

Vitamin B12 and Folate deficiency in Megaloblastic Anaemia diagnosed morphologically at the University Teaching Hospital, Lusaka, Zambia.

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ABSTRACT

Background: Vitamin B12 and folate deficiency is a well-known health problem world-wide. 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. The study aimed to determine vitamin B12 and folate levels in megaloblastic anaemia, diagnosed morphologically, in patients at the University Teaching.

Methods: This was a cross sectional 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. Vitamin B12 and folate concentrations were compared between groups using t-test.

Results: 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 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.

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 which further implicates vitamin B12 and folate in the disease process of megaloblastic anaemia.

Key words: Megaloblastic anaemia, pancytopenia, folate deficiency, Vitamin B12 deficiency, Zambia

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 (Carmel *et al.*, 2004; Antony *et al.*, 2005).

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 tetrahydrofolate, which enters the cells from plasma, to tetrahydrofolate, 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 or folate levels in Zambian patients, whether normal levels or in relation to anaemia. This study aimed to evaluate vitamin B12 and folate levels in megaloblastic anaemia, diagnosed morphologically to add to the existing knowledge in literature on megaloblastic anaemia.

METHODS

Study area and population

The study was conducted at the University Teaching Hospital (UTH) from February, 2016 to June, 2016. The study population included patient samples that had an MCV of greater than 95fl. Patient samples of all ages with a laboratory diagnosis of megaloblastic anaemia were targeted.

Study design and Sampling method

This was a case control study involving 80 participants (40 cases and 40 controls). 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 were 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°C for analysis on a later date. The comparison group was selected by means of frequency matching of the same proportional characteristics of age and sex.

Inclusion Criteria

All samples with a mean corpuscular volume (MCV) of more than 95fl and peripheral blood smears consistent with megaloblastic anaemia (macrocytosis on peripheral smear, tear drop poikilocytosis and hypersegmented neutrophils) were included. A control group of samples with an MCV between the ranges of 76.8fl – 95.0fl were also included.

Specimen preparation and Quality Control

In the laboratory, each specimen serial number was recorded on to a compilation summary sheet. Thereafter a full blood count done and peripheral smear were 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°C until the specimens were required for analysis.

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.

Vitamin B12 and Folate quantification

The Human Vitamin B12 and Folate ELISA (Enzyme-Linked Immuno-sorbent Assay), a quantitative competitive immunoassay manufactured and supplied by Neo-scientific group, United States of America was used.

Full Blood Count Test

Complete blood count tests were determined using Sysmex XT 4000 haematology analyzer manufactured by Sysmex Europe- supplied by Sonergy Diagnostics-Lusaka.

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 \pm 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.

Ethics Approval

This study was approved by the Zambia Biomedical Research Ethics Committee (UNZA BREC) (REF No.010-11-15) and permission to conduct the study was obtained from the UTH. 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.

RESULTS

Demographics characteristics showed that age group between 30-34 were most affected with megaloblastic anaemia. Overall it showed 35% (14/40) male and 65% (26/40) female

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% (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. 1).

Laboratory characteristics showed that vitamin B12 was the main cause of megaloblastic anaemia in our participants (Fig 2)

The study found that 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 $p=0.0001$ (Fig. 3). 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. 4).

DISCUSSION

Vitamin B12 and folic acid are essential dietary components for humans because they are required for DNA synthesis (Erslev *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 (Erslev *et al*, 1985). Hematopoietic 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.

The study showed that 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. (Fig 1). 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.

