Vitamin A, B_{12}, folate, ferritin and transferrin status of second and third trimester anaemic pregnant women attending antenatal care in west Showa zone, Ambo Ethiopia.

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RESEARCH THESIS APPROVAL

This is to certify that the thesis prepared by ***Teshome Bekele Elema,*** entitled: ***Vitamin A, B12, Folate, Iron biomarker and C-reactive protein status of second and third trimester anaemic pregnant women attending antenatal care in west showa zone, Ambo Ethiopia*** and Submitted in partial fulfillment of requirements for the degree of Master of Science complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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DEDICATION

This work is dedicated to my father who let the ground for my current educational careers, but not alive today to see the finals

In Evergreen Loving Memory of my Dearly Beloved Father, **Bekele Elema (Gaashee).** You're eager to see my achievement and a Rare Gem gone too soon; I will never forget your esteem love and care you nurtured me, I really dearly miss you very much. May your Gentle Soul Continue to Rest in Perfect Eternal Peace in Bosom of the Lord, Amen.

STATEMENT OF AUTHOR

I declare that this thesis is my bona fide work and all sources of materials used for this thesis have been duly acknowledged. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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List of abbreviations

ACOG:	The American College of Obstetricians and Gynecologists
AGP:	1-acid glycoprotein
ANC:	Antenatal Care
APP:	Acute phase protein
B_{12}:	Vitamin B12
CBC:	Complete blood count
cCRP:	Cardiac C-reactive protein
CDC:	Center of Disease control and prevention
CI:	Confidence interval
CRP:	Complement reactive protein
CSA:	Central Statistical Agency
DHS:	Demographic and Health survey
dL:	Deciliter
DNA:	Deoxyribonucleic acid
EAR:	Estimated average requirement
ECLIA:	Electro chemuniluminescence immune assay
EDTA:	Ethylenediamine tetra acctic acid
ELISA:	Enzyme Linked Immunosorbent assay
EPHI:	Ethiopian Public health Institution
ESR:	Erythrocyte sedimentation rate
FAD:	Folic acid deficiency
FBP:	folate binding protein
FDA:	Food and Drug Administration
FDSN:	Food science and Nutrition
FMOH:	Federal Ministry of Health
GDP:	Gross domestic Product
GI:	Gastro intestinal
Hb:	Hemoglobin concentration
HCT:	Hematocrit
Hcy:	Homocysteine
HELLP:	H- hemolysis EL- elevated liver enzymes LP- low platelets counts
HH:	Hereditary Hemochromatosis

HPLC:	High Performance Liquid Chromatography
hsCRP:	High sensitivity C-reactive protein
IBC	Iron binding capacity
ID:	Iron deficiency
IDA:	Iron deficiency anaemia
IF:	Intrinsic Factor
IFA:	Iron-Folic acid
INACG:	International Nutritional Anemia Consultative Group
IRMA:	Immunoradiometric assay
IU:	International Unit
LDL:	Lower detection limit
LSD	Least Significant Difference
MCV:	Mean cell volume
MI:	Micronutrient Initiative
MMA:	Methylmalonic acid
MRDR:	Modified relative dose response test
NADPH:	Nicotinamide adenine dinucleotide phosphate oxidase
NBD:	Neural Birth defects
NCCLS:	National Committee for Clinical Laboratory Standards
NCP:	Nutrition care process
ng:	Nanogram
NNP:	National Nutrition program
NPC:	National Population Commission
PAHO:	Pan American Health Organization
PEM:	Protein-Energy malnutrition
PGA:	Pteroylglutamic acid
Qn-CRP:	Quantitative C-reactive protein
Qt-CRP:	Qualitative C-reactive protein
RBC:	Red blood cell
RCOHA:	Review of the cost of Hungry in Africa
RDA:	Recommended dietary allowance
RDR:	Relative dose response test
RI:	Reduced iron

RNA:	Ribonucleic acid
RNI:	Recommended nutrient intake
sf:	Serum ferritin
SIVIN:	Integrated Nutritional Surveillance System
SNNPRS	Southern Nation's and Nationalities People's Regional State
SPSS:	Statistical package Software System/ solution
sTfR:	Solube transferrin receptor
TB:	Tuberculosis
THF:	Tetra hydro folate
TIBC:	Total iron binding capacity
TSP	Triple Super Phosphate
VAD:	Vitamin A deficiency
VMNIS:	Vitamin and Mineral Nutrition Information System
WHO:	World Health Organization
WRA:	Women of reproductive Age

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Vitamin A, B12, folate, ferritin and transferrin status of second and third trimester anaemic pregnant women attending antenatal care in Ambo Health centers and Hospital.

Teshome Bekele Elema

Addis Ababa University, 2015

ABSTRACT

Iron, folate and vitamin B12 play important roles in the healthy development and brain function of the fetus. The levels of these micronutrients in pregnant women are influenced among other factors by dietary habits. The purpose of this study was to determine the level of iron biomarkers, serum retinol, B12, C-reactive protein (CRP) and folate in second and third trimester pregnancies attending antenatal care in the west showa, Oromia region. One hundred four anaemic pregnant women were screened during their 2nd and 3rd prenatal visit. A socio-demographic characteristic of study subjects were obtained and Hemoglobin (Hb), serum retinol, serum ferritin (sf), serum transferrin (sTfR), folate, retinol and vitamin B_{12} levels were evaluated. Based on WHO guidelines, anaemia was defined as severe Hb<7g/L, moderate 7-9.9g/L and mild 9.9-10.9g/L; ID as sf <12 µg/L and sTfR >8.3g/L. Serum folate and B_{12} deficiencies were defined as levels <6.8 ng/ml and 150 pg/ml respectively. The mean age and gestational week was 24.6±5 years and 23.3±5.6 weeks respectively. The mean concentrations of Hb in serum were 10.5±1.2 g/dL, 30.3±23.6 µg/L for sf, 5.4±2.8g/L for sTfR, 1.69±2.02µmol/L for retinol, 7.6±3.5ng/ml for folate, 187.4±53.7pg/ml for vitamin B12 and 4.5±1.6 mg/L for CRP. IDA was present in 32.7%, VitB_{12} deficency in 26.9% and folic acid deficiency in 27.9% of anaemic pregnant women. Vitamin B12 and serum transferrin were associated with CRP (r=0.189; P=0.015). Iron, vitamin A, vitamin B12, and folate deficiencies as well as infections were relatively common in the anaemic pregnant women. Vitamin A and vitamin B12 supplementation in addition to iron must be considered in pregnant women living in the study areas.

Key words: Anaemia, Hemoglobin, ferritin, folate, vitamin B12, Vitamin A, pregnancy

1. Introduction

1.1 Background

Anemia is a public health concern that affecting 2 billion people worldwide (Zimmerman *et al.*, 2005). Anaemia affects disproportionately women in developing countries, and is an independent risk factor for decreased quality of life, increased morbidity and mortality, particularly for pregnant women. According to WHO, about half of the anaemia is believed to be due to iron deficiency anaemia (IDA). IDA is ranked 9th on the list of risk factors contributing to the global burden of disease (WHO, 2008). However, besides iron deficiency, deficits in several other micronutrients including vitamin A, folate, vitamin B12, and possibly zinc can cause anaemia (Samuel *et al.*, 2013; Bhandari and Banjara, 2015 and WHO/Unicef, 2004).

Diets in most developing countries are predominantly plant-based with little consumption of animal-source foods. This leads to an increased risk of multiple micronutrient deficiencies, especially at lifestages like pregnancy when physiological demands are substantially increased (WHO/FAO, 2003 and FAO, 2004). Besides, such plant-based diets also contain significant amount of mineral absorption inhibitors like phytate and polyphenols that impair the bioavailability of micronutrients. Despite WHO's recommendation that pregnant women take a prophylactic dose of 30-60 mg of iron/iron-folic acid supplements, adherence to these recommendations, particularly in developing countries like Ethiopia have been very low (Gebremedhin *et al.*, 2014).

The first 1,000 days, starting from conception to the child's second birthday, is a critical window of opportunity for nutritional interventions that aim to improve pregnancy, birth, and developmental outcomes. This is a period when a child's brain, organs, and body is rapidly developing (Samuel, 2013). Anaemia during pregnancy has been associated with complication in pregnancy outcomes and greater risk of low birth weight, which itself has been linked to adverse physical and cognitive development. It is therefore, crucial to prevent and correct anemia during pregnancy.

1.2 Statement of the problem

In Ethiopia, the prevalence of anaemia among pregnant women was reported to be 22 % (DHS, 2011), which suggests that anaemia is a moderate public health concern (WHO/Unicef/UNU, 2001) Although iron/iron-folic acid (IFA) supplements are available and recommended by health personnel, the adherence has been extremely low (Gebremedhin *et al.,* 2014). Although anaemia could also be due to other micronutrient deficiencies, multiple micronutrient supplements are not yet proposed and available for pregnant women. More importantly, little is known on the etiology of anaemia in most developing countries including Ethiopia. In Ethiopia, the leading cause for the poor adherence to iron supplements was reported to be side effects (Gebremedhin *et al.,* 2014; Pavord *et al.,* 2012; SAPPG, 2013).

The investigation of the etiology of anaemia during pregnancy by assessing serum levels of multiple micronutrients is necessary to identify nutritional deficiencies with certainty (Johnson *et al.,* 2010; Pasricha *et al.,* 2010; Fleming, 1989 and van den Broek, 1996). The purpose of this study was to investigate the vitamin A, B12, iron biomarkers, folate and CRP status among anaemic pregnant women attending ANC in Oromia regional state of west showa zone, Ethiopia

1.3 Objective

1.3.1 General objective:

- To determine the serum retinol, ferritin, transferrin, folate and B12 status of anaemic 2nd and 3rd trimester pregnant women attending ANC in Ambo Health centers and Hospital.

1.3.2 Specific objectives

- To investigate the prevalence of iron deficiency, vitamin A, B12, and folate status of anaemic 2nd and 3rd trimester pregnant women attending ANC in Ambo Health centers and Hospital.
- To investigate the relationship between vitamin A, B12, iron biomarkers, folate, and CRP with hemoglobin level among study subjects.

2. Literature review

2.1 Iron

2.1.1 Health effects, absorption, transportation and excretion of iron

Health effects: Of the 3 to 5 g of iron in the body, approximately 2 to 2.5g of iron is in hemoglobin, mostly in RBCs and red cell precursors. A moderate amount (ca.130) mg is in myoglobin, the oxygen-carrying protein of muscle. A small, 8 mg, but extremely important, pool is in tissue where iron is bound to several enzymes that require iron for full activity. These include peroxidases, cytochromes, and many of the Krebs cycle enzymes (Michael *et al.,* 2010). Catalase for the breakdown of hydrogen peroxide and myeloperoxidase found in neutrophils both require iron as cofactor. The ribonucleotide reductase and xanthine oxidase involved in RNA and DNA metabolism both require iron as cofactor (Ghosh, 2006). Iron is also stored as ferritin and hemosiderin, primarily in the bone marrow, spleen, and liver. Only 3 to 5 mg of iron is found in plasma, almost all of it associated with transferrin, albumin, and free hemoglobin (Rossi, 2005).

There are many other enzymes that require iron, and without sufficient quantities, these enzymes are prevented from performing as they should in energy metabolism, growth and proliferation, biotransformation of drugs, myelinogenesis, cell differentiation, and nutrient absorption. Deficiencies of iron are associated with anaemia but also akathisia, or "restless leg syndrome" (Michael *et al.,* 2010). Increasing the iron supplies to these patients has led to improvement and a reduction in symptoms (Nuttall and Klee, 2001).

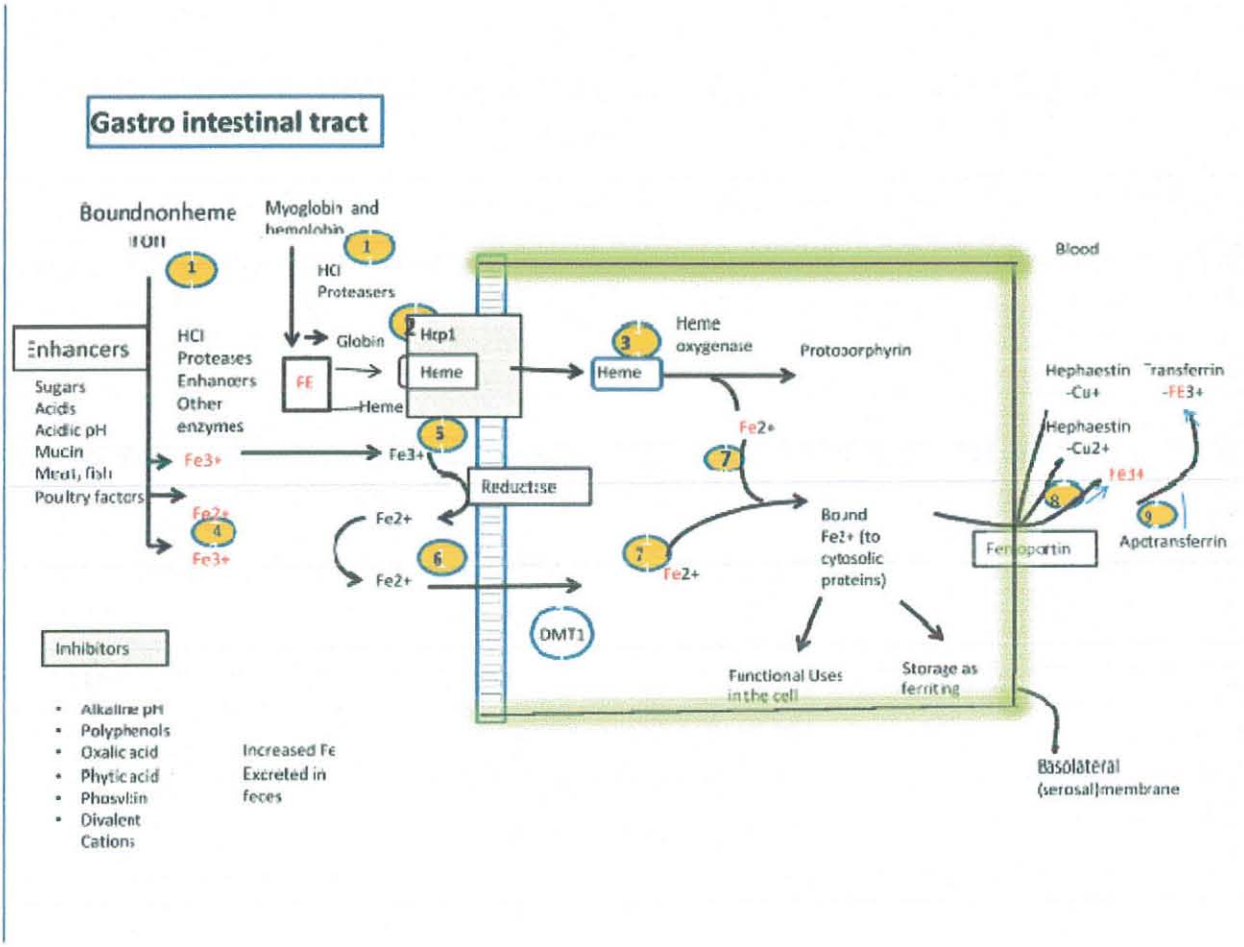
Iron is essential for life and a constituent of hemoglobin and other proteins, called enzymes. Iron participates in many important processes that facilitate oxygen transport and supply to the tissue and organs. In a healthy, well-nourished individual, iron balance occurs when the quantity of iron absorbed from the diet is sufficient to compensate daily iron loss, and to maintain adequate body iron stores. The iron balance can be disturbed by a variety of factors, such as low dietary iron intake, increased iron losses and increased iron requirements. A certain amount of iron is lost daily due to basal iron losses with epithelial cells from internal and external surfaces (Michael *et al.,* 2010). The Recommended daiy allowance (RDA) of iron ranges from 8gm/day for an adult over 51 years of age and to 18gm/day for 19–50 years of age females (Michael *et al.,* 2010; FNBIM, 2002; Underwood, 1990 and Bogden, 2000).

Absorption of iron from the intestine is the primary means of regulating the amount of iron within the body. Typically, only about 10% (1 g/day) of dietary iron is absorbed. To be absorbed by intestinal cells, iron must be in the form of Fe^{2+} (ferrous) oxidation state and bound to protein. Because $Fe^{3+}$is the predominant form of iron in foods, it must first be reduced to Fe^{2+} by agents such as vitamin C before it can be absorbed. In the intestinal mucosal cell, Fe^{3+} is bound by *apoferritin,* and then oxidized by *ceruloplasmin* to Fe^{3+} bound to ferritin (Michael *et al.*, 2010). From there, iron is absorbed into the blood by apotransferrin, which becomes transferrin as it binds two Fe^{3+} ions. In plasma, transferrin carries and releases Fe to the bone marrow, where it is incorporated into hemoglobin of red lood cells (RBCs). After about 4 months in circulation, red cells are degraded by the spleen, liver, and macrophages, which return Fe to the circulation, where it is bound and carried by transferrin for reuse. Ferroportin controls the release of iron from cells. The recently discovered peptide hormone hepcidin largely controls iron metabolism by its ability to modulate the release of iron from cells by inhibiting ferroportin (Rossi, 2005).

Iron regulation is primarily through modified absorption from the upper gastrointestinal tract. Absorption and transport capacity can be increased in conditions such as ID and anaemia. As iron is obtained in the form of non-haem iron from vegetables and as haem iron from meat; haem iron is absorbed about two to three times better than non-haem iron. A small amount of haem iron in the diet will improve absorption of non-haem iron and thus the diet composition is an important determinant of the amount of iron actually absorbed (Bhale *et al.*, 2013).

Transport and excretion: Iron absorption by the GI system will be affected by the level of iron present in the body and in ferritin storage form. Low levels of serum or body iron enhance absorption from the intestinal cells as the ferritin levels are low and the apotransferrin levels are high. During iron absorption and metabolism special protein helps the body to absorb iron from food. Mucosal ferritin protein receives iron from food and stores it in the mucosal cells of the small intestine. When the body needs iron, mucosal ferritin releases some iron to another protein called mucosal transferrin; again mucosal transferrin transfers the iron to another protein called blood transferrin which transports the iron to the rest of the body (Rady *et al.*, 2005). Ferritin is found mostly in the liver, but smaller amounts are also in bone marrow, the spleen, and muscles. Normally only a small amount is in the

blood, but this test can still help estimate the body's total iron stores. Stored iron is important because when iron intake is low, the body relies on ferritin to release the iron it needs.



HCL: Hydrochloric acid, **Fe^{2+}**: ferrous iron, **Fe^{3+}**: ferric iron, **DMT 1**: Divalent Metal Transporter 1

Figure 2.0: Iron digestion, absorption, enterocyte use and transport

Source: TINU MARY SAMUEL, Journal os south India. 2013 Pp. 31

Legend:

Step 1: iron is released from food bound components. HCl in the stomach reduces Fe^{3+} to Fe^{2+}. Step 2: Free heme is absorbed by heme carrier protein 1(located in proximal small intestine). In step 3: within the enterocyte, heme is catabolised by heme oxygenase to protoporphyrin & Fe^{2+}. Step 4: In the small intestine non heme iron may react with one or more inhibitors, which promotes fecal excretion of iron. In fifth step cytochrome b reductase 1 reduces Fe^{3+} to Fe^{2+}. Step 6: DMT 1 carries Fe^{2+} across the brush border membrane into the cytosol of the enterocyte. Step 7: Fe^{2+} may bind to polyrC binding protein for transport into cytosol; iron may also be used within the cell or stores as

a part of ferritin. Step 8: Ferroportin transports iron across the basolateral membrane. Iron transport is coupled with its oxidation to Fe^{3+} by hephaestin. At last step Fe^{3+} attaches to transferrin for transport in blood.

2.1.2 Iron loss

About one-third of iron is lost from the GI tract and a tiny fraction of iron is lost in urine or sweat. Significant iron loss occurs in women during menstruation. Median monthly loss of blood during menstruation has been estimated at 35 ml, which is equivalent to more than 12 mg of iron. Menstrual blood flow is increased by approximately 100% in women using intrauterine devices and reduced by about 50 percent in women using oral contraceptives (Madan *et al.*, 1989). Because of women's greater iron requirements due to menstruation, and also because they usually consume less food than men, woman's daily iron intake tends to be marginal, and they are therefore more likely to develop IDA than men. This is especially true in many developing countries where the general food consumption is reduced because of poverty, unwise agricultural practices, and other reasons. There are certain critical periods when iron requirements are significantly increased and the iron balance can be easily disturbed. Such situations include pregnancy and growth during infancy (Sharman, 2000).

2.1.3 Iron Losses in Pregnancy and lactation

The second and third trimesters of pregnancy constitute a major drain on the iron reserves of women of reproductive age (figure 2).

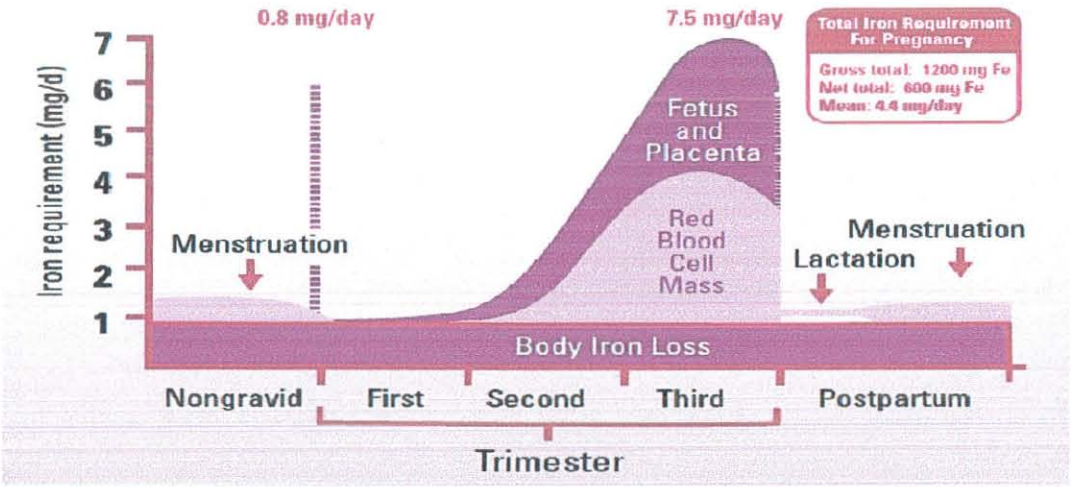


Figure 2.1: Daily iron requirements during pregnancy and postpartum

During these periods, the requirements of the growing fetus (ca. 270 mg), umbilical cord and placenta (90 mg) are especially great. There is an expansion of maternal RBC mass (ca. 450

mg), which raises the iron requirements even more. Expressed in terms of need for iron, these changes along with blood loss during delivery (150 mg) and basal iron losses (170 mg) are equivalent to about an additional 1,130 mg of iron (Sharma and Shankar, 2010).

