Note: This example serves as a guideline for PCR amplification. Optimal reaction condition such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.

   Note: If the mixture lets stand at RT for 1 - 2 min after adding water, the pellet is easily dissolved.

4. Add mineral oil (Optional).

   Note: this step is unnecessary when using a thermal cycler that employs a top heating method (general method).

5. Perform PCR of samples.

6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.
Suggested Cycling Parameters:

PCR cycle 30-40 (30 cycle used) Temperature PCR product size 100-500 bp

- Initial denaturation: 94 °C for 2 min
- Denaturation: 94 °C for 20 secs
- Annealing: 50 – 56 °C for 10 secs
- Extension: 65 – 72 °C for 20 – 30 secs
- Final extension: 72 °C Optional, normally, 2 – 5 min

PCR primers used in genotyping the polymorphisms.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II (G20210A) Common</td>
<td>5’-TCTAGAAACAGTTGCCCTGGCAG-3’</td>
<td>Mutant 5’-GCACTGGGAGCATTGAGGATT-3’ Gawish et al. (2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal 5’-GCACTGGGAGCATTGAGGATC-3’</td>
<td></td>
</tr>
<tr>
<td>Factor V (G1691A) Common</td>
<td>5’-CTTTCAGGCAGGAACAACACC-3’</td>
<td>Mutant 5’-TGGACAAAATACCTGTATACCTT-3’ Dajani et al. (2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal 5’-GGACAAAATACCTGTATTGCTC-3’</td>
<td></td>
</tr>
</tbody>
</table>

(5’TCA GGC AGG AAC AAC ACC AT-3’)

5’GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT 3
Chapter Four

4. Results and Discussion

4.1. Results:
This is a case control study done at Mohammed Ali Alshaikh (Saudi Hospital), Friendship Hospital and Omdurman Maternal Hospital, during the period from August 2013 to February 2018 to detect the association between acquired and hereditary thrombophilia and recurrent pregnancy loss. The participants in our study include 100 patients with two or more recurrent pregnancy losses (case group) and 51 healthy women with no history of miscarriage as (control group).

4.1.1. Demographical data:

4.1.1.1. Age distribution:
The ages of patients were between 20 – 45 years with the mean of 32±3.4 years and the mean age of control groups were 27.2 ± 5.8

Fig: 2
Age distribution of patients.
4.1.2. Clinical Data:

4.1.2.1. Frequency of recurrent abortion:

Fig: 3
Frequency of recurrent abortion for patients.

4.1.3. Laboratory Results:

4.1.3.1. FV Leiden gene mutation:

Heterozygous factor V Leiden (G1691A) gene mutation was found in 11 (11%) of patients with RM, and in 0 (0%) of controls, there was statistically significant differences when compare with the control group P. value (0.000), Table 1.

Homozygous Factor V Leiden (G1691A) gene mutation was seen in 9(9%) of patients with RM, and in 2(3.9)% of controls, there was statistically significant differences when compare with the control group P. value (0.000), Table 1. The relation between Factor V Leiden gene mutation and recurrent pregnancy loss was statistically significant, p value (0.000).
Table: 1
Distribution of factor V Leiden in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n=100)</th>
<th>Controls (n=51)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous (AA)</td>
<td>9 (9%)</td>
<td>0 (0%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>11 (11%)</td>
<td>2 (3.9%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>80 (80%)</td>
<td>49 (75.5%)</td>
<td></td>
</tr>
</tbody>
</table>

4.1.3.2. Prothrombin gene mutation:
Homzygous Factor II prothrombin (G20210A) gene mutation was seen in 11(11%) of patients with RM, and in 3(5.8%) of controls, statistically significant differences was found when compared with the control group P. value (0.000), Table 2.

Heterozygous Factor II prothrombin (G20210A) gene mutation was found in 5(5%) of patients with RM, and in 0(0%) of controls, there was statistically insignificant differences when compare with the control group P. value (0.000), Table 2.

