Obazelu et al., 2024
J. Basic Appl. Res biomed 10(1): 1-5

10.51152/jbarbiomed.v10i1.230

JBAAR
Journal of Basic and Applied Research in Biomedicine
ISSN 2413-7014

Original Article

Molecular Detection of Some Risk Factors of Thromboembolism Among Nigerian Women on Hormonal Contraceptives

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Received: 02-02-2024
Revised: 24-03-2024
Published: 02-04-2024

Keywords:
Contraceptives, Thromboembolism, Factor V Leiden, Prothrombin G20210A

Abstract: Hormonal contraceptives are commonly used by women of reproductive age and may be associated with an increased risk of thromboembolism. Any rise in the relative risk of venous thromboembolism related to specific contraceptive formulations could lead to a notably elevated absolute risk. This study seeks to evaluate the potential role of Factor V Leiden (FVL) and Prothrombin G20210A mutations as risk factors for thromboembolism in users of hormonal contraceptives in Benin City. The study population consisted of 50 non-contraceptive users, 50 non-hormonal contraceptive users, 50 progestin-only contraceptive users, and 50 combined oral contraceptive users recruited from Family Planning Clinics in Benin City. Each participant provided aseptically collected venous blood, with 5.0 mL collected in total. 0.5 mL was placed in a container containing 3.1% tri-sodium citrate anticoagulant for the manual determination of Prothrombin time (PT) and activated partial thromboplastin time (APTT). Additionally, 0.5 mL of venous blood was dispensed into a plain container with 0.25 mL DNA-RNA shield solution for the detection of Factor V Leiden and Prothrombin 20210A gene mutations using PCR Duplex Prothrombin 20210 and Factor V. Data analysis was conducted using the statistical package for social sciences (SPSS) version 17.0 software. The findings revealed that the overall mutation carriage rate (AA and AG) for the Prothrombin 20210A gene (5%) was more prevalent than FVL (3%) among the subjects. Furthermore, the mean PT and APTT (in seconds) were significantly lower ($p<0.001$) among women using combined hormonal contraceptives. These findings suggest a potential association between the presence of FVL and FII genes mutations and thromboembolism among Nigerian women using hormonal contraceptives.

Cite this article as: Obazelu, P.A., Emokpae, M.A. Erhabor, O.N. (2024) Molecular Detection of Some Risk Factors of Thromboembolism Among Nigerian Women on Hormonal Contraceptives. Journal of Basic and Applied Research in Biomedicine, 10(1): 1-5 10.51152/jbarbiomed.v10i1.230

INTRODUCTION

Although the prevalence is low, thromboembolism has been linked with the extensive utilization of oral contraceptives among reproductive-age women in our environment and if the relative risk of venous thromboembolism were to increase for some forms of contraception, it may lead to a substantial rise in the absolute risk (Dragoman et al., 2018). In terms of public health, both consumers and policymakers will benefit from molecular identification of such risk variables. Although venous thromboembolism (VTE) is uncommon in healthy reproductive-age women (about 5–10 events per 10,000 women-years), a study found that combined oral contraceptive usage has been reported to intensify the risk for VTE such as deep venous thrombosis and pulmonary embolism (Dragoman et al., 2018). Women utilizing a combination oral contraceptive with different types of progestogens exhibit a distinct susceptibility to venous thromboembolism (VTE), a risk previously attributed solely to the impact of estrogen on hemostatic factors (Dragoman et al., 2018).

Contraceptives refer to any method, including medication and devices, that may prevent an unintended pregnancy. Hormonal contraceptives usually contain one or more artificial female sex hormones; hormonal oral contraceptives that comprise both estrogen and progestin are termed combined hormonal contraceptives (CHCs) while those that have just progestin are named progestin-only contraceptives (POC) (Reid et al., 2011). Hormonal contraceptives are obtainable as injections, patches, implants, vaginal rings, pills (oral contraceptives), and intrauterine devices (Reid et al., 2011). These hormonal contraceptives have been linked with numerous side effects such as appetite changes, vaginal discharge, acne, breast soreness, fluctuations in menstrual flow, pain or spotting in between periods, inflammation of the gums, constipation or diarrhoea, mood fluctuations, hair development in uncommon places, headaches development of brown or black patches on the skin, nausea, stomach swelling, weight increase or reduction, blurred vision, cardiovascular diseases, cancers, leg enlargement or pain, and high chance of developing blood clots (Thrombosis) (Reid et al., 2011).

