Sudan University of Science and Technology

College of Graduate Studies

Detection of β-fibrinogen 455G/A Gene Mutation among Sudanese Patients with Deep Venous Thrombosis

الكشف عن طفرة جين البيتا فبرينوجين (G>A 455) لدى المرضى السودانين المصابين بخثرات الأوردة العميقة

A Dissertation submitted for partial fulfillment for requirement of Master degree in medical laboratory science (haematology and immunohaematology)

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الآية

بسم الله الرحمن الرحيم

قَالَ تَعَالَىٰ:

﴿لَا يُكَلِّفُ ٱللَّهُ نَفْسًا إِلَّا وُسْعَهَا ۚ لَهَا مَا كَسَبَتْ وَعَلَيْهَا مَا ٱكْتَسَبَتْ ۗ رَبَّنَا لَا تُؤَاخِذْنَآ إِن نَّسِينَآ أَوْ أَخْطَأْنَا ۚ رَبَّنَا وَلَا تَحْمِلْ عَلَيْنَآ إِصْرًا كَمَا حَمَلْتَهُۥ عَلَى ٱلَّذِينَ مِن قَبْلِنَا ۚ رَبَّنَا وَلَا تُحَمِّلْنَا مَا لَا طَاقَةَ لَنَا بِهِۦ ۖ وَٱعْفُ عَنَّا وَٱغْفِرْ لَنَا وَٱرْحَمْنَآ ۚ أَنتَ مَوْلَىٰنَا فَٱنصُرْنَا عَلَى ٱلْقَوْمِ ٱلْكَٰفِرِينَ ﴿٢٨٦﴾﴾

سورة البقرة الآية (٢٨٦)

Dedication

To my parents, family members, friends and all who have had positive impacts on my life I dedicate this research.

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Abstract

This is analytical a case control study conducted in Khartoum state during the period from Mars 2018 to September 2018, the study aimed to Detect the fibrinogen polymorphism G-455A in sample of Sudanese patient with deep vein thrombosis.

Forty patients diagnosed with DVT as case and forty healthy volunteers as control were included in this study. Venous blood samples were collected in EDTA containers, then DNA was isolated from peripheral blood leucocytes and β fibrinogen G455A polymorphism was detected by PCR. The data obtained was analyzed using SPSS program version 16.

The age of case group ranged between 20 and 90 years with mean age (43±18). Most of patients were first diagnosed by DVT between 20-40 years and the majority of them were female.

β fibrinogen gene wild type was seen in 35(43.75%) among case group and 36(45%) among control group, while mutant gene was seen in 5(6.25%) among case and 4(5%) among control group. However, there was no significant statistical differences (P- value 0.723).

β-fibrinogen 455G/A polymorphism was detected at 5 DVT patients (6.25%) all of them were homozygote (A/A) type, while in control group 3 out of 4 were heterozygote (3.75%) and 1 out of 4 was homozygote (1.25%). The difference did not Show the statistical significance (P-value=0.058).

Out of 40 patients confirmed to had DVT, A allele frequency of β fibrinogen -455G/A gene was 10(6.37%) and G allele frequency 70 (44.59%). While among control group A allele frequency was 5(3.18%) and G allele frequency 75 (47.77%). This result shows significant statistical differences (P-value=0.016).

The study concluded that β-fibrinogen 455G/A polymorphism was not linked to an increased risk for DVT, and neither did the heterozygous (A\G) nor homozygous (A\A) carriers show an increased risk for DVT.

المستخلص

أجريت دراسة تحليلة الحالة والحالة الضابطة هذه بولاية الخرطوم خلال الفترة من مارس ٢٠١٨ وحتى سبتمبر ٢٠١٨، هدفت هذه الدراسة لتحديد مدى إنتشار طفرة الجين بيتا فبرينوجين (G>A 455) في المرضى السودانين المصابين بمرض خثرات الأوردة العميقة بمستشفي ام درمان، وكذلك دراسة العلاقة بين الأنماط المختلفة لجين البيتا فبرينوجين (G>A 455) وحدوث خثرات الأوردة العميقة.

شملت الدراسة أربعين مصابا بمرض خثرات الأوردة العميقة وأربعين متطوعين أصحاء، تم جمع عينة من الدم الوريدي في حاوية تحتوي على الإيثايلين ثنائي الأمين رباعي حامض الإستريك، ثم عزل الحمض النووي منزوع الأوكسجين من كريات الدم البيضاء الموجودة في عينات الدم، وتم الكشف عن الطفرة بواسطة أليل البلمرة المتسلسل. ثم تم تحليل البيانات بإستخدام النسخة ١٦ من برنامج الحزم الإحصائية للعلوم الإجتماعية.

تراوحت أعمار المرضى بين ٢٠ و٩٠ عاما بمتوسط عمر ٤٣±الإنحراف المعياري ١٨، معظم المرضى كانوا بين ٢٠ و٤٠ عاما عندما تم تسخيصهم بالمرض للمرة الأولى وغالبيتهم كانوا من النساء.

وجد الجين الشائع لبيتا فبرينوجين (G>A 455) في ٣٥ بنسبة ٤٣,٧٥% من المرضى وفي ٣٦ بنسبة ٤٥% من العينة الضابطة، بينما وجدت الطفرة في جين البيتا فبرينوجين (G>A 455) في ٥ بنسبة ٦,٢٥% من المرضى و ٤ بنسبة ٥% من العينة الضابطة، مع عدم وجود علاقة ذات دلالة إحصائية (القيمة الإحتمالية ٠,٧٢٣).

عند الكشف عن الأنماط المختلفة لطفرة الجين بيتا فبرينوجين (G>A 455) وجد أن الخمسة مرضى جميعهم يحملون نمط اللاقحة المتجانسة من النوع (A\A)، أما في العينة الضابطة فثلاثة من أصل أربعة بنسبة ٣,٧٥ كان يحملون نمط اللاقحة غير المتجانسة (A\G) وواحد من أصل أربعة بنسبة ١,٢٥ كان يحمل نمط اللاقحة المتجانسة من النوع (A\A)، لكن لم يكن الإختلاف ذا دلالة إحصائية (القيمة الإحتمالية ,٠٥٨).

