University of Zambia
School of Medicine
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Evaluation of Vitamin B12 and Folate Levels in Megaloblastic Anaemia, diagnosed morphologically, at the University Teaching Hospital, Lusaka, Zambia.

A Dissertation Submitted to the University of Zambia, in Partial Fulfilment of the Requirements for the Master of Science Degree in Pathology (Haematology)

By

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Declaration

This work/dissertation in its present form has not been submitted or accepted previously for the award of a degree or diploma at the University of Zambia or any other tertiary institution. I declare that this Dissertation contains my own work and where other authors have been cited due acknowledgement has been given. I further declare that I followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Master of Science in Pathology (Haematology), University of Zambia guidelines.

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Abstract

Background: Vitamin B12 and folate deficiency is a well-known health problem. Deficiencies of folic acid and vitamin B12 are known to cause megaloblastic anaemia, which is characterised by presence of abnormally large erythrocyte precursor cells, megaloblasts, in the bone marrow and macrocytic red cells in the peripheral blood. These megaloblasts arise because of impaired deoxyribonucleic acid (DNA) synthesis followed by ineffective erythropoiesis. However, vitamin B12 or folate levels have not been described in Zambia, whether normal levels or in relation to anaemia.

Aims: The study aimed to determine vitamin B12 and folate levels in megaloblastic anaemia, diagnosed morphologically, in patients at the University Teaching.

Methods and results: This was a case control study which was undertaken at the University Teaching Hospital (UTH) in Lusaka, Zambia. Full blood count (FBC), Peripheral smears and ELISA were assessed on blood samples received from megaloblastic anaemia and non-anaemic patients. The age range was between 18 – 54 years (Mean age-31 years). Among the 40 megaloblastic patients, 35% (14/40) were male and 65% (26/40) were female with a male to female ratio of 1:1.9. Full blood count and peripheral smear findings revealed that bicytopenia was present in 22.5% (9/40) and pancytopenia in 72.5% (29/40) patients. Furthermore, the results showed megaloblastic anaemia participants had statistically significant lower median vitamin B12 concentration 175 (150-333)pg/ml than non-anaemic control participants 299.5 (238-571)pg/ml p=0.0001. Megaloblastic anaemia participants also had a statistically significant lower folate concentration (12.32± 2.28 ng/ml) than non-anaemic control participants (19.28 ± 2.84 ng/ml)p=0.029. Of the megaloblastic anaemia patients, vitamin B12 deficiency was in 60% (24/40), pure folate deficiency in 30% (12/40) and combined deficiency was observed in 15% (6/40) patients. A weak negative correlation was found between vitamin B12 and mean corpuscular volume but statistically significant (r= -0.0278, p =0.001). However, there was no statistical significant correlation between folate and mean corpuscular volume (r = -0.098, p = 0.326).

Conclusion – This study shows that majority of patients with megaloblastic anaemia, diagnosed morphologically, at the University Teaching Hospital have a deficiency of vitamin B12 deficiency.

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List of abbreviations

BREC	Biomedical Research Ethics Committee
CVD	Cardiovascular Disease
DATP	Deoxyadenosine Triphosphate
DCTP	Deoxycytidine Triphosphate
DGTP	Deoxyguanosine Triphosphate
DNA	Deoxyribonucleic Acid
DNTP	Deoxyribonucleoside Triphosphate
DTP	Deoxyuridine Triphosphate
DTTC	Deoxythymidine Triphosphate
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBC	Full Blood Count
FL	Femtoliter
HB	Haemoglobin
HRP	Horseradish Peroxidase
HIV	Human Immunodeficiency Virus
IF	Intrinsic Factor
MCV	Mean Corpuscular Volume
NTD	Neural Tube Defect
OD	Optimal Density
RBC	Red Blood Cell
THF	Tetrahydrofolate
TMP	Thymidine Monophosphate
TTP	Thymidine Triphosphate

UTH	University Teaching Hospital
UNZA	University of Zambia
WBC	White Blood Cell
WHO	World Health Organisation

Chapter 1: Introduction

1.1. Background

Megaloblastic anaemia is defined as a highly characteristic set of morphological changes which affect cells of the erythroid, myeloid and megakaryocytic lineages in the peripheral blood and bone marrow (Provan *et al.*, 2005). Macrocytic anaemia refers to a blood condition in which the red cells are abnormally large (mean corpuscular volume, MCV >95 femtoliter). There are several causes but they can be broadly subdivided into megaloblastic and non-megaloblastic, based on the appearance of developing erythroblasts in the bone marrow (Hoffbrand *et al.,* 2006).

Megaloblastic anaemia has been recognized as a clinical entity for over a century. Megaloblastic anaemia results from abnormal maturation of haematopoietic cells due to faulty DNA synthesis (Addison, 1849). The cause is usually deficiency of either cobalamin (vitamin B12) or folate but megaloblastic anaemia may arise because of genetic or acquired abnormalities affecting the function of metabolism of these vitamins. Two vitamins, cobalamin (vitamin B12) and folic acid are essential for DNA biosynthesis. Deficiency of either vitamin results in asynchrony in the maturation of the nucleus and cytoplasm of rapidly regenerating cells (Carmel *et al.*, 2004). In the haematopoietic system this asynchrony results in abnormal nuclear maturation with normal cytoplasmic maturation, apoptosis, ineffective erythropoiesis, intramedullary haemolysis, pancytopenia and typical morphological abnormalities in the blood and marrow cells (Khanduri *et al.,* 2007).

The conditions that give rise to megaloblastic changes share a common disparity in the synthesis or availability of the four immediate precursors of DNA that is deoxyribonucleoside triphosphates (dNTPs) i.e.deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP), required for ordered DNA replication during the S-phase of the cell cycle (Provan *et al.*, 2005).

DNA is formed by the polymerization of the four deoxyribonucleoside triphosphates. Folate deficiency is thought to cause megaloblastic anaemia by inhibiting thymidylate

synthesis, a rate limiting step in DNA synthesis in which thymidine monophosphate(TMP) is synthesized as this reaction requires a coenzyme (Hoffbrand *et al.*, 2006).

Folate is required in one of its coenzyme forms, 5, 10-methylene tetrahydrofolate (THF) polyglutamate, in the synthesis of thymidine monophosphate from its precursor deoxyuridine monophosphate (dUMP) (Bailey *et al.*, 2009). The human body needs folate to synthesize, repair, and methylate DNA as well as to act as a cofactor in certain biological reactions (Weinstein *et al*, 2003). It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy. Children and adults both require folate to produce healthy red blood cells and prevent anaemia. Vitamin B12 is needed to convert methyl THF, which enters the cells from plasma, to THF, from which polyglutamate forms of folate are synthesized (Banerjee *et al.*, 2003).

DNA synthesis is dependent on a key structure, thymidine triphosphate (TTP). This structure cannot be formed unless 5, 10-methylene THF polyglutamate is synthesized, and is required as a coenzyme. Vitamin B12 is the cofactor responsible for demethylating methyl tetrahydrofolate (Doig *et al.*, 2002). Sufficient quantities of vitamin B12 and folic acid are key to the formation of TTP. If TTP cannot be synthesized, then it is replaced by deoxyuridine triphosphate (dUTP). The synthesis of this component leads to nuclear fragmentation and destruction of cells and impaired cell division. For this reason, vitamin B12 and folic acid are essential elements in the DNA pathway (Doig *et al.*, 2002).

There is scarce data on vitamin B12 and folate levels have not been described in Zambians, whether normal levels or in relation to anaemia. This study aimed to evaluate vitamin B12 and folate levels in megaloblastic anaemia, diagnosed morphologically, and also the correlation of MCV with vitamin B12 and folate levels with the aim to add to the existing knowledge in literature on megaloblastic anaemia. The knowledge generated from this research shall also help establish a platform for further testing in megaloblastic suspected cases and introduction of the two tests in government institutions.

1.2. Statement of the problem

Anaemia is a major public health problem affecting 1·62 billion people globally (Mc lean *et al.*, 2009).Although the prevalence of anaemia is estimated at 9% in highly developed countries, prevalence in low developed countries is as high as 43% (Mclean *et al.*, 2009). Children and women of reproductive age are most at risk, with global anaemia prevalence estimates of 47% in children younger than 5 years, 42% in pregnant women, and 30% in non-pregnant women aged 15–49 years, and with Africa and Asia accounting for more than 85% of the absolute anaemia burden in high-risk groups. Anaemia is estimated to contribute to more than 115 000 maternal deaths and 591 000 prenatal deaths globally per year (Ezzati *et al.*, 2004).

The consequences of morbidity associated with chronic anaemia extend to loss of productivity from impaired work capacity, cognitive impairment, and increased susceptibility to infection, which also exerts a substantial economic burden (Ezzati *et al.*, 2004).

The global prevalence of vitamin B12 deficiency is unknown, but evidence from several developing countries suggests that deficiency is widespread and is present throughout life. In South America, at least 40% of children and adults were vitamin B12 deficient, with prevalence greater than 70% in Africa and Asia (Allen, 2004, 2009). However, how much this deficiency contributes to anaemia is unclear, with few data available for the haematological effect of increasing B12 intake at the population level (Metz, 2008).

The contribution of folate deficiency to anaemia at the population level is unknown because few global data exist, although it is thought to be low in developing countries (Metz, 2008). Review of epidemiological studies identified high prevalence of serum folate deficiency in specific subpopulations of Cuban men (64–89%) and Chilean women (25%) (Allen, 2004).

