HISTOMORPHOLOGY OF BONE MARROW FROM ADULT PANCYTOPENIC PATIENTS AT THE UNIVERSITY TEACHING HOSPITAL IN LUSAKA, ZAMBIA.

BY

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A dissertation submitted to the University of Zambia in partial fulfilment of the requirements for the award of the degree of Master of Medicine in Pathology.

THE UNIVERSITY OF ZAMBIA

LUSAKA

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DECLARATION

I, **Francis Kaoma Musonda** hereby declare that this dissertation is based on my original work and that where this is not the case acknowledgements indicate the source.

I further declare that this work has not been done before in Zambia and is hereby being submitted to the University of Zambia in partial fulfilment of the Master of Medicine in Pathology degree.

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Signed……. …….Date…….

DR. CLEMENCE MARIMO.

CERTIFICATE OF APPROVAL

This dissertation by Dr. **FRANCIS KAOMA MUSONDA** has been approved as partial fulfilment of the requirements for the award of the **Master of Medicine in Pathology** at the University of Zambia.

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ABSTRACT

Pancytopenia is a hematologic condition characterised by leukopenia, anaemia and thrombocytopenia. Pancytopenia is not a diagnosis and has to be qualified by determination of its cause. The aetiologies of pancytopenia are diverse, and study of bone marrow cytology and histology are key components that assist in the determination of the underlying cause. Pancytopenia is encountered regularly in medical practice in Zambia; however, no studies have been conducted on pancytopenia to date.

A total of 45 bone marrow biopsies were collected over the study period. In all cases the indication was pancytopenia that had been confirmed by a full blood count done at the UTH and the biopsy site was either the anterior superior iliac spine or the posterior superior iliac spine. Demographic and clinical details were obtained using data collection sheets and from review of patient records. The collected data was analysed using the Statistical Package for the Social Sciences (SPSS) version 21 and excel 2016 data analysis tool pack. A Chi square test was used to measure association between categorical variables and Student's t-test to measure association between categorical and numerical values. A p value of < 0.05 at 95% confidence interval was considered statistically significant.

There were 32 females (71%) and 13 males (29%), and the age ranged from 15 to 72 years with an average age of 35 years. Forty percent (n=18) of the study participants had human immunodeficiency virus (HIV) and all of these all were on highly active antiretroviral therapy (HAART). There were 6 histologic patterns found the commonest being megaloblastosis seen in 38% of the patients, followed by malignancy and myelodysplasia both at 17.0%. Bone marrow aplasia accounted for 13.0%, non-megaloblastic erythroid hyperplasia accounted for nine percent and myelofibrosis for four percent.

The bone marrow biopsies of the study population showed six histomorphologic pictures which in order of frequency were megaloblastosis, malignancy and myelodysplasia, bone marrow hypoplasia, non-megaloblastic erythroid hyperplasia and myelofibrosis.

Keywords: *Pancytopenia, Bone marrow, Trephine and Histomorphology*

DEDICATION

To the Musondas' past, present and future and all the children at Arthur Davidson's Childrens' Hospital who we lost suffering from undiagnosed pancytopenia, you were the motivation behind completion of this document.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome.
AML	Acute Myeloid Leukaemia
BMB	Bone marrow biopsy
FBC	Full blood count.
HAART	Highly active anti-retroviral therapy.
HIV	Human Immunodeficiency syndrome
MDS	Myelodysplastic syndrome.
NAAC	Normal age appropriate cellularity
UTH	University Teaching Hospital.
WCC	White cell count.

DEFINITIONS

Aplastic anaemia: Multiple cytopenia's with trilineage bone marrow hypoplasia in the absence of neoplasia and reticulin fibrosis, indicating a basic failure to produce normal hematopoietic elements.

Non-megaloblastic erythroid hyperplasia: Increase in cellularity of the erythroid component of the bone marrow. It is associated with destruction of erythrocytes in the peripheral circulation.

Megaloblastic anaemia: Heterogeneous group of disorders the common morphological hallmark of which is megaloblastosis.

Myelodysplastic syndrome: Clonal disorders of multipotent bone marrow stem cells with cytopenia(s), dysplasia in one or more of the major myeloid cell lines, ineffective or disorderly haematopoiesis.

Myelofibrosis: This is a condition characterised by increased reticulin content of the bone marrow, it may be primary or secondary.

Primary myelofibrosis: Clonal disorder arising from the neoplastic transformation of early hematopoietic stem cells that is characterised by characterized by anaemia, bone marrow fibrosis, extramedullary haematopoiesis, leukoerythroblastosis and teardrop-shaped red blood cells in peripheral blood and hepatosplenomegaly.

Secondary myelofibrosis: This is fibrosis of the bone marrow that occurs secondary to bone marrow pathology that is not primary myelofibrosis. The pathologies include primary myeloid neoplasms, malignant lymphomas, metastatic carcinomas, inflammatory reactions, granulomatous reactions, and osteopathies.

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CHAPTER ONE
BACKGROUND

1.1 Introduction

Pancytopenia is defined as a deficiency of all three cellular components of the peripheral blood, namely erythrocytes, leukocytes and platelets. The aetiologies of pancytopenia are numerous, and examination of bone marrow is a vital component in the algorithm employed to determine the cause as it supplies material to use in cytology, histology, microbiology and cytogenetics. The cause of pancytopenia in an adult is usually acquired rarely however it may be caused by an undiagnosed hereditary disease.[1]

The gold standard for investigation of the bone marrow of pancytopenic patients worldwide incorporates study of aspirates with study of the histology of the bone marrow biopsy as the two procedures that is, bone marrow aspiration and biopsy complement one another[2,3]. In Zambia bone marrow studies are currently largely restricted to aspirate studies and thus the morphologic patterns of the bone marrow architecture seen on biopsy in this patient population is not defined. This in turn means that the corresponding diagnostic information with regards to pancytopenia in Zambia is also lacking.

Causes of pancytopenia are influenced by geography, socioeconomic conditions and endemic illness,[1] and although the list of the underlying causes of pancytopenia is extensive the underlying mechanisms regardless of the primary pathology can broadly be divided into three, that is bone marrow infiltration or replacement, bone marrow aplasia and blood cell destruction/excessive sequestration [1]

Considered under Bone marrow infiltration and replacement are neoplastic conditions (leukaemia, lymphoma, multiple myeloma, myelodysplastic syndromes and metastatic cancers), myelofibrosis and infectious diseases (tuberculosis and fungal infections)[1,3]

Examples of diseases which share the underlying mechanism of bone marrow aplasia are nutritional disorders (deficiencies of vitamin B_{12} or folate), aplastic anaemia, infectious diseases (HIV, viral hepatitis, parvovirus B19), immune destruction and medications[1,3].

Pancytopenia in which the underlying mechanism is blood cell destruction can be seen in patients with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura and nutritional disorders. Excessive sequestration is the underlying mechanism in pancytopenia in a patient with hypersplenism[1,3]

It must be noted that some diseases will cause pancytopenia via a combination of all three previously stated mechanisms[1].

Variation in aetiology of pancytopenia and therefore on the bone marrow morphology is not only appreciated in different countries but also in different regions of a single country[4]. Review of literature states the commonest causes of pancytopenia stretching from Asia, through the Middle East and into North Africa and into eastern and southern Africa as megaloblastic anaemia, malignancy and aplastic anaemia[4-11]

Diagnoses that are quoted infrequently include Non-megaloblastic erythroid hyperplasia[6], myelodysplastic syndrome[4,10] , drug induced pancytopenia, Waldenstroms macroglobinaemia[12] and infectious diseases like tuberculosis, human immunodeficiency virus and malaria[4,6,13]

There have been no studies done on pancytopenia in Zambia.

1.2 Approach To A Bone Marrow Trephine Biopsy In A Case Of Pancytopenia

1.2.1 Assessment of Cellularity

The morphology of bone marrow in various pathological states can generally be divided into hypercellular and hypocellular[14-16]. These two broad categories can then be sub divided into subsequent fewer morphologic differentials by the pathologist via searching for dysplasia in any of the cell lines, prominent excess of cell lines, cells that demonstrate blastic morphology, proliferations of metastatic cells and assessment of fibrosis[14-16].

Hypercellular bone marrow

A hypercellular bone marrow is defined as an increase in the haematopoietic cells with a reduction in the number of adipocytes when corrected for age and is a finding seen in many diseases with or without pancytopenia[14-16]. The causes of pancytopenia based on a hypercellular morphology include ineffective haematopoiesis (e.g. megaloblastic anaemia, HIV), myelodysplasia, myeloproliferative disorders (e.g. primary myelofibrosis) and neoplasms (e.g. lymphoma, metastatic disease).[14-16]

Normocellular marrow

This is defined histologically as a marrow having its hematopoietic elements in their correct proportions for age.[14]

Hypocellular bone marrow

A hypocellular bone marrow is defined histologically as a reduction of hematopoietic cells with an increase in the resident adipose tissue.[14-16] When the initial morphologic assessment of the bone marrow of a pancytopenic patient is hypocellular, the differentials include, aplastic anaemia, chemotherapy, infection and hypocellular forms of myelodysplastic syndrome and haematologic neoplasms.[14-16]

1.2.2 Architectural Patterns after Assessment of Cellularity

Granulomatous inflammation

A granuloma is defined histologically as an aggregation of activated histiocytes with an associated peripheral rim of lymphocytes.[14-16] Granulomas of the bone marrow may take several unique morphologic appearances such as epithelioid (aggregates of epithelioid histiocytes infection or autoimmunity), ring (fibrin and inflammatory cells arranged around a central clear space, may be seen in Q fever though not specific), lipid (usually insignificant).[14] In 80 to 90 % of cases of granulomatous inflammation in the bone marrow (initially assessed on histology), a combination of histochemical stains, culture, serology, enzyme immunoassay of body fluids and molecular analysis may be used to derive a diagnosis.[16]

Fibrosis and the bone marrow

Diseases that result in fibrosis of the bone marrow can also cause pancytopenia[3], it may be primary or secondary and the mechanism is thought to be a combination of ineffective haematopoiesis and subsequent hypersplenism[3]. The causes are numerous and include malignancy, inflammatory reactions and osteopathies[3]. Fibrosis of the bone marrow can only be examined on histology making the trephine the only means of its determination. Fibrosis in the bone marrow is graded from 0 to 3 and has been shown to carry prognostic significance.[17]

1.3 The Eitiology And Pathophysiology Of Diseases Causing Pancytopenia

1.3.1 Normal Haematopoiesis

All blood cells are derived from pluripotent stem cells that occupy niches within the bone marrow and whose survival and normal function is dependent on maintenance of the delicate microenvironment also known as the haematopoietic inductive environment in which the stem cells, stromal cells and haematopoietic cells coexist and interact with one another.[18,19] Once formed in the bone marrow the blood cells move to the peripheral circulation. Disruption of the

stem cell and or its microenvironment or peripheral blood cell destruction or sequestration may result in pancytopenia. [1]

1.3.2 Derangements of haematopoiesis occurring in common causes of pancytopenia

Considering the consistency with which aplastic anaemia, megaloblastic anaemia and bone marrow malignancy appear in literature as occupying at least two of the top three or indeed all being amongst the top 3 causes of pancytopenia in various geographic locations[4-7,9-12,20], these conditions perhaps merit the first mention with respect to the current concepts regarding their pathophysiology. Aplastic anaemia is a bone marrow failure syndrome characterised by bone marrow hypoplasia and peripheral pancytopenia[21], theories attempting to explain this disorder suggest primary defects or insults to marrow stem cells or the bone marrow micro environment[22-24], and current evidence suggests that the underlying process is of an autoimmune nature[21-24]. Bone marrow morphology in these cases demonstrates a cellularity of less than 25% with replacement of the haematopoietic elements by adipose tissue.[21] Megaloblastic anaemia refers to a heterogenous group of disorders that have in common megaloblastosis, a morphology for which the hallmark is the megaloblastic erythroid cell. [25] The phenotypic changes seen are the mainly the result of impaired DNA synthesis though impaired RNA and protein synthesis do play a role.[25,26] Vitamin B_{12} and folic acid deficiency are the commonest causes of megaloblastic anaemia, however HIV infection and myelodysplastic syndrome can also cause megaloblastic anaemia via direct effect on DNA synthesis.[26] Carcinogenesis results from the accumulations of complimentary mutations in a stepwise fashion over time and these may be hereditary or sporadic.[16] The incidence of cancer varies with geography, age and race. Furthermore genetic background and interactions between environmental and genetic factors may be important determinants of cancer risk.[16] Bone marrow malignancy like malignancy occurring in other anatomic locations may be primary or metastatic and is predominantly sporadic that is**,** there is no identifiable inherited gene involved, but the cancers develop as a result of environmental factors.[27] Whatever the cause, the underlying mechanism of pancytopenia in malignancy is by bone marrow space infiltration/replacement.[1] Myelodysplastic syndrome refers to a heterogenous group of clonal haematopoietic disorders that are commonly found in the elderly.[28] All the disorders in this group are characterised by one or more peripheral blood cytopenias and the primary defect is disruption of haematopoiesis at the stem cell level.[28,29] In addition to being a discrete entity carrying with it its own morbidity and mortality it is important to recognise MDS as a subset of MDS patients may through the acquisition of additional driver mutations develop acute myeloid leukaemia (AML).[28,29] In MDS a mutant stem cell undergoes clonal proliferation

and disrupts the function of the normal stem cells in bone marrow environment.[28] The cause of the mutation may be genetic or environmental (cytotoxic drugs, radiation, viral infection and genotoxic chemicals).[28] Infectious diseases may cause pancytopenia via bone marrow infiltration/replacement (e.g. tuberculosis and fungal infections) or via bone marrow aplasia by direct disruption of stem cells in the bone marrow (e.g. HIV, viral hepatitis and parvovirus B19).[1]

The human Immunodeficiency virus has been shown to cause bone marrow failure and indeed pancytopenia.[1,3] HIV is cytotoxic to T-helper lymphocytes, which in turn leads to dysregulation of B cells and altered release of cytokines. HIV-infected T cells directly suppress growth of bone marrow progenitors, thus suppressing haemopoiesis. CD4, the cell-surface receptor target of HIV, is carried by T-helper lymphocytes, monocytes and microvascular endothelial cells which are prevalent in marrow. The infection of monocytes in the marrow further alters release of cytokines, which indirectly suppress the capacity for haemopoietic progenitor cells to adequately respond to anaemia and other peripheral cytopenias. This explains why in most patients with advanced HIV pancytopenia is the rule.[30] In Zambia, 13% of adults aged 15-49 years are infected with HIV,[31] in this clinical setting, the morphological picture may be hypercellular, normocellular or hypocellular[15] and the clinical context must be taken into consideration.

It is reasonable to assume that we could one day provide a study with a bigger sample size and more insight into pancytopenia in the HIV infected. It is hoped that this study will provide the initial data, knowledge and experience for such future studies. Bone marrow biopsy examination is of value in diagnosing opportunistic infections that infiltrate the bone marrow and may cause pancytopenia in patients with HIV, more especially in areas where HIV is endemic and in patients that present with fever.[13,32-34] Although higher diagnostic yields are achieved by culture of bone marrow, faster results are obtained by bone marrow histology.[32,34]

1.3.3 Bone marrow biopsy compared to bone marrow aspirate

Currently at the U.T.H most pancytopenic patients only undergo a bone marrow aspirate as part of marrow evaluation. The gold standard in centres across the globe have bone marrow biopsy done simultaneously with the aspirate as the two complement one another as aspirates are superior in terms of cytologic detail (e.g. cytoplasmic and nuclear features) and biopsy superior in providing an architectural detail (e.g. cellularity and bone marrow infiltration/replacement).[2] Some details that we may currently be missing by performing aspirate only are adequacy of sample, ability to detect involvement of bone marrow by granulomatous diseases like

tuberculosis[15] and metastatic non-hematologic malignancies.[2] In summary the two procedures must always be done together and the current management of pancytopenic patients' needs to be amended to always include the bone marrow biopsy.

1.4 Statement Of The Problem

Pancytopenia is a common problem and the type and frequency of its causes vary from region to region[3], and thus conclusions of the main causes in one country cannot be made based on the findings from other countries. At the time that this study was being done, there was, to our knowledge no documented data on the morphologic patterns of bone marrow pathology and their corresponding clinical diagnoses in patients with pancytopenia at the UTH. The negative implications of this include non-evidence-based allocation of resources for investigation and treatment of pancytopenic patients at the UTH (i.e. inadequate amounts of reagents for reticulocyte count and serum $B_{12,}$ treatment for aplastic anaemia and projections on human resource allocation and development to aid in faster diagnosis for pancytopenic patients).

This study was undertaken to help address this problem.

1.5 Study Justification

This is the first study in Zambia done to determine the morphological patterns of the bone marrows of adult patients with clinically diagnosed pancytopenia

Treatment of pancytopenic patients is determined by the diagnosis, therefore this study hopes to assist treating physicians formulate treatment protocols, identify uncommon aetiologies and advocate for the procurement of diagnostic equipment and medicines.

This study will also provide research material for future investigators who may wish to determine the answers that this current study will not i.e. molecular and cytogenetic studies as the paraffin blocks that we produced may be stored for many years and tests done in retrospective reviews.

1.6 Study Question

What are the histomorphologic patterns of bone marrow pathology of pancytopenic adult patients based on histology at the University Teaching Hospital?

1.7 Study Objectives

General objective

To describe the histomorphologic patterns of bone marrow pathology in pancytopenic adult patients at the U.T.H, in Lusaka.

Specific objective

1. To determine if there is any association between bone marrow histomorphology and HIV status in the study population.

1.8 Search Strategy

The material used in the literature review was obtained from The PubMed archives and references from standard anatomic pathology textbooks. Key words used were pancytopenia, bone marrow, trephine, morphology and causes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pancytopenia, a Worldwide Perspective

Variation in aetiology and therefore bone marrow morphology is not only appreciated in different countries but also in different regions within the same country[3] furthermore causes of pancytopenia are influenced by geography, socioeconomic conditions and endemic illnesses[1]. That said a review of data on pancytopenia from around the world revealed a recurring theme with aplastic anaemia, megaloblastic anaemia and bone marrow malignancy being the most common[4-6,8-12,20]. . Diagnoses that were infrequent but reported by more than one source included myelodysplastic syndrome, [4,7,35] Non-megaloblastic erythroid hyperplasia[12,36], metastatic carcinoma[12] and infectious diseases such as Kalar azar,[5,37,38] tuberculosis[4,39] , enteric fever[40,41], malaria[38] and HIV.[4,7]

The incidence of aplastic anaemia is generally low and figures quoted in literature include 2 cases per million population in Europe[42] and 4 cases per million population in Bangkok.[43] There are currently no accurate data on the incidence of aplastic anaemia in The United States of America[21]. Males and females are equally affected and the disease may occur at any age but demonstrates a bimodal distribution with one peak during childhood and another between the ages of 20 and 25.[21]

The incidence of megaloblastic anaemia is highest in countries in which malnutrition is rampant and regular vitamin supplementation is absent.[25,26] Haematopoietic neoplasms can cause pancytopenia in both children and adults and acute leukaemias are the most common of these.[3] Acute lymphoblastic leukaemia is the commonest childhood leukaemia, it is also common in adults[44] and it is generally a neoplasm of childhood whereas acute myeloid leukaemia is a disease of adults and constitutes up to 80% of adult acute leukaemias.[44] Chronic myeloid leukaemia and non-Hodgkin lymphomas can also cause pancytopenia but this occurrence is rare,[3] as is the finding of Hodgkin lymphoma being the cause of pancytopenia.[3] Plasma cell myeloma is stated as being a rare cause of pancytopenia with pancytopenia occurring in 10% of patients with this malignancy.[3] MDS occurs throughout the world and exhibits similar characteristics worldwide, that said incidence rates from the United States of America and

western Europe show similar incidence rates that range between 1.67 and 4.4 per 100000.[28]The average age of patients diagnosed with MDS is 65 and diagnoses in patients under the age of 50 though not impossible is uncommon.[28,29] The incidence of MDS at all ages is higher in males than it is in women.[28] Metastatic malignancy to the bone marrow can also cause pancytopenia through marrow space infiltration/replacement.[1] The commonest malignancies to metastasize to the bone marrow are lymphoma, and malignancies of the prostate, breast, lungs, thyroid, kidney, and stomach.[45,46]

2.2 Pancytopenia In Africa

A literature search on the topic of pancytopenia on the African continent yielded the following, in 1999 Savage et al in Zimbabwe carried out a study on pancytopenia in which 134 hospitalized patients were studied and they concluded that the top 3 causes of pancytopenia were aplastic anaemia, megaloblastic anaemia and AIDS, the prevalence of pancytopenia quoted in that study was 10.3%[7]. In a Kenyan study conducted in 2011 by N.A Ongeri, the main causes of pancytopenia in a sample population comprising adolescents and adults (n=139), the main causes were quoted as hypoplastic anaemia, megaloblastic anaemia and acute lymphoblastic leukaemia and the HIV and pancytopenia prevalence in that study were quoted as 7.9% and 15.8%[8]respectively. In 2012 Nafil et al in Morocco conducted a study on pancytopenic adults in that country, they found that the main causes of pancytopenia in that cohort to be Vitamin B_{12} deficiency (this causes a megaloblastic bone marrow morphology), malignancy and aplastic anaemia.[11] Dagdia et al carried out a study on 75 pancytopenic patients in Egypt in 2016 in that study the main causes of pancytopenia were megaloblastic anaemia, malignancy and aplastic anaemia[10].