نسبة وجود الأليل A الخاص بجين البيتا فبرينوجين (G>A 455) في المرضى ٦,٣٧% (١٠) وعدد الأليل G الخاص بجين البيتا فبرينوجين (G>A 455) ٧٠ بنسبة ٤٤,٥٩%، بينما عدد الأليل A الخاص بجين البيتا فبرينوجين (G>A 455) في العينة الضابطة ٥ بنسبة ٣,١٨% وعدد الأليل G الخاص بجين البيتا فبرينوجين (G>A 455) ٧٥ بنسبة ٤٧,٧٧% مع وجود علاقة ذات دلالة إحصائية (القيمة الإحتمالية ٠,٠١٦).

خلصت الدراسة إلى عدم وجود علاقة بين الطفرة بجين البيتا فبرينوجين (G>A 455) وأنماطه المختلفة سواءً كانت لاقحة متجانسة أو غير متجانسة وزيادة مخاطر الإصابة بخثرات الأوردة العميقة.

List of contents

List of tables

List of graphs

Chapter one

Introduction and literature review

1.1 Hemostasis:

A complex physiologic process that keeps circulating blood in a fluid state and then, when an injury occurs, produces a clot to stop the bleeding, confines the clot to the site of injury, and finally dissolves the clot as the wound heals. When hemostasis systems are out of balance, hemorrhage or thrombosis can be life-threatening (Fritsma and Fritsma, 2016).

Primary hemostasis, beginning after endothelial damage, comprises the process of platelet adhesion, activation and aggregation, to form a platelet plug at the site of injury Both endothelial cells and circulating platelets provide a source of von Willebrand factor (vWF) which binds to the collagen on the exposed sub-endothelial surface, and is then utilized for platelet binding via the glycoprotein Ib (GPIb) complex. The platelets undergo a shape change and then release their granules. A platelet plug is eventually formed. Secondary hemostasis involves the activation of the coagulation system and coagulation factors to stabilize the platelet plug (Bonar, *et al*. 2017).

Coagulation is initiated after vascular injury by the interaction of the membrane bound tissue factor (TF), exposed and activated by vascular injury, with plasma factor VII. The factor VIIa and TF (extrinsic factor Xase) complex activates both factor IX and factor X. Factor Xa in the absence of its cofactor, forms small amounts of thrombin from prothrombin. This is insufficient to initiate significant fibrin polymerization. The initiation pathway is rapidly inactivated by tissue factor pathway inhibitor (TFPI). Thrombin generation is now dependent on the traditional intrinsic pathway (Hoffbrand and Moss, 2016).

Factor VIII and V are converted to VIIIa and Va by the small amounts of thrombin generated during initiation. In this amplification phase the intrinsic Xase, formed by IXa and VIIIa on phospholipid surface in the presence of

Ca2+, activates sufficient Xa, which then, in combination with Va, PL and Ca2+, forms the prothrombinase complex and results in the explosive generation of thrombin which acts on fibrinogen to form the fibrin clot. Thrombin hydrolyses fibrinogen, releasing fibrinopeptides A and B to form fibrin monomers. Fibrin monomers link spontaneously by hydrogen bonds to form a loose insoluble fibrin polymer. Factor XIII is also activated by thrombin and stabilizes the fibrin polymers with the formation of covalent bond cross - links. Fibrinogen consists of two identical subunits, each containing three dissimilar polypeptide chains (α, β and γ) which are linked by disulphide bonds. After cleavage by thrombin of small fibrinopeptides A and B from the α and β chains, fibrin monomer consists of three paired α, β and γ chains which rapidly polymerise (Hoffbrand and Moss, 2016).

Fibrinolysis, the final stage of coagulation, fibrinolysis is the systematic, accelerating hydrolysis of fibrin by bound plasmin. Tissue Plasminogen Activator (TPA) and Urokinase Plasminogen Activator (UPA) activate fibrin-bound plasminogen several hours after thrombus formation, degrading fibrin and restoring normal blood flow during vascular repair. There is a delicate balance between activators and inhibitors, excessive fibrinolysis can cause bleeding due to premature clot lysis before wound healing is established, whereas inadequate fibrinolysis can lead to clot extension and thrombosis (Fritsma and Fritsma, 2016).

1.2 Thrombophilia:

A clinical condition involving a coagulation system, it is characterized by a hypercoagulation which predisposes to venous thromboembolism and subsequently, its serious complication (Sulima, *et al*. 2017).

1.2.1 Venous thrombosis (VTE):

is a complex and multifactorial disorder, in which a number of putative thrombophilic conditions interplay and finally contribute to propel the individual risk over a certain degree, ultimately culminating in the development of venous occlusive disorders, VTE usually entailing deep vein thrombosis (DVT), pulmonary embolism (PE), or both (Montagnana, *et al*. 2017).

Venous thrombosis occurs at an annual incidence of about 1 per 1000 adults. Rates increase sharply after around age 45 years, and are slightly higher in men than women in older age. About two-thirds of episodes manifest as DVT and one-third as PE with or without DVT. The major outcomes of venous thrombosis are death, recurrence, post-thrombotic syndrome and major bleeding due to anticoagulation. Thrombosis is also associated with impaired quality of life (Cushman, 2007).

1.2.1.1 Deep venous thrombosis:

A severe clinical entity characterized by the formation of thrombi in deep veins. DVT can complicate the course of a disease but might also be encountered in the absence of precipitating disorders. Thrombosis can take place in any section of the venous system, but arises most frequently in the deep veins of the leg (Sirlak and Inan, 2012).

DVT is caused by some combination of hypercoagulability either systemic or local, stasis of the venous blood, and injury to the vein wall intima, specifically the endothelium, and this known as Virchow’s triad (Malone and Agutter, 2008).

The prevalence of DVT in Africa varies between 2.4% and 9.6% in patients after surgery, and between 380 and 448 per 100 000 births per year in pregnant and postpartum women (Danwang, *et al*. 2017).

1.2.1.1.1 Risk factors:

An understanding of the risk factors for DVT is necessary in order to maximize the prevention of this disease in high risk individuals and groups of patients .Major risk factors for DVT include advancing age, obesity, previous venous thromboembolism, surgery, trauma, active cancer, acute medical illnesses, antiphospholipid syndrome, dyslipoproteinemia, nephrotic syndrome, paroxysmal nocturnal haemoglobinuria, myeloproliferative diseases, Behçet's syndrome, varicose veins, superficial vein thrombosis, congenital venous malformation, long-distance travel, prolonged bed rest, immobilization, limb paresis, chronic care facility stay Pregnancy/Puerperium, oral contraceptives, hormone replacement therapy, heparin-induced thrombocytopenia, central Venous catheter, vena cava filter, and intravenous drug abuse (Sirlak and Inan, 2012).