Multiple studies have shown that thromboembolism is more likely to occur in women who take combined hormonal contraceptives (CHCs), regardless of the mode of administration (Vinogradova et al., 2015). They increase the susceptibility to VTE by 2-3 times in comparison with non-users, however the overall susceptibility to VTE remains modest, notably for individuals without additional risk factors (Van Hylckama Vlieg et al., 2009). Thromboembolisms in women of reproductive age occur predominantly among women on hormonal contraception (Hugon-Rodin et al., 2014; Lidegaard, 2014). Multiple reports have documented a higher possibility of thromboembolism with combination oral contraceptives (COCs), notably those who include the progestin desogestrel, while also outlining the potential risks connected to the factor V Leiden mutation (Jick et al., 1995).

Factor V Leiden is a hereditary condition acknowledged as a fundamental contributor to Anti Protein C (APC) resistance. This mutation includes the replacement of arginine with glutamine at amino acid 506 (factor V Arg506 Gln or factor V Leiden) (G-to-A) at nucleotide 1691 in exon 10, which leads to the creation of a faulty factor V molecule (FVL), which is resistant to cleavage by APC (Van Vlijmen et al., 2016). Meanwhile, Prothrombin gene mutation is a blood clotting

condition causing the human body to produce too much Prothrombin hence making them susceptible to blood clot. A hereditary risk factor for thrombosis has been recognized in the form of sequence variation involving a G-to-A mutation at position 20,210 of the Prothrombin gene. This variation is associated with a 2-4 fold increased susceptibility to thromboembolic disease (Van Vlijmen et al., 2016).
Most bearers of the mutations do not immediately produce clinical indications, but stay asymptomatic. However, when subjected to any extra risk factors that cause stasis, such as oral contraceptives usage, or hormone replacement treatment, or other abnormalities of the artery wall, thromboembolism develops. The risk of experiencing thrombotic events is greatly elevated and could manifest clinically. This research intends to investigate Factor V Leiden (FVL) and ProthrombinG20210A mutations as risk factors for thromboembolism among users of hormonal contraceptives in Benin City.

MATERIALS AND METHODS

Study Design and Population

This cross-sectional study was carried out among women attending the Family Planning Clinics at Faith Mediplex Hospital, Central Hospital, and the Planned Parenthood Federation of Nigeria (PPFN) in Oredo Local Government Area, Benin City, Edo State. The focus was on women utilizing hormonal contraceptives.
The study population was 200 apparently healthy female subjects who consisted of 50 non-contraceptive users (control, group A), 50 non-hormonal contraceptives users (group B), 50 progestin-only contraceptives users (group C), and 50 combined oral contraceptives users (group D). The subjects were of reproductive age between 18 years to 45 years. The socio-demographic data were obtained using semi structured questionnaires.

Inclusion and Exclusion criteria

Female of reproductive age between 18 years to 45 years on contraceptives without chronic illness were recruited for the study. Hospitalized patients, surgical patients and pregnant women or those with chronic illnesses were excluded.

Ethical Consideration

Permission to carry out the research was authorized by the Ethics Committee on Human Subjects at the Edo State Ministry of Health in Benin City (Reference Number: HA.737/50, issued on 2nd March 2021). Prior to their inclusion in the study, informed consent for participation was obtained from each participant.

Method

Upon completion of the questionnaire and obtaining consent for the research, we collected 5.0 mL of venous blood from each individual using sterile procedures. This blood was gently mixed in a plain tube with 3.1% trisodium citrate anticoagulant (0.5 mL). Following a 15-minute centrifugation at 3000 rpm, the citrated plasma was transferred to another tube for prothrombin time (PT) and activated partial thromboplastin time (APTT) testing. Simultaneously, 0.5 mL of venous blood was placed into a plain sample tube containing 0.25 mL of DNA-RNA shield solution. Multiplex allele-specific PCR amplification was used for the molecular identification of Factor V Leiden and Prothrombin 20210 A gene mutation. The materials were stored at room temperature until the molecular analysis was completed.

