ASSOCIATION OF SERUMESTRADIOL LEVELS AND BONE MARROW ESTROGEN RECEPTOR DENSITIES IN ACUTE MYELOCYTIC LEUKEMIA PATIENTS IN MOI TEACHING AND REFERRAL HOSPITAL, KENYA

BY

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DECLARATION

I declare that the work presented herein is my original work and has not been presented for the award of any degree in any other university or institution.

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DEDICATION

This work is dedicated to my wife Caren Chepng'etich and my two sons, Lee Mutai Mbogori and Leonel Bii Mbogori for their support during the study period

ABSTRACT

Acute Myelocytic Leukemia (AML) is a cancer of the bone marrow characterized by arrested maturation along with uncontrolled proliferation of hematopoietic progenitor cells. Mostly, it affects persons of age 65years and above. In AML, there is absence or low amounts of Estrogen Receptors in the bone marrow. Various studies have associated Estradiol to the variation of ER. However, the exact role Estradiol is still not known in association with the onset and progression of AML. The main objective was to determine association of serum Estradiol levels and bone marrow Estrogen Receptor densities in AML patients attending Moi Teaching and Referral Hospital (MTRH) Kenya. The research was conducted atMTRH. A sample size of 17 case samples and 17 control samples was analysed. Bone marrow cell blocks from inpatients and outpatients were sectioned and stained for ER using anti-ER antibodies tagged with an orange dye for immunohistochemical analysis. ER percentages were arrived at by dividing the total number of ER-positive myelocytes by total number of myelocytes seen per field. Blood samples collected in plain tubes from the subjects and serum was collected. Estradiol was determined using competitive ELISA technique. The results showed ER densities (less or equal to 10%) in 12 out of 17 (70.6%) cases and 3 out of 17 (17.6%) controls. The mean percentage of Estrogen Receptors case samples was 7.65% and in control samples was 23.53%. The mean Estradiol concetration in case samples was found to be 128.94 pg/ml whereas in control samples was found to be 32.41pg/ml. The correlation of Estradiol and Estrogen Receptors in AML patients is -0.5952. In cases, the mean ER percentages for the males with AML was 8.89 while for females was 6.25. The control males had mean ER percentage of 50.44 while females had mean ER percentage of 12.125. Most of the cases were of the age between 25 and 31 which carried 41% of all the AML cases. The correlation of age and estrogen receptors is -0.262. In conclusion, the findings of this study that AML cases had very low estrogen receptor densities as compared to the non-AML control samples. Also, the Estradiol levels in cases samples were relatively high as compared to the control samples. Both in cases and controls, the higher the Estradiol the lower the ER. The two variables vary inversely. The study suggest that AML is associated with downregulation of ER in the bone marrow. It is therefore possible to adopt use of bone marrow ER and serum Estradiol as markers of AML.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

ALL	Acute lymphocytic leukemia
AML	Acute MyelocyticLeukemia
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen receptor
ERs	Estrogen receptors
FNA	Fine Needle Aspirate
IREC	Institutional Research and Ethics Committee
Jak	Janus kinase
MTRH	Moi Teaching and Referral Hospital
RNA	Ribonucleic acid
STAT	Signal transducer and activator of transcription
U.S.	United States of America

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Acute Myelocytic Leukemia (AML) is a cancer of the bone marrow in which hematopoietic precursors are arrested in an early development stage (Estey and Döhner, 2006). It is characterized by uncontrolled proliferation of hematopoietic progenitor cells(Gregory et al., 2009). Most AML subtypes are uniquely isolated from other related disorders of the blood by the manifestation of more than 20% immature cells (blasts) in the bone marrow (Gilliland, 2001).Persons of age 65 years and above are mostly affected by AML (Howlader et al., 2011; Stone, O'Donnell, and Sekeres, 2004).A two-year survey at Moi Teaching and Referral Hospital (MTRH) data on the incidence of leukemia and leukemia-like cancers has however, shown that 48 percent of the patients with AML were of age between 12 and 25 years (Mostert et al., 2012).

In AML, there is absence or low amounts of Estrogen Receptors(ER) (Li et al., 1999). ER have also been identified as negative regulators of the onset and progression of cancers(Sueyoshi, Yokomori, Korach, and Negishi, 1999; Yamashita and Iwase, 2002). Estrogen receptors are intracellular proteins that are activated by dimerizing with estrogen to initiate transcription of various genes.Down-regulation of these receptors leads to up-regulation of constitutive STAT5, which is an important oncoprotein in the progression of leukemia.

There are contradicting arguments that ER gene in leukemia cells is methylated at the CpG islands(Issa et al., 1996) or this gene is unmethylated (Rosen et al., 1983). Thus, it is not clear what leads to inadequate amounts of ER in leukemia cells.

Various studies have however, associated Estradiol to the variation of ER(Vladusic, Hornby, Guerra-Vladusic, and Lupu, 1998).In studies by Robertson (2002) and Borras (1996) there is consensus that estrogen influences the control mechanism of Estrogen Receptors through gene (Borrás et al., 1996; Robertson, Farnell, Lindahl, and Ing, 2002). The onset for AML for persons aged 12 to 25 years may be due to hormonal changes and inappropriate regulation mechanisms.Hormonal regulatory mechanisms are complex systems that are triggered by abundance or deficit of specific hormones. The system also involves precursor hormones as well as target cells. The most variable hormone in this age group is estrogen. Abundance of estrogen receptors may be triggered by low amounts of estrogen. The role of Estradiol is still not known in the onset and progression of AML

Estrogens are steroid hormones that control growth, specialization, and function in various cells in a broad range of target tissues in the human body. They are crucial in female reproductive system as they play a fundamental role in the formation and maintenance of the endometrium among other roles. During adolescence in females, these hormones are responsible for the development of secondary sexual characteristics. Estrogens consists of three hormones that include Estrone, Estriol, and Estradiol. The most potent and leading estrogen in humans is Estradiol (17β – estradiol)(Björnström and Sjöberg, 2005). In males, the primary sources of estrogen are adipose tissue and brain(Kula et al., 2005). Its function in males is in the

biosynthesis mechanism of testosterone. As men age, past the age of 55, testosterone decrease and Estradiol increase significantly (Kula et al., 2005). It is however not clear if variation in Estradiol in the two genders can assert significant physiological difference and disease exposure.

1.2 Statement of theProblem

Acute Myelocytic Leukemia has the highest prevalence of all the blood cancers in Moi Teaching and Referral Hospital.Global prevalence show that the most affected age bracket is persons above 65 years of age. However, in Moi Teaching and Referral Hospital, 48% of the patients with AML range between ages of 12 and 25 years.

Estrogen Receptors (ER) have been studied widely and seen to have a role in oncogenesis. Underexpression of estrogen receptors leads to overexpression of oncoproteins such as STAT5 and thus progression of AML. It is however not known how ER varies in AML patients in Kenya in comparison to non-cancer patients.

Estradiol is one of the main variants in the age group of 12 to 25 years and it causes ER variations in other cancers such as breast cancer. However, little has been done in the assessment of the role hormonal regulation plays in the onset and progression of AML, and nothing has been done to investigate how ER and Estradiol associate in AML patients. In addition nothing has been done to investigate sex as a variation factor of ER that has lead to the skewed prevalence of AML in the region.

1.3 Objectives

Main Objective

To determine the association betweenbone marrow Estrogen Receptor densities and serumEstradiol in AML patients attending Moi Teaching and Referral Hospital

Specific Objectives

1) To compare Estrogen Receptor percentages in AML population and non-cancer population
2) To compare Estradiol levels in AML population and non-cancer population
3) To determine the correlation between ER and Estradiol AML patients and in non-cancer patients
4) To determine the variation of ER and Estradiol in AML patients in relation to their gender
5) To determine the variation of ER and Estradiol in AML patients in relation to their ages

1.4 Hypotheses

H_0: Equal means of Estrogen receptor percentage among case and control samples

Ho: Equal means of Estradiol levels among cases and control samples

H0: there is no correlation between Estradiol and ER

H0: Equal means of Estrogen receptor percentage among AML cases

H0: Equal means of Estradiol levels among cases of varying ages

H0: correlation is equal to zero: there is no correlation between AML and age

1.5 Significance of the study

This research on the trend ER and Estradiol in AML will enhance the understanding of the treatment of cancer from its cause. Also based on the findings of this study that ER is low in Myeloid cells of AML patients, it is recommended that ER analysis be used as one of the diagnostic parameters of AML. In addition, Estradiol was discovered as a predisposing factor of AML and monitoring of this hormone as a likely cause of AML is recommended.