2.1.4 Importance of iron in pregnancy

The amount iron lost is greater than that which can be absorbed from diet; hence iron supplementation is necessary during pregnancy. After delivery, expanded red cell mass contracts and some iron returns to reserve sites (Samuel *et al.,* 2013). However, despite such return, the average cost of each pregnancy in terms of iron loss is still high, approximately 680 mg. The size of iron stores at the beginning of pregnancy plays an important role (Sharma and Shankar, 2010). Iron requirements are higher in mothers who begin pregnancy with depleted or low iron stores. Such situation is common in developing countries. Low birth intervals may negatively affect initial iron stores and increase the chance of ID during pregnancy (Samuel *et al.,* 2013).

2.1.5 Dietary iron content and bioavailability of iron

There are at least four dietary factors that determine iron status in humans: (1) food iron content, (2) Levels of iron absorption (the so-called bioavailability of iron), (3) the presence of nutritional factors that promote iron absorption, and (4) the presence of nutritional factors that inhibit iron absorption. According to food balance sheets, in developing countries the total iron in diet per capita usually varies between 13 and 21 mg/day, and most of this iron is provided by vegetables (WHO/FAO, 2003); while the amount of dietary iron is important in maintaining iron balance, perhaps the more important factor is the level of iron absorption, which determines the bioavailability of iron.

In humans, iron is absorbed in the upper intestine in two forms: heme iron and non-heme iron. Heme iron is easily absorbed (15% to 20%), and its absorption is unaffected by gastric acidity and dietary composition. Heme iron is of great nutritional significance and is called bioavailable. But, only 2 to 5% of non heme iron is absorbed. Absorption of non-heme iron requires three major factors: (a) an adequate gastric acidity; (b) the presence of dietary components that promote iron absorption, such as ascorbic acid or meat; and (c) the absence of factors that inhibit iron absorption, such as phytates and tannates (contained in tea, coffee, and cereals), phosphates, egg yolk, and isolated soy protein (Simpson *et al.,* 2010). Today, the typical diet of most inhabitants of developing countries is based on cereals or roots and

tubers, with little or no meat, fish, and foods rich in ascorbic acid, and with high concentrations of iron absorption inhibitors. It has been estimated that the average iron absorption of this type of diet is between 1% and 5% (Samuel *et al.*, 2013). Such a dietary pattern is a major reason for the high prevalence of iron deficiency anemia in economically deprived and developing countries.

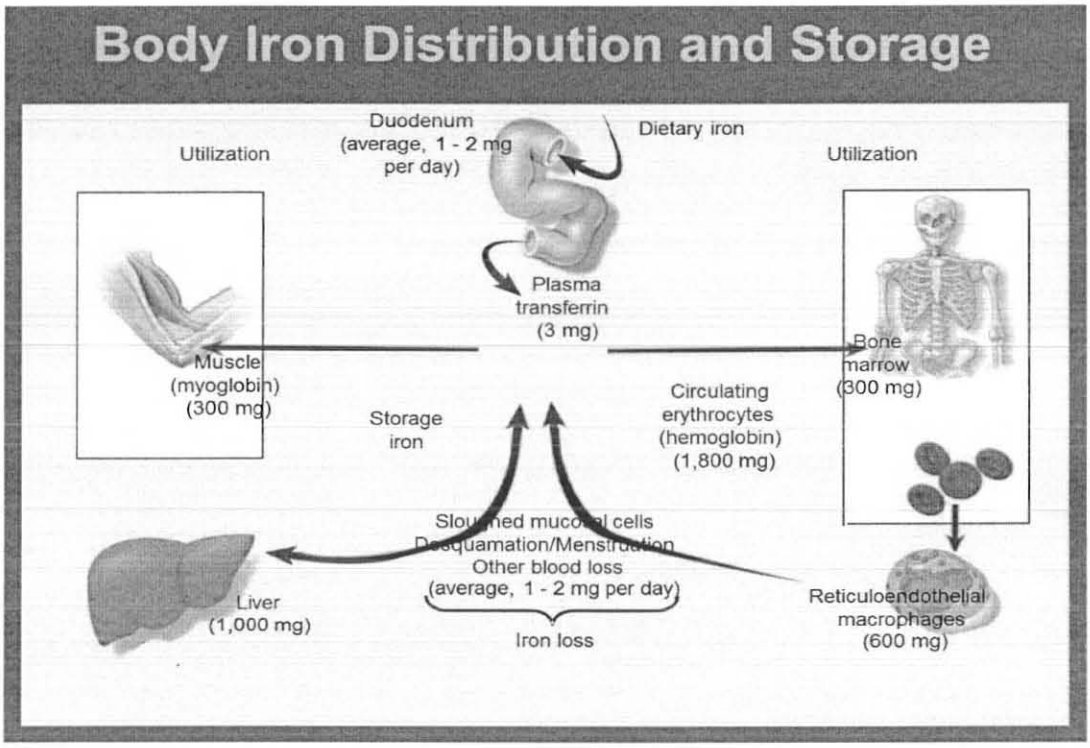


Figure 2.2: Body Iron Distribution and Storage

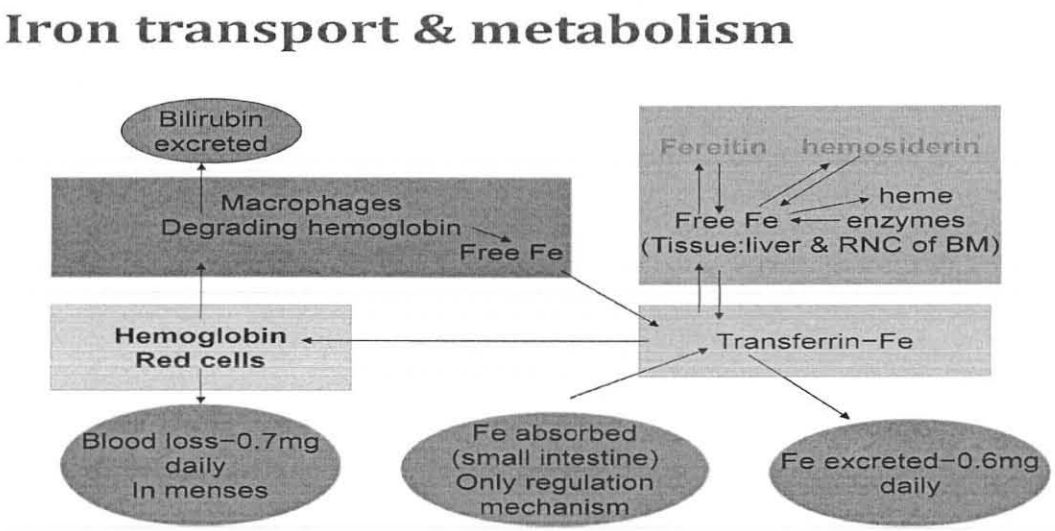


Figure 2.3: Iron transportation and metabolism

2.1.6 Iron Deficiency and iron deficiency anaemia in pregnancy

Increased blood loss, decreased dietary iron intake, or decreased release from ferritin may result in iron deficiency, ID. Reduction in iron stores usually precedes both a reduction in circulating iron and anaemia, as demonstrated by a decreased RBC count, mean corpuscular hemoglobin concentration, and microcytic RBCs (Michael *et al.,* 2010). Iron deficiency can be defined as a depletion of body iron stores and a restricted supply of iron to various tissues. Iron deficient anemia of pregnancy is a reduction of the concentration level of circulating hemoglobin below normal that occurs during pregnancy due to iron deficiency in a woman's body (Unicef/Unu/WHO, 2001 and Umeta *et al.,* 2008).

2.1.7 Toxicity of Iron

Iron overload states are collectively referred to as **hemochromatosis,** whether or not tissue damage is present. Primary Fe overload is most frequently associated with hereditary hemochromatosis (HH). HH is a single-gene homozygous recessive disorder leading to abnormally high Fe absorption, culminating in Fe overload. Secondary Fe overload may result from excessive dietary, medicinal, or transfusional Fe intake or be due to metabolic dysfunction. Hemosiderosis has been used to specifically designate a condition of iron overload as demonstrated by an increased serum iron and TIBC or transferrin, but without demonstrable tissue damage (Michael *et al.,* 2010). HH causes tissue accumulation of iron, affects liver function, and often leads to hyper pigmentation of the skin. Some conditions associated with severe hemochromatosis include diabetes mellitus, arthritis, cardiac arrhythmia or failure, cirrhosis, hypothyroidism, impotence, and liver cancer. Transferrin can be administered in the case of a transferrinemia (Weinberg, 1990 & Anghileri, 1995, Meneghini, 1997 and Halliwell, 1991). Fe (III), released from binding proteins, can enhance production of free radicals to cause oxidative damage. In iron-loaded individuals with thalassemia who are treated with chelators to bind and mobilize iron, intake of ascorbic acid may actually promote the generation of free radicals (Herbert, 1996).

2.2 Anaemia

Anaemia is not a disease, rather a symptom of various diseases. In the case of sickle cell anemia, a defect in the hemoglobin molecule changes the shape of the RBCs (Rady *et al*, 2005). Anaemia describes the condition in which the number of RBCs in the blood is low.

Any processes that can disrupt the normal life span of a RBC may cause anaemia. Normal life span of a RBC is typically around 120days (Michael *et al.,* 2005). The body needs the iron to make hemoglobin. The most common nutritional cause of anaemia is iron deficiency. It results from a combination of several factors: (1) inadequate iron intake and/or low dietary availability; (2) high physiologic demands in early childhood and pregnancy, and periods of rapid growth such as adolescence; (3) chronic iron losses from parasitic infections such as hookworm and schistomiasis; and (4) deficiencies of vitamin B12, folic acid, and vitamin A (Yip, 2008, Black *et al.*, 1994 and Van den Broek and Letsky, 2000). Non nutritional causes of anaemia include malaria, hemorrhage, inherited disorders, and various chronic diseases (Madan *et al.*, 1989). This form of anaemia occurs quite often in pre-menopausal women because women lose blood during menstruation (WHO, 2008 and Mohammed *et al.,* 2013).

2.2.1 Classification of anaemia

Generally there are three major types of anaemia based on the size of the RBCs: Microcytic anaemia if the RBC's are smaller than normal, MCV <80. If the RBC's are normal in size (but low in number) this is called normocytic anaemia, such as anaemia that accompanies chronic disease or anaemia related to kidney disease (Mohammed *et al.,* 2013). Third type is macrocytic anaemia if RBC's are larger than normal and major causes are pernicious anaemia mostly anaemia related to alcoholism (Michael *et al.,* 2005 and WHO, 2008). Anaemia is usually classified as either chronic or acute. Chronic anaemia occurs over a long period of time and symptoms typically begin slowly and progress gradually; whereas acute anaemia occurs quickly and symptoms can be abrupt and more distressing (Mohammed *et al.,* 2013).

2.2.2 Anaemia in Ethiopia and chronic infections

Anaemia in Ethiopia

Beside the prevalence of anaemia, globally 41.8% of the pregnant women are anaemic (Kazmi *et al.*, 2013). In Africa, 57.1% of the pregnant women were anaemic.Moreover, anaemia in pregnant women is a severe public health problem in Ethiopia; 62.7% of pregnant women were anaemic (Melku *et al.,* 2014 and WHO, 2008). Nutritionally, ID is the main

cause of anaemia throughout the world especially in WRA and particularly during pregnancy (Kazmi *et al.*, 2013). Many factors have been associated with the risk of ID in pregnancy, it is due to increased demand and decreased iron intake in diet and many other reasons (Kazmi *et al.*, 2013) such as nutritional status, socioeconomic variables, culture, age, parity, spacing of pregnancies (Williams and Wheby, 1992, Umniyati, 1997 and Tayrab *et al.*, 2013).

2.2.3 Stages in iron deficiency and iron deficiency anaemia

ID develops in three stages. The first stage is iron stores diminish. This is a reduction in the serum ferritin level, with no evidence of functional consequences (Bharati *et al.*, 2008). The second stage is characterized by a decrease in transporting iron (serum iron falls) and iron carrying protein transferring increases (an adaptation that enhance iron absorption) while in both the stages the level of hemoglobin is normal as shown in table 2.1 below. The last stage is when the lack of iron limits hemoglobin production. Now the hemoglobin precursors, erythrocyte protoporphyrin, begin to accumulate as hemoglobin and Hematocrit values decline. These cells can't carry oxygen from the lungs to the tissues which results fatigue, weakness, headaches, apathy, pallor and poor resistance to cold temperatures (Rady *et al.*, 2005). Iron deficient erythropoiesis occurs when the needs of the erythroid marrow for iron are no longer met, with a subsequent rise in erythrocyte protoporphyrin and serum transferrin receptor level; and finally, IDA is the most severe form associated with functional consequences. IDA is diagnosed when the Hb concentration is lower than the level considered normal for the person's age, gender, and physiological status (ie, below a statistically defined threshold of 2SD from the mean for a healthy population) (Michael et al., 2005).

Stages	Causes and summary of results	
Negative iron balance/iron depletion **Prelatent stage/ Stage 1**	Demands for or loss of iron exceed the body's ability to absorb iron from diet and starts Iron store depletion. Reduction in iron stores without reduced serum iron levels	Hb (N), MCV (N), iron absorption (↑), transferin saturation (N), serum ferritin (↓), marrow iron (↓)
Iron deficient erythropoiesis **Latent stage/ Stage 2**	After the depletion of Iron store,once transferrin saturation falls to 15–20%, Hb synthesis becomes impaired	Hb (N), MCV (N), TIBC (↑), serum ferritin (↓), transferrin saturation (↓), marrow iron (absent)
IDA Stage 3	Both Iron store and serum Iron depleted and Hb & hematocrit begin to fall, reflecting iron deficiency anemia, transferrin saturation at this poit is 10–15% with hypochromia/microcitosis.	Hb (↓), MCV (↓), TIBC (↑), serum ferritin (↓), transferrin saturation (↓), marrow iron (absent)

Table 2.1: Stages in Iron deficiency and their results

Sources: Imran Shahzad Anjum, MD

2.3 Laboratory Evaluation of Anaemia status

The physiological changes occurring during pregnancy affect both hematological and biochemical laboratory tests available for evaluating iron, folate, vitamin A, B12 and anaemia status (Kazmi *et al*, 2013).

2.4 Diagnosis of iron deficiencies and iron deficiency anaemia

Among the approaches used for the assessment of either iron nutritional status or the diagnosis of iron metabolism disorders, hemoglobin concentration and hematocrit measurement most frequently used even though these are least sensitive to ID. Disorders of iron metabolism are evaluated by measuring total iron and TIBC, percent saturation, transferrin, and ferritin from serum (Tofaletti *et al.,* 2004; Carl *et al*., 1999; Donald *et al*., 2001 and Tayrab *et al*., 2013),

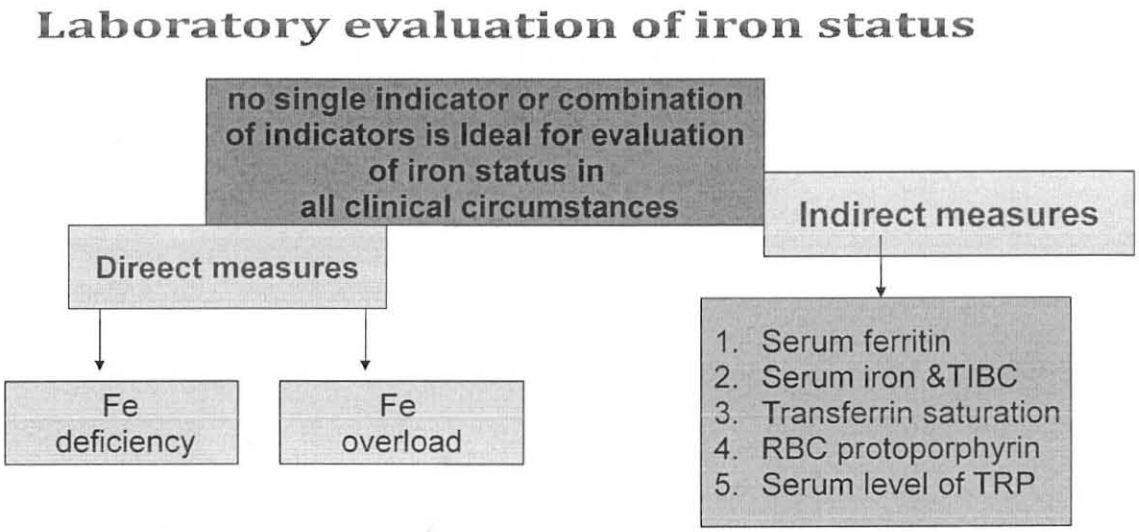


Figure 2.4 Laboratory evaluation of iron status

2.4.1 Ferritin

Ferritin is a water soluble protein-iron complex of molecular weight 465,000 (Bhale *et al.*, 2013). Ferritin is found in nearly all cells of the body. In hepatocyte and in the macrophage system of the bone marrow and other organs, ferritin provides a reserve of iron readily available for formation of hemoglobin and other haem proteins. Ferritin is the key to this important control of the amount of iron available to the body (Janne and Vihko, 1980).

Ferritin is a protein that stores iron and releases it in a controlled fashion (Bhale *et al.*, 2013). Hence, the body has a "buffer" against ID (Bhale *et al.*, 2013). Measures of serum ferritin provide an estimate of iron stores. Such information is most valuable to iron assessment. Ferritin levels are considered the gold standard for the diagnosis of IDA in pregnancy and the ferritin levels measured usually have a direct correlation with the total amount of iron stored in the body (Janne and Vihko, 1980 and Bhale *et al.*, 2013). If the ferritin level is low, there is a risk for lack of iron, which could lead to anaemia. In the setting of anaemia, low serum ferritin is the most specific laboratory test for IDA (Tayrab *et al.*, 2013).

2.4.1.1 Measurement of Serum Ferritin/Iron stores:

Ferritin is measured in serum by immunochemical methods, such as Immunoradiometric assay (IRMA), enzyme- linked immunosorbent assay (ELISA), and chemiluminescent techniques (Weinberg, 1990). Several manufacturers provide kits for measuring serum ferritin by either manual or automated means. Ferritin is decreased in IDA and increased in

iron overload, other conditions, such as chronic infections, malignancy, and viral hepatitis (Smith and Perry, 1995; Herbert and Shaw, 1996; Tofaletti *et al.*, 2004, Chiswell *et al.*, 1994 and Milne, 1999).

2.4.2 Total iron content (Serum iron) and measurement of serum iron concentration

The diagnosis of IDA using iron related parameter is associated with a number of challenges. Serum iron levels are not helpful by themselves because they vary with time of the day and due to various systemic insults (Adams, 2005). Measurement of total iron concentration refers specifically to the Fe^{+3} bound to transferrin and not to the iron circulating as free hemoglobin in serum. The specimen may be collected as serum without anticoagulant or as plasma with heparin. Oxalate, citrate, or EDTA binds Fe ions and all are unacceptable anticoagulants. Early morning sampling is preferred because of the diurnal variation in iron concentration. Specimens with visible hemolysis should be rejected. Spectrophotometric determinations have been adapted to automated analysis. These procedures generally have the following steps: Fe^{+3} is released from binding proteins by acidification, reduced to Fe^{+3} by ascorbate or a similar reducing agent, and complexed with a color reagent such as ferrozine, ferene, or bathophenanthroline. Another challenge is that although serum iron levels can be measured directly in the blood, but these levels increase immediately with iron supplementation (patient must stop supplements for 24 hours) (Ahlan *et al.*, 2001).

2.4.3 Total Iron-Binding Capacity and determination

TIBC refers to the amount of iron that could be bound by saturating transferrin and other minor iron-binding proteins present in the serum or plasma sample. Typically, about one-third of the iron binding sites on transferrin are saturated. TIBC is determined by adding sufficient Fe^{+3} to saturate the binding sites on transferrin, with the excess iron removed by addition of $MgCO_3$ to precipitate any Fe^{+3} remaining in solution. After centrifugation to remove the precipitated Fe^{+3}, the supernatant solution containing the soluble iron bound to proteins is analyzed for total iron content (Tayrab *et al.*, 2001).

2.4.4 Percent Saturation

The percent saturation, also called the transferrin saturation, is the ratio of serum iron to TIBC. Saturation or transferring saturation index or percent) is the most specific indicator of ID (Tayrab *et al.*, 2013 and Ahlan *et al.*, 2001).

2.4.5 Transferrin

Iron moves through the blood attached to a protein called transferrin. Transferrin is a glycoprotein with a molecular weight of 79570 daltons and with a biologic half-life of approximately 9 days (shorter than albumin). It consists of a polypeptide strand with two N-glycosidically linked oligosaccharide chains and exists in numerous isoforms. This test indicates how well that protein can carry iron in the blood (Goldman, 2011). The rate of synthesis in the liver can be altered in accordance with the body's iron requirements and iron reserves. It is synthesized in the liver and binds and transports ferric iron. When hepatocyte iron is absent or low, transferrin levels rise in proportion to the deficiency. It is an early indicator of ID, and the elevated transferrin is the last analyte to return to normal when ID is corrected. **IBC** is a measure of an iron that serum protein can combine and nearly all the binding capacity is due to transferrin. A decrease in transport iron characterizes the second stage of ID. This is revealed by an increase in the IBC of the protein transferrin and a decrease in serum iron. These changes are reflected by the transferrin saturation, which is calculated from the ratio of the two values (Ahlan *et al.,* 2001).

Normally; only about one third of the iron binding sites of transferrin are occupied by iron, so that serum transferrin is considered as reserve IBC. A decrease in IBC may be due to hemochromatosis and/or acute iron poisoning. Increased IBC appears in IDA (Donald *et al.,* 2001 and Buritis *et al.,* 2005). Transferrin rises as ID grows worse and falls as iron status improves. Markedly reduced transferrin levels indicate severe PEM; in mild-to-moderate PEM, transferrin levels may vary, limiting their usefulness (Rady *et al,* 2005). The half-life of transferrin is approximately one half that of albumin and the body pool is smaller than that of albumin; therefore, transferrin is more likely to indicate protein depletion before serum albumin concentration changes. Transferrin levels can be lowered by factors other than protein or energy deficiency, such as nephrotic syndrome, liver disorders, anaemia, and neoplastic disease (Ferguson *et al.,* 1992).