Estimation of Prothrombin Time

The prothrombin time was conducted by transferring 0.1ml of the citrated plasma samples into a glass test tube, followed by an incubation in a water bath set at 37°C for 2 minutes. Subsequently, 0.2ml of the thromboplastin/calcium chloride reagent (BIOLABO Diagnostics, Maizy, France), pre-warmed, was introduced into the test tube using an automatic pipette. Simultaneously, the stopwatch was initiated while gently moving the tube back and forth within the water bath. The stopwatch was stopped upon the initial detection of clot formation, and the result was recorded in seconds.

Estimation of Activated Partial Thromboplastin Time (APTT)

A mixture with a 1:9 ratio of anticoagulant to blood was prepared in a plain container and centrifuged for fifteen (15) minutes at 3000g using the bucket centrifuge to obtain platelet-poor plasma. Subsequently, 0.1 milliliters of the obtained plasma was transferred into a clean and dry tube. A 0.1ml aliquot of pre-warmed kaolin/platelet substitute (BIOLABO Diagnostics, Maizy, France) was then introduced into the test tube and incubated at 37°C for two (2) minutes. The sample underwent re-calcification with 0.1ml of 0.025M calcium chloride, and a stopwatch was immediately started while tilting the tube back and forth to observe clot formation. The stopwatch was halted upon the initial detection of clot formation, and the result was recorded in seconds.

Assay of Factor V Leiden and Prothrombin 20210A

DNA Isolation

Zymo research's Zymo Blood DNA extraction kit was used to obtain total genomic DNA. In a ZR Bashing BeadTM lysis tube, precisely 200 µL of blood sample was introduced. The sample was then mixed with around 750 uL of buffer. A five-minute vortex was performed on the resultant solution, followed by a one-minute centrifugation at 10,000x g. A Zymo-Spin™ llF filter was filled with around 400 uL of supernatant, and the tube was centrifuged at 8,000 x g for one minute. The filtrate in the collection tube was then mixed with 1200µL of Genomic lysis buffer. 800µL of this mixture was then transferred to a Zymo-Spin™ llCR column in a collection tube and centrifuged at 10,000 x g for a duration of one minute. The Zymo-Spin™ llCR column was filled with 200µL of DNA pre wash buffer in a fresh collection tube, and the flow through was disposed of. The mixture was then centrifuged at 10,000 x g for a duration of one minute. To extract the DNA, 100µL of DNA elution buffer was added to the column matrix of the Zymo-SpinTM llCR column, and it was centrifuged at 10,000 x g for 30 seconds after being moved to a clean 1.5 mL micro centrifuge tube. Following the addition of 600µL of Prep solution to a clean collection tube containing the Zymo-Spin™ ll-HRC filter, the tube was centrifuged at 8,000 x g for three minutes. Subsequently, the eluted DNA was moved to a Zymo-Spin™ ll-HRC filter and centrifuged at 13,000 x g for three minutes in a clean 1.5 mL micro centrifuge tube. For PCR application, the filtered DNA was used. Using absorbance measurements at 260 and 280 nm, the concentration and purity of the DNA were determined. The extracted DNA was kept in −200C for next stage of the test.

Primer design

The primers employed in this study were crafted using the web-based PRIMER program (version 3.0) available at http://workbench.sdsc.edu. To assess primer specificity, the "BLAST" program at http://www.ncbi.nlm.nih.gov/blast was utilized.
Primers used for the identification of the factor V Leiden mutation, as described by Blasczyk et al. (1996): Sense primer: 5' CTT TCA GGC AGG AACAAC ACC 3', Antisense primer: 5' GGA CAA AAT ACC TGTATT GCT C 3' and Antisense primer for mutated gene amplification: 5' TGG ACA AAA TACCTG TAT ACC TT 3'.
For the prothrombin variant identification, the sense primer (5' TCC GCC AGT GGA AGA TA) reported by Poort et al. (1996) was used. Additionally, new primers were designed to detect the prothrombin gene mutation (20210 G/A base change), differing only in the last nucleotide: Mutated gene primer: 5' CCA ATA AAA GTG ACT CTC AGC A 3', Physiological prothrombin gene primer: 5' CCA ATA AAA GTG ACT CTCAGC G3".