In this study most of megaloblastic patients had either bicytopenia 22.5% or pancytopenia 72.5% (Fig. 2). These findings are consistent with the findings of study performed by Khanduri *et al* in which 62% patient had pancytopenia showing megaloblastic anaemia to be the most

prevalent and cause of pancytopenia (Khanduri *et al.*, 2007). Similarly comparable studies of salvage *et al* and Iqbal *et al* all showed that megaloblastic anaemia was a major cause of pancytopenia (Savage *et al.*, 1994; Iqbal *et al.*, 2001). This is attributed to asynchrony between the maturation of cytoplasm and nuclei which lead to macrocytosis, immature nuclei and hypersegmentation 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 (Stabler., 2013). The ineffective erythropoiesis results in intramedullary haemolysis and release of lactate dehydrogenase (Stabler., 2013). Deficiency of folate or vitamin B12 (cobalamin) causes megaloblastic anemia, a disease characterized by pancytopenia due to the excessive apoptosis of hematopoietic progenitor cells (Mark *et al.*, 2000).

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. 3). 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 $p=0.001$ (Lindenbaum *et al.*, 1994). 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 2) of megaloblastic anaemia patients had vitamin B 12 deficiency. This is consistent with a recent study conducted in 95 subjects; 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 (Iqbal *et al.*, 2009). 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.*, 1988). Severe vitamin B12 deficiency could 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, 25% 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 (Chan *et al.*, 2007; Steven *et al.*, 2013). 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 al* have shown an association of neuropsychiatric disorder with vitamin B12 deficiency in the absence of anaemia or macrocytosis (Lindenbaum *et al.*, 1988). 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).

The study showed that the mean plasma folate concentration in megaloblastic anaemia was significantly lower (12.32 ± 2.28 ng/ml) compared to the non-anaemic (19.28 ± 2.84) participants $p=0.029$ (Fig.4). 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 lead to the intake of acid –suppressing medication (H₂ 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.*, 1994). This was at variance with our study which had 60% vitamin B12 deficiency and 30% folate deficiency (Table 2). 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 (Hashim *et al.*, 2006). 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% cases. Studies suggest that folate status may play a role in depression (Coppin *et al.*, 2005). The role in depression is due to their role in transmethylation reactions which are crucial for the formation of neurotransmitters (e.g. serotonin, epinephrine and purines) (Coppin *et al.*, 2005; Karakula *et al.*, 2009). Low levels of folate or vitamin B12 can disrupt transmethylation reactions leading to an accumulation of homocysteine (hyperhomocysteinemia) 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 (Coppin *et al.*, 2005; Karakula *et al.*, 2009).

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.

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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 B12 or folate deficiency (Hoffbrand *et al.*, 2006).

It is particularly important to detect vitamin B12 deficiency in women in the child-bearing age group because low maternal vitamin B12 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.

Limitation

A limitation for this study is that we 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. We could not do this in this study due to resource constraints and limited time.

List of abbreviations: MCV, Mean Corpuscular Volume; DNA, Deoxyribonucleic Acid; FBC, Full Blood Count; UTH, University Teaching Hospital; ELISA, Enzyme-Linked Immunosorbent Assay; DNTP, Deoxyribonucleoside Triphosphate; DATP, Deoxyadenosine

Triphosphate; DGTP, Deoxyguanosine Triphosphate; DCTP, Deoxycytidine Triphosphate; THF, Tetrahydrofolate.

Consent for publication

Not applicable

Availability of data and materials

All data analyzed in this study can be accessed on request to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Author contributions:

JN, FSK & TK conceived the study. JN performed data collection.

JN, FSK, TK, MS, SM, AM PN SM, CNP and MMM contributed to data analysis and interpretation. All authors read and approved the final manuscript.

SMM: was involved in experimental design, data analysis and interpretation, drafting and critical revision of manuscript, approved final draft.

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Fig 1: Demographic characteristic of megaloblastic anaemia