1.2.1.1.2 Signs and symptoms of DVT:

Clinical signs and symptoms of DVT are highly variable and unspecific but remain the cornerstone of diagnostic strategy. Symptoms include pain, swelling, increased skin veins visibility, erythema, and cyanosis accompanied by unexplained fever (Mazzolai, *et al*. 2017).

Chronic venous insufficiency secondary to venous dilation and valvular incompetence is a typical long-term DVT complication. At 5 years post DVT, symptoms may include: Night pain, pigmentation changes, pain with prolonged standing, venous ulceration, and edema (Quinn, 2017).

1.2.1.1.3 Diagnosis of DVT:

Clinical diagnosis of deep vein thrombosis is notoriously unreliable and therefore clinical probability scores have been developed to guide further

investigation and treatment. The most widely used is the Wells' score (which assigns an individual to one of three risk groups) or the modified Wells' score (which uses two risk groups). Patients with a score of 2 points or more should be offered a venous duplex ultrasound scan carried out within 4 h. The sensitivity of the Wells' score is reasonable for DVT (77%-98%) although specificity was less good (37%-58%). Sensitivity was higher (96%) and specificity lower (26%) in people with underlying malignancy (Bevis and Smith, 2016).

1.2.1.1.3.1 Duplex ultrasound:

Venous duplex ultrasound has been widely adopted as the first line investigation for suspected DVT. It is able to identify DVT of the leg with a sensitivity of 96.5 % for proximal (above knee) DVT and 71.2 % for calf DVT, both with a specificity of 94 % (Goodacre, *et al*. 2005).

1.2.1.1.3.2 Impedance plethysmography:

Used for patients at increased risk of DVT. Isotope-labeled fibrinogen imaging can demonstrate calf vein thromboses but is unreliable in demonstrating more proximal thrombosis (Bevis and Smith, 2016).

1.2.1.1.3.3 Venography:

Gold standard for the diagnosis of DVT in the calf and thigh, with sensitivity and specificity almost 100%, the detection of pelvic thrombi is poor, unless direct femoral vein puncture is performed (Quinn, 2017).

1.2.1.1.3.4 D-dimer:

D-dimers are fibrin degradation products produced during the immediate fibrinolytic response to thrombus formation. Negative D-dimer results, in combination with a low probability score, are increasingly being used to exclude the diagnosis of DVT. Sensitivity and Specificity varies according to

the assay method: ELISA, whole blood agglutination, and latex assay (Bevis and Smith, 2016).

1.2.1.1.3.5 Magnetic resonance imaging (MRI):

Used to image DVT, particularly in the pelvis (100% sensitivity, 95% specificity), where it is more sensitive than venography. MRI is equally sensitive for detection of DVT in the thigh but inferior in the calf (87% sensitivity and 97% specificity) (Quinn, 2017).

1.2.1.1.3.6 Computerised tomographic (CT):

CT venography is similarly advantageous as MRI over duplex ultrasound but does involve ionizing radiation and the use of intravenous contrast. It is reported to be 97 % sensitive and 100 % specific at determining lower limb DVT compared with duplex ultrasound (Frankel and Bundens, 2014).

1.2.1.1.4 Treatment of DVT:

The aim of treatment for DVT is to relieve the acute symptoms whilst reducing the risk of recurrent thrombosis and post-thrombotic syndrome:

1.2.1.1.4.1 Anticoagulation:

The initial anticoagulant regime for DVT can be a choice of either intravenous or subcutaneous un-fractionated heparin, low molecular weight Heparin, Fondaparinux, Rivaroxaban and Apixaban. Vitamin K antagonists such as Warfarin can be initiated simultaneously with heparin to a target international normalised ratio (INR) of 2.0–3.0 (Hansrani, *et al*. 2016).

1.2.1.1.4.2 Catheter directed thrombolysis:

It is more efficient than systemic lysis, mainly due to less bleeding, as thrombolytic agent is directly administered within the clot, Mechanical thrombus removal alone is not successful and needs adjuvant thrombolytic therapy (Mazzolai, *et al*. 2017).

1.2.1.1.4.3 Surgical thrombectomy:

Generally favored for acute clot removal, surgical thrombectomy may be considered for patients with iliofemoral DVT without phlegmasia cerulea dolens in whom thrombolytic therapy is contraindicated or in settings in which catheter-directed thrombolysis is unavailable (Liu, *et al*. 2015).

1.2.1.1.4.4 Vena cava filter:

It is may be used when anticoagulation is absolutely contraindicated in patients with newly diagnosed proximal DVT. One major complication is filter thrombosis. Therefore, anticoagulation should be started as soon as contraindications resolve and retrievable filter rapidly removed (Mazzolai, *et al*. 2017).

1.3 Fibrinogen:

Fibrinogen is a 340,000 Dalton glycoprotein synthesized in the liver. The normal plasma concentration of fibrinogen ranges from 200 to 400 mg/dL, the most concentrated of all the plasma procoagulants. Fibrinogen is an acute phase reactant protein, whose level increases in inflammation, infection, and other stress conditions. Platelet a-granules absorb, transport, and release abundant fibrinogen (Fritsma and Fritsma, 2016).

The fibrinogen gene cluster is located on chromosome 4q32 in the order β–α–γ, with β transcribed in the opposite direction to α and γ (Gomez and McVey, 2016).

The fibrinogen molecule is a mirror-image “trinodular” dimer, each half consisting of three non identical polypeptides, designated Aa, Bb, and g, united by disulfide bonds. The six N-terminals assemble to form a bulky central region called the E domain. The carboxyl terminals assemble at the outer ends of the molecule to form two D domains Thrombin cleaves fibrino-peptides A and B from the protruding N-termini of each of the two a and b chains of fibrinogen, reducing the overall molecular weight by 10,000 Daltons. The cleaved

fibrinogen is called fibrin monomer. The exposed fibrin monomer a and b chain ends (E domain) have an immediate affinity for portions of the D domain of neighboring monomers, spontaneously polymerizing to form fibrin polymer (Fritsma and Fritsma, 2016).