PCR conditions for factor V and Prothrombin Variant PCR

A PCR mixture with a final volume of 20µL was prepared, comprising 100 ng of genomic DNA, 60 mM Tris HCl (pH=8.8), 15 mM ammonium sulfate, 3.5 mM MgCl2, 0.2 mM of each dNTP, 1 pmol of the specific prothrombin primer, and 3 pmol of the factor V Leiden primer. The amplification process was conducted using the Eppendorf Mastercycler AG22331 (Hamburg, Germany).

The initial denaturation step at 94 °C for 3 minutes was followed by 35 cycles of amplification. Each cycle included denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The amplification concluded with a final extension at 72 °C for 10 minutes. By employing the method outlined by Poort et al. (1996), Hind III digestion was carried out and the prothrombin variant was detected by PCR.

Gel Electrophoresis

The product from the polymerase chain reaction was subjected to analysis through electrophoresis. This process occurred in a 0.1% agarose gel immersed in a solution of 0.5 × TBE buffer (which comprised of 2.6 g Tris base, 5 g Tris boric acid, and 2 ml of 0.5M EDTA, adjusted to pH 8.3 with sodium hydroxide pellets) and 3 µl of EZ-vision (VWR Life Science). Subsequently, the resulting bands representing the expression products were observed using a Blue-light-transilluminatorTm (Bluebox, USA).

Data Analysis

Data analysis was done using Statistical Package for Social Sciences (SPSS) programme, the collected data were arranged into tables, and analysed. A p-value less than 0.05 was considered significant.

RESULTS

Table 1 shows the the overall rate of mutation carriage (AA and AG) among the 150 women on contraceptives for Prothrombin 20210A and FVL gene mutants to be 12%, while 88% had homozygous wild genotype. Of the 18/150 (12%) with mutant genotypes, 15(10%) had genotype GA while 3(2%) had genotype AA. The variation in the frequencies of the genotypes was statistically significant (p<0.001).

Table 1: Frequency of combined genotypes of Prothrombin 20210A and FVL among the Study Subjects compared using Chi square

Genotype	Subjects on Contraceptives	Percentage (%)	P-Value
GG	132	88	
GA	15	10	0.001
AA	3	2	

GG= Homozygous wild type
GA= Heterozygous mutant
AA Homozygous mutant
P ≤ 0.001=extremely significant

Table 2 shows the Odd of causing thromboembolic risk among those with mutant genes was 29.20 (CI: 13.2, 38.6). Also, the frequency of Prothrombin 20210A mutant gene (8%) was higher than FVL (4%) among women on contraceptives. The PT and APTT of those with mutant genes were lower than those without mutation (p<0.001).

Table 2: The Prothrombin Time and Activated Partial Thromboplastin Time of subjects with Mutant Genes Compared with those without Mutant Genes on Contraceptives

Variables	Mutant Genes (GA + AA) n=18	Non-Mutant Genes (GG Wild type) n=132	P-Value
Prothrombin Time (Seconds)	14.2±0.2	16.3±0.3	0.001
Partial Thromboplastin Time (Seconds)	30.1±0.2	36.2±0.4	0.001
Odd Ratio (CI, 95%)	29.20(13.2, 38.6)		

GG= homozygous wild type
GA=heterozygous
AA= homozygous mutant
OR= Odds Ratio
CI= Confidence Intervals
P ≤ 0.001=extremely significant

Figure 2 shows the expression of genes as represented by the gel electrophoresis picture, and internal control (Beta {β} actin) of mRNA expression of Factor V Leiden of contraceptive naïve subjects (controls) (group A), non-hormonal contraceptive subjects (NHC) (group B), progestin-only contraceptive subjects (POC) (group C) and combined hormonal contraceptive subjects (CHC) (group D) represented on different bars of the bar chart. The result showed that mRNA FV Leiden expression was significantly higher (p<0.05) in group C and D than group A subjects and comparison between group B and C was also statistically significantly higher (p<0.05). Factor V Leiden mRNA was however highly expressed among subjects on progestin-only than subjects on combined hormonal contraceptives, those on non-hormonal contraceptive and control subjects.