CHAPTER TWO

LITERATURE REVIEW

2.1 Leukemia

Leukemia is a clonal proliferative condition of the multipotent hematopoietic stem cells that leads to irregular cell growth or differentiation to form colonies of non-functional cells (Bogdanov, 2009). In most cases, these cells are released into blood circulation as immature cells and in other cases, the cells appear normal. The cause of leukemia is the occurrence of oncogene expression in bone marrow or peripheral blood as a consequence of some chromosome translocations such as translocation between chromosomes 8 and 21; translocation between chromosomes 1 and 22; invasion of chromosome 16; and translocation between chromosomes 9 and 11 (Döhner and Döhner, 2008).

Transformation of proto-oncogenes to oncogenes and/or the loss of anti-oncogenes either provide the leukemic cell with a continuous proliferative process or prevent its normal differentiation and apoptosis. The oncogene and the corresponding normal proto-oncogene may exist side by side, but sometimes the oncogene will exert a dominant effect. Conversely, some mutations or rearrangements lead to loss of gene function, particularly in the case of anti-oncogenes that normally suppress cancer. Thus, both copies of the anti-oncogene must be inactivated for the cancer cell to gain a proliferative advantage (Gilliland, Jordan, and Felix, 2004).

2.2 Types of Leukemia

The World Health Organization has classified Leukemia on the basis of the cells that are affected as well as its causes (Harris et al., 2000). The classification of leukemia is also done on the basis of whether the condition is severe (acute), or that one can live with for a long time (chronic). In chronic leukemia, oncogenesis occurs in the peripheral blood. The leukemia cells emanate from mature, irregular cells. The cells increase in number and fail to initiate apoptosis. The cells age slowly. Acute leukemia, in contrast to the chronic leukemias, develops in the bone marrow from blasts. Blasts are undeveloped cells that divide repeatedly. In acute leukemia, these blasts don't stop dividing(Harris et al., 2000).

Further, they can be divided into two other types depending on the cell lineage that is affected. Myeloid leukemia, also known as myelocytic leukemia, progresses from myeloid cells. The disease can either be chronic, referred to as chronic myelogenous leukemia (CML) or acute, referred to as acute myeloid leukemia (AML). Lymphocytic leukemia advances from cells called lymphoblasts or lymphocytes. The disease can be acute, referred to as acute lymphocytic leukemia (ALL) or chronic, referred to as chronic lymphocytic leukemia (CLL) (Harris et al., 2000; Redaelli et al., 2003).

2.2.1 Acute Myeloid Leukemia (AML)

AML, a blood cancer that emanates from the bone marrow, is characterized by the rapid uncontrolled growth of immature blood cells called myelocytes. The average age at diagnosisglobally being adults of more than 65 years of age(Redaelli et al., 2003). However,

recent studies have shown that the age of diagnosis is shifting to younger patients in Moi Teaching and Referral Hospital (Mostert et al., 2012). The cause of this has not been explored.

Normally, blood cells are developed in the bone marrow in a methodical and controlled way. The multipotent hematopoietic stem cell divides into lymphoid progenitor which gives rise to lymphocytes and natural killer cells and myeloid progenitor which gives rise to granulocytes, red blood cells and thrombocytes(Jagannathan-Bogdan and Zon, 2013) as shown in figure 1 below.

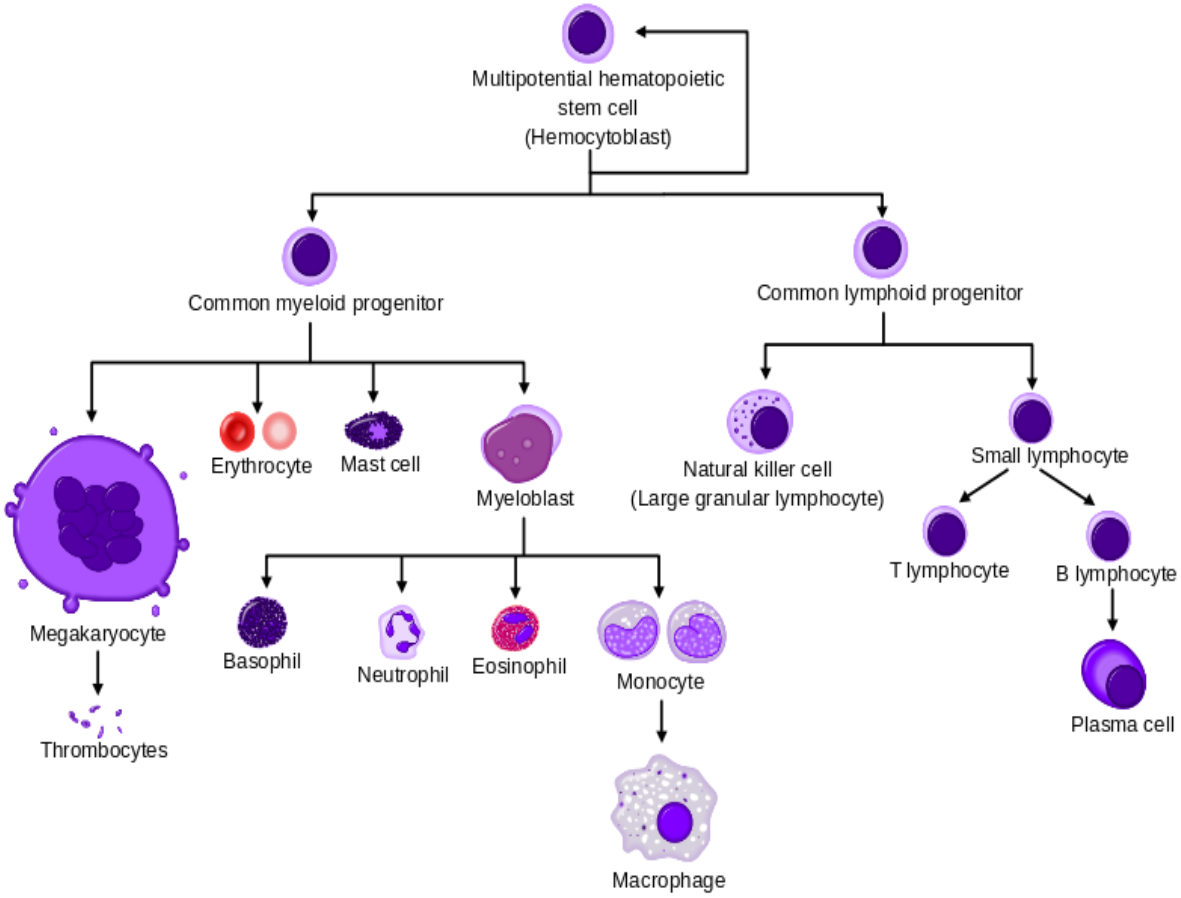


Figure 1: Hematopoiesis

In Acute Myeloid Leukemia, this process gets unchecked, and many abnormal leukemia cells of the myeloid lineage are released. Thesecells are unable to develop into normal efficient blood cells. They are immature and are often referred to as blast cells. In most types of AML, the leukemia cells are undeveloped white cells. However, in some less common types of AML, there are many immature platelets or immature red blood cells released in additional to the immature white blood cells(Estey and Döhner, 2006; Stone et al., 2004).

The immature cells stock up the bone marrow, eating up space that is needed to makenormal cells. Some leukemia cells 'spill over' into the blood and circulate around the body in the bloodstream. These leukemia cells do not mature, and so do not work correctly. This leads to a bigger risk of infection in addition to symptoms like bruising and anemia caused by insufficient healthy platelets and red blood cells being made (Stone et al., 2004).

Treatment is designedat bringing about prompt decrease or the destruction of all leukemia cells. However, AML responds to asmaller number of drugs than acute lymphocytic leukemia does. In addition, chemotherapy treatment often makes patients' conditions worse before they get better since the treatment kills the cancerous cells and a significant number non-cancerous cells in the myeloid cell lineage, thus resulting in fewer white blood cells, and most affected are neutrophils. Consequently, neutropenia increases the chances of infection. Thorough care is therefore taken to avert any infections that may occur while administering chemotherapy and in case they occur, they are quickly treated. Platelet and Red blood cell transfusions are invariably also needed(Xie, Davies, Xiang, Robison, and Ross, 2003).

Although it is the most widespread type of leukemia in adults, AML continues to have the least survival rate of all leukemias (Redaelli et al., 2003).Without treatment, 95% of the patients diagnosed with AML die within six months of the diagnosis. However, with treatment, between 20% and 40% of people survive at least five years, without any relapse. Because relapse almost at all times occurs within the first five years after first treatment, most patients who remain leukemia-free after five years are considered cured(Schoch et al., 2004). People who have the poorest prospects are those older than sixty-fiveyears because of their deteriorating cellular activity which leads to poorresult in patients age above 65 years, and thus, treatment aftermath deteriorates with age (Appelbaum et al., 2006).Also, prospects are poor in those who advance AML after undertaking radiation therapy and chemotherapy for other cancers, and those whose leukemia developed slowly over a period of months to years with abnormal blood counts.

