A CROSS SECTIONAL SURVEY ON THE PREVALENCE OF PROTEIN C DEFICIENCY AMONG PATIENTS PREVIOUSLY TREATED FOR VENOUS THROMBOEMBOLISM AT THE KENYATTA NATIONAL HOSPITAL

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A Dissertation Submitted in Partial Fulfillment to the Award of the Degree of Masters in Medicine in Internal Medicine, University of Nairobi

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LIST OF ABBREVIATIONS

APA	Antiphospholipid Antibodies
AT	Antithrombin
ART	Anti Retroviral Therapy
APCsr	Activated Protein C Sensitivity Ratio
CP	Cancer Procoagulant
CHD	Coronary Heart Disease
CMF	Cyclophosphamide/Methotrexate/5-Fluruoracil
CI	Confidence Interval
DIC	Disseminated Intravascular Coagulation
ETP	Endogenous Thrombin Potential
EPCOT	European Prospective Cohort on Thrombophilia
G-CSF	Granulocyte-Colony Stimulating Factor
HRT	Hormonal Replacement Therapy
HIV	Human Immunodeficiency Virus
INR	International Normalized Ratio
PGM	Prothrombin Mutation
PE	Pulmonary Embolism
TF	Tissue Factor
TNF	Tissue Necrosis Factor
VTE	Venous Thromboembolism

ABSTRACT

Inherited thrombophilic disorders are a relatively rare group of conditions but whose presence significantly increases the risk of venous thromboembolism. The prevalence of these disorders and the impact on risk of thrombosis have been well studied in other parts of the world but no local data is available.

This study proposes to determine the prevalence of Protein C deficiency among patients who were previously treated for venous thromboembolism at the Kenyatta National Hospital.

STUDY OBJECTIVE

To determine the prevalence of Protein C deficiency among patients with Venous Thromboembolism who were treated at Kenyatta National Hospital.

STUDY DESIGN

Cross Sectional Study

STUDY SITE

Hemato-Oncology Clinic

STUDY POPULATION

Patients who were previously treated for venous thromboembolism and have completed their prescribed duration of anticoagulation.

SAMPLING AND DATA COLECTION

Files of patients who were previously treated for venous thromboembolism were randomly selected from the Records Department and patients were contacted on phone and invited to enrol in the stud.Data will be entered into a study proforma and statistical analysis will be done using STATA Software-Texas 2010.

LITERATURE REVIEW

Introduction

Venous thromboembolism (VTE) is a term that collectively defines patients who present with either deep vein thrombosis (DVT) and/or Pulmonary Thromboembolism (PTE) [1].

Arterial and venous thrombosis are common conditions worldwide with risk of severe complications including pulmonary embolism, stroke, myocardial infarction, transient ischaemic attacks, arterial and venous retinal occlusive disease and gangrene of the extremities. In a study conducted in Worcester by Anderson et al in Massachusetts [2] and Silverstein et al in Minnesota[3], the incidence of venous thromboembolism was about 1 in 1000 per year. The mortality rate for pulmonary embolism continues to be high. In the international cooperative pulmonary embolism registry of 2454 patients, the three month mortality rate was 17.5 %[4].In a local study by Gikonyo, the average autopsy incidence of pulmonary embolism was found to be 5.4% [5].

While the acquired risk factors for deep vein thrombosis have been well studied, the impact of inherited factors has not been well studied especially in our setup. In a case control study conducted in Italy by Prandoni et al [6], inherited thrombophilia was equally distributed between patients with secondary and idiopathic deep vein thrombosis and was proved to be an independent from acquired risk factors. In a local study by Magada[7], hyperhomocysteinemia was found to be a relatively common risk factor for DVT in our population and is coexistent with other risk factors suggesting that it is additive in the production of disease. Other studies by Simeoni et al in Italy and Den Heijer et al in the Netherlands have shown similar findings [8, 9].

Virchow first described the risk factors for thrombosis in 1856, summarized in what is popularly known as Virchow's triad of stasis, hypercoagulability and endothelial injury [10].

Surgery

Surgery predisposes patients to thromboembolism even as late as one month post-operatively[11,12,13,14].This is especially seen in patients who undergo orthopaedic (hip and knee), thoracic, abdominal and genitourinary surgery. In patients with recurrent thromboembolic episodes or with a peri-operative thromboembolism complication, an elaborate medical and laboratory evaluation can reveal a definite diagnosis. For example, patients with protein S deficiency undergoing cardiac surgery belong to a high risk group.

Although rare, this and other coagulation disorders can be critical in cardiac surgery patients. In such patients, perioperative warfarin therapy with a target INR of 2 and incomplete protamine antagonism is recommended to minimize the risk of perioperative thromboembolic events [15].

Obesity

Obesity, in particular central obesity, has long been regarded as a risk factor for arterial as well as venous thrombosis. It more than doubles the risk in numerous studies particularly in combination with use of oral contraceptive pills or in the peri-operative period. The various mechanisms by which obesity promotes thrombosis include; the action of adipocytokines from adipose tissue e.g. leptin and adiponectin, increased activity of the coagulation cascade manifested by increased levels of plasminogen activator inhibitor (PAI-1).Other coagulation abnormalities include increased levels of Factor VII, VIII and vWF. Platelet aggregation is also enhanced in obese individuals. Obese people also have large numbers of circulating microvesicles (fragments of damaged cells that bear tissue factor [16]. Other pathogenic factors include decreased activity of the fibrinolytic system, enhanced inflammation, increased oxidative stress and disturbances of glucose and lipid metabolism associated with the metabolic syndrome [17].

Malignancy

Neoplastic cells can generate thrombin or synthesize various procoagulants like tissue factor(TF) and cancer procoagulant (CP) from malignant cells [18].CP is a cystein proteinase produced by different types of malignant cells that may directly activate clotting factor X without involvement of factor VII pathway [18].Yet, cancer cells may also produce tissue factor themselves thereby inducing a hyper activation of the factor VII pathway. Furthermore also, release of clotting factor X by malignant cells has been rarely described [19].Moreover, tissue factor can also be produced by endothelial cells or monocytes/macrophages because of the involvement of a cytokine network related to tumor growth, in particular TNF alfa and interleukin 1 beta [20].

A subclinical hypercoagulable state has already been described by several authors in literature. Several tests testify to the presence of an acquired hypercoagulable state in cancer such as increased levels of D-dimer, prothrombin fragment 1 and 2 and/or thrombin-antithrombin complexes [21].In a local study by Asaava, there was a significant association

between advanced breast cancer and DVT, elevated D- dimers and prolonged clotting times[22].There is evidence that some patients develop an acquired protein C resistance and and increased fibrinogen levels secondary to malignancy[23].

Venous thromboembolism has already been described in patients receiving chemotherapy. First literature is available since the 1980s and is focused mainly on patients with breast cancer especially in advanced stages of disease [24].In the following years, several studies have focused on the role of chemotherapy as an additional risk factor for VTE particularly in the treatment of haematological malignancies. Furthermore also, specific drugs have been implicated in the pathophysiology of acquired thrombophilia during chemotherapy. A common example has been given by chemotherapy for breast cancer based on the CMF regimen. CMF and also other chemotherapeutic regimens have been shown to reduce the levels of natural anticoagulants protein C and S especially if associated with administration of tamoxifen.[25,26] On the other hand, growth colony stimulating factor (G-CSF) used to fight chemotherapy induced neutropenia has also shown a prothrombotic action.[27,28,29]In the last ten years,Thalidomide has also been associated with thrombotic complications during chemotherapy although the pathophysiologic mechanisms are still unclear.[30,31,32,33]

Pregnancy

From 1979 to 1986, 2726 pregnancy associated deaths were reported in the United States[34].The risk of thrombosis is significantly higher during puerperium compared to the antenatal period.[35]More than half of all deep vein thrombosis during pregnancy occur during the first and second trimester.For women whose pregnancies resulted in live births, thrombotic pulmonary embolisms were the leading cause of death. Antenataly, multiple pregnancy is an important risk factor. Postnatally, women who have had a caesarean section, cardiac disease, delivery at gestational age of <36 weeks, a body mass index of equal or greater than 25 and a maternal age of greater than 35 were all found to have a higher incidence of venous thromboembolism.[36]

During pregnancy, the thrombogenic potential of inherited thrombophilia is increased because of pregnancy associated changes in several coagulation factors. These include increased resistance to activated protein C during the second and third trimester. There is also a decrease in Protein S activity decreases due to estrogen induced decreases in total

protein S and increases in the complement 4b binding protein which binds protein S. There is also an increase in levels of fibrinogen and Factors II VII and VIII and X.[37,38,39,40] The net effect of these pregnancy induced changes is to produce a hypercoagulable state that is more accentuated in women with inherited thrombophilia.