Figure 4 shows the expression of genes as represented by the gel electrophoresis picture, and internal control (Beta {β} actin) of mRNA expression of Prothrombin 20210A of contraceptive naïve subjects (controls) (group A), non-hormonal contraceptive subjects (NHC) (group B), progestin- only contraceptive subjects (POC) (group C) and combined hormonal contraceptive subjects (CHC) (group D) represented on the bar chart. The result showed that mRNA Prothrombin 20120A expression was significantly higher (p<0.05) in group D than subjects in groups A, B and C. Similarly, subjects on non-hormonal contraceptives (group B) was significantly higher than those on progestin-only contraceptives (p<0.05).

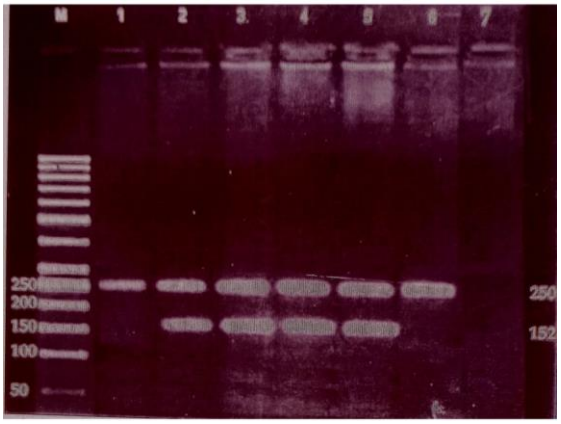

Figure 1: Shows the application of FVL; mutant A alleles in Lane 1,3,5, while Lane 2,4,6 indicate positive bands(152bp), and negative bands in Lane 1,6(G/G) wild genotype, while G/A genotype in lanes 3,4, A/A mutant genotype in Lane 5,6. M refers to molecular marker (band size 250 bps).

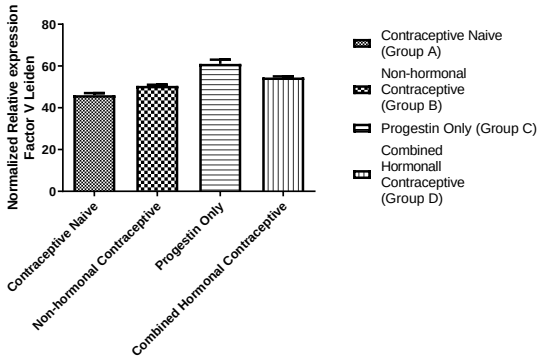

Figure 2: mRNA Expression of FV Leiden among the Studied Participants.

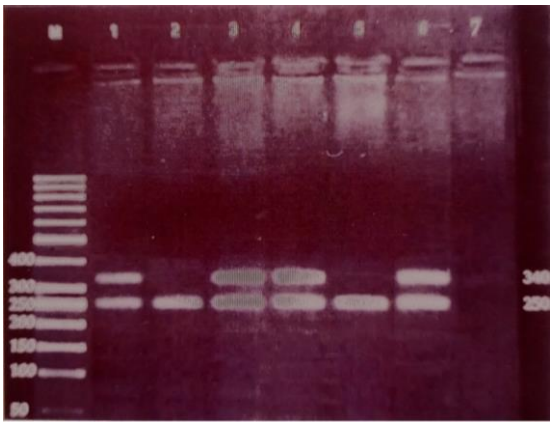

Figure 3: shows Prothrombin G20210A mutant alleles (lanes 1,3,5) and normal G alleles (lanes 2,4,6). Lanes 1,3,4,6 with negative bands in Lane 2. Lane 5(A/A) mutant genotype in Lanes 1,2, and G/A in lanes 3,4. G/G wild type in Lanes 5,6. Again, M indicates molecular marker. Band size 250bps.