2.4.5.1 Measurement of serum transferrin/ transports iron

Transferrin is measured by immunochemical methods such as nephelometry. Transferrin (TIBC) may also be decreased in chronic infections/ inflammatory conditions and malignancies. Transferrin is primarily monitored as an indicator of nutritional status. TfR can

be detected in serum; the measurement of sTfR has been introduced as a powerful tool for the diagnosis of ID in a variety of clinical situations (Huebers *et al.,* 1990; Baynes, 1996a; Punnonen *et al.,* 1997; Suominen *et al.,* 1997).

2.4.6 Folate

Folate is the generic term for components nutritionally and chemically similar to folic acid. **Folate,** also known as folacin or PGA in short, has a chemical name that would fit a flying dinosaur. Folate functions metabolically as coenzymes involved in various one-carbon transfer reactions. Folate in the diet is absorbed in the jejunum, and the excess is excreted in the urine and feces (Ronald *et al.,* 2008). Large quantities of folate are also synthesized by bacteria in the colon. Folate is one of the few nutrients whose nutritional status in humans can be defined from low dietary intakes and marginal deficiency to depleted tissue stores and clinical symptoms (megaloblastic anaemia). Food folates are primarily found in green and leafy vegetables, fruits, organ meats, and yeast. Boiling food and using large quantities of water result in folate destruction. Folate must be reduced to H_2 folate or Tetrahydrofolate (H_4 folate is the active coenzyme form of the vitamin) (Mahmood, 2014). Folates are also present in most foods with legumes (peanuts, cowpeas, peas, etc.), leafy greens, citrus (orange juice), some fruits, vegetables (broccoli, cauliflower), and liver considered to be good sources. Enriched cereal products are fortified at 140 μg 100 g−1 (Ronald *et al.,* 2008).

Folate chemistry is complex because of the many different molecular forms and the presence of polyglutamate-conjugated forms. Plasma folates exist predominantly in the monoglutamate form, polyglutamate forms in red cells, tissue and foods must be hydrolyzed before radio assay. Folate participates in formation of DNA required for rapid growth (Mahmood, 2014). For folate coenzyme to be effective (active form) the methyl group should be removed from inactive form of folate by the help of vitamin B12. Primary coenzyme of folic acid THF serve as a part of an enzyme complex that transfers one carbon compounds that arise during metabolism. This action helps convert vitamin B12 to one of its coenzyme forms and helps synthesize the DNA required for all rapidly growing cells (Rady *et al.,* 2005). Impaired DNA synthesis arises from decreased production of 5,10-methylene Tetrahydrofolate (5,10-CH_2H_4 folate), which is required for the synthesis of deoxythymidine monophosphate nucleotide. The small intestine prefers to absorb the "free" folate form-folate with only one glutamate attached (the monoglutamate form). Enzymes on the intestinal cell surfaces hydrolyze the

polyglutamate to monoglutamate and several glutamates. Then the monoglutamate is attached to a methyl group (-CH_3). Special transport system delivers the monoglutamate with its methyl group to the liver and other body cells (Mahmood, 2014).

Folate, vitamin B12, vitamin B6 and one carbon metabolism

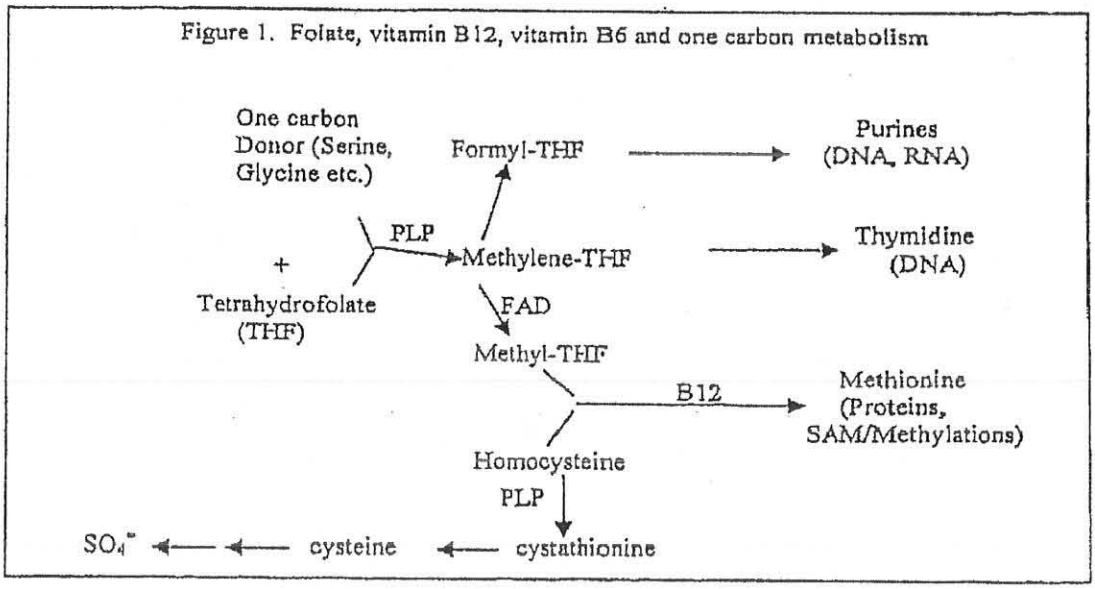


Figure 2.5: Folate, Vitamin B12, B6 and one carbon metabolism

Source: Selhub, J. 2002. The Journal of Nutrition, Health and Aging, 6: 40

Folate is required for the remethylation of homocysteine to methionine, which is dependent upon sufficient levels of 5-methyltetrahydrofolate (5-CH3-H4 folate) as the one-carbon donor (Figure 2.5). Folate deficiency impairs cell division and protein synthesis processes critical to growing tissues. Marginal deficiency produces general symptoms including tiredness, irritability, and decreased appetite. Severe deficiency produces megaloblastic anaemia or the production of large immature RBCs. Other symptoms include abdominal pain, diarrhea, ulcers in the mouth and pharynx, skin changes, hair loss, and neurological disorders such as dementia and depression (Gibson, 2005).

In a folate deficiency, the replacement of RBCs and GI tract cells falters. Not surprisingly, then, two of the first symptoms of a folate deficiency are anaemia and GI tract deterioration. The anaemia of folate deficiency is characterized by large, immature RBCs. Without folate,

DNA damage destroys many of the RBCs as they attempt to divide and mature (Koury and Ponka, 2004). The result is fewer, but larger, RBCs that cannot carry oxygen or travel through the capillaries as efficiently as normal RBCs. Plasma folate levels (5-CH3-H4 folate) rapidly fluctuate with recent intake; therefore, erythrocyte folate levels are considered a more reliable status index (Gibson, 2005). Folate depletion is characterized by a fall in the folate concentrations of RBCs (erythrocytes). As erythrocyte folate levels diminish, folate-deficiency anaemia develops. Patients receiving dialysis treatment rapidly lose folate.

Clinical conditions associated with FAD include megaloblastic anaemia, alcoholism, mal-absorption syndrome, carcinoma, liver disease, chronic hemodialysis, and hemolytic and sideroblastic anaemia (Bailey and Berry, 2005). Certain anticonvulsants and other drugs that interfere with folate metabolism include sulfasalazine, isoniazid, and cycloserine. Folate deficiency of dietary origin commonly occurs in older persons. Phenytoin (Dilantin) therapy accelerates folate excretion and interferes with folate absorption and metabolism. Alcohol interferes with folate's enterohepatic circulation, and methotrexate, a chemotherapeutic agent, inhibits the enzyme dihydrofolate reductase. The final stage of folate deficiency produces megaloblastic anaemia and hyper segmented neutrophils, which was described by Herbert *et al.,* (1996).

2.4.6.1 Folate toxicity

The increase in folate requirement during lactation results, in part, from the presence of high-affinity folate binders in milk. In women of childbearing age, 400 g of folate per day is recommended to prevent or reduce the incidence of neural tube defects (FNBIM, 2002). Folate has proven to be critical in reducing the risks of **NTDs** (Bailey and Berry, 2005). The brain and spinal cord develop from the **neural tube,** and defects in its orderly formation during the early weeks of pregnancy may result in various central nervous system disorders and death.

Low levels of serum folate can occur with use of oral contraceptives. There are no known cases of folate toxicity; the RDA is 400 g/day for adult males and females (FNBIM, 2002). The major clinical symptom of folate deficiency is megaloblastic anaemia. Folate and vitamin B12 are closely related metabolically. The hematologic changes that result from deficiency of either vitamin are indistinguishable (Michael *et al.,* 2010).

2.4.6.2 Measurement of serum folate

Among the several test the deoxuridine suppression test provides a measure of functional folate deficiency and can provide a distinction between folic acid and vitamin B12 deficiencies. The test is based on the folate requirement for conversion of deoxyuridine to deoxythymidine. The test is not popular for routine clinical use, because it is tedious to perform while not offering diagnostic advantage over red cell folate measures. The histidine load test, based on the increased unitary excretion of formiminoglutamate after an oral histidine load, is not widely used because it is rather tedious and responds to vitamin B12 as well as folate deficiencies (Mahmood, 2014). When folate deficiency develops, serum levels fall first, followed by a decrease in erythrocyte folate levels and ultimately hematologic manifestation (Simpson *et al.*, 2010).

Measuring both serum and erythrocyte levels is helpful because serum levels indicate circulating folate and erythrocyte levels better approximate stores. But, serum contains endogenous binding proteins that can bind folate and result in falsely low serum folate concentration measurements. Folate in the serum is almost exclusively present in the monoglutamate form; however, in RBCs, it is in the polyglutamate form and as high-molecular-weight complexes (Simpson *et al.*, 2010). Serum folate levels stabilize after a few weeks at about 7 nmol/L when subjects are on a folate-deficient diet, Herbert *et al.*, (1996) indicating acute status but not the level of tissue stores (Gibson, 2005). Serum folate is the most sensitive measure of early folate depletion of which values less than 3ng/mL represents a low dietary intake or negative folate balance (Gibson, 2005).

2.4.7 Vitamin B12

Vitamin B12 is a water-soluble vitamin. It is one of the "B complex vitamins," which play roles in RBC formation, nerve cell maintenance, and methyl donation in DNA synthesis (Ola et al., 2011). Vitamin B12 (cobalamin) is an organic complex that contains a cobalt ion in its structure (Kumar *et al.*, 2010). This vitamin is an important coenzyme for cell development and growth, and its deficiency leads to weakness, fatigue, nausea, constipation, weight loss, pernicious anaemia and nerve degeneration (Bernard *et al.*, 1998). Peculiarly, vitamin B12 is essential for the human diet, but cannot be synthesized by the body. The primary dietary sources for vitamin B12 are from animal products (e.g., meat, eggs, and milk). Therefore,

total vegetarian diets are likely to be deficient or low in vitamin B12 (Mahmood, 2014). Animals derive vitamin B12 from intestinal microbial synthesis.

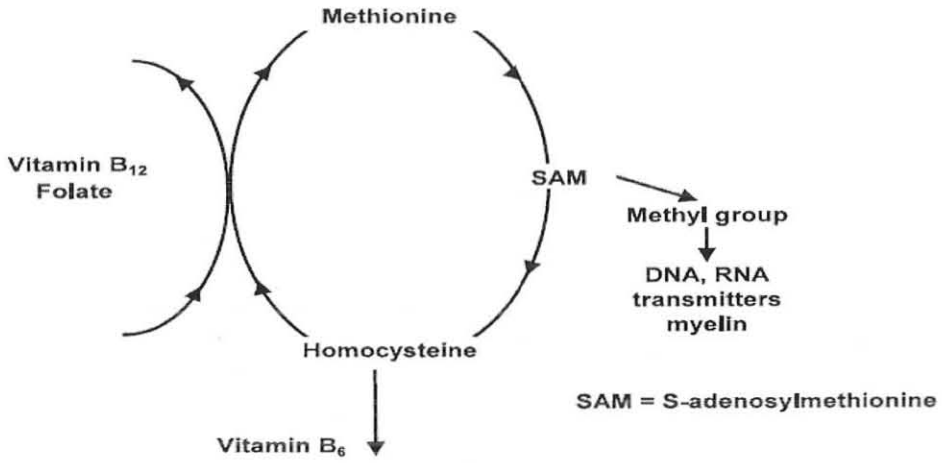


Figure 2.6: Interaction betqeen Folate, Vitamin B12 and Homocysteine

Source: Scandinavian Journal of nutririon 2003 47 (3), 132

2.4.7.1 Absorption and metabolism of vitamin B12

Intestinal absorption of vitamin B12 takes place in the ileum and is mediated by a unique binding protein called intrinsic factor (IF), which is a protein secreted by gastric parietal cells. Vitamin B12 participates as a coenzyme in enzymatic reactions necessary for hematopoiesis and fatty acid metabolism. Excess vitamin B12 is excreted in the urine. Vitamin B12 bears a corrin ring (containing pyrroles similar to porphyrin) linked to a central cobalt atom (Mahmood, 2014). The active cofactor forms of vitamin B12 are methylcobalamin and deoxyadenosylcobalamin.

Most vitamin B12 absorption occurs through a complex with IF. This IF–B12 complex binds with specific ileal receptors (Sharma and Shankar, 2010).

Without the help of vitamin B12 folate becomes trapped inside cells in its methyl form, unavailable to support DNA synthesis and cell growth. Like folate, vitamin B12 follows the enterohepatic circulation route. It is continually secreted into bile and delivered to the intestine, where it is reabsorbed (Stabler and Allen, 2004). Because vitamin B12 is required to convert folate to its active form, one of the most obvious vitamin B12-deficiency symptoms is the anaemia of folate deficiency (Mahmood, 2014).

2.4.7.2 Effects of vitamin B12 deficiency

The term pernicious anaemia is now most commonly applied to vitamin B12 deficiency resulting from lack of **intrinsic factor**. A loss of B12 also occurs in individuals infected with fish tapeworm or because of malabsorption diseases, such as sprue or celiac disease. Vitamin B12 and Folate are closely related: each depends on the other for activation. Recall that vitamin B12 removes a methyl group to activate the folate coenzyme. When folate gives up its methyl group, the vitamin B12 coenzyme becomes activated (Pawlak *et al.*, 2014).

The regeneration of the amino acid methionine and the synthesis of DNA and RNA depend on the both folate and vitamin B12. In the body, methionine serves as a methyl (CH_3) donor. In doing so, methionine can be converted to other amino acids. In addition, without any help from folate, vitamin B12 maintains the sheath that surrounds and protects nerve fibers and promotes their normal growth. Bone cell activity and metabolism also depend on vitamin B12. Close relationships between folate and vitamin B12 deficiencies result from the involvement of the vitamins in DNA synthesis (Karnaze and Carmel 1990).

Megaloblastic anaemia produced by deficiencies of folate and vitamin B12 are characterized by abnormally large megaloblasts in the bone marrow and abnormally large red cells in the blood (Gibson, 2005). Deficiency of vitamin B12 causes the major disorders includes hematological, neurological, and gastrointestinal symptoms/disorders (FNBIM, 2002).

Hematological symptoms commonly referred to as pernicious anemia include megaloblastic anaemia with diminished energy, fatigue, shortness of breath, and heart palpitations (FNBIM, 2002 and Gibson, 2005). Etiology is similar to megaloblastic anaemia associated with folate deficiency and arises from interferences with DNA synthesis (Pawlak, Lester and Babatunde, 2014). Since folate supplementation can mask hematological changes due to lack of vitamin B12 but not reverse neurological damage associated with the deficiency, conservative approaches have been used in the initiation of folic acid supplementation programs. Hematological effects can be reversed by vitamin B12 treatment (FNBIM, 2002).

The neurologic manifestations are variable and may be subtle. For this reason, vitamin B12 deficiency should be considered a cause of any unexplained macrocytic anaemia or neurologic disorder, especially in an older person. Neurological effects include tingling and numbness in the arms and legs, motor disturbances (gait), and various cognitive changes (loss

of memory, disorientation, dementia, and mood changes). Other effects include visual disturbances, insomnia, impotency, and impaired bowel and bladder control. Neurological effects can be reversed by treatment, but reversal depends on duration and extent of the neurological damage. Gastrointestinal effects include appetite loss, sore tongue, flatulence, and constipation (FNBIM, 2002).

2.4.7.3 Toxicity of vitamin B12

Toxicity of B12 has not been reported and the average daily diet contains 3–30 g of vitamin B12, of which 1–5 g is absorbed. The frequency of dietary deficiency increases with age, occurring in more than 0.5% of people older than age 60, although the symptoms resulting from dietary deficiency are rare. However, compared to other vitamins, the daily requirement of vitamin B12 is relatively low. Cobalamin refers to a large group of cobalt containing compounds (FNBIM, 2000).

2.4.7.4 Measurement of serum vitamin B12

As folate deficiency progresses and low serum levels persist, folate stores decline, resulting in folate depletion. Because low erythrocyte folate concentrations also occur with vitamin B_{12} deficiency, serum B_{12} concentrations must also be measured for determination deficiency of anaemia (Dilshad *et al.*, 2010). The determination of Cobalamin in human serum at picogram level becomes necessary for the detection of its deficiency (Oh and Brown, 2003). Several non-radio isotopic assays for vitamin B12 have been developed for routine laboratory use. The determination of serum B_{12} levels provides the best test for routine screening of cobalamin status (Lok *et al.*, 2012 and Kazmi *et al.*, 2013).

2.4.8 Vitamin A

Vitamin A is fat soluble and believed to be essential micronutrient for normal embryogenesis, hematopoiesis, cell growth, reproduction and epithelial differentiation. Vitamin A and related retinoic acids are a group of compounds essential for vision, cellular differentiation, growth, reproduction, and immune system function (Tanumihardjo, 2002).

2.4.8.1 Absorption of vitamin A

A clearly defined physiologic role for retinol is in vision. Retinol is oxidized in the rods of the eye to retinal, which, when complexed with opsin, forms rhodopsin, allowing dim-light

vision. Vitamin A and D act through specific nuclear receptors in the regulation of cell proliferation (Anke *et al.,* 1994 and Milne, 1999). In VAD states, epithelial cells (cells in the outer skin layers and cells in the lining of the gastrointestinal, respiratory, and urogenital tracts) become dry and keratinized. VAD is most common among children living in non-industrialized countries and is usually a result of insufficient dietary intake (Tanumihardjo, 2002).

Deficiency may also occur because of chronic fat malabsorption or impaired liver function or may be associated with severe stress and protein malnutrition. Premature infants are born with lower serum retinol levels, as well as lower hepatic stores of retinol; therefore, these newborns are treated with vitamin A as a preventive measure (ATSDR, 2003; Ronald, 2008; Machlin and Huni, 1994 and van den Berg, 1996). While IDA and VAD are both significant problems in many countries, research establishing an interaction between iron and vitamin A could have major implications for treatment of anaemia that is due to nutritional status. Numerous studies using humans have supported the notion that vitamin A has an impact on iron status and, in turn, IDA. Early research suggested that people deficient in vitamin A were prone to anaemia that was reversed when sufficient doses of vitamin A were taken (Karyadi and Bloem, 1996). Many studies in humans have been performed in countries where nutritional anaemia and VAD are major public health problems (FNBIM, 2002).

VAD is a severe public health problem in Ethiopia affecting around 61% of children 6-59 months of age in the 11 regions of the country (EDHS, 2012). The situation is probably worse in emergency affected areas. Clinical VAD, untreated can lead to childhood blindness and it is likely that VAD is one of the major contributing factors to the high under-five mortality rate of Ethiopia (174 per 1000, UNICEF). In Ethiopia, 18 percent of all women reported having some form of night blindness during their last pregnancy (ANCB, 2001). VAD results when body stores are used up either because too little vitamin A is present in the foods, or there is insufficient absorption of vitamin A from foods. VAD can also result from rapid utilization of vitamin A during illnesses (particularly measles, diarrhoea and fevers), pregnancy and lactation and during phases of rapid growth in young children. The immune systems become weak and illness is more common and more severe, increasing under-five death rates. The eye could be damaged with appearance of lesions, and when severe, blindness can occur. There is an increased risk of a woman dying during pregnancy or during the first three months after delivery (Hamdy, Aleem and Shazly, 2013).

2.4.8.2 Vitamin A in pregnancy

In pregnancy, extra vitamin A is required for growth and tissue maintenance in the fetus, for providing it with reserves and for maternal metabolism. Basal requirements in pregnancy are 370 μg per day, which increase during lactation to 450 μg per day (Van den Broek, 2003). On the other hand, a relationship has been suggested between the incidence of birth defects and high vitamin A intakes during pregnancy with an apparent threshold of 10,000 IU per day (WHO, 1998, Mills *et al.,* 1997 and Rothman *et al.,* 1995). However daily doses of up to 10,000 IU or weekly doses of 25,000 IU after day 60 of pregnancy are safe, especially in areas where VAD is common (WHO, 1998). Vitamin A is known to play a role in hematopoiesis, and anaemia is a common consequence of VAD (Semba and Bloem, 2002).

Vitamin A supplementation during pregnancy was found to improve maternal Hb. A diet devoid of vitamin A results in decreased hemoglobin levels (Hodges *et al.,* 1980). Antenatal supplementation with both iron and vitamin A was shown to reduce anemia prevalence in a study from Indonesia (Suharno *et al.,* 1993), but other studies conducted in sub-Saharan Africa were not able to obtain the same positive result (Van den Broek *et al.,* 2003). The mechanism whereby vitamin A supplementation could improve hemoglobin and iron status has not been fully elucidated but it has been suggested that vitamin A is required for the mobilization and utilization of iron for hemoglobin synthesis (Bloem, 1990). The one suggested mechanism is VAD decreases transferrin synthesis and thus reduces iron transport to the bone marrow, reduces bone marrow iron uptake; impairs the differentiation of blood cells due to lack of retinoic acid and impairs mobilization of iron from ferritin stores (Kifle Habte *et al.,* 2014).

An important and large study from Nepal had generated discussion about the role of vitamin A in the possible reduction in maternal mortality (West *et al.,* 1999). A clear biological explanation could not be given and anaemia was not studied as an outcome in this trial. Further trials are currently under way to address these issues (Van den Broek, 2003).