Obstetric Complications

Studies have evaluated the association between thrombophilia and adverse pregnancy outcomes, but the results are frequently contradictory, populations heterogenous and the absolute risk small when any is found.[41,42] There is contradictory literature on the association between maternal inherited thrombophilia and recurrent spontaneous abortion occurring in the first trimester. A meta analysis of 31 case- control, cohorts and cross sectional studies calculated the pooled odds ratio (OR) with a 95% confidence interval (CI) by random effects model. The results showed that Factor V Leiden and Prothrombin gene mutation were significantly associated with early recurrent pregnancy loss and late non recurrent pregnancy loss while Protein C and Antithrombin deficiencies were not significantly associated with pregnancy loss.[43]

The European Prospective Cohort on Thrombophilia (EPCOT) evaluated 843 women with thrombophilia, 571 of whom had 1524 pregnancies compared with 541 control women, 395 of whom had 1019 pregnancies. They defined miscarriage as a fetal loss less than 28 weeks of gestation and stillbirth as a fetal loss >28 weeks of gestation. The study showed that the rate of fetal loss was significantly increased among women with thrombophilia. However, the OR was stadistically significant only for still birth with highest risk for Antithrombin, Protein C, Protein S and Factor V Leiden in decreasing order. A dose-response effect was also noted with multiple inherited defects.[44,45]

The EPCOT study also showed that the presence in inherited thrombophilia was protective for early pregnancy loss defined as loss before 10 weeks and was associated with increased risk of loss after 10 weeks.This paradoxical observation that maternal thrombophilias are protective of early loss is not unexpected. Early pregnancy is normally associated with a low oxygen environment (oxygen pressures of 17+/- 6.9 mm Hg and 60.7 +/- 8.5 mm Hg at 8 to 10 and 13 weeks of gestation, respectively), trophoblast plugging of the intervillous space, and low Doppler flow of the uteroplacental circulation.[46,47] Oxygen may actually be harmful

during the embryonic period.[48] Thus, the adverse effect of maternal thrombophilias on uteroplacental blood flow and oxygen delivery would be expected to be harmful to the late, but not early, first trimester pregnancy.

Oral Contraceptives

The risk of pulmonary pulmonary embolism among users of oral contraceptives is about three times that of non users. The risk among current users is primarily determined by duration of disease. Most users of oral contraceptives use the second generation formulations that contain levonorgestrel, norgestrel or norgestrieone as the progesterone and low dose estrogen (<50mg).Third generation oral contraceptives contain desodestrel, gestodene or norgestimate as the progesterone in combination with low dose estrogen. These third generation contraceptives attenuate the androgenic effects of hirsutism and acne. An excess risk of non fatal venous thromboembolism has been associated with the new generation oral contraceptives containing low dose estrogen and the third generation progestagens desogestrel and gestadene compared to levonorgestrel.[49,50] Several studies have shown an association between inherited thrombophilic disorders and use of oral contraceptive pills. In young females, homozygosity for Factor V Leiden, Prothrombin mutation and Protein c deficiency have been associated with increased risk for venous thromboembolism among young women on oral contraceptive.[51,52]

Hormone Replacement Therapy

Hormone replacement therapy (HRT) was increasingly promoted over the last 40 years to improve quality of life, and to reduce the risks of osteoporotic fractures and coronary heart disease (CHD). In recent years, observational studies, randomized trials and systematic reviews of such trials have shown that HRT does not reduce, but actually increases cardiovascular risk. HRT increases the relative risks of venous thromboembolism (twofold), and of fatal or disabling stroke (by 50%); whilst increasing the early risk of myocardial infarction and having no protective effect against CHD on longer term use. The risk is higher near the start of therapy compared to after long term use. Possible mechanisms for these increased cardiovascular risks include down-regulation of several inhibitory pathways of blood coagulation, resulting in increased coagulation activation, which promotes venous and arterial thrombosis.[53,54,55,56,57,58]

Thrombin generation is significantly increased in women using the oral formulations compared to transdermal formulations.[59]This is probably mediated by the hepatic first pass metabolism of estrone, the main metabolite of oral estradiol, which is avoided in the transdermal route..The effect of estrone on thrombin generation may provide an explanation for the higher thrombotic risk seen in women on oral formulations. Certain small subgroups of patients should be treated specifically with oral HRT, for example those with lipid and lipoprotein abnormalities and glucose intolerance. Others should be treated preferably with the transdermal route especially those with a personal or family history of venous thrombosis. The risk of developing DVT may be higher in users of combined oestrogen-progestin HRT compared to users of estrogen only.[60,61]

HIV Infection

HIV infection is known to be associated with endothelial dysfunction which predisposes to venous thrombosis.The same cytokines responsible for endothelial activation are up-regulated in the course of HIV infection.[62,63]These cytokines, including tumor necrosis factor a, interleukin-1, and interleukin-6, activate coagulation and down-regulate the production of fibrinolytic proteins . Increased concentrations of procoagulant proteins and decreased concentrations of anticoagulant proteins have been identified as risk factors for venous and arterial thrombosis.[64,65] Of these proteins, factor VIII and fibrinogen are acutephase proteins that become risk factors when their concentrations remain increased for a prolonged time . Low concentrations of protein C have been reported in various infections, possibly because of the consumption of protein C in its role as an antiinflammatory mediator.[66] Inherited protein C deficiency is a strong risk factor for venous thrombosis , but whether acquired deficiency is also a risk factor is unknown. Inherited protein S deficiency is another risk factor for venous thrombosis, and possibly for arterial thrombosis as well . Approximately 60% of protein S is bound to complement Gib-binding protein, but only free protein S is active as an anticoagulant. During infections, the concentration of C4b-binding protein increases up to 400% of its typical concentration.[67] Some small studies have shown decreased concentrations of both protein S and protein C in HIV-infected patients.[68,69] Recently, a larger study of 94 HIV-infected women showed that an advancing HIV infection was associated with high factor VIII concentrations and a decrease in protein S activity.[70]

HIV infection is also associated with coagulation abnormalities that significantly increase the risk of thrombosis. Venous thromboembolic events have been independently associated with

increased plasma levels of P-selectin, D-dimers and hyaluronic acid. Venous thromboembolic events were also related to nadir CD4 cell counts, lifetime history of multiple opportunistic infections, CMV disease and CMV viremia, immunological AIDS, active infection and provocation (recent hospitalization, surgery or trauma).[71,72,73]

Natural Anticoagulant Deficiencies (Protein C/S Antithrombin)

Hereditary thrombophilia is defined as an enhanced inherited tendency to form intravascular thrombi, which may be arterial or venous that characteristically occurs in young age (before 45 years) and tends to recur.

