Determination of the risk of Venous thromboembolism (VTE) in women on injectable contraceptives using d-dimer, fibrinogen levels and platelet count.

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ABSTRACT

Venous thromboembolism is a disease that includes both deep vein thrombosis and pulmonary embolism. It is a common lethal disorder that affects hospitalized and non-hospitalized patients. This condition however is often overlooked. Oral contraceptive use is a widely-accepted risk factors for venous thromboembolism. The role of injectable contraceptives as a risk factor for venous thromboembolism is controversial. The aim of the study was to determine the risk of thrombosis associated with the use of injectable contraceptives in Port Harcourt, Nigeria. This is a hospital based cross sectional comparative study, in which d-dimer, fibrinogen levels and platelet count were evaluated in serum, citrated plasma and EDTA samples of 80 women on injectable contraceptives and 80 controls who were not on hormonal contraceptives. D-dimer was analyzed using ELISA based methods, fibrinogen was analyzed using modified Clauss method while platelet count was assessed using a full blood count auto analyzer. The mean d-dimer levels between the cases and controls did not differ significantly (p-value 0.143). The frequency of occurrence of an elevated d-dimer level was also not significant (p-value 1.000). The mean fibrinogen levels in cases and controls were not statistically significant. (p-value 0.145). None of the cases nor controls had a high level of fibrinogen. Although the mean platelet count was normal in both populations, the cases had a significantly lower platelet count (214.16 ± 65.42 X 10⁹/L) compared to the controls (244.18 ± 77.34 X 10⁹/L), p = 0.03; however the frequency of having a high platelet count was not significant. Normal platelet counts, d-dimer and fibrinogen suggest that the risk of thrombosis in women on injectable contraceptives is not increased, therefore injectable contraceptive use may not be associated with increased thrombotic risk. It is therefore recommended that routine screening of women on injectable contraceptives for VTE may be unnecessary. Larger population-based local studies to validate these results are required.

Keywords: Venous thromboembolism, contraceptives, d-dimer, fibrinogen levels and platelet count.
VENOUS THROMBOEMBOLISM (VTE) is a disease condition characterized by the formation of blood clots in the vein [1, 2, 3]. This may manifest itself either as deep vein thrombosis or pulmonary embolism [4, 5, 6]. VTE occurs both in hospitalized and non-hospitalized patients [4, 5]. The overall prevalence of VTE in Nigeria is estimated to be 2.9%. Risk factors for the development of VTE include increased age, immobility, cancer, hormonal contraceptives, obesity, lower limb and pelvic surgery, lower limb trauma and vascular diseases [6]. Generally, increased levels of some coagulation factors (factor VII, factor VIII, von Willebrand factor, prothrombin) and defects in the natural anticoagulants (Protein C, Protein S and antithrombin) are associated with an increased risk of thrombosis [7]. Previous studies have shown that hormonal contraceptives have effects on thrombus formation, especially the combined oral contraceptive pills. Injectable contraceptives were reported to be associated with a small risk of venous thromboembolism [8, 9, 10]. There are controversies on the association between thromboembolism and progesterone-only (injectable contraceptives). One school of thought believes there is an association. While the other does not. There is however a paucity of studies done in our environment on the risk of developing thrombosis with the use of injectable contraceptives [11].

PROGESTERONE-ONLY HORMONAL CONTRACEPTION

Hormonal contraceptive usage has been associated with changes in some haemostatic parameters. Studies have shown an association between combined oral contraceptives (COC) usage and an increased risk of venous thromboembolism (VTE) [12]. VTE could present as deep vein thrombosis or pulmonary embolism. The use of COC increases the risk of VTE by two to three folds. The effect of VTE on non-COC users has been underestimated. This literature review aims to provide an overview of haemostasis and its abnormalities, detailing hormonal contraceptives and thrombotic effects. It also cites observational studies showing an increased risk of VTE induced by injectables contraceptives [13].

COAGULATION SYSTEM

Haemostasis involves the body’s physiological response to injury, through cessation of bleeding, thus ensuring that blood is kept in its fluid state and the vascular integrity preserved. Procoagulant and anticoagulant factors, as well as the vessel wall, soluble and cell bound constituents are required to maintain blood in a fluid state in an intact vasculature. When haemostasis fails, haemorrhage or thrombosis may occur. The haemostatic system comprises of platelet aggregation, coagulation and
fibrinolysis also known as primary, secondary and tertiary haemostasis [14]. In primary haemostasis, once there is a disruption in the vascular endothelium which may be due to trauma, inflammation, neoplasm or toxins, the vessel responds by contracting to reduce blood flow and to increase platelet interaction with vessel wall which is necessary for platelet plug formation. Platelets are attracted to and adhere to the subendothelial extracellular matrix that became exposed as a result of injury. This platelet adherence is mediated by von Willebrand [15].