Table: 2
Distribution of Prothrombin G20210A in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n=100)</th>
<th>Controls (n=51)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous (AA)</td>
<td>11 (11%)</td>
<td>3 (5.8%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>5 (5%)</td>
<td>0 (0%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>84 (84%)</td>
<td>48 (82.4%)</td>
<td></td>
</tr>
</tbody>
</table>

4.1.3.3. Combined Thrombophilia:
Combined thrombophilia in our study patients (homozygous Factor V Leiden and homozygous prothrombin G20210A gene mutations) were found together in one patient 1% (double mutation). While heterozygous and homozygous gene mutation of FV Leiden together with prothrombin gene were detected in 4% patients with RPL. In addition, heterozygous Factor V Leiden G1691A and heterozygous prothrombin G20210A gene mutations also were detected in 2 (2%) of patients. Table 3.
Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FV</th>
<th>FII</th>
<th>frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous (AA)</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>Homo/Hetero (AA/AG)</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
</tbody>
</table>

4.1.3.4. Antiphospholipid antibodies:

Antiphospholipid antibodies (IgG) were detected in 10(10%) of women with RPL, and in 2(3.9%) of controls. P value (0.000). Antiphospholipid antibodies (IgM) were detected in 11(10%) of women with RPL, and in 1(1.9%) of controls, P value (0.007).

Table 4 and table 5.

Table 4
Distribution of Antiphospholipid Antibodies (IgG) in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Antiphospholipid</th>
<th>Patients (n=100)</th>
<th>Controls (n=51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>41 (41%)</td>
<td>22 (43.1%)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (10%)</td>
<td>2 (3.8%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Borderline</td>
<td>49 (49%)</td>
<td>27 (52.9%)</td>
<td></td>
</tr>
</tbody>
</table>

(-ve) less than 6.65 u/ml, (borderline) btw 6.62 – 45.18 u/ml & (+ve) more than 45.18 u/ml.

The borderline group for antiphospholipid antibodies IgG and IgM include the patients with recurrent miscarriage RM, that have been developed specific antibodies against the phospholipid with concentration between negative and positive level of controls. These groups have high prevalence among patients with IgG, IgM antiphospholipid antibodies and the control groups table (4) and (5).
Table: 5
Distribution of Antiphospholipid Antibodies (IgM) in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Antiphospholipid</th>
<th>Patients (n=100)</th>
<th>Controls (n=51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>30 (30%)</td>
<td>16 (31.4%)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (10%)</td>
<td>2 (3.8%)</td>
<td>0.007</td>
</tr>
<tr>
<td>Borderline</td>
<td>59 (59%)</td>
<td>35 (68.6%)</td>
<td></td>
</tr>
</tbody>
</table>

(-ve) less than 15.0 u/ml, (borderline) btw 15.1 – 59.99 u/ml & (+ve) more than 59.9 u/ml.

4.1.3.5. Anticardiolipin antibodies:

Anticardiolipin antibodies IgG were positive in 12(12%) of women with RPL, and in 3(5.8%) of controls, there was statistically significant difference when compared with controls, P value (0.000) table (6). The prevalence of IgM anticardiolipin antibodies were positive in 7(7%) of patients with RPL, and in 2(3.9%) of controls, P value (0.000). There was statistically significant correlation between the presence of anticardiolipin IgG antibodies and RPL, P value (0.000). In addition, between anticardiolipin IgM antibodies and RPL, P value (0.000) table (7).

Table: 6
Distribution of Anticardiolipin Antibodies (IgG) in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Anticardiolipin</th>
<th>Patients (n =100)</th>
<th>Controls (n=51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>67 (67%)</td>
<td>26 (51%)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12 (12%)</td>
<td>3 (5.8%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Borderline</td>
<td>21 (21%)</td>
<td>22 (46%)</td>
<td></td>
</tr>
</tbody>
</table>

(-ve) less than 30.0 u/ml, (borderline) btw 33.1 – 237.9 u/ml & (+ve) more than 237.9 u/ml.

Table: 7
Distribution of Anticardiolipin Antibodies (IgM) in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Anticardiolipin</th>
<th>Patients (n =100)</th>
<th>Controls (n=51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>89 (89%)</td>
<td>46 (90.2%)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>7 (7%)</td>
<td>2 (3.8%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Borderline</td>
<td>4 (4%)</td>
<td>5 (9.8%)</td>
<td></td>
</tr>
</tbody>
</table>

(-ve) less than 44.0 u/ml, (borderline) btw 44.1 – 169.9 u/ml & (+ve) more than169.9 u/ml.
The serum of patients and controls that contain specific antibodies against the cardioliopin with level between negative and positive results of controls were classified into a borderline group. Table 5 and table 6.