2.3 Pancytopenia in Zambia

There were no published works on pancytopenia in Zambia.

CHAPTER THREE

METHODOLOGY

3.1 Study Design and Site

This study was a descriptive retrospective cross-sectional study. It was done at the University Teaching Hospital over an eight-month period at the hospital's Department of Pathology and Microbiology.

3.2 Study Population

The study population comprised adult male and female patients admitted to the U.T.H within the study period with a finding of pancytopenia on FBC. Pancytopenia was determined as per U.T.H protocol as a white cell count less than 4.0 $x10^9$/L, haemoglobin less than 10.0 g/dL and platelet count less than 150 x 10^9/L.

3.3 Sampling Frame

INCLUSION CRITERIA

Patients 15 years and above with;

1. Bone marrow biopsies done, the indication for which was proven pancytopenia determined by a full blood count performed at the U.T.H.
2. Bone marrow biopsies done on patients who have pancytopenia and are not responding to therapy based on diagnosis that did not include bone marrow biopsy

EXCLUSION CRITERIA

1. Bone marrow biopsies not done at U.T.H.
2. Bone marrow biopsies done on patients without pancytopenia.
3. Bone marrow biopsies done on patients on chemotherapy.

3.4 Sample Size

The sample size was established using the formula:

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Where it is assumed that;

Z is the Z score and is equal to 1.96 for a 95% confidence interval

p is prevalence and equal to 0.0118 (calculated using the U.T.H disa lab system records to obtain 200 bone marrow biopsies in year 2017 divided by 17130 adult patients admitted to medical ward in 2017, figure obtained from U.T.H official records)

d (the margin of error) is equal to 0.05.

Thus, using this formula, a sample size of 18 would be statistically significant.

3.5 Sampling Method

Convenience sampling method was used.

3.6 Tissue Processing

All the samples collected were initially placed in 10% neutral buffered formalin for 24 hours to achieve fixation. After this the samples were placed in 10% formic acid solution for another 24 hours to decalcify them. After decalcification the samples then followed the standard U.T.H tissue processing protocol (appendix A). The samples were then sliced to a thickness of 3 micrometres. The following stains were employed (S.O.Ps, appendix B)

1. Haematoxylin and eosin, standard basic histology stain to highlight the basic architecture and cell population and cytomorphology.
2. Reticulin to assess the amount of reticulin deposition and screen for fungi.

There was no immunohistochemistry used in this study.

After staining the slides were examined using an Olympus® BX50 light microscope.

3.7 Data Collection Plan and Tools

The bone marrow biopsies were collected from patients as they were requested by the attending physicians in the haematology unit. The results were released following the current U.T.H standards for pathological reports in which a reviewing consultant pathologist is the final authorizer. These records were reviewed from the U.T.H Disa database and were found using the software's search function by entering text to search 'BONE MARROW TREPHINE PANCYTOPENIA' and the stipulated time period that the samples were collected (application wxdisa; version; 04.16.04.836, database; DisaLab via MSSQL_Disalab).

Accompanying results for patients FBC and differential count, CD4 count and viral load (where applicable and if done) were obtained from the U.T.H Disa database and their reference numbers in the Disa database will be included in the data collection tool (appendix C).

For this study, the values for pancytopenia and fever were as defined as per U.T.H reference values (appendix D).

3.8 Data Management and Storage

Once processed (see appendix D) the biopsies were stored as paraffin embedded blocks and labelled which permits retrospective review in future studies. The slides prepared from these samples were also archived. Once the result was reviewed and authorized, it then became a permanent part of the Disa database and an electronic time stamp was added to that result showing the date, time and authorizer of the said result. Only pathologists registered to the Disa system by the U.T.H can amend reports and this action is also recorded. Altering results that have been entered is difficult and an audit trail is readily available to identify any person altering the results. The results once stored on the Disa system become subject to the same security protocols of all other medical results on the U.T.H Disa system.

3.9 Data Analysis Plan

Data collected was analysed using the Microsoft excel 2016 data analysis tool pack and SPSS version 21. Descriptive statistics were used to define the study population and as continuous variables. Frequency listings were used for qualitative variables. A Chi square test was employed to determine associations between variables in the data. A p-value less than 0.05 was considered as significant.

3.10 Ethical Approval

Study approval was granted by ERES CONVERGE IRB, Reference Number 2017-Jun-023 (appendix A).

CHAPTER FOUR

RESULTS

4.1 Characteristics Of Patients At Presentation

The clinical characteristics of the study participants at the time of presentation are summarised in Table 1 below;

Table 1. Clinical characteristics at presentation.

Table 1. Clinical characteristics at time of presentation, all patients (n=45)

Parameter	Value
Age; I. Mean II. Inter quartile range III. Median IV. Range	I. 35 years II. 17 III. 32 years IV. 15 – 72 years.
Sex; n (%) I. Male II. Female	I. 13 (29.0 %) II. 32 (71.0%)
HIV positive; n (%)	18(40 %)
Number of positive on HAART; n (%)	18 (100 %)
Fever at presentation; n	0
Splenomegaly; n (%)	2 (4.0%)

The mean age of the sample population was 35 years with a median of 32 years and a range of 31 to 40 years. Forty percent (n=18) of the population were HIV positive and all of these were documented as being on HAART. Only 8 (44.0%) of the participants with HIV had a documented CD4 count result done at U.T.H and stored on the disa system (some patients had their CD4 counts done outside the UTH). Review of medical charts showed that none of the enrolled patients presented with fever and 2 (4.44%) had splenomegaly.

Table 2. Age and gender distribution. All patients.

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	12 (27.0%)	10(20.0%)	3 (7.0%)
26-35	14 (31.0%)	10 (22.0%)	4 (9.0%)
36-45	8 (18.0%)	5 (11.0%)	3 (7.0%)
46-55	8 (18.0%)	7 (16.0%)	1 (2.0%)
56-65	2 (4.0%)	0	2 (4.0%)
66-75	1 (2.0%)	0	1 (2.0%)
Totals	**45 (100.0%)**	**32 (69.0%)**	**13 (31.0%)**

A total of 45 patients were considered eligible for this study, of these 13 (29.0%) were male and 32 (71.0%) were female as shown in table 2.

Table 3 shows the bone marrow histomorphology and the corresponding final diagnosis from all 45 patients.

Table 3. Bone marrow histomorphology and final diagnosis

Bone marrow histomorphology (n, %)	Final diagnosis (n, %)
Megaloblastosis (17, 38.0%)	Megaloblastic anaemia (17, 38.0%)
Marrow infiltration (8, 18.0%)	Malignancy (8, 18.0%)
Dysplastic marrow (8, 18.0%)	HIV associated dysplasia (6, 13.0%) and myelodysplastic syndrome (2, 4.0%)
Hypoplastic marrow (6, 13.0%)	Aplastic anaemia (6, 13.0%)
Non megaloblastic erythroid hyperplasia (4, 9.0%)	Splenomegaly (2, 4.0%) and ? sepsis (2, 4.0%)
Fibrosis (2, 4.0%)	Myelofibrosis (2, 4.0%)

The gender distribution of the observed histomorphologic findings are shown in figure 1 below.

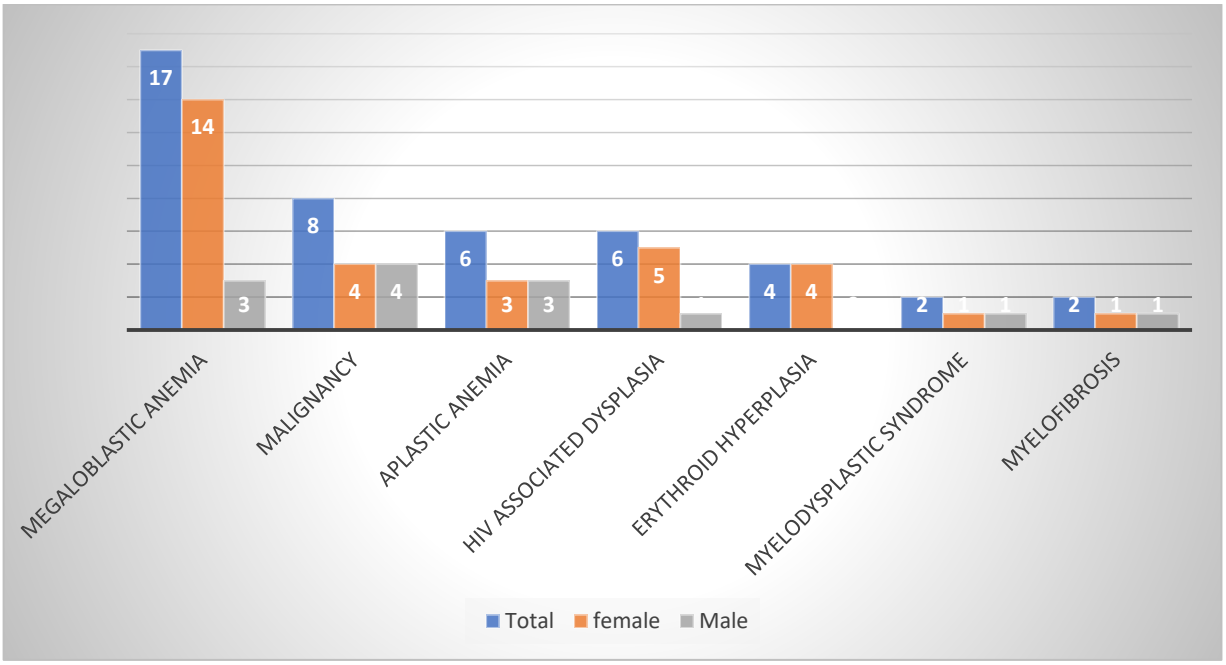


Figure 1. Column chart depicting Gender distribution of morphologic findings

The frequencies of each morphologic finding are shown in figure 2.

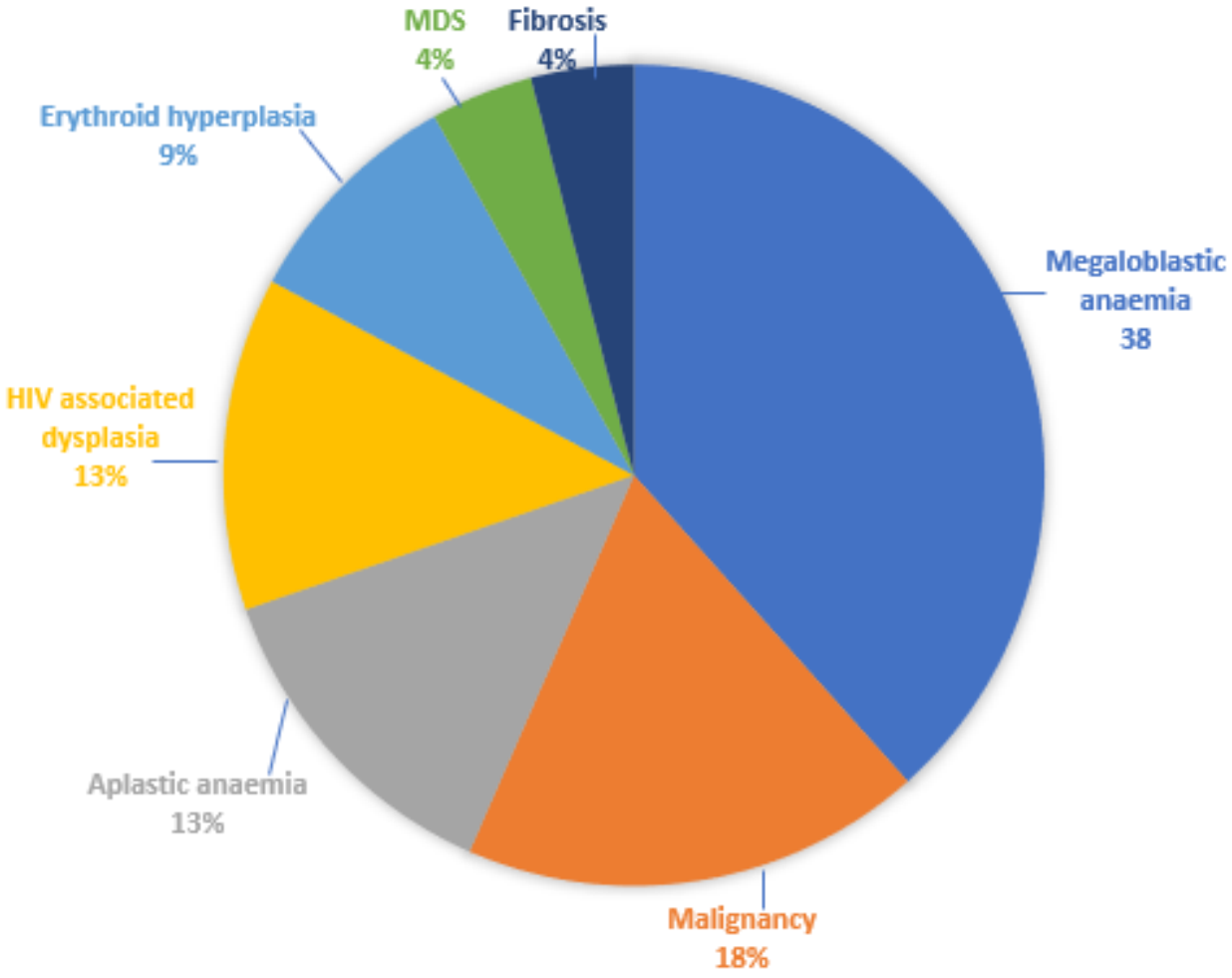


Figure 2. Pie chart showing distribution of morphologic findings

The haematologic characteristics for the enrolled patients are summarised in Table 4.

Table 4. Haematologic characteristics of sample population

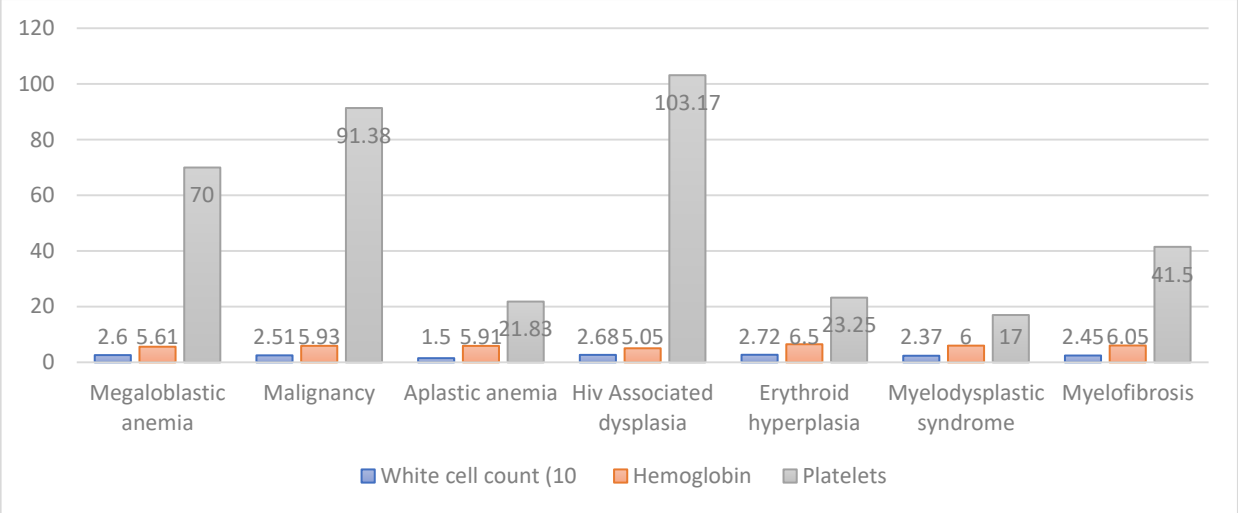
Parameter	**Value (x 10^9/L)**
White cell count (x 10^9)	
Mean	2.43
Median	2.40
Range	2.05 – 2.82
Haemoglobin (g/dL)	
Mean	5.75
Median	5.0
Range	5.00 – 6.50
Platelet count (x 10^9)	
Mean	64.28
Median	46
Range	48.81 -79.75
Neutrophil count (x 10^9)	
Mean	1.02
Median	0.825
Range	0.826 – 1.22
Lymphocyte count (x 10^9)	
Mean	0.88
Median	0.8
Range	0.72 – 1.05
Monocyte count (x 10^9)	
Mean	0.26
Median	0.25
Range	0.20 – 0.32

The haematologic parameters defining pancytopenia by gender are shown in table 5

Table 5. Haematologic characteristics of total sample population by gender.

Parameter	**Male (n=13)**	**Female (n=32)**
White cell count (x10^9/L)		
Mean	2.41	2.45
Median	2.49	2.4
Range	1.93 – 3.04	1.89 – 2.90
Haemoglobin (g/dL)		
Mean	4.76	5.84
Median	5	5.2
Range	3.95 – 6.04	4.28 – 6.12
Platelet count (x10^9/L)		
Mean	58.15	66.77
Median	41	46.5
Range	11.11 – 70.89	27.43 – 65.57
Neutrophil count (x10^9/L)		
Mean	1.04	0.92
Median	0.81	0.81
Range	0.44 – 1.18	0.61 – 1.01
Lymphocyte count (x10^9/L)		
Mean	1.10	0.79
Median	0.88	0.76
Range	0.49 – 1.27	0.58 – 0.93
Monocytes (x10^9/L)		
Mean	0.31	0.24
Median	0.28	0.20
Range	0.18 – 0.38	0.12 – 0.27
Eosinophils (x10^9/L)		
Mean	0.18	0.060
Median	0.03	0.03
Range	0.00- 0.19	0.00 – 0.064
Basophil count (x10^9/L)		
Mean	0.079	0.035
Median	0.01	0.01
Range	0.00 – 0.14	0.00 – 0.061

Figure 3. Column chart showing comparison of definers of pancytopenia, median values.



Comparison of the determinant hematologic parameters across the diagnoses that is white cell count, haemoglobin level and platelet count revealed that aplastic anaemia had the lowest median white cell count of 5.91 $x10^9$/L. The lowest median haemoglobin value was seen in HIV induced dysplasia with 5.05g/dL and the lowest median platelet count seen in myelodysplastic syndrome with 17 x 10^9/L.

4.2 Bone Marrow Morphology

4.2.1 Megaloblastic Anemia

Megaloblastic anaemia which is characterised morphologically by a hypercellular marrow composed of a polymorphous cellular population that demonstrate megaloblastic erythroid hyperplasia, delayed nuclear maturation and blast forms in the erythroid series, was the most common histologic finding and accounted for 37.78% (n=17) of the total study population. Of these 82.35% (n=14) were female and 17.64% (n=3) were male.

The clinical characteristics of the enrolled patients with megaloblastic anaemia are summarized in table 6.

Table 6. Clinical characteristics: Megaloblastic anaemia

Parameter	Value
II. Median III. Range (95% confidence level)	II. 28.0 years III. 24.0 – 38.0 years.
Sex; n (%) I. Male II. Female	 I. 3 (17.64%) II. 14 (82.35%)
HIV positive; n (%)	5(29.42%)
Number of positive on HAART; n (%)	5 (100 %)
Fever at presentation;	0
Splenomegaly;	0

Positive HIV status was documented in 29.0 %(n=5) of the patients with a finding of megaloblastic anaemia.

The age and gender distribution for the megaloblastic anaemia population are summarised in table 7 below.

