HAEMOSTATIC DISORDERS IN PATIENTS WITH BREAST CANCER AT KENYATTA NATIONAL HOSPITAL, KENYA

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DECLARATION

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I **Dr. Peter K. Asaava,** declare that this dissertation for master of medicine in human pathology is my original work and has not, to the best of my knowledge, been presented by any other individual at any other institution of higher learning.

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This dissertation for Master of Medicine in Human Pathology is submitted with our approval.

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DEDICATION

This work is dedicated to my daughter Audrey B. Asaava and my friend and partner June D. Otieno.

ACKNOWLEDGEMENTS

I wish to acknowledge with gratitude all those who have contributed in any way directly or indirectly to the successful completion of this dissertation. Special thanks go to my supervisors: Professor J. Githanga, Dr. R Pamnani and Dr. G. Kiarie who guided me as I compiled this work. I am also deeply grateful to Mrs Karanja who assisted me in the oncology unit and Mr Jackson Ireri for his professional and tireless work in analysis of the samples in the university of Nairobi haematology laboratory. For those whom I have not mentioned by name including the statistician, my friends and family who provided me the necessary support for the completion of this work, I am deeply grateful.

ABBREVIATIONS

APTT:	Activated partial thromboplastin time
AT:	Antithrombin
BMA:	Bone marrow aspirate
CVC:	Central venous catheter
CMF:	Cyclophosphamide, methotrexate, 5-flourouracil
CCF:	Congestive cardiac failure
DVT:	Deep venous thrombosis
DIC:	Disseminated intravascular coagulation
ELISA:	Enzyme linked immunosorbent assay
ENT:	Ear nose throat
FDPs:	Fibrin degradation products
FHG:	Full haemogram
MBChB:	Bachelor of Medicine and Bachelor of Surgery
Mls:	Milliliter
PT:	Prothrombin time
PBF:	Peripheral blood film
SD:	Standard deviation
TF:	Tissue factor
TT:	Thrombin time
TAT:	Thrombin antithrombin
UON:	University of Nairobi
VTE:	Venous thromboembolism
vWD:	von Willebrand Disease
RBC:	Red blood cell
WBC:	White blood cell
Hb:	Haemoglobin

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ABSTRACT

Background

Haemostatic disorders (thrombosis and haemorrhage) are increasingly being investigated and assessed as factors that influence outcome of treatment of solid tumors including breast cancer. There is little data available on prevalence and influence of these disorders in patients with breast cancer locally.

Objective

To determine and describe haemostatic disorders in patients with breast cancer at Kenyatta National hospital (KNH), Nairobi.

Design and Setting

A cross-sectional descriptive study conducted at KNH oncology unit and the University of Nairobi (UON) haematology laboratory.

Methods

One hundred and three (103) eligible patients were studied. Data was obtained by direct interviews, clinical examination and from patient files. Full haemogram (FHG), peripheral blood film (PBF), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, thrombin time (TT) and d-dimers were assayed at the UON haematology laboratory.

Data handling

Data collected and that generated from the laboratory tests was analysed using Statistical Package for Social Sciences (SPSS) Version 15 software for analysis and presented as proportions and percentages in form of tables and charts. Correlations between variables were determined where appropriate.

Results

A total of 103 patients were recruited into the study 93.2% of whom were female and the majority in the 36-45 year age group (31.1%). Ninety three percent (93%) had invasive ductal carcinoma and 6.8% with lobular carcinoma. One patient had lower limb deep venous thrombosis and none had clinical features of haemorrhage (petechiae, purpura, and easy

bruisability). During the course of treatment, 93.2% had had surgery. Fifty eight percent and 31.1% were on chemotherapy and radiotherapy respectively. Abnormal clotting times were observed in 21.3% and elevated d-dimers in 7.7% of the participants. There was a statistically significant association between abnormal clotting times, elevated d-dimers and elevated fibrinogen levels with advanced disease (stage IV).

Conclusions

1. Haemostatic disorders are present in patients on management for breast cancer at KNH at a rate of 21.3%
2. The disorders are present at low prevalence compared to other studies done in other centers
3. Late stage of disease (Stage IV) is associated with likelihood of haemostatic disorder development

Recommendations

1. This study demonstrated that there are coagulation disorders (DVT, elevated d-dimers, prolonged clotting time) in patients with cancer with significant association with late stage disease. It is therefore recommended that coagulation disorders be screened for in patients with breast cancer in late stage disease.
2. It is recommended that the study be undertaken where the haemostatic parameters will be assayed on automated equipment to improve accuracy e.g. use of ELISA to identify d-dimers in thrombosis, automated PT, aPTT and thrombin time.
3. Other similar studies enrolled participants who were newly diagnosed and not on treatment. A similar study to be undertaken using similar criteria so as to delineate effect of disease and treatment as independent and related variables on haemostasis.
4. To conduct a prospective study in Kenyan patients with quantifiable objective endpoints such as venography and/or Doppler studies.

INTRODUCTION

Breast cancer is the commonest malignancy among females in Kenya (23.3%) and the least among males 0.7% (1). Haemostatic disorders (thrombosis and haemorrhage) are the second commonest cause of morbidity and mortality in patients with solid tumours (23%), after infections (36%). Other causes of morbidity and mortality include organ failure due to metastasis, cachexia, infarction and carcinomatosis (2). Local data on causes of morbidity and mortality are not available.

Cancer and haemostasis demonstrate an increasingly important association that was first described by Trousseau in 1865. He postulated the link between migratory thrombophlebitis and malignancy (3). The haemostatic disorders include thrombosis and haemorrhage with thrombosis being the most common disorder which is observed in 15% of patients with cancer (4, 5). The spectrum of thrombosis includes deep venous thrombosis, superficial vein thrombosis, migratory thrombophlebitis, pulmonary embolism, central venous devices thrombosis and arterial thrombosis (6). Clinically overt thrombotic disorders are demonstrated in 28.7% of affected patients, in contrast to 50% rate at autopsy (7, 8).

Hemorrhagic disorders are less common and occur in the setting of disseminated intravascular coagulation (DIC) and in patients on treatment or prophylaxis for venous thromboembolism (VTE) as well as in patients with wide spread metastasis and liver disease (9, 10). Haemorrhage may present as mucosal petechiae/ecchymosis, skin purpura or bleeds.

The pathogenesis of these haemostatic disorders is multifactorial with interactions between tumour and haemostatic system influencing underlying disease and development of complications. The main mechanisms involve: platelet abnormalities (thrombocytopenia, thrombocytosis and platelet function abnormalities), abnormalities in coagulation factors, activation of coagulation and acquired thrombophilia e.g. antithrombin (AT) deficiency, protein C and S deficiency, tissue factor pathway inhibitor deficiency and elaboration of pro-inflammatory cytokines (11,12).

In breast cancer, the main haemostatic disorder is thrombosis with 5-6% of patients developing thrombosis. The major risk factors include: older age, female gender, postmenopausal age, late stage disease, surgery, combination of tamoxifen and chemotherapy treatment and comorbidity (13, 14).

Despite being the second commonest cause of morbidity and mortality in patients with solid malignancies, haemostatic disorders remain under recognized and under treated. Haemostatic disorders are indicators of poor prognosis, recurrent hospital admissions and increased probability of death (15).

LITERATURE REVIEW

Breast Cancer

Breast cancer is the commonest malignancy among females in Kenya accounting for 23% of all female malignancies. Among males, it's the least common malignancy accounting for 0.7% of all male malignancies. In sub-Saharan Africa, male breast cancer accounts for 1-5.7% of breast cancer prevalence and 1% in Europe (16, 17). Globally, female breast cancer accounts for the second commonest cancer after skin cancer with a prevalence of 10.4%. In Kenya and sub-Saharan Africa, age at presentation is a decade younger and presentation is at a more advanced stage (19).