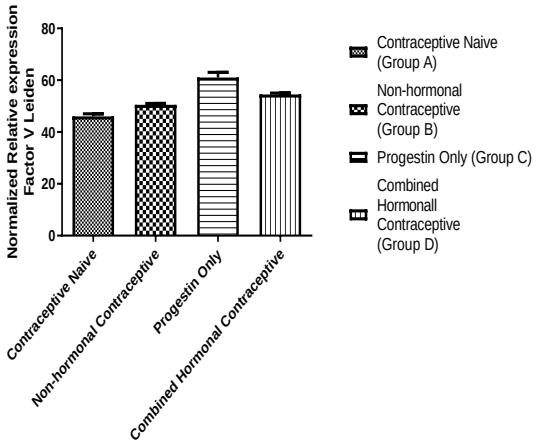

Figure 4: mRNA Expression of Prothrombin G20120A among the Studied Participant

DISCUSSION

Factor V Leiden (FVL) and the prothrombin 20210A mutation are the prevailing genetic polymorphisms that make individuals more susceptible to venous thromboembolism (VTE). Poort et al. (1996) first identified the Prothrombin 20210A mutation, which is a single nucleotide change of adenine for guanine in the 3´-untranslated region of the gene. Higher prothrombin levels have been reported in people with the Prothrombin 2021A gene mutation (Chudej and Plamenova, 2016). Additionally, the level of prothrombin itself raises the risk of thrombosis. According to the original study by Poort et al. (1996), the mutation was identified in 18.3% of individuals with a familial or previous occurrence of VTE, 6.2% of participants undergoing their initial VTE episode, and 2.3% of individuals in the control group. Research indicates that women with the prothrombin 20210A mutation who make use oral contraceptives are more prone to a higher risk of developing VTE (Chudej and Plamenova, 2016). Although the use of contraceptives is common among Nigerians in Benin City, it is unclear if such a mutation exists in our case. Congenital thrombophilia and venous thromboembolism have been found to be between 24 and 37% in the Caucasian population. However, in patients with congenital thrombophilia, thrombosis can frequently be brought on by external factors, which can disrupt the labile haemostatic balance and trigger a never-ending cycle of thromboembolic complications. Conversely, it has been shown that smoking, cardiovascular disease, and the use of contraceptives are risk factors related with acquired thrombophilia. It has also been noted that spontaneous thrombosis is uncommon in the general population (Chudej and Plamenova, 2016).

Predicting thrombotic events remains challenging, despite a strong correlation observed between the prothrombin G20210A mutation and an increased likelihood of thrombosis in the presence of certain risk factors, such as the utilization of combined hormonal contraceptives (Momot et al., 2019).
In this research, it was discovered that in the combined genotypes of Prothrombin G20210A and FVL among the people on contraceptives, the homozygous wild type (GG) had a higher frequency compared to the heterozygous mutant (GA) and homozygous mutant (AA). This result is in agreement with that published in research among Saudi women with recurring pregnancy loss, where the researchers noticed that the overall carriage rate of mutation (AA and AG) for Prothrombin G2021A was higher in individuals compared to FVL (Gawish and Al-Khamees, 2013).

Prothrombin time and Activated Partial Thromboplastin time were lower in participants with mutant gene carriage (GA+ AA) on contraceptives than in the non-mutant (GG wild type), and the differences were significant ($p<0.001$). This may be symptomatic of an increased susceptibility to hypercoagulable condition. This is consistent with recent research showing patients are at an elevated risk of thrombosis if their PT and APTT values are shortened (abnormal) (Korte et al., 2000; Lowe et al., 2000). According to Momot et al. (2019), the incidence of VTE might be nine times higher than in wild type FVL G1691G persons when additional risk factors such pregnancy and/or the use of combined hormonal contraceptives are present. While a few researchers have noted that shorter PT and APTT are thought to pose potential risks for the development of VTE, recent studies indicate that there is no correlation between reduced PT and APTT and the occurrence of thromboembolic events. The researchers observed that the APTT and PT may be shortened by increased quantities of any of the blood clotting factors, including fibrinogen, factors II, VIII, IX, XI, VII, and patients' clinical characteristics (Shervinrad et al., 2019). According to Shervinrad et al. (2019), it was determined that patient follow-up is essential in order to characterise the natural history of those with shorter PT and APTT.