1.3.1 Fibrinogen function:

Fibrinogen is a coagulation or inflammatory biomarker strongly associated with atherogenesis (Zhang, *et al*. 2014). Fibrinogenisan abundant plasma protein, highly susceptible to such oxidative modifications, and is therefore a potential marker for oxidative protein damage (Meredith, *et al*. 2014). Also fibrinogen plays an essential role in the hemostatic system to cause atherosclerosis and thrombosis by bridging activated platelets and being the key substrate for thrombin to establish a consolidating fibrin network (Sorensen, 2012).

1.3.2 Pathophysiology:

The function and quantity of plasma fibrinogen can be altered by both inherited and acquired disorders. Increased levels of fibrinogen can be seen in pregnancy, acute or early liver disease, and many acute illnesses. Acquired fibrinogen deficiencies can be due to consumptive states (e.g. placental abruption), fibrinolytic therapy, hemodilution and severe liver disease (Verhovsek, *et al*. 2008).

Fibrinogen abnormalities can also be inherited in an autosomal pattern and occur in two main patterns: hypo/afibrinogenemia and dysfibrinogenemia. Afibrinogenemia is a very rare disorder that occurs when any one of the three genes coding for the alpha, beta, or gamma chains that make up the fibrinogen molecule is mutated, if the mutation is sufficient to disrupt the formation or secretion of any of the three chains, afibrinogenemia results. Dysfibrinogenemia is also rare but is more common than afibrinogenemia. The dysfibrinogens are the result of mutations in the genes encoding fibrinogen. 90% are missense mutations, and the majority of patients are heterozygous for the disorder.

Several hundred mutations have been recorded, many of which result in neither a hemorrhagic nor a thrombotic state. However, other dysfibrinogens are associated with bleeding episodes, and a few may be associated with venous or arterial thrombosis (Martin, *et al*. 2018).

hayperfibrinogenaemia has been reported to be associated with increase risk of DVT. The B fibrinogen gene G-455A polymorphism has been reported to confer susceptibility to thromboembolic diseases and increased plasma fibrinogen levels (Fajar, 2016).

1.3.3 β-fibrinogen gene 455 G/A polymorphism:

A common G>A polymorphism at position -455 in the promoter region of the β-fibrinogen gene has been associated with elevated fibrinogen levels for the AA homozygotes (Wypasek, *et al*. 2012).

Homozygote of the A allele of the fibrinogen beta -455-G>A gene polymorphism, which is caused by a Guanine-to-Adenine substitution at position -455 in the 5¯ promoter region of the fibrinogen beta gene, show higher plasma fibrinogen levels than subjects with the GG genotype. Carriers of the -455-A allele at this polymorphic site, about 20% of the population, have been suggested to have about 7–10% higher fibrinogen levels than those homozygous for the -455G allele (Leander, *et al*. 2002).

Few previous studies done on β fibrinogen G-455A mutation in DVT, both (Renner, *et al*. 2002; Camilleri and Cohen, 2005) found that fibrinogen-elevating FGβ G-455A gene polymorphism is not linked to an increased risk for DVT.

1.4 Rationale:

There is a high incidence DVT in Sudan but studies concerning the role of fibrinogen mutation G-455A in patients with DVT are lacking, this study aimed to evaluate the role of fibrinogen mutation G-455A in the etiology of DVT among Sudanese patients for purposes of screening those at risk, thus achieving good prevention of DVT and improving management of patients with DVT.

1.5 Objective:

1.5.1 General objective:

To determine the frequency of fibrinogen mutation G-455A in sample of Sudanese patient with deep vein thrombosis.

1.5.2 Specific objective:

- To assess fibrinogen level in cases and control.
- To detect mutation in specific gene G-455A.
- To correlate level of fibrinogen with demographic factors collected from study population.

Chapter two

Materials and methods

2.1 Study design:

This is a case control study.

2.2 Study area:

The study was conducted in Khartoum state in order to determine the prevalence of genetic mutations that predispose thrombophilia among deep venous thrombosis patients.

3.3 Study duration:

This study was carried out during the period from Mars 2018 to September 2018.

2.4 Study population:

Forty patients diagnosed with DVT, twenty-six females and fourteen males; all had been recruited as study group. forty healthy volunteers were included as control group. They were chosen in accordance to the gender and age of the study group. Both case and control group were unrelated and randomly selected.

2.4.1 Inclusion criteria:

Confirmed DVT patients were included. The diagnosis done by clinical assessment of risk factors and physical findings followed by Doppler ultrasound confirmation. Forty normal individuals who did not experience any episodes of deep venous thrombosis nor had a history of thrombophilia were included as control group.

2.4.2 Exclusion criteria:

Patients of other thrombosis than deep vein thrombosis (e.g fatty embolism) were excluded.

2.5 Sampling:

Blood samples were collected under sterile condition and the vein puncture was well dressed. Two milliliter of blood was drawn from each participant, and poured in ethylene diamine tetra acetic acid (EDTA) container which used for the molecular techniques. EDTA blood were stored at minus seventy degrees Celsius tell the time of examinations.

2.6 Ethical consideration:

The volunteers were informed by the aim of this study and they were aware with the consequences of the research and their written consent was taken.

2.7 Methodology:

2.7.1 Molecular biology:

2.7.1.1 DNA extraction:

2.7.1.1.1 Red blood cells lyses buffer:

This solution is composed of: chlorine (NH4CL), potassium bicarbonate (KHCO3) and EDTA 5%, all ingredients dissolved in one liter distilled water and kept at room temperature.

2.7.1.1.2 White cells lysis buffer:

WBC lysis buffer prepared by dissolving four chemicals in 500 distilled water. These chemicals include: Tris-Hcl, EDTA, Nacl and SDS.

2.7.1.1.3 Guanidine hydrochloride:

Strong chaotropic agent used for the denaturation and subsequent refolding of proteins. Prepared by weighing 57.2 grams of powder and dissolved in 100 ml distilled water.

2.7.1.1.4 Proteinase K:

Proteinase K is commonly used in molecular biology to digest protein and remove contamination from preparations of nucleic acids. Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that

might otherwise degrade the DNA or RNA during purification (Crowe JS**,** Cooper HJ).

2.7.1.1.5 Chloroform -20:

Chloroform ensures phase separation of the two liquids because chloroform is miscible with phenol and it has a higher density (1.47 g/cm3) than phenol; it forces a sharper separation of the organic and aqueous phases thereby assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase (Piotr C and Nicoletta S).