Table 7. Age and gender distribution. Megaloblastic anaemia

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	7 (41.0%)	5(29.0%)	2 (12.0%)
26-35	7 (41.0%)	6 (35.0%)	1 (6.0%)
36-45	1 (6.0%)	1 (6.0%)	0
46-55	1 (6.0%)	1 (6.0%)	0
56-65	0	0	0
66-75	1 (6.0%)	1 (6.0)	0
Totals	**17 (100.0%)**	**14 (82.0%)**	**3 (18.0%)**

Haematologic characteristics for the megaloblastic anaemia population are shown in table 8 below.

Table 8. Haematologic characteristics of sample population: Megaloblastic anaemia

Parameter	**Value (x 10^9/L)**
White cell count (x 10^9)	
Mean	2.59
Median	2.77
Range	2.07 – 3.10
Haemoglobin (g/dL)	
Mean	5.61
Median	4.9
Range	4.44 – 6.78
Platelet count (x 10^9)	
Mean	70.69
Median	56
Range	45.30 -96.06
Neutrophil count (x 10^9)	
Mean	1.15
Median	0.82
Range	0.78 – 1.52
Lymphocyte count (x 10^9)	
Mean	0.89
Median	0.9
Range	0.72 – 1.05
Monocyte count (x 10^9)	
Mean	0.17
Median	0.12
Range	0.20 – 0.32

Haematologic characteristics by gender for the megaloblastic anaemia population are shown below in table 9.

Table 9: Haematologic characteristics by gender Megaloblastic anaemia.

Parameter	**Male (n=3)**	**Female (n=14)**
White cell count (x10^9/L)		
Mean	2.29	2.65
Median	2.66	2.89
Range	1.22 – 2.99	0.89 – 4.01
Haemoglobin (g/dL)		
Mean	5.2	5.7
Median	5	4.8
Range	3.6 -7	2.5 – 8.9
Platelet count (x10^9/L)		
Mean	66.33	71.62
Median	58	54.35
Range	23 - 118	12 – 149
Neutrophil count (x10^9/L)		
Mean	0.85	0.98
Median	0.79	0.81
Range	0.53 – 1.22	0.31 – 2.19
Lymphocyte count (x10^9/L)		
Mean	0.93	0.88
Median	0.88	0.91
Range	0.56 – 1.37	0.13 – 2.68
Monocytes (x10^9/L)		
Mean	0.20	1.18
Median	0.13	0.12
Range	0.093 – 0.37	0.01 – 14.37
Eosinophils (x10^9/L)		
Mean	0.27	0.044
Median	0.02	0.037
Range	0 – 0.8	0 – 0.1
Basophil count (x10^9/L)		
Mean	0.27	0.059
Median	0.01	0
Range	0 – 0.8	0.0.8

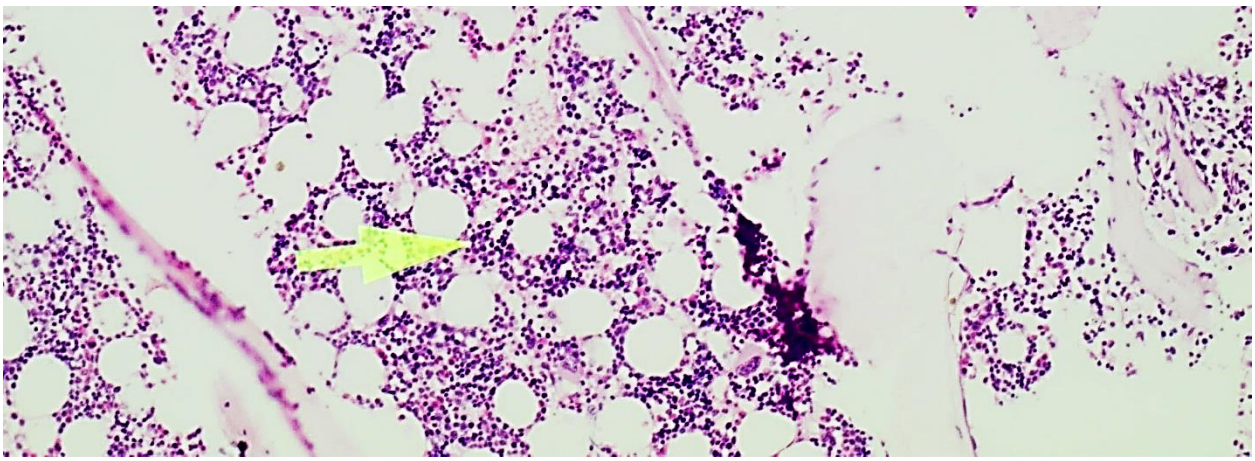
Table 10 demonstrates p-values for the megaloblastic anaemia population.

Table 10: p-values megaloblastic anaemia, chi square test and students t-test.

Parameter	**p-value**
Age	0.14
White cell count	0.55
Haemoglobin	0.86
Platelet count	0.52
Neutrophil count	0.91
Lymphocyte count	0.95
Eosinophil count	0.99
Monocyte count	0.99
Basophil count	0.0.09
HIV status	0.259
Gender	0.195

Image 1 below shows a micrograph of the bone marrow of a patient with megaloblastic anaemia.

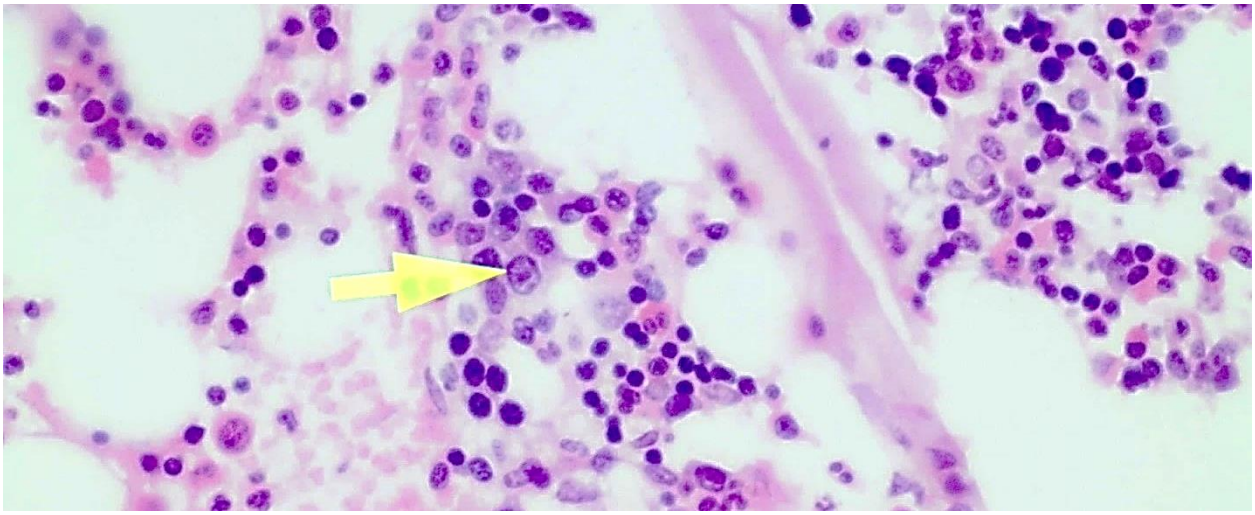
Image 1. Microscopy, megaloblastic anaemia



Low power view showing a cellular bone marrow in a patient with pancytopenia. Different patterns of staining depict different cell types (polymorphous proliferation). The arrow demonstrates erythroid cells which are small and dark staining. The patient was an HIV negative 27-year-old female and the histologic diagnosis was Megaloblastic anaemia (H&E, ×40).

Image 2 below shows another micrograph of the bone marrow of a patient with megaloblastic anaemia.

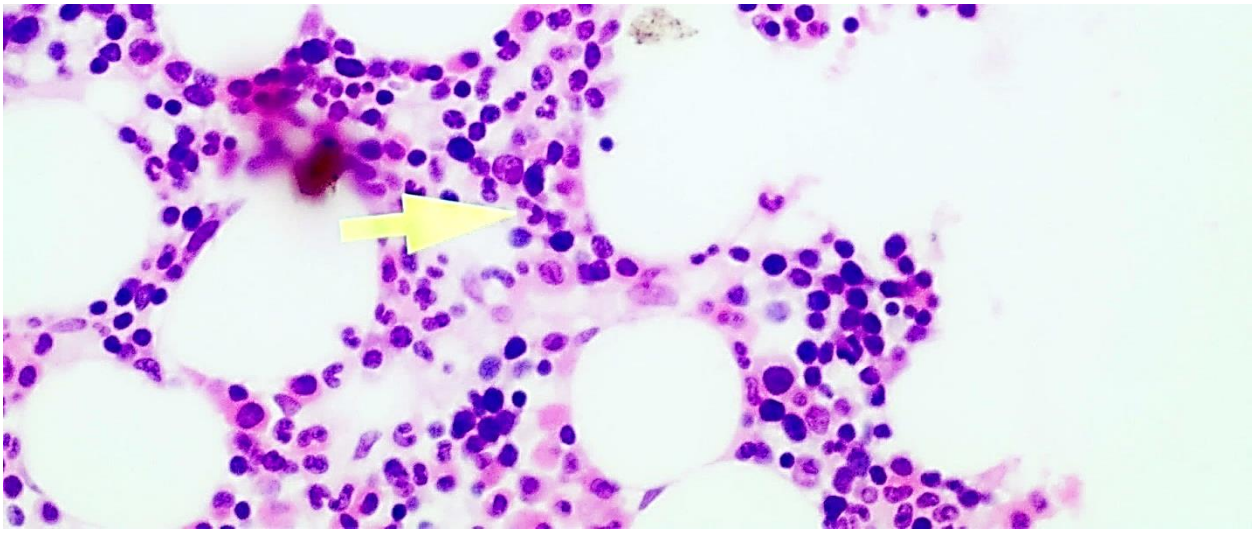
Image 2. Microscopy, megaloblastic anaemia



Same case as image 1 at higher magnification. A megaloblastic erythroid precursor (arrow) characterised by large size, large nucleus with clear nucleoplasm and single prominent round nucleolus, careful inspection reveals several such cells in this field (H&E, ×400).

Image 3 below shows another micrograph of the bone marrow of a patient with megaloblastic anaemia, this one at high magnification.

Image 3. Microscopy, megaloblastic anaemia



Same case as image 1 showing a giant band cell (arrow), another characteristic feature of megaloblastic anaemia (H&E, ×400).

4.4 Malignancy

Morphology consistent with malignancy was found in 18 % (n=8) of the study population with equal gender distribution. The malignancies included acute leukaemia 50% (n= 4), metastatic disease to bone marrow 25% (n=2) and plasma cell dyscrasia 25% (n=2). Twenty five percent (n=2) were positive for HIV. Their characteristics are summarized in Table. 11.

Table 11. Clinical characteristics at presentation: Malignancy

Parameter	Value
Age; I. Mean II. Median III. Range	 I. 41.0 years II. 39.0 years III. 15.0 – 72.0 years.
Sex; n (%) I. Male II. Female	 I. 4 (50.0 %) II. 4 (50.0%)
HIV positive; n (%)	2(25 %)
Number of positive on HAART; n (%)	2 (100 %)
Fever at presentation; n	0
Splenomegaly; n (%)	0 (0.0%)

Eighteen percent of the study population were found with bone marrow malignancy.

The age and gender distribution for the patients with malignancy in the study population are shown below in table 12.

Table 12. Age and gender distribution. Malignancy

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	1 (12.5%)	1(12.5%)	0
26-35	1 (12.5%)	0	1 (12.5%)
36-45	3 (37.5%)	2 (25.0%)	1 (12.5%)
46-55	2 (25.0%)	1 (12.5%)	1 (12.5%)
56-65	0	0	0
66-75	1 (12.5%)	0	1 (12.5%)
Totals	**8 (100.0%)**	**4 (50%)**	**4 (50%)**

Figure four below is a pie chart depicting the proportions of malignancy found within the study population.

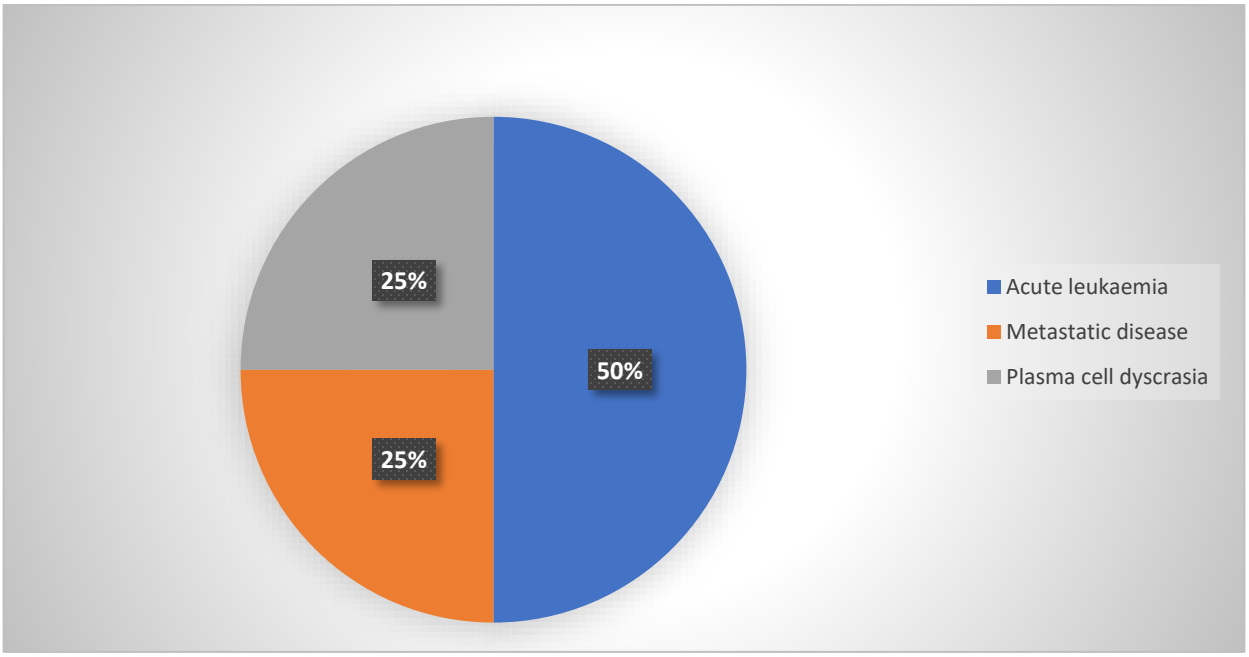


Figure 4. Pie chart of proportions of malignancy found in the study population

The hematologic characteristics for the patients with malignancy in the study population are shown in table 13.

Table 13 Hematologic characteristics: Malignancy

Parameter	**Value (x 10^9/L)**
White cell count (x 10^9)	
Mean	2.51
Median	2.61
Range	1.59 – 3.44
Haemoglobin (g/dL)	
Mean	5.94
Median	5.65
Range	4.33 – 7.54
Platelet count (x 10^9)	
Mean	91.38
Median	94
Range	49.08 -133.67
Neutrophil count (x 10^9)	
Mean	0.87
Median	0.45
Range	0.20 – 1.54
Lymphocyte count (x 10^9)	
Mean	1.19
Median	1.14
Range	0.50 – 1.88
Monocyte count (x 10^9)	
Mean	0.43
Median	0.34
Range	0.21 – 0.65
Eosinophil count (x 10^9)	
Mean	0.16
Median	0.075
Range	0 – 0.32
Basophil count (x 10^9)	
Mean	0.031
Median	0.015
Range	0.00 – 0.067

The haematologic characteristics by gender for the patients with malignancy in the study population are shown below in table 14.

Table 14. Haematologic characteristics by gender Malignancy.

Parameter	**Male (n=4)**	**Female (n=4)**
White cell count (x10^9/L)		
Mean	3.19	1.84
Median	3.3	1.95
Range	2.14 – 4.00	0.66 – 2.82
Haemoglobin (g/dL)		
Mean	5.6	6.3
Median	5.7	6.4
Range	3.2 – 7.7	4.2 – 8.4
Platelet count (x10^9/L)		
Mean	96.25	86.5
Median	112.5	76.5
Range	11 – 149	45 - 148
Neutrophil count (x10^9/L)		
Mean	1.43	0.31
Median	1.68	0.34
Range	0.3 – 2.07	0.08 – 0.5
Lymphocyte count (x10^9/L)		
Mean	1.59	0.79
Median	1.45	0.89
Range	0.55 – 2.9	0.18 – 1.2
Monocytes (x10^9/L)		
Mean	0.46	0.40
Median	0.42	0.33
Range	0.24 – 0.75	0.09 – 0.85
Eosinophils (x10^9/L)		
Mean	0.15	0.17
Median	0.09	0.07
Range	0.02 – 0.4	0 – 0.52
Basophil count (x10^9/L)		
Mean	0.04	0.02
Median	0.02	0.02
Range	0 – 0.13	0 – 0.05

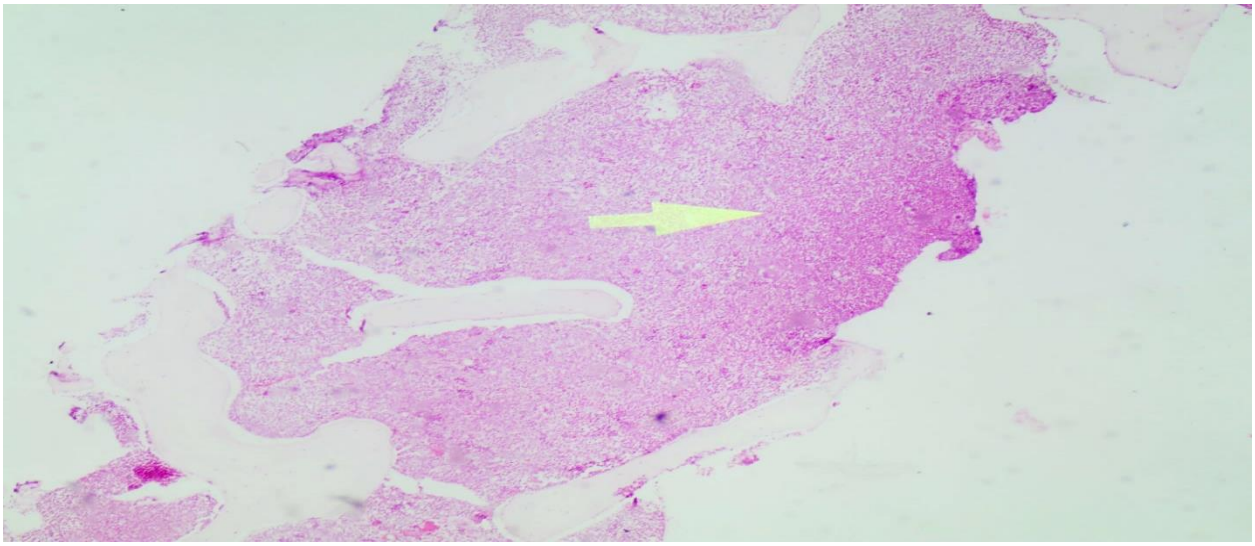
The p values for the patients with malignancy in the study population are shown below in table 15

Table 15. p-values malignancy, chi square test and students t-test.

Parameter	**p-value**
Age	0.20
White cell count	0.85
Haemoglobin	0.60
Platelet count	0.10
Neutrophil count	0.68
Lymphocyte count	0.08
Eosinophil count	0.29
Monocyte count (Mean	**0.00**
Basophil count	0.73
HIV status	0.34

Image 4 below shows a micrograph of the bone marrow of a patient with malignancy.