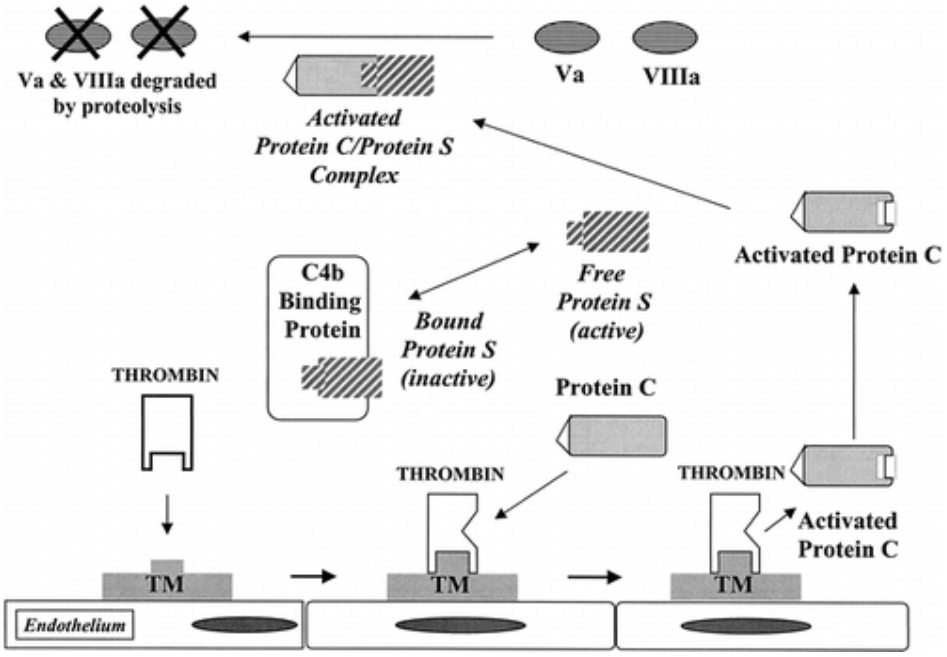


Figure 1 shows the basic pathophysiology of Protein C and S deficiencies that result in a hypercoagulable state. A Japanese study by Sakata et al [74] compared the prevalence of protein C and antithrombin deficiency among patients with deep vein thrombosis with that of normal subjects. Protein C deficiency was found to have a 5 % prevalence while that of antithrombin was 6.5%. The prevalence in the deep vein thrombosis group was statistically higher than that of the general population indicating that each deficiency is a severe risk factor for deep vein thrombosis in the Japanese population. Similar studies done in Europe (Italy) by Stefano et al have shown a prevalence of protein C deficiency of ranging from and 2.4 % to 3.2%.[75,76]

African literature on thrombophilic disorders is scanty. One study done in Cotonu, (Benin,), by Houennais et al, was a case control study of fifty four patients in each arm, Protein C deficiency was found to have a prevalence of 9.3%.[77] A possible explanation for the marginally higher prevalences observed in African patients is that most commercial assays for determination of these thrombophilic disorders use reference values obtained from causcasian subjects. One study done to determine the normal levels for Protein C, S ,Antithrombin and Lupus anticoagulant found that Africans have lower levels of these proteins and this may account for the higher prevalences observed in African subjects[78] Several case control studies showed no difference in the prevalence of these deficiencies among patients with arterial thrombosis compared to matched controls.[78,79,80,81] The lack of association between these deficiencies and arterial occlusive events could not be explained by the mere low prevalences of these deficiencies.

The population based ARIC conducted among individuals with a mean age of 54,the incidence of VTE not due to cancer was 3.36 times higher in patients with low Protein C levels compared to that occurring in individuals with higher Protein C values[82].This RR estimate is similar to that obtained from other population based studies. In particular, the Leiden thrombophilia study[83], showed a RR of 3.1, and an Oxford study [84,] which showed a RR of 2.9.

Acquired Protein C Deficiency

Protein C deficiency may be acquired and caused by increased consumption (e.g overt DIC, severe infection without DIC , acute VTE) or by decreased synthesis of the active protein as occurs with administration of vitamin K antagonists like warfarin. Severe hepatic dysfunction and prematurity are other causes. Rarely, antiphospholipid antibodies (APA) may cause an acquired Protein C deficiency via antibody mediated clearance.[85] It is recommended that screening for Protein C not be done until resolution of the acute phase which is considered to be six months from the onset of illness. If a patient is on long term warfarin and protein c deficiency needs to be screened for, warfarin can be substituted with heparin for two weeks after which testing can be done.[86]

LABORATORY ANALYSIS OF PROTEIN C

Two types of protein C deficiency states are recognized. In type I deficiency the plasma

concentration of protein C is reduced both in functional and immunological assays, reflecting a genetic defect causing a reduced biosynthesis of protein C. Type II deficiency is characterized by normal protein C antigen levels, but with decreased functional activity. This type of defect reflects the synthesis of abnormal molecules with reduced function. Type I deficiency is the most common type of disorder.[87] The laboratory evaluation of protein C is the only definitive way of diagnosing hereditary protein C deficiency in thrombophilic patients. Various types of assays have been developed and some are available in commercial kit form. Protein C is measured using either a functional assay, that tries to evaluate the biological activity of protein C, or an immunological assay, which determines the total amount of protein C related material in plasma. Each assay has a number of pros and cons. However, for the routine screening of hereditary protein C deficiency, a functional activity assay is generally recommended. This approach will detect low activity levels associated with both reduced(Type I) as well as dysfunctional protein C (Type II).[88,89]

ACTIVITY ASSAYS

Numerous activity assays have been described that use different types of activators and detection methodologies.[90] .The majority of the proposed methods can be divided into three major steps: i) isolation of protein C from plasma, ii) protein C activation, and iii) measurement of APC using either synthetic substrates or clotting-based assays.[91]

A) The Isolation Step

In the first generation of functional protein C assays, the activation of protein C was achieved either by thrombin alone, or by the thrombin-thrombomodulin complex. These reagents required an adsorption step prior to the protein C activation in order to isolate protein C from its plasma inhibitors and other interfering substances. The surface binding of protein C was obtained using either immunoadsorption techniques or insoluble salts (e.g barium citrate or aluminium hydroxide). The latter procedure exploited the ability of protein C and other vitamin K-dependent proteins to bind to insoluble salts via the Gla-domain. Once protein C was isolated and activated, the thrombin excess has to be quenched or removed by specific thrombin inhibitors before the protein C activity could be quantified accurately. In general, these multi-step assays have been shown to be specific for protein C but are time-consuming and unsuitable for clinical use.[92]

B) Use of Snake Venom Activator

The determination of protein C was greatly facilitated by the use of a specific protein C activator. The activator is a serine protease isolated and purified from the venom of the southern copperhead snake, *Agkistrodon Contortrix*.[93] It rapidly activates both human and bovine protein C, probably via the same mechanism as thrombin, without interfering with other coagulation factors. The activation reaction is especially effective in the absence of calcium ions and conditions of low ionic strength. Furthermore, the venom activator does not hydrolyze the chromogenic substrates for protein C to any significant extent. Since the activation is rapid, it minimizes the efficiency of the protein C inhibitors and thus eliminates the need for isolating protein C in an adsorption step.[94]

C) Measurement Of Activated Protein C (APC)

APC can be measured using either chromogenic substrates or clotting-based techniques. Chromogenic substrates are small synthetic peptides that mimic the cleavage site of a natural substrate.[95]The peptides are generally composed of a sequence of 2-4 amino acids with the chromogen,4-nitroaniline (pNA) attached to the end. When the chromogenic substrate is incubated with a proteolytic enzyme, such as APC, it is cleaved and pNA (yellow colour) is released. The release is measured at a wavelength of 405 nm, either during the reaction in a photometer cuvette (kinetic method), or discontinuously by stopping the reaction with acetic or citric acid (end-point method). The photometric signal is proportional to the enzyme activity in a properly-designed assay. Substrates to be used in a chromogenic assay for protein C must be specific for the enzyme, and activators and contaminating factors should not cleave the substrate. One of the most suitable chromogenic substrates available for the assay of protein C activated, appears to be S-2366™. The substrate has been shown to have little sensitivity to the isolated venom activator, although it is significantly hydrolyzed by normal plasma mixed with the activator.[96,97]

Clotting assays for the determination of protein C use the ability of activated protein C to prolong the clotting time. A widely-used method is the activated partial thromboplastin time, APTT . The use of a protein C-deficient plasma in these assays negates the possibility that deficiencies of other plasma proteins (e.g. protein S) are the cause of APTT prolongation.