2.2.2 Epidemiology of Acute Myeloid Leukemia

According to Jemal (2012) the global, the incidence of AML is uppermost in the U.S., Australia, and Western Europe (Siegel, Naishadham, and Jemal, 2012). It accounts for around 25% of all hematological malignancies in adults in the Western countries and thus is the most recurrent form of leukemia(Greenlee, Hill-Harmon, Murray, and Thun, 2001). The American Cancer Society estimates that 6350 men and 5580 women in the United States had AML in 2006 (Siegel et al., 2012).

The incidence of AML varies with gender. AML in adults has a slight female prevalence in most countries. In 2000–2003, the age-adjusted prevalence of AML in the United States was an

average of 3.7 per 100,000 for both males and females, 4.6 per 100,000 for females and 3.0 per 100,000 for males. The incidence rate of U.S. men is considerably higher than the incidence rates reported for men in all other countries (Jemal, Thomas, Murray, and Thun, 2002).

As much as leukemia has been viewed as a first world disease over years, there has been a steady increase in the incidence of leukemia in Africa. A study carried out in Nigeria between 1990 and 2004 estimated that out of 160 leukemia new cases 51.6% were AML. The real trigger of leukemia was not known(Omoti, Awodu, and Bazuaye, 2007).

Little has been researched on the epidemiology of Acute Myeloid Leukemia in Kenya. However, according to research conducted in Moi Teaching and Referral Hospital, out of 436 cancer cases that were diagnosed in Western Kenya, AML accounts for 4%,and it was the most common form of blood cancer.The two-year survey at Moi Teaching and Referral Hospital (MTRH) data on the incidence of leukemia and leukemia-like cancers also, showed that 48 percent of the patients with AML were of age between 12 and 25 years (Mostert et al., 2012).

2.3 Estrogen and Estrogen receptors

Estrogens are among the most variable hormones in persons of age between 12 and 25 years. They control growthand function in a wide range of target tissues in the human body. They consist of three hormones which include estrone, estriol and estradiol. The most powerful and principal estrogen in humans is estradiol (17β – estradiol), but lower levels of the estrone and estriol are also present (Björnström and Sjöberg, 2005).

Estrogens are mainly produced by developing follicles in the ovaries, by the corpus luteum, and by the placenta. Some estrogen is also produced in smaller amounts by other tissues which include adrenal glands, the liver, and the breasts. Fat cells also produce estrogen making them the key source of estrogen in menopause women(Kula et al., 2005). Although estrogens exist in males as well as females, it is found in higher amounts in women, within reproducing age. They are generally significant in the female for thedevelopment of secondary sex features, which are the major differences between men and women that do not relate to the propagative system. In women, these features include breasts, a broadened pelvis, and increased quantities of body fat in the thigh, hip region, and buttock. Estrogen also is responsible for the fact that women have smoother skin and less facial hair than men (Goumidi et al., 2011).

Estrogen exerts its effects through several pathways. Estrogen receptors (ER) are crucial intracellular proteins through which estrogen mediates its effects. These receptors are ligand-activated transcription factors. The classical mechanism of ER action entails estrogen binding to intracellular estrogen receptors in the nucleus, causing the receptors to dimerize and bind to reaction components known as estrogen response elements (EREs) which are positioned in the promoters of target genes(Cotoi et al., 2014).This also leads to recruitment of coactivator proteinsthus activating transcription of the genes. Proof for signaling pathways that diverge from this traditional model has emerged. Studies now show that ERs can regulate gene expression by a number of distinct mechanisms (O'Lone, Frith, Karlsson, and Hansen, 2004). ERs can also control gene expression without binding straight to DNA by modulating the role of other classes of transcription factors via protein-protein interactions in the nucleus (O'Lone et al., 2004).

Although estrogens exert some of their effects through the action of ERs on gene expression, a number of other estrogens effects are so fast that they cannot depend on the stimulation of RNA and protein synthesis. These actions, better known as nongenomic actions, are mediated through a protein kinase activation cascades that are initiated by membrane-associated ER to take effect later in the nucleus (Losel et al., 2003). However, nongenomic actions of estrogens may indirectly influence gene expression, via the activation of signal transduction pathways that ultimately act on target transcription factors. ERs regulate genes, such as the β-casein gene, that has signal transducer and activator of transcription (STAT) 5 binding sites (Björnström and Sjöberg, 2005). STAT5-dependent transcription is suppressed by ERs activated by 17β-estradiol (Stoecklin et al., 1999).

2.4 Signal transducers and activators of transcription (STAT)

Signal transducers and activators of transcription (STAT) moleculesare hidden cytoplasmic transcription factors that are activated in reaction to a large number of cytokines, growth factors, and hormones. The binding of the ligand to its cognate cytokine receptor induces activation of receptor-associated members of the Janus kinase (Jak) family, which sequentially phosphorylate a conserved tyrosine residue at the C-terminal region of STATs. Activation of STATs call for phosphorylation of their tyrosine residues by either the receptors that habitually display receptor-associated kinase or an intrinsic tyrosine kinase activity. The activated STATs form dimers that move into the nucleus and start transcription of the growth factor/cytokine-responsive genes (Darnell, 1997). In addition to basic tyrosine phosphorylation, the transcriptional activity of

STATs can be regulated by serine phosphorylation of the transactivation domain(Shah et al., 2013).

STATs play an important role in promoting cell proliferation and survival, both normal and malignant. Members of the STAT family have been studied and designated STAT1 to STAT6 (Decker and Kovarik, 2000; Kovarik et al., 2001). Numerous biochemical and genetic studies have demonstrated that constitutive activation of STATs, such as STAT5, is essential for cellular transformation and oncogenesis (Heath and Cross, 2004). Constitutive STAT activation might either be due to the constitutive activation of cytokine receptors triggered by autocrine growth factors or due to alterations in specific upstream tyrosine kinases occasioning in constitutive activity of these kinases.

STAT5 comprises of two distinct components designated STAT5A and STAT5B which are extensively similar, 94% identical, at the protein level (Ambrosio et al., 2002). However, gene knock-out mouse models clearly demonstrated that STAT5A and STAT5B possess some distinct, non-overlapping functional properties (Ambrosio et al., 2002; Buitenhuis et al., 2004). Although the constitutive activation of STAT5 has been reported in AML, the exact role of STAT5A versus STAT5B in malignant cell transformation is at present much less understood.

Various studies have suggested that STATs are constitutively tyrosine phosphorylated and stimulated in AML blasts, which might add to the leukemic phenotype (Gouilleux-Gruart et al., 1996; Xia, Baer, Block, Baumann, and Wetzler, 1998). Constitutive STAT activation may be due either to mutations in precise upstream tyrosine kinases resulting in constitutive activity of these

kinases or due to the constitutive stimulation of cytokine receptors activated by autocrine growth factors.

2.5 Regulation of estrogen receptor density

Several factors have been linked to regulation of ER in various cancers. Inactivation of ER gene expression is associated with de novo methylation of a cluster of CpG sites (CpG island) located in and around the promoter element of the gene.In the colon, the ER gene displays progressive, age-related CpG island methylation in normal-appearing colonic mucosa, and this methylation appears to be one of the earliest molecular modifications in the development of colorectal neoplasia (Issa et al., 1996).While methylation of CpG islands is the cause of down-regulation of ER in colon cancers, some hematological cancers like CLL show no sign of methylation (Rosen et al., 1983) and thus other mechanisms may be involved. Prolactin has also been linked to the regulation of Estrogen Receptors in the ovaries (Frasor and Gibori, 2003).

Various studies have associated Estradiol to the variation of ER. Estradiol has been seen to increase mRNA for ER by 30 to 40 folds in T47D breast cancer cells (Vladusic et al., 1998) while it down-regulates ER in MCF-7 breast cancer cells.There is a hypothesized association of ER and estradiol in AML patients given the fact that estrogen is one of the most variable factors across the ages and sexes and that global prevalence of AML is high in patients above 65 years and in males.

2.6 Measurement of ER using Immunohistochemistry techniques

Immunohistochemistry has been used extensively as a favorable diagnostic technique in intracellular proteins in histological tissues. Several approaches have been thereafter attempted for non-tissue specimen. These include direct smears whereby Fine Needle Aspirate (FNA) material is air-dried and fixed in formalin. The cost of production of this technique is low. However, the cells of interest are dispersed, and thus more ER antibodies are needed to cover the whole slide, and fewer cells will be stained. Cytospins are same as direct smears, but cell population is concentrated by centrifugation of the sample prior to smear preparation. Cell blocks have widely beenpreferred for cytological specimens. The cells are concentrated and processed in histological cassettes and a cell block made and embedded in paraffin wax. These cells are then sectioned like histological specimens. Storage of the samples is less space consuming and easily retrievable (Skoog and Tani, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Area of study

The research was conducted at Moi Teaching and Referral Hospital (MTRH) which is located in Uasin Gishu County in Kenya. This is a national hospital which attends to patients mostly from three provinces which lie in the western Kenya:these are the former Rift Valley, Western and Nyanza Provinces. The hospital has oncology clinics that specialize in treating cancer patients including leukemia patients. The hospital bed capacity is 550 beds and an elaborate oncology clinic for outpatients and referral patients.