According to the available laboratory records reviewed between 2013 and 2015 at UTH in Zambia, there has been an increase of 7% of the patients that report to the hospital who present with macrocytic anaemia (UTH lab records). The deficiencies that lead to macrocytic anaemia affect all rapidly growing tissues. After the marrow, the next most

affected tissues are the epithelial cell surfaces of the mouth, stomach, small intestine, respiratory, urinary and female genital tracts (Czeizel *et al.*, 2004).

The gonads are also affected and infertility is common in both men and women with either deficiency. Maternal folate deficiency has been implicated as a cause of prematurity and both folate and cobalamin deficiencies have been implicated in recurrent foetal loss (Czeizel *et al.*, 2004).

1.3. Justification of the study

Effective treatment and management of megaloblastic anaemia requires a deep insight into the disease. Most of the knowledge that has come to surface in Zambia is from studies that were done elsewhere. Megaloblastic anaemia has substantial impact with its related complications.

Scarcity of complete data records to make definite conclusions proves challenging in determining the incidence of megaloblastic anaemia. MCV value above the upper limit of normal or those that differ significantly from the patient baseline values require further clinical and laboratory assessment to determine the underlining cause of macrocytosis.

Currently, megaloblastic anaemia is diagnosed using red cell indices from a full blood count, a peripheral smear and bone marrow examination in some cases, which may lead to substantial morbidity if unrecognized or misdiagnosed. Owing to this, this research proposed a study that would provide evidence based information on vitamin B12 and folate levels which will help in the definitive diagnosis of megaloblastic anaemia. The undertaking of this study was to determine vitamin B12 and folate levels with a view that the findings will enhance the proper management of the patients. Vitamin B12 and folate are of considerable relevance because they are pathophysiologically linked to serious medical problems such as cardiovascular disease (CVD) and neural tube defect (NTD).

1.4. Research question

What are the distributions of vitamin B12 and folate levels of patients diagnosed with megaloblastic anaemia on morphology at the University Teaching Hospital?

1.5. Objectives

1.5.1. General objective

To determine the plasma levels of vitamin B12 and folate in patients with megaloblastic anaemia, diagnosed morphologically, at the University Teaching Hospital.

1.5.2. Specific objectives

1.5.2.1 To compare the plasma concentration levels of vitamin B12 and folate between megaloblastic anaemia patients and non-anaemic controls.

1.5.2.2 To describe the laboratory and demographic characteristics associated with megaloblastic anaemia patients.

1.5.2.3 To determine the relationships between mean corpuscular volume, plasma levels of vitamin B12 and folate in megaloblastic anaemia.

Chapter 2: Literature review

Vitamin B12 (cobalamin) is a water-soluble vitamin that is crucial to normal neurologic function, red blood cell production, and DNA synthesis. Vitamin B12 is essential for three enzymatic processes: the conversion of homocysteine to methionine; the conversion of methylmalonic acid to succinyl coenzyme A; and the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, a process necessary for DNA synthesis and red blood cell production (Evatt *et al.*, 2010).

Folate is necessary for the production and maintenance of new cells, for DNA synthesis and RNA synthesis, and for preventing changes to DNA, and, thus, for preventing cancer (Kamen,1997). It is especially important during periods of frequent cell division and growth, such as infancy and pregnancy. Thus, folate deficiency hinders DNA synthesis and cell division, affecting hematopoietic cells and neoplasms the most because of their greater frequency of cell division (Figueiredo *et al.*, 2009).

The deficiency of these vitamins leads to the increase in the MCV of the red blood cells. The normal values of the MCV are as shown in the table below.

Table 1: Reference ranges of MCV for different age groups

Age(years)	**Male**	**Female**
2- 5	76.8 – 83.3	77.7 – 84.1
6-11	78.2 – 83.9	79.5 – 85.2
12-17	80.8 – 86.6	82.1 – 87.7
18-Adults	81.2 – 94.0	78.5 – 95.0

(www.pubinfor.vcu.edu.haematology reference ranges)

A study in Framingham, United States of America was done to determine the prevalence of cobalamin deficiency in the elderly population. Serum concentration of cobalamin and folate were measured in 548 surviving members of the original Framingham study cohort which comprised of 176 controls (89 females and 87 males). Serum cobalamin concentration analysed by radioimmunoassay of <258pmol were found in 222 (40.5%) subjects compared to 98 (17.9%) of young control subjects (P <0.001). The mean serum concentration of vitamin B12 was 623pg/ml, lower than that of the healthy control

subjects which was 673pg/ml. The mean serum folate concentration in 548 elderly subjects was 18ng/ml versus 11.1ng/ml that of the controls. Low concentrations of both Vitamin B12 and folate levels were found in 82 (15%) subjects (Lindenbaum *et al.*, 1994).

A research at Royal Free Hospital, London was conducted on the effect of folate analogues and vitaminB12 on the provision of thymine nucleotide for DNA synthesis in megaloblastic anaemia. The role of vitamin B12 in the folate dependant biosynthesis of thymine nucleotide was controversial. In an attempt to clarify this, three methods were used to assess the relative efficacy of vitamin B12 and various folate analogues in titrated concentration at correcting de novo thymidylate synthesis by megaloblastic human marrow cells. The methods were the deoxyuridine (dU) suppression test analyses the reduction in (^{3}H)-thymidine labelling of DNA by unlabelled (dU), incorporation into DNA and the accumulation of (6-^{3}H)-deoxyuridine monophosphate. The methods gave similar results (Taheri *et al.*, 1982).

A report that was reviewed in Pennsylvania at St Luke's Hospital, Bethlehem showed that vitamin B12 is a common cause of megaloblastic anaemia and a variety of neuropsychiatric symptoms. Most of the older people who had a vitamin B12 deficiency also showed an elevation of serum homocysteine. The true prevalence of vitamin B12 deficiency is difficult to estimate because reports are based on values that vary with inclusion criteria and individual laboratory methodology. Although the classic hematologic expression of vitamin B deficiency is a megaloblastic macrocytic anaemia characterized by an elevated mean corpuscular volume and mean corpuscular haemoglobin, and a peripheral smear containing macroovalocytes and hypersegmented neutrophils, up to 28 percent of affected patients may have a normal haemoglobin level, and up to 17 per cent may have a normal mean corpuscular volume (Langan *et al.*, 2011).

Khanduri *et al* conducted a study in India on the prevalence and causative factors of megaloblastic anaemia. All patients presenting to the hospital over a period of six months with haemoglobin (Hb) <10g/dl and/or MCV of >95 and blood findings consistent with megaloblatosis were included in the study. Out of the 175 patients who were enrolled,

only 120 samples were assayed. Vitamin B12 and folate were analysed by an enzyme immunoassay. Results showed cobalamin deficiency in 78 patients (65%), combined cobalamin and folate deficiency in 20 patients(16%) and pure folate deficiency in 8 patients(6%).The peak incidence of megaloblastic anaemia was in the age of 10-30 with female predominance of 71% (Khanduri *et al.*, 2007).

Vitamin B12 deficiency is a major cause of megaloblastic anaemia in patients in Pakistan. In order to find out the contribution of folate and vitamin B12 deficiency in megaloblastic anaemia, a retrospective cohort study was conducted at Aga Khan University hospital in which a total of 220 patients (101 female and 119 male) who presented themselves with Macrocytic anaemia were enrolled. The diagnosis was based on MCV of >96fl performed by coulter counters. Serum levels of folate and Vitamin B12 were carried by Dual count Kit. The results showed that Sixty-nine percent of the patients had severe anaemia (Hb<8 gm/dl). Mean±SEM values of haemoglobin, serum folate and serum B12 were not significantly different between males and females (Hb 6.4±0.3 gm/dl vs 6.3±0.3 gm/dl; folate 6.9±0.8 ?g/ml vs 7.8±1 ?g/ml; B12 259±65 ?g/ml vs 225±45 ?g/ml, respectively). Linear regression analysis showed that serum folate was inversely related with the mean corpuscular volume (MCV, p=0.04). Folate deficiency was 43.4%, while vitamin B12 deficiency was 78.5% in these patients. Seventy-one percent of folate-deficient patients had vitamin B12 deficiency as well, while 26.1% of patients with vitamin B12 deficiency had a co-occurrence of folate deficiency (Iqbal *et al.*, 2009).

A cross sectional study at a Tertiary Care hospital in Pakistan was done to determine frequency of vitamin B12 deficiency in subjects with anaemia and elevated mean corpuscular volume. Of the 113 subjects, 37(32.7%) were male and 76(67%) were female. The full blood count (FBC) was performed on Sysmex KX 21. Of the 113, 48(42.4%) had normal vitamin B12 levels and 65(57.5%) subjects were found to be vitamin B12 deficient. Mean corpuscular volume as high as 139fl and vitamin as low as <30pg/ml were found. Significant negative correlation was found between vitamin B12 and MCV (Nizamani *et al.*, 2004).

The determination of MCV, if it was an unreliable screening parameter to assess vitamin B12 deficiency, in the diagnosis of megaloblastic anaemia was done in India in which 71 men and 46 women were enrolled and vitamin B12 was analysed by chemiluminescence. Of the 94 cases with reduced serum vitamin B12, 26 showed macrocytosis and hypersegmented neutrophils were present in 24patients. Twenty-six patients showed a raised MCV, 50 patients had an MCV within the reference range and 28 had low MCV (Bhatia *et al.*, 2012).