Haemostatic Disorders

In the course of illness 15% of all cancer patients develop clinically apparent thrombosis (20). Major bleeding episodes occur in 8% of the patients with solid tumours (21).In breast cancer, thromboembolic disease rates vary from 5-10% (22). This rate is in contrast with the autopsy rate of 50%. Bleeding episodes are associated with DIC, metastasis, and treatment with anticoagulant therapy. Locally, no data is available on prevalence and risk factors for development of haemostatic disorders with breast cancer.

Pathogenesis

Interaction of tumour cells and the haemostatic system is the basis of the development of the disorders. The disorders may be due to the cancer, its treatment or both. Alterations in haemostasis commonly accompany the progression of malignancy and any component of the haemostatic system may be affected by disease or treatment. Most changes are subtle and few result in signs and symptoms (23).

Risk factors for thrombosis

Risk factors for development of thrombosis include (24, 25):

- Pharmacologic: erythropoietin, hormone modulators such as tamoxifen, antiangiogenic medication like thalidomide, chemotherapy e.g. cyclophosphamides, methotrexate and fluorouracil combination
- Patient characteristics: postmenopausal age, female gender, black ethnicity, comorbid conditions e.g. sepsis

- Disease characteristics: late stage disease, duration of cancer, metastasis to liver
- Mechanical: surgery, central venous catheters

Risk factors for haemorrhage

They include liver metastasis, bone marrow infiltration and anticoagulant therapy

Mechanisms causing haemorrhage

Sepsis and wide spread metastasis may lead to bleeding by provoking development of DIC which is a rare disorder in cancer patients. If it develops, it is rapidly progressive and fatal (26, 27). Haemorrhage may also be due to platelet abnormalities (quantitative and qualitative). Thrombocytopenia occurs in up to 11% of patients with untreated malignancy (28). Thrombocytopenia may be due to bone marrow infiltration, chemotherapy, radiotherapy, sepsis or DIC. Risk of bleeding depends on extent of thrombocytopenia and may be localized or generalized e.g. petechiae, ecchymosis, epistaxis, oral bleeding, gastrointestinal bleeding and genitourinary bleeding (28). Thrombocytopathies which are a cause of functional platelets disorders may be due to acquired von Willebrand disease (vWD), uraemia in patients with renal failure and coagulation factor deficiency due to metastasis are risk factors for haemorrhage (28).

Mechanisms causing thrombosis

Cancer stimulates a procoagulant state with activation of coagulation which is demonstrated by presence of elevated d-dimers, prothrombin fragment 1+2, elevated thrombin-anti thrombin complex, and deficiencies of protein C, S and antithrombin (29, 30). The procoagulant state is due to production of fibrinolytic procoagulant substances and cytokines which are only elaborated by malignant cells. Cancer procoagulant activates factor X independently contributing to procoagulant state of cancer. Tissue factor is another mediator of coagulation activation. It is constantly being expressed without regulatory control with resultant effect of constant activation of coagulation. Decrease in levels of natural anticoagulants (antithrombin, protein C and protein S) due to metastasis to the liver contributes to the overall procoagulant environment (31, 32). Increased plasminogen inhibitor and impaired fibrinolytic activity enhance prothrombotic mechanisms of the tumour (33). Several factors like surgery, chemotherapy e.g. cyclophosphamide, methotrexate and 5-

flourouracil (CMF) combination treatment in breast cancer, comorbidity and use of antiangiogenic treatment further aggravates the procoagulant state (34). (Figure 1)

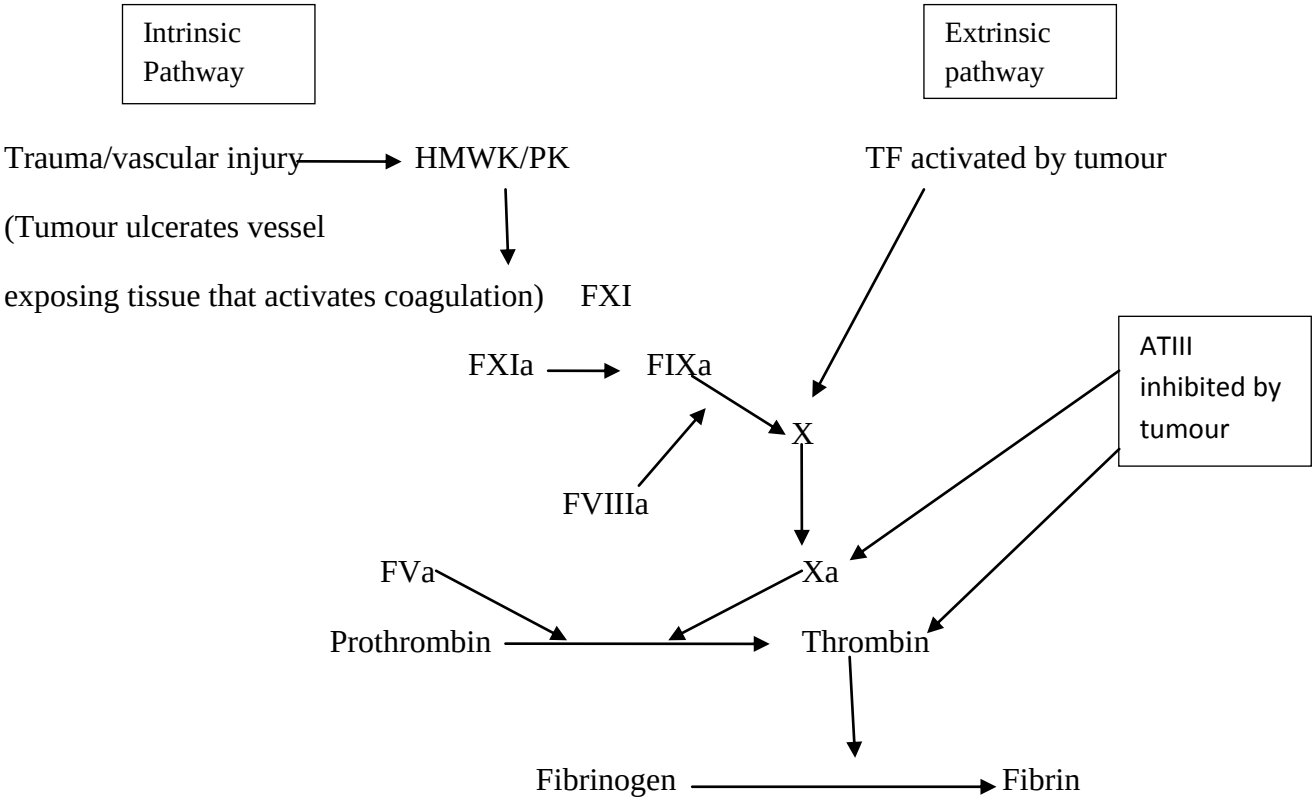


Figure 1: Prothrombotic effects of a tumour cell

Haemostasis in breast cancer

Haemostasis in breast cancer is further modulated by stage of disease with early disease associated with 0.1% of VTE and late disease 17% of VTE. This is directly proportional to tumour burden and metastasis. Menopause is also associated with increased risk of development of thrombosis. Specific cancer treatments have been demonstrated to activate coagulation towards a thrombotic state. Combination of CMF and tamoxifen therapy increase procoagulant activity with studies demonstrating increase in markers of in vivo coagulation like thrombin anti-thrombin (TAT) and d-dimers with a decrease in natural anticoagulants protein C, Protein S, and antithrombin (35). Combination of chemotherapy and tamoxifen increases VTE risk 2-7 fold. In combination with antiangiogenic treatment e.g. thalidomide, the risk markedly increases to 27% (36, 37).

Haemostatic disorders have a negative impact on treatment and outcome of breast cancer treatment. Fatal and non-fatal bleeding events and VTE may occur. A British study in 1994,

demonstrated that low dose warfarin prevents occurrence of VTE events (38, 39). Several studies have demonstrated improvement in treatment outcome and regression of disease in patients put on low molecular weight heparin independent of its action as an anticoagulant hence need to identify patients with activated coagulation (40).