3.2 Study Design

The study design was case-control study. Blood and bone marrow Samples from AML subjects were used as the cases while blood and bone marrow samples from non cancer subjects was used as controls

3.3 Inclusion criteria

Samples of bone marrow and blood from patients that were confirmed to have Acute Myeloid Leukemia.Samples of bone marrow and blood from patients without leukemia or any other known cancer (as controls).

3.4 Sample size

The sample size formula (Kasiulevičius, Šapoka, and Filipavičiūtė, 2006) used is given by:

$$n = \left(\frac{Z_{1-\alpha/2} + \sqrt{(+m\bar{P}(-\bar{P})} + Z_{\beta}\sqrt{p_1(-p_1) + mp_0(-p_0)}}{(p_1 - p_0)} \right)^2$$

$$Where\ \ \bar{P} = \left(\frac{p_1 + \frac{p_0}{m}}{1 + 1/m} \right) \ and\ \ p_1 = \left(\frac{p_0\psi}{1 + p_0(\psi - 1)} \right)$$

α - Type I error = 0.05,

$\beta = 1 - power$,

ψ - odds ratio (odds ratio of exposures between cases and controls) = 1,

m-number of control subjects per case = 1,

p_1 - The probability of exposure in controls = 20% (Birkenkamp *et al.*, 2001),

$p0$ -The probability of exposure among the cases predicted to be 70% (Birkenkamp *et al.*, 2001).

$Z_{1-\alpha/2}$ is the standard normal deviate for probability $(-\alpha/2)$.

Therefore, the sample size was calculated to be 17

This means 17 case samples and 17 control samples were studied to be able to reject or accept the null hypothesis that this odds ratio equals 1 with probability 0.8. The Type I error probability related to the test of this null hypothesis is 0.05.

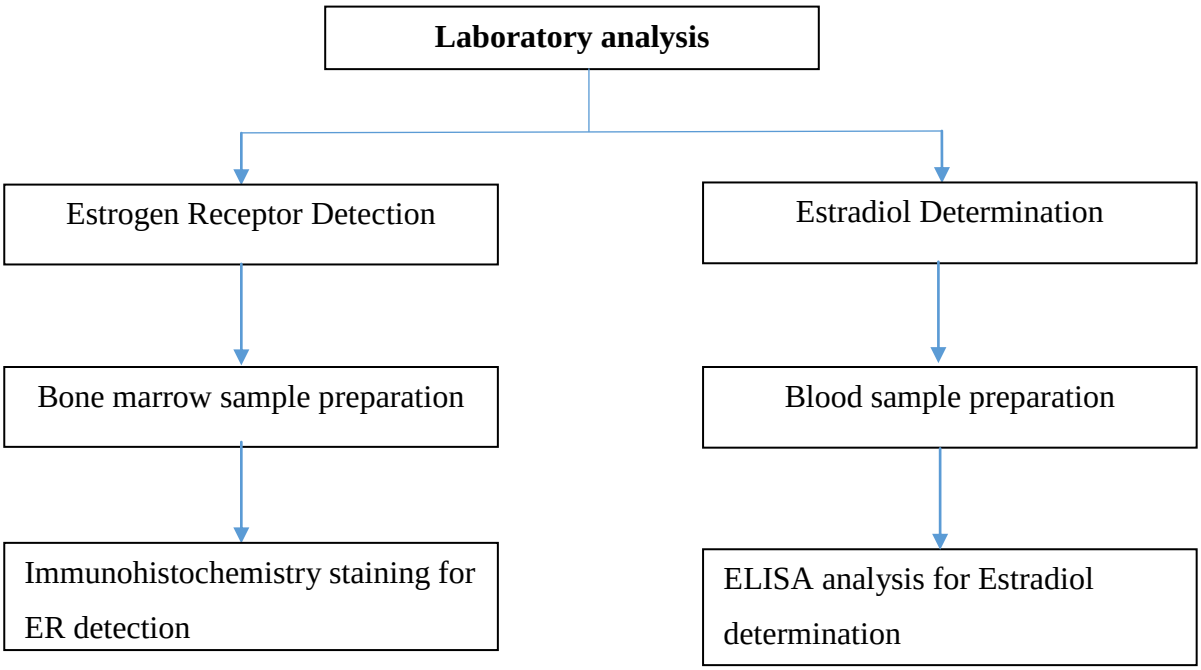
3.5 Study methods

3.5.1 Specimen collection

A consecutive sampling technique was used to acquire the samples until the required sample size for the study was achieved. The research relied on blood samples and bone marrow samples from each leukemia patients recruited to the study as well as from non-cancer personsas determined by the hospital Hematologist.Blood Samples were collected by the phlebotomist into aplain2ml vaccutainer®. 1ml of bone marrow sample wasaspirated from the sternum bone of each patient and put in an anticoagulated tube by the resident clinician. A biodata sheet was generated to categorize the gender and age of each patient.

3.5.1.1 Laboratory analysis of bone marrow and blood specimen

Bone marrow and blood samples collected were analysed using immunohistochemistry technique and Enzyme Linked Immunosorbent Assay (ELISA) respectively.



3.5.2 Bone marrow sample processing for immunohistochemistry

Bone marrow samples collected were processed for ER detection as follows

3.5.2.1 Bone marrow specimen preparationfor Immunohistochemistry

The bone marrow specimen collected in anticoagulated bottles were centrifugedfor three minutes at 3000 rpm using ROTOFIX 32A centrifuge (HETTICH, Germany). The supernatant was discarded. To remove any leftover serum, the pellets were washed three times, each time by adding 3mls of 10% normal salineand centrifuging at 3000rpm for 3mins and discarding the supernatant. 10% formaldehyde was added within five hours of collection to fix the cells. The pellets were picked, placed on a blotting paper which was folded and put into a tissue cassette. The cells in the tissue cassettes were dehydrated by soaking them for two hours in each of the ascending alcohol concentration of 10%, 20%, 50%, 70%, 90% and 100% alcohol. The cell

blocks were prepared from these cells by placing them on the bottom of the tissue holder and filling it with molten paraffin wax. A tissue cassette was then mounted to hold the wax firm ready for sectioning. The bone marrow cell blocks were sectioned using Leitz Leica 1512 microtome (W. Nuhsbam, USA). Wax holding the cells was removed by floating the sectioned cell block film in water bath set at 60^{o}Cbefore mounting them on microscope slides.

3.5.2.2 Immunohistochemistry staining for Estrogen Receptors in cell blocks

The immunohistochemistry assay was done on 4 μm sections cut from the blocks and float-mounted on adhesive microscope slides. The antigens were retrieved by boiling the mounted slides in 0.1 M citrate buffer (pH 6.0) for 90 min. Endogenous peroxidase was blocked with 0.1% sodium azide and 0.3% hydrogen peroxide. Non-specific protein binding was blockedusing 10% ovalbumin. Primary mouse monoclonal antibody 6F11 against the ER (Vector Laboratories, Burlingame, CA) was added and left for 2 hours to bind to the ER. The excess primary antibody was then washed using distilled water and biotinylated rabbit monoclonal antibody against the primary mouse immunoglobulin G (Dako Corp, Carpenteria, CA) was added and left for 30 minutes. An enzyme conjugate of streptavidin–horseradish peroxidase (Dako) was added and left for 30 minutes. The excess conjugate was washed, and 0.03% hydrogen peroxide together with 1 mg/mL diaminobenzidine was added for colourdevelopment. methyl green then added as a counterstain. Human endocervix tissue was processed together with the cell blocks and used as a positive control. The negative control consisted of nonimmune mouse immunoglobulin G substituted for the primary ER antibody.

The slides viewed using a light microscope at X100 and X400.Cells with more than 60% of ER coverage were considered as ER positive cells and show brown pigmentation caused by the dye on the nucleui of the myelocytes.On the other hand, the ER negative cells showlight blue nucleui. The total number of ER-positive myelocytes and ER-negative myelocytes was calculated using a hemocytometer. ER percentages were arrived at by dividing the total number of ER-positive myelocytes by total number of myelocytes seen per field.