2.7.1.1.6 Ethanol:

DNA is insoluble in alcohols (Ethanol and Isopropanol), therefore 100% alcohol used for the precipitation so we get good amount of DNA. Washing with 70% alcohol is to remove the excess of salts that might have come along with the extraction buffers (i.e. the excess of salts dissolve in the 30% of water).

2.7.1.1.7 DNA extraction protocol:

The genomic DNA was isolated from peripheral blood leucocytes using Guanidine Chloroform Extraction Method. The protocol was performed in three days. First day started with washing whole blood in RBC lysis buffer after thawing at room temperature. Blood placed in sterile falcon tube, lysis buffer added up to the 10 ml mark, tubes centrifuged at 6000 rpm for 15 minutes. After centrifugation supernatant was discarded. These steps were repeated several times until the supernatant became clear. After that 2ml of WBC lysis buffer, 1ml guanidine, 300 µl ammonium acetate and 25 µl proteinase K were added to the billet. Last step of day one was the incubation of tubes at 37 ^{0}C for overnight.

On the second day tubes were brought to the room temperature. Two ml of chilled chloroform added to each tube. After that, tubes were agitated in a vortex and centrifuged for 5 minutes at 2500 rpm. Three layers were obtained. The upper layer transferred to a new falcon tube containing 10 ml chilled

ethanol previously kept at -20. Then all tubes shaken very well and the second day finalized by placing tubes at -20 for overnight.

On day three, tubes were centrifuged and the ethanol discarded, tubes inverted on dry gauze for two hours. When they are dry tubes washed in 70 % ethanol, inverted on gauze until they were dry. After that 100 µl of doubled distilled water added and the mixture was transferred to appindorf tube. Finally, the product kept at -20. (**Piotr Chomczynski and Nicoletta)**

2.7.1.2 Beta-fibrinogen 455 G/A gene polymorphism:

2.7.1.2.1 Primers design and preparation:

Primers were adopted from (Jeddi-Tehrani, *et al.* 2011). They were prepared as described above. Table (2.1.) shows primer design.

2.7.1.2.2 PCR protocol:

The beta fibrinogen G455A polymorphism was detected by conventional PCR. The protocol was started with initial denaturation at 94 ^{0}C for 5 minutes. Then the amplification was performed with 36 cycles (denaturation at 95 ^{0}C for 15 seconds, annealing at 53 ^{0}C for 45 seconds, extension at 72 ^{0}C for 30 seconds). After that a final step of extension for 5 minutes at 72 ^{0}C was done (Jeddi-Tehrani, *et al*. 2011).

2.7.1.2.3 Detection of PCR product:

The PCR product was applied on 1.5 % agarose gel electrophoresis to which ethidium bromide was added. The 495 bp band was digested with Hae III restriction enzyme. A 5 µl from the product was incubated with 5units Hae III restriction enzyme at 37^{0}C for 18 hours. After that the enzyme reaction was inhibited by raising the temperature to 65^{0}C. Finally, the mixture applied on 3% agarose. The wild type of the gene had two restriction site resulting into three fragments, 383 bp, 86 bp and 26 bp. while the mutant gene had a restriction site resulting into to fragments 469 bp and 26 bp (Jeddi-Tehrani, *et al.* 2011).

Table (2.1): Primers design and restriction enzyme used for detection of polymorphisms:

Polymorphism	**Restriction enzyme**	**Primers**	**PCR product (bp)**	**RFLP products (bp)**
Fibrinogen G455A	Hae III	F5′GCTTGTGGGAAATGAAGGAA3′ R5′GGCAACCACTAAAATCGTGA3′	495	383, 86, 26[a]. 469, 26[b].

[a] RFLP product for normal allele.
[b] RFLP product for mutant allele.

2.8 Data analysis and presentation:

Data was analyzed with using SPSS software program version 16.0 and Chi-square test were used P-value were calculated. Statistical significance was set as <0.05. Data were reported as mean (±SD) for continuous variables or as percentage, and presented in form of tables and graphs.

Chapter three

Results

A total of 80 participants were included in this study; 40 cases have DVT and 40 control. The number of male among case group was 14 (17.5%) and female 26 (32.5%). While among control group male number was 30 (37.5%) and female 10 (12.5%). The number of female patients was significantly higher than male patients and male sex predominated in the control group, as showed in table (3.1).

The age of case group ranged between 20 and 90 years with mean age (43±18). Most of patients were first diagnosed by DVT between 20-40 years and the majority of them were female, as showed in graph (3.1).

Regarding the types and frequency of risk factors among case group, 5\26 females (12.5%) were pregnant, 13\26 (32.5%) females used contraceptive pills, 3\14 (7.5%) males were smoker, 6\40 (15%) patients underwent surgery, 5\40 (12.5%) patients were obese and 6\40 (15%) patients had diabetes, as showed in table (3.2).

Detection of β fibrinogen gene mutation revealed that 9 (11.25%) of study population harboring the mutant gene, while the reminder 71(88.75%) harboring the wild type gene, as showed in graph (3.2). Wild type was seen in 35(43.75%) among case group and 36(45%) among control group, while mutant gene was seen in 5(6.25%) among case and 4(5%) among control group. However, there was no statistical differences between two study groups (P-value 0.723), as showed in table (3.3).

β-fibrinogen 455G/A polymorphism was detected at 5 patients (6.25%) in whole study population, 0(0%) of them were heterozygote (G/A) and 5(6.25%) out of them were homozygote (A/A) type, while in control group 3 out of 4 were heterozygote (3.75%) and 1 out of 4 was homozygote (1.25%). Although the prevalence of this polymorphism was slightly higher in case group, the

difference did not reach the statistical significance (P-value=0.058), as showed in table (3.4).

Out of 40 patients confirmed to had DVT, A allele frequency of β fibrinogen -455G/A gene was 10(6.37%) and G allele frequency 70 (44.59%). While among control group A allele frequency was 5(3.18%) and G allele frequency 75 (47.77%). This result shows significant statistical differences (P-value=0.016), as showed in table (3.5).