APTT assays for protein C are generally less precise and show a greater variability than chromogenic protein C assays. This is probably due to the influence of high levels of factor VIII (an acute phase protein), and/or the variable phospholipid reagent quality and composition. The interference may result in erroneously low protein C levels.[98]

IMMUNOLOGIC ASSAYS

Immunological assays for protein C are usually based on the use of monoclonal antibodies against protein C and include Electroimmunoassays (EIA)[99],Radioimmunoassays (RIA),[100]and Enzyme-linked immunosorbent assays,(ELISA)[101].The advantages of these assays are their specificity, reproducibility and accuracy. However, since immunological assays measure all types of protein C molecules in plasma without evaluating their function, they will not detect dysfunctional molecules (type II deficiency).[102]

PROTEIN C TEST PERFORMANCE

To determine the performance and frequency of protein C reagents currently used by clinical laboratories, the North American Specialized Coagulation Laboratory Association (NASCOLA) analyzed protein C proficiency testing data from 6 surveys conducted in 2009 and 2010. Inter-laboratory coefficients of variation (CV) for commonly used reagents on a survey with normal protein C ranged from 8% to 12% for antigenic assays, from 4% to 7% for chromogenic activity assays, and from 7% to 22% for clot-based activity assays. CVs for commonly used reagents on specimens with abnormal protein C ranged from 15% to 24% for antigenic, 4% to 11% for chromogenic, and 10% to 17% for clot-based assays (averaged across 3 surveys). Some reagents were used by relatively few laboratories and therefore additional study may be needed for those reagents. For all commonly used reagents, biases were usually small and often not statistically significant. All assessed reagents were clinically accurate, and were considered acceptable options for a specialized coagulation laboratory.[103]

STUDY JUSTIFICATION

Given the high mortality and morbidity rate rate from venous thromboembolism, studies on risk factors for venous thrombosis are needed in order to enhance early intervention and

proper management of patients through lifestyle modification and therapy for modifiable risk factors and prophylactic anticoagulant therapy for the non modifiable factors.

Secondly, there is paucity of data on the prevalence of these disorders in our setup. One local study has evaluated homocysteine levels in patients with deep vein thrombosis. This study will inform us on the prevalence of protein C in our setup as well as its associated co morbidities. It will be of interest to know how our local data compares with that observed in other parts of the world where such studies have already been done.

Thirdly, studies like this one evaluating congenital protein deficiencies and defects can serve as a launching pad for further studies that will describe the exact mutations resulting in the observed phenotypes. Such studies will describe the clinical features associated with different mutations, frequency of occurrence of each particular mutation, the associated geographical/ethnic distribution as well as other demographic variables.

RESEARCH QUESTION

What is the magnitude of Protein C deficiency among patients with venous thromboembolism on follow-up at Kenyatta National Hospital?

SPECIFIC OBJECTIVES

1.To determine the prevalence of Protein C deficiency among patients with venous thromboembolism.

2. To document the risk factor pattern among patients with venous thromboembolism

MATERIALS AND METHODS

Study Design

This was a cross sectional study.

Study Area

The study was carried out at the Kenyatta National Hospital Hemato-Oncology clinic.

Study Population

The study population consisted of patients who were previously diagnosed with venous thromboembolism and have completed the prescribed duration of anticoagulation

Case Definition

1.Patients who had a confirmed diagnosis of either or both of:

a) Deep venous thrombosis
b) Pulmonary Thromboembolism

2. Diagnosis was confirmed both clinically, and radiological, using one or more of the following modalities.
    a. Doppler Ultrasound
    b. CT Pulmonary Angiography
    c. V/Q Scan
3. Patients who have completed the prescribed duration of anticoagulant therapy.

Outcome Variable

Protein C Activity Level (%)

Normal: 70%-140%

Low Abnormal: 1%-69%

High Abnormal: >140%

Inclusion Criteria

1. Patients with a history of deep vein thrombosis and or pulmonary thromboembolism
2. Duly signed consent
3. Age 13years and older

Exclusion Criteria

1. Current use of warfarin (Vitamin K antagonist medication)
2. Acute phase venous thrombosis (within 6 months of diagnosis)
3. Clinical evidence of chronic liver disease.

Sampling Technique

A simple random technique (by Moore and Mc Cabe) was used

Sample Size Calculation

The sample size formula is: $N = \frac{Z^2 p(1-p)}{e^2}$

Z= confidence interval of 95% (Standard value of 1.96)

P= Estimated prevalence (5%) Prevalence Study by Sakata

E= margin of error (4%)

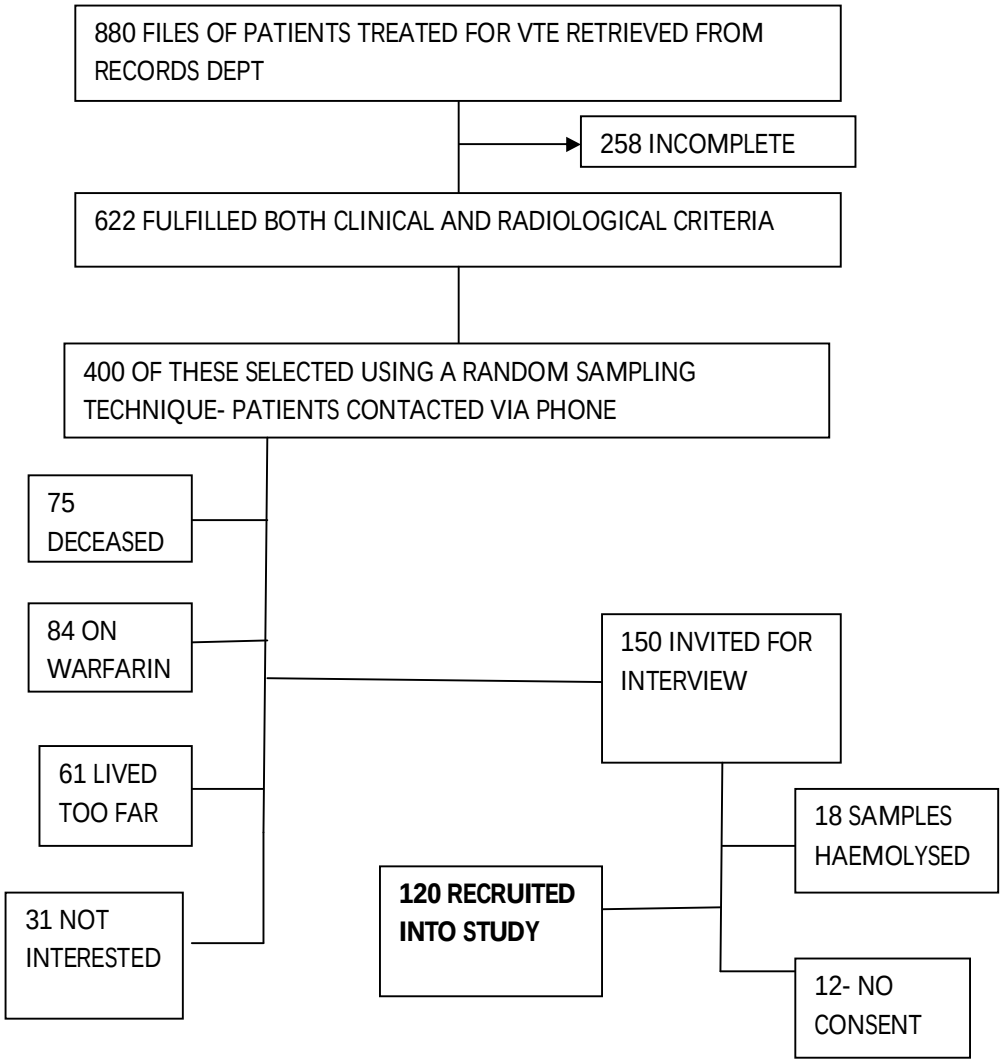
N= Minimum sample size = **115**

PROCEDURE AND DATA COLLECTION

1. Files of patients who were previously diagnosed and treated for venous thromboembolism were retrieved from the medical records and statistics department.
2. The principal investigator and his assistant went through the medical notes to verify the diagnosis was made in the standard way to fulfil the case definition criteria.
3. A Simple random sampling technique (Moore and Mc Cabe) was used to sample individual files that fulfilled the inclusion criteria.
4. Patients were contacted using the contact telephone numbers given in every file.
5. A rapport was established and the purpose / reason for the study will be explained carefully to every study subject.
6. The study subjects were invited to come for an interview with the principal investigator at the Hemato-Oncology clinic at a convenient date.
7. Upon meeting the study subject, information on the study was given and upon obtaining consent a study questionnaire was administered to the study subject.