Blood processing for Enzyme LinkedImmunosorbert Assay (ELISA)

Blood samples collected were processed for Estradiol determination as follows

3.5.2.3 Blood specimenpreparationforELISA

Blood samples collected from the subjects in were left for 10min to clot. They were then centrifuged at 3000rpm for 3min using ROTOFIX 32A centrifuge (HETTICH, Germany). The Serum, (supernatant) was collected and used for Estradiol determination.

3.5.2.4 ELISA analysis for Estradiol levels determinition in serum

Estradiol was analysed using competitive ELISA technique, as per the procedure from ELISA kit lot. 13003 (Human® Company, Germany). The kit contains microwells mounted with anti Estradiol antibodies. 200µl of the serum was added to the wells and incubated for 2hrs at 37^{o}C. Excess serum was pipetted and discurded. The microwells were washed (8 cycles) using ELx50 ELISA washer(BIOTEK, USA). 100µl of Enzyme conjugate provided in the ELISA kit was added to the microwells and incubated for 1hr at 37^{o}C. the washing procedure was then done as

earlier described 100µl of substrate was added to the microwells and incubated for 30min at 37^{o}C. The reaction was then stopped using 1M sulphuric acid. The concentrations of estradiol in the samples were determined using ELx800 ELISA reader (BIOTEK, USA) and recorded in picograms per mililiter (pg/ml)

ER percentages and Estradiol levels were compared among the cases and further compared with the controls to determine the association of estrogen to the amount of estrogen receptors and in relation to age and gender as well

3.6 Data management

Data analysis was performed using SPSS. Skewed continuous variables were summarized as median (Lower quartile, Upper quartile) while the normally distributed continuous variables were summarized as mean (standard deviation). Categorical variables were summarized as frequencies (percentage) and the association between the result, and the independent variables were assessed using the correlation. The case and control means were tested using t-test to accept or reject the null hypothesis. All these variables, continuous, as well as the categorical, were presented in a table separately for the cases and controls. The frequency of some of these variables in the controls may help to judge whether they are likely to represent the population from which the cases arise.

3.7 Ethical Considerations

Before commencement of the study,ethics approval was sought from Maseno University and Moi University , Moi Teaching and Referral Hospital Institutional Research and Ethics Committee (IREC). Copy of letter of approval is annexed in appendix 1.

CHAPTER FOUR

RESULTS

4.1 Estrogen Receptors (ER) in case and control populations

The ER positive (controls) sample slides

When viewed using a light microscope (mg X100), the background showed blue-green colour of the counterstain with clusters of myelocytes. The myelocytes present showed brown colour of the ER stain as shown in plate 1. Viewed using a light (mg X400) the number of ER stained myelocyte were tallied using a cytometer and given as a percentage of the total myelocytes seen as shown in plate 2.

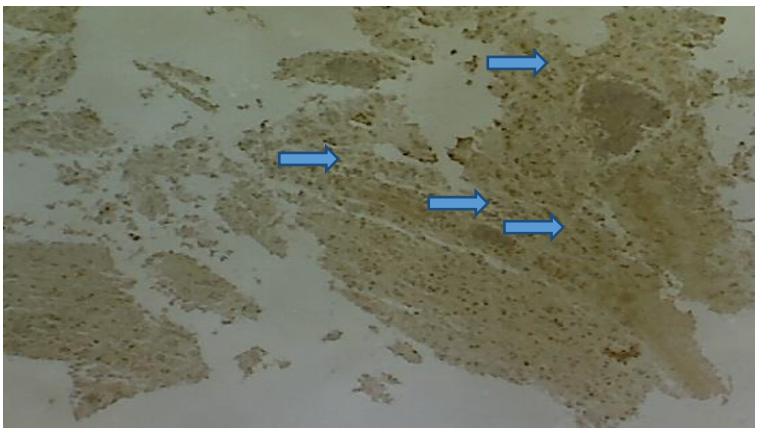


Plate 1: A micrograph of ER positive cells from a control sample. The brown spots (arrows)are nuclei of myelocytes stained with anti-ER mouse monoclonal antibodies that are conjugated with a brown dye (Mg X100).

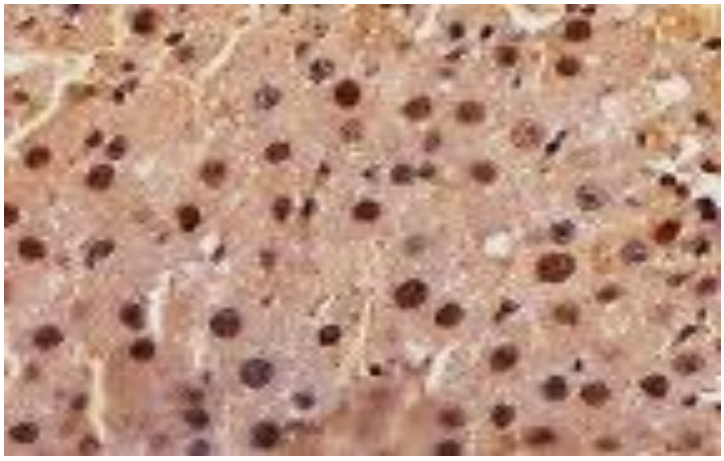


Plate 2:A micrograph of ER positive cells (Mg X400).

The ER negative (cases) sample slides

When viewed using a light microscope (X100), the background showed blue-green colour of the counterstain with clusters of myelocytes as shown in plate 3. Few myelocytes that were present showed brown colour of the ER stain as shown in figure 3 and figure 4.

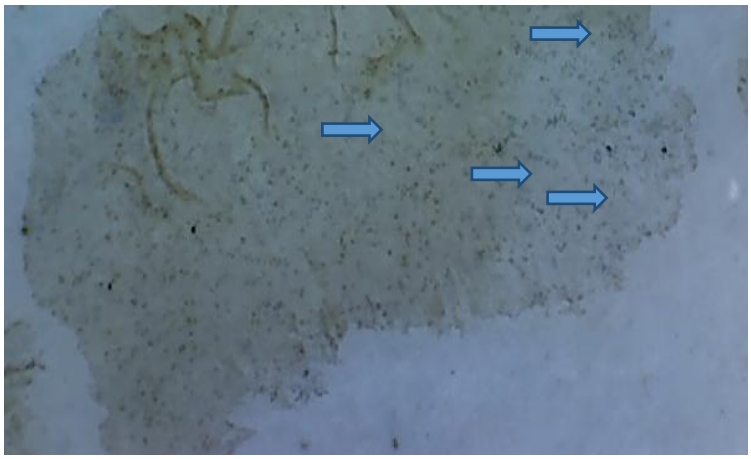


Plate 3: A micrograph of ER negative cells from a case sample. The blue spots (arrows) are nuclei of myelocytes (Mg X100).

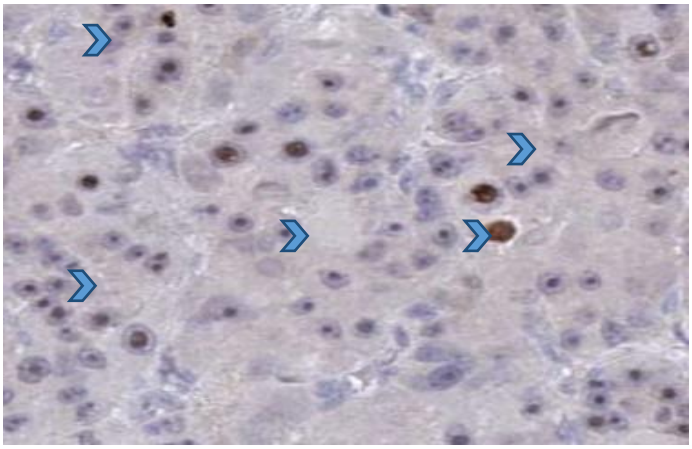


Plate 4: A micrograph showing ER negative (arrow heads) cells (Mg X400)

Frequency of ER in cases and controls

The number of cases with ER which was less than 10% was found to be 12 (70.6%) for cases and 3 (17.6%) for controls. There were no cases that had ER percentage greater than 60% for cases while controls had 2 as shown in table 1 below.

Table 1: Number of cases and controls within various ER percentage groups

ER percentage	Cases		Controls	
	In numbers	In percentages	In numbers	In percentages
0-9	12	70.6	3	17.6
10-19	2	11.8	7	41.2
20-29	1	5.9	2	11.8
30-39	0	0	1	5.9
40-49	1	5.9	0	0
50-59	1	5.9	2	11.8
60-69	0	0	0	0
70-79	0	0	1	5.9
80-89	0	0	1	5.9
Total	**17**	**100**	**17**	**100**

The number of cases with ER which was less than 10% was found to be 12 for cases and 3 for controls. There were no cases that had ER greater than 60% for cases while controls had 2 as shown in table 1 below. The mean percentage of Estrogen Receptors case samples was 7.65% and in controlsamples was 23.53%.