A prospective study in Tunisia showed that the majority of megaloblastic anaemia results from deficiencies of either vitamin B12 or folate. A total of 478 both male and female were enrolled with consecutive patients with megaloblastic changes in the bone marrow smears. Serum cobalamin and folate levels were determined by radioimmunoassay methods. Out of the 439 patients in which blood cobalamin and folate were determined, 430(98%) expressed low vitamin B12 levels. Pure folate deficiency was observed in only 6(1.2%) patients. Vitamin B12 deficiency was slightly higher in men than women with 21.5% of being less than the age of 30 years at the time of diagnosis (Maktouf *et al.*, 2006).

Another study was conducted in Zimbabwe to determine if vitamin B12 deficiency was the primary cause of megaloblastic anaemia. A total of 144 consecutive Zimbabwean patients with megaloblastic haematopoiesis were evaluated. Vitamin B12 deficiency was diagnosed in 86.1% of the patients. Isolated folate deficiency accounted for only 5% and neurologic dysfunction was noted in 70.2% of Vitamin B12 deficient patients, especially those who had an Hb of <6g/dl (Savage *et al.*, 2008).

As seen above, most studies show similar findings with vitamin B12 deficiency been predominant as compared to folate deficiency. Nonetheless, there is a disparity in correlation with MCV. Therefore, the results in the research will be used to determine the concentration difference between the two groups and a correlation with the MCV.

Chapter 3: Methodology

3.1. Study design and study site

A case control design was used to determine vitamin B12 and folate levels from blood samples with a diagnosis of megaloblastic anaemia collected from routine samples of the haematology laboratory at UTH.

3.2. Target population

All patient samples of all ages with a laboratory diagnosis of megaloblastic anaemia.

3.3. Study population

All samples that met the inclusion criteria were part of the study. These were samples that had an MCV of greater than 95fl. The study sample and the comparison group were categorically matched for age and sex in order to minimize bias.

3.4. Sample size

A total sample size of 88 samples (44 cases and 44 controls) was calculated using the formula for determination of sample size for comparative research studies between two groups as given below;

$$N = \frac{4\sigma^2(z_{crit} + z_{pwr})^2}{D^2},$$

$$N = \frac{4 \times 10^2 \times (1.960 + 1.282)^2}{(6.9)^2}$$

N = **88.258**

N = **88** samples.

Where; *N* was the total sample size (the sum of the sizes of both comparison groups), s was 10; the assumed SD of each group (assumed to be equal for both groups), the z_{crit} value was 1.960 as given in tables for Standard Normal Deviation (z_{crit}) corresponding to the desired significance criterion of 0.05 or 95% confidence interval (CI), the z_{pwr} value was 1.282 as given in Standard Normal Deviate (z_{pwr}) tables corresponding to 90% statistical power, and *D* was the minimum expected difference between the two means which was estimated at 6.9 (Folate concentration 18ng/ml versus 11.1ng/ml). Both z_{crit} and z_{pwr} were cut-off points along the x axis of a standard normal probability distribution that demarcate probabilities matching the specified significance criterion and statistical power, respectively. The two groups that made up *N* were assumed to be equal in number, also that the outcome variable of a comparative study was a continuous value for which means were compared, and the two-tailed statistical analysis was used (Eng, 2003). However, only **80** participants' samples (**40 cases and 40 controls**) were analysed due to inadequate reagents.

3.5. Sampling methods

Convenience sampling in which consecutive samples with megaloblastic anaemia diagnosed morphologically and found to meet the inclusion criteria (given below) were included into the study sample. At least 4mls of anticoagulated (EDTA vacutainer) routine blood samples received in haematology laboratory. A full blood count was done and a peripheral smear examined. The plasma was separated from the blood cellular components and stored at a temperature of -20^0C for analysis on a later date. The comparison group was selected by means of frequency matching of the same proportional characteristics (age and sex) as the study sample.

3.5.1. Inclusion criteria

The inclusion criteria for the study were:

1. Mean corpuscular volume (MCV) more than 95fl.
2. Peripheral blood smears consistent with megaloblastic anaemia (macrocytosis on peripheral smear, tear drop poikilocytosis and hypersegmented neutrophils).

3.5.2. Exclusion criteria

1. All blood samples that were not diagnosed as having megaloblastic anaemia.
2. All patients on vitamin B12 and folic acid supplementation.

3.5.3. Controls

1. All blood samples without any form of anaemia with an MCV between the range of 76.8fl – 95.0fl.

3.6. Material and methods

3.6.1. Specimen preparation and storage

In the laboratory, each specimen serial number was recorded on to a compilation summary sheet. Thereafter a full blood count done and peripheral smear examined. The blood specimen was centrifuged at 3000 revolutions per minute (3000 rpm) in order to separate the plasma (supernatant) from the blood cellular components (sediment). Only supernatant (plasma) was then meticulously collected from the Ethylene diamine tetra-acetic acid (EDTA)vacutainer using pipettes and transferred to 2ml plastic cryovial containers with sealable screw caps which was stored in a freezer at -20^{o}C until the specimens were required for analysis.

3.6.2. Quality control

To ensure accurate and reliable results, quality control was performed on all the analytical instruments and analysers used for any purpose during specimen analysis according to the UTH quality control guidelines. Quality control included equipment calibrations and analytical control runs on every analyser before each test analysis.

3.7. Specimen analysis

3.7.1. Vitamin B12 ELISA test protocol

Plasma vitamin B12 concentration was determined by a competitive immunoassay. The Neoplate provided was coated with a Vitamin B12 specific antibody. Standards or samples were co-incubated in wells along with a vitamin B12 conjugate. Vitamin B12 in standards or samples competed with vitamin B12 –horseradish peroxidase (HRP) conjugate for binding to the plate bound antibody. Higher levels of vitamin B12 from standards or sample led to the decreased vitamin B12-HRP conjugate binding and reduced signal. Captured vitamin B12-HRP was quantitatively detected by the incubation with HRP substrates (solution A and B). Binding of the vitamin B12-HRP was visualised by the production of colorimetric reaction products that could be measured by absorbance at 450nm.ELISA plates were read using the VersaMax PLUS Rom v1.23 ELISA plate reader.

3.7.1.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The Neoplate was brought to room temperature before opening. The wash solution concentrate (25×) was diluted with 1990 mL of distilled water.

3.7.1.2. Assay Procedure

Prior to use, all reagents were thoroughly mixed taking care not to create any foam within the vials. 100 µL of SAMPLE and STANDARD (1 to 6) were added in duplicate to the appropriate wells in the supplied Neoplate. Then 50 µL of enzyme solution was added to each well and mixed well. The plate was then covered and incubated for 1 hour at 37^0C in a humid chamber. After incubation, each well was washed 5times with 300 µL 1X WASH SOLUTION per well. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid at each step. Then 50 µL of substrate A was added to each well followed by addition of 50µL solution B. The plate

was then covered and incubated for 30 minutes at room temperature in the dark at room temperature for 30 minutes avoiding direct exposure to light. After incubation, 50 µL of STOP REAGENT was added to each well and the contents mixed well. The optical density (O.D.) was immediately read at 450 nm.

3.7.1.3. Data processing

The O.D. of other non-zero standards were divided by that of the zero standards, and then multiplied by 100 (used as X variables). Then, the base 10 logarithm of other standard concentration was calculated (taken as Y variables). A standard curve was generated from these variables in Microsoft Excel 2011 for Mac.

FIGURE 1: Vitamin B12 Calibration Curve

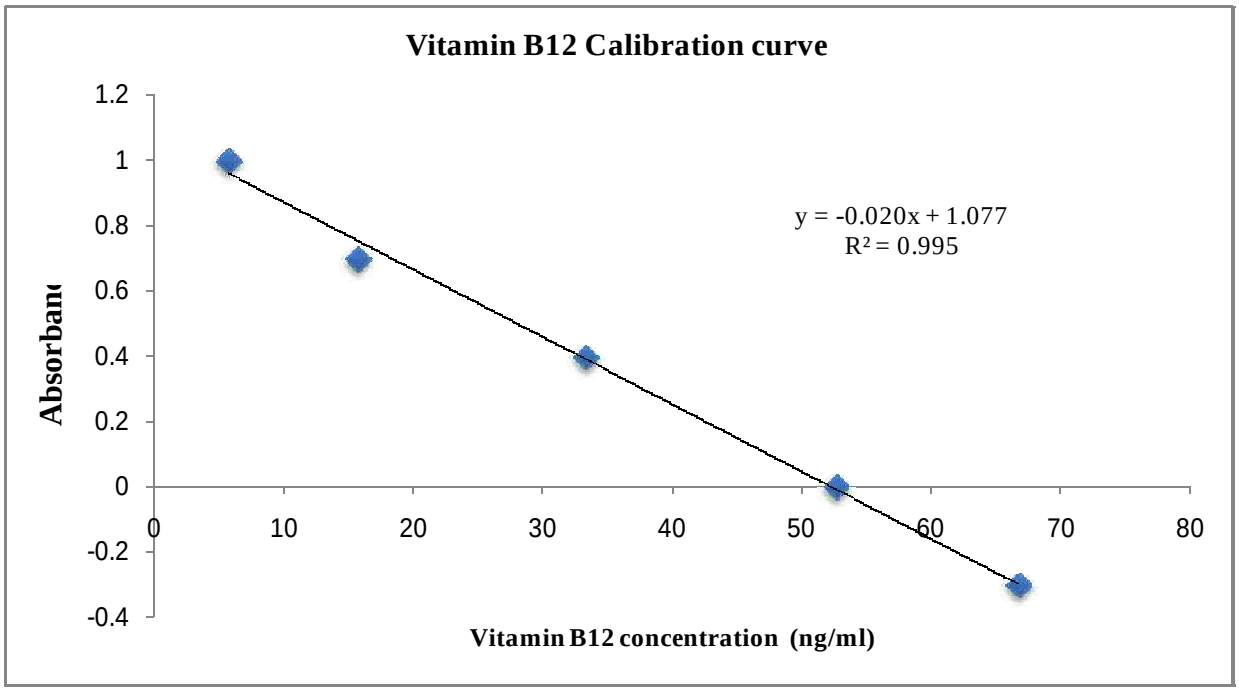

Fig. 1: Vitamin B12 calibration curve plotted from concentrations against standard absorbances (O.Ds). The regression equation was used to calculate sample concentration from their respective O.Ds.