Table (3.1): Shows the distribution of sex among case and control groups:

Sex	Sample		Total
	Case N(%)	Control N(%)	
Male	14 (17.5%)	30 (37.5%)	44 (55%)
Female	26 (32.5%)	10 (12.5%)	36 (45%)
Total	40 (50%)	40 (50%)	80 (100%)

Graph (3.1): Show the distribution of cases among age groups:

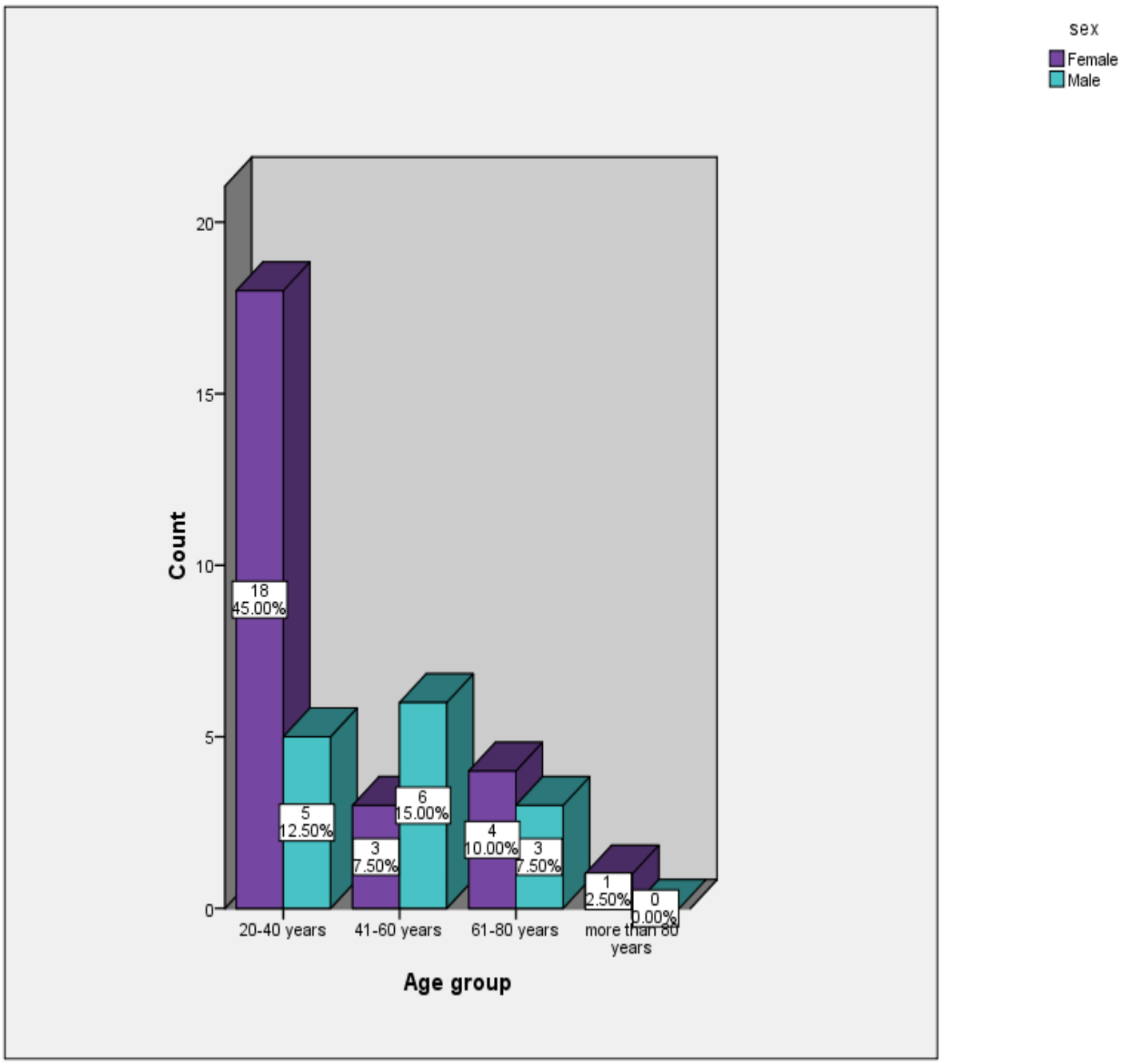

Table (3.2): Shows the frequency of risk factors among case group:

Risk factors	case						Total
	Male N(%)		Female N(%)		Total N(%)		
	Yes	No	Yes	No	Yes	No	
Pregnancy	-	14(35%)	5(12.5%)	21(52.5%)	5(12.5%)	35(87.5%)	40
Contraceptive	-	14(35%)	13(32.5%)	13(32.5%)	13(32.5%)	27(67.5%)	40
Surgery	3(7.5%)	11(27.5%)	3(7.5%)	23(57.5%)	6(15%)	34(85%)	40
Smoking	3(7.5%)	11(27.5%)	0(0%)	26(35%)	3(7.5%)	37(92.5%)	40
Obesity	2(5%)	12(30%)	3(7.5%)	23(57.5%)	5(12.5%)	35(87.5%)	40
Diabetes mellitus	2(5%)	12(30%)	4(10%)	22(55%)	6(15%)	34(85%)	40

Graph (3.2): Shows the distribution of mutation among study sample:

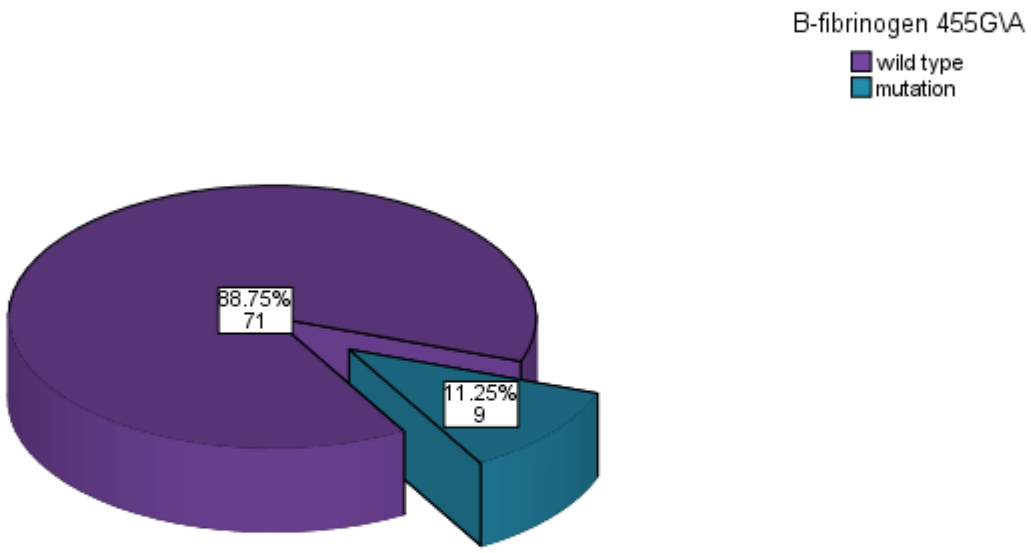

Table (3.3): Shows the frequency and association between β fibrinogen -455G/A mutation and study groups