Table 2: Mean ER percentages in case and control samples

ER percentage	Cases		Controls	
	Number of cases **(N=17)**	Mean ER percentage	Number of controls **(N=17)**	Mean ER percentage
0-9	12	0	3	0
10-19	2	10	7	11.4
20-29	1	20	2	20
30-39	0	0	1	30
40-49	1	40	0	0
50-59	1	50	2	50
60-69	0	0	0	0
70-79	0	0	1	70
80-89	0	0	1	80
Mean (%)		**7.65**		**23.53**

Variation of Estrogen Receptor percentages in cases and in controls

The mean percentage of ER in cases was 7.65 and that of controls was 23.53. This canbe seen in the table below.

Statistics Table

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Estrogen Receptor percentage	AML	17	7.65	15.219	3.691
	Non-cancer	17	23.53	24.416	5.922

The hypothesis tested was;

H_0: Equal means of Estrogen receptor percentage among case and control samples

H_1: Unequal means of Estrogen receptor percentage among case and control samples

		t-test for Equality of Means						
		T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Estrogen Receptor percentage	Equal means assumed	-2.276	32	0.030	-15.882	6.978	-30.096	-1.669
	Equal means not assumed	-2.276	26.802	0.031	-15.882	6.978	-30.205	-1.560

P value is 0.03 andis smaller than 0.05, the null hypothesis is therefore rejected at 5% level of significance. The means for receptor percentages for cases and controls are significantly different.

4.2 Estradiol levels incasesas compared tocontrols

The mean of Estradiol in case samples was found to be 128.94 pg/ml whereas in control sampleswas found to be 32.41 pg/ml as shown in Table3 below

Table 3: Estradiol levels in the cases and controls

Estradiol concenrations (pg/ml)	Number of AML subjects (cases)	Number of non- AML subjects (controls)
0-19	0	11
20-39	2	2
40-59	0	1
60-79	0	1
80-99	2	1
100-119	4	0
120-139	4	0
140-159	1	0
160-179	1	0
180-199	1	0
Above 200	2	1
Total	17	17
Mean concentration (pg/ml)	128.94	32.41

Variation of Estradiol levels in cases and control samples

From the descriptive statistics as presented in the table below, AML patients have higher levels of estrogen as compared to the non-cancer patients.

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Estrogen levels	AML	17	128.94	61.076	14.813
	Non-cancer	17	32.41	54.735	13.275

The hypothesis was tested using t-test;

Ho: Equal means of Estradiol levels among cases and control samples

H1: unequal means of Estradiol levels among cases and control samples

T-test outputs are shown in the table below;

		t-test for Equality of Means						
		t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Estrogen levels	Equal means assumed	4.853	32	.000	96.529	19.891	56.012	137.046
	Equal means not assumed	4.853	31.623	.000	96.529	19.891	55.993	137.065

The P value of 0.000 is significantly smaller than 0.05; this implies that the null hypothesis was rejected, and the alternative accepted at 5% level of significance. Estradiol levels are significantly different for AML and non-cancer cases.

4.3 The correlation between Estradiol and Estrogen receptor

Correlation between Estradiol and Estrogen receptor in cases

The samples from AML patients that had the estrogen receptor values ranging from 0 to 50% and Estradiol levels ranging from 23 pg/ml to 274 pg/ml as shown in the table4 below.

Table 4: Correlation between Estradiol and ER in cases

ER percentages for AML (case)	Estradiol levels in pg/ml for AML(Cases)
0	162
0	103
0	155
0	104
0	117
0	274
0	136
0	121
0	132
0	220
0	132
0	191
10	38
10	100
20	99
40	85
50	23

To test the hypothesis

H0: correlation=0: no correlation between Estradiol and ER

H1: Correlation is not equal to zero: there exists a correlation between Estradiol and ER

Correlations

		Estradiol levels	Estrogen Receptor percentage
Estradiol levels	Pearson Correlation	1	-0.5952
	Sig. (2-tailed)		0.01
	N	17	17
Estrogen Receptor percentage	Pearson Correlation	-0.5952	1
	Sig. (2-tailed)	0.01	
	N	17	17

The correlation of Estradiol and Estrogen Receptors in AML patients is -0.5952, which is a negative correlation.This means that they are inversely proportional.As the Estradiol levels increase the ER percentages and vice versa.

The p value is 0.01, which is less than 0.05, thus rejection of the no correlation null hypothesis that estrogen receptor percentages are not dependent on Estradiol. That is estrogen receptor percentages are influenced by the estrogen of the subjects

Correlation between Estradiol and Estrogen receptor in controls

Most of the samples that were tested for non-cancer patients showed Estrogen receptor values that were more than 10% and lower Estradiol levels. The concentration of the two markers was seen to be inversely proportional as shown in the table5 below.

Table 5: Covariance between Estradiol and ER in controls

ER percentages for non-cancer (controls)	Estradiol levels in pg/ml for non-cancer (Controls)
30	9
50	24
50	4
0	91
0	41
80	1
10	25
10	10
15	3
10	67
10	4
20	15
70	12
0	222
20	0
10	11
15	12

To test the hypothesis

H0: correlation=0: no correlation between Estradiol and ER

H1: correlation is not equal to zero: there exists acorrelation between Estradiol and ER

Correlations

		Estradiol levels	Estrogen Receptor percentage
Estradiol levels	Pearson Correlation	1	-0.4059
	Sig. (2-tailed)		0.03
	N	17	17
Estrogen Receptor percentage	Pearson Correlation	-0.4059	1
	Sig. (2-tailed)	0.03	
	N	17	17

The correlation of Estradiol and Estrogen Receptors in non-cancer patients is -0.4059, which is a weak negative correlation.This means that they are inversely proportional.As the Estradiol levels increase, the ER percentages decrease, and vice versa.

The p-value is 0.03 and is less than 0.05, thus arejection of the no correlation null hypothesis that estrogen receptor percentages are not dependent on Estradiol. That is estrogen receptor percentages are influenced by the estrogen of the subjects

4.4 Estradiol levels and Estrogen receptors variation in relation to gender

Estrogen receptor percentages and gender

In cases, the mean estrogen receptor percentages for the males with AML was 8.89 while for females was 6.25. The control males had mean ER percentage of 50.44 while females had mean ER percentage of 12.125 as shown in the table6 and figure 2 below

Table 6: Estrogen receptors in cases and controls in relation to gender

	Male	**Female**
AML (cases)	8.89	6.25
Non cancer (controls)	50.44	12.125

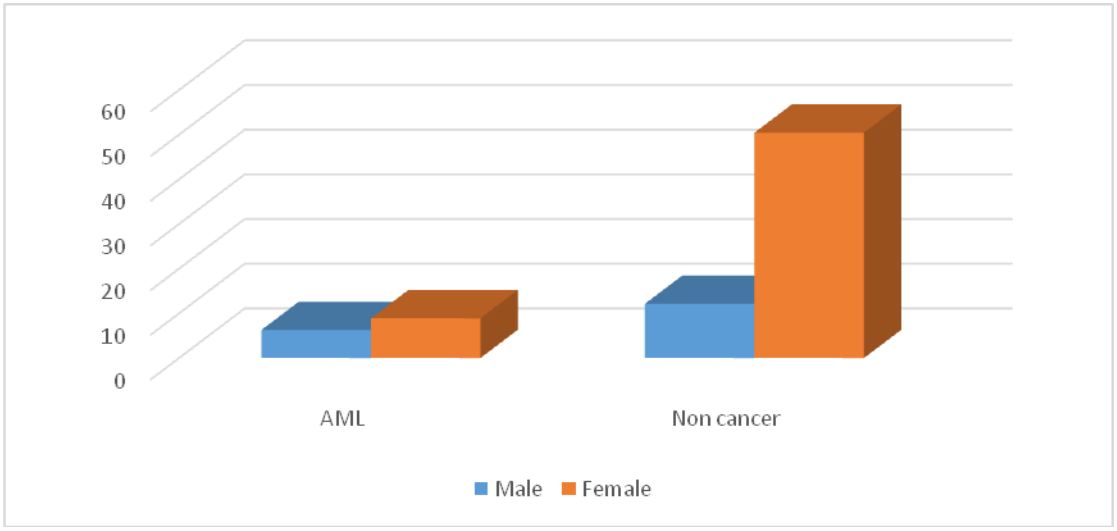


Figure 2: Estrogen receptors in cases and controls in relation to gender

To test of there was a difference in means for Estrogen Receptors in AML a t-test was conducted and p value calculated at 95% confidence level and t-test outputs were as shown below

H0: Equal means of Estrogen receptor percentage among AML cases
H1: unequal means of Estrogen receptor percentage among AML cases

		t-test for Equality of Means						
		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Estrogen Receptor percentage	Equal means assumed	-0.148	32	.884	-1.111	7.532	-16.453	14.231
	Equal means not assumed	-0.148	31.945	.883	-1.111	7.496	-16.380	14.158

The p-value is 0.884, which is way above 0.05. The null hypothesis is therefore accepted at 5% level of significance. Estrogen receptors are independent of gender. That is Estrogen receptor percentages do not vary with the genderin AML patients.