To calculate results: the sample O.D. was processed as follows: O.D. of sample divided by that of standard 0, then multiplied by 100, to get Y values using the formulation y = -0.020x + 1.077. To get the concentration of samples: 10 was powered to Y value (10^{Y}).

3.7.1.4. Sensitivity and Specificity

The sensitivity, minimum detectable dose of vitamin B12 using this Human vitamin B12 ELISA kit is approximately 0.1ng/mL. The assay has high sensitivity and excellent specificity for detection of vitamin B12. No significant cross-reactivity or interference between vitamin B12 and any homologous proteins has been observed. Species cross-reactivity has not been specifically detected.

3.7.2. Folate ELISA test protocol

Plasma folate concentration was determined by a competitive immunoassay. The Neoplate provided was coated with a folate specific antibody. Standards or samples were co-incubated in wells along with a folate conjugate. Folate in standards or samples competed with folate –horse radish peroxidase (HRP) conjugate for binding to the plate bound antibody. Higher levels of folate from standards or sample led to the decreased folate-HRP conjugate binding and reduced signal. Captured folate-HRP was quantitatively detected by the incubation with HRP substrates (solution A and B). Binding of the folate-HRP was visualised by the production of colorimetric reaction products that could be measured by absorbance at 450nm. ELISA plates were read using theVersa MaxPLUS Rom v1.23 ELISA plate reader.

3.7.2.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The Neoplate was brought to room temperature before opening. The wash solution concentrate (25×) was diluted with 1990 mL of distilled water.

3.7.2.2. Assay Procedure

Prior to use, all reagents were thoroughly mixed taking care not to create any foam within the vials. 100 µL of SAMPLE and STANDARD (1 to 6) were added in duplicate to the appropriate wells in the supplied Neoplate. Then 50 µL of enzyme solution was added to each well and mixed well. The plate was then covered and incubated for 1 hour at 37^0C in a humid chamber. After incubation, each well was washed 5times with 300 µL 1X WASH SOLUTION per well. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid at each step. Then 50 µL of substrate A was added to each well followed by addition of 50µL solution B. The plate was then covered and incubated for 30 minutes at room temperature in the dark at room temperature for 30 minutes avoiding direct exposure to light. After incubation, 50 µL of STOP REAGENT was added to each well and the contents mixed well. The optical density (O.D.) was immediately read at 450 nm.

3.7.2.3. Data processing

The O.D. of other non-zero standards were divided by that of the zero standards, and then multiplied by 100 (used as X variables). Then, the base 10 logarithm of other standard concentration was calculated (taken as Y variables). A standard curve was generated from these variables in Microsoft Excel 2011 for Mac.

FIGURE 2: Folate Calibration Curve

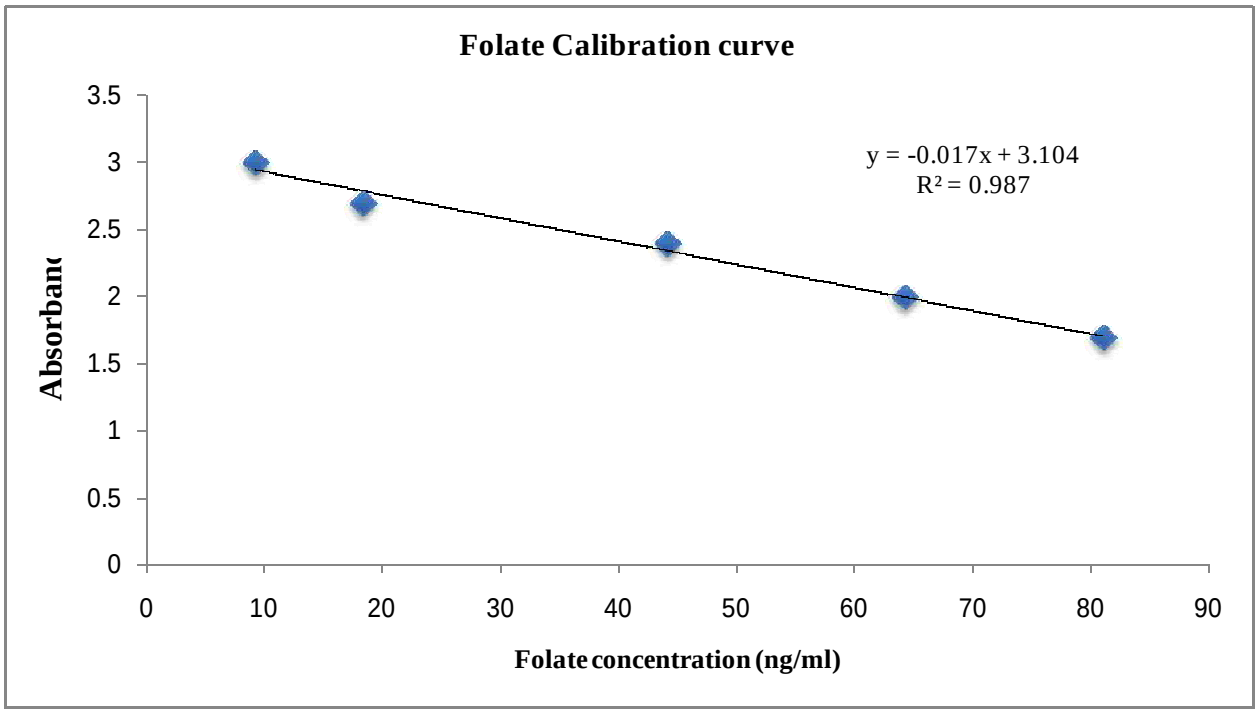

Fig. 2: Folate calibration curve plotted from concentrations against standard absorbances (O.Ds). The regression equation was used to calculate sample concentration from their respective O.Ds.

3.7.2.4. Sensitivity and Specificity

The sensitivity, minimum detectable dose of folate using this Human Folate ELISA kit is approximately 1.0ng/mL. The assay has high sensitivity and excellent specificity for detection of folate. No significant cross-reactivity or interference between folate and any homologous proteins has been observed.Species cross-reactivity has not been specifically detected.

3.7.3. Full blood Count Test Protocol

Complete blood count tests were determined using Sysmex XT 4000 haematology analyzer manufactured by Sysmex Europe- supplied by Sonergy Diagnostics-Lusaka. The machine counts the numbers and types of different cells (WBC, RBC, platelets) within the blood. It then prints out the results using an in built printer that is connected to it. The two main sensors used in this analyzer are light detectors and electrical impedance that passes through the blood thereby analyzing data about the size and aspects of light as they pass through the cells called forward and light scatter.

3.7.3.1. Handling

The cap of the reagent cassette was removed and all foam removed using a Pasteur pipette before placing the cassette into the Sysmex XT 4000 haematology analyzer compartment. The analyzer was then calibrated after which controls were run and passed before running the specimens.

3.7.4. Peripheral blood Smear

Blood films were made by placing a drop of blood from an EDTA container on one end of a slide, and using a spreader slide dispersed the blood over the slide's length. The slide was left to air dry, after which the blood was fixed to the slide by immersing it briefly in methanol. After fixation, the slide was stained using May-Grunwald Giemsa stain and transferred into buffer water. It was left to air dry and later examined the body of smear where the cells were lined singly and evenly distributed.

3.8. Ethical considerations and permissions

3.8.1. Ethical considerations

Ethical clearance was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) before the commencement of the study. Patient information and results were confidential and access to this information was restricted to the researcher and supervisors. There was no direct contact with the patient as only routine samples were used for the study. Demographic data such as age, sex, clinical data and vitamin supplementations were extracted from the files. The specimen container or any other material for the patients was assigned a serial number hence they were identified by a unique study identifier. In no way was the participant's name or file number linked to the specimen or research results.

3.8.2. Ethical permission

The research proposal was submitted to the University of Zambia Biomedical Research Ethics Committee (UNZA-BREC) for approval and was approved Assurance No.FWA00000338 IRB00001131 of IORG0000774, Ref. No.010-11-15 obtained on 4th February, 2016

Permission to conduct the study in the Haematology laboratory was sought from the Medical Superintendent at the University Teaching Hospital.

Permission to use equipment and facilities in the Department of Pathology and Microbiology in the UTH was obtained from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital, and permission to use the ELISA equipment and laboratory facilities in the KS Research Laboratory was obtained from the KS Research Laboratory Head of Department.