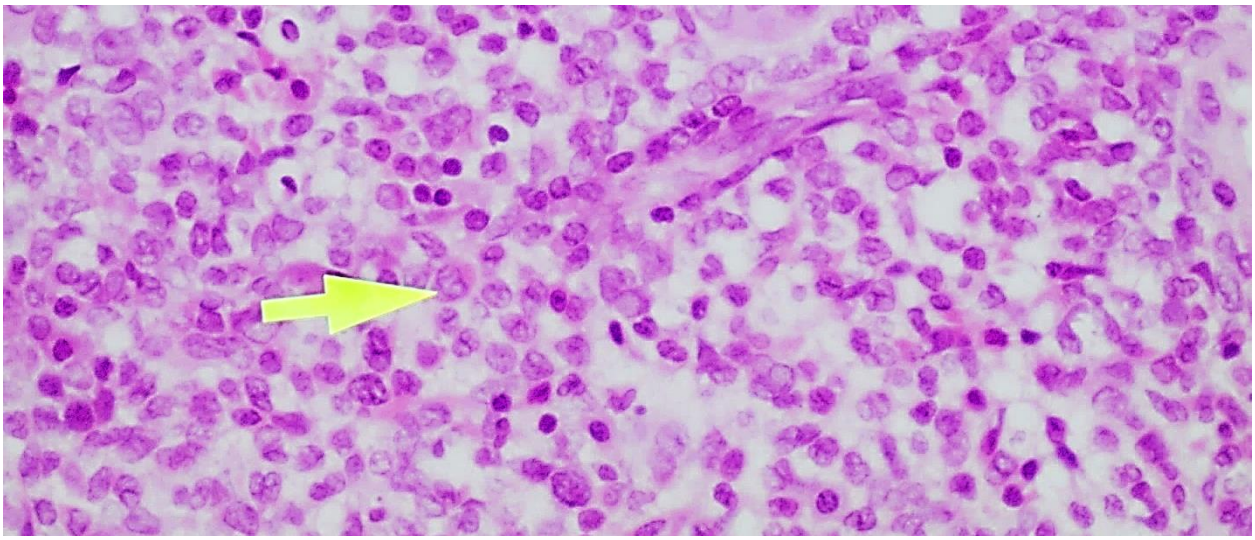
Image 4. Acute leukaemia.



Low power view showing a hypercellular (almost 100% cellularity) marrow from a 36-year-old HIV negative female who presented with pancytopenia, note in this case the monochromic staining pattern indicative of a monomorphic infiltrate (H&E, ×40).

Image 5 below shows below shows another micrograph of the bone marrow the same patient from image 4.

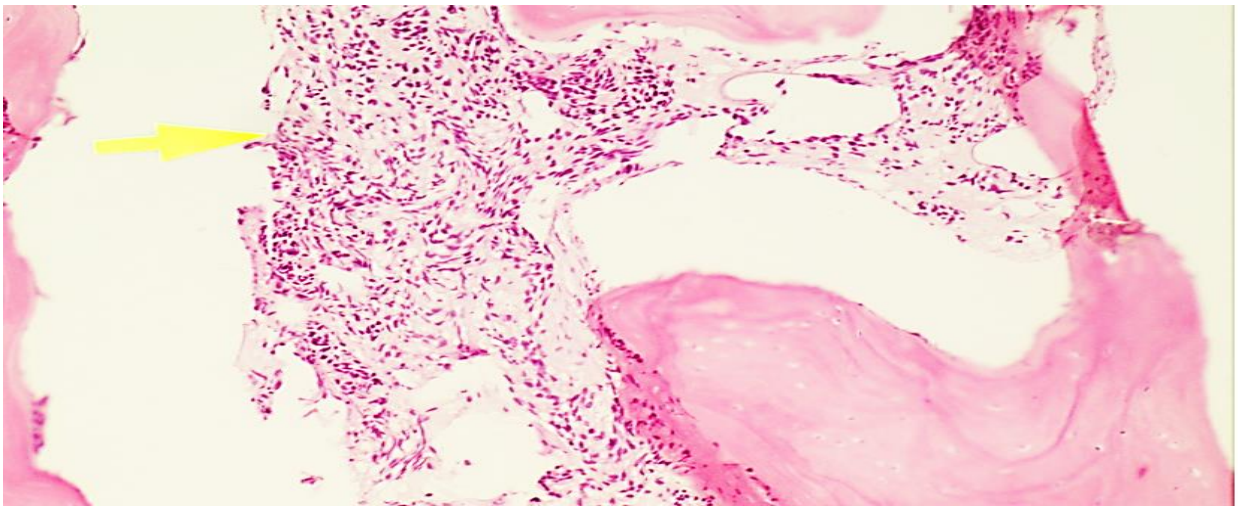
Image 5. Microscopy, acute leukaemia.



Same case in image 4 showing a neoplasm composed of a monomorphic proliferation of round to oval cells with abundant cytoplasm and clefted nuclei with inconspicuous nucleoli. The diagnosis was acute promyelocytic leukaemia hyper-granular type (H&E, ×400).

Image 6 below shows a micrograph of the bone marrow of one of the patients found with malignancy.

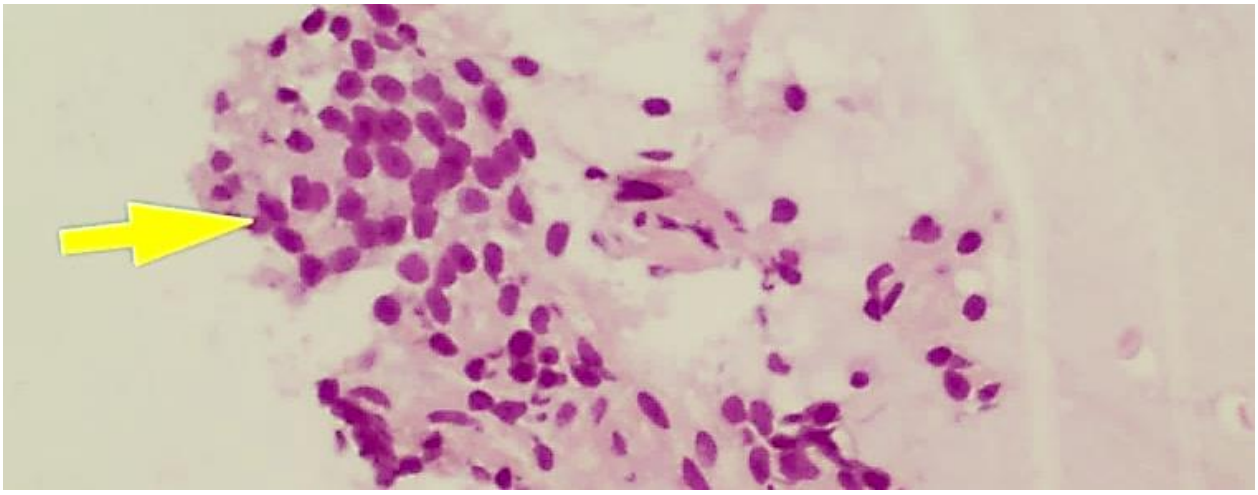
Image 6. Microscopy, metastatic prostatic carcinoma.



Low power view of bone marrow from a 72-year-old male who presented with paraplegia and pancytopenia. Note the fibrosis and monomorphic tumour cells that have replaced hematopoietic cell space (H&E, ×40).

Image 7 below shows a micrograph of a higher magnification of bone marrow of the same patient with prostatic adenocarcinoma.

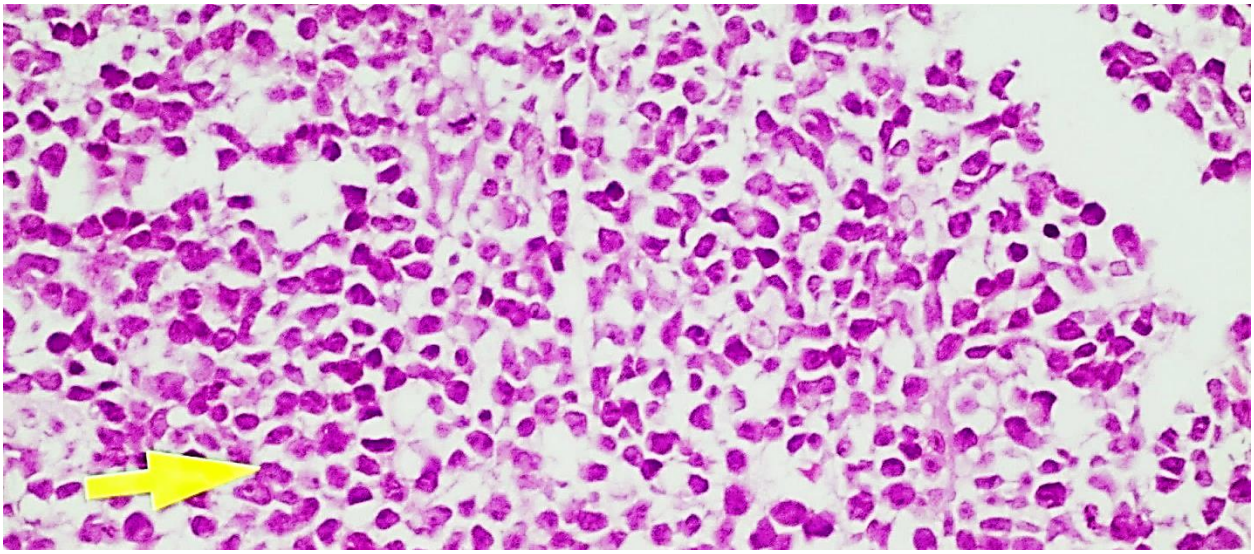
Image 7. Microscopy, prostatic adenocarcinoma.



Higher magnification of image 6, focusing on tumour cells. This case was diagnosed as a metastatic prostatic adenocarcinoma after ancillary studies (H&E, ×400).

Image 8 below shows a micrograph of the bone marrow of one of the patients found with malignancy.

Image 8. Microscopy, acute lymphoid leukaemia



High power magnification of trephine biopsy from a 36-year-old HIV positive male that presented with pancytopenia. The caption demonstrates a monomorphic proliferation of small to medium sized round to oval cells, some had open chromatin and prominent nucleoli as shown by the arrow (H&E, ×400).

4.5 Aplastic Anaemia

The histologic finding of hypocellular marrow consistent with aplastic anaemia was found in 13% (n=6) of the study population. Gender distribution and HIV status were equal. The appearance was characterised by a bone marrow that was hypocellular for age with a reduction in all three haematopoietic cell elements and an increase in adipose tissue. The cellular component was represented by inflammatory infiltrates of histiocytes, plasma cells and lymphocytes were seen in focal areas.

The clinical characteristics for the aplastic anaemia population are shown below in table 16.

Table 16. Clinical characteristics, aplastic anaemia

Parameter	Value
Age; I. Mean II. Median III. Range (95% confidence level)	 I. 32.0 years II. 33.0 years III. 21.0 – 43.0 years.
Sex; n (%) I. Male II. Female	 I. 3 (50.0 %) II. 3 (50.0%)
HIV positive; n (%)	3 (50 %)
Number of positive on HAART; n (%)	3 (100 %)
Fever at presentation; n	0
Splenomegaly; n (%)	0 (0.0%)

Age and gender distribution for the aplastic anaemia population are shown below in table 17.

Table 17 Age and gender distribution. Aplastic anaemia

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	2 (33.3%)	1(16.7%)	1 (16.7%)
26-35	1 (16.7%)	0	1 (16.7%)
36-45	2 (33.3%)	1 (16.7%)	1 (16.7%)
46-55	1 (16.7%)	1 (16.7%)	0
56-65	0	0	0
66-75	0	0	0
Totals	**6 (100.0%)**	**3 (50.0%)**	**3 (50.0%)**

The presenting haematologic characteristics for the patients with aplastic anaemia in the study population are shown below in table 18.

Table 18 Presenting hematologic characteristics: Aplastic anaemia all patients

Parameter	**Value (x 10^9/L)**
White cell count (x 10^9) Mean Median Range	 1.50 1.17 0.72 -2.27
Haemoglobin (g/dL) Mean	 5.92

Median	4.85
Range	2.77 – 9.06
Platelet count (x 10^9)	
Mean	21.83
Median	16.5
Range	2.35 – 41.31
Neutrophil count (x 10^9)	
Mean	0.95
Median	0.86
Range	0.43 – 1.47
Lymphocyte count (x 10^9)	
Mean	0.64
Median	0.65
Range	0.53 – 0.74
Monocyte count (x 10^9)	
Mean	0.19
Median	0.23
Range	0.088 – 0.29
Eosinophil count (x 10^9)	
Mean	0.16
Median	0.075
Range	0 – 0.40
Basophil count (x 10^9)	
Mean	0.013
Median	0.015
Range	0.00 – 0.026

The haematologic characteristics for the patients with aplastic anaemia are shown table 19.

Table 19: Haematologic characteristics by gender Aplastic anaemia.

Parameter	**Male (n=3)**	**Female (n=3)**
White cell count (x10^9/L)		
Mean	1.93	1.07
Median	1.78	1.09
Range	1.13 – 2.88	0.9 – 1.21
Haemoglobin (g/dL)		
Mean	3.6	4.9
Median	3.9	4.6

Range	1.9 – 5.1	4.3 – 5.7
Platelet count (x10^9/L)		
Mean	24.3	19.3
Median	26	7
Range	6 – 41	5 – 46
Neutrophil count (x10^9/L)		
Mean	0.91	0.48
Median	0.81	0.34
Range	0.58 – 1.34	0.34 – 0.76
Lymphocyte count (x10^9/L)		
Mean	0.66	0.68
Median	0.65	0.64
Range	0.65 – 0.67	0.55 – 0.85
Monocytes (x10^9/L)		
Mean	0.23	0.14
Median	0.23	0.18
Range	0.22 – 0.25	0 – 0.26
Eosinophils (x10^9/L)		
Mean	0.23	0.08
Median	0.07	0.08
Range	0 – 0.62	0.02 – 0.14
Basophil count (x10^9/L)		
Mean	0.0	0.02
Median	0.0	0.02
Range	0 – 0.02	0.01 – 0.03

Table 20 shows the p values calculated for the aplastic anaemia population.

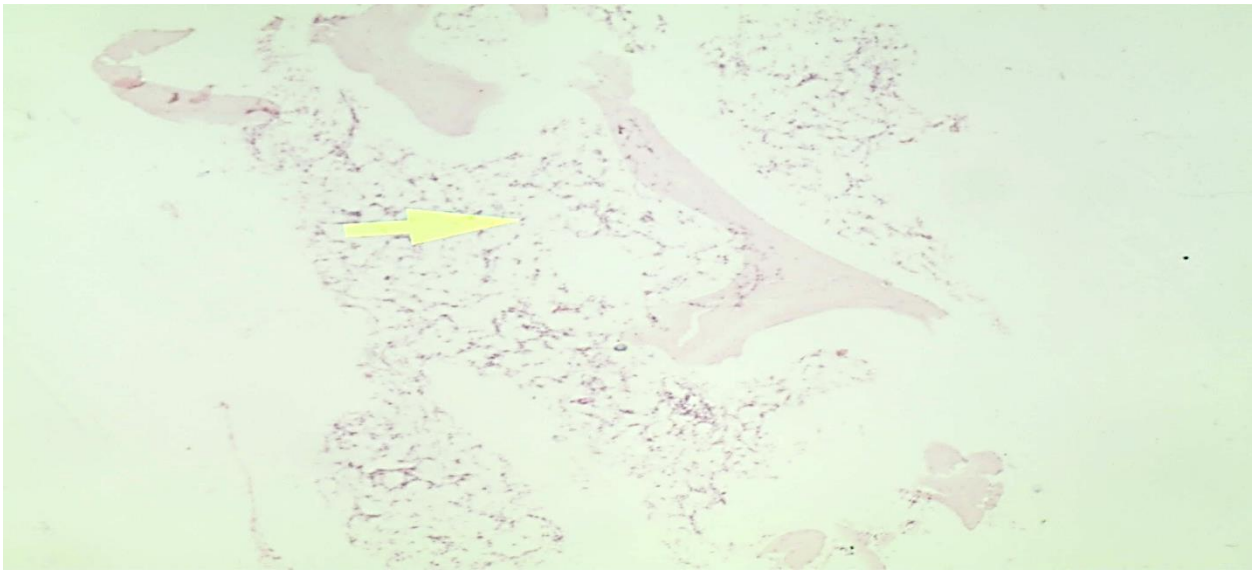
Table 20. p-values aplastic anaemia, chi square test and students t-test.

Parameter	**p-value**
Age	0.52
White cell count	0.05
Haemoglobin	0.16
Platelet count (median 16.5)	**0.03**

Neutrophil count	0.49
Lymphocyte count	0.31
Eosinophil count	0.39
Monocyte count	0.29
Basophil count	0.56
HIV status	0.591

A low power micrograph is shown in image 9

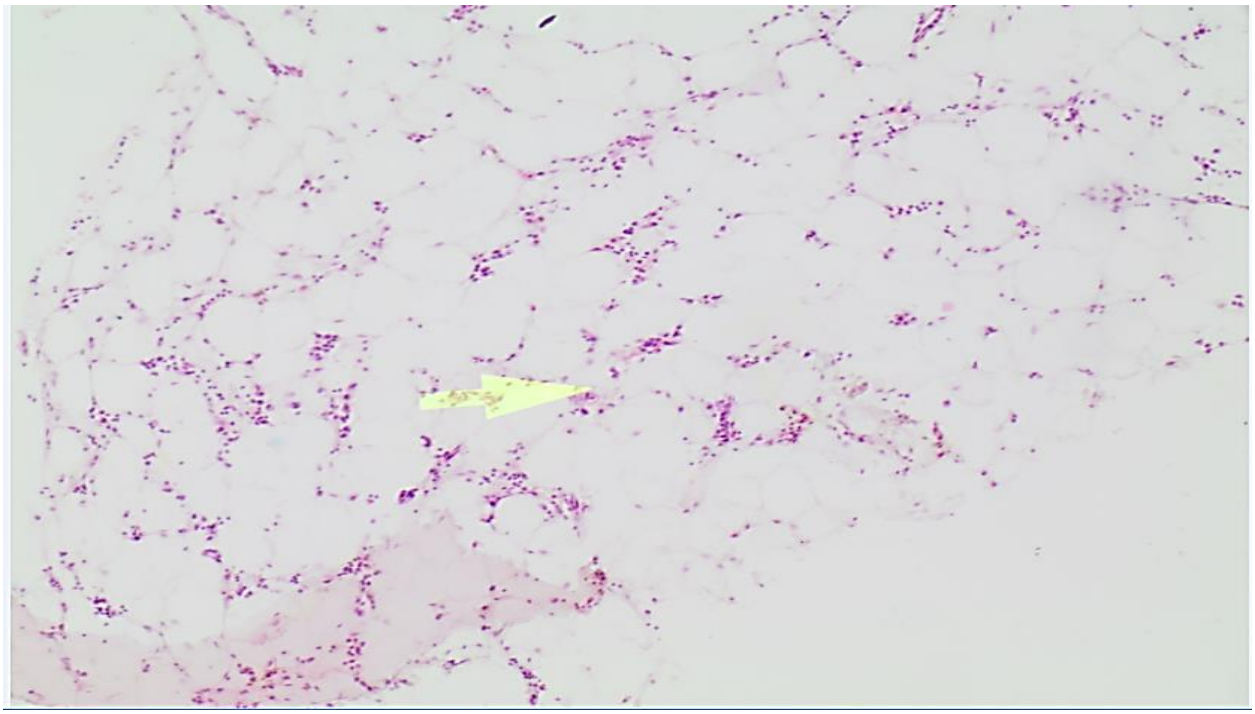
***Image 9. Microscopy, aplastic anaemia*.**



Low power view of bone marrow from an HIV negative 20-year-old male with long standing pancytopenia. Note the profound hypocellularity of the bone marrow and prominence of adipocytes. Expected cellularity at the age of 20 is approximately 80%. (H&E, ×40).

Aplastic anaemia is shown again in image 10.

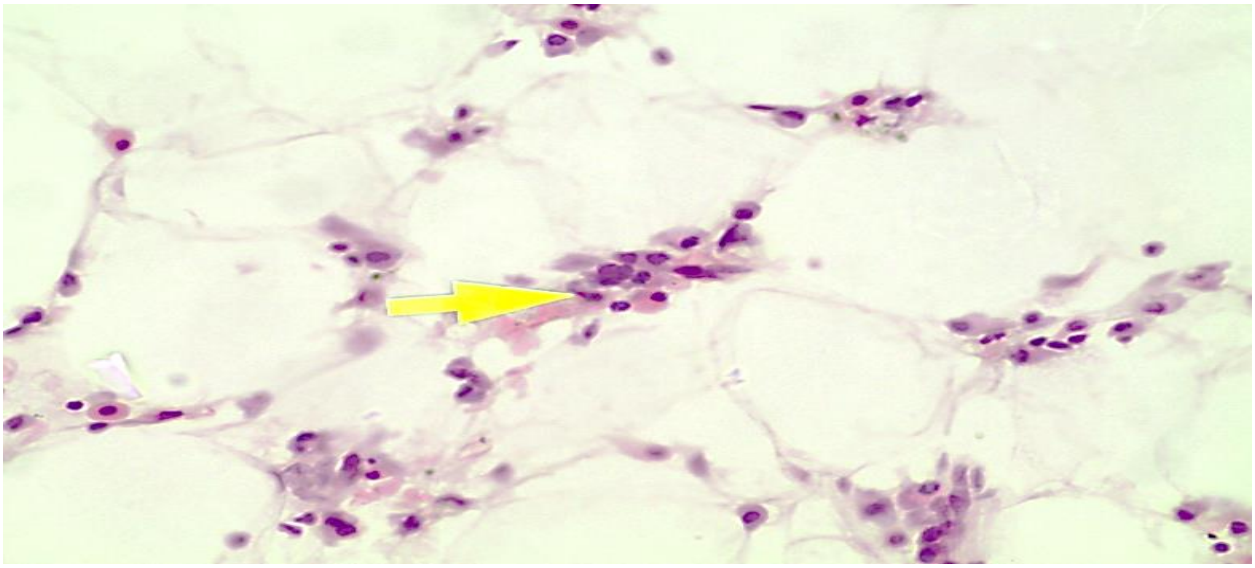
Image 10. Microscopy, aplastic anaemia.