β fibrinogen	Sample		Total N(%)	P-value
	Case N(%)	Control N(%)		
Wild type	35(43.75%)	36(45%)	71(88.75%)	0.723
Mutant	5(6.25%)	4(5%)	9(11.25%)	

Table (3.4): Shows genotypic distribution of β fibrinogen -455G/A polymorphism in different studied groups

Genotype	Sample		Total N(%)	P-value
	Case N(%)	Control N(%)		
A/A	5(6.25%)	1(1.25%)	6(7.5%)	0.058
G/G	35(43.75%)	36(45%)	71(88.75%)	
G/A	0(0%)	3(3.75%)	3(3.75%)	

Table (3.5): Shows allele frequencies of β fibrinogen -455G/A polymorphism in different studied groups

Allele	Sample		P-value
	Case N(%)	Control N(%)	
A	10(6.37%)	5(3.18%)	0.016
G	70(44.59%)	75(47.77%)	

Chapter four

Discussion, conclusion and recommendation

4.1 Discussion:

Elevated fibrinogen levels have been linked to increased risk for deep venous thrombosis, although it is not clear whether fibrinogen is causal or rather a marker for the presence of other risk factors. A common G/A polymorphism in the gene for the fibrinogen beta-chain (FGB G-455A) is associated with elevated fibrinogen levels. The present study was designed to evaluate the role of fibrinogen mutation G-455A in the etiology of DVT among Sudanese patients.

In this study a total of 80 participants were included 40 cases have DVT and 40 control, the mean age of DVT patients was (43±18). Most of patients were first diagnosed by DVT between 20-40 years and the majority of them were female, with decrease in frequencies with increasing age, an observation that disagree with Engber, *et al*. (2010), who founded that the incidence of VT increase sharply with age, it is very rare in young individuals but increase in elderly. This may attribute to small sample size in this study and differ in type and frequencies of risk factors. However, this study agrees with Engber, *et al*. (2010) in part that there is a complex relationship between the incidence of VTE and sex, as it is modified by age. Women have higher rates of VTE than men in those aged <55 years, which corresponds to being premenopausal and the effect of estrogen as a risk factor for VTE. Men have higher incidence of VTE among those aged 60 to 80 years, and then among those aged 80+ years, women again have a higher incidence than men.

In the present study, the FGB G-455A gene polymorphism was not linked to the presence of DVT, and neither did the heterozygous nor homozygous carriers show an increased risk for DVT. Although the prevalence of this polymorphism

was slightly higher in case group compared to control, this result was similar to other studies conducted by (Koster, *et al*. 1994; Renner, *et al*. 2002; Camilleri and Cohen, 2005; Tiedje, *et al*. 2011).

This study showed that there were significant statistical differences (P-value=0.016) in frequencies of A and G allele between case and control with A allele frequency higher in case than control a founding that differ from Koster, *et al*. (1994), who found that A allele was associated unexpectedly with reduced VT risk than G allele. Also Cushman, *et al*. (2007), found that A allele was associated with lower VT risk, and homozygote A\A had a 40% lower risk, while A\G had a 23% lower risk of VT, but it did not reach statistical significant. (Renner, *et al*. 2002), also found that A allele was slightly more in control (27.4) than case (26.4) but it did not reach statistical significant (P-value=0.69).

4.2 Conclusion:

On the basis of result this study concluded that the β fibrinogen -455G/A gene polymorphism was not linked to the presence of DVT, and neither did the heterozygous (A\G) nor homozygous (A\A) carriers show an increased risk for DVT.

4.3 Recommendation:

According to the result, the study recommends:

- Further study should be done with large sample size.
- Further study is needed regarding the relation of fibrinogen levels, functions and other fibrinogen gene cluster variants.

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Appendices

Appendix I Questionnaire:

Sudan University of Science and Technology

College of Graduate studies

Detection of β-fibrinogen 455G/A Gene Mutation among Sudanese Patients with Deep Venous Thrombosis

2018

Name:…… No:……..

Gender:…….

Age:……..

Residence:…….

Phone No: ……

Diagnosis:……..

Time when first diagnosed by DVT:……..