Although VAD is recognized to cause anaemia, 'VAD anaemia' lacks complete characterization as a distinct clinical entity. Vitamin A appears to be involved in the pathogenesis of anaemia through diverse biological mechanisms, such as the its influence on growth and differentiation of erythrocyte progenitor cells, its effect on immunity to infection

and reduction of the anemia of infection, and mobilization of iron stores from tissues. Epidemiological surveys show that the prevalence of anaemia is high in populations affected by VAD in developing countries (Semba and Bloem, 2002). A close association between VAD and anaemia has been shown in many nutritional surveys from around the world, and perhaps this is not surprising, given the widespread prevalence of nutritional anaemia and VAD in developing countries (Bloem, 1995). Most of these epidemiological surveys did not identify the underlying causes of anaemia, and often the proportion of subjects with concurrent VAD and anaemia are not stated (Semba and Bloem, 2002).

Observational studies showed that serum retinol was positively associated with hemoglobin, Hematocrit, and serum iron (Bloem *et al.,* 1989). Some studies also showed that vitamin A may be required for the mobilization and utilization of iron for hemoglobin synthesis, and thus contribute to erythropoiesis (Bloem *et al.,* 1990). A study conducted in Indonesia showed that antenatal iron and vitamin A supplements were better than iron alone (Suharno *et al.,* 1993). The requirements of vitamin A are increased during pregnancy. A daily intake of 800 μg retinol equivalents was recommended as a safe level of vitamin A for pregnant women (FAO/WHO, 2004). However studies in Malawi did not show a similar effect (Galloway, 2003). Hodges's study indicated that iron stores are unavailable for erythropoiesis during VAD which implies that vitamin A plays a role in the release of iron from the liver (Hodges *et al.,* 1980). The data of an experimental study showed that in a population with low socioeconomic status, vitamin A supplementation (3.0mg/d) can improve the hematological condition (hemoglobin increased by 9 g/L) of anemic children (Mejía and Chew, 1988).

2.4.8.3 Toxicity of vitamin A

When ingested in high doses, either chronically or acutely, vitamin A causes many toxic manifestations and may ultimately lead to liver damage due to hypervitaminosis. High doses of vitamin A may be obtained from excessive ingestion of vitamin supplements or large amounts of liver or fish oils, which are rich in vitamin A. Carotenoids, however, are not known to be toxic because of a reduced efficiency of carotene absorption at high doses and limited conversion to vitamin A. The RDA of vitamin A is 700 g per day for adult females. Toxicity is usually assessed by measuring retinyl ester levels in serum rather than retinol, which is accomplished by HPLC (Kratz *et al.,* 2002 and Rossi, 2005).

2.4.8.4 Measurement of vitamin A: Retinol

Human status assessment methods include dietary assessment, assessment of content of liver, plasma, and breast milk, and functional assessment by dark adaptation and conjunctival impression cytology (Ronald, 2008; van den Berg, 1996). Biochemical tests include the relative dose response test (RDR) and the modified relative dose response test (MRDR) that estimate liver stores of vitamin A. Such tests are useful to assess marginal VAD. Plasma retinol concentration is a commonly used measure of status (FNBIM, 2002 and Gibson, 2005). More recent clinical studies of deficiency use serum retinol concentration as the status indicator (Ramakrishnan and Darnton, 2002; Singh and West, 2004).

Measurement of retinol is the most common means of assessing vitamin A status in the clinical setting (Dilshad et al, 2010), but because of the serum retinol level is homeostatically regulated it does not fall to low level until liver stores of the vitamin are nearly exhausted. Hence the test does not differentiate vitamin A status over a wide range of nutriture from marginally deficient to near tissue saturation. The test is most useful for detecting over tissue depletion of vitamin A in individual and assessing the prevalence of VAD in population. Serum retinol values less than 0.70µmol/L generally indicate low dietary intake and tissue stores of the vitamin, where as values below a cutoff of 1.05 µmol/l has been proposed to reflect low vitamin A status among pregnant and lactating women (WHO, 2008; Gibson, 2005 and Ronald, 2008).

2.5 Chronic infections

Several species of worms contribute to anemia in developing countries, with hookworms and schistosomes being the most common. Both cause significant blood loss in the host, which leads to iron deficiency and anemia (Hansen 1983 and Huo *et al.*, 2011). Anaemia can be caused by chronic infections (TB, HIV/AIDS, Malaria, and Hookworm). Anemia of Inflammatory Conditions, Hemoglobin apathies (Sickle cell anemia, Sideroblastic anemia, etc.). The relationship between infection and anaemia varies with the nature of the disease. Chronic inflammation is also associated with anaemia due to swelling of tissues, rather than nutritional ID (Galloway, 2003).

HIV infection is also strongly linked with anaemia through a variety of mechanisms. These include chronic disease and inflammation; increased metabolic and nutritional needs (iron);

poor intake of iron and other nutrients due to reduced appetite and anorexia; mal-absorption of nutrients; and direct suppression of RBC production. Researchers in Malawi found that HIV-infection was significantly more prevalent in anaemic pregnant women (47%) compared with the overall antenatal population (30%), and was associated with greater severity of anaemia (Van den Broek, 1996).

2.5.1 Assessment of inflammation

Nutritional surveys use APP biomarkers such as CRP and AGP to identify the influence of inflammation on the distribution of iron status biomarkers. CRP is an acute phase protein produced by the liver in response to inflammation, infection and tissue injury. But, qualitative CRP analysis was not effective in identifying persons who had other indications of mild inflammation. Increased CRP concentrations occur much earlier than with other acute phase reactants- within 4 to 6 hours- and this rapid response to trauma or infection are the distinguishing feature of CRP. Therefore, its level in the blood increases if there is any inflammation in the body. In addition, CRP levels return to normal quickly at the end of an acute episode making CRP useful for both the detection of acute episodes as well as in treatment monitoring. But, the CRP level does not provide any specifics about the inflammatory process going on in the body (such as the location of the inflammation) (Emedicine, 2009).

2.5.2 Measurement of C-reactive protein

Conventional CRP assays include qualitative, semi-quantitative and quantitative assays, with indications for use for evaluation of infection, tissue injury, and inflammatory disorders. These assays provide information for the diagnosis, therapy, and monitoring of inflammatory diseases. In apparently healthy person's blood CRP levels are below 5 mg/L, while in various conditions this threshold is often exceeded within 4-8 hours after an acute inflammatory event, with CRP values reaching approximately 20 to 500 mg/L. CRP rises in concentration 4–6 hours before other acute phase reactants begin to rise (Boosalis *et al.,* 1989 and Boosalis *et al.,* 1996). CRP is a more sensitive and more reliable indicator of acute inflammatory processes than the erythrocyte sedimentation rate (ESR) and leukocyte count. CRP can increase dramatically up to 1,000 times after tissue injuries, which are more than two or three orders of magnitude greater than any other acute-phase reactant and after the disease has subsided CRP values rapidly fall and reach the reference interval often days before ESR has returned to normal (Boosalis *et al.,* 1996).

3.0 Materials and methods

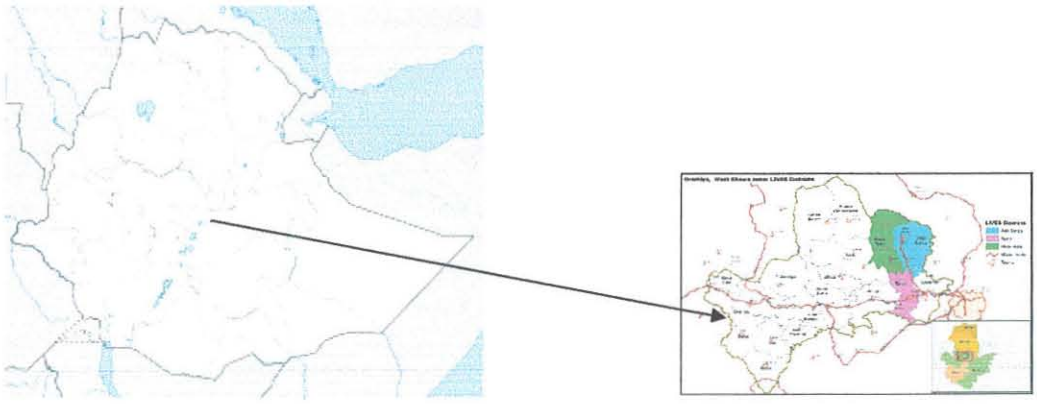
3.1 Study design

This cross-sectional study was carried out between December 22, 2014 and June 09, 2015. The study population was composed of pregnant women attending the ambulatory pregnancy clinic during second and third trimester.

3.2 Study Area

The present research work was carried out in Ambo, western Shoa zone of Oromia regional state, located 115 km from Addis Ababa. The area is found at a longitude of 37^0 32' to 38^0 3' E, and latitude of 8^0 47' to 9^0 20' N and the altitude range is from 1900 to 2275 meters above sea level. The climatic condition of the area is 23% highland, 60% midland, and 17% lowland. It has an annual rain fall ranging from 800 – 1000 mm and temperature ranging from 20°C – 29°C. Agriculture is the main occupation of the population of the area.

Ambo is popular for its immense natural gifts including excellent climate which provides comfortable living and working environment. Ambo is also famous for its mineral water widely consumed in Ethiopia. Besides, the town also hold the pioneer higher learning institution i.e. Ambo University. Afan Oromo is the most spoken language here and the largest ethnic group in the town is also Oromo. The town has one zonal hospital and two health centres, one Maternal Health Center and all the antenatal clinics of these institutions were not used for the study.



Map of West Shoa zone with selected districts colored

Source: Zonal diagnosis and intervention plan West Shoa, Oromia

3.2.1 Source Population

24,634 were men and 23,537 were women.

3.2.2 Study population

The study population consisted of a sample of all second and third trimester pregnant women attending ANC at Ambo hospital and two health centers.

3.2.3 Inclusion criteria

All second and third trimester pregnant women attending ANC at Ambo hospital and health centers.

3.2.4 Exclusion criteria

Any woman with pregnancy-related complications such as history of diabetes mellitus, hypertension, those on iron supplements, history of blood transfusion within the last 3 months were excluded

3.3 Dependent Variable

Hemoglobin level

3.4 Independent Variable

1. Socio-demographic and socio-economic (age, Marital status, ethnicity, education, income, occupation, availability of latrine, washing after toilet, and water resource)
2. Maternal related characteristics and pregnancy complication (abortion, loss of blood, GA, place of delivery, interval of delivery, contraceptive, malaria infection)
3. Serum retinol, B12, folate, ferritin and transferrin

3.5 Sample size and sampling technique

Sample size was determined based on single population proportion formula with a 95% CI (d=0.95), 5% margin of error, and assumption that among pregnant women attended ANC 50% of pregnant women were anemic during second and third trimester (p=49.1%). The sample size for quantitative analyses is calculated using the following formula $n= Z^2p (1-p)/d^2$ Z= 1.96: Three health institutions were selected from 3 kebeles using the random sampling system. A proportional allocation was employed to obtain the sample size from each health institutions.

3.6 Ethical consideration

The research proposal was first approved at the center for Food Science and Nutrition; it was ethically cleared by Institutional Health Research Ethical Reviewer Board of Faculty of Natural Science. The research again ethically cleared from Oromia Health Research Ethical Review Committee.

3.7 Sample Collection and Methods

3.7.1 Socio-demographic characteristics

Verbal informed consent was obtained from all the participants and a semi-structured questionnaire was given in Afan Oromo language to gather information including age, time of previous gestation, and obtain socio-demographic information, present and past history in nutritional assessment of anaemic pregnant women and dietary habit.

3.8 Management/handling of the experimental unit and sample collection process

Blood sample (ca. 4ml) was drawn into vacutainer tube without anticoagulants by medical laboratory technicians from the anecubital vein. Gestational ages were assessed by the reported last menstrual period and examination of fundal height by experienced midwives at maternal and child health, MCH center of each center and were expressed in weeks.

The sample collected into tube was allowed to clot and centrifuged at 3000 × g, for 15 min to produce serum for biochemical analysis and frozen at -20°C until assayed (Dilshad *et al.*, 2010). Blood samples were stored in the dark immediately after being drawn (Broek and Letsky, 2008). Sample collected into a tube was allowed to store and transported within maximum of 3 days to both EPHI and FDSN program Laboratory session for biochemical analysis of serum transferrin, serum ferritin, vitamin A, B12, CRP and folate.

3.9 Laboratory Analysis

3.9.1 Script Roche Elecsys 2010 chemistry analyzer, Cobas Integra 400 Plus Analyzer

COBAS INTEGRA 400 plus is a fully automated, random access, sample discrete, multi-analyte analyzer. This table top system employs four different technologies namely absorption photometry, fluorescence polarization immuno assay (FPIA), Immuno-turbidimetry & Potentiometry.

Due to the multiple technologies employed the one single platform, the system can accommodate wide menu with over 100 types of assays on board.

Four different measuring technologies on single platform: Fluorescence Polarization, Immuno Turbidimetry, Absorbance Photometry and Potentiometry (direct & indirect)

Clot detection system

Ready to use reagents in a unique reagent carrier

Long reagent on-board stability

Minimal calibration frequency (typically each lot)

Barcode reading of all relevant reagent information

3.9.2 Hemoglobin test

Hemoglobin tests were performed from fresh blood sample before centrifugation. Separate vacutainer tube with out EDTA was used for whole blood samples collection for analysis of complete blood count (CBC) by ADVIA 2120i 5 Part Cell Counter (SIEMENS), and centrifuged on the same day of collection with in a maximum of four hour. At Ambo town health center from the fresh blood sample Hematocrit was determined by centrifuging heparinized blood in a capillary tube (also known as a micro hematocrit tube) at 2500 RPM for 15 minutes.

Altitude adjustment

Hemoglobin levels was used to define anaemia by adjusting for altitude according WHO Hb adjustment formula (Sulivan et al., 2008)

Hb adjustment = -0.032 x (Altitude x 0.0032808) + 0.022 x (Altitude x 0.0032808)2. Hb adjustment was made by using approximately 2100m above sea level and high altitude area. Anaemia was then defined as Hgb < 10.9 g/dl in pregnant women (Melaku *et al.,* 2008 and INACG, 1985). After adjustment severe, moderate and mild anaemia were defined as Hgb below 7gm/dl, 7-9.9 gm/dl and 9.9-10.9 gm/dl respectively (WHO, 2008).

3.9.3 Extraction of retinol

A 300µL of serum sample was denatured by mixing with 300µL of ethanol containing 300µL of saline solution to standard and 300µL of retinol acetate (0.5 µg/mL) as an internal standard. The entire rack of tubes was vigorously mixed by hand for 30 seconds, using a vertical shaking motion. HPLC grade Hexane (2.4mL) was then added, and the tubes were capped. The tubes were vortexed for 45 seconds, and centrifuged at 3000rpm for 15 minutes, 800µL of the supernatant (layer) was carefully pippeted off twice. The extract was evaporated to dryness under nitrogen gas and the residue was reconstituted immediately in 800µL methanol (mobile phase). All solvent and chemicals used were HPLC grade and all mobile phases were filtered through 0.45µm pore size filtered.

3.9.4 Serum retinol determination

Measurement of serum retinol concentration was done by HPLC (Shimadzu LC-20AD) using reversed-phase column. Briefly, chromatography was carried out on a 6mm X15 cm CLC-ODS reverse phase column (Shimpack, Shimadzu Japan) using a Supelco LC-18 HPLC column (150X4.6mm; i.d. 5 µm beads). Inert all-trans retinyl acetate was used as an ISTD. The analytes were separated on a Supelco LC-18 HPLC column (150X4.6mm; i.d. 5 µm beads) at a flow rate of 1.5 mL/min, by using UV-VIS detector; with detection at 325 nm. The temperature is ambient; Run time was 10 minutes and retention time app. 3-5 min.

3.9.4.1 Method development

Quantitative internal standard method was used and target peaks were quantitated by adding an internal standard substance, ISTD to both the standard and unknown sample. This method gives stable results and eliminates injection errors. A specific amount of ISTD substance is added to the standard sample that contains a know quantity of the target component. The standard sample was analyzed and a calibration curve was created from the peak area ratio ($A_{vit\text{-}A}/A_{IS}$) and component concentration ratio between the target and ISTD peak. The area ratio between the target and ISTD peaks is obtained from the unknown sample that contains the same ISTD substance. Using the concentration ratio acquired from the calibration curve above, the target concentration was quantitated. By using the same calibration curve (y=mx+b) the concentrations of retinol in a serum sample was easily determined after calculation of its peak height ratio.

3.9.4.1 Linearity, Repeatability and analytical recovery

Calibration curves were generated using standard solutions of concentrations of 5µg/dL to 60µg/dL. The measure of linearity was Pearson's linear correlation coefficient (r) for parameters with regular distribution was tested and a correlation coefficient (R^2 = 0.9985) was obtained for both compounds. Additionally, peak area repeatability for a concentration of about 50 µg/dL for (n = 6). The analytical recovery of retinol from serum was determined by adding known quantities of retinol to serum and analyzing using the following formula.

%Recovery= (Ct/Ca)X100

Where Ct is the calculated/recovered concentration sample and Ca standard concentration added/spiked. The relative standard deviations (RSDs) value for intra-day and inter-day repeatability was less than 3.01% and 14.62%, respectively which falls in the acceptable region (Shim *et al.*, 2013).

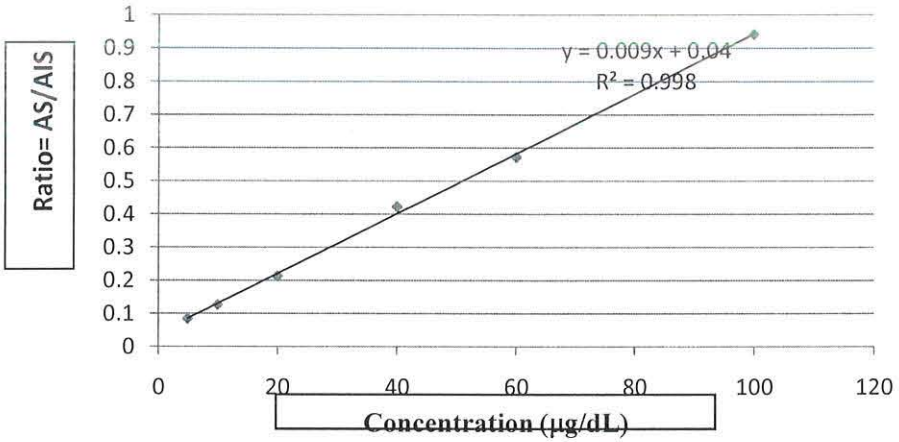


Figure 3.2 Sample calibration curve for area ratio versus concentration of retinol

Calculation for Response factor	Calculation for concentration of sample
RF= $Conc._{Vit}A_{IS}/A_{vit}$	$Conc(\mu g/dL)=RF.(A_{IS}/A_{vit})$
RF= Response factor	$Conc._{Vit}$ =Concentration of Vit A in the sample
$Conc._{Vit}$ =Concentration of Vit A in STD	A_{IS}= Area of ISTD in the Sample
A_{IS}= Area of ISTD in the STD	A_{vit}= Area of vit A in the Sampple
A_{vit}= Area of vit A in the STD	
The amount of vitamin A in the sample was calculated using RF determined in the calibration run	

Sample Analytical recovery of Retinol from serum using HPLC

Code	Initial conc. (µmol/L)	Spiked (µmol/L)	Measured (µmol/L)	Recovered (µmol/L)	Recovery (%)
53078	1.049	1.708	1.556	1.417	82.96
171	0.581	1.708	2.282	1.301	76.17
54931	0.8933	1.708	1.926	1.4327	83.88
201	0.5217	1.708	2.036	1.5143	88.66
56284	0.8759	1.708	2.343	1.4671	85.895
10025	2.0822	1.708	3.672	1.5898	93.08

Table 3.1 Analytical recovery of retinol from serum using HPLC

2.9.5 Serum Ferritin

Serum ferritin was measured using Elecsys 2010, Roche diagnostics, GmbH, Germany, an immunoassay analyzer using the Electrochemiluminescence immunoassay (ECLIA). According to Roche diagnostic laboratory the measuring range 0.500-2000 µg/L (ng/mL) was (defined by the lower detection limit and the maximum of the master curve). Samples with ferritin concentrations above the measuring range were diluted with diluent universal by the cobas e 411 analyzers. The cobas e411 software automatically takes the dilution into account when calculating the sample concentration. The expected values for normal measurement were 15-150 µg/L (34-337 pmol/L or 15-150 ng/mL) for women 17-60 years (Elecsys and cobas e 411 analyzers manual 2012).

Based on the iron cut off points, iron deficiency of ferritin (<12 µg/L). The first stage iron depletion ferritin level is between 30-50 µg/L and the value lies between 12-30µg/L is second stage iron depletion. While ferritin value is <30 µg/L indicates a low iron status that is small or no iron reserve (WHO, 2008).

3.9.6 Serum Transferrin

A variety of methods were available for determining transferrin including radial immunodiffusion, nephelometry and turbidimetry. The Roche transferrin assay is based on the immunological agglutination principle (Suominen *et al.,* 1997; Mast *et al.,* 1998). In this analysis serum transferrin was determined using human serum transferrin enzyme immunoassay test kit by immunological analysis instrument (COBAS Integra 400 plus Roche Diagnostic, GmbH, Germany) (Dilshad *et al.,* 2010). According to Roche diagnostic laboratory; the normal measuring range is 2.0-3.6 g/L (1.26-65.5 µmol/L, 10-520 mg/dL). The samples having higher concentrations via the rerun function was determined. The LDL of the test was 0.1 g/L (1.26 µmol/L, 10 mg/dL). The expected values for normal measurement was 2.71-3.91 g/L (34.2-49.2 µmol/L; 271-391 mg/dL).

3.9.7 Serum Folate and B12

Serum vitamin B12 and folate concentrations were measured using an electrochemiluminescence immunoassay (ECLIA) (Elecsys 2010 Hitachi; Roche Diagnostics) (Dilshad *et al.,* 2010 and Erhabor, 2013). According to the WHO folate cut off point a serum folate concentration of >20ng/ml (>45.3 nmol/L), 6-20 ng/ml (13.5-45.3nmol), 3-5.9 ng/ml (6.8-13.4 nmol/L) and concentration <3 ng/ml (<6.8 nmol/L) are indicative of elevated, normal, moderate and severe folate deficiency (Gibson *et al.*, 2008; WHO, 2012). Samples with folate concentrations above the measuring range can be diluted with diluents universal.