Current focus is now on relating elevated levels of d-dimers and distant metastasis (lymph-vascular invasion) with persistence of haemostasis activation as a strong predictor of recurrence and poor outcome. Plasma d-dimer levels are markers of lymph-vascular invasion, clinical stage and lymph node involvement in operable breast cancer. Correlation suggests that detectable fibrin degradation products are a clinically important marker for lymph-vascular invasion and early tumour metastasis in breast cancer (41). Laboratory methods that can be used to demonstrate thrombo-haemorrhagic complications include: prothrombin time, thrombin time, activated partial thromboplastin time, fibrinogen, d-dimers and platelet count. Surveys employing prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrin degradation products (FDP), fibrinogen levels and total blood count show anomalies in more than 50% of cancer patients and in over 90% of patients with metastatic disease (42). Not all demonstrable abnormalities in these parameters are associated with clinically apparent signs and symptoms of haemostatic disorders.

Laboratory investigations

Thrombin time is used to detect deficiency or abnormality of fibrinogen, inhibition of thrombin by inhibitors and FDPs e.g. in DIC and heparin therapy. Prothrombin time identifies deficiency or inhibition of one or more of the following coagulation factors: VII, X, V, II and fibrinogen. Prolonged prothrombin time is commonly due to liver disease or warfarin therapy. APPT prolongation indicates deficiency or inhibition of one or more of the following coagulation factors: XII, XI, IX, VIII, X, V, II or vWF, heparin therapy and liver disease (43). Thrombocytopaenia is a common complication of disease and /or treatment hence a blood count including a platelet count, PBF and bone marrow aspirate (BMA) smear examination may be required. Fibrinolysis and/or thrombosis can be identified and assessed by determining fibrinogen levels, d-dimers and/or FDPs levels (44). The specificity of these assays is lowered by hepatic disease, renal insufficiency, surgery, septicaemia, stroke and major trauma. High values of d-dimers or FDPs are therefore, not confirmatory of disease as compared to low values which are confirmatory of lack of procoagulant activity and ongoing fibrinolysis. D-dimer assays are used for exclusion of DVT or diagnosis of DIC (45). More

sensitive assays for thrombosis include prothrombin fragments 1+2, thrombin-antithrombin complex (TAT) and factor assays for factor VIII, protein C and protein S levels (46). Haemostatic disorders in patients with solid malignancies are increasingly being used as prognostic factors in management of these patients (47). Systemic activation of clotting system occurs frequently and correlates with tumour burden, clinical progression and response to chemotherapy. Development of these haemostatic complications is a poor prognostic factor and evidence (clinical or laboratory) may herald relapse or progressive disease (48). Despite treatment, there is a high risk of recurrence of thrombo-haemorrhagic complications in patients with solid malignancies hence close follow up is recommended. Anticoagulants are now used as an adjunct to cytotoxic therapy and to increase survival therefore there is a need to identify patients who develop thrombosis (49).

STUDY JUSTIFICATION

Haemostatic disorders are independent predictors of poor outcome in patients with malignancy and the second commonest cause of morbidity and mortality in patients with cancer after infections. However, prevalence of these disorders in patients with malignancy has not been established locally therefore recognition and timely interventions may be delayed. This delay usually results in hospital admissions due to thrombosis and haemorrhage with increased morbidity and mortality.

Since breast cancer is the commonest malignancy in females locally this study aims to describe prevalence of these complications in patients with breast cancer (16, 17).

RESEARCH QUESTION

What are the haemostatic disorders in patients with breast cancer at Kenyatta National Hospital (KNH)?

BROAD OBJECTIVE

To determine and describe the haemostatic disorders in patients with breast cancer at KNH

SPECIFIC OBJECTIVES

1. To establish prevalence of abnormal haemostatic parameters (TT, PT, APPT, fibrinogen levels, d-dimers, platelet count) in patients with breast cancer at KNH
2. To determine any correlation between patient characteristics (sex, stage of disease, treatment modality, co morbidity) and abnormal haemostatic parameters

METHODOLOGY: MATERIALS AND METHODS

STUDY DESIGN:

A cross sectional descriptive study that was conducted from November 2009 to April 2010.

STUDY POPULATION

Patients who had breast cancer and were receiving treatment at KNH.

STUDY SITE

Kenyatta National Hospital (oncology unit clinic, breast clinic, surgical and medical wards)

SAMPLE SIZE

Sample size: $n = \frac{Z^2 P(1-P)}{d^2}$

Where

- n is minimum number of patients with breast cancer who develop haemostatic disorders
- Z is the standard of mean corresponding to a 95% confidence interval
- P is the prevalence of thromboembolism in patients with breast cancer in this a prevalence of 7% is used
- d is the margin of error with degree of precision set at +/- 5%

The number was $\frac{1.96^2 x 0.07(1-0.07)}{0.05^2} = 103$

Inclusion criteria:

1. Patients who had consented
2. Patients who had a histology diagnosis of breast cancer
3. Patients who had a diagnosis for breast cancer and a known diagnosis of a comorbidity e.g. heart disease, HIV, Infections

Exclusion criteria

1. Patients with known hereditary haemostatic disorders.

2. Patients on anticoagulant therapy for haemostatic disorders (acquired or congenital).

3. Patients who did not give consent.

Recruitment of study participants and the process of consenting

The study participants were recruited at the radio-oncology clinic, breast clinic, medical and surgical wards at the KNH. To take part in the study the potential participants were informed of the importance, merits and demerits of the study and the need to give an informed consent (Appendix I). The identified breast cancer patients were approached and informed about the study. Informed consent was then obtained to use their demographic and clinical data obtained from their clinical records. The participant was interviewed, examined, records reviewed for relevant data and specimens collected by the principle investigator. Participants who were on treatment for breast cancer and who had not started treatment were all included in the study.

Clinical Procedures

Demographic and clinical data was then recorded in the questionnaires (Appendix II). The interval between treatment modality initiation (mastectomy, chemotherapy and radiotherapy) and current study did not influence recruitment of a participant into the study. In addition to clinical history and clinical physical examination, features determined and confirmed by imaging techniques like Doppler studies were included where they were done and features recorded in the questionnaire such as deep venous thrombosis. Review of recruited patients' file records was done to establish treatment modality and stage of the disease. The TNM (Tumour Node Metastasis) classification for staging and treatment system was used (Appendix VI). No information on receptor analysis (hormonal or protein receptors) e.g. Human epidermal growth factor 2 (Her2) receptor was gathered.

Laboratory Procedures

Consumables for the tests

1ml CryoGen ^{tm} vials

Micropipette tips

Plain glass tubes

Microscope slides

K_3EDTA 7.2mg Vacutainer tubes 4ml Akuret [tm] LOT/Batch number: C249. Expiry 2011-02.

9NC Coagulation sodium citrate 3.2% 3.5ml VACUETTE©. LOT A0905006. Expiry:2010-05

Specimen collection and storage

Six(6) mls of blood was obtained aseptically by venipuncture and 2.5mls aliquot was put into a sterile K_3EDTA (Tripotassium ethylenediamine-tetra acetic acid) vacutainer tube for full blood count and peripheral blood film (PBF) and 3.5mls into sterile trisodium citrated vacutainer specimen tubes in a ratio of one part of anticoagulant and nine parts of blood for coagulation tests. The samples once put into the vacutainer tubes were gently mixed and transported to the UON haematology unit laboratory within one hour of collection. The samples for haemostasis were immediately centrifuged at 1500g for 5 minutes to prepare platelet poor plasma and aliquot into two cryovials and stored at -70^0c for one month.

Assay procedure

Once in the laboratory, a full blood count (FBC) was immediately performed using Cell-Dyn 1300® automated cell counter and a peripheral blood smear immediately prepared. The films were stained with Leishman's stain and were used to validate platelet counts in the thrombocytopenic range and ensure thrombocytopaenia was not due to platelet aggregation, agglutination or satellitism.