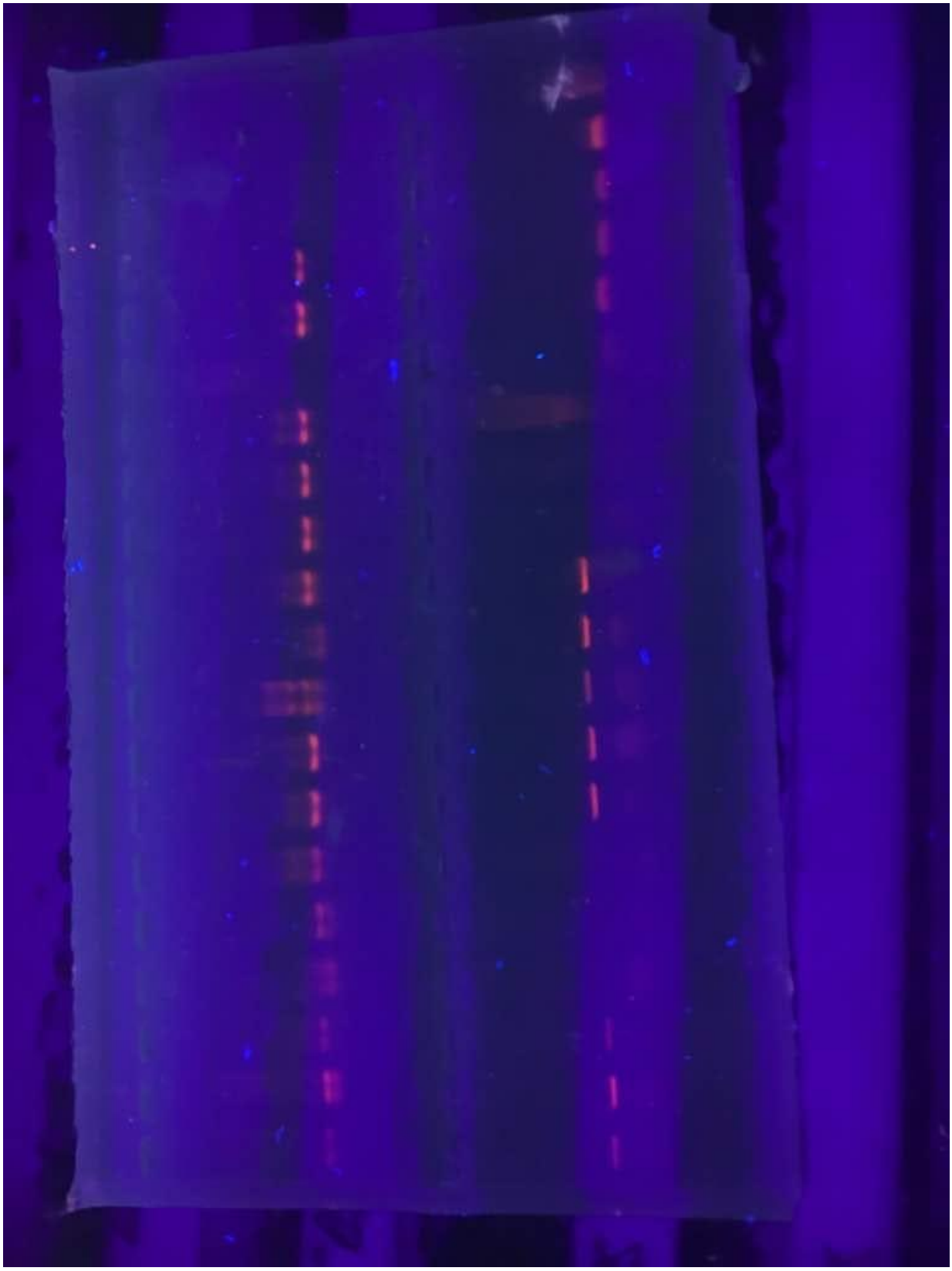
Treatments:……..

Risk factors:

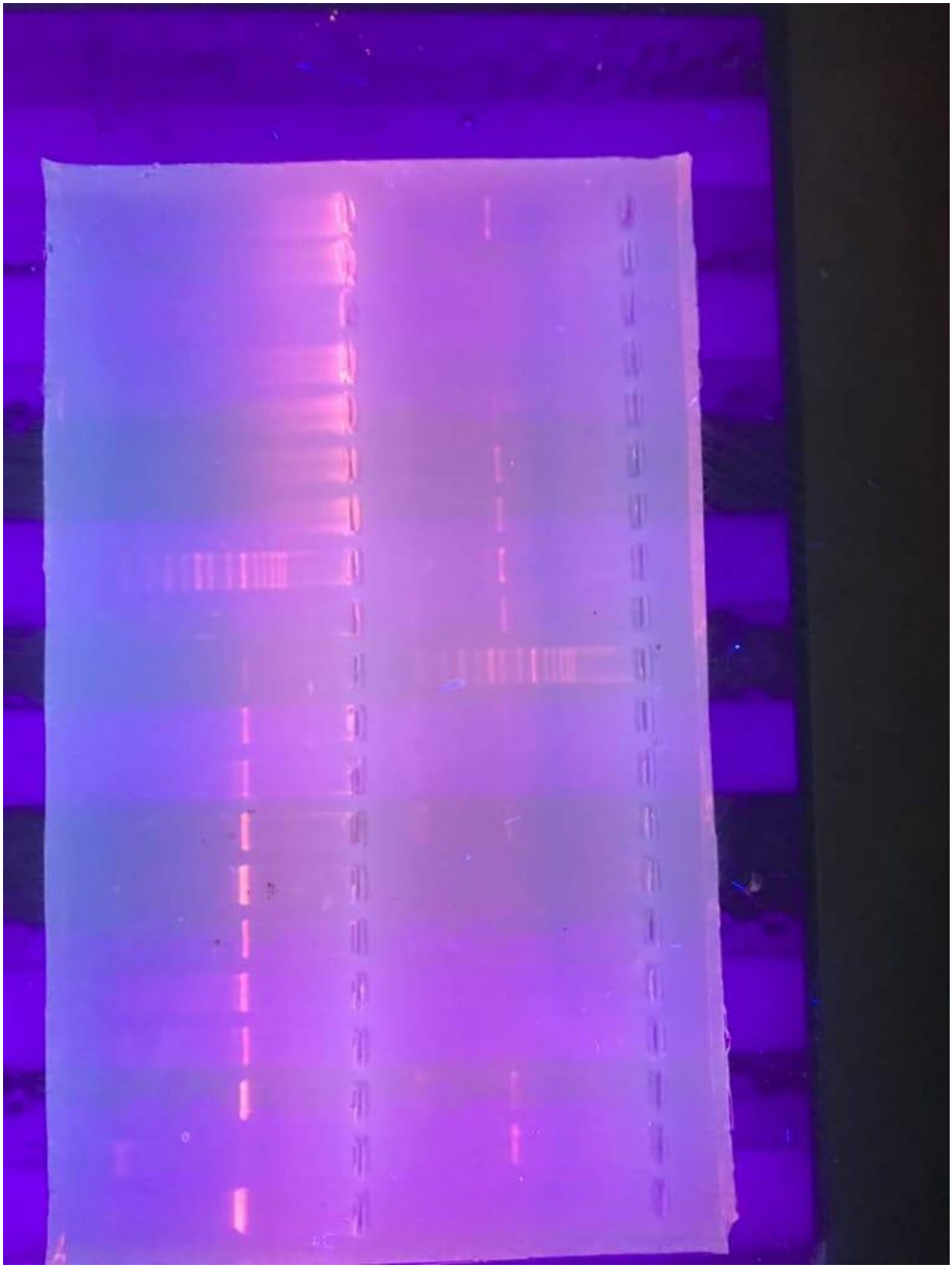
Pregnancy ☐ Contraceptive pills ☐ Surgery ☐

Diabetes ☐ Smoking ☐ Obesity ☐

Signature ……. Date ……



fibrinogen 455G/A Gene Mutation



fibrinogen 455G/A Gene Mutation