8. The antecubital fossa was used to draw 4 millilitres of blood using an aseptic technique and samples were filled into citrated tubes and centrifuged at 3000 rpm within 30 minutes of collection as per the protein c kit specifications.
9. Plasma was separated and all plasma samples were frozen at -25°C awaiting batch processing.
10. Samples were thawed at a controlled temperature of 37°C and immediately processed using the ACL-8000 Coagulation Analyzer. An S-2366 Chromogenic Assay for Protein C (HemosIL) was used with Quality Control runs after every 20 specimens, and Special Quality Control runs on all low abnormal readings.

FLOW CHART FOR PATIENT SELECTION AND RECRUITEMENT



DATA MANAGEMENT

The data collected was verified, cleaned and entered into a Microsoft Access database and analysis done using SPSS version 17.0.The descriptive characteristics of the patients were summarized using socio-demographic factors. Continous variables such as age were represented as mean and standard deviation while categorical variables such a gender, occupation and level of education are presented as proportions. Presentation of data was done using frequency tables, graphs and pie charts. Protein C activity level is expressed as a proportion

STUDY FEASIBILITY

A pilot survey to this effect. 20 files were randomly selected and using the contacts given in the files, patients were called and asked if they are available and wiling to participate in the study.12 of the patients called were ready and available to come for testing, 3 are on lifelong warfarin and therefore ineligible, 2 had passed away and 2 were unreachable.

It was estimated that 10 patients would be recruited per week

PRESENTATION OF STUDY PROPOSAL

The study protocol was presented to the full Department of Medicine Research Committee and was passed**.**

ETHICAL CONSIDERATIONS

Permission to carry out this study was sought from the Kenyatta National Hospital /University of Nairobi Research and Ethic Committee before data collection commenced. Informed consent was obtained from all study participants and all benefits and risks were explained. Transport fare was re-imbursed to all study participants Confidentiality was maintained at all times and data obtained was only used for the purposes of the study. The results of the tests done were communicated directly to the study participants via mobile phone. Those participants found to have a deficiency of protein c were requested to attend the haematology clinic for a review with a haematologist.

RESULTS

Between 19th March and 24th August 2012, 880 files of patients who have had been previously treated for venous thromboembolism were retrieved. Of these, 622 (70.6%) had both clinical and radiological documentation of the diagnosis. We randomly 400 of these files underwent random sampling and the patients were contacted using the phone numbers given in all files. Seventy-five patients were deceased, eighty four are on lifelong warfarin and therefore ineligible to participate. Another sixty-one could not be recruited due to logistical problems like distance while another thirty patients were not interested. A total of one hundred and fifty patients came for the interview. Of these, twelve did not give consent. A total of one hundred and thirty eight patients were recruited into the study and had the study questionnaire administered and blood samples drawn. Eighteen samples haemolysed leaving one hundred and twenty for analysis. The baseline characteristics are shown in Table 1 below.

Table 1: Socio-demographic Characteristics

Variable	Frequency (%)
Sex	
Male	26 (21.7)
Female	94 (78.3)
Age	
Mean (SD)	36.9 (8.9)
Range	23.0-67.0
Marital status	
Married	66 (55.0)
Single	54 (45.0)
Education	
Primary	27 (22.5)
Secondary	55 (45.8)
Tertiary	47 (39.1)
Occupation	
Employed	72 (60.0)
Unemployed	48(40.0)
Geographical Distribution	
Nairobi and Environs	95 (79)
Upcountry	25 (21)

The study population was young with a mean age of 36.9 years and the range being from 23 years to 67 years.

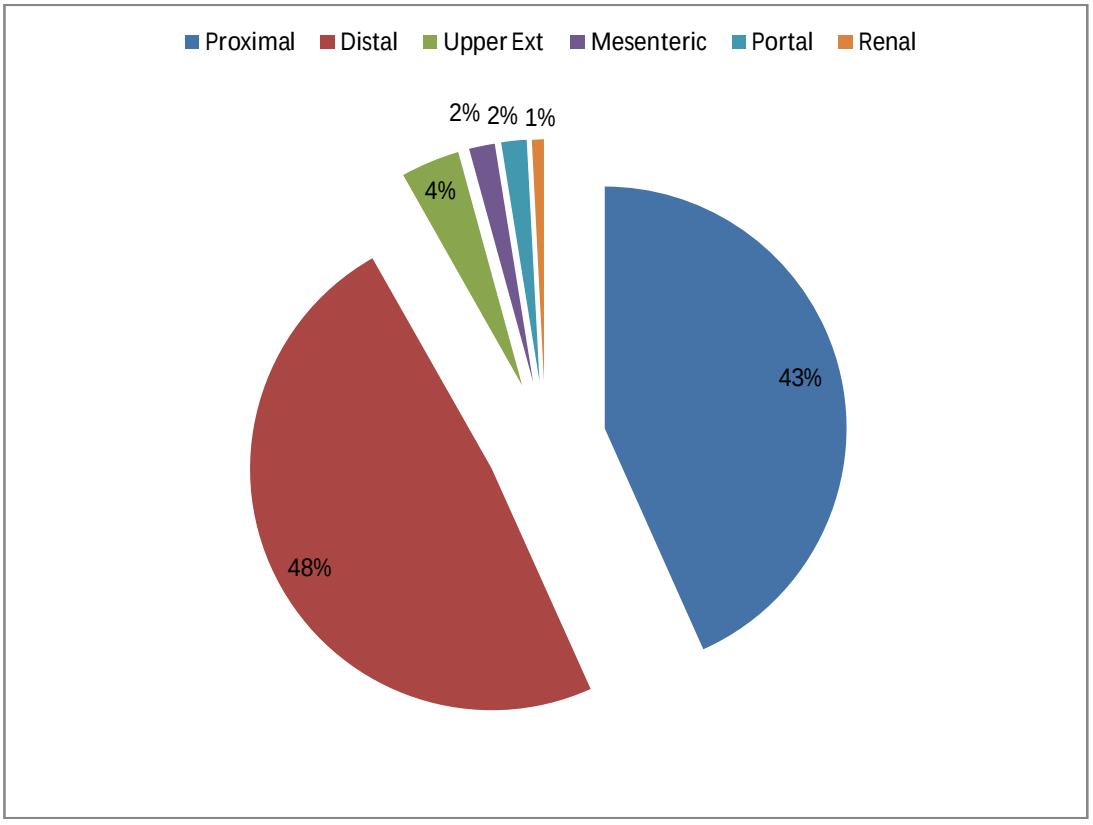
In this study, 94 (78%) of the study participants were females .Slightly over half (55%) of them were married and 102 (84.9%) had post primary level of education. A majority of these patients (79%) were from Nairobi and surrounding towns.

Two hundred and fifty patients could not be recruited into the study due to various reasons. About one third (33.6%) were on warfarin .Another 75(30%) patients had passed away while 61(24.4%) could not come because of logistical problems like distance or financial constraints. Thirty patients (12.2%) were not interested in participating.

Looking at the socio-demographic characteristics of the group that was not recruited into the study ,170 (70.8%) were females. The mean age was 39.7(32%) years and the ages ranged from twenty six to sixty five years. One hundred and fifteen (46%) of these were married . One hundred and sixty five (66%) were from Nairobi and surrounding towns. The level of education could not be ascertained in this group as this information is not part of the data given in the file.