For the haemostasis tests (PT, TT, fibrinogen, aPTT, d-dimers), samples, reagents and controls (normal and abnormal) were brought to the appropriate assay temperatures. The tests were then done according to the manufacturer's instructions (Appendix III).

The full blood count was assayed on the automated Cell-Dyn 1300®. PT, TT and aPTT were assayed manually as per the manufacturer's instructions on the appended inserts. Fibrinogen was determined manually using the Clauss technique (Appendix III). D-dimers were determined semi quantitatively using the rapid agglutination assay utilizing latex beads coupled with highly specific d-dimer monoclonal antibody (Appendix III).

QUALITY ASSURANCE

Internal Quality Control

To ensure the results of the study were valid, quality assurance measures were undertaken.

Specimen collection

- Specimens: Venous blood was drawn aseptically from the cubital vein
- Lipaemic and/ or haemolysed samples were excluded
- Specimens were correctly labeled at site of collection and same number used to identify the sample throughout the analytical process
- Specimens were collected into appropriate specimen tubes (EDTA tubes & Sodium Citrate tubes) , in appropriate volumes and mixed well
- Specimens were immediately transported to the laboratory within 30 minutes where they were sorted.
- Samples in EDTA tubes were then immediately analysed within 10 minutes to yield a full blood count on the Cell-Dyne 1300® automated cell counter and a PBF blood film was prepared.
- Samples for haemostasis were centrifuged at 1500g to prepare platelet poor plasma and the plasma stored at -78^0C

All laboratory tests were performed in the UON haematology laboratory by qualified technical personnel.

Internal quality control was ensured by running control specimens with each batch of specimens analyzed. The full blood counts were determined on automated equipment with proper service records and calibration. The PBF results were confirmed by two consultant pathologists independently as internal quality procedure. PT, TT and aPTT were assayed in duplicate as a quality control. Refreezing and thawing of specimens was not done once they were thawed. Haemolysed and lipaemic samples were excluded. Freshly reconstituted reagents, all within dates were used. Normal controls, abnormal controls and calibrants provided in the reagent kits were run for each batch of specimens done.

Analytical process

- Full blood count was determined on an automated system immediately sample was received in the laboratory.
- A peripheral blood film was also immediately prepared
- Haemostasis
- Kits stored at 2-8^{0}C were used. They were verified before use to ensure that they were not expired and that they were appropriate for the tests.
- Samples were thawed once and not refrozen
- Assay procedure was as per the manufacturer's instructions
- Instruments were well calibrated
- Calibrants, normal controls and abnormal controls were run in duplicate with each batch of specimens
- Specimens were run in duplicate and a mean established
- Interpretation was as per the UON haematology laboratory guidelines

Post analytical

Data was entered into a data sheet with values entered in corresponding data space of participant

Ethical considerations

The study was undertaken after approval was obtained from the KNH/UON ethics and research committee (Appendix V).The participants were informed that their participation was voluntary and no benefits were to be expected however, test results were relayed to the doctor for use in management where applicable. Informed consent was obtained prior to participation.

Data management

Data was obtained through direct interviews, clinical examination and review of patient records to obtain information on staging and treatment. The data collected and that generated from the laboratory were entered into the data collection tool (questionnaire and data sheets). The data was transferred from data sheets to SPSS version 15 statistical software for analysis. Summary for the statistics was determined and presented as proportions and percentages in the form of tables, graphs and pie charts. Variables were correlated for any significant association using Fisher's Exact T test. A p value of <0.05 was considered significant.

Role of principle investigator:

Enrolling study participants into study, taking history, examining participants and reviewing treatment charts and clinical notes for treatments administered.

Performing some of the haemostasis tests in conjunction with qualified technical personnel and examining all peripheral blood films before confirmation by a haematopathologist.

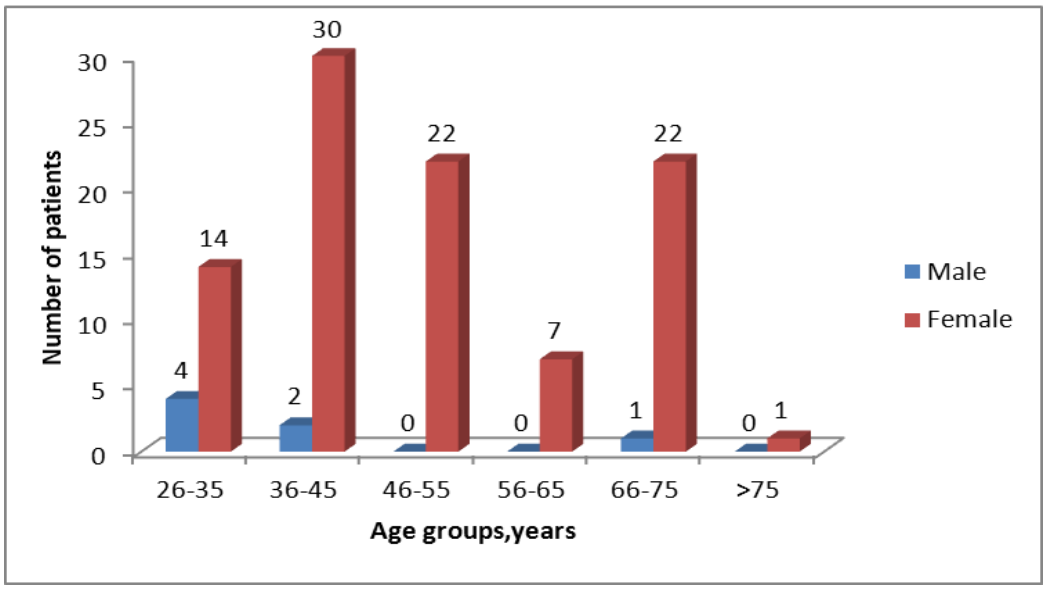
Sample exclusion: No samples were excluded.

RESULTS

Patient demographics

A total of 103 participants were recruited into the study of whom the female gender accounted for 93.2%. Majority, 31.1% of the study participants were aged between 36 and 45 years. The mean age was 48.20 years. The median was 46 years and the mode was 44 years (Figure 2)

Figure 2: Age-sex distribution of study participants



Clinical characteristics

Diagnosis

Most of the females, 88.3% and all the males had a diagnosis of invasive ductal carcinoma of the breast. Of the females, 4.8% had a diagnosis of lobular carcinoma.

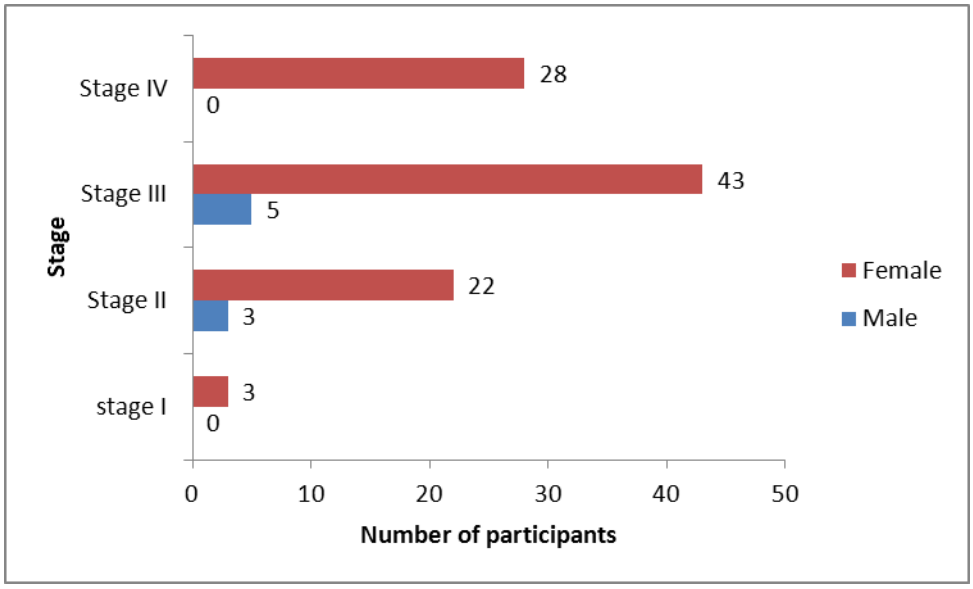
Co morbidities

Co morbidities were observed in six of the study participants and all were female. Of the co morbidities observed, five (5) cases were of HIV and one case of heart disease.