3.9. Data analysis

Data was analysed with IBM SPSS Statistical version 21 for Mac and Microsoft Excel 2011 for Mac and results summarized onto tables and graphs. Data was expressed as mean ± SEM for normally distributed continuous variables or median (interquartile range) for non-normally distributed variables. Normality was assessed using the Shapiro and Wilk statistic and the normality plots.

The *t*-test was used to compare values of plasma vitamin B12 concentration and folate concentration between the two groups (Megaloblastic anaemia vs. Non anaemic group), and any other possible confounder. All statistical tests were performed at 5% significance level or 95% confidence interval with p-value of <0.05 to determine statistical significance.

Bivariate linear regression and correlation coefficients were used to assess correlation between MCV, plasma vitamin B12 and folate levels in megaloblastic anaemia. The Bivariate linear regression data on MCV vs. vitamin B12 and MCV vs folate was plotted and presented on scatter graphs.

Chapter 4: Results

4.1. Vitamin B12 and Folate Concentration Differences

The study found that megaloblastic anaemia participants had statistically significant lower median vitamin B12 concentration 175(10-812) pg/ml than non-anaemic control participants 299.5(114-897) pg/ml) p=0.0001 (Fig. 3A and Table 2). Megaloblastic anaemia participants also had a statistically significant lower mean folate concentration (12.32±2.28 ng/ml) than non-anaemic control participants (19.28 ± 2.84 ng/ml)p=0.029 (Fig. 3B and Table 2).

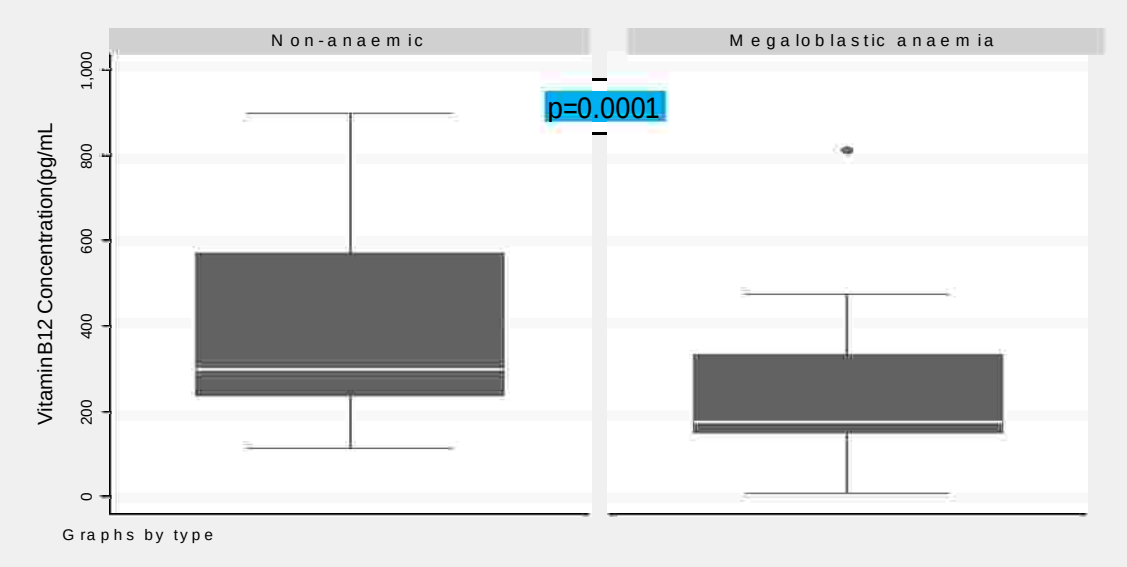

Figure 3A: Median Vitamin B12 concentration for megaloblastic anaemia participants 175(150-333) pg/ml was lower than for the non-anaemic control participants 299.5(238-571)pg/ml.

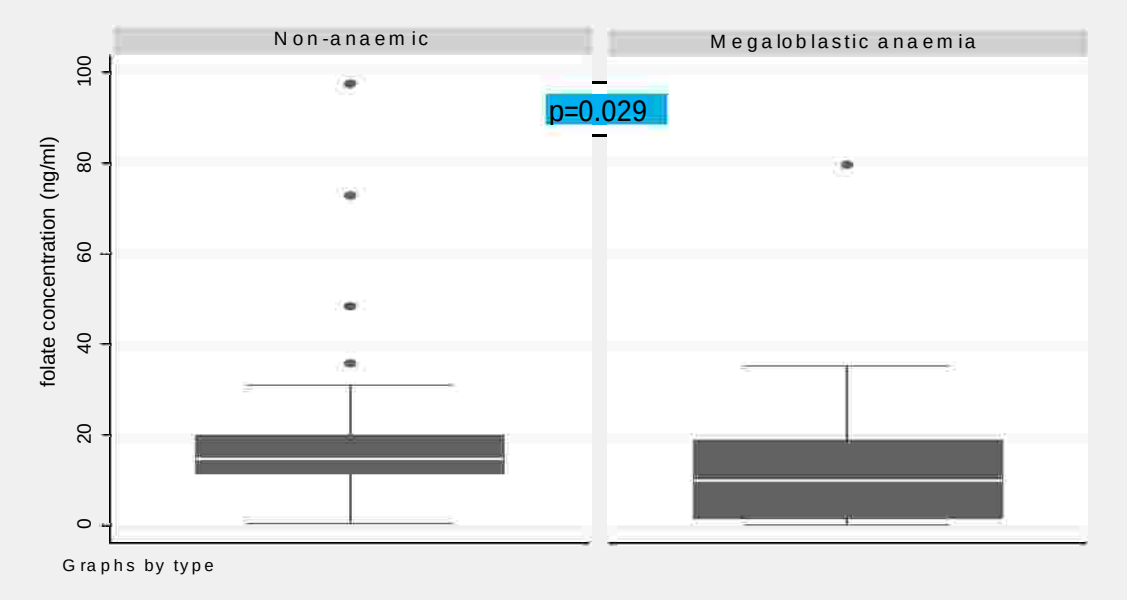

Figure 3B: Mean Folate concentration for megaloblastic anaemia participants (12.32± 2.28ng/ml)was lower than for the non- anaemic control participants (19.28 ± 2.84 ng/ml).

TABLE 2: Comparisons for Vitamin B12 and Folate between groups

Variable	Megaloblastic anaemia	Non anaemic controls	P-value
Vitamin B12	175(150-333)	299.5(238-571)	0.0001
Folate	12.32± 2.28	19.28 ± 2.84	0.029

Table 2: Comparison of p-values for vitamin B12 and folate concentrations between megaloblastic anaemia and non-anaemic control groups significant at the 0.05 level. Vitamin B12 in megaloblastic anaemia was significantly lower than in non-anaemic controls. Folate in megaloblastic anaemia participants was significantly lower than in non-anaemic controls.

4.2. Laboratory and demographic characteristics in megaloblastic anaemia

The study found that out of the 40 megaloblastic anaemia participants who were analysed,full blood count results revealed that bicytopenia was present in 22.5% (9/40) and pancytopenia in 72.5% (29/40) patients. Vitamin B12 deficiency was present in 60% (24/40), folate deficiency was present in 30% (12/40) and combined deficiency in 15% (6/40) (Fig. 4A).

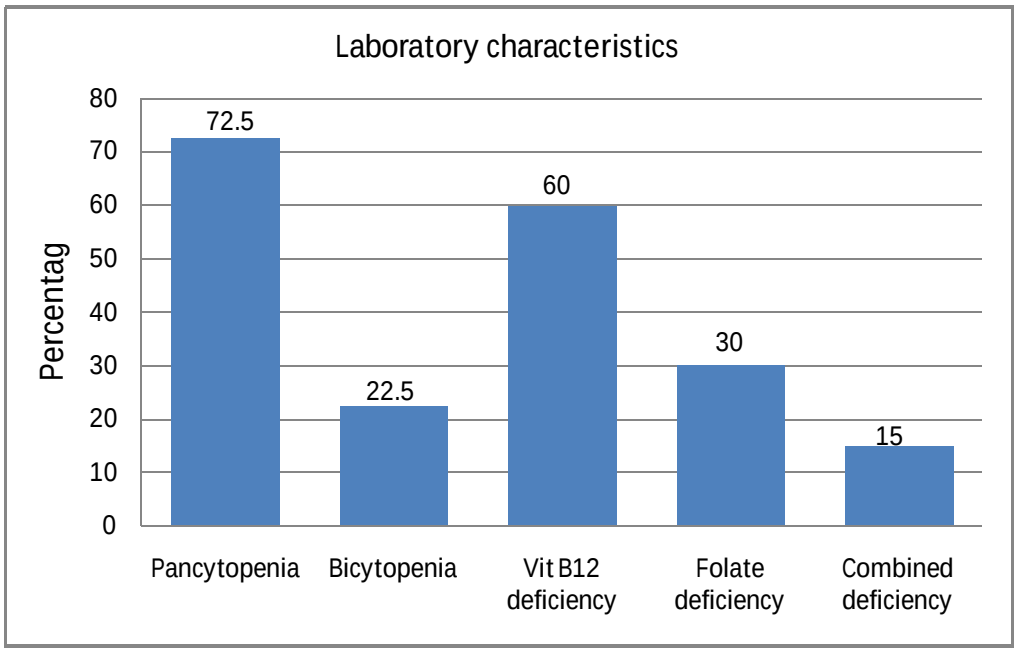

Figure 4A. Laboratory characteristics showed that vitamin B12 was the main cause of megaloblastic anaemia in our participants.