OBJECTIVE OF THE RESEARCH
To determine the risk of VTE in women on injectable contraceptives using d-dimer, fibrinogen levels and platelet count.

RATIONALE FOR STUDY
This study seeks to assess the risk of venous thromboembolism in women on injectable contraceptives living in Port Harcourt attending University of Port Harcourt Teaching Hospital and Braithwaite Memorial Hospital family planning unit, thereby providing a baseline data for monitoring women on injectable contraceptives.

MATERIALS AND METHODS
Study Location
This study was conducted at the University of Port Harcourt Teaching Hospital (UPTH), a Federal Government tertiary institution and Braithwaite Memorial Specialist Hospital (BMSH), a state owned tertiary institution. Both tertiary health institutions are situated in Port Harcourt, Rivers State and have several specialties including Haematology, Obstetrics & Gynaecology and Paediatrics. These hospitals also serve as the main referral centres for Rivers State and its environs.

STUDY DESIGN
This is a cross sectional comparative study.

STUDY POPULATION
Women on injectable contraceptives attending the family planning clinic in two tertiary health care centres – University of Port Harcourt Teaching Hospital and Braithwaite Memorial Hospital in Port Harcourt metropolis comprised the subjects. While the controls comprised of apparently healthy women between the ages of 18 and 45 who were not on any form of contraceptives and who gave their informed consent to participate.

Inclusion Criteria for the Cases
1. Attendees of the family planning clinic on injectable contraceptives.
2. Non-pregnant women of reproductive age (18-45) who have been on injectable contraceptives.
Exclusion Criteria for the Cases

1. Women with chronic illness (except hypertension and diabetes mellitus), malnutrition or on anticoagulant therapy.

The controls were apparently healthy women (except those with a history of diabetes mellitus or hypertension) between the ages of 18-45 who gave consent to participate in the study and had never been on hormonal contraceptives.

ETHICAL CONSIDERATION

Ethical approval was obtained from University of Port Harcourt Teaching Hospital. The study objectives and procedure for sample collection and follow up was explained to each participant before signing the consent form. Confidentiality and animosity were maintained in the course of the research.

SAMPLE SIZE ESTIMATION

Consecutive sampling was used.

The sample size was calculated from the formula $n = \frac{Z^2pq}{d^2}$. Where $n = \text{the required sample size (when population is } >10,000)$. $Z= \text{the standard normal deviation, usually set at 1.96 which corresponds to 95% confidence interval.}$

$P= \text{the proportion of the target population estimated to have a particular characteristics.}$ In this research, data from United Kingdom revealed that 3% of women are on injectables, there are varying or controversial literature concerning the number of women on injectable contraceptives in Nigeria. Some studies gave 7.9% while others gave 3.8%. However due to absence of local prevalence in Port Harcourt, 5% prevalence was used to calculate the sample size. $Q = 1.0 - p = 1.0 - 0.05 = 0.95$ $D = \text{the degree of accuracy which is usually set at 0.05.}$ With this formula $n = 72$ Applying this formula, a minimum sample size of 72 was determined. However given 10% non-response rate, a minimum sample size of appropriately 80 was recruited into the study. The control sample was taken from women within the ages of 18-45 years that are not on hormonal contraceptives.

SAMPLE COLLECTION AND PREPARATION

After filling the questionnaire, venous blood was collected by venipuncture following standard sterile procedure into 2 vacuum tubes. Blood measuring 5ml was put into an EDTA bottle and 4.5ml into a bottle containing 0.5ml of 0.129M sodium citrate to ensure adequate blood-anticoagulant ratios. Samples were properly mixed after sample collection. Samples were also collected for C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).
levels assessment, as controls for d-dimer and fibrinogen testing to rule out acute phase reaction. Those with high CRP or ESR values were excluded. The sample in the EDTA bottle was used to evaluate the platelet count of the participants. Samples in the sodium citrate bottle which was for fibrinogen assay and d-dimer testing were centrifuged at 3500 rpm for seven minutes and stored at room temperature. If testing was not done within four hours of collection, the plasma was separated immediately and stored at -20°C for two weeks.