Same case as image 9 at medium magnification. Cellularity in this case was approximately 10%. The cellular elements (arrow) were inflammatory and not haematopoietic (H&E, ×100).

A high power magnification of bone marrow in aplastic anaemia is shown in image 11.

Image 11. Microscopy, aplastic anaemia..



High power view of the case in image 9. The cellular elements in-between the adipocytes are inflammatory and not hematopoietic (H&E, ×400).

4.6 HIV Associated Dysplasia

These were characterised by a hypercellular marrow with a polymorphous proliferation of hematopoietic elements. The key features on histology are Non-megaloblastic erythroid hyperplasia and dysplasia in the megakaryocytic series. In a patient with HIV in whom megaloblastic anaemia and MDS had been excluded this histology was considered diagnostic for HIV associated dysplasia. Unfortunately cytogenetic confirmation was technically beyond us.

As shown in Table 1, 40% (n=18) of the study population were HIV positive. The histologic findings by disease are shown in the chart below.

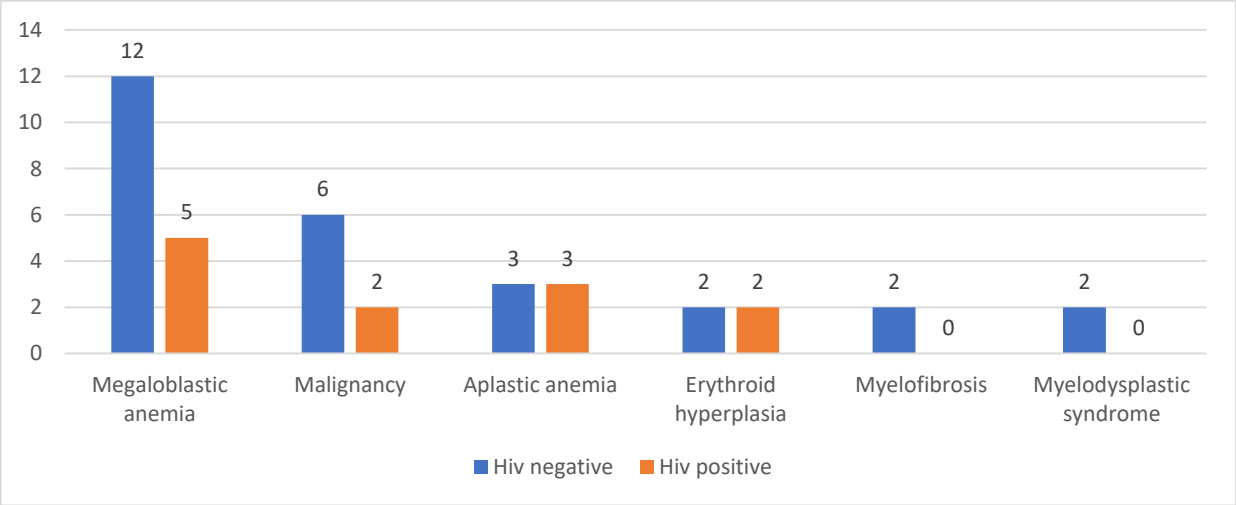


Figure 5. Column chart showing morphologic distribution by HIV status

Only 8 (44.0%) of the participants with HIV had a documented CD4 count result done at U.T.H and stored on the disa system. Those available were as follows.

The 8 available CD4 counts are shown in table 21

Table 21: Available CD4 counts

Morphologic pattern	**CD4 count. (x 10^6/L)**
Megaloblastosis	458
Bone marrow hypoplasia	751
Bone marrow hypoplasia	180
Bone marrow hypoplasia	12
HIV associated dysplasia	1
HIV associated dysplasia	230
HIV associated dysplasia	466
HIV associated dysplasia	74

As 66.0% of the CD4 counts were not available no statistically valid conclusions could be drawn regarding the CD4 counts in our study population.

Table 22 shows p-values calculated in the patients with myelodysplasia secondary to HIV.

Table 22 HIV and morphologic pattern, p-values by chi square test.

Morphologic pattern	**p-value**
Megaloblastic anaemia	0.259
Malignancy	0.34
Aplastic anaemia	0.591
Non-megaloblastic erythroid hyperplasia	0.669
Myelofibrosis	0.44
Myelodysplastic syndrome	0.44

All morphologies had a p value > 0.05, thus no association of histology to HIV status was present.

Opportunistic Infections

There was no histologic evidence of opportunistic infections as cause for pancytopenia in the study population.

Haematologic characteristic at presentation in the HIV associated dysplasia group are shown in table 23.

Table 23 Presenting hematologic characteristics: HIV associated dysplasia

Parameter	**Value (x 10^9/L)**
White cell count (x10^9)	
Mean	2.68
Median	2.035
Range	0.00 – 5.39
Haemoglobin (g/dL)	
Mean	5.05
Median	4.55
Range	1.90 – 8.20
Platelet count (x10^9)	
Mean	103.17
Median	129.5
Range	40.72 – 165.62
Neutrophil count (x10^9)	
Mean	1.71
Median	0.99
Range	0.00 – 4.19
Lymphocyte count (x10^9)	
Mean	0.62
Median	0.66
Range	0.78 – 0.92
Monocyte count (x10^9)	
Mean	0.29
Median	0.27
Range	0.093 – 0.50
Eosinophil count (x10^9)	
Mean	0.005
Median	0
Range	0 – 0.013
Basophil count (x10^9)	
Mean	0.01
Median	0.01
Range	0.00 – 0.021

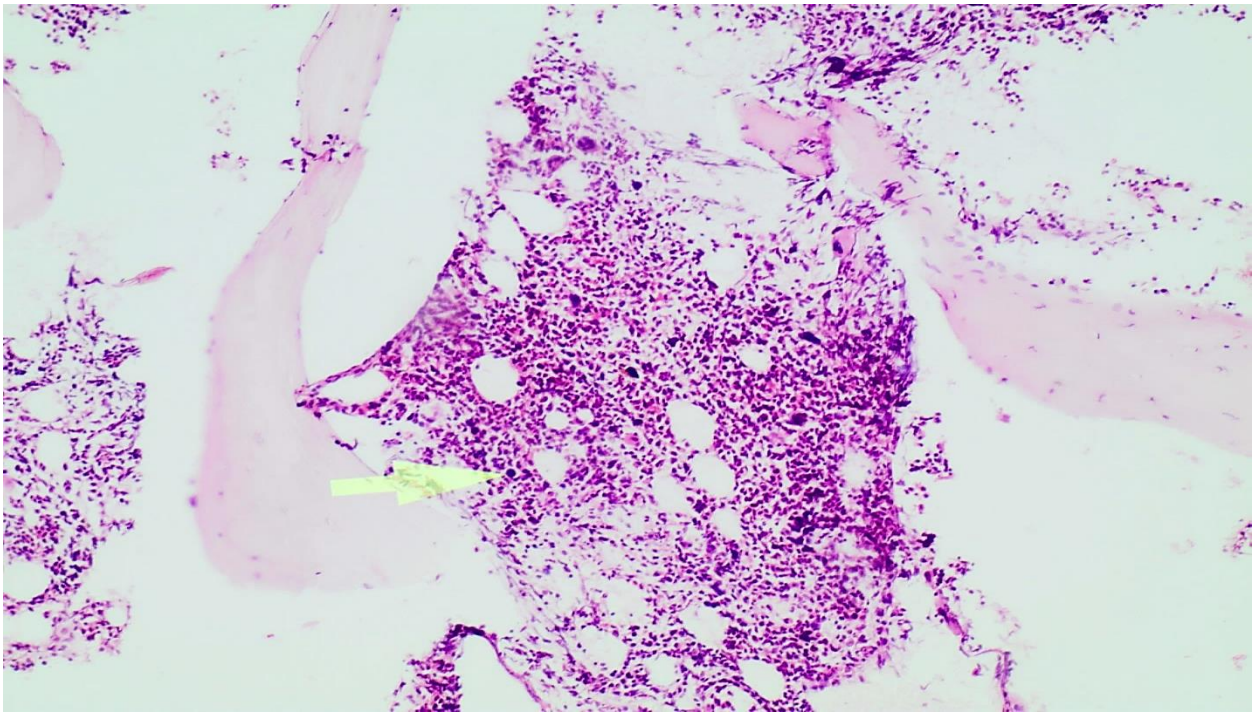
Table 24 depicts haematologic characteristics by gender in the HIV associated dysplasia group.

Table 24: Haematologic characteristics by gender, HIV associated dysplasia.

Parameter	**Male (n=1)**	**Female (n=5)**
White cell count (x10^9/L)		
Mean	1.21	2.97
Median	1.21	2.5
Range		0.32 – 7.6
Haemoglobin (g/dL)		
Mean	6.5	4.8
Median	6.5	3.6
Range		2.2 – 10.1
Platelet count (x10^9/L)		
Mean		
Median	57	112.4
Range	57	135
		4 – 149
Neutrophil count (x10^9/L)		
Mean	0.2	0.87
Median	0.2	0.66
Range		0.1 – 1.62
Lymphocyte count (x10^9/L)		
Mean	0..7	0.61
Median	0.7	0.63
Range		0.22 – 1.00
Monocytes (x10^9/L)		
Mean	0.28	0.30
Median	0.28	0.26
Range		0 – 0.59
Eosinophils (x10^9/L)		
Mean	0.02	0.002
Median	0.02	0.0
Range		0 – 0.01
Basophil count (x10^9/L)		
Mean	0.01	0.01
Median	0.01	0.01
Range		0 – 0.03

Image 12 is a micrograph of bone marrow from a patient with HIV associated dysplasia.

Image 12. Microscopy, HIV associated dysplasia



Low power view of a trephine biopsy obtained from a 40-year-old HIV positive female patient, clinicians wished to exclude sepsis. Note the hyper-cellularity and abnormally spaced and dysplastic megakaryocytes one such is shown by the arrow (H&E, ×40).

The p-values in the HIV associated dysplasia patients are listed in table 25.

Table. 25 p-values HIV associated dysplasia, chi square test and students t-test.

Parameter	**p-value**
Age	1.0
White cell count	0.62
Haemoglobin	0.60
Platelet count	0.05
Neutrophil count	0.38
Lymphocyte count	0.22
Eosinophil count	0.14
Monocyte count	0.72
Basophil count	0.252
Gender	0.53

4.7 Non-Megaloblastic Erythroid Hyperplasia

The clinical characteristics of this patient population are summarised in table 26

Table 26. Clinical characteristics. Non-megaloblastic erythroid hyperplasia

Parameter	Value
Age; I. Mean II. Median III. Range (95% confidence level)	 I. 33.0 years II. 33.0 years III. 31.0 – 40.0 years.
Sex; n (%) I. Male II. Female	 I. 0 (0.0 %) II. 4 (100%)
HIV positive; n (%)	2(50%)
Number of positive on HAART; n (%)	2 (100 %)
Fever at presentation; n	0
Splenomegaly; n (%)	2 (50%)

In table 27 the age and gender distribution are shown

Table 27. Age and gender distribution. Non-megaloblastic erythroid hyperplasia

Age range, years	Frequency over all	Frequency female	Frequency male
15-25	2 (50%)	2 (50%)	0
26-35	0	0	0
36-45	1 (25%)	1 (25%)	0
46-55	1 (25%)	1 (25%)	0
56-65	0	0	0
66-75	0	0	0
Totals	**4 (100.0%)**	**4 (100.0%)**	**0**

Table 28 below shows the presenting hematologic characteristics in patients with non-megaloblastic erythroid hyperplasia

Table 28 Presenting hematologic characteristics: Non-megaloblastic erythroid hyperplasia

Parameter	**Value (x 10^9/L)**
White cell count (x10^9)	
Mean	2.72
Median	2.65
Range	0.66 -4.78
Haemoglobin (g/dL)	
Mean	6.5
Median	6.7
Range	0.72 – 12.28
Platelet count (x10^9)	
Mean	23.25
Median	20.5
Range	0 – 56.79
Neutrophil count (x10^9)	
Mean	1.45
Median	1.36
Range	0.82 – 2.07
Lymphocyte count (x10^9)	
Mean	0.80
Median	0.74
Range	0.00 – 1.61
Monocyte count (x10^9)	
Mean	0.33
Median	0.31
Range	0.019 – 0.64
Eosinophil count (x10^9)	
Mean	0.078
Median	0.075
Range	0 – 0.18
Basophil count (x10^9)	
Mean	0.0175
Median	0.015
Range	0.00 – 0.045

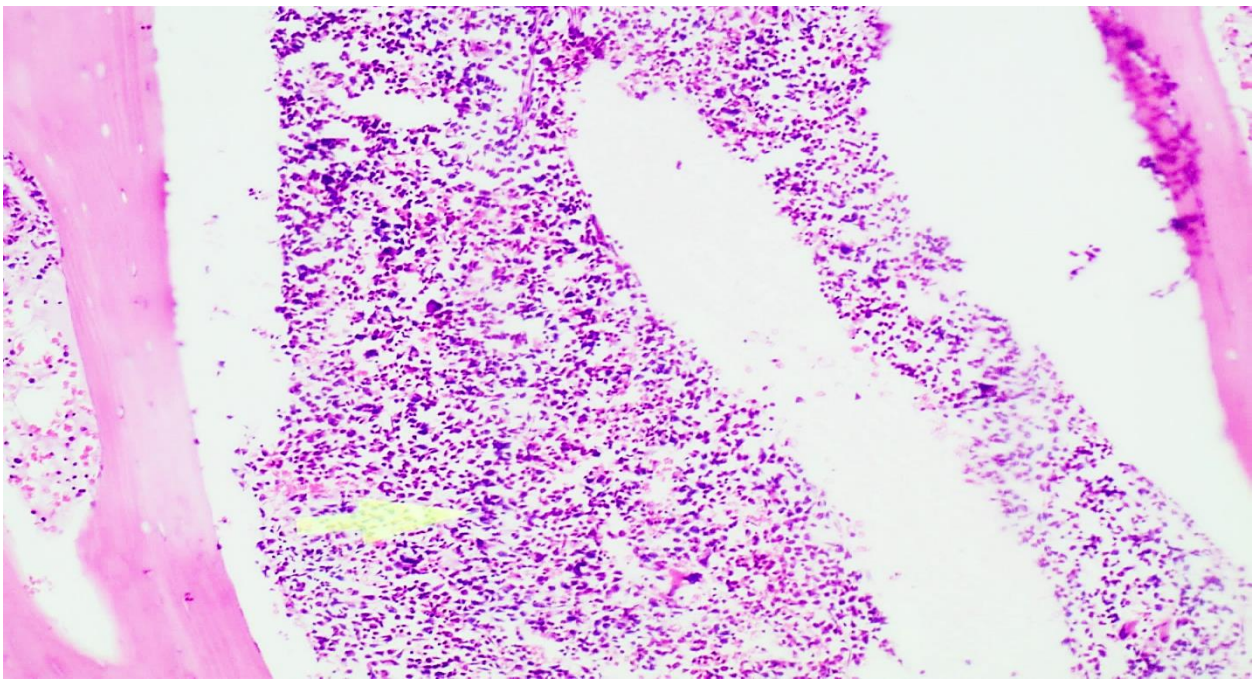
Table 29 below shows the p-values calculated in patients with non-megaloblastic erythroid hyperplasia

Table. 29. p-values non megaloblastic erythroid hyperplasia, chi square test and students t-test.

Parameter	**p-value**
Age	0.53
White cell count	0.80
Haemoglobin	0.44
Platelet count	0.20
Neutrophil count	0.25
Lymphocyte count	0.69
Eosinophil count	0.81
Monocyte count	0.69
Basophil count	0.82
HIV status	0.669
Gender	0.428

Image 13 below shows a micrograph of the bone marrow of a patient with non-megaloblastic erythroid hyperplasia

Image 13. Microscopy, Non-megaloblastic erythroid hyperplasia



Low power view of a trephine biopsy from a 50-year-old HIV negative female who was admitted with splenomegaly and pancytopenia, she was being investigated for hematologic malignancy. Expected cellularity in this age group is approximately 50%, in this case it was more than 90% Note the increased numbers of dark staining erythroid islands, one such is depicted by arrow (H&E, ×40).

4.8 Myelodysplastic Syndrome

Histologically myelodysplastic syndrome demonstrates hyper-cellularity with dysplastic features of the haematopoietic cells. On histology the easiest of these dysplastic features is megakaryocytes that are small, clustered and have nuclear abnormalities that include hyperchromasia and, mono/hypo-lobulation. The clinical characteristic of these patients are shown in table 30.

Table. 30. Clinical characteristics Myelodysplastic syndrome

Parameter	Value
Age; I. Mean II. Median	 I. 59.0 years II. 59.0 years
Sex; n (%) I. Male II. Female	 I. 1 (50 %) II. 1 (50%)
HIV positive; n (%)	0
Number of positive on HAART; n (%)	0
Fever at presentation; n	0
Splenomegaly; n (%)	0

Two patients (4.0%) were found to have histology consistent with myelodysplastic syndrome. One was a male aged 63 and the other a female aged 54. Both had a history of recurrent admissions for pancytopenia requiring blood transfusions and both were HIV negative and had normal serum iron and folic acid levels.

Table 31 below shows the p-values calculated in patients with non-megaloblastic erythroid hyperplasia.

Table 31. Age and gender distribution. Myelodysplastic syndrome

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	0	0	0
26-35	0	0	0
36-45	0	0	0
46-55	0	0	0
56-65	2	1	1
66-75	0	0	0
Totals	**2 (100.0%)**	**1 (50.0%)**	**1 (50.0%)**

Table 32 below shows presenting hematologic characteristics of patients with myelodysplastic syndrome

Table 32. Presenting hematologic characteristics: Myelodysplastic syndrome

Parameter	**Value (x 10^9/L)**
White cell count (x10^9)	
Mean	2.37
Median	2.37
Range	
Haemoglobin (g/dL)	
Mean	6
Median	6.
Range	
Platelet count (x10^9)	
Mean	17
Median	17
Range	
Neutrophil count (x10^9)	
Mean	1.28
Median	1.28
Range	
Lymphocyte count (x10^9)	
Mean	1.11

Median Range	1.11
Monocyte count (x10^9) Mean Median Range	 0.38 0.38
Eosinophil count (x10^9) Mean Median Range	 0.015 0.015
Basophil count (x10^9) Mean Median Range	 0.005 0.005

Myelodysplastic syndrome haematologic characteristics n=2

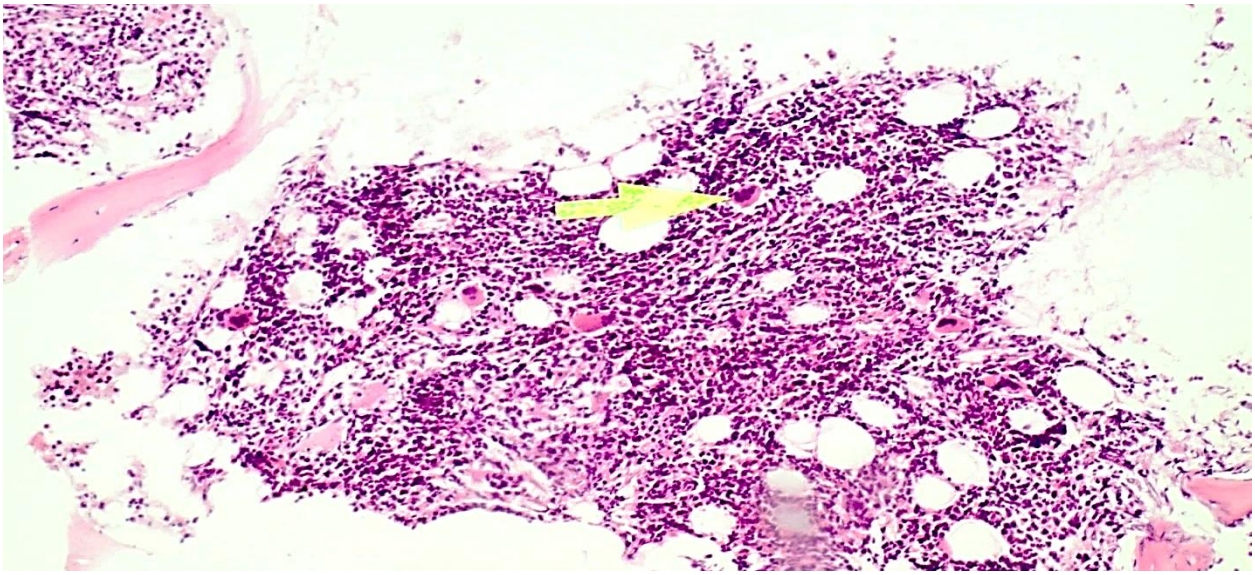
Presenting hematologic characteristics of patients with myelodysplastic syndrome are shown in table 33.