4.1.3.6. Basic coagulation screening:

The screening tests for coagulation system were done by prothrombin time PT and activated partial thromboplastin time APTT. 8(8%) of women with RPL had prolonged APTT, and 0(0%) of controls, there was statistically significant correlation when compared with the control group P. value (0.000) table 8. The prolonged APTT results were indicating for the presence of antiphospholipid antibodies interfered with APTT, Table 8.

On the other hand, PT was prolonged in 6 (6%) of women with RPL, and in 0(0%) of controls, there was statistically significant correlation when compared with the control group P. value (0.000) as shown in table 8, whereas the remaining patients (94%) and controls (100%) had normal PT.

4.1.3.7. Platelet counts:

Four percent (4%) of women with RPL, had thrombocytopenia, and 0(0%) of controls, there was statistically significant correlation when compared with the control group P. value (0.000) as shown in table 8, whereas; the remaining patients (96%) and controls (100%) had normal platelet counts. The correlation between the presence of thrombocytopenia and RPL statistically was significant. P value (0.000), Table 8.

Table: 8
Distribution of PT, APTT, and Platelet counts in Women with recurrent pregnancy loss and control Women.

<table>
<thead>
<tr>
<th>Test</th>
<th>Patients (n=100)</th>
<th>Controls (n=51)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (prolonged)</td>
<td>6 (6%)</td>
<td>0 (0%)</td>
<td>0.000</td>
</tr>
<tr>
<td>APTT (prolonged)</td>
<td>8 (8%)</td>
<td>0 (0%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Platelet counts (&lt;150000c/cu mm)</td>
<td>4 (4%)</td>
<td>0 (0%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Platelet counts (&gt;400000c/cu mm)</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

(Prolonged mean more than 14 sec. for PT and 35 sec. for APTT).
Table: 9
Distribution of abortion and stillbirth by trimester in Women with recurrent pregnancy loss.

<table>
<thead>
<tr>
<th>Abortion</th>
<th>Patients (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester</td>
<td>35 (35%)</td>
</tr>
<tr>
<td>Second trimester</td>
<td>7 (7%)</td>
</tr>
<tr>
<td>Third trimester</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>First and second trimester</td>
<td>39 (39%)</td>
</tr>
<tr>
<td>First and third trimester</td>
<td>5 (5%)</td>
</tr>
<tr>
<td>Second and third trimester</td>
<td>7 (7%)</td>
</tr>
<tr>
<td>First and second and third trimester</td>
<td>5 (5%)</td>
</tr>
</tbody>
</table>
4.2. Discussion:

Our study reports the frequency of inherited and acquired thrombophilia in a group of Sudanese women with RPL. The cause of RPL is largely unclear. Epidemiological studies suggested that it might be multifactorial with involvement of environmental and genetic factors.

Thrombophilia in its hereditary or acquired form has been considered as a possible cause of RPL. The rate of thrombophilia varies from study to another due to different selection criteria of the patients.

Deficiency of clotting inhibitors, such as protein S, protein C and antithrombin III has been associated with RPL since 1996. (Preston F E, et al, 1996)

Inherited thrombophilia may be due to a gene defect or deficiency of clotting inhibitors leading to a hypercoagulable tendency.

Gene defect frequently associated with RPL in our current study are factor V Leiden G1691A and prothrombin G20210A gene.

The prevalence of homozygous factor V Leiden mutation in our study was seen in 9(9%) of patients with RPL and in 0(0%) of controls, there was statistically significant correlation when compared with the control group. P value (0.000).

Heterozygous factor V Leiden gene mutation was detected in 11(11%) of patients with RPL and in 2(3.8%) of controls, and there was statistically significant correlation when compare with the control group. P value (0.000).

Our findings are in agreement with different studies in the literature review.