Materials
1) Auto haematology analyzer (model: BC0800)
2) Glass test tubes
3) Stop clock
4) Water bath
5) Precision pipettes (capable of delivering between 0-200ul)
6) Racks
7) Microplate reader capable of measuring absorbance at 450nm
8) Precision pipettes to deliver 2ul to 1ml volumes
9) Adjustable 1-25ml pipettes for reagent preparation
10) 100ml and 1litre graduated cylinders
11) Absorbent paper
12) Distilled water or deionized water
13) Log-log graph paper or computer and software for ELISA data analysis
14) Test tubes to prepare standard or sample dilutions

Reagent
1) Calibration/standard plasma
2) Control plasma
3) Bovine Thrombin solution
4) Imidazole Buffer
5) Distilled water
6) D-dimer Microplate of 96 wells coated with antihuman d-dimer
7) Wash Buffer Concentrate
8) Assay Diluent buffer
9) Standard Protein of Human d-dimer
10) Detection Antibody d-dimer of biotinylated anti-human d-dimer
11) HRP-Streptavidin Concentrate
12) TMB One-step Substrate Reagent
13) Stop solution (Sulfuric acid)

METHODOLOGY
D-Dimer and Fibrinogen testing was carried out in duplicate for all the samples. Full blood count was carried out on the sample in an EDTA bottle using an autoanalyzer. The normal reference range of platelet count is 90-350 x 10^9/L.
Okpara test virtually insensitive to heparin (10 I.U./mL) and enhance clot detection.

Fibrinogen assay procedure

1) Standard plasma was diluted with imidazole buffer solution. This gave a range of fibrinogen concentration (i.e. 1 in 5, 1 in 10, 1 in 20 and 1 in 40).

2) 200ul of the solution was warmed in a water bath, at 37°C.

3) 100ul of thrombin solution was added and the clotting time measured.

4) A plot of the clotting time against fibrinogen concentration in seconds and g/l respectively was made on a log/log graph paper.

5) 1 in 10 dilution of each test samples was made with imidazole buffer solution.

6) 200ul of this test sample dilutions was clotted with 100ul of thrombin solution and clotting times was noted.

7) The fibrinogen concentrations of each test sample was read off the graph.

Normal range approximately 1.5 - 4.0g/l

D - Dimer Assay

D-dimer testing was measured with IMUCLONE® D-dimer ELISA method following the manufacturers instruction

1) 100ul of sample was added to each well

2) The microplate was incubated at room temperature for 2.5 hours

3) The microplate was washed 4 times with wash solution, decanted and blotted with absorbent paper.

4) 100ul prepared biotin antibody was added to each well.

5) The microplate was then incubated at room temperature for 1 hour.

6) The microplate was once again washed 4 times with wash solution, decanted and blotted with absorbent paper.

7) 100ul prepared Streptavidin solution was then added.

8) Thereafter the microplate was incubated at room temperature for 45 minutes.

9) Subsequently it washed 4 times with wash solution, decanted and blotted with absorbent paper.

10) 100ul TMB One-Step Substrate Reagent was added to each well.

11) The microplate was then incubated at room temperature in the dark for 30 minutes.

12) 50ul of Stop solution was added to each well.

13) The Absorbance was read at 450nm immediately.

Plasma normal level < 200ng/ml

PRECAUTIONS

1) Contact was avoided with the skin and mouth, even when the reagent did not contain reactive components.
Donor blood and materials used for the preparations were handled as potentially infectious agents, although the blood had been found to be sero-negative to HIV, HCV and HbsAg.

DATA ANALYSIS

Data entry and analysis were done using the Statistical Package for Social Sciences (SPSS) software version 22.0. The quantitative variables such as d-dimer, fibrinogen and platelet values were summarized as means ± standard deviation while frequencies and proportions were used to summarize categorical variables.

Differences in means between groups (women on injectable and controls) were compared using the student t-test. Chi-square and Fishers exact test were used as appropriate to compare differences in proportions. Probability values less than 0.05 (p < 0.05) were considered as significant.

RESULTS

A total of 160 women were recruited for this study, comprising of 80 women on injectable contraceptives (cases) and 80 women not on any form of contraceptives (controls). Both groups were within the 18-45 years age bracket. The study was conducted over a period of 24 months (July 2015 to June 2017).