Table 33. p-values myelodysplastic syndrome, chi square test and students t-test.

Parameter	**p-value**
Age (median 58.5)	**0.02**
White cell count	0.94
Haemoglobin	0.78
Platelet count	0.19
Neutrophil count	0.40
Lymphocyte count	0.55
Eosinophil count	0.48
Monocyte count	**0.03**
Basophil count	0.70
HIV status	0.44
Gender	0.5

Image 14 shows a micrograph of bone marrow from a patient with myelodysplastic syndrome.

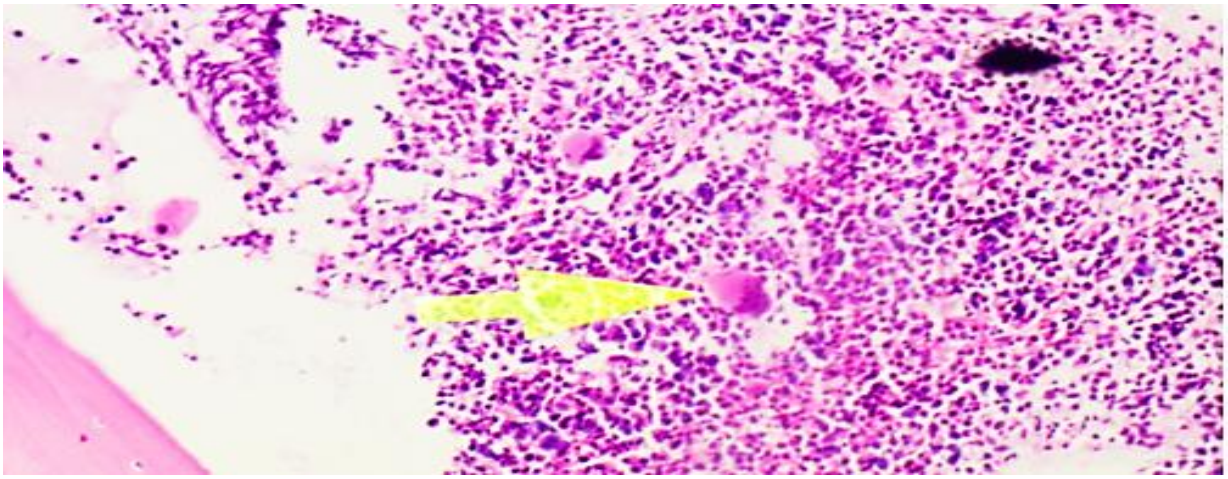
Image 14. Microscopy, myelodysplastic syndrome.



Low power view of bone marrow from a 63-year-old HIV negative male with a history of long-standing pancytopenia. Expected cellularity at this age is approximately 40%, this marrow was hypercellular and had dysplastic features in the megakaryocytes. The patient had normal serum vitamin B_{12} and folic acid levels (H&E, ×40).

Image 15 shows a micrograph of bone marrow from the previous patient with myelodysplastic syndrome at a higher magnification.

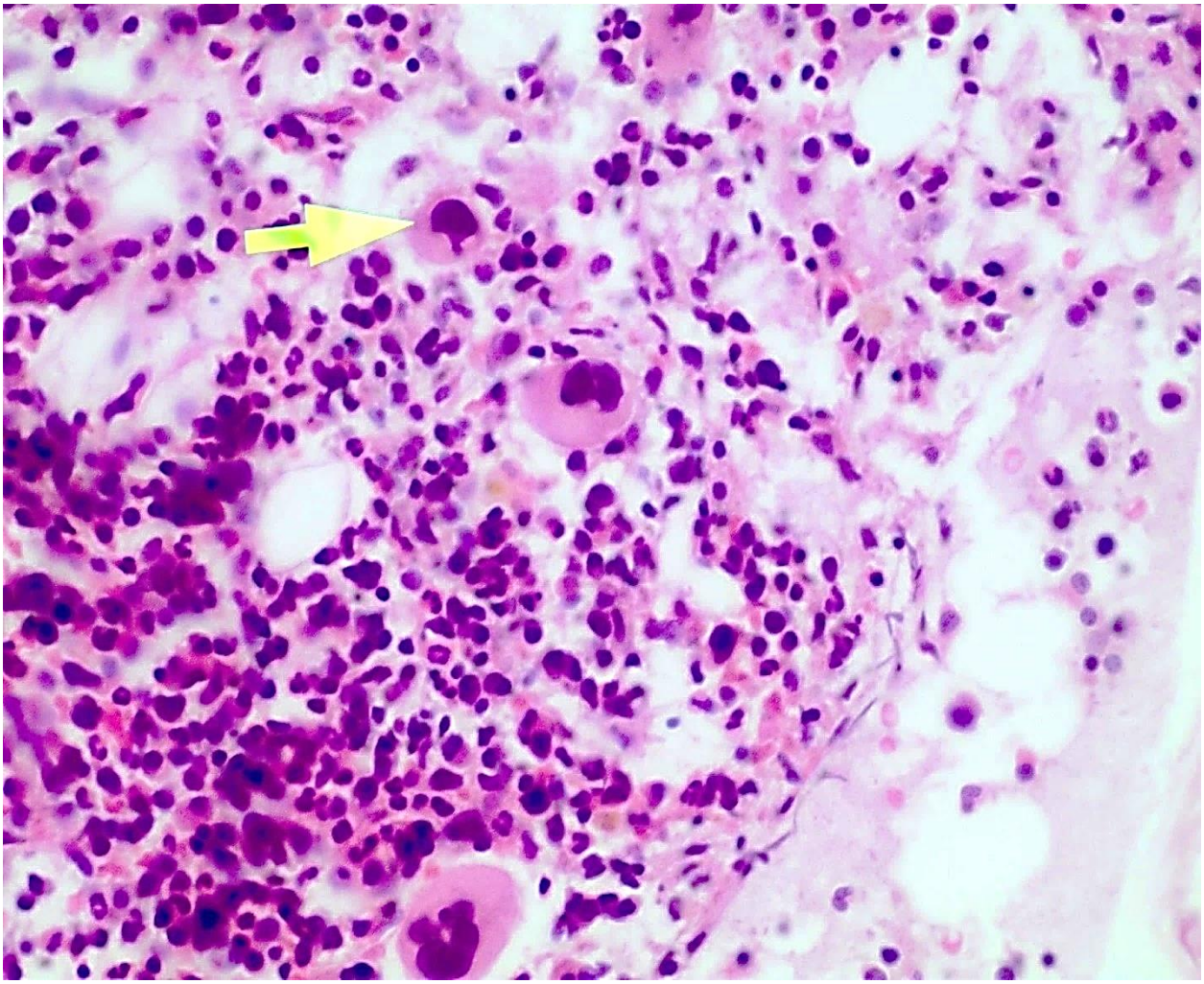
Image 15. Microscopy, myelodysplastic syndrome



Medium power magnification of image 14. Abnormal megakaryocytes and erythroid hyperplasia are noted (H&E, ×100).

Image 16 shows a micrograph of bone marrow from a patient with myelodysplastic syndrome at high magnification.

Image 16. Microscopy, myelodysplastic syndrome



High power view of case from image 14. The megakaryocytes demonstrate dysplasia i.e. clustering, small size and hypo-lobulation (H&E, ×400).

4.9 Myelofibrosis

Histologically myelofibrosis is seen as obliteration of the normal marrow space and its haematopoietic cells and their gradual replacement by fibroconnective tissue. Two patients (4.0% of the study population) were found with myelofibrosis, one was a male aged 27 who had been chronically ill with an undiagnosed illness and the other was a 52-year-old female also with a chronic undiagnosed illness.

Table 34 lists the clinical characteristics of patients with myelofibrosis.

Table 34: Clinical characteristics myelofibrosis

Parameter	Value
Age; I. Mean II. Median	 I. 39.5 years II. 39.5 years
Sex; n (%) I. Male II. Female	 I. 1 (50 %) II. 1 (50%)
HIV positive; n (%)	0
Number of positive on HAART; n (%)	0
Fever at presentation; n	0
Splenomegaly; n (%)	0

Table 35 lists the Age and gender distribution of patients with myelofibrosis.

Table 35. Age and gender distribution. Myelofibrosis

Age range, years	**Frequency over all**	**Frequency female**	**Frequency male**
15-25	0	0	1
26-35	0	0	0
36-45	0	0	0
46-55	0	0	0
56-65	2	1	0
66-75	0	0	0
Totals	**2 (100.0%)**	**1 (50.0%)**	**1 (50.0%)**

Table 36 shows the presenting haematologic characteristics of the patients with myelofibrosis.

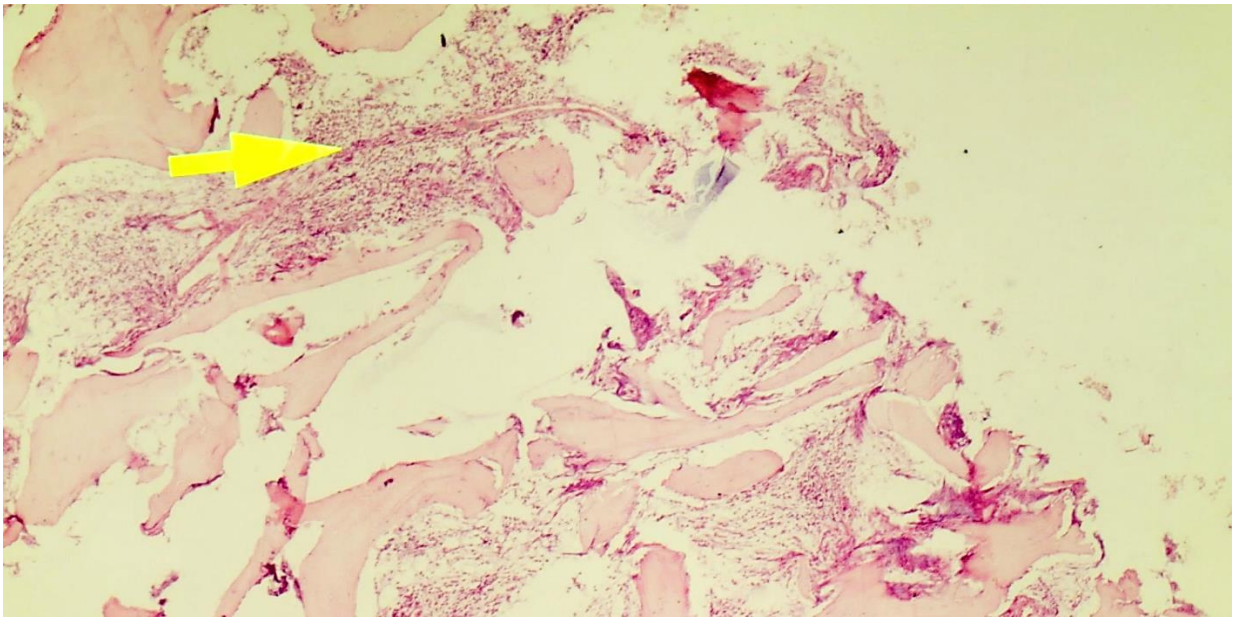
Table. 36 Presenting hematologic characteristics: Myelofibrosis

Parameter	**Value (x 10^9/L)**
White cell count (x10^9)	
Mean	2.45
Median	2.45
Range	
Haemoglobin (g/dL)	
Mean	6.05
Median	6.05
Range	
Platelet count (x10^9)	
Mean	41.5
Median	41.5
Range	
Neutrophil count (x10^9)	
Mean	0.84
Median	0.84
Range	
Lymphocyte count (x10^9)	
Mean	0.96
Median	0.96
Range	
Monocyte count (x10^9)	
Mean	0.32
Median	0.32
Range	
Eosinophil count (x10^9)	
Mean	0.11
Median	0.11
Range	
Basophil count (x10^9)	
Mean	0.025
Median	0.025
Range	

Myelofibrosis haematologic parameters, n=2.

The image below shows bone marrow from one of the patients in this patient population.

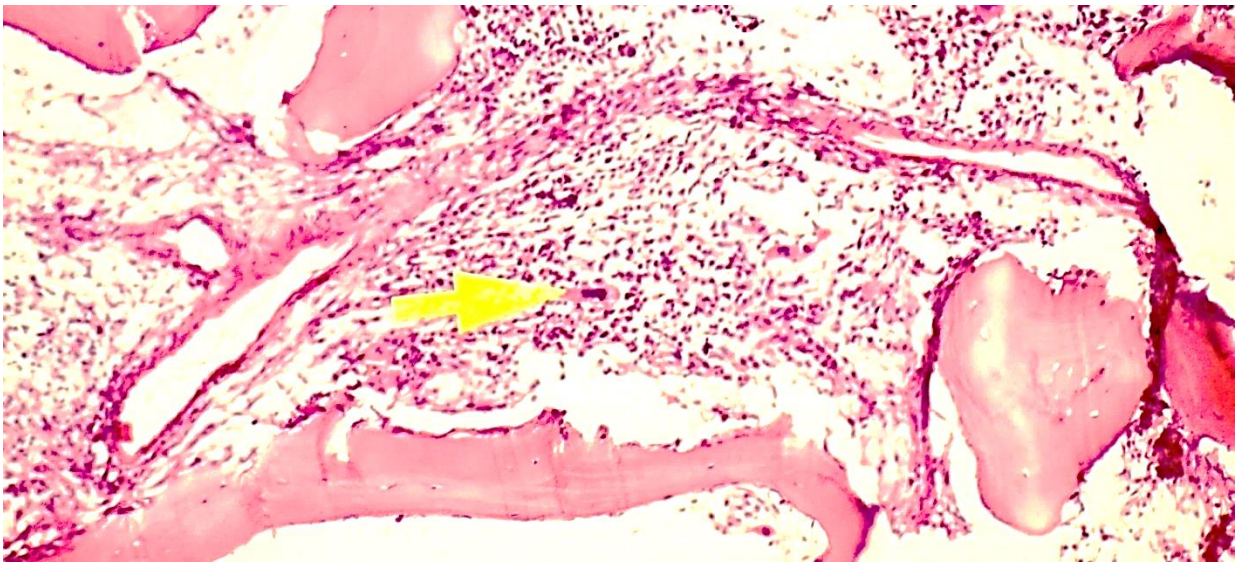
Image 17. Microscopy, myelofibrosis



Bone marrow trephine from a 52-year-old HIV negative female who presented with a long-standing undiagnosed illness and pancytopenia. The marrow space is replaced by fibrous connective tissue (H&E, ×40).

Image 18 below shows bone marrow from one of the patients in this patient population .

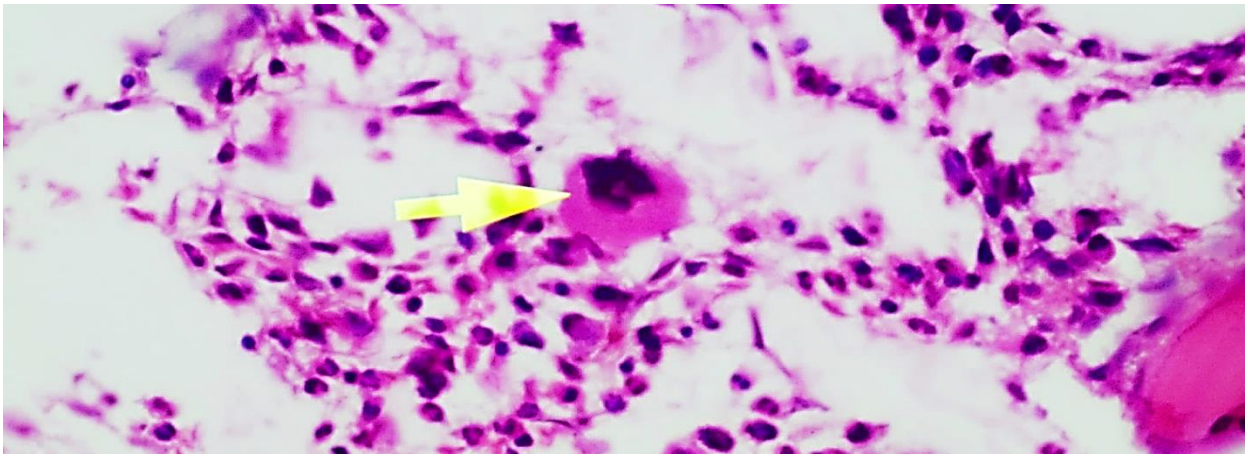
Image 18. Microscopy, myelofibrosis



Intermediate power view of case in image 17. Abnormal megakaryocytes can be seen dispersed in the fibro connective tissue (H&E, ×40).

The image below shows bone marrow from one of the patients in this patient population at high magnification.

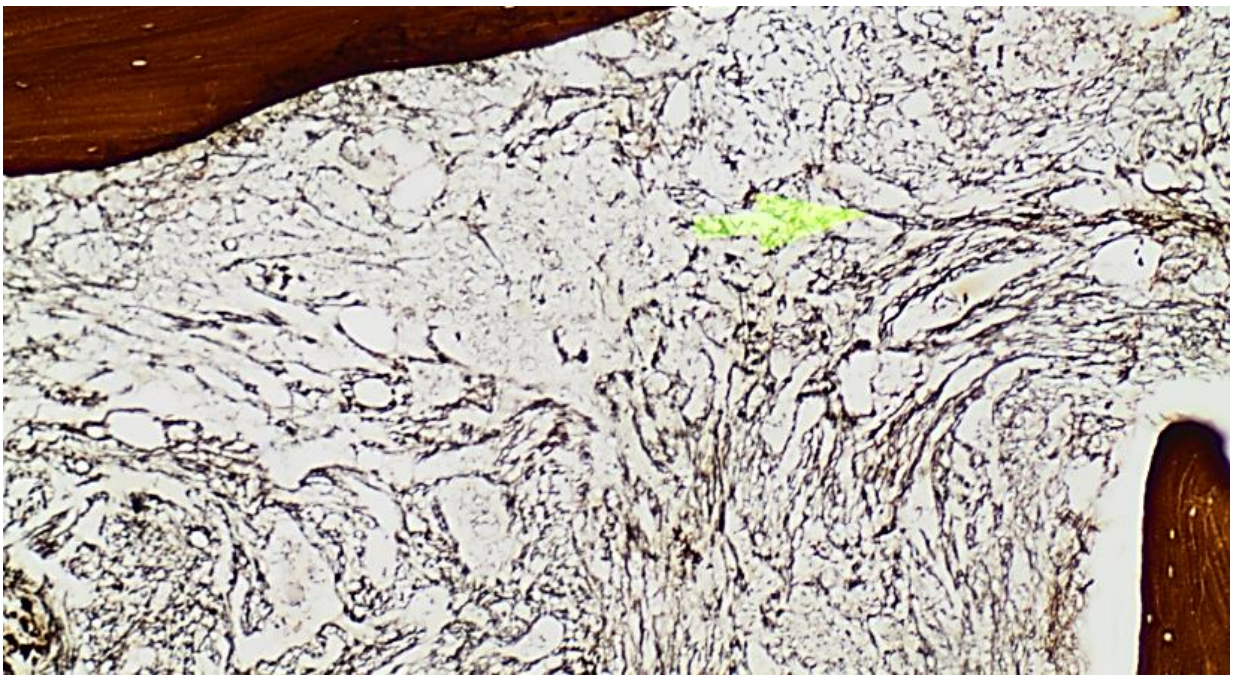
Image 19. Microscopy, myelofibrosis



High power view of case in image 18. An abnormal megakaryocyte is seen here, note the small size and abnormal nuclear lobulation (H&E, ×400).