For analysis of vitamin B12 the analyzer automatically calculates the analyte concentration of each sample (either in pmol/L or pg/mL). The normal measuring range for COBAS analyzer, 197-866 pg/mL or 145.386-639.108 pmol/L (defined by the LDL and the maximum of the master curve). Serum vitamin B12 was considered as severe deficient when serum vitamin B12 was <150 pg/mL, moderate when lies between 150-200 pg/mL.

3.9.8 Serum C - reactive protein

Various assay methods are available for CRP determination, such as nephelometry and turbidimetry. The Roche CRP assay is based on the principle of particle-enhanced immunological agglutination. In this study serum sample was used for colorimetric determination of CRP using the Nephelometry Assay method by Cobas Integra e 411 by using Liquid, ready-to-use reagents (Kimberly *et al.,* 2003). In apparently healthy person's blood CRP levels are below 5 mg/L, while in various conditions this threshold is often exceeded within four to eight hours after an acute inflammatory event, with CRP values reaching approximately 20 to 500 mg/L (WHO, 2008). The LOQ was determined using the result of functional sensitivity testing.

3.10 Statistical Analysis

The data were coded and analyzed with the Statistical analyses using SPSS (version 20; SPSS Inc., Chicago, IL) software (Sherry and Tanumihardjo, 2002 and Dilshad *et al.,* 2010).

Test of Normality

Shapiro-Wilk test was used to test normality of data. From the normality test, the p-value is <0.05, we reject the null hypothesis and conclude that the data comes from a normal distribution. The Pearson correlation coefficient was used to measures the strength of the linear relationship between normally distributed variables.

Based on WHO and international guidelines, anaemia was defined as hemoglobin <11.9 g/dl, and ID as ferritin <12 µg/L and sTfR as >8.3 g/L (Gibson, 2005). Serum folate and vitamin B12 deficiencies were defined as levels below 3ng/ml and 150 pg/ml respectively. Cutoff point for CRP and serum retinol were >5mg/L and <0.7µmol/L respectively (Hamdy, Aleem and Shazly, 2013). Descriptive analyses of percentages of categorical variables was reported and $p<0.05$ at $\alpha=0.05$ denoted a statistically significant difference in all statistical comparisons. The results were expressed as mean ± SD. Correlation coefficients were determined by linear regression analysis (Rama *et al.,* 2004, Sherry *et al.,* 2002). To analyze the differences between groups, the independent student t test was used for continuous variables, among mild and moderate anaemic participant and between second and third trimester anaemic pregnant women. Pearson's correlation was used to examine correlation between hemoglobin and serum retinol concentrations (WHO, 2008).

4. Result and Discussion

4.1 Result and Discussion

4.1 Socio-demographic characteristics, Correlation and Regression analysis

4.1.1 Socio-demographic characteristics:

Socio-demography of mothers (n=206) attended ANC during the study period is presented in **Table 4.1**. The mean ± SD of gestational age was 23.3 ± 5.6 weeks, mean age of the pregnant women was 24.6±5.03. All of the study participants were Christians and 95.6% were married. Only 57.8% had their own latrine and 50% had formal education. Based on the socio-demographic data collected, 35 (17%) of pregnant women were infected with malaria at least once in the past two years.

Table: 4.1 Selected socio-demographic characteristics of the pregnant woman attending ANC

Characteristics		**mean ±SD**	**(N=206)**	**(%)**
Age of Participant		24.6 ± 5.03		
	15-19		82	39.8
	20-25		50	24.3
	26-30		52	25.2
	31+		22	10.7
Marital Status	Married		197	95.6
Gestational Age		23.3 ± 5.6		
	14-26wks (2^{nd})		137	66.5
	27-37 wks (3^{rd})		69	33.5
Occupation	Farmer		62	30.1
	House wife		113	54.9
	Merchant		31	15
Educational Status	Illiterate		75	36.4
	Read and Write		28	13.6
	With formal education		103	50
Owns Latrine			119	57.8

Maternal under-nutrition diminishes a woman's productivity, causing repercussions for herself, her family, her community, and the broader society. Daba *et al.,* (2013) showed that educational level, monthly income and nutrition information during pregnancy were identified as important predictors of knowledge of women on nutrition during pregnancy among the study participants. Due to the third trimester covers the 28^{th} week onwards till

delivery the severity of anaemia is directly proportional to the occurrence of complications in pregnancy. Etiologic pattern is often complex in second and third trimester.

4.1.2 Pregnancy history and complications in the current pregnancy

Based on the prevous pregnancy history only 1.9% had a delivery interval of less than 2 years and 91 (44.2%) was pregnant for the first time. More than one-third of the study participants (34.5%) had received information about nutrition (Table 4.2); and about 12.6% of participants had blood loss during previous pregnancy.

Table 4.2: Maternal pregnancy history (N=206)

Characteristics	Frequency	%
First pregnancy	91	44.2
Previous deliveries		
Health Institution	50	24.3
Home	65	31.6
Information about Nutrition	71	34.5
Blood Loss	26	12.6
Interval between babies		
<2 year	2	1.9
>2 year	115	55.8

Based on the previous pregnancy history delivery recorded in the interval between babies, correlation was only observed in use of contraceptive and history of blood loss with hemoglobin value. According to the study of Obse *et al.*, (2013) the magnitude of anaemia was determined and told that in decreasing gap between previous birth, increases the magnitude of anaemia. In this finding there was no association between previous birth and hemoglobin status. This might be due to all the participant's history of birth interval was greater than two years and the study respondents are from the similar status.

4.1.3 Complications related to the current pregnancy

Table 4.3: Complications related to the current pregnancy (n=206)

Characteristics	Frequency †	%
Use of Contraceptive	126	61.2
Iron or Folate Supplementation		
Iron Supplementation	25‡	23.6
Iron-folic acid supplementation	41‡	39.4
No Supplementation	38	36.5
Sign of Symptoms		
Persistent swelling of feet, hands or face	24	11.7
Increasing breathing, especially on routine activity	71	34.5
Headaches	57	27.7
Blurring of Vision	29	14.1
Fever temperature >38°C	6	2.9
High Colored urine in the past two weeks	10	4.9
No Symptoms	9	4.4

†data in use of contraceptive include all the 206 pregnant women in 2nd and 3rd trimester

‡ Out of 104 none of pregnant women took IFA suppmentation more than one month.

As shown in **Table 4.3,** 36.5% of study participants did not take any iron or iron-folic acid supplementation at all. Hence, not more than one month; 63.5% of the participants took either iron/iron-folic acid supplementation. Increased breathing, especially on routine activity 71 (34.5%) and headaches 57 (27.7%) were the two leading sign of symptoms and 14.1% had blurred vision during current pregnancy. Anaemia during pregnancy has been attributed not only to increased iron requirements during the second and the third trimester of gestation, but also due to effects of physiological state as also decribed in study of pregnant women in Ugandan (Baingana *et al.,* 2014).

4.1.4 Dietary assessment and nutritional status of respondents

Regarding food consumption and dietary pattern, 87.9% (n=181) of the participants had at least three meals a day (**Table 4.4**). Few 5.3% missed their breakfast during fasting period. Calcium and zinc rich foods such as fish, cabbage and eggs were consumed by 42.7% (n=88) of the study group. Animal source foods were consumed by less than half of the respondents. According to Gibsn *et al.,* (2008) the study conducted in pregnant farmers of Sidama, Southern Ethiopia a high prevalence of protein and zinc deficiency was observed and two-third of pregnant women had anaemia, in the area were no cellular animal products consumed, 13% had iron deficiency anaemia, 33% had depleted iron stores, and 27% had low plasma retinol. This study might have an advantage over the previous study but, due to all participants are from the same group (anaemic) still no correlation and any prediction was obtained from the current data.

Table 4.4: Reported food groups consumed during the period of current pregnancy (N=206)

Characteristics	Frequency	%
Frequency of meals per day		
2 per day	25	12.1
3 per day	181	87.9
Flesh foods	96	46.6
Dairy	70	34
Vitamin A rich foods	72	35
Ca & Zn rich foods	88	42.7
Level of Fasting		
Deprivation of animal source foods except fish	30	14.6
Deprivation of all animal source foods	32	15.5
Deprivation of animal Source foods and no breakfast	11	5.3
No fasting	62	59.6
Drink alcohol during pregnancy	51	24.8

4.1.5 Anaemia and micronutrient status of pregnant women:

For comparison of each micronutririent status categorized by age; the mean±SD of serum retinol was low in the younger pregnant women, (1.40±1.94) than older pregnant women, (1.66±1.96); where as the mean ± SD of serum ferritn and transferrin was low in the middle age of pregnant women betwe age of 20-30 years. But, serum folic acid and CRP status were recorded lower in the older pregnant women. The study conducted by Kefiyalw *et al.*, (2014) stated that the prevalence of anaemia was higher in pregnant women in the age group of 18-26. This might be due to the participant group in the study were inclusion of all status of pregnant women (anaemin & nn anaemin). In the current study prevalence of anaemia had comparatively closer hemoglobin. One way ANOVA showed as significant difference in mean comparisons of vitamin B12.

According to the present finding, severity of anaemia almost increases as the age of the respondent's increases; this finding also supported by Obse *et al.*, (2013). No statistically significant difference was observed between serum retinol concentrations of moderately anaemic compared to mildly anaemic mothers. Pearson correlation coefficients were significant between serum folate and serum B12 (r = 0.265; P = 0.015), the non significant difference between moderate and mild mothers regarding serum folate and B12 concentrations indicates that folic acid deficiency and vitamin B12 deficiency is not the only cause of anemia during pregnancy.

Table 4.5 Micronutrient status of pregnant women by age category, one way ANOVA (n=104)

	15-19 yrs		20-25 yrs		26-30 yrs		30+ yrs			95% Confidence Interval of the Difference	
	Mean + SD								P-value	upper	lower
Hemoglobin	9.80	0.60	9.64	0.56	9.7	0.60	9.40	0.94	0.180	9.41	10.07
Serum Retinol	1.40	1.94	1.74	1.92	1.8	2.32	1.66	1.96	0.952	0.32	2.46
Serum ferritin	28.56	22.00	28.01	23.62	33	22.20	34.62	30.00	0.736	16.40	40.74
sTfR	6.01	3.71	5.15	2.40	4.9	2.89	6.53	2.27	0.215	4.00	8.07
sFA	8.41	3.72	7.70	4.00	7.6	2.83	6.09	2.97	0.340	6.35	10.46
Serum B12	206.9	81.0	178.25	40.30	197	58.33	173.0	36.65	0.006*	162.01	251.72
Serum CRP	5.08	1.86	4.35	1.52	4.6	1.81	3.82	0.84	0.302	4.05	6.11

* The mean difference is significant at the p≤ 0.05

4.1.6 Prevalence of micronutrient deficiencies among pregnant women attending ANC

The mean hemoglobin value was 10.5±1.2 g/dL. Mean ± SD value of serum ferritin, transferrin, retinol, folate, B12 and CRP was noted to be 30.3±23.6 (µg/L), 5.4±2.8 (g/L), 1.7±2.0 (µmol/L), 7.6±3.5 (ng/mL), 187.4 ± 53.7 (pg/mL) and 4.5±1.6 (mg/L), respectively. The prevalence micronutrients was 34 (32.7%) due to ferritin <12µg/L, 23 (22.1%) due to transferrin >8.3g/L, 42 (40.4%) due to VAD based on serum retinol<0.7µmol/L, 28 (26.9%) due to vitamin B12 deficiency based on serum B12<150pg/mL, and in 29 (27.9%) due to FAD based on serum folate <6.8ng/mL and 24 (23.08%) had acute inflammation based on the serum CRP≥ 5mg/L. This study was agreed with deficiency of vitamin B12 deficiency that conducted in southern Ethiopia by Gibson *et al.,* (2008) and in Nepal (Bhandari and Banjara, 2015).

TABLE 4.6: Prevalence of micronutrient deficiencies among anaemic pregnant women attending (N=104)

Parameter	N	%	Mean + SD
Altitude adjusted Hb	206		10.5± 1.2
Hb (<10.9g/dL)			9.7±0.66
Serum ferritin	104		30.3±23.6
IDA for sf<12 µg/L)	34	32.7	
Serum Transferrin	104		5.4±2.8
IDA for sTfR >8.3g/L)	23	22.1	
Serum Retinol	206		1.69±2.03
VAD (< 0.7µmol/L)	42	40.4	
Serum Folic acid	104		7.6±3.5
FAD (<6.8ng/ml)	29	27.9	
Serum Vitamin B_{12}			187.4±53.7
Vit B12 deficiency (<150pg/mL)	28	26.9	
Serum CRP			4.5±1.6
Presence of inflammation CRP (>5.0mg/L)	24	23.1	

Hb: hemoglobin, IDA: iron deficiency anaemia, VAD: vitamin A deficient; FAD: folic acid deficient

4.1.7 The comparisons of the micronutrients status of among moderate and mild anaemic pregnant women

The mean micronutrient status of pregnant women categorized by the severity of anemia (mild or moderate) is presented in table 4.7. Except for serum transferrin, no statistically significant difference was observed between mild and moderate anaemic pregnant women.

Table 4.7: Micronutrient status of pregnant women with mild and moderate anaemia (N=104)

	Types of anaemia					95% CI of the difference	
	Moderate		Mild				
	N	Mean+SD	N	Mean + SD	p-value	lower	upper
Serum Retinol	59	1.5±2.0	45	1.9±2.1	0.276	-1.232	0.356
Serum ferritin	59	30.1±23.9	45	30.6±23.5	0.906	-0.986	8.746
Serum transferrin	59	5.9±2.9	45	4.7±2.4	0.029*	0.119	2.21
Serum FA	59	7.9±3.7	45	7.2±3.4	0.296	-0.653	2.122
Serum B12	59	178.8±46.5	45	198.6±60.5	0.072	-41.41	1.838
Serum CRP	59	4.5±1.7	45	4.4±1.5	0.642	-0.487	0.786

* The mean difference is significant at the $p \leq 0.05$

4.1.8 Comparisons of the micronutrients status among second and third trimester anaemic pregnant women

A comparison of the micronutrient status of the pregnant women by GA is presented in **Table 4.8.** Except for CRP values, no significant difference in serum micronutrient composition was observed between the second and the third trimester. Infection/inflammation as indicated by elevated CRP values was higher in the third trimester. In the severity of anaemia categorizing in GA the severity of anaemia was high in the third trimester in which this data was agreed and supported by Obse *et al.,* (2013) again.

From the study conducted by Alene and Dohe (2014) in eastern Ethiopia pregnant women in the second and third trimesters were more likely to be affected by anaemia as compared to pregnant women in the first trimester of which was supported by this study. Because excluding the first pregnancy was causes to increase prevalence of anaemia to be high (68.3%); again supported by Gebremedhin *et al.,* (2014).

Table 4.8: Micronutrient status of pregnant women between second and third trimester anaemic women (N=104)

Gestational Age	14-26wks (2nd trimester, N=74)	14-26wks (3rd trimester, N=30)		95% CI of the Difference	
	Mean + SD		p-alue	Lower	Upper
Hemoglobin	9.64+0.65	9.65+0.6	0.900	-0.29	0.25
Serum Retinol	1.6+2.0	1.9+2.1	0.514	-1.16	0.59
Serum ferritin	30.6+22.64	29.5+26.2	0.834	-9.09	11.25
Serum Transferrin	5.0+2.3	6.2+3.64	0.115	-2.60	0.293
Serum Folic acid	8.0+3.6	6.7+3.3	0.105	-.263	2.75
Serum Vitamin B12	182.3+45.54	200+69.1	0.206	-45.28	10.07
Serum CRP	4.2+1.4	5.0+2.0	.050*	-1.60	.00000

* The mean difference is significant at the $p \leq 0.05$

4.1.9 Prevalence of IDA (based on Ferritin and Transferrin), vitamin A, folate and vitamin B12 and C-reactive protein of pregnant women attending ANC

Among the anaemic pregnant women (n= 104), ~71% were in the second trimester, whereas 29 % were in their third trimester of pregnancy. From the 2nd and 3rd trimester pregnant women more than half, 55% and 60 % respectively were moderately anaemic (**Table 4.9**). About 42 % of the pregnant women had some form of mild (19.8 %) or moderate (21.8 %) VAD (serum retinol < 0.7mol/L). About one-third of the anaemic pregnant women were IDA, 32.7% of which were severe, 31.7 % were first stage iron depletion, and 18.3 % are second stage of iron depletion. Based on serum transferrin only 20 (19.3%) were anaemic. Folate and B12 was severe in 18 and 28 women, possible deficiency in 11 and 46, and 74 and 30 women had normal range respectively. But, 24 (34%) had high inflammation based on serum CRP.

Micronutrient deficiency, also known as "hidden hunger", because it is less visible to the naked eye, is an additional, yet related issue in Ethiopia. Regarding the infection of pregnant women based on serum CRP the prevalence of inflammation was very high in comparison to study conducted by Gibson *et al.,* (2008) in southern Ethiopia. In a study conducted by Baingana *et al.,* (2008) in Uganda similar result was reported. According to the Baingana *et al.,* (2008) CRP concentration was markedly raised and nearly the two-third of the women

had elevated CRP. In Malawi also the same result was recorded which agreed with the finding of the present study (Van den Broek and Letsky, 2008). The contradiction of findings with Gibson *et al.,* (2008) might be due differences in study participants (only anaemic pregnant included in this study).

Table 4.9: Prevalence of IDA (based on Ferritin and Transferrin), vitamin A, folate and vitamin B12 and C-reactive protein of pregnant women attending ANC

	Tot.	Sub Total		Sum %	Moderate anaemia, Hb<9.9 %	Mild anaemia Hb<10.9 (N=45) %
GA*	206	74	14-26wks	71.15	41(69.5)	33(73.3)
		30	27-37wks	28.85	18(30.5)	1(26.7)
Retinol	104	20	Severe	19.23	15(14.4)	5(4.8)
		22	Mild	21.15	14(13.5)	8(7.7)
		62	ND	59.62	30(28.9)	32(30.8)
IDA	104	34	SF<12	32.69	20(19.3)	14(13.5)
based on		33	SF 30-50	31.73	18(17.3)	15(14.4)
SF		19	SF12-30	18.27	11(10.6)	8(7.7)
		18	SF 50-150	17.31	10(9.6)	8(7.7)
STRf	104	84	sTRf<8.3	80.77	43(41.4)	41(39.4)
		20	sTRf>8.3	19.23	16(15.4)	4(3.8)
FA		18	Severe	17.31	8(7.7)	10(9.6)
		11	Possible	10.58	5(4.8)	6(5.8)
		74	Normal	71.15	45(43.3)	29(27.9)
B12	104	28	Severe	26.92	18(30.5)	10(9.6)
		46	Moderate	44.23	29(27.9)	17(16.4)
		30	Normal	28.85	12(11.5)	18(17.3)
CRP	104	80	sCRP<5	76.92	47(45.2)	33(31.7)
		24	sCRP>5	23.08	12(11.5)	12(11.5)

ND: Non deficient, 14-26wks = second trimester; 27-37wks = third trimester

*Gestationa age data included for all the 206 participants

4.1.10 Micronutrient interrelations

The Pearson correlation (Table 4.10) showed a significant positive association between serum retinol and hemoglobin value (r=0.232; p=0.018). There was also a significant negative association between the age of a women and IFA suplementation (r=-0.208; p=0.034). The negative association of age of women and iron folic acid supplementation may indicates that the older women awareness of IFA supplementation was low compared with the younger women.On the other hand, serum CRP was positively correlated with vitamin B12 (r=0.263; p=0.007).