Social Demographics

The largest number of cases (39 cases) were found in the 31-40 years group, constituting 48.8% of that population. The 21-30 year group constituted the largest number (48) among the controls. There was a significant difference between the ages of the cases and controls (p-value- 0.0001). Ninety nine percent of cases were married while 85.00% of controls were single. Tertiary level of education was attained by 46 (57.50%) of the cases while all the controls had tertiary level of education. Eighty five percent of the cases were employed while 80.0% of the controls were unemployed as shown in table I.

Risk of VTE based on D-dimer, Fibrinogen and Platelet Count in cases and controls

Among the women on injectable contraceptives using elevated d-dimer levels, one (1.2%) of the case group was found to be at risk of VTE, while two (2.5%) of the controls were at risk. None of the cases nor control had a high fibrinogen level therefore none was at risk of VTE. Among the 80 cases, one (1.2%) was at risk using elevated platelet count as a risk factor, and three (3.8%) of the controls were at risk. There was no correlation between the two groups (P = 0.620) as shown in table II.

Risk of VTE among cases and controls

One of the 80 women on injectable contraceptives (cases) was at risk of VTE is one (1.2%) while 3(3.8%) of the controls were at risk of VTE, based on d-
dimer, fibrinogen levels and platelet count. The result showed no statistically significant difference between the cases and controls (P = 0.620), see Table III.

Relationship between age and risk of venous thromboembolism among cases

In the case group, only 5.30% of the 21-30 years age category was at risk of VTE. Other age groups were not associated with any risk factor for venous thromboembolism (P = 0.238), see Table IV.

Table I: Socio-demographic Characteristics of Respondents

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤20 years</td>
<td>0 (0.00)</td>
<td>19 (23.80)</td>
<td>19 (11.90)</td>
</tr>
<tr>
<td>21 - 30 years</td>
<td>19 (23.80)</td>
<td>48 (60.00)</td>
<td>67 (41.90)</td>
</tr>
<tr>
<td>31 - 40 years</td>
<td>39 (48.80)</td>
<td>7 (8.80)</td>
<td>46 (28.80)</td>
</tr>
<tr>
<td>&gt;40 years</td>
<td>22 (27.50)</td>
<td>6 (7.50)</td>
<td>28 (17.50)</td>
</tr>
</tbody>
</table>

Chi square = 62.956; p-value = 0.0001*

<table>
<thead>
<tr>
<th>Marital status</th>
<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>1 (1.20)</td>
<td>68 (85.00)</td>
<td>69 (43.10)</td>
</tr>
<tr>
<td>Married</td>
<td>79 (98.80)</td>
<td>12 (15.00)</td>
<td>91 (56.90)</td>
</tr>
</tbody>
</table>

Chi square = 114.388; p-value = 0.0001*

<table>
<thead>
<tr>
<th>Educational level</th>
<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>9 (11.30)</td>
<td>0 (0.00)</td>
<td>9 (5.70)</td>
</tr>
<tr>
<td>Secondary</td>
<td>25 (31.20)</td>
<td>0 (0.00)</td>
<td>25 (15.70)</td>
</tr>
<tr>
<td>Tertiary</td>
<td>46 (57.50)</td>
<td>80 (100.00)</td>
<td>126 (78.80)</td>
</tr>
</tbody>
</table>

Fisher’s exact test = 50.030; p-value = 0.0001*

<table>
<thead>
<tr>
<th>Employment status</th>
<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unemployed</td>
<td>12 (15.00)</td>
<td>64 (80.00)</td>
<td>76 (47.50)</td>
</tr>
<tr>
<td>Employed</td>
<td>68 (85.00)</td>
<td>16 (20.00)</td>
<td>42 (26.20)</td>
</tr>
</tbody>
</table>