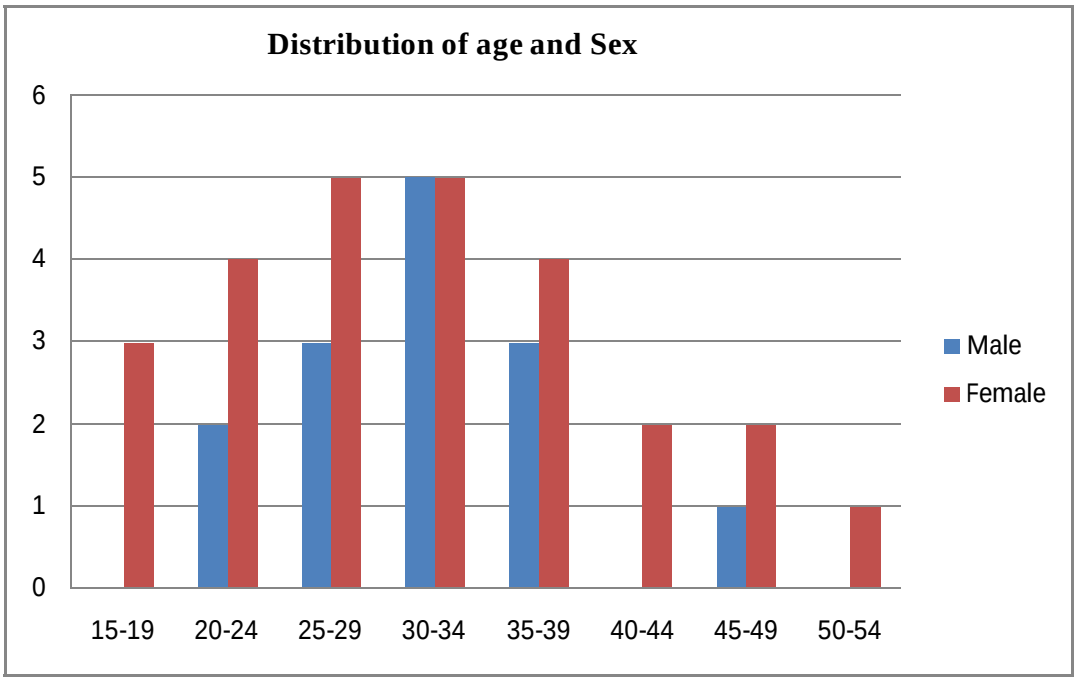

Fig 4B: Demographics characteristics showed that age group between 30-34 was most affected with megaloblastic anaemia and it was equally distributed among men and women. Overall distribution was 35% (14/40) male and 65% (26/40) female.

4.3. Linear Regression of MCV vs. Vitamin B12 and MCV vs. Folate

Bivariate linear regression analysis of MCV vs. Vitamin B12 showed a negative correlation and statistically significant (r= -0.0278, p =0.001) (Fig. 5A and Table 4). MCV vs. Folate showed a negative correlation and statistically not significant (r = -0.098, p = 0.326) (Fig. 5B and Table 3).

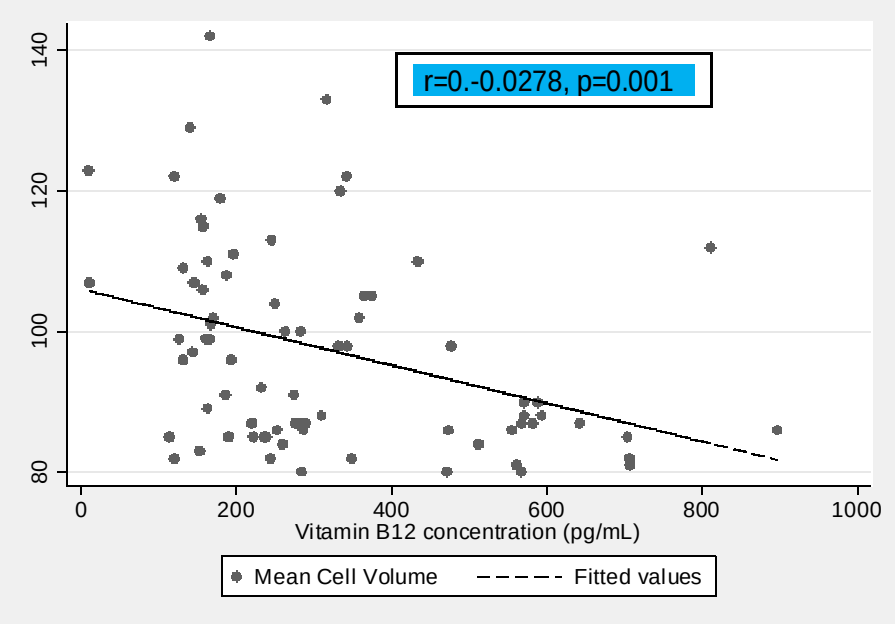

Figure 5A: There was a weak negative correlation between mean cell volume and vitamin B12 concentration. Vitamin B12 was inversely proportion with MCV.

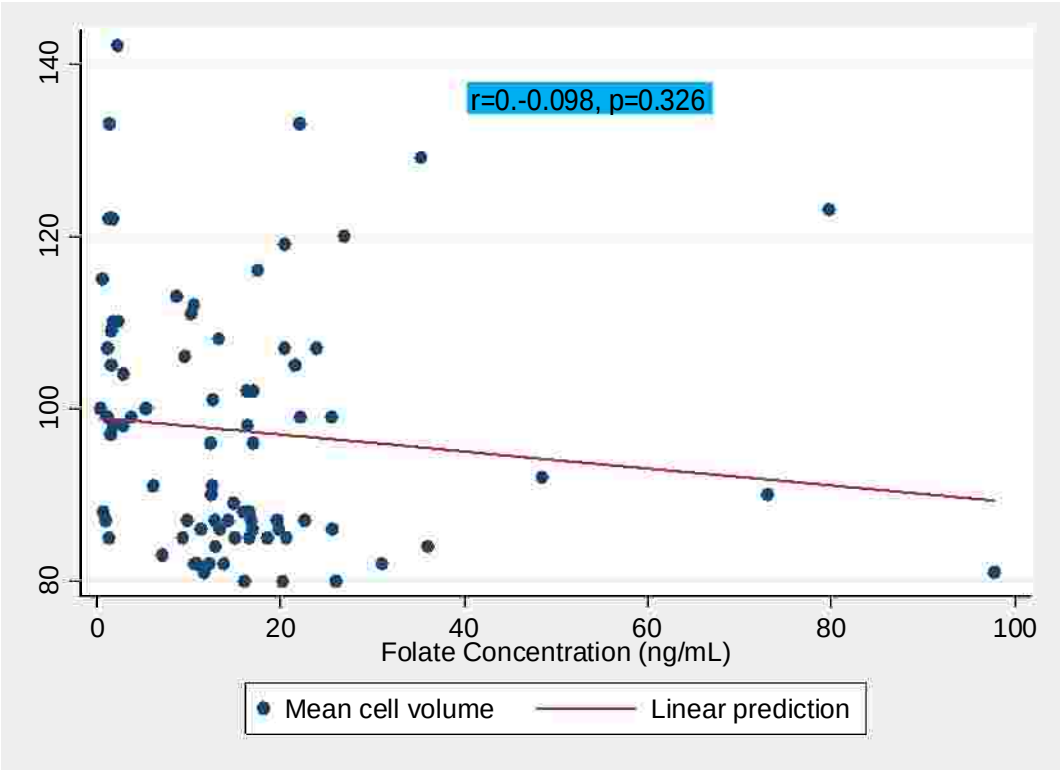

Figure 5B: There was a weak correlation between mean cell volume and folate concentrate. Folate was inversely proportion with MCV

Table 3: Linear Regression of MCV vs. Vitamin B12 and MCV vs. Folate

Independent variable	**Dependent variable**	**R value**	**P=value**
Vitamin B12	Mean cell volume	-0.0278	0.001
Folate	Mean cell volume	-0.098	0.326

Chapter 5: Discussion

Vitamin B12 and folic acid are essential dietary components for humans because they are required for DNA synthesis (Erlgy *et al*, 1985). Deficiency of vitamin B12 and folate disturbs the rapid proliferation of bone marrow with the resultant ineffective erythropoiesis which causes the formation of large immature red blood cells known as megaloblasts (Erlgy *et al*, 1985). Haematopoietic cells having a very high turnover are especially sensitive to deficiencies of folate and vitamin B12.With derangement of DNA synthesis due to deficiencies of these vitamins megaloblastic anaemia ensues.

Vitamin B12

In this study, it was observed that Vitamin B12 concentration in the megaloblastic anaemia patients was significantly lower (175pg/ml) compared to the non-anaemic controls, (299.5pg/ml), p=0.0001(Fig. 3A and Table 2). There was a significant difference between the two groups as shown by the p-value. This finding is similar to the findings of the study done in the USA by Lindenbaum *et al* in which the subjects had a lower vitamin B12 compared to the controls with a p=0.001. A decrease in vitamin B12 is thought to be caused by dietary lack, terminal ileum surgery, intrinsic factor deficiency, Helicobacter pylori infection, pancreatitis and bacterial overgrowth syndrome. Among these the commonest cause is deficiency of intrinsic factor (IF) to which vitamin B12 binds before it is absorbed in the tissue. There is an autoimmune attack on the gastric mucosa leading to atrophy of the stomach. The wall of the stomach becomes thin with the plasma cell and lymphoid infiltrate of lamina propria. There is achlorhydria and secretion of IF is absent (Hoffbrand *et al.*, 2006).