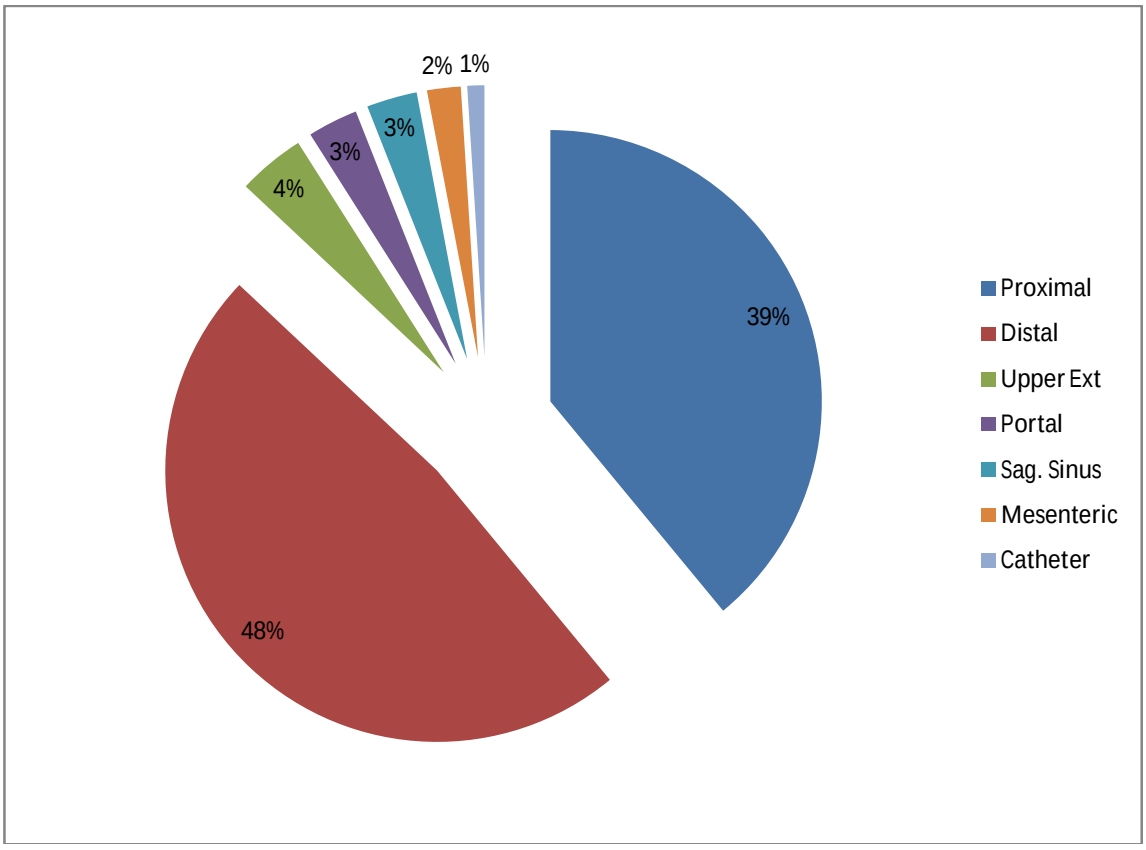
In summary, the socio-demographic characteristics of the non participants were largely similar to those of the study participants in terms of gender, marital status and geographical distribution. However, this group had a higher mean age and a majority of the non participants (58%), were not engaged in any economic activity.

Figure 2: Baseline Characteristics: Site of DVT



We found that 58 (48%) patients were treated for distal DVT while 52 (43%) were treated for proximal DVT. Thrombosis in unusual sites constituted the remaining 9% of patients. Specifically, 5 (4%)patients had upper extremity DVT ,2 (2.4%) patients had portal vein thrombosis, another 2 (2.4%) had mesenteric vein thrombosis and 1 (0.8%) patient had renal vein thrombosis .Figure 2.

Figure 3. Baseline Characteristics- Site of DVT among the non participants



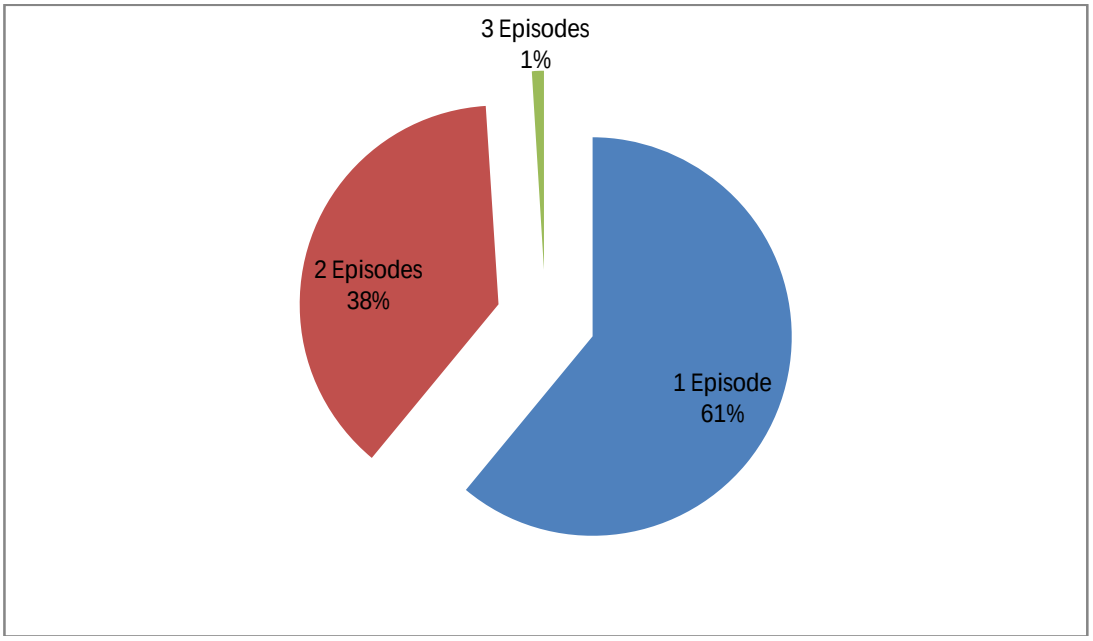
Among the non participants, distal DVT was the most common form of presentation (48%) followed by proximal DVT (39%).Seven patients (3%), were treated for saggital sinus thrombosis and 3(1%) patients were treated for subclavian catheter related upper extremity thrombosis. Figure 3.

BASELINE CHARACTERISTICS-NUMBER OF THROMBOTIC EPISODES

This study found that 46(38%)patients were treated for recurrent DVT. The most common number of recurrences was 2(38%). Only 1(0.8%) patient had been hospitalized thrice for DVT . Figure 4.

Among the study non participants, 78 (31%) of patients had recurrent disease but no patient had more than 2 thrombotic episodes.

Figure 4: Baseline Characteristics- Number of Thrombotic Episodes



A total of 120 specimens were analyzed for protein c levels. Ten (8.3%) patients were found to have deficient protein c activity levels. The mean protein c activity level was 88.4% and the values ranged from 27% to 128%. Table 2.

Table 2: Statistical Analysis of Protein C Values in the Study Population

Variable	Frequency (%)	
Protein C levels n=120		
Mean (SD)	88.4 (19.)	
Median	79.5	
Range	27-128	
Deficiency n= 10		
Deficient (<70%)	10 (8.3)	(95% CI 3.3,14.2)
Normal	110 (91.7)	

Table 3: Protein C Deficiency Patient Summary

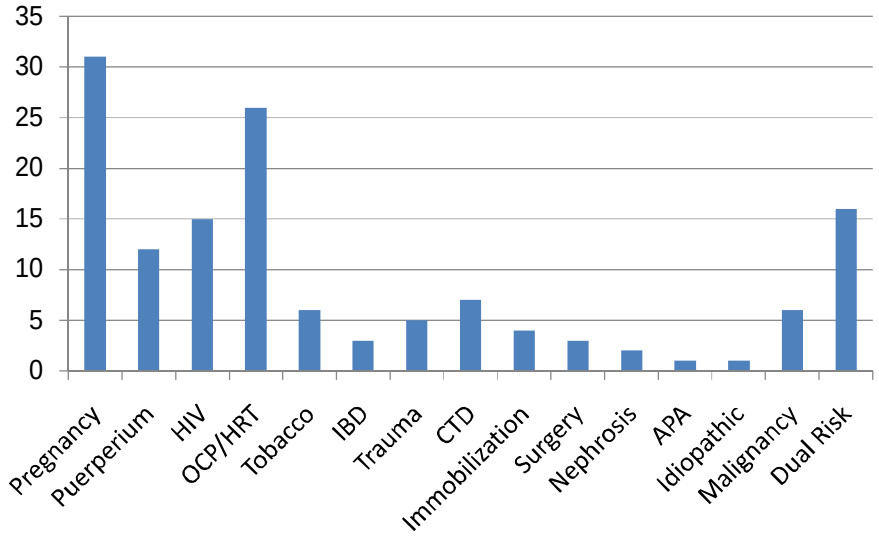
AGE	SEX	MORBIDITY	RISK FACTOR
32	Female	Recurrent Proximal DVT	OCP
29	Female	Proximal DVT	OCP
28	Female	Recurrent Distal DVT	OCP
34	Female	Distal DVT	OCP
28	Female	Proximal DVT and 1 pregnancy loss	Pregnancy
37	Female	Proximal DVT	Pregnancy
29	Female	Distal DVT and 2 pregnancy losses	Pregnancy
39	Female	Recurrent proximal DVT	HIV
31	Female	Distal DVT	HIV
32	Male	Proximal DVT	Idiopathic