Kovalevsky G (2013) who conducted a study among Russian women with RPL, he reported that significant association between RPL and FVL gene mutation was found and his statistical analysis show 2.0 (95% confidence interval, 1.5-2.7). P. value (0.001). (Kovalevsky G, et al, 2013)

Our results were also in agreement with Gihan and Osama, (2013) when they studied the causes of RPL among 142 Saudi women with 2 or more RPL and 72 healthy controls, they found significant difference in prothrombin gene mutation when compared with controls, P. value (0.0001), and concluded that recurrent pregnancy loss
among Saudi women was strongly associated with thrombophilic mutations related to prothrombin gene. (Gihan EH, et al, 2013)

Concerning FV Leiden gene mutation in this study, our results did not agree with different studies from different countries.

Our findings were not in agreement with a study done by Majid, (2013) among Iranian women with recurrent pregnancy loss. He reported that no FV Leiden gene mutation in cases and controls was found, and concluded that FV Leiden gene mutation is not play a significant role in recurrent pregnancy loss in Iranian women. (Majid, et.al, 2013)

Omneya M. Osman (2015) conducted a research on inherited thrombophilia and early pregnancy loss among Egyptian women. 100 patients of unexplained RPL and 43 healthy controls were investigated for inherited thrombophilic factors FV Leaden G1691A, prothrombin G20210A and MTHFR gene mutation. She found that heterozygous FVL was found in 58% of patients with RPL and in 41.9% of controls. P. value (0.105). (Omneya M. Osman, et al, 2015)

Also,Yıldız Gazi (2012), conducted a study on inherited thrombophilia with recurrent pregnancy loss in Turkish women. He reported that no statistically significant difference was found between the study and the control groups in terms of the prevalence of Factor V Leiden. P. value (0.534) (Yıldız G, et al, 2012)

Alireza. Parand (2013) conducted a study on inherited thrombophilia and recurrent pregnancy loss, among 90 randomly selected Iranian women with three or more consecutive miscarriage and 44 healthy women with no history of pregnancy loss as controls, to determine the frequency of FV Leaden, prothrombin gene mutation as well as protein C and protein S deficiency. He found that heterozygous FVL was found in 15(13.5%) of patients with RPL and in 6(2.6%) of controls. P. value (0.081). (Alireza. Parand, et al, 2013)

Asaad M A. (2016) has conducted a study on point mutation in factor V Leiden G1691A and prothrombin G20210A gene mutation and effect on coagulation profile and frequency of recurrent pregnancy loss among Sudanese women. He found that the prevalence of heterozygous FVL gene mutation in women with RPL was found to be 8% but in control group, it found to be 6.4%. P. value (0.66). (Asaad M A. et al, 2016)
Prothrombin gene G20210A mutation was found in 16(16) % of women with recurrent pregnancy loss RPL, and in 3(5.8) % of all controls. There was statistically significant correlation when compared with controls. P value (0.000).

Homozygous prothrombin gene mutation was detected in 11(11%) of patients with RPL, and in 3(5.8%) of controls. Statistically significant differences between patients and the controls were found. P. value (0.000).

Heterozygous prothrombin gene mutation was found in 5(5%) of patients with RPL, and in 0(0%) of controls. There was statistically significant correlation when compared with controls. P value (0.000).

Our findings are in agreement with different studies in the literature review.

Kovalevsky G (2013) who conducted a study among Russian women with RPL, he reported that significant association between RPL and prothrombin G20210A gene mutation was found and his statistical analysis show 2.0 (95% confidence interval, 1.0-4.0), P. value (0.03). (Kovalevsky G, et al, 2013)

Our results were also in agreement with Gihan and Osama, (2013) when they studied the causes of RPL among 142 Saudi women with 2 or more RPL and 72 healthy controls, significant differences in prothrombin gene mutation when compared with controls was found, P. value (0.0001), and concluded that recurrent pregnancy loss among Saudi women was strongly associated with thrombophilic mutations related to prothrombin gene mutation. (Gihan EH, et al, 2013)

Our findings were not in agreement with different studies in the literature review one of these done by Omneya M. Osman (2015). She conducted a research on inherited thrombophilia and early pregnancy loss among Egyptian women. 100 patients of unexplained RPL and 43 healthy controls were investigated for inherited thrombophilic factors FV Leaden G1691A, prothrombin G20210A and MTHFR gene mutation. She found that heterozygous prothrombin gene mutation was found in 11% of patients and in 4.7% of controls. P. value (0.34). (Omneya M. Osman, et al, 2015)