Disease stage

The majority of the female study participants, (45%) and 71 % male study participants had stage III disease (figure 3).

Figure 3: Tumour Staging of study participants (n=103)



Treatment modalities

Majority of study participants had had therapeutic surgery (mastectomy)-65%, 58.3% were on chemotherapy and 10.6% were not on any treatment (figure 4).

Majority of the participants, (67.9%) in stage III and IV were on chemotherapy. The commonest chemotherapeutic combination, (85.7%) was cyclophosphamide, methotrexate and 5-fluorouracil (CMF). No study participant recruited was on tamoxifen

Blood cellular component characteristics of study participants

Haemoglobin

The mean haemoglobin (Hb) was 14.2g/dl and values ranged from 8.6-19.0g/dl. (Median 14.5g/dl, SD 2.14). Of the 103 participants, 5.8% had Hb level between 10 and 11g/dl, and 1.9% with levels less than 9g/d (Table 1).

Red blood cells

The mean red blood cell (RBC) count of the 103 participants was 5.3 x 10^{12}/L. The counts ranged from 3.10 to 7.03x10^{12}/L (Table 1).

White Blood Cells

The mean white blood cell count was 8.1x10^{9}/L (Reference Range 4.1-10.9x10^{9}/L, SD 0.8 and a median of 5.0. The counts ranged from 3.10x10^{9}/L-13.3x10^{9}/L (Table 1).

Platelets

Of the 103 participants, 2.9% had platelet counts less than $140x10^9$/L with the lowest count being $106x10^9$/L. Majority of the participants, 82.5% had platelet counts within the UON-hematology laboratory reference range ($140\text{-}440x10^9$/L).Of the 103 participants, 14.5% had counts above the upper reference limit with the highest count being $721x10^9$/L. (Mean : $350.1x10^9$/L, SD 124.8,median $328.0x10^9$/L (Table 1).

Table 1: Haematology Cellular Parameters

Parameter	Range	Mean	Median	SD
RBC $x10^{12}$/L	3.1-7.1	5.3	5.4	0.8
Haemoglobin(g/dl)	8.6-19.0	14.2	14.5	2.1
White blood cells $x10^9$/L	3.1-13.3	8.1	5.0	0.8
Platelets $x10^9$/L	106-721	350	328	124

Peripheral Blood Film Parameters

Red blood Cell Morphology:

Majority of the participants, 68.9%, had normal peripheral blood film features, 9.7% had normocytic hypochromatic features on film, 10.7% had microcytic hypochromatic features and, 11.6% had anisopoikilocytosis with mostly schistocytes seen.

White blood cell morphology:

Thirteen participants had leucopaenia on automated count with normal morphology and distribution of leucocytes on peripheral blood film.

Platelet parameters:

The three participants who had thrombocytopaenia on automated count were confirmed to have thrombocytopaenia on peripheral blood film examination. None of the specimens with thrombocytopenia on automated count had any platelet agglutination, aggregation or satelitism.

Haemostasis parameters of study participants

Of the 103 patients, 27.1% had one or more abnormal haemostatic parameters with the majority of the participants, 72.8% having all haemostatic parameters within the reference limits of the assays.

Activated Partial Thromboplastin Time (aPTT)

Of the 103 participants, 3.8% had prolongation in aPTT only. Of the four participants, 75% were females. All were on chemotherapy. Three, were in stage III, one in stage IV and none in stage I nor II. There was no statistically significant association between prolonged aPTT and any variable (gender, age, staging of disease and treatment).

Thrombin time

Thrombin time was prolonged in 5.8% of the participants but not as a single parameter. It was prolonged in association with either prolonged PT or aPTT or both. All participants were female, and were in stage IV, one in stage III, and one in stage II. One participant was not on any treatment and 83.3% were on chemotherapy. There was no statistically significant association between prolonged TT and any variable (gender, age, staging of disease and treatment).

Prothrombin Time (PT)

Of the 103 participants, 8.7% had only the PT being prolonged of whom 88.8% were female. Majority were in stage IV. Most were on chemotherapy, none on radiotherapy and two were not on any treatment. There was a statistically significant association between stage IV disease and having a prolonged prothrombin time (p-value=0.01) (Table 2).

Haemostatic disorders

One (0.9%) patient had lower limb deep calf veins deep venous thrombosis confirmed clinically and by Doppler studies. This participant was in stage IV disease with vertebral metastasis and on a combination of radiotherapy and chemotherapy (CMF) and bed ridden. No surgery had been performed on the patient. None of the patients had any evidence of bleeding like petechiae and ecchymosis.

Table 2: Relationship between prolonged Prothrombin time and gender, Stage of Disease and Treatment (n=9).

Characteristic	Number (%)	P-value	95% confidence interval
Sex			
Male	1(11.1%)	0.96	0.03-11.8
Female	8(88.8%)		
Stage			
I	0	0.66	0.05-13.1
II	1(11.1%)	0.7	0.01-9.8
III	2(22.2%)	0.25	0.03-11.76
IV	6(66.6%)	**0.01**	0.04-17.9
Treatment			
Chemotherapy	7(77.7%)	0.56	0.38-12.4
Radiotherapy	0	0.2	0.04-1.77
Not on treatment	2(22.2%)	0.49	0.05-4.2

D-dimers

D-dimers were elevated in 7.7% of the participants of whom 87.5% were females. Majority of them, (75%) were in stage IV. Of the participants with elevated d-dimers, 75% were on chemotherapy and 25% on radiotherapy. The highest d-dimer titer was 1.6-3.2 mg/L (two participants), and three had the lowest titers of d-dimers (0.2-0.4mg/L). There is a statistically significant association between being in stage IV disease (p-value=0.003) and having elevated d-dimers (Table 3).

Table 3: Correlation of D-dimers with Sex, Age, Treatment, Stage of Disease

Patient characteristic	**Number**	**P value(Fisher's exact T test) (95% confidence interval)**
Sex		
Male	1	0.44(0.03-18.4)
Female	7	
Treatment		
Radiotherapy	2	0.75(0.74-3.45)
Chemotherapy	6	0.8(0.9-4.9)
Surgery	7	0.25(0.85-3.1)
Stage		
I	0	1(0.05-0.85)
II	0	0.30(0.09-0.7)
III	2	0.19(0.1-0.9)
IV	**6**	**0.03(1.78-33.3)**
Age group(years)		
26-35	0	0.34(0.34-2.75)
36-45	0	0.49(0.12-2.23)
46-55	3	0.36(0.27-3.3)
56-65	4	0.06(0.29-2.33)
66-75	1	0.4(0.04-2.41)
>76	0	1(0.31-2.45)

Low platelet count.

Three participants had mild thrombocytopenia (platelet count less than $140x10^9$/L). The least was $106x10^9$/L. Two of them were in stage III and one was in stage IV. One was on

radiotherapy, one on chemotherapy and one on no treatment. One participant had fibrinogen, PT, TT and aPTT within the reference ranges but with elevated d-dimers

Fibrinogen

As shown in table 4 below, 18 study participants had elevated fibrinogen levels of whom females were 14 (77.7%). Majority of them (66.6%), were on chemotherapy. Ten (55.5%) were in stage IV. There was a statistically significant association between having elevated fibrinogen levels and gender (p-value=0.01). There was also a statistically significant association between elevated fibrinogen and being in stage IV. Low fibrinogen values were not observed.