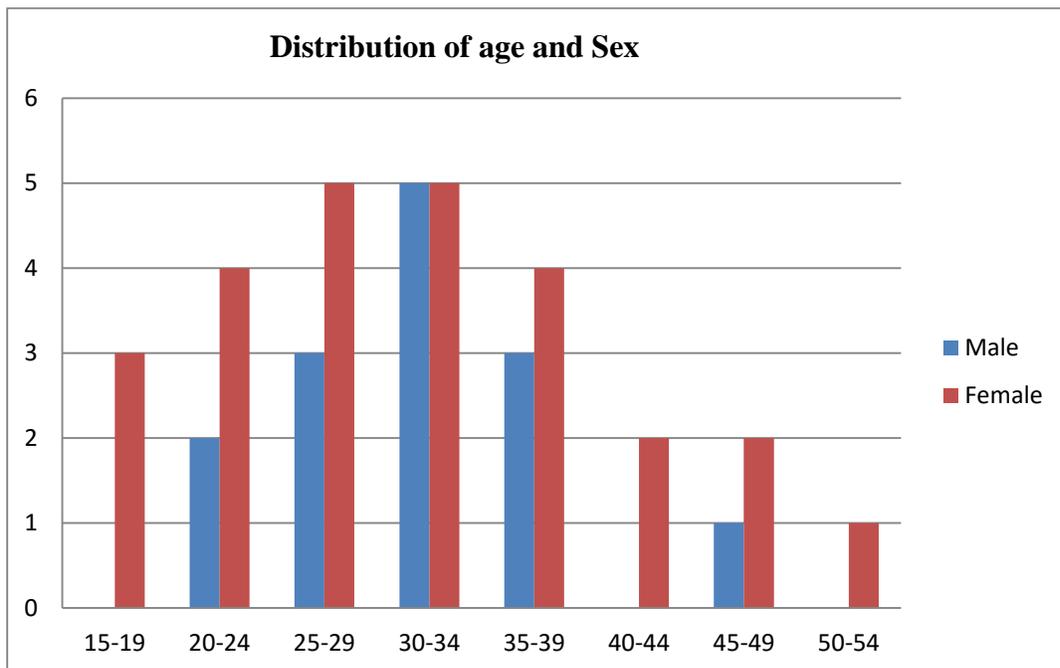
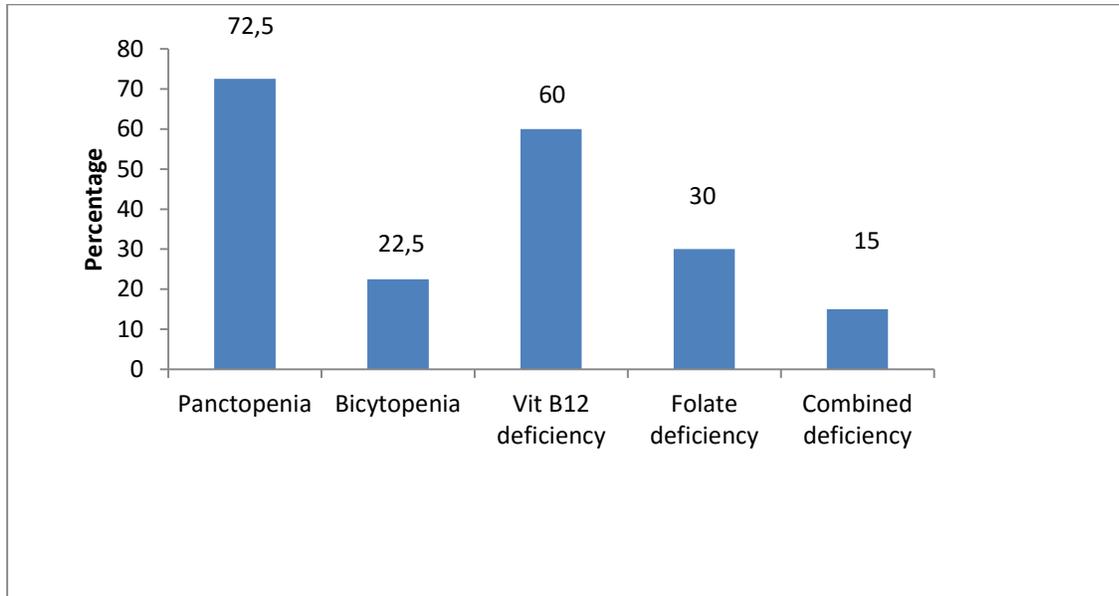