The image below shows bone marrow from one of the patients with myelofibrosis, this one depicts use of a special stain.

Image 20. Microscopy, myelofibrosis



Low power view of myelofibrosis, the arrow shows areas with grade 4 fibrosis (Reticulin stain, ×20).

The p values calculated for the myelofibrosis patients is shown below in table 34.

Table 34. p-values Myelofibrosis, chi square test and students t-test.

Parameter	**p-value**
Age	0.70
White cell count	0.99
Haemoglobin	0.76
Platelet count	0.53
Neutrophil count	0.78
Lymphocyte count	0.84
Eosinophil count	0.92
Monocyte count	0.69
Basophil count	0.83
HIV status	0.44
Gender	0.5

CHAPTER FIVE

DISCUSSION

5.1 Clinical Characteristics

In this study 45 participants were enrolled, their ages ranged from 15 to 72 years with a median age of 32 years. There were 13 males and 32 females (male to female ratio 1:2.5). Comparisons of characteristics of the participants enrolled in this study to those enrolled in other similar studies are outlined in Table. 35.

Table 35 Comparison of patient characteristics with other pancytopenia studies

Authors (year) **Number of participants (n)**	**Country, Region**	**Age range/ years**	**Male to female ratio**
Gayathri BN, Rao KS n = 104	India, Asia	2 – 80	1.2:1
Khunger JM et al (2002) n = 200	India, Asia	2 – 70	1.2:1
Kumar R et al (2001) n = 166	India, Asia	12 – 73	2.1:1
Dasgupta S et al (2015) n = 248	India, Asia	3 – 84	1.7:1
Tilak V et al (1999) n = 77	India, Asia	5 – 70	1.14:1
Yokus O, Gedik H (2016) n = 137	Turkey, Eastern Europe/ Asia	17 – 95	1.01:1
Kishor K et al (2001) n = 50	India, Asia	3 - 69	1:3.1
Musonda F (2018), this study n = 45 participants	Zambia, South central Africa	15 – 75	1:2.5

This study only enrolled patients enrolled to the adult medical wards, this is because although there is overlap in the pathologies that may cause pancytopenia across a wide age range, the causes in adults are generally acquired[1] whilst included as causes in children are congenital diseases like Fanconi anaemia and dyskeratosis congenita[3] which are very rarely diagnosed in adulthood. Combining the pathologies in adults with those in children would give skewed results of the frequencies of disease as for example aplastic anaemia and acute leukaemia are the most common causes of pancytopenia in paediatric patients whilst myelodysplastic syndrome, chronic leukaemia and megaloblastic anaemia are more common in adults, pooling results would therefore imply that these conditions occur at a higher frequency in the opposing age groups. Consequently more meaningful data is obtained by separating the causes in the paediatric population from those in the adult population as done by Kumar et al and Yokus and Gedik who also excluded the paediatric population. Other similar studies quoted included the paediatiric population, undoubtedly this would give a larger sample size however the issue of skewed data raised above would be an issue.

5.2 Bone Marrow Morphology

A total of six histo-morphologies were observed in our study population. Listed in order of frequency, they were as follows.

1. Megaloblastosis (38.0%)
2. Malignancy (18.0%)
3. Myelodysplasia (18.0%)
4. Bone marrow aplasia (13.0%)
5. Non-megaloblastic erythroid hyperplasia (8.0%) and
6. Bone marrow fibrosis (4.0%).

Table 36 summarises the bone marrow findings in this study with those of similar studies

Table 36 Comparison of bone marrow findings in pancytopenia with some similar studies.

Authors (year) **Number of participants (n)**	**Country, Region**	**Bone marrow morphology**	**Percentage %**
B.N Gayathri, K.S Rao (2011) n = 104	India, Asia	i. Megaloblastic anaemia ii. Malignancy iii. Aplastic anaemia	i. 74. ii. 5.0 iii. 18.0
Dasgupta S et al (2015) n = 248	India, Asia	i. Aplastic anaemia ii. Megaloblastic anaemia iii. Malignancy iv. MDS	i. 33.0 ii. 21.0 iii. 5.0 iv. 2.0
Kumar R et al (2001) n = 166	India, Asia	i. Aplastic anaemia ii. Megaloblastic anaemia iii. Malignancy iv. Erythroid hyperplasia	i. 30.0 ii. 22.0 iii. 18.0 iv. 11.0
Yokus O, Gedik H (2016) n = 137	Turkey, Europe/Asia (Middle east)	i. Megaloblastic anaemia ii. Malignancy iii. MDS iv. Aplastic anaemia	i. 17.0 ii. 13.0 iii. 13 iv. 8.0
Dagdia K et al (2016) n = 75	Egypt, North Africa	i. Megaloblastic anaemia ii. Malignancy iii. Aplastic anaemia iv. MDS	i. 29.0 ii. 23.0 iii. 19.0 iv. 8.0
Nafil H et al (2012) n = 118	Morocco, North Africa	i. Megaloblastic anaemia ii. Malignancy iii. Aplastic anaemia	i. 32.0 ii. 24.0 iii. 15.0
Ongeri NA (2011) n = 139	Kenya, East Africa	i. Aplastic anaemia ii. Malignancy iii. Megaloblastic anaemia	i. 27.0 ii. >17.0 iii. 17.0
Savage DG et all (1999) n = 134	Zimbabwe, Southern Africa	i. Megaloblastic anaemia ii. Aplastic anaemia iii. AIDS	- - -

Musonda F (2018), **This study** n = 45	Zambia, South Central Africa.	i. Megaloblastic anaemia ii. Malignancy iii. Aplastic anaemia/HIV associated dysplasia iv. Non-megaloblastic erythroid hyperplasia v. MDS/Myelofibrosis	i. 38.0 ii. 18.0 iii. 13.0 iv. 9.0 v. 4.0

Bone marrow examination combined with a detailed clinical history, is often required for an accurate diagnosis in the determination of the cause of pancytopenia.[3] The finding in this study of the top three bone marrow histomorphologies being consistent with megaloblastic anaemia (38.0%), malignancy (18.0%) and aplastic anaemia (13.0%) is consistent with literature which states that these three diagnoses are in the top three causes of pancytopenia across a wide geographic distribution.[6,8,20,25,28] Considering that megaloblastic anaemia and aplastic anaemia do have an epidemiologic aspect associated with the prevailing environment,[21,22,25,26] this result is in keeping with literature, as several counties in these geographic locations share within them areas with a socio-economic environment similar to that found in Zambia.[47] The histomorphology of myelodysplasia was demonstrated in 8 (18.0%) of the study population, 6 of these were HIV positive and below the age of 50, the remaining 2 were HIV negative and aged 54 years and above. The patients with marrow dysplasia who were HIV negative and above 54 years and had had megaloblastic anaemia excluded were concluded as myelodysplastic syndrome which is a rare disease of the elderly, and very rare before the age of 50 years.[28] HIV has been shown to directly cause bone marrow failure[1,3] and indeed it has been shown that myelodysplasia may be the first manifestation of HIV disease,[48] furthermore literature states that pancytopenia occurs as a rule in advanced HIV infection,[30] therefore given the ages (less than 50 years) of the HIV positive patients with myelodysplasia those cases were concluded as HIV induced myelodysplasia. This would be in tandem with the findings of a study on pancytopenia done in Zimbabwe which listed HIV as the third most common cause of pancytopenia.[7] A search for other published studies that were similar to this one and done in Africa revealed that these were few and did not list HIV as a cause,[10,11]a possible explanation for this may be the low incidences of HIV documented in the countries in which those studies were conducted (Egypt and

Morocco).[49,50] If however pancytopenia does occur as a rule in advanced HIV infection a mention of HIV as a cause of pancytopenia would be expected no matter how low the frequency. In this study non megaloblastic erythroid hyperplasia was found in 4 patients (9.0%), this low frequency is similar to that quoted in similar studies done in India,[4-6] other studies however did quote this histomorphologic finding in the top 3 bone marrow morphologies in pancytopenic patients in their studies.[51,52] These two opposing sets of results can be explained by the fact that non megaloblastic erythroid hyperplasia is a bone marrow histomorphology associated with peripheral destruction of the cellular components of blood and not a diagnosis, therefore, any pathology causing the peripheral destruction or sequestration of the cellular components of blood can result in the histologic picture of erythroid hyperplasia, these are varied and include any pathology causing haemolytic anaemia, hypoxia and renal disease and also include hereditary diseases like hemoglobinopathies and familial polycythaemia.[53] The epidemiology of non-megaloblastic erythroid hyperplasia is therefore influenced by the epidemiology of the said pathologies The patients with a histomorphology of myelofibrosis (4.0%) had both suffered from chronic illness associated with persistent hematologic abnormalities, we were however unable to determine the primary pathology due to our inability to perform the appropriate ancillary studies. There was no histologic evidence of infiltration of the bone marrow by opportunistic infections (OIs) found in this study, this is consistent with literature stating that the incidence of OIs has greatly reduced since the advent of HAART[54] and all patients with HIV in this study were on HAART, literature also states that the chances of positive morphologic evidence of OIs (i.e. granulomata, lympho-histiocytic infiltrates) being found in the bone marrow are increased if a patient presents with fever[32] and none of our cohort presented with fever.

With regards to tests for association significant results were obtained in the tests involving monocyte count and malignancy, monocyte count and MDS, platelet count and aplastic anaemia and age and MDS (see results section). No literature supporting a relationship between the level of depression of white cell count and differential, haemoglobin or platelet count and any of the histomorphologies described in this study has been described. This result may however mean that in our population a pancytopenic patient with an exceedingly low monocyte count may have bone marrow malignancy or myelodysplastic syndrome. Likewise lower platelet counts in a pancytopenic patient may be a pointer to aplastic anaemia**.** [1] Myelodysplastic syndrome is associated with elderly patients and is rare before the age of 50 years,[28] therefore the finding of

an association between this morphology and age is consistent with literature and MDS must be investigated for in an elderly patient presenting with pancytopenia.

CHAPTER SIX

CONCLUSION

The histomorphology of the bone marrows from pancytopenic adults at the University Teaching Hospital in Lusaka in order of frequency is megaloblastosis, malignancy, myelodysplasia, bone marrow aplasia, non-megaloblastic erythroid hyperplasia and myelofibrosis. No opportunistic infections were identified in the study population.

6.1 Study Limitations

A major limitation was our inability to collect all CD4 counts and viral loads from the HIV positive population in our cohort left us unable to corelate CD4/viral load to bone marrow histomorphology. The fact that the study was centred in Lusaka only is a limitation as it means that although the histomorphology of pancytopenia across the different geographic locations in Zambia can be inferred from this study's results, region specific data is still lacking.

6.2 Recommendations

6.2.1 Training and Research

There is a need to train haematologists, haemato-oncologists and pathologists to assist with early diagnosis of pancytopenic patients and to conduct research and collect data with regards to this patient population in our setting. Furthermore, stakeholders are advised to implement strategies that will make ancillary studies that is serum folic acid, serum vitamin B_{12}, flow cytometry, immunohistochemistry and cytogenetic investigations readily available in order to fully and rapidly diagnose these patients and institute targeted and timely treatment.

6.2.2 Investigative Approach To Pancytopenia

Investment in less invasive technology that helps diagnose the cause of pancytopenia is a must as bone marrow aspiration and biopsy cause discomfort to the patient and are costly and time-consuming procedures that must only be done when no other option is available. As this study has shown megaloblastosis and non-megaloblastic erythroid hyperplasia account for 47.0% (with a 95% confidence interval) of cases of pancytopenia at the U.T.H, these can be diagnosed by performing an FBC, a reticulocyte count and serum Vit B_{12} and folic acid levels, these can all be

measured by drawing a blood sample. Therefore, stakeholders must invest in making reticulocyte count, serum Vit B_{12} and folic acid tests reliably available.

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APPENDICES

PART A: ETHICS APPROVAL

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3rd August, 2017

Ref. No. 2017-Jun-023

The Principal Investigator
Dr. Francis Kaoma Musonda
Department of Pathology and Microbiology
University Teaching Hospital
LUSAKA, ZAMBIA.

Dear Dr. Musonda,

RE: THE MORPHOLOGIC SPECTRUM OF BONE MARROW BIOPSY HISTOLOGY FROM ADULT PANCYTOPENIC PATIENTS AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, ZAMBIA.

Reference is made to your resubmission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2017-Jun-023**
Approval and Expiry Date	Approval Date: 1st August, 2017	Expiry Date: 31st July, 2018
Protocol Version and Date	Version-Nil	31st July, 2018
Information Sheet, Consent Forms and Dates	• N/A	31st July, 2018
Consent form ID and Date	Version-Nil	31st July, 2018
Recruitment Materials	Nil	31st July, 2018
Other Study Documents	Checklist.	31st July, 2018
Number of participants approved for study	150	31st July, 2018

Where Research Ethics and Science Converge

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Prof. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

PART B: PARTICIPANT INFORMATION SHEET

Study title: **Histomorphology Of Bone Marrow from Adult Pancytopenic Patients At The University Teaching Hospital In Lusaka, Zambia.**

Locality: **University Teaching Hospital, Lusaka, Zambia.**

Lead Investigator: **Dr Francis Kaoma Musonda**

EREZ reference number:

Contact phone number:

You are invited to take part in a study on pancytopenia, for the condition for which you/your next of kin are admitted. Whether or not you take part is your choice. If you choose not to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

Before you decide you may want to talk about the study with other people, such as family, friends, or other healthcare providers. Feel free to do this.

This Participant Information Sheet will help you decide if you'd like to take part and we will go through it with you and answer any questions you may have.

If you agree to take part in this study, you will be asked to sign the Consent Form which will be provided to you by the health practitioner taking you through this information sheet. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

Please make sure you have read (or have had this document read to you by someone you trust) and understood all the content of this information sheet.

Why am I doing this project?

The project is part of the requirements for my master's degree course at the University of Zambia. It is hoped that the project will provide useful information for healthcare professionals about pancytopenia, the condition which you/your next of kin is being treated for at U.T.H.

What is expected of you if you agree to take part?

If you agree to take part we will collect at least one bone marrow biopsy from you/your next of kin. This specimen is required to help your doctors understand your condition and hopefully help in your treatment.

How much of your time will participation involve?

The amount of your time that will be required is the time taken to collect the biopsy/biopsies. The preliminary results will be brought to you within 3 days. If other studies are required it may take longer but this will be communicated to you.

How will we ensure your confidentiality?

Your names will not be used in any of the results generated, and your biopsy will be identified by a number only. For this study we assure you will remain anonymous.

Are there any advantages of your taking part?

Yes, these include

1. A thorough investigation of your bone marrow. Your condition requires your bone marrow be assessed.

2. If all factors involved are at optimum, you will receive your results within three days.

3. More than ample analgesia will be provided.

4. We sincerely hope that because of this procedure a diagnosis for your condition will be determined.

Are there any disadvantages of taking part?

There are no disadvantages.

What are the risks involved?

The procedure carries a risk of haemorrhage, fracture and infection.

Researcher:

Dr Francis Kaoma Musonda

Supervisor:

Dr Clemence Marimo

PART C: CONSENT FORM

I (name of patient/ next of kin)_____ hereby grant permission for at least one bone marrow biopsy to be done on me/my next of kin. It has been explained to me that the sample will be used for diagnostic, scientific and educational purposes as the University Teaching Hospital or/and University of Zambia deem fit.

This authority is granted subject to the following restriction (if none, write NONE);

_____

_____

I am the patient/ next of kin to the patient and entitled by law to control the deposition of the specimen.

Signature (or Thumb print)		
Relationship (when applicable) :	Date:	Time:
Postal address:		
Mobile number:		

Witness (Name):	Sign (or Thumb print):
Date:	Time:
Postal address:	
Mobile number:	

For official use

Consent obtained by (full name):
Designation:

Date:	Signature:

PART D: DATA COLLECTION TOOL

AGE		
SEX		
STUDY NUMBER		
HIV STATUS		
FEVER at admission	**Y N**	**Value**
TOWN/CITY		
FBC DISA NUMBER(S)		**Date(s)**
CD4/VIRAL LOAD **where applicable**		
Splenomegaly		
Drug history		

Summary of clinical findings and clinical diagnosis ……

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REFERENCE RANGES (AS PER STANDARD U.T.H PRACTICE)

Fever

Defined as armpit temperature of ≥ 38^{o}C.

PART E HAEMATOLOGIC PARAMETERS

Haematologic parameters. Males

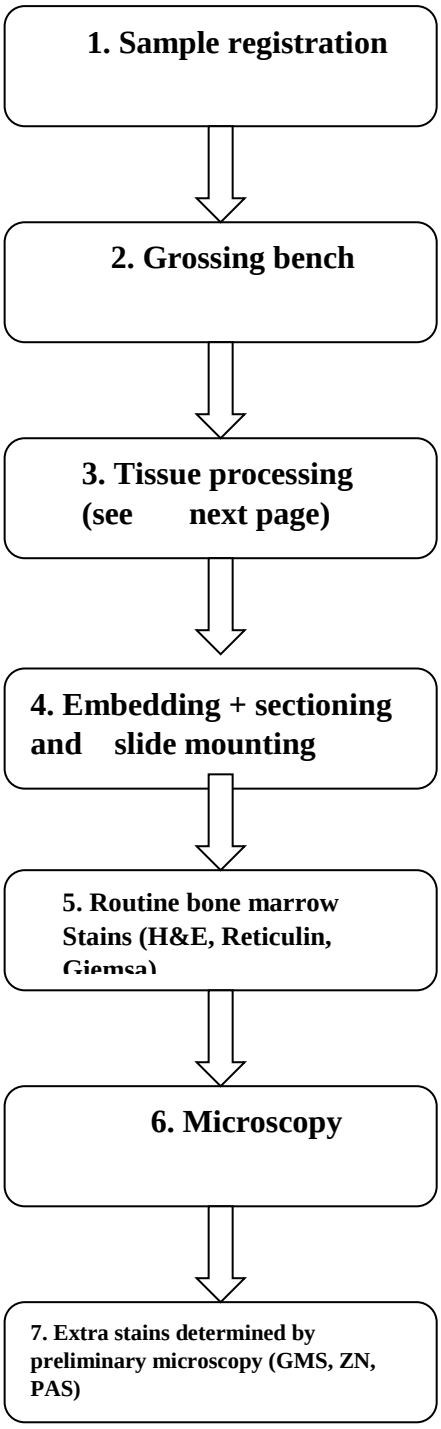
Parameter	Value	Units
White cell count	4 - 11	x 10^9/L
Haemoglobin	14.3 – 18.3	g/dL
Platelet count	150 – 400	x 10^9/L
Lymphocyte count	1.0 - 4.0	x 10^9/L
Monocytes count	0.18 - 0.8	x 10^9/L
Eosinophil count	0.00-0.45	x 10^9/L
Neutrophil count	2.0-7.5	x 10^9/L
Basophil count	0.00-0.2	x 10^9/L

Haematologic parameters. Females

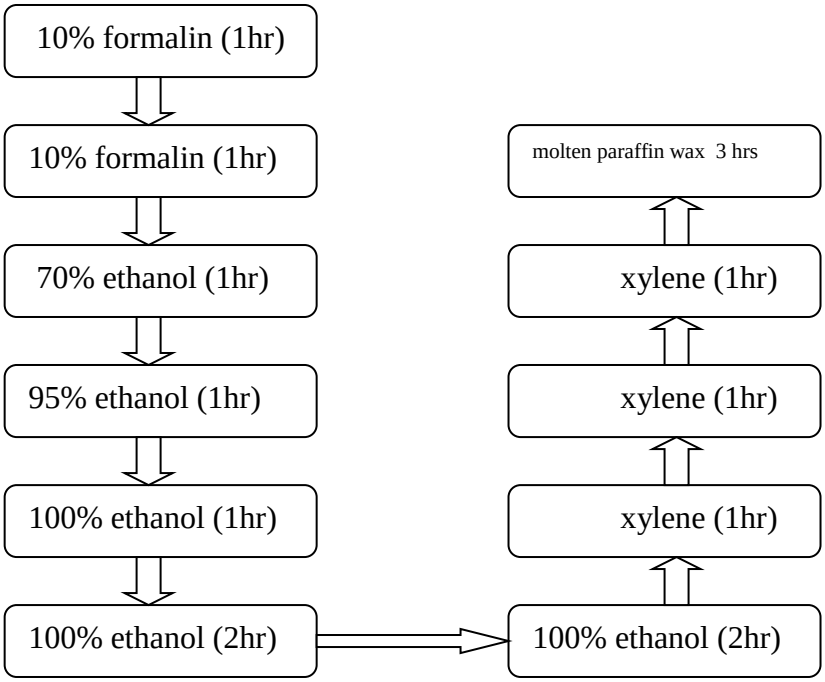
Parameter	Value	Units
White cell count	4 – 11	x 10^9/L
Haemoglobin	12.0 -15.0	g/dL
Platelet count	150 – 400	x 10^9/L
Lymphocyte count	1.0 - 4.0	x 10^9/L
Monocytes count	0.18 - 0.8	x 10^9/L
Eosinophil count	0.00-0.45	x 10^9/L
Neutrophil count	2.0-7.5	x 10^9/L
Basophil count	0.00-0.2	x 10^9/L

STANDARD OPERATING PROCEDURES (SOPs)

TISSUE PROCESSING FLOW CHART



STEPS IN AUTOMATED TISSUE PROCESSOR



THE HAMMERSMITH PROTOCOL

Specimens of bone marrow trephine biopsy (BMT) are transported and fixed in acetic acid-zinc-formalin fixative, decalcified in 10% formic acid-5% formaldehyde and processed with other specimens to paraffin-wax embedding. Sections, are cut by experienced histotechnologists and used for haematoxylin and eosin, Giemsa, reticulin silver and other histological stains. Further, all immunohistochemical procedures used in the laboratory, including double immunostaining, can be used on these sections with no or minimal modifications. About 10,000 BMT specimens have been analysed using this procedure since 1997 and diseases involving the bone marrow have been classified successfully. More recently, standardised polymerase chain reaction-based analysis and mRNA in situ hybridisation studies have been conducted. Excellent morphology with good antigen, DNA and RNA preservation is offered by the Hammersmith Protocol.