Table 4: Correlation of Elevated Fibrinogen with Sex, Treatment Type and Stage of Disease

Patient characteristic	Number	P value(Fisher's Exact T test)
Sex		
Male	4	**0.01(0.017-14.1)**
Female	14	
Treatment		
Radiotherapy	4	0.54(0.93-1.2)
Chemotherapy	12	0.54(0.94-2.6)
Surgery	10	0.08(1.1-2.9)
Stage		
I	0	1(0.82-1.7)
II	3	0.55(0.95-2.3)
III	5	0.06(1.3-2.1)
IV	10	**0.002(1.59-16.22)**

Correlation of chemotherapy treatment and haemostatic parameters

As indicated in tables 3, 4 and 5 there was no statistically significant association between chemotherapy and prolonged PT, elevated d-dimers and elevated fibrinogen levels respectively. This lack of association with chemotherapy was also seen with prolonged aPTT.

DISCUSSION

Most of the study participants were female (93.2%) with majority (29.1%) in the 36-45 years age group. Males accounted for 6.7% of the participants and in the 26-35 years age group. Majority of the patients were diagnosed on histology and had infiltrating ductal carcinoma (95.1%) and 4.9% had lobular carcinoma. None of the male patients had lobular carcinoma. This trend is comparable to the data in the Nairobi cancer registry (1) which indicates that majority of patients with breast cancer are females and occur mostly in the ages between 30 and 65 years. They presented mostly (74.7%) in the late stage disease (stages III & IV) with the fewest in stage I. Age of presentation is a decade younger than the patients in Western countries who present at an earlier stage but are a decade older as described in other studies (19). Gakwaya et al in Uganda found a female to male ratio of 24:1, with a peak age of 30-39 years (50). The comparable earlier age of presentation in Kenyan patients with breast cancer may be due to specific genetic features of breast cancer in Kenya and Uganda that have not been characterized and described. These unknown genetic characteristics may be interacting with certain environmental factors that trigger expression of the genes associated with breast cancer that result in development of breast cancer a decade earlier than in patients in Europe and North America.

One study participant (0.9%) had DVT. These figures are lower than what has been observed in other studies. Some studies demonstrated a rate of up to 15% for venous thrombosis (51). Rickles et al described thromboembolic disease in 5-10% of the participants (22). Other studies showed prevalences that vary with stage of breast cancer in the patients. Stage I had the lowest rates at 0.9%, stage II, 7% and stage IV, 17 % (52). These studies enrolled treatment naïve subjects who were then enrolled for surgery and chemotherapy after baseline hematological parameters were determined. Surgery is a significant risk factor for thrombosis with general surgery and orthopedic surgery imparting the greatest risk. Schönmann et al found a VTE rate of 6% in post-operative patients with non-metastatic cancer (53). In this study subjects were recruited with no regard to time and type of surgery which may explain the low prevalence since the patients may have recovered from the inflammatory effects of surgery. Therapeutic surgery (mastectomy) was the commonest mode of surgery undertaken and patients were later enrolled into chemotherapy and radiotherapy. At the time of the study, 58.3% of the patients were on chemotherapy, 31.1% on radiotherapy and 10.6% were not on any treatment. These findings are similar to most studies done where patients undergo mastectomy prior to chemotherapy and/or radiotherapy (53).

Haemoglobin, WBC and Platelet counts were mostly within normal limits. Three patients (2.9%) had mild thrombocytopaenia which is a low prevalence compared to other studies done in which the thrombocytopaenia rates are up to 11% in patients with solid malignancies (28). Wu Y., et al described a rate varying from 21.9% to 64.2% depending on treatment. In this study 15 (14.6%) had thrombocytosis which is a common feature demonstrated in patients with solid malignancies (28).

In this study, the prevalence of prolonged clotting times (PT, aPTT, TT) are low compared to studies done on solid tumours with some studies reporting prevalence of 50% in early tumour stages (I,II) and up to 90% in metastatic disease (54). Elevated d-dimers which are markers of ongoing thrombosis and fibrinolysis were demonstrated in 8 (7.7%) of the patients. Prevalence from other studies vary widely between 5-18% as described in a publication by Graham et al (55).Georf-Friedrich et al demonstrated elevated d-dimers in 6% of patients who were started on chemotherapy soon after surgery (53). The latter prevalence is comparable to the prevalence of this study. There was a significant association between elevated d-dimers and late stage of disease (p value =0.03) and is in keeping with the procoagulant effect of disease proportional to tumour burden. The latter varies with treatment hence the rate will vary proportional to number of patients in various stages of treatment (56, 57).

Elevated fibrinogen is a non-specific marker of haemostasis disorders. It is elevated in 50-90% of patients with solid tumours, with marked elevation demonstrated in patients with late stage malignancy. This study demonstrated a lower prevalence of elevated fibrinogen levels and none of the patients had decreased fibrinogen levels below 150mg/dl. Elevated fibrinogen levels were significantly associated with male sex and stage IV disease with p value of 0.01 and 0.02 respectively. Late stage disease with a larger tumour burden, elaborates a greater of amount of inflammatory cytokines that stimulate an inflammatory process with increased yield of fibrinogen hence the elevated levels in late stage disease (58).

The level of fibrinogen, platelets and more specific markers of in vivo coagulation TAT, prothrombin fragment 1+2 and d-dimers parallel stage of disease and disease activity. Other markers like factor V, VII, IX, XI, and factor Xa assays are also elevated in late stage disease as demonstrated by Edwards et al (59) but in this study they were not determined due to limitation of cost.

Gender had a significant association with elevated fibrinogen levels with a p value of 0.01. Treatment type e.g. radiotherapy, surgery or chemotherapy did not have any significant association with any of the abnormal haemostatic parameters which is in contrast to other studies done elsewhere which demonstrated an association between treatment and abnormal haemostasis in malignancy. Graham et al demonstrated an association between prothrombotic states in breast cancer and treatment modalities with rates varying from 5% to 18% in various studies reviewed in the article (55). Surgery was used in treatment of early disease was a predictor of thromboembolic disease which was not significantly demonstrated in this study due to long duration of time lapse between surgery and enrollment in to the study. A further contributing factor was that some of the patients were already on treatment which had reduced tumour burden.

Stage of disease had significant association with abnormal coagulation parameters like elevated d-dimers, elevated fibrinogen levels and prolonged PT. There was significant association between stage IV disease and abnormal haemostatic parameters. This is comparable with other studies where stage IV and metastasis is associated with abnormal haemostatic parameters. This is associated with tumour burden, activation of the coagulation system and deficiency of antithrombotic factors. Sakeer et al demonstrated association between advanced stages of disease with abnormal haemostatic parameters (60).

The effect of treatment in breast cancer has been investigated and some treatment modalities found to activate coagulation and hence hypercoagulability. In breast cancer, combination of tamoxifen and chemotherapy e.g. cyclophosphamide, methotrexate fluorouracil (CMF) independently increase risk of VTE (35, 61). Post-menopausal age and use of antiangiogenic drugs increase risk 2-7 fold. This study could not significantly ascertain effect of specific chemotherapy treatment types because of extremely low numbers of participants on the treatments. Radiotherapy contributes to haemostatic disorders through damage of vessels which can lead to stasis and phlebitis increasing the risk of thrombosis. Radiotherapy also did not have any significant association with abnormal haemostatic parameters (62).

The prevalence of DIC in most solid tumours varies between 1-11% with a post mortem rate of up to 75% (9). In this study however, none of the participants had features of DIC. This can be attributed to the fact that the fibrinogen levels, platelet count, PT and aPTT were assayed once only. Sensitivity is improved if the parameters are assayed serially to detect

decline in platelet count and fibrinogen levels and prolongation of the clotting times as described by the British Committee on standards in haematology (63).

This study generally demonstrated low prevalence of abnormal haemostatic parameters tested at 27.1% which is lower than other studies done elsewhere. These low rates could be due to recruiting patients who were already long into treatment and yet their staging had not been assessed and changed to match disease regression due to treatment. This study recruited patients with no specific timeline between surgery and enrollment into treatment by chemotherapy or radiotherapy. Baseline haemostasis parameters were also not established to assess the effect of surgery, chemotherapy and radiotherapy upon commencing treatment. In other studies patients were followed up from diagnosis to surgery and eventual cytotoxic treatment.