Factor V Leiden is a genetic mutation that causes thrombophilia, a heightened tendency to generate abnormal blood clots that can obstruct blood vessels. Those with the Factor V Leiden mutation face an elevated risk of experiencing deep venous thrombosis. In contrast, another research discovered that within a group of 500 apparently healthy persons who had the FV Leiden G1691A mutation, there was no increase in the incidence of thrombosis when compared to normal zygote individuals (Momot et al., 2019). This research found that the FV Leiden gene was more expressed in progestin-only and combined oral contraceptive participants compared to contraceptive naive ones. This may indicate an increased predisposition to hypercoagulability in these hormonal contraception users. This is consistent with recent research (Ahmed et al., 2022). Previous research (Vandenbroucke et al., 1994; Martinelli et al., 1999; Cushman et al., 2004; Hugon-Rodin et al., 2018) have shown that people using combined oral contraceptives had higher mRNA expression of the FV Leiden mutant gene than those using progestin-only and non-hormonal contraceptives. The existence of a mutation in the Factor V Leiden gene has been linked to resistance against activated protein C, leading to an elevated susceptibility to thrombosis. This current research also found that the FV Leiden gene was more expressed in progestin-only participants than in non-hormonal contraceptive users, suggesting that progestin-only contraception could potentially increase the risk of VTE among some Nigerians.

The presence of the Prothrombin G20210A mutation is a recognized genetic risk factor for thrombosis. The Prothrombin G20210A mutation raises the clotting factor Prothrombin levels in carriers' blood, increasing their susceptibility to clotting (hypercoagulability). This research also looked at the thrombotic risk related with hormonal contraception usage and Prothrombin G20210A. All comparisons demonstrated that mRNA Prothrombin 20120A expression was greater in combined oral contraceptive participants compared to contraceptive naive subjects. This might indicate that these people using combined oral contraceptives are more prone to hypercoagulability. This result was consistent with prior research (Martinelli et al., 1999), but differed with a study by Ahmed et al., (2022), which found no significant difference between patients using combined oral contraceptives and controls.

It is known that pregnancy and COC cause a rise in Prothrombin activity within blood plasma. The rise in Prothrombin G20210A mutation among oral contraception users is likely to appear as thrombosis. Some scientists have estimated that the significant cutoff value of Prothrombin activity is 174.8% or above, which may predict thrombosis in 90.4% of instances (Momot et al., 2019).

CONCLUSION

Data from this study have indicate that Factor V Leiden mutant gene was mostly expressed in subjects on progestin only contraceptive and combined oral contraceptive, while prothrombin 2021A gene mutant was mostly expressed among women on combined-oral contraceptive and non-hormonal contraceptive. There may be an association between the

presence of FVL and FII genes mutation and thromboembolism among Nigerian women on hormonal contraceptives. It will be of public health importance to evaluate potential users of hormonal contraceptives to enable the identification of those at risk of thromboembolism.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data Availability Statement: All available data are presented in this paper.

Conflicts of Interest: The authors declare no conflicts of interest.

REFERENCES

Ahmed, O.A.B., Hassan, F.M., Asad, M., Asdaq, S.M.B., Alsalman, A.J., Al Mohaini, M., Alamri, S.A., Alsanie, W.F., Alhomrani, M., Hawaj, M.A., & Imran, M. (2022). The role of factor V Leiden and prothrombin G20210A mutations for clotting in Sudanese women under oral contraceptive use. *Journal of King Saud University- Science*, *34*(2), 1-7.

Blasczyk, R., Ritter, M., & Thiede, C. (1996). Simple and Rapid Detection of Factor V Leiden by Allele Specific PCR Amplification. *Thrombosis and Haemostasis*, *75*, 757-759.

Chudej, J., & Plameňová, I. (2016). Prothrombin Gene 20210. A Mutation in Slovak Population, *Vnitřni Lékarstvi*, *62*(4), 281–286.

Cushman, M., Kuller, L.H., Prentice, R., Rodabough, R.J, Psaty, B.M, Stafford, R.S, Sidney, S., & Rosendaal, F. R. (2004). Women's Health Initiative Investigators. Estrogen plus progestin and risk of venous thrombosis. *Journal of the American Medical Association*, *292*(13), 1573-80.

Dragoman, M. V., Tepper, N. K., Fu, R., Curtis, K. M., Chou, R., & Gaffield, M. E. (2018). A systematic review and meta-analysis of venous thrombosis risk among users of combined oral contraception. *International Journal of Gynecology and Obstetrics*, *141*(3), 287- 294.