Chi square = 79.960; p-value = 0.0001*

*Statistically significant
Table II: Risk of VTE Based on D-dimer, Fibrinogen and Platelet Count in Cases and Controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>D-dimer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not at risk</td>
<td>79 (98.80)</td>
<td>78 (97.50)</td>
<td>157 (98.10)</td>
</tr>
<tr>
<td>At risk (&gt;200g/l)</td>
<td>1 (1.20)</td>
<td>2 (2.50)</td>
<td>3 (1.90)</td>
</tr>
<tr>
<td><em>p-value = 1.000</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not at risk</td>
<td>80(100.00)</td>
<td>80(100.00)</td>
<td>160(100.00)</td>
</tr>
<tr>
<td>At risk (&gt;4.0g/l)</td>
<td>0(00.00)</td>
<td>0(00.00)</td>
<td>0(00.00)</td>
</tr>
<tr>
<td>Platelet count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not at risk</td>
<td>79 (98.80)</td>
<td>79 (96.20)</td>
<td>156 (97.50)</td>
</tr>
<tr>
<td>At risk (&gt;350X10⁹/L)</td>
<td>1(1.20)</td>
<td>3 (3.80)</td>
<td>4 (2.50)</td>
</tr>
<tr>
<td><em>p-value = 0.620</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*None of the cases nor controls were at risk of VTE*

Table III: Risk of VTE among Cases and Controls

<table>
<thead>
<tr>
<th>Category</th>
<th>Yes</th>
<th>No</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Cases</td>
<td>1(1.20)</td>
<td>79(98.80)</td>
<td>80 (100.00)</td>
</tr>
<tr>
<td>Control</td>
<td>3(3.80)</td>
<td>77(96.20)</td>
<td>80 (100.00)</td>
</tr>
<tr>
<td>Total</td>
<td>4(2.50)</td>
<td>156 (97.50)</td>
<td>160 (100.00)</td>
</tr>
</tbody>
</table>

*p-value = 0.620*
Table IV: Relationship between Age and Risk of VTE among Cases

<table>
<thead>
<tr>
<th>Variables</th>
<th>Yes n (%)</th>
<th>No n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 – 30 years</td>
<td>1 (5.30)</td>
<td>18 (94.70)</td>
<td>19 (100.00)</td>
</tr>
<tr>
<td>31 – 40 years</td>
<td>0 (0.00)</td>
<td>39 (100.00)</td>
<td>39 (100.00)</td>
</tr>
<tr>
<td>&gt;40 years</td>
<td>0 (0.00)</td>
<td>22 (100.00)</td>
<td>22 (100.00)</td>
</tr>
<tr>
<td>Total</td>
<td>1 (1.20)</td>
<td>79 (98.8)</td>
<td>80 (100.0)</td>
</tr>
</tbody>
</table>

Fisher’s exact = 2.672; p-value = 0.238

DISCUSSION

Venous thromboembolism is associated with high morbidity and mortality rates. Injectable contraceptives are generally believed not to be associated with any thrombotic risk, but there are conflicting reports on thrombotic risk in women on injectable contraceptives [6]. Some researchers have reported an increased risk of venous thromboembolism. However most of the available studies were conducted in non-African populations. There is paucity of studies on this in our environment. In this study of apparently healthy women, between the ages of 18 and 45 years, the mean ages of the cases and controls were significantly different with the cases being older than the controls, although they were both within the age range specified for the study. More married women were on injectable contraceptives than those in the control group, only one single woman was on injectable contraceptives. Using these blood coagulation parameters, it was observed that the frequency of being at risk using high platelet count, d-dimer and fibrinogen were not statistically significant. Based on elevated d-dimer levels, more controls were at risk for VTE than the cases. With regards to the platelet count, there were also more controls at risk for VTE (platelet count >350 X 10⁹/L) than the cases. However, none of the cases nor controls had a risk for VTE based on fibrinogen levels. This is because they all had values <4.0g/L. Several studies [10, 11, 13] evaluated the association between injectable contraceptives and thrombotic risk in a population where thromboembolism had already occurred. Their observation was the use of injectables was a risk factor for developing VTE. Unlike this index study where risk for thrombosis was assessed based on some haemostatic parameters of apparently healthy women who were on injectable contraceptives, they obtained their results from a population with various predisposing factors.
**CONCLUSION**

In conclusion, women on injectable contraceptives in Port Harcourt Nigeria do not have a higher risk of VTE than non-contraceptive users.

**RECOMMENDATION**

The routine screening of women on injectable contraceptives for the risk of venous thromboembolism using serum D-dimer, fibrinogen levels and platelet count is unjustified. Only those with other risk factors should be screened. A larger scale study is highly recommended to help buttress the observations of this study.

**REFERENCES**

13. World Health Organization. Cardiovascular disease and use of oral and injectable progestogen-only contraceptives