Laboratory markers of thrombosis and /or haemorrhagic tendencies in malignancy are neither sensitive nor specific enough to be used alone as diagnostic tools (64). These tests may predict thrombosis but will not differentiate venous or arterial thrombi. Careful clinical evaluation and use of imaging studies like Doppler Studies, magnetic resonance imaging and venography with haemostatic parameter correlation greatly increases accuracy of diagnosis (64). The studies quoted earlier used a combination of Doppler imaging, venography and sensitive haemostatic assays like assay of thrombin-antithrombin levels and protein C: antithrombin complex assays that were correlated with d-dimer levels to improve sensitivity and specificity (22, 51, 52, 53). This study relied on d-dimer levels and/or thrombocytopaenia to predict thrombosis. However, latex and ELISA d-dimer levels have been associated with high false negative rate especially if there are co morbidities that elevate d-dimer levels. With latex assays the false negative rates range between 25 to 40%. This underestimation occurs when d-dimer levels are in the range of 0.2-0.4mg/L (65, 66, 67). Assay of d-dimer levels with ELISA is a sensitive method that can accurately rule out the diagnosis of thrombosis with a predictive value of 98-100% when the d-dimer levels are less than 0.2mg/L and it is the appropriate modality to confirm that a patient had no thrombosis. This would have reduced need to rely on Doppler imaging or venography to rule out thrombosis and reduce costs (65, 66, 67). ELISA was not used in this study because of limited funding. Thrombin-antithrombin complex assay is also a sensitive and accurate marker of thrombosis with a rate of 86% sensitivity however an affordable assay for use in this study could not be identified as compared to the slide agglutination method used in this research (68).

CONCLUSIONS

1. Haemostatic disorders are present in patients on management for breast cancer at KNH at a rate of 21.3%
2. The disorders are present at low prevalences compared to other studies done in other centres
3. Late stage of disease (Stage IV) is associated in general with a likelihood of haemostatic disorder

RECOMMENDATIONS

1. This study demonstrated that there are coagulation disorders (DVT, elevated d-dimers, prolonged clotting time) in patients with cancer with significant association with late stage disease. It is therefore recommended that coagulation disorders be screened for in patients with breast cancer in late stage disease.
2. It is recommended that the study be undertaken where the haemostatic parameters will be assayed on automated equipment to improve accuracy e.g. use of ELISA to identify d-dimers in thrombosis, automated PT, aPTT and thrombin time.
3. Other similar studies enrolled participants who were newly diagnosed and not on treatment. A similar study to be undertaken using similar criteria so as to delineate effect of disease and treatment as independent and related variables on haemostasis and to correlate with venography and/or Doppler studies.
4. To conduct a prospective study in Kenyan patients with quantifiable objective endpoints such as venography and/or Doppler studies.

LIMITATIONS

1. Limited financial resources to be able to use automated laboratory coagulation assays that are more accurate than manual assays
2. In this study, subjects were recruited with no regard to time and type of surgery which may explain the low prevalence since the patients may have recovered from the inflammatory effects of surgery
3. No information on receptor analysis (hormonal or protein receptors) e.g. Human epidermal growth factor 2 (Her2) receptor was gathered
4. Unavailability of doppler studies to identify subclinical thrombosis

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APPENDICES

APPENDIX I: Informed Consent Form

TITLE: To describe haemostatic disorders in patients with breast cancer at Kenyatta National hospital

You have been diagnosed with breast cancer which is associated with occurrence of thrombo-haemorrhagic complications that vary with disease stage and type of treatment you are receiving. The main risk of thrombosis is pulmonary embolism which is the second commonest cause of illness and death among cancer patients. I am carrying out a study to describe which types of these disorders are present among breast cancer patients attending Kenyatta National Hospital. My research assistant or I will cleanly and aseptically draw 6mls of blood by venipuncture from a vein in your arm/forearm. Every precaution will be taken to reduce pain, swelling or any bleeding that may occur. The blood will be collected into sterile vacutainer tubes and taken to UON haematology laboratory for analysis at no cost to you. The results and their significance will be communicated to you through your attending doctor for follow up. Participation in this study is voluntary.

I ……. hereby do voluntarily consent to take part in the research being undertaken by Dr. Peter Asaava, the nature of which has been explained to me by him.

Date:……

Participant signature:……

Principle Investigator Questionnaire:……..

If you have any concerns about this research please contact any of the following:

Principle investigator: Dr. Asaava Peter 0721259180

My study supervisors:

Dr. Kiarie Gladwell 0724053501

Dr. Ritesh Pamnani 0733743792

Dr. Jessie Githanga 0721245721

You may also contact the chairperson KNH Ethical and research committee: 202725452

APPENDIX II: Questionnaire

1.0 Patient characteristics

1.1 Study number: ……

1.2 Hospital number: ……

1.3 Study site:

1.3.1Breast clinic ☐

1.3.2 Oncology ☐

1.3.3Surgical Wards ☐

1.3.4 Medical Wards ☐

1.4 Age: ……

1.5Sex:

1.5.1Male ☐

1.5.2 Female ☐

1.6 Histologic Diagnosis:

1.6.1 Invasive Ductal Carcinoma ☐

1.6.2 Lobular carcinoma ☐

1.6.3 Others ☐

1.7 Staging:

1.7.1 No staging ☐

1.7.2 Stage I ☐

1.7.3 Stage II ☐

1.7.4 Stage III ☐

1.7.5 Stage IV ☐

2.1 Comorbidity (HIV/AIDS, Infections, Dehydration, Malnutrition, CCF, others)

2.1.1 None ☐

2.1.2 Present ☐

2.2 DVT:

2.2.1 Upper limb(s) ☐

2.2.2 Lower Limb(s) ☐

2.2.3 None ☐

2.3 Skin/mucosal purpura, easy bruisability:

2.3.1Yes ☐

2.3.2 No ☐

2.4 Surgery

2.4.1 Mastectomy ☐

2.4.2 Biopsy ☐

2.4.3 No surgery ☐

2.5 Treatment

2.5.1 Radiotherapy ☐

2.5.2 Chemotherapy:

2.5.2.1 CA5FU
2.5.2.2 Tamoxifen
2.5.2.3 CM5FU
2.5.2.4 Cisplatin + paclitaxel
2.5.2.5 Adriamycin,5FU,dexamethasone
2.5.2.6 Cisplatin,vincristine,adriamycin,cyclophosphamide
2.5.2.7 Vinorelbine, dexamethasone, cyclophosphamide
2.5.2.8 Docetaxel, cyclophosphamide

3.1 RBC Count: 3.2 WBC Count: 3.3 Platelet Count: 3.4 Hb:

3.5 PBF Features

PLATELETES	
4.5.14 Count 4.5.15 Aggregation	

3.6 Coagulation findings

3.6.1 Prothrombin time(seconds)	
3.6.2 Thrombin time(seconds)	
3.6.3 aPTT(seconds)	
3.6.4 Fibrinogen (g/L)	
3.6.5 d-dimer(mg/ml)	

APPENDIX III: Laboratory Procedures

Peripheral Blood film preparation

Stain preparation: 1.5g of Leishman's powder was added to 500ml of methanol, mixed well and incubated at 37^0C overnight.

Method

Thin film was made and air dried

Film was then flooded with Leishmann stain after two minutes water was added and film stained for seven minutes

The film was then washed in buffered water for two minutes and air dried.

Red blood cell fragments, platelet agglutination, aggregation and satelitism were examined for.

Manual determination of prothrombin time (PT, QUICK Test)

HEMOSTAT THROMBOPLASTIN-SI (HUMAN ^{TM})

Reagents: Rabbit brain thromboplastin extract, $CaCl_2$

Reagents confirmed not to be expired and stored at 4^oC.

The thromboplastin reagent (lyophilized rabbit brain extract) was reconstituted with 2mls of distilled water and mixed well. It was used within seven days of reconstitution.