Estradiollevels and gender

The Estradiol levels for the males and the females with AML were found to be close compared to those of non-cancer patients as shown in the table7 and figure 3 below

Table 7: Estradiol levels in cases and controls in relation to gender

	Male	Female
AML	114.125	142.111
Non cancer	12.125	50.444

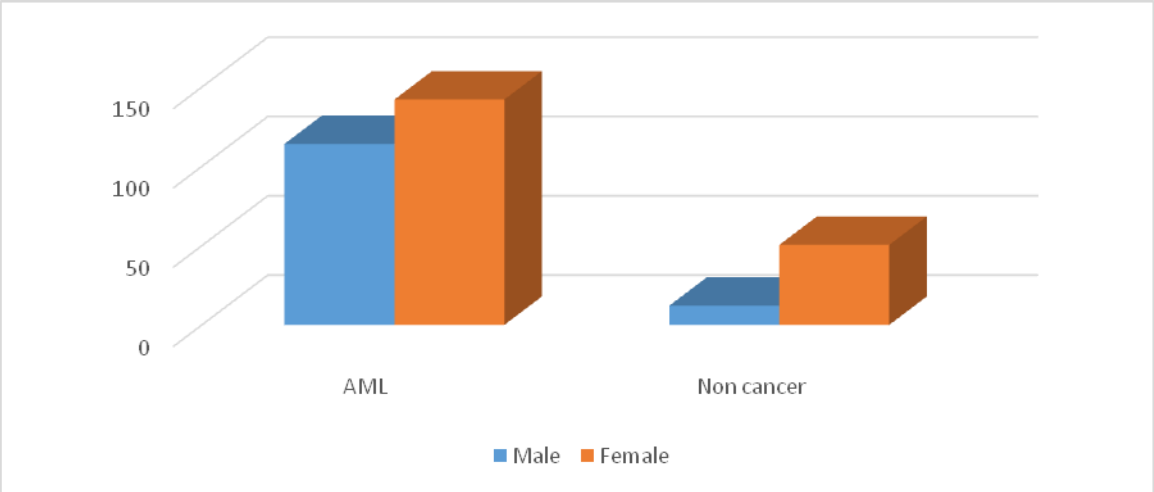


Figure 3: Estradiol levels in cases and controls in relation to gender

To test of there was a difference in means for Estrogen Receptors in AML a t-test was conducted and p-value calculated at 95% confidence level, and t-test outputs were as shown below

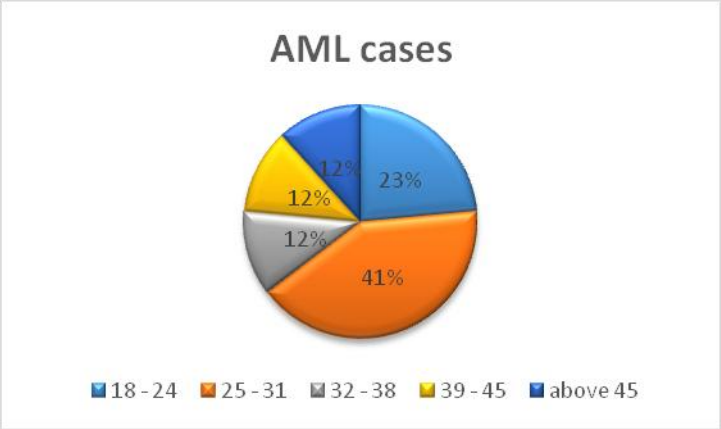
H0: Equal means of Estradiol levels among male and female AML cases
H1: unequal means of Estradiol levels among male and female AML cases

		The t-test for Equality of Means						
		T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Estrogen levels	Equal means assumed	-1.296	32	.204	-33.153	25.591	-85.279	18.974
	Equal means not assumed	-1.331	28.831	.194	-33.153	24.914	-84.121	17.815

A p-value of 0.204 is greater than 0.05, we, therefore, accept the null hypothesis that Estrogen levels are not significantly different with respect to gender. That is estrogen levels do not vary with respect to gender.

4.5 Estrogen levels and receptor percentages with respect to ages

From the research it was found out that most of the AML cases were patients of the age between 25 and 31 which carried 41% of all the AML patients that were incorporated in the study as shown in the chart below.



Correlation of cases with age

To check how age correlates with AML, correlation analysis was done, and the results summarized below;

Correlations

		Condition	Age
Condition	Pearson Correlation	1	.046
	Sig. (2-tailed)		.798
	N	34	34
Age	Pearson Correlation	.046	1
	Sig. (2-tailed)	.798	
	N	34	34

It was discovered that there was a positive correlation between age and existence of AML of 0.046. As age advances there are greater chances of getting AML. However, the P value is 0.798 which is greater than 0.05, this implies acceptance of the null hypothesis. We can therefore confirm that there is no correlation between ages and existence of AML among AML patients.

Correlation of estrogen receptors with age

The case samples from persons of the age between 18 and 24 years had mean ER percentage of 27.5% while the control samples of the same age group had mean ER of 32% as shown in table 8 below

Table 8: Mean ER percentages in case and control samples in relation to their ages

Age	Mean ER percentages for AML (case)	Mean ER percentages for non-cancer (control)
18-24	27.5	32.5
25-31	1.4	19.3
32-38	0.0	45.0
39-45	5.0	10.0
above 45	0.0	12.5

To test the hypothesis

H0: correlation=0 against an alternative

H1: correlation is not equal to zero

Correlations			
		Age	Estrogen Receptor percentage
Age	Pearson Correlation	1	-0.262
	Sig. (2-tailed)		0.134
	N	34	34
Estrogen Receptor percentage	Pearson Correlation	-0.262	1
	Sig. (2-tailed)	0.134	
	N	34	34

The correlation of age and estrogen receptors is -0.262, which is a weak negative correlation. The p value is 0.134 which is greater than 0.05, thus acceptance of the no correlation null

hypothesis that estrogen receptor percentages are not dependent on age. i.e. estrogen receptor percentages are not influenced by the ages of the subjects

Correlation of Estradiol levels with age

The cases of the age between 18 to 24 years had mean estradiol levels of 118pg/ml while the controls of the same age group had a mean of 42.25 pg/ml. The cases aged above 45years had estradiol mean levels of 161 pg/ml while the controls of the same age had mean levels of 11.50 pg/ml as shown in table 9 below

Table 9: Estradiol levels in cases and controls in different age groups

Age	Estradiol levels for Cases(pg/ml)	Estradiol levels for Controls(pg/ml)
18-24	118.00	42.25
25-31	132.43	21.57
32-38	126.50	13.50
39-45	160.00	111.00
above 45	161.50	11.50

To test the hypothesis

H0: correlation is equal to zero: there is no correlation between Estradiol levels and age

H1: correlation is not equal to zero: there is a correlation between Estradiol levels and age

Correlations

		Age	Estrogen levels
Age	Pearson Correlation	1	0.148
	Sig. (2-tailed)		0.402
	N	34	34
Estrogen levels	Pearson Correlation	0.148	1
	Sig. (2-tailed)	0.402	
	N	34	34

It was discovered that there was a positive correlation of age and existence of AML of 0.148. As age advances there are greater chances of getting AML. However, the P value is 0.798 which is greater than 0.05, this implies acceptance of the null hypothesis. We can therefore confirm that there is no correlation between ages and existence of AML among AML patients.

Correlation of 0.148, implies a weak correlation between age and estrogen levels, the p values of 0.402 which is greater than 0.05 further confirms the acceptance of the no correlation null hypothesis, thus there is no correlation between age and estrogen levels.

CHAPTER FIVE

DISCUSSION

5.1Introduction

The study was meant to demonstrate the association of estradiol and estrogen receptors in Acute Myeloid Leukemia (AML) subjects (cases) and in non-cancer subjects (controls). AML, in a population, may be secondary to downregulation of estrogen receptors which upregulates oncoproteins responsible for AML manifestation. Little is known about the role of Estradiolin Estrogen Receptor regulation in AML patients as compared to non-cancer subjects. It is on this basis that the current study gives an in-depth analysis of the variation of Estradiol and Estrogen Receptors and their correlation in AML patients as compared to non-cancer subjects, who were controls.