Our results regarding prothrombin gene G20210A mutation did not agree with Yıldız Gazi, (2012), who conducted a study about inherited thrombophilia with recurrent pregnancy loss in Turkish women. He found that statistically insignificant difference
was found between patients and control groups in terms of the prevalence of prothrombin G20210A gene mutation. P. value (0.452). (Yildiz G, et al, 2012)

Our results were different with the findings of Alireza. Parand (2013) who conducted a study on inherited thrombophilia and recurrent pregnancy loss, among 90 randomly selected Iranian women with three or more consecutive miscarriage and 44 healthy women with no history of pregnancy loss as controls, he determine the frequency of FV Leaden, prothrombin gene mutation as well as protein C and protein S deficiency. He found that heterozygous prothrombin gene was found in 2(0.9%) of patients and in 1(0.4%) of controls. P. value (0.58). (Alireza. Parand, et al, 2013)

Also regarding prothrombin gene mutation, our findings were not in agreement with a study done by Majid, (2013) among Iranian women with recurrent pregnancy loss. He reported that no prothrombin gene mutation in cases and controls was found, and concluded that prothrombin gene G20210A mutation is not play a significant role in recurrent pregnancy loss in Iranian women. (Majid, et.al, 2013)

Asaad M A. (2016) has conducted a study on point mutation in factor V Leiden G1691A and prothrombin G20210A gene mutation and effect on coagulation profile and frequency of recurrent pregnancy loss among Sudanese women. He found that the prevalence of heterozygous prothrombin gene was 3% among cases with RPL, but no mutant gene was detected among control group. P. value (0.091). (Asaad M A. et al, 2016)

The prevalence of IgG antiphospholipid antibodies was 10(10%) in our patients and in 2(3.8%) of controls, there was statistically significant deference between the patients and controls. P value (0.000). The relation between antiphospholipid (IgG) antibodies and recurrent pregnancy loss in our study was statistically significant. P value (0.000).

The prevalence of IgM antiphospholipid antibodies in our patients was 11(11%) and in 1(1.9%) of controls. P value (0.007). The IgM antibodies was detected in the serum of 59% of patients that were divided into sub groups named as borderline group and in 68% of borderline group of the controls. The relation between antiphospholipid (IgM) and recurrent pregnancy loss in our study was statistically significant. P value (0.007).

Our results obtained were in agreement with the results of Ahmed Bolad, (2013) who studied and determined antiphospholipid antibodies and thrombophilia among
Sudanese women with recurrent pregnancy loss, he found that the frequency of APA was 20% of patients with RPL. P. value (0.03). (Ahmed B, et al, 2013)

Our results concerning antiphospholipid antibodies (APA) were in agreement with Vora SA (2008), who study 430 Indian women with RPL and 100 normal control women when screened for the presence of antiphospholipid antibodies (APA), anticardiolipin antibodies (ACA), and annexin V. He found that antiphospholipid antibodies APA was positive in 12.2% of patients with RPL. P. value (<0.05). (Vora SA, et al, 2008)

Also our results were similar with Sater MS (2013), who conducted a study involving 277 women with RPL and 288 healthy control women. Antiphospholipid IgG antibodies, anticardiolipin IgG/IgM antibodies and anti prothrombin IgM antibodies were measured. Antiphospholipid IgG antibodies were found in 33.5% patients with RPL, and in 1.5% of controls. P. value (0.001). (Sater MS, et al, 3013)

Anticardiolipin antibodies IgG in this study was detected in 12(12%) of patients with recurrent pregnancy loss RPL, and in 3(5.8%) of controls, and there was statistically significant correlation when compared with controls. P value (0.000).

Anticardiolipin antibodies IgM in our study was detected in 7(7%) of patients with recurrent pregnancy loss RPL, and in 2(3.8%) of controls, and there was statistically significant correlation when compared with controls. P value (0.000).