References

KN Naresh, et al, 2006. Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol

The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The *in situ* oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate.

Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the over staining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed "differentiation". Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "bluing-up". The haematin demonstrates cell nuclei.

Full cellular detail is obtained by counterstaining with the eosin mixture. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenated fluorosceins). The mechanism of their staining is not fully understood, but is believed to be of an electrostatic nature. Visualisations most acceptable to the histologist are obtained by applying the dyes in acidic conditions, whereby more intense specific colourations are obtained, the more acidic tissue components taking up the dye to a greater intensity, hence the addition of acetic acid.

Technical Points

1. (step 2) - The length of time necessary to over-stain the tissues will depend upon fixation and the type of alum haematoxylin employed. Lillie Mayer's alum haematoxylin-formalin fixed tissues should take 5 minutes.

Tissue Type	Haematoxylin	Acid alcohol 0.3%	Eosin	Comment
Routine tissues	4 minutes	See technical point 2	2 minutes	
Renal biopsies	10 minutes	1-2 seconds	2-4 minutes	Check staining
Decals	10 minutes	1-2 seconds	30 seconds	Check staining after blueing. Hx step may need to be repeated if prolonged decal.

2. (Step 4) - Differentiation with acid alcohol requires some practical experience to ascertain the correct end-point, since the acid solution alters the colour of the tissue to red. The correct end-point is when, after blueing up, the background is almost colourless. For renal biopsy sections, two quick dips in 0.3% acid alcohol are all that is required

3. (Step 6) - If Scott's tap water substitute is employed; blueing up is achieved in a much shorter time.

4. (Step 8) - Eosin is highly soluble in water. Over-staining is removed by washing in running water.

5. Fixation - Not critical. Acidic fixatives will give a more eosinophilic result. Picric acid containing fixatives give an overall enhanced result. Acidic decalcifying fluids give poor nuclear staining.

6. Renal biopsies - 10% buffered formalin. Sections cut at 2 micrometers

Method

1. Bring sections to distilled water
2. Stain nuclei with the alum haematoxylin (see note)
3. Rinse in running tap water
4. Differentiate with 0.3% acid alcohol (see note)
5. Rinse in running tap water
6. Rinse in Scott's tap water substitute (see note)
7. Rinse in tap water
8. Stain with eosin 2 mins
9. Dehydrate, clear and mount.

Results

Collagen.....pale pink

Muscle.....deep pink

Acidophilic cytoplasm.....red

Basophilic cytoplasm.....purple

Nuclei.....blue

Erythrocytes.....cherry red

Reagent Formulae

1. Lillie Mayer alum haematoxylin

aluminium ammonium sulphate -----	200	g
haematoxylin (CI 75290) ----------	20	g
ethanol ----------	40	ml
sodium iodate ----------	4	g
acetic acid ----------	80	ml
glycerol ----------	1200	ml
distilled water ----------	2800	ml

In a 4L Erlenmeyer flask, to 1000 mls of the distilled water add the aluminium ammonium sulphate. Place the flask on a heater/stirrer, turn on the heater and allow to mix until the alum dissolves - this takes about 15 minutes. Remove the flask from the heater/mixer, allow cooling, and then adding the remaining 1800 mls distilled water - this will further cool the solution. Add the haematoxylin powder to the alcohol and dissolve as much of the powder as possible by shaking for a few minutes. Pour the strong alcoholic solution of haematoxylin into the cooled alum solution and stir to ensure all the powder is dissolved, preferably overnight. Add the sodium iodate, acetic acid, and finally the glycerol. Mix well, plug loosely and store. It is appropriate to make up a batch of the required amount, dependent upon the usage rate.

2. Acid alcohol 0.3% Acid Alcohol

commercial grade ethanol -----	2800	ml
distilled water ----------	1200	ml

conc. hydrochloric acid --------- 12 ml

In a sufficiently large container, add the acid to the water, then add the alcohol and mix thoroughly. The generation of fine bubbles is an indication that mixing is thorough.

3. Scott's tap water substitute

sodium hydrogen carbonate	---	10	gm
magnesium sulphate	----------	100	gm
distilled water	----------	5	L

Dissolve the salts in the water. Store stock solutions at room temperature.

4. alc. acetified eosin/phloxine TQEH

1% eosin Y (CI 45380)	----------	400	ml
1% aqphloxine (CI 45405)	-----	40	ml
95% alcohol	----------	3100	ml
glacial acetic acid	----------	16 ml	

Mix the above reagents together, and stir well. The solution keeps well.

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PERIODIC ACID SCHIFF STAINING PROTOCOL:

Purpose

This stain is used for the demonstration of glycogen, mucin, and fungi; it is used for detection of glycogen in tissues such as liver, cardiac and skeletal muscle on formalin-fixed, paraffin-embedded tissue sections, and may be used for frozen sections as well. The glycogen, mucin, and fungi will be stained purple and the nuclei will be stained blue. Tissue sections are first oxidized by periodic acid. The oxidative process results in the formation of aldehyde groupings through carbon-to-carbon bond cleavage. Free hydroxyl groups should be present for oxidation to take place. Oxidation is completed when it reaches the aldehyde stage. The aldehyde groups are detected by the Schiff reagent. A colorless, unstable di-aldehyde compound is formed and then transformed to the colored final product by restoration of the quinoid chromophoric grouping.

Equipment, Reagents, Supplies

Equipment	Reagents	Supplies	PPE
Staining rack	Schiff's reagent	Positive control slide	Lab Coat
	1% Periodic Acid	Slides of interest	Gloves
	Distilled Water, Mayers Haematoxylin	Filter paper	Safety Goggles
	Xylene, Ethyl Alcohol,		

	running tap water		

REAGENT PREPARATION

Periodic acid solution

Periodic acid	1 g
Distilled water	100 ml

Preparation of Schiff reagent

Dissolve 1 g of basic fuchsin and 1.9 g of sodium metabisulfite ($Na_2S_2O_5$) in 100 ml of 0.15 M hydrochloric acid (HCl). Shake the solution at intervals or on a mechanical shaker for 2 hours. The solution should be clear and yellow to light brown in color.

Add 500 mg of activated charcoal and shake for 1 to 2 minutes. Filter the solution through a No. 1 Whatman filter into a bottle. The filtered solution should be clear and colorless. If the solution is yellow, repeat the charcoal decolorization using a fresh lot of activated charcoal. Store at 4°C. Solution is stable for several months.

Specimen

Formalin fixed paraffin sections

Special safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to. Special safety procedures must be followed when performing any procedure with corrosive reagents according to the Histology Safety Manual (HIS-SFT-v1).

Step-by step Procedure

Step #	Instruction
	Deparaffinize and hydrate to water.
	Rinse in distilled water
	Oxidize in 1% periodic acid solution for 5 minutes.
	Rinse in several changes of distilled water.
	Place in Schiff reagent for 15 - 20 minutes (Sections become light pink colour during this step).
	Rinse in running tap water for 5 minutes (Immediately sections turn dark pink colour).
	Stain the nuclei with Mayer's Haematoxylin for 1 minute. Differentiate and blue the sections.
	Wash in tap water for 5 minutes.
	Dehydrate in graded alcohol, clear in xylene (2 minutes each) and coverslip using a synthetic mounting medium DPX.

	Examine the control slide for the positive features and quality of stain.

Quality Control

The PAS stain with diastase or -amylase digestion has histochemical specificity for

Glycogen. Skeletal muscle normally contains glycogen and is often recommended as a Positive control tissue. Kidney tissue can be used as a control demonstrating the features of the basement membrane (Tissue should be sectioned at 2µm).

Schiff Reagent QC:

Test for Schiff reagent:

Pour 10 ml of 37% formalin into a watch glass. To this add a few drops of the Schiff reagent to be tested. A good Schiff reagent will rapidly turn a red-purple color. A deteriorating Schiff reagent will give a delayed reaction and the color produced will be a deep blue-purple.

All solutions must be made in distilled water

Use of any counter stain other than water weak Haematoxylin may mask results.

Reference range/Test Interpretation

Glycogen, neutral/sialomucins and some basement membranes ------red/purple

Fungi/various glycoprotein's---------- red/purple

Background ---------- blue

Nuclei ---------- blue

Notes, Limitations and Anything Else

The intensity of stain is dependent to some extent on the length of treatment with the periodic acid and Schiff reagent. For basement membranes, a longer time in periodic acid (10 minutes) and Schiff reagent (20 minutes) may give better results.

Earlier descriptions of the PAS procedure frequently recommended post-Schiff bisulfite rinses for the reduction of background. This is not necessary provided the slides are adequately rinsed in tap water.

Fixatives containing glutaraldehyde should be avoided if tissues are to be stained with the PAS technique. This is because glutaraldehyde contains two aldehyde groups; tissues fixed in glutaraldehyde contain free aldehyde groups capable of undergoing the Schiff reaction. This results in non-specific background staining.

Staining of glycolipids may be detected when frozen sections are used. In addition, staining of unsaturated lipids may occur in some cases due to the oxidation of carbon-to-carbon double bonds to produce Schiff-reactive aldehyde groups. However, glycolipids and unsaturated lipids rarely interfere with interpretation of results obtained from paraffin-embedded tissues as a significant loss of these molecules likely occurs during tissue processing.

Reference

1. http://www.ihcworld.com/_protocols/special_stains/pas.htm

2. John D. Bancroft (2007) Theory and Practice of Histological Techniques 6th Edition.

ZIEHL NEELSEN STAINING PROTOCOL:

1.0 Purpose

To demonstrate acid fast bacteria belonging to the genus mycobacterium.

1.2 Principle

Mycobacterium possess a capsule containing a long chain fatty acid (mycolic acid) that makes them hydrophobic. The fatty capsule influences the penetration and resistance to removal of the stain by acid alcohol (acid fastness).The fatty capsule takes up the carbol fuchsin (primary stain)

and resists decolorization (differentiation with acid alcohol) by acid alcohol. The speed with which the primary stain is removed by differentiation is proportional to the extent of the fatty coat. Either methylene blue or malachite green can be used as a counter stain. The principle is based on the primary stain that binds cell wall mycolic acids and the intense decolorization by acid alcohol does not release primary stain from the cell wall of AFB. The counter stain provides the contrasting background.

Equipment, Reagents, Supplies, Personal Protective Equipment (PPE)

Equipment	Reagents	Supplies	PPE
Staining Rack Staining trough	Filtered Carbol Fuchsin Methylene blue or Malachite green 1% Acid Alcohol Alcohol Xylene Tap water Distilled water	Filter paper	Gloves Proper PPE

PREPARATION OF REAGENTS

1. CARBOL FUCHSIN

Basic fuchsin	*1g*
Absolute alcohol	*10ml*

5% aqueous phenol solution	*100mls*

Dissolve carbol fuchsin in absolute alcohol then add 5% phenol solution. Mix well and filter before use.

2. DECOLOURIZING SOLUTION

70% Ethanol	*99mls*
Hydrochloric acid	*1ml*
Mix well.	

3. 0.2% METHYLENE BLUE

Methylene blue	*2mg*
Distilled water	*100mls*
Mix well	

Specimen

Formalin fixed paraffin wax sections on frosted slides preferred. Sections must be appropriately cut, adequately dried.

Safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to.

Calibration procedures

NA

Procedure Step-by step

Describe the procedure step-by-step either in free text or table format:

Step #	Action
	Take test and control sections to distilled water
	Flood sections with filtered carbol fuchsin and stain for 30 minutes
	Wash well in tap water
	Differentiate well in 1% acid alcohol until sections are pale pink and red colour stops running (this usually only takes 2-5 dips).
	Wash well in tap water for tap water for 5 minutes then dip in distilled water
	Counter stain in 0.2% working methylene blue solution or malachite green until sections is pale blue or green (30 seconds -1 minute)
	Rinse in tap water then dip in distilled water
	Blot dry the slides after washing in water after the methylene blue counterstain. This will reduce the dehydration time and hence less leaching of the methylene blue.
	Dehydrate in alcohol then clear in Xylene and mount in DPX.

Quality Control

Always stain with a positive slide known to demonstrate the expected tubercle bacilli.

Avoid over – counterstaining as scanty organisms can easily be obscured

Before differentiation with acid alcohol, slides should be washed with 70% alcohol for about a minute to remove majority of the stain and this will reduce the differentiation time.

For easy and quick deparaffinization, sections should be place in Xylene right from the hot plate/oven while they are still warm

Calculation of results

Results are indicated as either positive or negative.

Reference range/Test Interpretation

Features	Result
Mycobacteria, hair shafts, Russell bodies, Splendore-Hoeppli immunoglobulins around actinomyces, fungal organisms	Red/Magenta
Background (Counterstain dependant)	Pale blue/Green

Alert/critical values, where appropriate

NA

Notes, Limitations and Anything Else

The blue counterstain may be patchy if extensive caseation is present. Care should be taken to avoid over-counterstaining as scant organisms can easily be obscured.

Decalcification using strong acids can destroy acid-fastness; formic acid is recommended.

Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if color blindness causes a recognition problem.

Reference

Bancroft J.D. Stevens, A. Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 1982.

Bancroft, J.D.; Cook, H.C. Manual of Histological Techniques, Churchill, Livingstone, London, 1984.

Bancroft JD, Gamble M, Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 2008.

Cook H.C.; Manual Histological Demonstration Techniques; Butterworths, 1974.

Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.

1. Principle

Chromic acid oxidation forms aldehydes from fungal cell wall polysaccharide components, which are subsequently demonstrated by reduction of an alkaline hexamine-silver complex. The reaction may be compared to that of the periodic acid Schiff reaction, (see PAS).

Grocott's alkaline hexamine-silver solution represents a vehicle which, upon reduction, precipitates nascent silver ions, thus blackening the site. This is known as an "argentaffin reaction".

Argentaffin reaction - *the ability of a silver complex solution to blacken a tissue element without the need of a reducing bath.* The term is adjectival and is applied to many methods, (eg von Kossa). The term "argentaffin reaction" should therefore not be used as a proper name.

2. Technical Points

1. A known positive control section must be used to ensure correct differentiation has been achieved.

2. Reagents should be prepared in a fume hood.

3. Method

1. Bring sections to distilled water.

2. Oxidise with 4% aq chromic acid at room temperature 1 hr

3. Wash in water for a few seconds.

4. Treat sections with 1% sodium metabisulphitee 1 min

5. Wash in running tap water 3 mins

6. Rinse thoroughly in distilled water.

7. Place in pre-heated working silver solution in a water bath at 60°C for 15 to 20 mins until section turns yellowish-brown (Check microscopically after washing in distilled water – fungi should be dark brown).

8. Rinse well in distilled water

9. Tone sections with 0.2% gold chloride 2 mins

10. Rinse in distilled water

11. Treat sections with 2% sodium thiosulphate 2 mins

12. Wash with running tap water 5 mins

13. Counterstain in working light green 15 sec

14. Rinse excess light green off slide with alcohol

15. Dehydrate, clear and mount.

4. **Results**

Fungi, Pneumocystis carnii, histoplasma spp ----------black

Inner parts of mycelia and hyphae ----------old rose

Leishmaniaspp, toxoplasma spp ----------negative

Mucin----------dark grey

Background ----------pale green

5. Reagent Formulae

Wear protective clothing, gloves and safety glasses when preparing reagents.

1. 4% aq Chromic Acid

Chromium trioxide (analytical) ---- 4 g

Distilled water ---------- 100 ml

2. Silver solution

3% methenamine (= hexamine) ---- 23 ml

5% silver nitrate ---------- 1.25 ml

5% borax (sodium tetraborate) ---- 3 ml

Distilled water ---------- 25 ml

3. 0.2% aq Sodium chloroaurate (yellow gold chloride)

Gold Chloride (analytical) ---------- 1.0 g

Distilled water ---------- 500 ml

4. 2% aq Sodium thiosulphate (hypo)

Sodium thiosulphate ---------- 2.0 g

Distilled water ---------- 100 ml

5. Working light green

1% light green (CI 42095) in 1% acetic acid --- 10 ml

Distilled water ---------- 40 ml

6. **References**

Grocott, R.G. 1955, A stain for fungi in tissue sections and smears. American Journal of Clinical Pathology, V25, p975

Luna L.G. Histopathological Methods and colour atlas of special stains and tissue artefacts, American Histo Labs Inc, Publications Division 1992.

GIEMSA - SHEEHAN'S MODIFIED MAY-GRUNWALD

PURPOSE To permit differentiation of cells present in hematopoietic tissue. The stain is also used for the demonstration of some microorganisms.

PRINCIPLE: The "neutral" dyes combining the basic dye methylene blue and the acid dye eosin, give a wide color range when staining. The pH of the staining solution is critical and ideally should be adjusted for

different fixatives. More acid pH levels give more selective chromatin staining and less cytoplasmic basophilia; less acid pH levels give denser nuclei and increased cytoplasmic basophilia. The pH range should be between 6.4 and 6.9.

CONTROL: Spleen

FIXATIVE: 10% formalin, B-5 fixative.

TECHNIQUE: Cut paraffin sections 4-5 microns.

EQUIPMENT: Rinse glassware in DI water. Coplin jars, filter paper, staining rack, pipettes.

SAFETY/PPE: Wear gloves, goggles and lab coat. Avoid contact and inhalation of dyes and chemicals.

REAGENTS:

WRIGHT STAIN:

Commercial

GIEMSA STAIN:

Commercial

PHOSPHATE BUFFER, pH 6.8:

Sodium phosphate, di 0.3 gm

Sodium phosphate, mono 0.7 gm

Distilled water 100.0 ml

Store in the refrigerator, stable for

1 year.

MICROORGANISMS

SHEEHAN'S GIEMSA Page 2 of 2

GIEMSA STAIN:

Phosphate buffer 50.0 ml

Giemsa stain 2.5 ml

Methanol, acetone free 2.5 ml

Make fresh, filter, discard after

use.

ACETIC WATER:

Acetic acid 1.0 ml

Distilled water 400.0 ml

Stable for 1 year.

PROCEDURE:

1. Deparaffinize, bring to absolute alcohol.

2. Methanol, three changes.

3. Place slide on staining rack, cover with Wright stain, 5 minutes.

4. Do not drain off stain, add an equal amount of distilled water until a metallic sheen appears. Leave for 5 minutes.

5. Place slides directly into the Giemsa solution, for 45 minutes, room temperature.

6. Differentiate and dehydrate in the following:

acetic water 3 dips

distilled water 2 dips

95% alcohol 3 dips

100% alcohol 3 dips

100% alcohol 3 dips

xylene 3 changes

Coverslip

RESULTS:

Nuclei blue

Cytoplasm pale blue

Rickettsias reddish purple

Erythrocytes yellowish pink

REFERENCE:

Sheehan D, Hrapchak B, Theory and practice of Histotechnology, 2nd Ed, 1980, pp155-156, Battelle Press, Ohio

Carson F, Histotechnology: A Self-Instructional Text, 1990, pp110-112,

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