5.2 Estrogen Receptors (ER) in case and control populations

This study shows that absence or very low amounts of estrogen receptors in the cases as compared to the controls. 12 out of 17 cases had less than 10% of the ER percentage compared to 3 out of the 17 control samples. The mean ER percentage for the case population was 7.65% which was quite low compared to that of the control population which was 23.53%.The ER means were testedby using t-test and found out that the two sample means were significantly different. This was in line with a study by Li (1999) which showed that there was low or absent estrogen receptor in AML cases (Li et al., 1999). Thismeans that most of the cases in the study were as a result of down-regulation of ER.

It is known that down-regulation of ER leads to up-regulation of and oncoprotein STAT5, which mainly presents in myelocytic cells (Björnström and Sjöberg, 2005).The cells that lack or have very low ER density accumulate in the bone marrow hindering production of normal cells. Overall cancer manifestation is caused by the accumulation of defective cells that overcome the immune mechanisms.

5.3 Estradiol concentration in case and control populations

From the study, it was observed that estradiol in the cases was higher than that of the controls. The mean concentration of Estradiol in the cases was 128.94 pg/ml while that of controls was 32.41pg/ml. The t-test analysis showed that there was a significant difference between cases and controls. In normal population, the reference range for estradiol is 10 to 200 pg/ml (Folkerd, Lonning, and Dowsett, 2014). And although some studies have reported the reference ranges of Estradiol in AML to be the same as that of normal subjects (Molgaard-Hansen et al., 2012), this study shows that the Estradiol levels for the cases were closer to the upper margin. This is may be the cause lower levels of ER in the cases. The estradiol levels for the controls were nevertheless found to be low but within the normal acceptable ranges. The balance between the hormone and the receptors was well maintained.

5.4 Correlation between Estrogen Receptors (ER) and estradiol concentration in case and control populations

In cases it was observed that there was inverse proportion trend in the percentages of ER and levels of Estradiol. The patients who had less than 10% of myelocytes with ER, had more than

100 pg/ml of estradiol.As the estrogen receptor percentages increased, the estradiol levels decreased. This is because the Estradiol down-regulates estrogen receptors. Very high levels of Estradiol keep the levels of ER low and thus initiation and progression of Acute Myelocytic Leukemia of STAT5 pathway. The control samples also had negative correlation, however, the values were not widely dispersed as seen in AML.

5.4.1 Variation of Estrogen Receptors (ER) and estradiol in cases and controls in relation to gender

There was a significant difference between the cases and the controlsas demonstrated by T-test.The mean Estradiol levels of the AML patients were 114.125 pg/ml for males and 142.111 pg/ml for females. This was relatively high as compared to the expected values (which are 15-60 pg/ml for males and 70-120pg/ml for females)(Robertson et al., 2002). This study cannot establish the reason this occurrence. However, these high levels could have been responsible for down-regulation of the ER levels in the AML cases. Studies show that ER ligand binding by Estradiol down-regulates ER (Valley and Solodin, 2008). The control samples showed normal levels of Estradiol (less than 55 pg/ml for males and less than 105 pg/ml for females) (Robertson et al., 2002).

In the current study, the estrogen receptor percentage means for males and females were almost equal in AML patients and highly dispersed in controls. Women are known to produce more Estradiol,which is directed to other parts of the body leaving little Estradiol to be utilized in the bone marrow. This thus leaves the myelocytes in females with less Estrogen Receptors than the

males. However, there is no significant difference between estrogen receptors of males and females with AML. The Estradiol levels also had asignificant difference between the two genders.

5.4.2 Variation of Estrogen Receptors (ER) and estradiol in cases in relation to age

A shift of AML prevalence from persons above 65 years to younger patients was seen. Most of the AML cases were between the age of 25-31, which was 41% of the cases, and 18-24,which was 23% of the total AML cases. This is because variations in Estradiol are more in these age brackets than the other older ages(Khosla, 2013).Unlike the traditional statistics whereby AML was known to affect persons above 65 years old (Howlader et al., 2011; Stone et al., 2004), the new trend was also seen in a study by Mosert et al., (2012) that showed that 48% of AML cases were less than 25 years of age. It was also observed that age and AML had a weak positive correlation of 0.046. This means that though many AML cases were reported among the youth, there were higher chances of acquiring AML as age advanced.

The mean estradiol concetration for AML in persons above 45 years of age was 161pg/ml, which was the highest of all the age groups. Comparing this to the mean estrogen receptor percentages, this group had mean ER percentage of 0. In this age group, it is expected that the normal range of estradiol to be 5-15pg/ml (Robertson et al., 2002). The controls on the other hand has a mean concentration of 11.5 pg/ml. The same trend was seen in other age groups whereby there was very high estradiol levels were recorded in cases and normal estradiol levels were recorded in controls. This shows that upregulation of estradiol had caused down-regulation of ER and thus causing AML through out all the age groups.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSION, AND RECOMMENDATIONS

6.1 Summary

Acute Myelocytic Leukemia has various pathways in which it can start. One of the pathways is via down-regulation of ER which leads to up-regulation of oncoproteins, such as STAT5, which are responsible for AML progression. From the study it was found out that mean percentage of ER in bone marrow samples of cases was 7.65%. On the other hand, mean ER percentage from the control bone marrow samples, which were collected from subjects with no known cancer, was 23.53%. Comparing the two means it was clear that there was a significant difference between the two. This demonstarates that most of AML patients in Moi Teaching and Referral Hospital have down-regulated ER which could be the cause of AML.

Estadiol is a major regulator of ER. However, it has not been studied in AML patients to find out how the hormone presents in these patients. Blood samples were collected from cases and controls and analysed for estradiol. The mean estradiol concentration from both populations was calculated and compared. It was found out that cases had a mean concentration of 128.94 pg/ml while the controls had a mean of 32.41 pg/ml. T-test analysis showed that the mean concentration of estradiol in cases was significantly high.

Analysing case by case using correlation, it was observed that there was a negative correlation of -0.5952,thus inverse proportion trend in the percentages of ER and concentration of Estradiol. As the estrogen receptor percentages increased, the estradiol levels decreased. This is because the

Estradiol down-regulates estrogen receptors. Very high levels of Estradiol keep the levels of ER low and thus initiation and progression of Acute Myelocytic Leukemia of STAT5 pathway. Unlike in AML, the non-cancer patients had a random trend of the Estradiol.

The incidence of AML varies with gender. Females have slightly higher prevalence than males(Jemal, Thomas, Murray, and Thun, 2002). From the study it was seen that the meanestrogen receptor percentages in females was 6.25% which was slightly lower than 8.89% in males. In the control population, the mean of the females was 12.125% while that of males was 50.44%. High incedence of AML females could be as an outcome of low ER.

Literature has showed that in Moi Teaching and referral Hospital, the trend of AML prevalence has been seen to shift from 65 years to less than 25 yrears of age (Mostert et al., 2012).From this study it was seen that 41% of the AML cases were below 25 years of age.

Estrogen Receptor studies are a new direction of cancer research that help the researchers to understand the aetiology, progression, treatment, prevention and prognosis of the Acute myeloid leukemia. The studies of the Estradiol and estrogen receptors interactions aid the researchers to realize the possible causes of inadequate ER in the body.

6.2 Conclusion

In conclusion,

i. Most of the AML samples that were tested for the estrogen receptors showed very low percentages of the estrogen receptors compared to the non-AML samples. Thus these

cases could be as a result of down-regulation of ER and thus possible up-regulation of oncoproteins such as STAT5.

ii. The Estradiol levels in cases samples were relatively high as compared to the control samples. Estradiol could be downregulating ER in AML patients

iii. Both in cases and controls, the higher the Estradiol the lower the ER. The two variables covary inversely.

iv. Females have higher Estradiol levels and lower ER levels thus consequently have more reported cases of AML.However, Gender, on the other hand, does not influence the commencement AML.

v. The most affected age group is individuals between 25 years and 31 years of age.

6.3 Recommendations from the study

i. ER analysis be adopted as a diagnostic method to determine the cause of AML

ii. Estradiol should be monitored since it is a predesposing factor of AML

iii. Non Estrogen contraceptives should be used since chances in estrogen tampers with ER

6.4 Recommendation for further research

i. More studiesbe carried out to develop ER as a possible target of chemotherapy

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APPENDIX

Appendix 1: IREC approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
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Reference: IREC/2013/88
Approval Number: 0001065

23rd September, 2013

Elius Mbogori,
Moi Teaching and Referral Hospital,
P.O. Box 3-30100,
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
APPROVED
23 SEP 2013
P.O. Box 4606 ELDORET

Dear Mr. Mbogori,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:-

"Association of Estradiol and Estrogen Receptors in Acute Myelocytic Leukemia Patients".

Your proposal has been granted a Formal Approval Number: **FAN: IREC 1065** on 23rd September, 2013. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 22nd September, 2014. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc Director - MTRH Dean - SOM Dean - SON
Principal - CHS Dean - SPH Dean - SOD