Gawish, G., & Al-Khamees, O. (2013). Molecular Characterization of Factor V Leiden G1691A and Prothrombin G20210A Mutations in Saudi Females with Recurrent Pregnancy Loss. *Journal of Blood Disorders and Transfusion*, *4*(6), 165-169.

Hugon-Rodin, J., Gompel, A., & Plu-Bureau, G. (2014). Epidemiology of hormonal contraceptives-related venous thromboembolism. *European Journal of Endocrinology*, *171*, 221–230.

Hugon-Rodin, J., Horellou, M.H., Conard, J., Gompel, A., & Plu-Bureau, G. (2018). Type of Combined Contraceptives, Factor V Leiden Mutation and Risk of Venous Thromboembolism. *Thrombosis and Haemostasis*, *118*(5), 922-928.

Jick, H., Jick, S.S., Gurewich, V., Myers, M.W., & Vasilakis, C. (1995). Risk of idiopathic cardiovascular death and nonfatal venous thromboembolism in women using oral contraceptives with differing progestagen components. *Lancet*, *346*(8990), 1589-1593.

Korte, W., Clarke, S., & Lefkowitz, J. B. (2000). Short Activated Partial Thromboplastin Times are Related to Increased Thrombin Generation and an Increased Risk for Thromboembolism. *American Journal of Clinical Pathology*, *113*(1), 123–127.

Lidegaard. O. (2014). Hormonal Contraception, thrombosis and age. *Expert Opinion on Drug Safety*, *13*(10), 1353-1360.

Lowe, G., Woodward, M., Vessey, M., Rumley, A., Gough, P., & Daly, E. (2000). Thrombotic Variables and Risk of Idiopathic Venous Thromboembolism in Women Aged 45-64 Years. Relationships to Hormone Replacement Therapy. *Thrombosis and Haemostasis*, *83*(4), 530–535.

Martinelli, I., Taioli, E., Bucciarelli, P., Akhavan, S., & Mannucci, P.M. (1999). Interaction between the G20210A mutation of the prothrombin gene and oral contraceptive use in deep vein thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *19*(3),700-3.

Momot, A. P., Nikolaeva, M., Yasafova, N. N., Zainulina, M. S., Momot, K. A., & Taranenko, I. A. (2019). Clinical and laboratory manifestations of the prothrombin gene mutation in women of reproductive age. *Journal of Blood Medicine*, *10*, 255-263.

Poort, S. R., Rosendaal, F. R., Reitsma, P. H., & Bertina, R. M. (1996). "A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis". *Blood*, *88*(10), 3698–3703.

Reid, R., Leyland, N., Wolfman, W., Alkire, C., Awadalla, A., Best, C., Dunn, S., Lemyre, M., Marcoux, V., Menard, C., Potestio, F., Rittenberg, D., Singh, S., & Senikas, V. (2011). SOGC clinical practice guidelines: oral contraceptives and the risk of venous thromboembolism: an update. *International Journal of Gynaecology and Obstetrics*, *112*, 252 – 256.

Shervinrad, M, Tormey, C., & Siddon, A (2019). PT and aPTT Are Not Predictors of Thromboembolic Events. *American Journal of Clinical Pathology*,*152*, S1-S36.

Van Hylckama Vlieg, A., Helmerhorst, F. M., Vandenbroucke, J. P., Doggen, C. J., & Rosendaal, F. R. (2009). The venous thrombotic risk of oral contraceptives, effects of oestrogen dose and progestogen type: results of the MEGA case-control study. *Clinical Research Education*, *13*, 339.

Van Vlijmen, E. F., Wiewel-Verschueren, S., Monster, T. B., & Meijer, K. (2016). Combined oral contraceptives, thrombophilia and the risk of venous thromboembolism: a systematic review and meta-analysis. *Journal of thrombosis and haemostasis*, *14*(7), 1393-1403.

Vandenbroucke J.P., Koste,r T., Briët, E., Reitsma, P.H., Bertina, R.M., & Rosendaal, F.R. (1994). Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation. *Lancet*, *344*(8935), 1453-1457.

Vinogradova, Y., Coupland, C., & Hippisley-Cox, J. (2015). Use of combined oral contraceptives and risk of venous thromboembolism: nested case-control studies using the Research and CPRD databases. *British Medical Journal*, *350*, 2135- 2140.