Nine of the ten (90%) patients with protein c deficiency were females. The most common risk factor among these patients was use of oral contraceptive pills (40%) while one patient had DVT of unknown cause. Of note is that two patients(20%) had experienced pregnancy losses occurring independently from the DVT episodes. Only three (30%) patients had recurrent DVT. Table 3.

RISK FACTOR PATTERN IN THE STUDY POPULATION

The most prevalent risk factor in the study population was pregnancy and puerprium that comprised of 35.8% of the study population. Use of OCP and HIV infection were also common triggers of DVT comprising of 21.6% and 12.5% of the study population respectively. The patients who had developed DVT following surgery (3.3%) had all undergone orthopaedic surgery. One patient (0.8%) had positive anti-phospholipid antibodies (APA). Seven patients (5.8%) developed DVT secondary to malignancy with solid tumours being the most common malignancies. Specifically,3 had breast cancer,2 had Prostate cancer and 2 had chronic myeloid leukemia .Sixteen patients (13.3%) had dual risk factors with HIV infection and OCP use being the most common combination. Figure

Figure 5: Risk Factors In The Study Population



DISCUSSION

This study set out to determine the prevalence of protein c deficiency in a population of patients with venous thromboembolism and to document the risk factors found in these patients. We found a protein c deficiency prevalence of 8.3% that is similar to other studies done on this topic. In a Japanese study by Sakata et al, a prevalence of 5% was found while in Italy, De Stefano et al found a prevalence of 3.2% (84,85).In African Houenass et al carried out a case control study in Benin and found a prevalence of 9.3%.[86]

One possible explanation for these narrow differences is that the reference values for thrombophilic disorders have been derived from studies carried out on Caucasian subjects so the normal reference values in Africans have not been established. To this effect, one study was done to determine levels of Protein C, S, Antithrombin III and Lupus anticoagulant, compared the levels of these proteins in healthy North Americans, Brazilians and Nigerians. This study showed that the West Africans had lower levels of these proteins and these differences were statistically significant .This study concluded that failure to account for these ethnic variations in normal levels of different thrombophilic markers may lead to an over-diagnosis of these deficiencies in African subjects.[87]

Genetic polymorphisms vary appreciably within and across racial groups. These genetic variations may explain the observed differences in thrombophilic markers across racial groups. Protein C has over 160 different mutations that encode for it and this varied genetics has been proposed as one of the reasons for variations in the phenotypes seen for protein c deficiency as well as the different prevalences in different races.[87] Studies are however needed to further characterize these different mutations.

Laboratory aspects may also in part explain he observed differences. In this study a chromogenic assay was used. This type of assay is has higher sensitivity and specificity for both low levels (Type 1 deficiency) as well as functional abnormalities (Type 2 deficiency). However inter-laboratory co-efficients of variation for abnormal Protein C levels vary between 4% to 11%% for chromogenic assays which may play a part in the observed differences.[113]

Although our study population comprised largely of females (78%), gender is not an independent risk factor for venous thromboembolism. There is good quality evidence that the

magnitude of venous thromboembolic risk is related to specific factors that affect women, e.g. pregnancy, use of OCPs or HRT, but not to the gender per se.[114]In this study, women were more enthusiastic about participating and this also may have influenced the largely female participation .Whether a persons' sex predisposes to recurrent venous thrombosis is not clearly known. One prospective study carried out on patients with DVT without out any known inherited or acquired risk factors showed that males had a higher relative risk for recurrent venous thrombosis compared to females.[115]Another possible explanation for the female preponderance in our study population could be that the females were generally more enthusiastic about participating in the study and easily perceived the benefit of participating in the study compared to males.

Protein C deficiency has been associated with initial venous thrombosis occurring at an early age, generally before the age of 40.The mean age of the study population in this study was 36.9 years while the protein c deficient group had a mean age of 31 years. The findings of this study are therefore consistent with what has been documented in literature.

In this study, the majority of patients (91%) had experienced lower limb DVT. In the Protein C deficiency group, all patients had experienced lower limb DVT only. Inherited thrombophilia plays an important role in the etiology of lower limb deep venous thrombosis being found in about one third of patients with the disease.[116]This study found a 4% prevalence of upper extremity deep venous thrombosis. Studies on upper limb DVT are less solid because of the rarity of this thrombotic manifestation. In this study, patients with upper extremity DVT developed it after subclavian catheter insertion which per se justifies thrombus formation. Studies on upper limb DVT not related to catheter insertion have included small series of patients. It appears that inherited thrombophilia are less frequently found in patients with upper limb DVT than lower limb DVT with the prevalence of Factor V Leiden at less than 10%, which is less than half the prevalence reported in lower limb DVT.[118,119]Among the patients who were not recruited into the study,10 (4%) of them experienced upper extremity DVT. Of these, 3(30%)were unrelated to catheter insertion which implies that catheter insertion is the main risk factor for upper extremity DVT in our set-up. In this group of patients, transient risk factors like immobilization and OCP use are rarely linked to upper extremity DVT and this can be atleast partly explained by the absence of valves and therefore stasis in the deep veins of the arm.[119] In these patients with upper limb DVT in the absence of an indwelling catheter, one may consider possible extraneous manual work like weight lifting (effort syndrome).Anatomic abnormalities such as thoracic outlet

compression or anomalous musculofascial bands may aggravate the intrinsic compression of the veins during muscular contraction. Such aggravation leads to endothelial damage, fibrosis, and reduced blood flow, any of which may contribute to thrombus formation.[116]

Abdominal DVT can present as either Budd Chiari Syndrome (IVC) or splanchnic thrombosis which may affect the portal or mesenteric veins. In this study, we found a minority of patients (4%) with abdominal vein thrombosis. Two patients (2%) had mesenteric vein thrombosis while a similar number (2%) had portal vein thrombosis .Denninger et al carried out a study that linked abdominal vein thrombosis to natural anticoagulant deficiencies like Protein C deficiency and Antithrombin deficiency.[119] Jansen et al carried out a case control study in India that showed that Protein C deficiency is the second most common inherited risk factor for either Budd Chiari Syndrome or Portal Vein Thrombosis, after Factor V Leiden.[120] In this study, the patients with abdominal vein thrombosis all had acquired risk factors as the predisposing condition with malignancies (Chronic Myeloid Leukemia) being responsible in three of the four patients. In the Protein C deficiency group, there were no cases of abdominal vein thrombosis which could be explained at least in part by the small numbers in this study.