Table 4 10: Correlation of selected socio demographic characteristics and biochemical analysis (N=104) value (r= -0.260*; p=0.047);

Correlations

		Age	GA	IFA Supple	Milk	Hemoglobin	Ser. retinol	Serum ferritin	sTfR	Serum FA	Serum B12	Serum CRP
Age of woman	r	1	.086	**-.208***	-.067	**-0.260***	.038	.075	.015	-.098	.001	-.142
	Sig. (2-tailed)		.386	**.034**	.497	**0.047**	.700	.446	.884	.322	.990	.150
GA	R	.086	1	-.070	.021	.067	.087	-.082	.117	-.147	.174	.175
	Sig. (2-tailed)	.386		.483	.835	.497	.378	.405	.238	.136	.077	.075
IFA Supplementation	R	-.208*	-.070	1	.272**	.195*	.107	.135	-.022	.156	-.140	-.033
	Sig. (2-tailed)	.034	.483		.005	.048	.278	.171	.822	.114	.155	.737
Milk and its Product	R	-.067	.021	.272**	1	.119	.051	-.050	-.014	-.064	-.110	-.105
	Sig. (2-tailed)	.497	.835	.005		.228	.608	.614	.888	.517	.264	.288
Adjusted Hemoglobin	R	-0.260*	.067	.195*	.119	1	**0.232***	.063	-.304**	.007	.087	-.025
	Sig. (2-tailed)	0.047	.497	.048	.228		**0.018**	.523	.002	.945	.381	.802
Serum retinol	R	.038	.087	.107	.051	0.232*	1	-.023	.012	.055	.109	-.031
	Sig. (2-tailed)	.700	.378	.278	.608	0.018		.820	.902	.576	.269	.753
Serum ferritin	R	.075	-.082	.135	-.050	.063	-.023	1	-.048	.011	-.090	-.180
	Sig. (2-tailed)	.446	.405	.171	.614	.523	.820		.630	.910	.364	.067
Serum Transferrin	R	.015	.117	-.022	-.014	-.304**	.012	-.048	1	-.054	.189	.141
	Sig. (2-tailed)	.884	.238	.822	.888	.002	.902	.630		.587	.055	.154
Serum Folic acid	R	-.098	-.147	.156	-.064	.007	.055	.011	-.054	1	-.137	-.093
	Sig. (2-tailed)	.322	.136	.114	.517	.945	.576	.910	.587		.165	.348
Serum Vitamin B12	R	.001	.174	-.140	-.110	.087	.109	-.090	.189	-.137	1	**.263****
	Sig. (2-tailed)	.990	.077	.155	.264	.381	.269	.364	.055	.165		**.007**
Serum CRP	r	-.142	.175	-.033	-.105	-.025	-.031	-.180	.141	-.093	.263**	1
	Sig. (2-tailed)	.150	.075	.737	.288	.802	.753	.067	.154	.348	.007	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 4.11a: Correlation analysis of biochemical test with severity of anaemia

Type of severity	Moderate (N=59)							Mild (N=45)						
	Hb	Retinol	Frt	sTfr	FA	B12	CRP	Hb	Retinol	Frt	sTfr	FA	B12	CRP
Variables (n)	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value
Adjusted Hb	-	0.115 /0.385	0.13/ 0.327	-0.24/ 0.067	0.145/ 0.274	-0.107/ 0.422	-0.043/ 0.749	-	0.151 /0.321	-0.70 0.647	-0.229 /0.129	0.070 /0.649	-0.010 0.947	0.242 /0.11
Serum Retinol	-	-	-0.040/ 0.766	0.113/ 0.393	-0.083/ 0.532	0.084/ 0.527	0.057/ 0.668	-	-	-0.004 /0.98	-0.083 /0.586	0.273 /0.07	0.100 0.512	-0.147 /0.335
Serum frT	-	-	-	-0.106/ 0.424	0.033/ 0.805	-0.134/ 0.310	-0.92/ 0.145	-	-	-	0.050 /0.743	-0.017 /0.913	-0.053 0.727	-0.162 /0.289
Serum Trsf	-	-	-	-	-0.118/ 0.375	0.472** /0.000	0.310* /0.017	-	-	-	-	-0.009 /0.953	-0.040 /0.792	-0.185 /0.223
Serum FA	-	-	-	-	-	-0.244/ 0.062	-0.074/ 0.579	-	-	-	-	-	0.010 /0.948	-0.138 /0.366
Serum Vitamin B12 result	-	-	-	-	-	-	0.322* /0.013	-	-	-	-	-	-	0.237 0.118

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Hb= Hemoglobin value (g/dL), Retinol= Serum retinol in (μmol/L), Frt= Serum ferritin, sTfr= serum transferrin, FA= Folic acid

Table 4.11b: Correlation analysis of selected socio-demographic with severity of anaemia

Type of severity	Moderate							Mild						
Variables (n)	Hb% r/p-value	Retinol r/p-value	Frt r/p-value	sTfr r/p-value	FA r/p-value	B12 r/p-value	CRP r/p-value	Hb r/p-value	Retinol r/p-value	Frt r/p-value	sTfr r/p-value	FA r/p-value	B12 r/p-value	CRP r/p-value
Age of woman	-0.260* /0.047	0.173 /0.191	-0.047 /0.722	-0.033 /0.815	0.161 /0.222	-0.075 /0.572	-0.10 /0.223	-0.057 /0.710	-0.137 /0.369	0.242 0.109	0.096 /0.529	-0.006 /0.970	0.075 0.623	-0.113 0.462
GA	0.145 /0.272	0.160 /0.226	-0.173 0.191	0.202 /0.124	-0.133 /0.314	0.065 /0.624	0.045 /0.736	0.069 0.653	0.005 0.973	0.032 /0.836	-0.013 /0.933	-0.173 0.257	0.292 /0.051	0.360* /0.015
delivery place	0.363** /0.005	-0.014 /0.917	-0.065 /0.627	0.138 /0.296	0.049 /0.712	-0.168 /0.202	-0.008 /0.953	-0.133 0.384	-0.014 0.930	-0.210 /0.166	0.003 0.984	-0.255 0.091	-0.008 /0.961	-0.303* /0.043
IFA	0.339** /0.009	0.078 /0.557	0.240 /0.067	0.112 /0.40	0.310* /0.017	-0.228 /0.083	-0.036 0.788	-0.048 /0.754	0.135 /0.377	-0.006 /0.970	-0.213 /0.161	-0.048 /0.753	-0.077 0.613	-0.025 /0.872
Animal Product	0.119 /0.371	-0.052 /0.698	-0.038 0.774	-0.040 0.763	-0.156 /0.239	-0.40 /0.711	0.010 0.939	-0.028 /0.856	-0.236 /0.118	0.118 /0.440	-0.012 /0.940	0.228 /0.132	0.096 /0.532	0.167 /0.273
Vitamin A rich food	-0.011 /0.932	-0.188 /0.155	-0.038 /0.775	0.017 /0.592	0.067 /0.617	0.126 /0.319	0.141 /0.288	-0.096 /0.529	0.078 /0.608	0.105 /0.493	-0.276 /0.067	0.176 /0.247	-0.141 /0.357	-0.117 0.443
Level of Fasting	-0.459** /0.000	-0.075 /0.574	0.124 /0.349	0.081 -0.542	0.127 /0.336	-0.187 0.155	0.019 /0.886	-0.186 0.222	0.014 0.927	0.171 0.261	-0.275 /0.067	0.036 /0.816	0.116 /0.449	-0.020 /0.899
Infection of Malaria	-0.209 /0.113	-0.049 /0.712	0.069 /0.604	0.157 0.236	-0.131 /0.323	-0.003 /0.982	-0.313 /0.016	-0.223 /0.141	-0.104 /0.496	0.101 0.507	0.180 /0.237	-0.037 /0.808	-0.407* 0.005	-0.564** 0.000
Blood Loss	-0.242 /0.065	0.031 /0.815	-0.260* /0.047	0.026 /0.846	- 0.072 /0.586	-0.080 /0.545	0.192 /0.145	0.024 /0.874	-0.192 /0.206	-0.044 /0.773	0.101 /0.511	0.151 /0.323	-0.013 /0.933	0.132 /0.388

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Hb= Hemoglobin value (g/dL), Retinol= Serum retinol in (μmol/L), Frt= Serum ferritin, sTfr= serum transferrin, FA= Folic acid

In the Table 4.12a and 4.12b the correlation analysis of selected socio-demographic with prevalence of anaemia with adequate iron store (anaemic only based on Hb value) as a dependent variable there are not a significant correlation between selected socio demographic data and biochemical analysis in both IDA with and without considering the values of serum ferritin.

Table 4.12a Correlation analysis of biochemical test with prevalence of IDA

Iron biomarker	**Non IDA**							**IDA**						
	Hb	Retinol	Frt	sTfr	FA	B12	CRP	Hb	Retinol	Frt	sTfr	FA	B12	CRP
	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value
Adjusted Hb		0.158	0.051	-0.112	0.067	0.168	0.005		0.164	-0.512	-0.522**	-0.074	-0.083	-0.049
	-	/0.188	/0.674	/0.354	/0.576	/0.162	/0.965	-	/0.362	/0.002	/0.002	/0.682	/0.645	/0.788
Serum Retinol			0.010	-0.065	0.108	0.144	-0.084			0.328	0.087	-0.025	0.043	0.035
	-	-	/0.934	/0.589	/0.372	0.230	/0.484	-	-	/0.063	/0.631	0.891	/0.813	/0.845
Serum frT				0.176	0.008	-0.173	-0.200				0.273	0.174	-0.111	-0.164
	-	-	-	/0.141	/0.950	/0.149	/0.094	-	-	-	/0.124	/0.334	/0.538	/0.362
Serum Trsf					0.008	0.112	-0.144					-0.137	0.438**	0.467**
	-	-	-	-	/0.950	/0.351	/0.230	-	-	-	-	/0.447	/0.011	/0.006
Serum FA						-0.065	-0.082						-0.309	-0.109
	-	-	-	-	-	/0.589	/0.499	-	-	-	-	-	/0.080	0.547
Serum Vitamin B12							0.127							0.604**
result	-	-	-	-		-	/0.291	-	-	-	-	-	-	0.000

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Hb= Hemoglobin value (g/dL), Retinol= Serum retinol in (μmol/L), Frt= Serum ferritin, sTfr= serum transferrin, FA= Folic acid

Table 4.12b Correlation analysis of selected socio-demographic with prevalence of IDA

Iron biomarker	Anemic with adequate iron store (n= 58)							IDA (n=45)						
	Hb	Retinol	Frt	sTfr	FA	B12	CRP	Hb	Retinol	Frt	sTfr	FA	B12	CRP
	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value	r/p-value
Age of woman	0.041	0.006	-0.025	-0.056	-0.145	0.024	-0.058	-.423**	0.122	0.410*	0.228	-0.025	-0.089	-0.252
	/0.733	/0.958	/0.836	/0.645	/0.228	/0.844	0.632	/0.014	/0.499	/0.018	/0.203	/0.889	/0.623	0.158
GA	0.154	0.043	0.017	0.043	-0.054	0.189	0.054	-0.051	0.161	-0.093	0.151	-0.341	0.178	0.384*
	/0.201	/0.725	0.885	/0.722	/0.652	/0.115	/0.657	/0.778	/0.371	/0.606	/0.402	/0.052	/0.321	/0.027
delivery place	0.104	0.000	0.058	0.016	0.033	-0.111	-0.186	0.307	-0.104	-0.090	0.016	-0.296	0.029	-0.111
	/0.389	0.998	/0.629	/0.894	/0.782	0.359	0.120	/0.082	/0.564	/0.617	0.931	/0.095	/0.873	0.539
IFA	0.115	0.133	0.224	-0.055	0.185	-0.117	-0.047	0.319	0.061	-0.027	0.015	0.110	-0.202	-0.016
	0.339	/0.269	/0.061	0.646	/0.123	/0.331	/0.696	/0.070	/0.737	/0.881	/0.936	/0.543	/0.260	/0.931
Animal Product	0.275*	-0.043	0.045	-0.108	0.079	0.067	0.092	0.013	-0.249	-0.276	0.012	-0.173	0.013	0.023
	/0.020	/0.724	/0.712	0.370	0.515	/0.580	/0.447	/0.942	/0.162	/0.120	/0.947	/0.337	/0.941	/0.899
Vitamin A rich food	0.051	-0.051	0.048	-0.190	0.208	-0.083	0.009	-0.094	-0.104	-0.099	0.126	-0.063	0.221	0.081
	/0.673	/0.676	/0.694	/0.112	/0.082	/0.492	/0.938	/0.601	/0.563	/0.584	/0.485	/0.728	/0.217	/0.653
Level of Fasting	0.356**	0.011	0.285*	-0.081	0.202	-0.011	-0.001	0.313	-0.093	-0.062	-0.162	-0.171	0.015	-0.030
	0.002	/0.927	0.016	/0.500	/0.091	/0.929	/0.992	/0.076	/0.606	/0.731	0.367	/0.341	/0.935	/0.867
Infection of Malaria	-0.244*	-0.187	0.196	0.168	-0.005	-0.366**	-0.473**	-0.300	0.101	0.185	0.207	-0.186	0.091	-0.335
	0.041	/0.117	/0.101	/0.162	/0.970	0.002	/0.000	/0.090	/0.576	/0.303	/0.249	/0.301	/0.616	/0.057
Blood Loss	-0.134	-0.059	-0.127	-0.70	0.090	-0.078	0.168	-0.302	-0.177	0.010	0.265	-0.043	-0.010	0.146
	/0.267	/0.626	/0.290	/0.562	/0.454	/0.520	/0.162	/0.088	/0.326	/0.956	/0.136	/0.812	/0.954	/0.418

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Hb= Hemoglobin value (g/dL), Retinol= Serum retinol in (μmol/L), Frt= Serum ferritin, sTfr= serum transferrin, FA= Folic acid

4.1.11 Correlation and regression analysis of Hb, VAD and B12

Logistic regression analysis was used to assess association between hemoglobin and retinol. Regression analysis of serum retinol with altitude adjusted hemoglobin provided the equation of Hb=9.561+0.048(Retinol), with test of slope 95% CI of the diference. This regression equation tells that by assuming the other variable constant an increase of 1 μmol/L in the retinol concentration was significantly associated with an increase of 0.048 g/L haemoglobin. Again in moderately anaemic pregnant women there was a correlation between serum transferrin and vitamin B12. There was also a negative correlation between age of women and hemoglobin value (r= -0.260*; p=0.047); where as there was a positive correlation between IFA supplementation and Hb. This show that iron and FAD might be the causes for depletion of Hb. Surprisingly that there was a positive correlation between IFA supplementation and serum folic acid. Hemoglobin was associated with vitamin A according to study conducted in Wolayita, Southern Ethiopia (Hiwot *et al.*, 2014). And the same result was found by Geremedhin *et al.*, (2014).

Factors affecting and predictors in severity of anaemia

From the correlation analysis of serum transferrin and serum FA with other biochemical analysis is presented in Table 4.13a and Table 4.13b. Positive correlation was found between serum transferrin and serum B12 (r=0.472; p=0.000); again between serum CRP and serum B12 (r=0.322; p=0.013) in moderately anaemic pregnant women. Gebremedhin *et al.*, (2014) found that from the interaction of micronutrients in associating with Hb value synergistic interactions were not witnessed across the vitamin A and ID. This was supported by this study. According to Gebremedhin *et al.*, (2014) in level indicates infection/inflammation only 8.4% of the pregnant women had elevated serum CRP (CRP≥6mg/l). This study was agreed with the interaction between iron and vitamin A but, there was contradicted discussion regarding inflammation. This might be due to variation in cutoff points. A previous study shows that high prevalence of anaemia: IDA and ID amongst women aged 31-35 years and involvement of women in income generating activities was associated with reduced risk of anaemia.

4.1.11 Correlation and regression analysis of Hb, VAD and B12

Logistic regression analysis was used to assess association between hemoglobin and retinol. Regression analysis of serum retinol with altitude adjusted hemoglobin provided the equation of Hb=9.561+0.048(Retinol), with test of slope 95% CI of the diference. This regression equation tells that by assuming the other variable constant an increase of 1 μmol/L in the retinol concentration was significantly associated with an increase of 0.048 g/L haemoglobin. Again in moderately anaemic pregnant women there was a correlation between serum transferrin and vitamin B12. There was also a negative correlation between age of women and hemoglobin value (r= -0.260*; p=0.047); where as there was a positive correlation between IFA supplementation and Hb. This show that iron and FAD might be the causes for depletion of Hb. Surprisingly that there was a positive correlation between IFA supplementation and serum folic acid. Hemoglobin was associated with vitamin A according to study conducted in Wolayita, Southern Ethiopia (Hiwot *et al.*, 2014). And the same result was found by Geremedhin *et al.*, (2014).

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Table 4.13a: Correlation analysis of serum transferrin and other biochemical analysis

Variables	Transferrin	Moderate			Mild	
	N	Pearson's r	p-value	N	Pearson's r	p-value
Hemoglobin	59	-.240	.067	45	-.229	.129
Serum Folic acid	59	-.118	.375	45	-.009	.953
Serum Vitamin B12	59	0.472*	.000	45	-.040	.792
Serum CRP	59	0.310*	.017	45	-.185	.223

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.13b: Correlation analysis of serum FA and other biochemical analysis

Variables	Serum FA	Moderate			Mild	
	N	Pearson's r	p-value	N	Pearson's r	p-value
Serum Vitamin B12	59	-.244	.062	45	.010	.948
Serum CRP	59	-.074	.579	45	-.138	.366
	Serum B12					
Serum CRP	59	.322*	.013	45	.237	.118

*. Correlation is significant at the 0.05 level (2

Among up to date finding conducted in the southern Ethiopia by Gibson *et al.*, (2008) showed that none of the biochemical indices of folate or vitamin B-12 status, plasma retinol were significant predictors of hemoglobin concentrations. This finding was not supported by this study. This might be due to frequently consumption of folate rich fermented enset in southern Ethiopia. Because in Sidama Zone of Southern Ethiopia, maize (*Zea mays L.)* and fermented enset (*Enset ventricosum*) products are the major staple foods, contributing up to 90% of energy. In the current study the older the woman had the least hemoglobin (9.40±0.94), serum folate (6.09±2.97) and CRP (3.8±0.8) but relatively had higher serum retinol (1.66±1.96), ferritin (34.6±30.0), transferrin (6.53±2.3).

4.3 Limitation of the study

This study has a few limitations, first, the nature of the study being a cross-sectional study design; it does not show risk factors in detail. Second, it is a hospital based study with a relatively small sample size. Lack of comparison group (aneamic with non anaemic). The pregnancy complications such as hypertension, blood sugar level and urinary albumin was not determined; even that study participants were not recalled their complications such as hypertension, blood sugar level and urinary albumin and no further interpretation of analysis was done in such complication factors. The current status of the mother somewhere else than Ambo

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Table 4.13b: Correlation analysis of serum FA and other biochemical analysis

Variables	Serum FA	Moderate			Mild	
	N	Pearson's r	p-value	N	Pearson's r	p-value
Serum Vitamin B12	59	-.244	.062	45	.010	.948
Serum CRP	59	-.074	.579	45	-.138	.366
	Serum B12					
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health centers and Ambo hospital was not included. And lack of anthropometric measurement of participants and their infants' are also other limitation.

5. Conclusions and Recommendation

5.1 Conclusion

In conclusion, this study helped us to understand the association between the level of anaemia and the biosocial factors. Women in developing countries have a high prevalence of ID but also tend to be deficient in other micronutrients such as zinc, Iodine, Vitamin A, C, B12, folate. The current study has several advantages over previous Ethiopian studies that examined micronutrient status during pregnancy including study area.

The prevalence of anaemia among third trimester anaemic pregnant women in this study compared with second trimester anaemic pregnant women was high. Hemoglobin values were found to be significantly associated with serum retinol positively. Study participants had high inflammation/infection based on serum CRP>5mg/l. Not only hemoglobin mean concentration of other micronutrients comparison between older and younger the older pregnant women were observed more deficient almost in all micronutrients than younger women. The high prevalence of vitamin B12 deficiency more than expected might be due to less amount of B12 required and the supplementation of folic acid which causes to increase in B12 because they have the interrelation between them. The present study has shown that clinical study of micronutrients iron biomarkers and vitamins deficiency is a hidden risk for pregnant women especially in second and third trimester.

5.2 Recommendation

The necessity for vitamin B12 supplementation needs to be confirmed with prospective randomized trials from different regions of our country before the introduction of a fortification program for prevention of neural tube defect. According to Baingana *et al.,* (2014) the recent development of analytical methods for analysis of hepcidin in biological samples has provided an opportunity to include hepcidin as a novel biomarker reflecting iron status and to that hepcidin concentration was positively correlated with ferritin and this test was recommended to be included in the future research. Further research on risk factors of anaemia, which include rural residents, should be conducted to strengthen and broaden these findings.

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Appendix: A

Table 1: SI unit, Conversion factors and alternative units of analyte

Analyte	Normal Range	SI unit	Conversion factor	Alternative unit
Hemoglobin concentration		g/L		
Retinol	µmol/L	µmol/L	x 0.286	mg/L
Serum folate		nmol/L	x 0.441	µg/L
Serum vitamin B12		pmol/L	x 1.357	ng/L
Ferritin	15-150 µg/L	µg/L	µg/L x 2.247 = pmol/L µmol/L x 445000 = ng/mL, µg/L = ng/mL,	pmol/L µmol/L ng/mL=µg/L
Transferrin	2.71-3.91 g/L	g/L	mg/dL x 0.01 = g/L, g/L x 100 = mg/dL, g/L x 12.6 = µmol/L µmol/L x 0.0796 = g/L).	mg/dL µmol/L µmol/L x 0.0796 = g/L).
Serum Folate	8.6-37.0 ng/mL	ng/mL	nmol/L x 0.44 = ng/mL, ng/mL x 2.27 = nmol/L.	nmol/L ng/mL
Serum B12	150-200 pg/mL	pg/mL	pmol/L x 1.36 = pg/mL and pg/mL x 0.738 = pmol/L	pmol/L pg/mL
C-reactive protein	<6.81 mg/L	mg/L	mg/L x 9.52 = nmol/L, mg/dL x 95.2 = nmol/L, mg/L x 0.1 = mg/dL, mg/dL x 10 = mg/L, mg/dL x 0.01 = g/L and g/L x 100 = mg/dL.	mg/L nmol/L, mg/dL g/L

Source: Sonja Nicholson, Katie Dearnley, Birgit Teucher and Alison Lennox (2008/2009 – 2009/10). National Diet and Nutrition Survey Headline results from Years 1 and 2 (combined) of the Rolling Programme

Appendix: B

Ethical clearance from AAU College of Natural Science Ethics review Board

COLLEGE OF NATURAL SCIENCES
Addis Ababa University

OFFICE OF THE DEAN
የዲን ጽ/ቤት

የተፈጥሮ ሣይንስ ኮሌጅ
አዲስ አበባ ዩኒቨርሲቲ

Ref: ቁጥር: CNSDO/216/07/15
Date: ቀን: January 13, 2015

To Whom It May Concern

The Ethical Committee of the College of Natural Sciences in its meeting held on 22/12/2015 (Minutes No.12) has examined the project entitled **"Vitamin a, B12, Folate and Hemoglobin Status of Third Trimester Pregnant Women Attending Care"** by Teshome Bekele (Department of Center for food Science and Nutrition) for ethical approval.

The Proposal is approved for implementation. The decision will remain valid until December 21, 2015.

With regards,

Negussie Retta, (Professor)
Dean, College of Natural Sciences

Encl:

- RERC Minutes

ሥ"ልኩTel: +251-11-123-9472
ፋክስFax: +251-11-123-9469
ፖ"ሣ"ቁ/POBox: 1176, Addis Ababa, Ethiopia
ኢ"ሜ"ል/Email: dean_cns@aau.edu.et

Please quote our reference number in your correspondence.

"Examine all things; hold fast that which is good"

"ሁሉን መርምሩ፣ መልካሙን ያዙ"

Appendix: C

Ethics Review Board attachment of minute

January 08, 2015

Professor Negussie Reta
Dean, College of Natural Sciences
Addis Ababa University

Subject **College Research Ethics Review Board (IRB) Decision**

Dear Professor Negussie,
The College IRB in its meeting on December 22, 2014 has evaluated research proposals by the following principal investigators and approved for implementation.

1. Dejene Getachew
2. Destaw Damtie
3. Getachew Kebede
4. Hadra Ali
5. Solomon Balemi
6. Teshome Bekele

Enclosed please find Minutes of IRB Meeting.