Fig 2: Laboratory characteristics



Vitamin B12 Median Concentrations

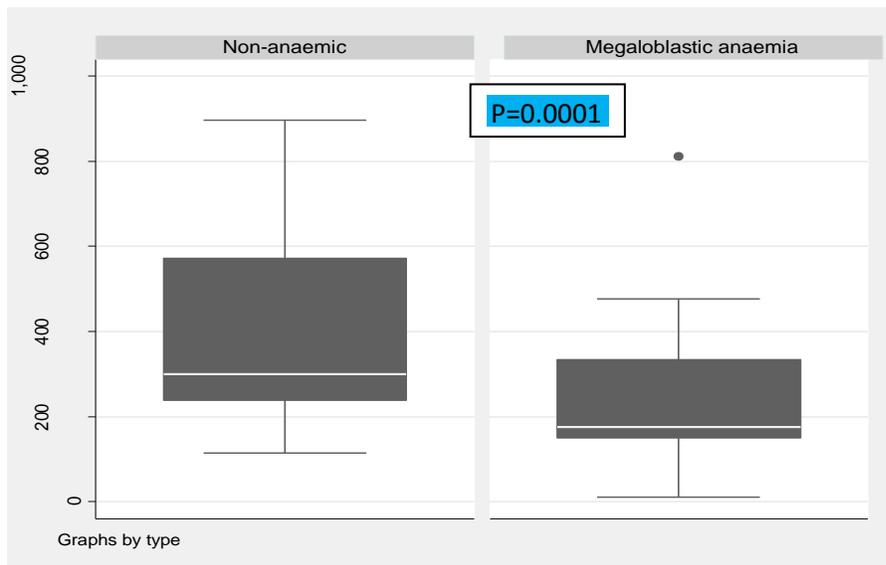


Figure 3: Median Vitamin B12 concentration for megaloblastic anaemia participants 175(150-333) pg/ml was lower than for the non-anaemic control participants 299.5(238-571)pg/ml.

Folate Mean Concentrations

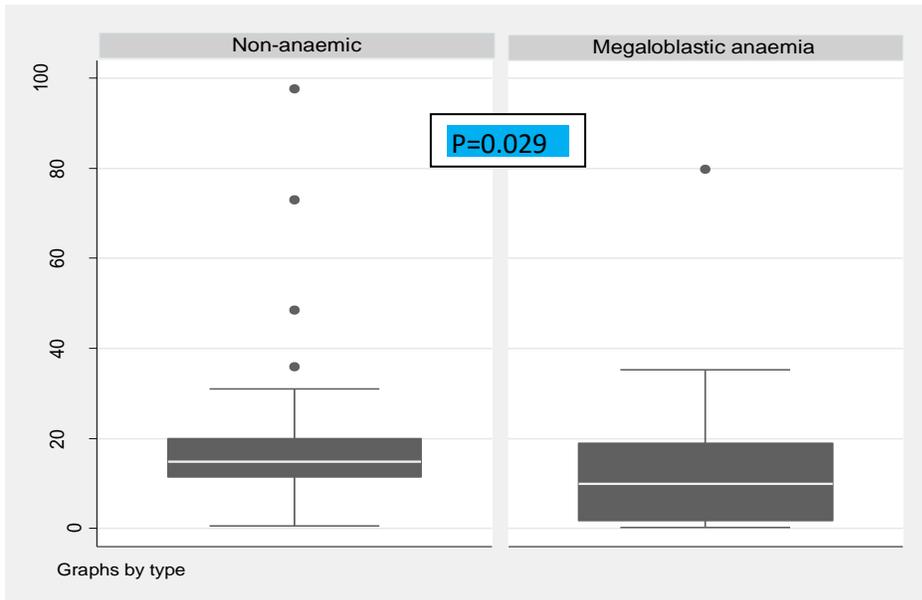


Figure 4: Mean Folate concentration for megaloblastic anaemia participants (12.32 ± 2.28 ng/ml) was lower than for the non- anaemic control participants (19.28 ± 2.84 ng/ml).