Specimen processing:

The frozen plasma was thawed rapidly at 37^oC and assayed but not refrozen.

The specimen was examined to ensure that it was not turbid, icteric, lipaemic or haemolysed.

Method

Duplicate tests were done on test plasma and control plasma. Controls (normal and abnormal) were tested with each batch of test plasma.

The reagents were prewarmed to 37^0C

0.1ml of plasma/control was pipetted into a prewarmed test tube and then pre-warmed 0.1ml thromboplastin reagent added and incubated for 5minutes at 37^0C. 0.1ml of $CaCl_2$ was then added and timer started. The time taken for clot formation was recorded. The average of the duplicate tests was then established.

Manual determination of Activated Partial Thromboplastin Time

Reagents:

Normal/abnormal control plasma

Test plasma

Kaolin reagent

Phospholipid reagent

$CaCl_2$

The commercial reagents were confirmed not to have expired.

Method

Duplicate tests of test and control plasma are done. Controls were done in each batch of tests.

0.1ml of plasma/control and 0.2ml of kaolin Phospholipid solution mixture is incubated at 37^0c for ten minutes and then 0.1ml of $CaCl_2$ is added and timer started.

Time taken for mixture to clot is recorded. The average of the duplicated tests was recorded to the nearest 0.1ml seconds.

Manual determination of thrombin time

Reagents:

Normal/abnormal control plasma

Test plasma frozen and thawed to 37^oC.

Thrombin solution

The commercial reagents were confirmed not to have expired.

Method

Duplicate tests of test and control plasma are done. Controls were done in each batch of tests.

0.1ml of plasma/control and 0.2ml of thrombin solution mixture is incubated at 37^0c and stop timer started immediately

Time taken for mixture to clot is recorded. The average of the duplicated tests was recorded to the nearest 0.1ml seconds.

Manual determination of plasma fibrinogen

(HEMOSTAT FIBRINOGEN-Human ^{tm})/CLAUS TECHNIQUE

Reagents: Bovine thrombin solution, calibrants with known level of fibrinogen calibrated against international reference standard, buffered saline and test/control plasma

Reagents confirmed not to be expired and stored at 4^{o}C.

The bovine thrombin solution reagent was reconstituted with 2mls of distilled water and mixed well. It was used within seven days of reconstitution while stored at 4^{0}C.

Specimen processing:

The frozen plasma was thawed rapidly at 37^{o}C and assayed but not refrozen.

The specimen was examined to ensure that it was not turbid, icteric, lipaemic or haemolysed.

Method

A calibration curve was prepared by making dilutions of calibration solution in buffer to give range of fibrinogen concentrations, i.e. 1 in 5, 1 in 10, 1 in 20, and 1 in 40. 0.2 ml of each dilution is warmed to 37^{o}C and 0.1ml of the thrombin solution added and clotting time measured. Each test is done in duplicate. The clotting times in seconds are read against the fibrinogen concentration in g/l.

1 in 10 dilutions of each test and control plasma are made and 0.2ml of the dilutions is clotted with 0.1ml of the thrombin solution.

The fibrinogen level is read directly of the graph.

Manual determination of d-dimer levels

Dimertest®

Is a rapid agglutination assay utilizing latex beads coupled with a highly specific d-dimer monoclonal antibody. XL-FDP present in a plasma sample bind to coated latex beads which results in visible agglutination occurring when the concentration of d-dimer is above the threshold of detection of the assay.

Reagents: Test plasma, negative and positive control plasma, latex reagent

Method:

Frozen plasma is thawed to 37^{0}C

0.1ml of control plasma, test plasma are placed on a card

To each 0.1ml of latex reagent and the test card rocked to mix.

Agglutination is checked at exactly three minutes

For samples that agglutinated dilutions were done to quantify and results reported in mg/L.

APPENDIX IV: Interpretation of Haemostasis Values

The cut-off values for various haemostatic tests based on control values and reference values were as follows (Table 5):

Table 5: Laboratory cut-off values

Parameter	Mean Control value/reference range	Upper Cut-off	Lower cut off
Prothrombin time	14 seconds	18seconds	10seconds
Activated partial thromboplastin time	31.5 seconds	38.5 seconds	21seconds
Thrombin time	16.4 seconds	19seconds	-
Fibrinogen	150-350mg/dl	<150mg/dl >350mg/dl	-
D-dimers	<0.2mg/L	≥0.2mg/L	-

Any laboratory value (s) above the cut-off value(s) were considered abnormal. For the fibrinogen levels, any level below 150mg/dl was also considered abnormal. A platelet count less than $140x10^9$/L was considered thrombocytopaenia.

The control values were used to ensure the analytic process was within limits. If controls were not agreeable to set values, the cause of the error was sought and rectified.

APPENDIX V: Ethical Clearance

APPENDIX VI: Breast Cancer Staging and Treatment Protocol

Pathologic Staging (based on information available to the pathologist) (pTNM)

TNM Descriptors (required only if applicable) (select all that apply)

___ m (multiple foci of invasive carcinoma)

___ r (recurrent)

___ y (post treatment)

Primary Tumour (Invasive Carcinoma) (pT)

___ pTx: Primary tumour cannot be assessed

___ pT0: No evidence of primary tumour

___ pTis (DCIS): Ductal carcinoma in situ

___ pTis (LCIS): Lobular carcinoma in situ

___ pTis (Paget): Paget disease of the nipple *not* associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma

pT1: Tumour ≤20 mm in greatest dimension

___ pT1mi: Tumour ≤1 mm in greatest dimension (micro-invasion)

___ pT1a: Tumour >1 mm but ≤5 mm in greatest dimension

___ pT1b: Tumour >5 mm but ≤10 mm in greatest dimension

___ pT1c: Tumour >10 mm but ≤20 mm in greatest dimension

___ pT2: Tumour >20 mm but ≤50 mm in greatest dimension

___ pT3: Tumour >50 mm in greatest dimension

pT4: Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules). *Note:* Invasion of the dermis alone does not qualify as pT4.

___ pT4a: Extension to chest wall, not including only pectoralis muscle adherence/invasion

___ pT4b: Ulceration and/or ipsilateral satellite nodules and/or oedema (including peau d'orange) of the skin which do not meet the criteria for inflammatory carcinoma

___ pT4c: Both T4a and T4b

___ pT4d: Inflammatory carcinoma

Regional Lymph Nodes (pN) (choose a category based on lymph nodes received with the specimen; immunohistochemistry and/or molecular studies are not required)

Modifier (required only if applicable)

___ (sn): Only sentinel node(s) evaluated. If 6 or more nodes (sentinel or non sentinel) are removed, this modifier should not be used.

Category (pN)

___ pNX: Regional lymph nodes cannot be assessed (e.g., previously removed, or not removed for pathologic study)

___ pN0: No regional lymph node metastasis identified histologically

___ pN0 (i-): No regional lymph node metastases histologically, negative IHC

___ pN0 (i+): Malignant cells in regional lymph node(s) no greater than 0.2 mm and no more than 200 cells (detected by H&E or IHC including ITC)

___ pN0 (mol-): No regional lymph node metastases histologically, negative molecular findings (reverse transcriptase polymerase chain reaction [RT-PCR])

___ pN0 (mol+): Positive molecular findings (RT-PCR), but no regional lymph node metastases detected by histology or IHC

___ pN1mi: Micrometastasis (greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm).

___ pN1a: Metastases in 1 to 3 axillary lymph nodes, at least 1 metastasis greater than 2.0 mm

___ pN2a: Metastases in 4 to 9 axillary lymph nodes (at least 1 tumour deposit greater than 2.0 mm)

___ pN3a: Metastases in 10 or more axillary lymph nodes (at least 1 tumour deposit greater than 2.0 mm)

Distant Metastasis (pM) (required only if confirmed pathologically in this case)

___ pM1: Distant detectable metastasis as histologically proven larger than 0.2 mm