With best regards,

Chairperson
Hassen Mamo (PhD)

cc
1. Dejene Getachew
2. Destaw Damtie
3. Getachew Kebede
4. Hadra Ali
5. Solomon Balemi
6. Teshome Bekele

Appendix: D

Letter of support from Amboo Hospital

Qajeelcha Eegumsa Fayyaa Godina Shawaa Lixaa Hospitaala Amboo
በምዕራብ ሸዋ ዞን ጤና መምሪያ
የአምቦ ሆስፒታል

ቁጥር አ/ሆ/ኸ9-157/2080/2007
ቀን 07/04/2007

ለአዲስ አበባ ዩኒቨርሲቲ

አዲስ አበባ

ጉዳዩ:-ለትብብሩ ፍቃደኞች መሆናችንን ማሳወቅ ይሆናል::

ከላይ በርእሱ እንደተጠቀሰው ተማሪ ተሾመ በቀለ በአዲስ አበባ ዩኒቨርሲቲ የምግብ ሳይንስና ኒዩትሪሽን ማስከል መሆናቸውንና በሶስተኛው መንፈቅ የእርግዝና ጊዜ የቅድመ ወሊድ ክትትል የሚያደርጉ እናቶች የቫይታሚን ሲ፤ ቢ12፤ ፎሊት እና የደም ማነስ ስርጭት ምክኒያቶች በሚል ርእስ ላይ የመመረቂያ ፅሁፍ ድጋፍ እንዲደረግላቸው በደብዳቤ ቁጥር QMF/421/D/2 በቀን 07/04/07 የድጋፍ ደብዳቤ ከም/ሸዋ ዞን ተጽፎላቸዋል::

ስለዚህ እኛ በሆስፒታላችን ይህንን ምርምር እንዲሰሩ ፍቃደኛ መሆናችንን በትህትና እናሳውቃለን::

ከሰላምታ ጋር

Dr. Dessale Iikedu

Appendix: E

Letter of Support from Zonal Health Bureau

Lakk Q&F/421/012

Guyyaa 27/04/07

Waajjira E/Fayyaa Magaala Amboo tiif

Hospitaala Amboo tiif

Amboo

Dhimmii :- Deegarsa gaafachuu ilaala

Mata duree irratti akkuma ibsamuuf yaalameetti Tashoomaa Bqqalaa barataa Yunivarisitii Addis Ababaa kan ta'an barumsa eebba isaaf Dhaabbilee fayyaa Magaala Amboo keessaa kan jiran Buufataalee fi Hospitaala Amboo irratti dubartoota tajaajila dahumsa duraa hordofan haqina Vitamine A, B12 Folat fi Anemia irratti qorannoo akka gaggeessuu danda'an xalayaa lakk. 211/07/14 guyyaa 3/4/ 2007 barreesaniin nu gafatanii jiru. Haaluma kanaan isiinis dhimma kana beektanii qorannoo iddoo gaggeesanitti deegarsa barbaachiisaa ta'ee akka gootaniif ni gaafana.

Nagaa wajjin

ABAATEE ARGAAWU

Health Officer (Bsc, MPH)

Qind GFM fi PEF

Appendix: F Consent form (English)

Consent form

Title: Vitamin A, B12, folate, and hemoglobin status in third trimester pregnant women of West showa Zone of Oromia Region, Ambo Health Center.

Principal Investigator: Teshome Bekele

Institutions: Food Science and Nutrition, Addis Ababa University

Introduction

Folate has proven to be critical in reducing the risk of neural tube defects. The brain and spinal cord development from the neural tube, and defects in its orderly formation during the early weeks of pregnancy may result in various central nervous system disorders and death. I have decided to study food science and nutrition at Addis Ababa University. This is because my country is among one of the world's food insecure countries and still our people are not aware of nutrition even if nutrition plays a significant role in their life. Most evidences support that many chronic diseases such as heart disease, cancer and cardiovascular diseases are highly linked with poor nutrition and conversely, that consuming nutritious and functional foods can reduce the risk of these diseases. My study is thus an opportunity to investigate the incidence of Iron Deficiency Anemia among pregnant women in with the hope of generating evidenced- based data than can improve the quality of antenatal services offered. Maternal micronutrient nutrition is an important determinant of size and body composition of the fetus. Maternal iron, iodine, calcium, folate, vitamin A, and vitamin C nutrition all influence offspring size.

In Ethiopia, the incidence of malnutrition is relatively very high and also most statistical data revealed that malnutrition is a greatest single cause of child death in the country. Previously, the government has been designing strategies to double production as a sole solution for food insecurity and malnutrition. However, even if there were improvements in the production, there is little reduction in malnutrition. Therefore, my aim as a citizen of Ethiopia is to address the issue of malnutrition and make great contribution to the ambitious plan of the government through research and teaching.

Objective of the Study:

The objectives of this study will be to determine the serum retinol, serum ferritin, serum transferrin, C-protein reaction (CRP), serum B12 and hemoglobin status in 3rd trimester anemic pregnant women. Specifically 1) To evaluate Iron Deficiency in pregnant women, 2) To evaluate Vitamin A deficiency of anemic and non anemic in third trimester pregnant women, 3) To investigate the relationship between anaemia and folate, B12 and vitamin A status of pregnant women and 4) To investigate folate and B12 status of pregnant mothers

Procedures

If you agree to participate, we will conduct the research on blood samples collected from you and will be analyzed in Addis Ababa University or Ethiopian Public Health Institute in addition we will ask you about your food intake pattern, health and information related to your social and demographic characteristics. The interview will only take 10-20 minutes; it will be transported to Addis Ababa for analysis.

Risks: nothing harm full will come to you, as the method only involves analysis of the serum retinol, serum folate, B12 and hemoglobin level of your blood. If you have questions about your rights as a participant of research, you may contact to Mr. Teshome Bekele :(0911543975 or 0942574564) or by E-mail elemabekele@gmail.com/eteshomebekele@yahoo.com Or Dr.Kaleab Baye: (0911890489) or by E-mail kaleabbaye@gmail.com

Benefits

There are no direct benefits to you. Therefore taking iron supplements before assessing iron status is clearly unwise; hemoglobin tests alone would fail to make the distinction because excess iron accumulates in storage. However the findings will possibly help others. The finds has significant benefits for the understanding of Iron Deficiency Anemia is a major public health problem particularly among pregnant women in developing countries with adverse effects on the mother and the new born it is considered as the single most prevalent nutritional deficiency. Worldwide my study will aim to assess the problem/limitations of health care practitioners to treat Iron Deficiency Anemia with other micronutrient deficiency. It contribute to the strategic objective of National Nutrition Program to reduce the prevalence of stunting up to 30%; of

chronic under nutrition in women of reproductive age to 19% by 2015 by supplementing an appropriate micronutrients for only those deficient in specific micronutrients and Saves the cost of hungry which is estimated about 12% more of Global Development Program for only anemia cases in Ethiopia.

Cost

There is no cost to for participant.

Compensation

There will be no compensation for participating

Participant Rights

If I have said things that are not clear to you, you may ask without hesitation and I will answer. You may feel free and ask questions. Your participation in the study is entirely voluntary and up to you to decide. There is no penalty if you do not agree to participate. If you don't agree to participate, you can say no without worry. You are not under any obligation to participate in this research project; there are no negative consequences to deciding not to participate your health centre and extension health worker will continue to provide health service to you as usual.

If you do agree to participate, you are not obliged to answer specific questions or to provide information you do not wish to give. You have the right to not answer specific questions but continue as a participant. If you choose to participate and have agreed to have the interviews; yet, you can withdraw from the interview by stating that you have decided to withdraw.

In addition, you can withdraw from the project up until the point when I provide the summary report of the interviews. There will be no negative consequences to withdrawing from the research project. You can state your intention to withdraw from the project by contacting me, Teshome Bekele (the researcher), whose contact information is provided at the end of this form. If you choose to withdraw from the project please indicate whether you want the previously collected data destroyed or returned to you.

Confidentiality

Test result and any information will be kept private .only the research team will have access to your information. When we write a report every ones information will be put together so that information about you or any other individual cannot be seen your blood will be identified with random numbers on serum vials. A list with the names and numbers will be kept in a private, locked file cabinet.

Persons to contact

If you have any questions, you can ask at any time if you have additional question about the study you may contact to Mr. Teshome Bekele at phone number (0911543975) E-mail. **elemabekele@gmail.com.**

Or Dr. Kaleab Baye (0911890489) E-mail **kaleabbaye@gmail.com**

If you agree to participate in the study, please sign or give your left thumb impression at the space indicated below.

Thank you for your cooperation.

Signature:

Mother's name_____

The study has been explained to me and my questions have been answered to my satisfaction.

I agree to participate in this study.

_____ _____ _____

Signature of the left thumb impression printed name Date

_____ _____ _____

Signature of study representative Printed name Date

Appendix: G **Consent form (Afan Oromo)**

Uunkaa Walii galtee

Mata-dure: Qorannoo Haadholii sadarkaa Vitamin A, Vitamin B12, Folic Aciidii fi sadarkaa dhiiga irratti argamu yeroo tajaajila hordoffii da'umsaa magaalaa Amboo Buufata Fayyaa Ambootti gaaggefamu.

Qorannoo Gaaggessaan: Tashoomaa Baqqala

Instituutii: Food Science and Nutrition, University Finfinnee

Seensa

Foolik aciidiin rakkoo sammuu daa'immaanii irra gahuu danda'uuf furmaataa gaarii akka ta'e qorannoodhaan mirkanaayeera(neural tube defect). Guddinni sammuu fi ribuu dugdaa dhibee kana irraa hambisuuf baayye gargaara keessattuu ji'oottan jalqabaa ulfaa irratti. Qorannoo kana ademsisuuf kan na kakkaase sababa biyyi kooo addunyaa kana irraa biyoota hanqina midhaaniitiin midhaamte keessaa ishee tokko ta'uu ishii fi sababa hanga ammaatti hubannoon hangami akka midhaan jireenya keenya keessatti miidhaa fidu sirriitti hubachuu dhabuuti.

Akka ragaaleen ibsanitti dhibeen akka onnee, Kaanserii fi k.k.f hanqinna nyaataa waliin hidhannoo cimaa akka qaban ni ibsa. Qorannoon kiyyaa carraa guddaa sababa ka'umsa hanqinna dhiigaa dubartoota ulfaa raga dhugaa fi haqa qabeessa ta'e dhiyeessuun tajaajila haadholii ulfaa sirriitti gargaara. Hanqinna nyaataa keessa Ironii, Ayoodinii, Foolik aciidii, Vitaamin A, Vitamin C kanneen haadhaa fi daa'immaan midhaan.

Itoophiyaatti ka'umsi hanqina nyaataa sadarkaa ol'aanaa irraa akka jiruu fi sababni hanqinna nyaataa du'a daa'immaaniitiif sababa tokkoofi tokko ta'e qofaa dha. Kanaan dura mootummaan istraateejiin du'a haadholii hir'isuu baayyee hojii irra oolchaa jira. Haa ta'uu malee carraaqiin kun hanga ta'e hir'isaa jiraatulle hanga ammaatti miidhaan hanqina nyaata ammas guutummaa guutuutti furmaata hin arganne. Kanaafuu, kaayyoon koo akka dhalattaa biyyaatti dhimma hanqinna nyaataa kana hir'isuuf tattaafatuu fi karoora mootummaan dhimma kana irratti baasegalmaan ga'uus gahee koo qorannoo fi barsiisuun ni baha.

Haala Qoranichaa

Yoo ati qorannoo kana irratti himaachuuf fedhintaa kee nuu ibsite dhiiga qorannoo kanaaf ta'u sirraa fudhachuun laboratoorii keessatti qorannoon ni adeemsifama. Gama biraatiin odeeffannoo waa'ee haala sirna nyaataa keessaanii yoo guddatee daqiiqaa 15 hanga 25tiif afaaniin isin gaafachuun sababoota dhibee kanaaf ta';uu danda'an jedhamanii yaadaman qorachuuf ni gargaara.

Miidhaa

Miidhaa ilaalchisee midhaa tokkolle sirratti hin geessisu, jechuun takkaa dhiigni keessan fudhatamnaan laboratoorii keessatti adeemsifama malee wanti isin wajjiin walitti fidu tokkolle hin jiru. Gaaffilee adda addaa yoo isinti uumamte bilbila kootiin 09111543975 ykn 0942574564 imeeliin koo elemabekele@gmail.com/eteshomebekele@yahoo.com Karaa biraatiin Dr. Kaleab Baye (09111890489) imeeliin isaanii immoo kaleabbaye@gmail.com gaafachuun ni danda'ama.

Bu'aa:

Bu'aan kallattidhaan argamuu hin jiru. Wanti beekkamuu qabuu yeroo ulfaa keessatti atoo sababa ka'umsa dhibee hanqina dhiigaa kun dhufeen hin beekin ykn qorannoo dhiigaa hanqinna Ironii qofaan murteeffamuun Ironii fudhachuun sirrii miti maliif jennaan yoo Ironii baay'inaa nafa keessa jiraate dhibeen haadha fi daa'immaan irra gahu cimaa waan ta'eef. Kana jechuun qorannoon kun alkallaattiidhaan sababa ka'umsa dhibee kanaa ni ibsa, biyyoota guddataa jirani irratti sababni dhibee kanaa maal akka ta'e beekuuf carraa ni uuma. Addunyaaf immoo beektonnii dhibee kana wal'aanuuf carraaqan bu'aa baayye irraa argatu. Karaa biraatiin sababa qancara daa'immaaniitii fi baasii Itoophiyaa keessatti qofa waa'ee beelaatiin sagantaa guddinaa Walii gala iraa 12% ba'u ni hir'isa.

Baassii

Baasiin namni qorannoo kana irratti hirmaatu baasu tokkollee hin jiru.

Bakka Bu'ummaa

Sababa dhiigni keessan qorannoof fudhatameen bakka bu'ummaan wanti kaffalamu hin jiru.

Mirga Hirmaattotaa

Wanti gaaffii uumu yoo jiraate sodaa fi shakkii tokko malee akka na gaafattan isin ni jajjabeessa gaaffii keessan hundaaf deebiin ni kennama. Hirmaachuuf murteessuun mirga keessan keessan qofa. Yoo fedhintaa hin qabaanne hin ta'u jechuun deebii kennuu ni dandeessu. Ogeessi fayyaa fi Exteenshiiniin fayyaa buufata fayyaa keessanii tajaajila barbaachisu hundumaa isiniif ni laatu.

Mirkanneessan keessan

Qorannoo fi deebiin/firiin qorannoo keessanii garee qoranichaa adeemsisuu (buufata qorannoo)f malee qaama biraatiif dabarfamee hin kennamu. Qoranichi erga adeemsifamee bu'aan argamee booda gabaasni yoo dhiyaatu walitti cuunfamee waan dhiyaatuuf qabxiin eenyuu kan eenyu akka ta'e hin beekkamu kana jechuun firii qorannoo nama dhunfaa namni biraa beekuu hin danda'u. Koodiin kennameefii laakkofsa qofaan galmee keessatti hidhamee taa'a.

Qaamni Dhimmichi ilaallatu

Akka carraa wanti gaaffii uumu yoo jiraate yeroo barbaaddanittin gaafachuu ni dandeessu. Obboo Teshoomee BeqqeleeLakk. Bilbilaa (0911543975) E-mail. **elemabekele@gmail.com.**
Or Dr. Kaleab Baye Lakk. Bilbilaa (0911890489) E-mail **kaleabbaye@gmail.com**

Yoo qorannoo kana irratti hirmaachuuf fedhintaa qabaattan maqaa fi mallaattoo keessanii asii gaditti mirkaneessa.

Qorannoo kanairratti waan hirmaattaniif galatooma

Mallattoo

Maqaa Haadhaa_____

Qoranichi naa ibsamee ifa naa ta'eera. Qorannoo kanairratti hirmaachuuf waliigaleera.

_____	_____	_____
Mallattoo	Maqaa barreeffamaan	Guyyaa
_____	_____	_____
Mallattoo bakka bu'aa qoranichaa	Maqaa barreeffamaan	Guyyaa

Appendix: H: English version Questionnaire

A questionnaire for the Nutritional Assessment and practices of second and third trimester pregnant Women
Hello, my name is _____. I am a student of Food Science and Nutrition in Addis Ababa University. I am interested in gathering information about the opinions and practices of pregnant women with iron-deficient anemia. This information is important for elaborating strategies to improve quality of care provided at prenatal clinics. Your participation in this survey will contribute to this purpose. The information you are going to provide will be used only in scientific purposes, your name will remain confidential. The interview will take 15 minutes.
Do you agree to be interviewed? **A. Yes** **B. No** Thank you, let's start.

A. Socio-demographic, economic and lifestyle characteristics				Code_____
1.0 Personal information				Address_____
Name (Optional)_____				
1.1	Age_____Height__Cm			
1.2	Occupation:	A. Farmer	B. Housewife	C. Merchant
1.3	What is your monthly income?	_____		
1.4	What is your educational status?	A. literature	B. Read & write	C. Educated
	If Educated	A.1^{0}school	B.2^{0}school	C. 30/University
1.5	What is your marital status?	A. Married	B. Single	C. Other
1.6	What is your religion?	A. Christian	B. Muslim	C. Other
1.7	Do you have latrine?		A. Yes	B. No
	If you have latrine do you wash your hand after latrine?		A. Yes	B. No
1.8	What is the source of your drinking	A. Wells	B. River water	D. Spring water

	water?	water	C. Tap water	E. Other
1.9	Do you wear Shoe?		A. Yes	B. No
2.0	**The nature of complications during pregnancy**			
2.1	What is your gestational age?		_____	
2.2	How many children do you have?		_____	
2.3	Where did you deliver your last babies?		**A. H/institute**	**B. Home**
2.4	Do you have source of information about nutrition in pregnancy?		**A. Yes**	**B. No**
2.5	If yes, what is the source of your information?		A. Radio/Televion	B. Newsletter/Internet
			C. Community advocacy/H.extension worker	
			D. School	E. specify Other,
2.6	Was there any blood loss in your previous delivery?		A. Yes	B. No
2.7	Did you follow antenatal care in your previous pregnancy?		A. Yes	B. No
2.8	What there any blood loss in your current pregnancy?		A. Yes	B. No
2.9	How many months (weeks) pregnant are you?		_____Months	_____weeks
2.10	Is it your first pregnancy?		A. Yes	B. No
2.11	How many times have you been pregnant?		A. Yes	B. No
2.12	At what interval did you deliver your babies?		**A. < 2 yrs**	**B>2 yrs**
2.13	was there any abortion in your pregnancy?		A. Yes	B. No

2.14	Do you use contraceptive?		A. Yes	B. No
2.15	If yes, what kind of infection or diseases?		_____	_____
2.16	Are you taking any medication?		A. Yes	B. No
2.17	Did you become infected with malaria for the last one year?		A. Yes	B. No
2.18	Do you have anti-malaria bed net?		A. Yes	B. No
2.19	If you say yes for question 2.17 do you use frequently?		A. Yes	B. No
2.20	Did you have nausea / vomiting at the beginning of the pregnancy?		A. Yes	B. No
2.21	Have you taken iron supplement the current pregnancy?		A. Yes	B. No
2.22	If yes, what kind of supplementation?		1. Iron sup	3. Anti-acid
			2. IFA	4.Other (specify)
2.23	If you are taking iron/iron-folic acid supplement, when did you start it?_____			
2.24	How often do (did) you take it? In tablet		A. 1 tab/day	B. 2tab/day
			C. 3 tab/day	D. other (specify)
2.25	Notice any changes in your appetite since you became pregnant?		A. Yes	B. No
2.26	If yes, specify (please note any increase/decrease)		Increase	Decrease
2.27	Do (did) you experience some symptoms like:	A. Persistent swelling of feet, hands	B.Increasing breathless, especially on routine activity	C. Headaches

		or face			
		D. Blurring of Vision	E. Fever) temperature > 38	F. High colored urine in the past two weeks.	
3.0	**Type of foods/Twenty four hour recall food questionnaire**				
3.1	Number of meals per day?___	1/day	2/day	3/day	
3.2	How often do (did) you eat the following meals?	3.2a. Breakfast	1.Always	2.Sometimes	3. Do not eat
		3.2b Lunch	1.Always	2.Sometimes	3. Do not eat
		3.2c. Dinner	1.Always	2.Sometimes	3. Do not eat
3.3	Did you eat the following foods in the past two weeks?		A. Yes	B. No	
3.4	Iron rich foods such as, Meat and meat products, eggs, bread, green leafy vegetables, pulses and fruits		A. Yes	B. No	
3.4.1	If you eat such products how many times?		A. Daily	B. every2day	
			C Every week	D. Once a month	
3.5	Animal source foods such as, milk, yogurt and cheese		A. Yes	B. No	
3.5.1	If you eat such products how many times?		A. Daily	B. every2day	
			C Every week	D. Once a month	
3.6	Vitamin A-rich foods such as, (papaya, Mango, Carrot and green leafy vegetables		A. Yes	B. No	
3.6.	If you eat such products how many times?		A. Daily	B. every2day	

1			C Every week	D. Once a month
3.7	Calcium rich foods such as, dairy products, cabbage, eggs and		A. Yes	B. No
3.7.1	If you eat such products how many times?		A. Daily	B. every2day
			C Every week	D. Once a month
3.8	Zinc rich foods such as, maize, meat, fish and beans		A. Yes	B. No
3.8.1	If you eat such products how many times?		A. Daily	B. every2day
			C Every week	D. Once a month
3.9	Did you practice any fasting since you became pregnant?		A. Yes	B. No
3.10	When do you fast?	A. Per month	B. Per year	C. per weak
3.11	For the question number 3.5 how many times?	A. 1	B.2	D.4
			C.3	E.>4
3.12	If per day only		A. Only meat	B<6hrs
			C1/2 day	D. Full day
3.13	Level of fasting?	Deprivation of animal source food except fish		Deprivation of all animal source food
3.14		Deprivation of animal source food + no breakfast		Deprivation of animal source food + fasting until 3.00 Pm
3.15	Did you drink tea/coffee daily in the past two weeks?		A. Yes	B. No

3.16	If yes, how many cups did you drink on average per day?			_____
3.17	If yes, to question number 3.8, how often did you practice to drink?	A. Before meal	B. With meal	C. Immediately after meal
			C. Approximately 1hr after meal	D. Do not know
3.18	Did you drink the following beverages in the past two weeks?		A. Yes	B. No
3.19	**If your answer in 3.18 is yes,**			
3.20	Please specify the number of times and amount you had.	Beer	Wine	Liquor
		Local liquor (Areqe)	D. Carbonated beverages	Tella
			Other specify	No._____Amount
3.21	Please take time and try to fill the next table accordingly.			

Interviewer_____ ID number_____ Interview date:_____ Mon Tue Wed Thru Fri Sat Sun	Location _____
Probe for sickness: 1. Yes 2. No If yes, did the sickness affect appetite? 1. Yes 2. No If yes, 1. Increase 2. Decrease	
Was it a feast day? 1. Yes 2. No Was it a fasting day? 1. Yes 2. No	

Thank you for taking time to fill this survey!!!!