Our results concerning anticardiolipin IgG antibodies were in line with Safinaz A (2016) who studied eighty-three pregnant Sudanese women with history of RPL and eighty healthy controls. They were tested for anticardiolipin IgG antibodies. She found that the frequency of positive anticardiolipin antibody among Sudanese women with RPL was 5(6%) while it was 0.00% in control group. P. value (0.000). (Safinaz. A,et al, 2016)

Our results obtained were in agreement with the results obtained by Ahmed Bolad, (2013) who reported that significant correlation between the presence of APA and ACA/IgG and RPL was found. P. value (0.03) and (0.04) respectively. (Ahmed B, et al, 2013)

Our findings regarding anticardiolipin antibodies (ACA) were in agreement with Vora SA (2008), who study 430 Indian women with RPL and 100 normal control women
when screened for the presence of antiphospholipid antibodies (APA), anticardiolipin antibodies (ACA), and annexin V. He found that ACA IgG/IgM antibodies were detected in 27.9% of patients with RPL. P. value (<0.05). (Vora SA, et al, 2008)

Also our results were similar with Sater MS (2013), who conducted a study involving 277 women with RPL and 288 healthy control women. Antiphospholipid IgG antibodies, anticardiolipin IgG/IgM antibodies and anti prothrombin IgM antibodies were measured. Anticardiolipin IgG antibodies were seen in 36.5% of patients with RPL and in 4.5% of controls. P. value (0.001). (Sater MS, et al, 3013)

Our results were in line with Safinaz A (2016), who tested eighty-three pregnant Sudanese women with history of RPL, who attended to hospital and eighty healthy controls for anticardiolipin IgG antibodies. She found that the frequency of positive anticardiolipin antibody among Sudanese women with RPL was 5 (6%) while it was 0.00% in control group. P. value (0.000). (Safinaz. A, et al, 2016)

The role of APA in reproductive failure appears to be more diverse. The adhesion properties of phospholipids play a major role in the physiology of reproduction; APA can interfere with the phospholipid adhesion, which may result in reproductive failure. ACA are known to be associated with specific autoimmune diseases such as SLE and with overt thromboembolic phenomena, including recurrent pregnancy loss. The mechanism of action is thought to be an increase in the hypercoagulable state via inability to activate protein C, inhibition of prostacyclin, and endothelial wall and platelet membrane damage.

The role of platelets as an important etiology in RPL had been explored in this study. The thrombocytopenia was found in 4(4%) of women with RPL in our study, and in 0(0%) of controls. P value (0.000).

These results were in line to the reported frequency of the thrombocytopenia (8%) in 100 Sudanese women with history of RPL by Ahmed Bolad (2013); thrombocytopenia in those women was presumably due to anti-platelet auto-antibodies directed against platelet-bound B2GPI. The correlation between the presence of thrombocytopenia and recurrent miscarriage was significant. P. value (0.001). (Ahmed Bolad, et al, 2013).
Prolonged APTT in women with RPL in our study was found in 8(8%) and in 0(0%) of controls, were affected significantly, P value (0.000), where prothrombin time PT is found in 6% of women with RPL and in 0% of controls, P value (0.000).

This is similar to the normal results reported by O. A Awodu (2003) among 100 Nigerian women with recurrent pregnancy loss to assess haemostasis by using activated partial thromboplastin time APTT. Prolonged APTT was seen in 3% of women with RPL. P. value (0.018) (O A Awodu, et.al., 2003)

Our results concerning APTT were in line with that reported by Ahmed Bolad (2013), who mentioned that APTT was prolonged in 8% of patients with RPL. Significant correlation obtained when compared with controls. P. value (0.018). (Ahmed B, et al, 2013)

Our results regarding APTT were in agreement with study reported by Cauchi (2003) and others in 145 French showed that only 7% of women with recurrent pregnancy loss had prolonged APTT. P. value (0.019) and that was due to the presence of antiphospholipid antibodies. (Cauchi, MN, et al, 2003)

The obtained results of current study regarding PT and APTT were not agree with Asaad M A (2016), who mentioned that prothrombin time (PT) and activated partial thromboplastin time (APTT) in women with recurrent pregnancy loss (RPL) were not affected significantly. P. value (0.93) and (0.69), respectively. (Asaad M A. et al, 2016)
5. Conclusion & Recommendations

5.1. Conclusion:

Concerning hereditary and acquired thrombophilia the study proved that:

- FV Leiden G1691A and prothrombin G20210A gene mutation was significantly associated with recurrent pregnancy loss (RPL).

- Our analysis show significant association between antiphospholipid antibodies, anticardiolipin antibodies, and recurrent pregnancy loss (RPL).