In the study population, we did not find any patient with cerebral vein thrombosis. However, in the group of patients who were not recruited we found 7 (3%) patients with cerebral vein thrombosis. These patients were not eligible for recruitment because of concurrent warfarin use. The most common risk factor in this group of patients was oral contraceptive pill use (57%).Other risk factors identified in this group of patients were pregnancy and puerperium. There is evidence showing that the prevalence of inherited thrombophilia in patients with cerebral vein thrombosis is similar, or higher to that found in patients with lower limb DVT cases.[116]In one meta-analysis of seventeen studies, the Prothrombin gene mutation and Factor V Leiden were the most common inherited risk factors among patients with cerebral vein thrombosis.[121] Among transient risk factors, a crucial role has the use of oral contraceptive pills present at the time of CVT in upto 85% of patients in some series while pregnancy and puerperium were present in 30-35% of patients.[122]

Renal vein thrombosis was present in only 1 (1.2%) of the study population and this patient was on follow-up for nephrotic syndrome. None of the patients in the Protein C deficiency group had developed renal vein thrombosis. Renal vein thrombosis has generally been

associated with loss of Antithrombin III through urine in patients with proteinuria. Some studies have shown that serum levels of AT III are below 70% of normal in patients with proteinuria who develop thrombotic phenomena.[116] Protein C is a much larger molecule compared to AT and is not readily lost through urine so protein c deficiency may not directly directly result in thrombotic disease but it may play a complementary role through loss of inhibition of Factor V and VIII.[123] However, several studies have linked homozygous Protein C deficiency occurring in neonates with renal vein thrombosis and is fatal in most cases.[124]

This study found that the Protein C deficiency group had 4 patients who developed thrombotic episodes while on oral contraceptive pills. The available data has shown that young women with FVL, Prothrombin mutation or Protein c deficiency, who are on estrogen containing oral contraceptive pills have a markedly increased risk of venous thromboembolism with the highest risk being for Factor V Leiden [58,59]. Three patients in this group developed DVT during pregnancy and two of these three patients had pregnancy losses in late pregnancy. There is evidence that has shown a clear association between Protein C deficiency and pregnancy complications like venous thromboembolism and recurrent miscarriages. One large study, the EPCOT study prospectively evaluated 843 women with thrombophilia. The highest risk for late pregnancy loss was seen in women who had Protein C and Antithrombin deficiencies[44].

There were 2 patients in the Protein C deficiency group who had HIV infection when they developed venous thrombosis. Several coagulation abnormalities have been described in HIV positive patients that predispose to venous thrombosis. These include deficiencies in Protein C, S, increased levels of D-dimers, P-Selectin and Factor VIII levels[77]. It is however unclear whether these deficiencies are real or they represent a consumptive coagulapathy state [78]. Of note is that apart from these altered coagulation parameters, other factors have been shown to influence thromboembolic disease in patients with HIV infection. These include; nadir CD4 counts, active infection and CMV viraemia[80,81].One patient in the Protein C deficiency group had idiopathic venous thrombosis. Inherited thrombophilic disorders have been strongly associated with idiopathic deep venous thrombosis[82].

In this study, recurrent DVT occurred in 39% of the population. In the group with recurrent DVT, the most common number of recurrences was 2 (98%) while only 1 (2%) patient had been hospitalized thrice. Some of the reasons why these patients, many of whom should have

been on long term anticoagulation were not on any medication include; lack of information from their primary care givers as well as fatigue related to taking daily pills and frequent laboratory testing. This study found pregnancy, oral contraceptive use and HIV infection to be the most common documented risk factors for venous thromboembolism. This study was carried out in a referral centre where high risk pregnancies are managed so this may explain why pregnancy was a more common risk factor. HIV infection is more prevalent in the African continent and this may reflect in higher numbers of HIV related venous thromboembolic disease observed in this study. A survival bias may also have affected the number of patients enrolled with venous thromboembolism due to malignancy or pulmonary thromboembolism as most of these patients were either deceased or too sick to come. Dual risk factors were seen in 16(13.3%) of the study population. Oral contraceptive pill use and HIV infection was the most common combination of risk factors found. This highlights the need for primary care givers to give adequate information to HIV positive patients on the added risk conferred to them by use of oral contraceptive pills and other risk factors like tobacco and immobilization.

CONCLUSIONS

Protein C deficiency occurred in 8.3% of this study population. The most common risk factors for VTE found in this study were pregnancy and puerperium, oral contraceptive use and HIV infection in decreasing order of frequency.

RECOMMENDATIONS

1. Studies are needed to identify the different mutations that occur in Protein C deficient persons and the phenotypes that these mutations encode for.
2. Larger studies that will enable to make correlations between Protein C deficiency and other risk factors like pregnancy and oral contraceptive pill use.
3. Adequate information be given to patients with venous thromboembolism on other acquired risk factors that may increase their risk of recurrent disease.
4. A study on upper limb DVT among ICU patients with central venous catheters should be carried out.

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APPENDICES

Appendix I : STATEMENT OF INFORMATION

Prevalence of Protein C Deficiency among patients with venous thromboembolism at the Kenyatta National Hospital

Purpose Of The Study:

I, Dr. Charles Mwai Ngare, am undertaking this study on the prevalence of Protein C deficiency among patients with venous thromboembolism previously treated at the Kenyatta National Hospital. This is a novel study that will hive useful information on this relatively rare disorder and will help estimate the risk of recurrence of this disease in this group of patients.

Right to refuse

Your participation in this research is voluntary. You are free to withdraw from the interview at any time and you shall not be discriminated upon. You are free to ask any questions and have a right to satisfactory answers before you sign the consent form.

If you agree to participate in this survey may you kindly sign on the consent form?

Benefits

You will not be charged for any of the lab tests.

The findings of the physical examination and laboratory tests will form part of your usual care. You will be provided with transport funds to travel to and from you home on the scheduled interview date.

Appendix 2: Study Proforma

PATIENT CODE……

DATE OF INTERVIEW……

NAME……

PHYSICAL ADRRESS……

TELEPHONE NUMBER……

DATE OF BIRTH……

SOCIO DEMOGRAPHIC DATA

Gender M ☐ **F** ☐

Marital Status Single ☐ **Married** ☐

Level of Education None ☐ **Primary** ☐ **College** ☐

Occupation Employed ☐ **Unemployed** ☐

PHYSICAL EXAMINATION

PALLOUR ☐ JAUNDICE ☐

OEDEMA ☐ ORAL THRUSH ☐

LYMPHADENOPATHY ☐ CYANOS ☐

DOCUMENTED RISK FACTORS

TOBACCO ☐ **CONNECTIVE TISSUE DISEASE** ☐

PREGNANCY ☐ **NEPHROSIS** ☐

PUERPERIUM ☐ **IMMOBILIZATION** ☐

HIV ☐ **TRAUMA** ☐

SURGERY ☐ TYPE OF SURGERY……

MALIGNANCY ☐ TYPE OF MALIGNANCY……

ANTIPHOSPHOLIPID ANTIBODIES ☐

INFLAMMATORY BOWEL DISEASE ☐

INDWELLING CATHETER ☐ **OCP/HRT** ☐

IDIOPATHIC ☐ **SICKE CELL DISEASE** ☐

POLYCYTHEMIA VERA ☐ **TAMOXIFEN** ☐

Appendix III: Laboratory Analysis

4 ml of blood was drawn using a vacuum technique. Blood was filled into citrated vaccutainers.

Specimens will then be centrifuged at 3000 rpm for 10 min to separate the plasma.

Plasma specimens were labelled clearly and frozen at -25*C awaiting batch processing.

An automated Chromogenic assay **(HemosIL)** for Protein C was used for quantitative determination of Protein C levels.

Internal Quality Control was run after every 20 specimens to ensure reliability of results.

Appendix IV: CONSENT FORM

I……..consent to participate in the study on; PREVALENCE OF PROTEIN C DEFICIENCY AMONG PATIENTS WITH VENOUS THROMBOEMBOLISM AT THE KENYATTA NATIONAL HOSPITAL. I do this with the knowledge of the purposes of the study and the procedures have been explained to me clearly by DR.CHARLES MWAI NGARE. I 'am also aware that I can withdraw from this study without losing any benefits and quality of care of my medical condition.

Anticipated risks include slight bleeding when blood is drawn.

Date and time of interview.....

Telephone Contact (Where possible).....

Signature of Study Subject.....

Signature of witness .....

If you have any questions during the course of the study, you may contact the following.

Dr. Charles Mwai

Mobile number 0721426068/ 0734826934

OR

The Chairman of the Ethical and Review committee

Kenyatta National Hospital

Tel 020-2726300/0722-829500/0733-606400 Ext, 44102

Appendix V: List of Tables

Appendix VI: List of Figures