In our study it was revealed that 60% (Fig. 4A) of megaloblastic anaemia patients had vitamin B 12 deficiency. This is consistent with a recent study conducted in 95 subjects in Pakistan in which vitamin B12 deficiency was reported in 72.6% of the study population (Almed *et al.*, 2012). In a retrospective study conducted by Iqbal et al at Alhl Khan Hospital, Karachi, vitamin B12 deficiency in vegetarians and non-vegetarians was 78% and 85% respectively. It has been demonstrated from other studies that even in the

absence of anaemia that vitamin B12 deficiency may have deleterious effects in the nervous system (Healton *et al.,* 1992). Severe vitamin B12 deficiency can cause progressive neuropathy affecting the peripheral sensory nerves and potential lateral column damage (Hoffbrand *et al.,* 2006). Neuropathy is symmetrical and affects the lower limbs more than the upper limbs.

In this study among the cases who exhibited megaloblastic changes in the blood, 10% neither had any deficiency. This could have been attributed to transcobalamin deficiency which is an essential plasma protein responsible for transferring vitamin B12 in the cell of the bone marrow and other tissue. Transcobalamin deficiency causes megaloblastic anaemia because of failure of vitamin B12 to enter the marrow from the plasma but the plasma vitamin B12 levels are normal (Hoffbrand *et al.,* 2006).

We are mindful of the fact that the inclusion of patients in this study was based on the high MCV (95fl); however concomitant deficiency of iron (which is common in Zambia) would impair identification of several cases of vitamin B12 or folate deficiency on the basis of macrocytosis only. (Stevens *et al.,* 2013, Chang *et al.,* 2007). Applying world health organization (WHO) criteria, 92.5% of the cases were found to be severely anaemic (Hb ?8.0g/dl). This indicates that all the patients came to the hospital when the disease had already worsened and could have had these deficiencies for several months. With apparently vitamin B12 deficiency close to 10% in the non-anaemic controls, it is suggested the plasma vitamin B12 screening should be considered for individuals showing minor symptoms of deficiency. Studies conducted by Lindebaum *et a*l have shown an association of neuropsychiatric disorder with vitamin B12 deficiency in the absence of anaemia or macrocytosis. Early screening for vitamin B12 would prevent the high cost of late treatment of irreversible neuropsychiatric disorders arising from vitamin B12 deficiency (Robert *et al,* 2003).

Folate

The study showed that mean plasma folate concentration in megaloblastic anaemia was significantly lower (12.32±2.28ng/ml) compared to the non-anaemic participants (19.28±2.84) p=0.029 (Fig.3B and Table 2). This is thought to have been due to a poor

dietary intake of folate alone or a combination with a condition of increased folate utilization or malabsorption. Gastritis, nausea and vomiting were present in most of the patients. The lining of the gastrointestinal tract becomes atrophic in megaloblastosis (Marcuard *et al.,* 1994). Atrophic mucosa and subsequent malabsorption of vitamins worsen megaloblastic anaemia. A history of gastritis could have led to the intake of acid –suppressing medication (H_2 receptor antagonist and proton inhibitor). The drug plays a role in malabsorption (Marcuard *et al.,* 1994).

A hospital based study conducted in Zimbabwe reported 86.1% vitamin B12 deficiency and 5% folate deficiency (Salvage et al, 2008). This was at variance with our study which had 60% vitamin B12 deficiency and 30% folate deficiency (Table 3). Folate deficiency in our study could have been attributed to increased demand, when the dietary folate intake is inadequate. Another hospital based study done; Hashim *et al* reported 76% frequency of folate and vitamin B12 deficiencies. The results are not consistent with the present study. The difference most probably is because of the small sample size (n=50) and the dietary habits of the two populations.

Based on the western literature, there is a perception that folate deficiency is the main cause of megaloblastic anaemia (Khanduri *et* al., 2007). This is at variance with our study which only accounted for 30%. Studies suggest that folate status may play a role in depression (Coppen *et* al., 2005).The role in depression is due to their role in transmethylating reactions which are crucial for the formation of neurotransmitters (e.g. serotonin, epinephrine and purines). Low levels of folate or vitamin B12 can disrupt transmethylation reactions leading to an accumulation of homocysteine (hyperhomocysteinamia) and to impaired metabolism of neurotransmitters (phospholipids, myline and receptors). High homocysteine levels in the blood can lead to vascular injury by oxidative mechanisms which contribute to cerebral dysfunction. All these can lead to the development of various disorders including depression (Coppen *et al.*, 2005).

Laboratory and demographic characteristics

In this study most of megaloblastic patients had either bicytopenia 22.5% or pancytopenia 72.5 %.(Fig. 4A). These findings in are consistent with the findings of study performed by Uma *et al* in which 62% patient had pancytopenia showing megaloblastic anaemia to be the most prevalent and cause of pancytopenia. Similarly comparable studies of salvage et al and Iqbal et al all showed that megaloblastic anaemia was a major cause of pancytopenia. This is attributed to asynchrony between the maturation of cytoplasm and nuclei which lead to macrocytosis, immature nuclei and hyper segmentation in granulocytes in the peripheral blood. In megaloblastic cells, there is a delayed maturation of nuclei with normal cytoplasm development. The bone marrow becomes hypercellular and dysplastic mimicking acute leukaemia. The ineffective erythropoiesis results in intramedullary haemolysis and release of lactate dehydrogenase (Stable, 2013).The premature death of cells which decreases the output of cells from the bone marrow leads to pancytopenia.

The study participants were between 18 – 54 years (Mean age-31 years). Among the 40 megaloblastic patients, 35% (14/40) were male and 65% (26/40) were female with a male to female ratio of 1:1.9. (Fig. 4B). In Caucasian and Chinese populations megaloblastic anaemia is reported to occur in older groups with an equal sex ratio or male predominance (Cha *et al.*, 1998).In contrast our study revealed 1:1.9 sex ratio and a predominance of female. This could be due to increased demand during growth spurt, puberty and child bearing.

Correlation between MCV, vitamin B12 and folate

The study demonstrated that there was a weak negative correlation between vitamin B12 and mean cell volume. Linear regression results showed that mean cell volume was inversely proportional to vitamin B12. (r= -0.0278, p =0.001) (Fig. 5A and Table 3).This was at variant with a study done by Nizamani *et al* which also showed a significant negative correlation between vitamin B12 and MCV (r = -0.79, p=0.0001). However, macrocytosis can occur when there is increased RBC production secondary to peripheral blood cell destruction (i.e., haemolysis) or loss (i.e., haemorrhage), leading to

reticulocytosis. Reticulocytes are incompletely processed RBCs and, therefore, are slightly larger than the average RBC (Ramsey et al, 2007). Punneeta *et al* in India which both patients who had macrocytosis or microcytosis had vitamin B12 deficiency. Calmel *et al* also did show in his study that iron deficiency is associated with megaloblastic anaemia.

The study also demonstrated that folate had a negative correlation with MCV but not statistically significant (r = -0.098, p = 0.326) (Fig. 5B and Table 3). This was at variance with the study that was done by Iqbal *et al* which showed that folate was inversely related with the MCV. Linear regression analysis showed that serum folate was inversely related with the mean corpuscular volume (MCV, p=0.04). Spearman's correlation analysis indicated an inverse mild association between MCV and folate (correlation coefficient= -0.18).This could have been attributed to the small proportion of participants who were folate deficient in our study as compared to the 43.4% in their study.

5.1. Conclusion

This study showed that the levels of vitamin B12 and folate were lower in patients with megaloblastic anaemia, diagnosed morphologically, presenting at the University Teaching Hospital. The predominant deficiency was vitamin B12 which was the major factor leading to megaloblastic anaemia. It was further shown that pancytopenia which is a laboratory feature of megaloblastic anaemia was the most frequent feature of patients presenting with megaloblastic anaemia at the UTH, Lusaka Zambia.

5.2 Implication and Recommendation

Megaloblastic anaemia has been recognized as a clinical entity for over a century. Severe vitamin B12 deficiency can cause a progressive neuropathy affecting the peripheral sensory nerves and posterior and lateral columns. Folate deficiency in the mother predisposes to neural tube defect (NTD) (anencephaly, spina bifida or encephalocoele) in

the foetus. Sterility is frequent in either sex with severe vitamin B12 or folate deficiency (Hoffbrand *et al.*, 2006).

It is particularly important to detect folate deficiency in women in the child-bearing age group because low maternal folate status is associated with a significantly increased risk for neural tube defects. For a laboratory diagnosis of megaloblastic anaemia, a full blood count with red cell indices, examination of a well stained blood film and assay of the 2 vitamins are sufficient to make a definitive diagnosis. The findings of this study also suggest that megaloblastic anaemia must be an important differential diagnosis in patients presenting with pancytopenia.

5.3. Limitations/ Weaknesses and Assumptions

- The study did not have a follow up programme to assess the implications of low levels of vitamin B12 and folate in megaloblastic anaemia patients. A follow up programme could have provided morbidity and mortality data for our participants.
- With the vitamin B12 and folate result obtained, it could have been better to run a profile of methylmalonic acid and homocysteine in order to compare the results and see if they correspond with each other.
- The study did not provide any data pertaining to incidence and prevalence of megaloblastic anaemia in Zambia.
- Some record files were not completely filled in.