- PT, APTT, and platelet counts in women with recurrent pregnancy loss (RPL) were affected significantly.
5.2. Recommendations:

- More health education to pregnant women to improve their conditions to prevent DVT and RPL.

- Patients with RPL should be top priority in clinical situation so they could consume and have baby.

- Increase the awareness among doctors as well as patients with the relation between recurrent pregnancy loss and thrombophilia.

- Patients with pregnancy loss should be investigated routinely for antiphospholipid syndrome.

- Patients with recurrent pregnancy loss should be referred to specialized clinics or research laboratory where they can be investigated for hereditary thrombophilia and antiphospholipid syndrome.

- A study involving a bigger number of women with recurrent pregnancy loss to determine the relation between adverse pregnancy outcome and acquired or hereditary thrombophilia is needed.

- Patients with recurrent fetal loss should be tested for clotting disorders, even in the absence of clinical signs because there were some studies concluded that many positive thrombophilic causative finding without any clinical signs.

- Evaluation of thrombophilia may be useful in the improvement of gynecological care of women with recurrent pregnancy loss and accurate knowledge of all significant complications in these women regarding thrombophilia and formulate a plan to diagnosis and treatment of these conditions.
References


Empson, M; Lassere, M; Craig, J; Scott, J (Apr 18, 2005). "Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant.". Cochrane database of systematic reviews (Online) (2): CD002859.


Wong, LF; Porter, TF; Scott, JR (Oct 21, 2014). "Immunotherapy for recurrent miscarriage". The Cochrane database of systematic reviews. 10 (10): 112.


Appendices

Appendix 1


Questionnaire

Personal Data:
Name (optional) …………………… Age …… yrs  Number …………
Residence ………………………………………………………………….
Occupation …………………. Phone …………………
Consanguinity:  No ( )  Second degree ( )  Third Degree ( )

Obstetric History:
No. of pregnancies ( ) No of living babies ( ) No of lost babies ( )
No of macerated stillbirth MSB (……… Wk) ( ) any cause?
No of fresh stillbirth FSB (……wks) ( ) Any cause?
No of early newborn death END (from birth to 1wk) ( ) any cause?
No of later newborn death LND (2 – 4 Wk) ( ) any cause?
No of later death LND (28 days) ( ) any cause?
No of abortion (loss before 24 wk) ( ) First trimester ( ) second trimester ( ) second trimester ( )

Thrombophilia:
History of DVT: yes ( ) No ( ). If yes number of times ( ).

Family history of DVT: yes ( ) No ( )

**Lab investigations:**

Platelet counts ................................................................. c/cumm

PT: ( ) sec APTT: ( ) sec.

Antiphospholipid antibodies (IgG):
Positive ( ) Negative ( ) Borderline ( )

Antiphospholipid antibodies (IgM):
Positive ( ) Negative ( ) Borderline ( )

Anticardiolipin antibodies (IgG):
Positive ( ) Negative ( ) Borderline ( )

Anticardiolipin antibodies (IgM):
Positive ( ) Negative ( ) Borderline ( )

Factor V leaden A1691G gene:
Wild type ( ) homozygous GG ( ) heterozygous AG ( )

Factor II prothrombin A20210G gene:
Wild type ( ) homozygous GG ( ) heterozygous AG ( )

Date …. /……… / ………………… Signature ……………………...
Appendix 2

Consanguinity for patients

Fig: 4
Consanguinity of patients and their couples.

Fig: 5
Times of recurrent abortion in patients.
Appendix 3

Sample size:

100 patients and 51 healthy controls.

Samples were calculated according to the following equation:

Since the global reported-risk of recurrent miscarriage was 1%; by applying the classical equation to estimate the sample size

\[ n = \left( \frac{Z\sigma^2}{d} \right)^2 \]

\( Z = \) was the value of specified level of significance, in this study the level of significance was 1% which gives a value of \( Z = 0.799 \)

\( \sigma = \) was the standard deviation of the cases, the value of S.D was estimated to be 2.5.

\( d = \) was the difference between the case mean and control mean was estimated not to exceed 0.5.

Hence, applying these values

\[ n = \left( 0.799 \times 2.5^2 / 0.5 \right)^2 = 100 \text{ women} \]