5.4. Future Directions

With respect to the above considerations, more supportive and definitive investigations are required. Follow up studies may be warranted to determine possible underlying causes of megaloblastic anaemia such as autoimmune diseases, pernicious anaemia and malabsorption syndromes.

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Chapter 7: Appendices

Appendix 1: Inclusion criteria explained

- Laboratory confirmed cases with megaloblastic anaemia were included in the study.
- Patients who were on treatment for human immunodeficiency virus (HIV) with reverse transcriptase inhibitor were excluded as this causes macrocytosis because they interfere with DNA production which may lead to megaloblastic changes.
- All patients with laboratory confirmed megaloblastic anaemia but on vitamin B12 or folate supplementation were excluded as this would interfere with our analysis.
- All patients on methotrexate were excluded as they antagonize with the function of folate resulting in megaloblastic changes.
- Blood samples with an MCV of >95fl but rouleaux formation seen on smears were excluded as this could have been due to cold agglutinin.

Appendix 2: Table 4 Megaloblastic anaemia Raw Data

Participant ID	Sex	Age	HB	WBC	RBC	PLT	MCV	Vitamin B12 pg/mL	Folate ng/mL
1	F	24	3.2	3.03	0.66	34	142	166	2.23
2	F	35	4.1	4.43	2.8	334	110	163	2.26
3	F	18	4.6	4.33	1.66	38	110	434	1.67
4	F	22	9.5	8.29	3.28	127	98	332	2.79
5	M	22	8.1	2.79	2.66	67	107	145	23.93
6	F	44	4.4	2.28	121	55	107	11	1.1
7	F	36	8.1	2.24	2.19	7	108	187	13.23
8	M	27	7.7	2.44	2.41	84	97	144	1.4
9	F	28	6.1	1.19	2.29	44	107	146	20.39
10	M	27	9.5	2.4	3.64	68	104	250	2.78
11	F	18	4.3	3.28	1.54	67	99	167	25.45
12	M	28	5.8	3.99	1.75	82	100	283	5.3
13	M	31	4.8	3.35	1.18	99	119	179	20.44
14	F	34	9.2	9.77	3.12	215	112	812	10.47
15	F	26	5.5	1.68	2.13	87	99	163	1.05
16	F	19	2	3.55	0.55	55	123	10	79.68
17	F	32	1.7	2.83	0.45	30	116	155	17.47
18	F	36	4.4	1.12	1.45	96	102	359	16.25
19	F	25	9.8	3.14	2.4	78	99	161	3.7
20	M	32	4	2.13	1.22	68	98	477	1.67
21	F	54	3.9	4.28	1.17	87	111	197	10.21
22	M	28	2.8	3.19	0.91	95	115	157	0.65
23	M	35	3.3	1.06	1.03	25	105	375	1.45
24	F	32	1.6	2.04	1.67	80	122	343	1.22
25	F	41	1.7	3.92	0.44	31	129	141	35.29
26	F	36	4.1	6.7	1.1	39	122	120	1.67
27	M	34	1.2	2.53	1.2	43	120	334	26.9
28	M	32	5.3	2.69	1.64	42	105	365	21.48
29	M	32	5	2.55	1.55	56	100	263	0.34
30	F	23	2.8	1.3	1.64	50	106	157	9.54
31	F	32	3.4	5.85	2.82	86	133	435	1.27
32	M	22	4.4	6.68	1.72	22	96	193	12.33
33	M	36	3.4	1.42	2.34	87	133	317	22
34	F	20	3.6	1.52	2.54	76	102	171	17.03
35	F	30	1.7	4.83	1.77	87	96	132	17
36	F	37	5.3	3.95	1.03	70	98	344	16.35
37	M	45	4	2.46	1.08	36	113	246	8.61
38	M	36	0.85	6.31	0.84	91	109	132	1.49
39	F	23	4.4	1.35	0.98	98	99	127	22.06
40	F	46	9.9	1.37	2.88	60	101	168	12.61

Appendix 3: Table 5 Non anaemic control Raw Data

Participant ID	Sex	Age	HB	WBC	RBC	PLT	MCV	Vitamin B12 pg/mL	Folate ng/mL
100	F	21	13.9	5.95	4.89	326	87	568	14.29
101	F	21	12.5	9	4.51	285	87	220	12.77
103	F	17	12.8	5.74	4.7	197	84	260	12.93
104	F	26	13.2	6.07	4.82	215	86	287	16.87
105	F	33	13.1	8.74	4.95	224	87	582	9.81
106	M	38	13.4	6.98	5.2	172	85	114	16.62
107	M	26	16.2	7.84	5.54	182	86	252	25.62
108	F	16	13.4	5.86	4.62	301	87	280	0.94
109	F	47	12.9	4.55	4.31	304	91	186	12.46
110	F	21	15.1	4.81	5.43	305	86	897	11.27
111	F	23	12.9	7.17	4.65	316	85	240	20.59
112	M	27	15	8.06	5.33	278	85	704	14.97
113	M	31	15.2	7.14	6.14	234	80	471	25.99
114	F	27	13.1	4.01	4.53	139	88	571	16.6
115	F	25	12.6	9	4.48	209	87	276	16.84
116	M	32	15.6	9.08	5.41	343	88	594	0.61
117	F	20	13	6.33	4.56	285	89	163	14.76
118	F	32	13.1	8.22	4.71	305	91	275	6.14
119	F	23	12.5	8.1	4.41	237	82	706	13.83
120	M	20	13.8	5.05	4.7	152	88	310	15.91
121	M	28	14	5.02	4.8	240	82	245	10.8
122	M	32	16.4	7.26	5.39	257	92	233	48.47
123	F	40	12.5	5.63	4.16	212	90	588	73.03
124	F	18	13.5	5.68	4.82	290	85	236	1.18
125	M	31	14.2	7.01	5	213	80	568	20.24
126	M	32	14.5	6.12	5.1	261	82	708	12.17
127	M	37	13.5	6.18	4.74	351	83	153	7.08
128	M	33	16.1	6.57	6.55	288	87	289	19.59
129	F	26	13.6	4.99	4.67	255	87	643	22.59
130	F	39	14	6.43	4.88	325	85	190	18.48
131	M	43	14.5	5.23	5.02	207	90	571	12.44
132	F	59	14.2	4.28	4.89	153	86	555	19.82
133	M	35	16.1	4.76	5.75	259	86	707	97.74
134	F	39	12.7	6.1	4.61	303	82	349	30.91
135	M	42	15.5	8.37	5.47	350	85	222	9.34
136	F	28	12.9	6.72	4.7	302	81	562	11.61
137	F	31	13.9	5.47	4.8	283	80	285	16.04
138	F	34	13.5	9.46	4.8	214	82	120	10.64
139	F	32	12.9	5.37	4.55	227	84	512	35.96
140	F	41	13.8	4.41	4.79	340	86	474	13.41

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02nd October, 2015

Mr. Jacob Ndhlovu
Department of Pathology & Microbiology
School of Medicine
UNZA
LUSAKA

Dear Mr. Ndhlovu,

RE: GRADUATE PROPOSAL PRESENTATION FORUM

Following the presentation of your dissertation entitled **"Evaluation of Vitamin B12 and Folate Levels in Megaloblastic Anaemias at the University Teaching Hospital, Lusaka, Zambia"** your supervisor has confirmed that the necessary corrections to your research proposal have been done.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Assurance No. FWA00000338
IRB00001131 of IORG0000774

4th February, 2016.

Our Ref: 010-11-15.

Mr. Jacob Ndhlovu,
University of Zambia,
School of Medicine,
Department of Pathology and Microbiology,
P.O Box 50110,
Lusaka.

Dear Mr. Ndhlovu,

RE: RESUBMITTED RESEARCH PROPOSAL "EVALUATION OF VITAMIN B12 AND FOLATE LEVELS IN MEGALOBLASTIC ANAEMIA AT THE UNIVERSITY TEACHING HOSPITAL ZAMBIA" (REF. No. 010-11-15)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 28th January, 2016. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

M.C Maimbolwa PhD
CHAIRPERSON

Date of approval: 4th February, 2016. **Date of expiry:** 3rd February, 2017.

University of Zambia
School of Medicine
Department of Pathology and Microbiology
LUSAKA,

21TH October 2015.

REPUBLIC OF ZAMBIA
SENIOR MEDICAL SUPERINTENDENT
22 OCT 2015
UNIVERSITY TEACHING HOSPITAL
PRIVATE BAG RW1X, LUSAKA

The Senior Medical Superintendent
University Teaching Hospital
LUSAKA.

Dear Sir,

RE: REQUEST FOR PERMISSION TO CONDUCT THE RESEARCH PROJECT AT THE UNIVERSITY TEACHING HOSPITAL (UTH).

I am a student pursuing a master of science in pathology (haematology) at the University of Zambia, school of medicine.

Am writing to request for permission to conduct my research at your institution titled **"Evaluation of Vitamin B12 and Folate Levels in Megaloblastic Anaemia at the University Teaching Hospital, Lusaka, Zambia".**

Please find attached a copy of my project proposal containing all the necessary information. The school has approved the research proposal and Ethical approval will be sought from UNZABREC upon your consideration of my request.

Yours sincerely

Jacob Ndhlovu.

Approved
Principal Pharmacist
PMA