Appendix: I: Afan Oromo version Questionnaire

Gaaffii fi Deebii Afaanii gulaalli Muuxannoo fi Sirna Nyaataa Haadholii mana yaalaa Hordofanii yeroo da'uumsa I assniitti				
Akkam Jirtu, Ani Maqaan koo _____jedhamaa barataa Yuniversiitiii Finfinnee sagantaa Nyaataa fi Sirna nyaataa irraa. Waa'ee muuxannoo fi carraalee mala nyaataa irratti odeeffannoo haadholii ulfaa kan hanqinna dhiigaa qabanii walitii guuruuf gammachuun guddaan natti dhaga'ama. Odeeffannoon kun istraateejii qulqullinna haadholii ulfaatiif kennamu irratti gar malee faayidaa qabeessa. Kunimmoo kan galma gahuu danda'uun hirmaannaa keessan qofaan. Odeeffannoon isin nuu kennitan kan ta'u tajaajila qorannoo qofaaf yoo ta'u maqaan keessaan icciitiidhaan qabama eenyulle beekuu hin danda'u. Gaaffiif deebiin kun yoo guddate daqiiqaa 15 hanga 20tii				
Gaaffiif deebii kana itti fufuuf fedhintaa qabduu? Galatoomaa itti fufuu ni dandeenya			**A. Eeyyeen**	**B. MIti**
A. dhuunfaa Ilaalchisesee gaaffii gaafatamu				Mallttoo/Koodii
1.0 Odeeffannoo dhuunfaa				Iddoo/addaa_____
Maqaa (Dirqamaa miti)_____ _____				
1.1	Umrii_____Dheerinna____Cm			
1.2	Hojii:	**A. Q/Bulta**	**B. Ha/manaa**	**C. Daldaltuu**
1.3	Galii dhunfaa ji'aan?	_____		
1.4	Sadarkaa barumsaa keessan?	**A. Hin bar**	**B. Bar fi Dub**	**C. kan baratte**
	Yo kan baratte ta'e	**A.Sad 1faa**	**B. Sad 2ffaa**	**C. Koll/Yuniversiti**
1.5	Haala maatii?	A.heerumte	B.hin eerumne	C. kan biraa
1.6	Amantaa?	A.kristaana	B. Musliima	C. kan biraa
1.7	Mana ficaanii qabduu?		A. Eeyyen	B. miti
	Yoo qabaattan isa booda harka ni dhiqattu		A. Eeyyen	B. miti
1.8	Maddi bishaanii keessan hoo?	A.kan harkifamu	B. Bis. ya'u C. kan banba	D. Madda E. kan bir
1.9	Kophee qabduu?		A. Eeyyen	B. miti
2.0	**Haalawwan yeroo ulfaa kana doorsisan**			
2.1	Amma ji'a meeqaffaa keessan?		_____	

2.2	Ijoollee meeqa qabdu?	_____	
2.3	Kanaan dura ijoollee keessan eessatti deessan?	**A. Buf fayyaa**	**B. Manatti**
2.4	Odeeffannoo mala nyaataa kanaan dura qabduu?	A. Eeyyen	B. miti
2.5	Maddii odeeffannoo nyaataa keessan hoo?	A. Raadoo/TV	B.Gaazexa/Interneet
		C. Exteenshiinii fayyaa	
		D. M/barumsaa	E. kan biraa yo ta'e
2.6	Dhiigaa kanaan dura mudatee turee?	A. Eeyyen	B. miti
2.7	Ulfa kanaan duraa iraatti nama yaala hordoftanii turtan moo?	A. Eeyyen	B. miti
2.8	Ulfa kana irraatti dhiigni jige hoo jiraa?	A. Eeyyen	B. miti
2.9	Amma ji'a meeqafaa Keessan jalqabdanii?	_____Ji'aan	_____torbaaniin
2.10	Ulfaa yeroo jalqabaa keessan moo??	A. Eeyyen	B. miti
2.11	Yeroo meeqaffaaf ulfooftan?	A. Eeyyen	B. miti
2.12	Garaagarummaan ijoollee keessan itti deessan meeqa?	**A. < 2 yrs**	**B>2 yrs**
2.13	Kanaan dura ulf isinirraa bahe jiraa moo?	A. Eeyyen	B. miti
2.14	Karoora matiitti fayyadamtanii turtan moo?	A. Eeyyen	B. miti
2.15	Dhibee buusan faa qabamtanii turtan moo?	_____	_____
2.16	Yoo jiraate qorichi fayyadamtani jiraa?	A. Eeyyen	B. miti
2.17	Waggaa tokkoon darbee keessatti dhibee dhukkuba buusa qabduu?	A. Eeyyen	B. miti
2.18	Marabii dhibee bookee hir'isu qabduu?	A. Eeyyen	B. miti
2.19	Gaaffii 2.18 eeyyen yoo ta'e yeroo hunda fayyadamtu moo?	A. Eeyyen	B. miti
2.20	Nyaanni isin deddeebisuun yeroo jalqabaa ture moo?	A. Eeyyen	B. miti
2.21	Qorichii biraa ulfa kana irratti fayyadmatan jira moo?	A. Eeyyen	B. miti
2.22	Yoo jiraate gosa isaa addaan haabaasanu	1. Iron	3. farra aciidii
		2.Ironfi fooliki	4.kan bira____

2.23	Yoo jalqabdanii jiraattan guyyaa itti jalqabdan				
2.24	Yeroo akkam akkamiitti fudhattu qorichicha		A. 1 tab/day	B. 2tab/day	
			C. 3 tab/day	D. other (specify)	
2.25	Fedhintaa nyaataa keessan irratti dhiibbaan fide jiraa?		A. Eeyyen	B. miti	
2.26	Yoo jiraatee hir'ataa deeme moo dabataan		dabalaa	Hir'acha	
2.27	Mallaattoo dhibee asii gadiitiin akka tasaa qabamtanii jirtan moo?	A. furdinna qaama	B.rakkina hafura fudhacu	C. bowwoo	
		D. rakkoo ijaa	E. ho'I qaama > 38	F. diimmachu bifa fincaanii.	
3.0	**Gaaffilee midhaan nyaataa yeroo gabaabaa keessatti argatan qabachiisuuf**				
3.1	Lakkoofta baay'ina nyaataa argatan	1/day	2/day	3/day	
3.2	Gaaffii 3.1 tiif haa tarreesanii	3.2a. ciree	1.Always	2.darbee	3. hin ny
		3.2b. ciree	1.Always	2.darbee	3. hin ny
		3.2c. ciree	1.Always	2.darbee	3. hin ny
3.3	Gosa nyaataa asii gaditti ibsame kana keessa yoo jiraate?		A. Eeyyen	B. miti	
3.4	Ironii dhaan guutamaa kan ta'a kan akka Foonii, Raafu, hanqaaquu, koolaa fi fuduraa fi kudraa adda addaa		A. Eeyyen	B. miti	
3.4.1	Yoo jiraatee yeroo akkamiitti nyaattan?		A. yero hunda	B. duyyaa lamaan	
			C torbanitti	D. ji'atti	
3.5	Nyaataa loonii keessaammoo aannan, dhadhaa fi itittuu?		A. Eeyyen	B. miti	
3.5.1	Yoo jiraatee yeroo akkamiitti nyaattan?		A. yero hunda	B. duyyaa lamaan	
			C torbanitti	D. ji'atti	
3.6	Vitamin A dhaan kan beekkaman kan akka paapaya, Maango, karotii fi K.Kf		A. Yes	B. No	
3.6.1	Yoo jiraatee yeroo akkamiitti nyaattan?		A. yero hunda	B. duyyaa lamaan	
			C torbanitti	D. ji'atti	
3.7	Calcium dhaan kan guutaman hoo kan akka aannanii,		A. Eeyyen	B. miti	

3.7.1	Yoo jiraatee yeroo akkamiitti nyaattan?		A. yero hunda	B. duyyaa lamaan
			C torbanitti	D. ji'atti
3.8	Zinc dhaan kan guutaman kan akka,badalla, qurxummii?		A. Eeyyen	B. miti
3.8.1	Yoo jiraatee yeroo akkamiitti nyaattan?		A. yero hunda	B. duyyaa lamaan
			C torbanitti	D. ji'atti
3.9	Ulfa kana irraatti xoomiin adda adddaa jira turee?		A. Eeyyen	B. miti
3.10	Yeroo akkamii soomtu?	A. Ji'atti	B. woggaati	C. torbanitti
3.11	Deebii armaan olitti deebistaniif yeroo meeqatti?	A. 1	B.2	D.4
			C.3	E.>4
3.12	Guyyaatti yoo ta'e akka armaan gadiiitti haa ibsan?		A. foon qofa	B<6hrs
			C1/2 day	D. guyyaa guutuu
3.13	Sadarkaan xoomii keessani hoo maal fakkaata?	Qurxummii malee foon hunda		Foon hundaa
		Ciree atoo hin nyaattin Foon hundaa		Ciree atoo hin nyaattin Foon hunda hanga sa. 9
3.15	Shaayii fi buna hoo ni dhugduu?		A. Eeyyen	B. miti
3.16	Yoo dhugdan ta'e yero akamiitti akka dhugdan tarreess?		_____	
3.17	If yes, to question number 3.8, how often did you practice to drink?	Nyata dura	Nyata woliin	battaluma nyaataatti
			Nyaatan sa .1 booda	Hin beeku
3.18	Dhugaatti keessaa hook an yaaltani jiraa?		A. Eeyyen	B. miti
3.19	Deebiin eeyyen yoo ta;e gosa dhugaatii dhugdanii			
3.20	Dhugaatii kana mmoo ammam aka dhugdan nutty himaa	Biira	Woyiniii	Dhugaati addaa
		(Areqe)	D. lallaafa	xallaa
			kanbiraa	Baay'ina lak____
3.21	Maalloo ogeessi fayyaa gabatee itti aanuu akka hin irraanfanne			

Hubachiisa: Dhuma gaafffif deebiin irratti uunkaan kunsirriitti haa guutamu.

Namni gaafate_____ Lakk addaa_____ Guyyaa gaaffi:_____ Wix Kib Rob Kam Jim S.xiq Dil	Iddoon_____
Dhukkubsataan dibeedhaaf: 1. eeyyen 2. miti Yoo jiraate fedhii nyaataa 1. Ni qaba 2. Hin qabu Yoo qabaate 1. Dabalaa deema 2. Hir'achaa deema	
Yeroon nyaata dhabe? 1. jira 2. hin jiru Sooma hoo? 1. Ni sooma 2. Hin soomu	
Maqaa_____ Mallattoo_____ Guyyaa:_____	

Appendix: J

Table 2: Clinical Sample and Data collection form summary for assessment and practices of second and third trimester pregnant Women attending ANC

Place_____ Hos/Health center

Code	Card NO	Client name	Age	Gestinal Age	Hb	MCV	PB		Remark

Appendix K:

Sample tables of cross tabs, correlations, regression, chi-square ANOVA

Table 3: Cross tabulation

Gestational Age * Vitamin A Def Description Cross tabulation

IDA based on cutoff			Vitamin A Def Description			Total
			severe	mild	Non deficienct	
.00	Gestational Age	17-26wks	12	13	27	52
		27-37 wks	2	3	14	19
	Total		14	16	41	71
Normal	Gestational Age	17-26wks	4	6	12	22
		27-37 wks	2	0	9	11
	Total		6	6	21	33

Gestational Age * Hb Description Cross tabulation

IDA based on cutoff			Hb Description		Total
			Moderate	Mild	
.00	Gestational Age	17-26wks	28	24	52
		27-37 wks	12	7	19
	Total		40	31	71
Normal	Gestational Age	17-26wks	13	9	22
		27-37 wks	6	5	11
	Total		19	14	33

Gestational Age * Frt_IDA Crosstabulation

Count

IDA based on cutoff			Frt_IDA				Total
			1.00	2.00	3.00	4.00	
.00	Gestational Age	17-26wks	1	23	15	13	52
		27-37 wks	0	10	4	5	19
	Total		1	33	19	18	71
Normal	Gestational Age	17-26wks	22				22
		27-37 wks	11				11
	Total		33				33

Gestational Age * sTrf higher 8.3 Crosstabulation

Count

IDA based on cutoff			sTrf higher 8.3		Total
			.00	1.00	
.00	Gestational Age	17-26wks	45	7	52
		27-37 wks	16	3	19
	Total		61	10	71
Normal	Gestational Age	17-26wks	19	3	22
		27-37 wks	4	7	11
	Total		23	10	33

Hb Description * Vitamin A Def Description Crosstabulation

Count

IDA based on cutoff			Vitamin A Def Description			Total
			severe	mild	Non deficienct	
.00	Hb Description	mild	11	9	20	40
		moderate	3	7	21	31
	Total		14	16	41	71
normal	Hb Description	mild	4	5	10	19
		moderate	2	1	11	14
	Total		6	6	21	33

Hb Description * Vit B12 deficiency based on cutoff Crosstabulation

Count

IDA based on cutoff			Vit B12 deficiency based on cutoff			Total
			Severe deficiency of Vit B12	Moderate deficiency of Vit B12	Normal range of B12	
.00	Hb Description	Moderate	13	18	9	40
		Mild	8	8	15	31
	Total		21	26	24	71
normal	Hb Description	Moderate	5	11	3	19
		Mild	2	9	3	14
	Total		7	20	6	33

Vitamin A Deficiency * Frt_IDA Crosstabulation

Count

IDA based on cutoff			Frt_IDA				Total
			1.00	2.00	3.00	4.00	
.00	Vitamin A Deficiency	non VAd	1	19	9	12	41
		VAD	0	14	10	6	30
	Total		1	33	19	18	71
normal	Vitamin A Deficiency	non VAd	21				21
		VAD	12				12
	Total		33				33

Vitamin A Deficiency * Vit B12 deficiency based on cutoff Crosstabulation

Count

IDA based on cutoff			Vit B12 deficiency based on cutoff			Total
			Severe deficiency of Vit B12	Moderate deficiency of Vit B12	Normal range of B12	
.00	Vitamin A Deficiency	non VAd	15	12	14	41
		VAD	6	14	10	30
	Total		21	26	24	71
normal	Vitamin A Deficiency	non VAd	5	12	4	21
		VAD	2	8	2	12
	Total		7	20	6	33

Vitamin A Deficiency * Presence of Inflammation Crosstabulation

Count

IDA based on cutoff			Presence of Inflammation		Total
			.00	1.00	
.00	Vitamin A Deficiency	non VAd	33	8	41
		VAD	24	6	30
	Total		57	14	71
normal	Vitamin A Deficiency	non VAd	15	6	21
		VAD	8	4	12
	Total		23	10	33

Table 4: Chi-Square Tests

IDA based on cutoff		Value	df	Asymp. Sig. (2-sided)
.00	Pearson Chi-Square	71.000[a]	2	.000
	Likelihood Ratio	96.716	2	.000
	Linear-by-Linear Association	58.316	1	.000
	N of Valid Cases	71		
normal	Pearson Chi-Square	33.000[b]	2	.000
	Likelihood Ratio	43.262	2	.000
	Linear-by-Linear Association	27.243	1	.000
	N of Valid Cases	33		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.92.

b. 4 cells (66.7%) have expected count less than 5. The minimum expected count is 2.18.

Symmetric Measures

IDA based on cutoff			Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
.00	Interval by Interval	Pearson's R	-.913	.011	-18.557	.000[c]
	Ordinal by Ordinal	Spearman Correlation	-.964	.013	-29.945	.000[c]
	N of Valid Cases		71			
normal	Interval by Interval	Pearson's R	-.923	.016	-13.325	.000[c]
	Ordinal by Ordinal	Spearman Correlation	-.975	.014	-24.428	.000[c]
	N of Valid Cases		33			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.

Crosstab

IDA based on cutoff	Frt_IDA				Total
	1.00	2.00	3.00	4.00	

.00	Vitamin A Deficiency	non VAd	1	19	9	12	41
		VAD	0	14	10	6	30
	Total		1	33	19	18	71
normal	Vitamin A Deficiency	non VAd	21				21
		VAD	12				12
	Total		33				33

Chi-Square Tests

IDA based on cutoff		Value	df	Asymp. Sig. (2-sided)
.00	Pearson Chi-Square	2.158[a]	3	.540
	Likelihood Ratio	2.527	3	.470
	Linear-by-Linear Association	.053	1	.818
	N of Valid Cases	71		
normal	Pearson Chi-Square	.[b]		
	N of Valid Cases	33		

a. 2 cells (25.0%) have expected count less than 5. The minimum expected count is .42.

b. No statistics are computed because Frt_IDA is a constant.

Chi-Square Tests

IDA based on cutoff		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
.00	Pearson Chi-Square	.716[a]	1	.397		
	Continuity Correction[b]	.251	1	.616		
	Likelihood Ratio	.740	1	.390		
	Fisher's Exact Test				.502	.313
	Linear-by-Linear Association	.706	1	.401		
	N of Valid Cases	71				
normal	Pearson Chi-Square	.251[c]	1	.616		
	Continuity Correction[b]	.012	1	.914		
	Likelihood Ratio	.255	1	.613		
	Fisher's Exact Test				.710	.463
	Linear-by-Linear Association	.243	1	.622		
	N of Valid Cases	33				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.23.

Crosstab

Count

IDA based on cutoff			FAD below 5.9		Total
			.00	FAD	
.00	Vitamin A Deficiency	non VAd	29	12	41
		VAD	23	7	30
	Total		52	19	71
normal	Vitamin A Deficiency	non VAd	15	6	21
		VAD	9	3	12
	Total		24	9	33

Crosstab

Count

IDA based on cutoff			Vit B12 deficiency based on cutoff			Total
			Severe deficiency of Vit B12	Moderate deficiency of Vit B12	Normal range of B12	
.00	Vitamin A Deficiency	non VAd	15	12	14	41
		VAD	6	14	10	30
	Total		21	26	24	71
normal	Vitamin A Deficiency	non VAd	5	12	4	21
		VAD	2	8	2	12
	Total		7	20	6	33

Chi-Square Tests

IDA based on cutoff		Value	df	Asymp. Sig. (2-sided)
.00	Pearson Chi-Square	3.047[a]	2	.218
	Likelihood Ratio	3.098	2	.213
	Linear-by-Linear Association	.672	1	.412
	N of Valid Cases	71		
normal	Pearson Chi-Square	.322[b]	2	.851
	Likelihood Ratio	.327	2	.849
	Linear-by-Linear Association	.043	1	.836
	N of Valid Cases	33		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.87.

b. 4 cells (66.7%) have expected count less than 5. The minimum expected count is 2.18.

Chi-Square Tests

IDA based on cutoff		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
.00	Pearson Chi-Square	.003[a]	1	.959		
	Continuity Correction[b]	.000	1	1.000		
	Likelihood Ratio	.003	1	.959		
	Fisher's Exact Test				1.000	.595
	Linear-by-Linear Association	.003	1	.960		
	N of Valid Cases	71				
normal	Pearson Chi-Square	.082[c]	1	.775		
	Continuity Correction[b]	.000	1	1.000		
	Likelihood Ratio	.081	1	.775		
	Fisher's Exact Test				1.000	.537
	Linear-by-Linear Association	.080	1	.778		
	N of Valid Cases	33				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.92.

b. Computed only for a 2x2 table

c. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.64.

Table 5: Regression Analysis of CRP and B12

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.263[a]	.069	.060	52.02237

a. Predictors: (Constant), Serum CRP result

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	20560.994	1	20560.994	7.597	.007[b]
	Residual	276045.346	102	2706.327		
	Total	296606.340	103			

a. Dependent Variable: Serum Vitamin B12 result

b. Predictors: (Constant), Serum CRP result

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	148.396	15.027		9.876	.000
	Serum CRP result	8.740	3.171	.263	2.756	.007

a. Dependent Variable: Serum Vitamin B12 result

Regression Analysis of VAD and B12

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.155[a]	.024	.014	.61801

a. Predictors: (Constant), Serum Vitamin A result

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.959	1	.959	2.510	.116[b]
	Residual	38.958	102	.382		
	Total	39.917	103			

a. Dependent Variable: Altitude adjusted Hemoglobin

b. Predictors: (Constant), Serum Vitamin A result

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	9.561	.079		120.770	.000
	Serum Vitamin A result	.048	.030	.155	1.584	.116

a. Dependent Variable: Altitude adjusted Hemoglobin

Correlations

Hb Description			Age of woman	Age
Moderate	Age of woman	Pearson Correlation	1	.956**

		Sig. (2-tailed)		.000
		N	59	59
	Age	Pearson Correlation	.956**	1
		Sig. (2-tailed)	.000	
		N	59	59
	Age of woman	Pearson Correlation	1	.954**
		Sig. (2-tailed)		.000
		N	45	45
Mild	Age	Pearson Correlation	.954**	1
		Sig. (2-tailed)	.000	
		N	45	45

**. Correlation is significant at the 0.01 level (2-tailed).

Vit B12 deficiency based on cutoff

Hb Description			Frequency	Percent	Valid Percent	Cumulative Percent
mild	Valid	Severe deficiency of Vit B12	18	30.5	30.5	30.5
		Moderate deficiency of Vit B12	29	49.2	49.2	79.7
		Normal range of B12	12	20.3	20.3	100.0
		Total	59	100.0	100.0	
moderate	Valid	Severe deficiency of Vit B12	10	22.2	22.2	22.2
		Moderate deficiency of Vit B12	17	37.8	37.8	60.0
		Normal range of B12	18	40.0	40.0	100.0
		Total	45	100.0	100.0	

Table 6: Regression to test linearity of Calibration curve

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	1.000[a]	1.000	.999	.54137

a. Predictors: (Constant), Peak Area of STD

Tabe 7 ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2079.121	1	2079.121	7094.051	.000[b]
	Residual	.879	3	.293		
	Total	2080.000	4			

a. Dependent Variable: Concentration of STD

b. Predictors: (Constant), Peak Area of STD

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.711	.408		-1.741	.180
	Peak Area of STD	2.009E-005	.000	1.000	84.226	.000

a. Dependent Variable: Concentration of STD

Next day

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.994[a]	.988	.984	2.92109

a. Predictors: (Constant), Peak Area of STD

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2054.402	1	2054.402	240.767	.001[b]
	Residual	25.598	3	8.533		
	Total	2080.000	4			

a. Dependent Variable: Concentration of STD; b. Predictors: (Constant), Peak Area of STD

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.234	2.113		.584	.600
	Peak Area of STD	11.471	.739	.994	15.517	.001

a. Dependent Variable: Concentration of STD

Model Summary

Mode l	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.999[a]	.997	.997	2.10927

a. Predictors: (Constant), Peak Area of STD

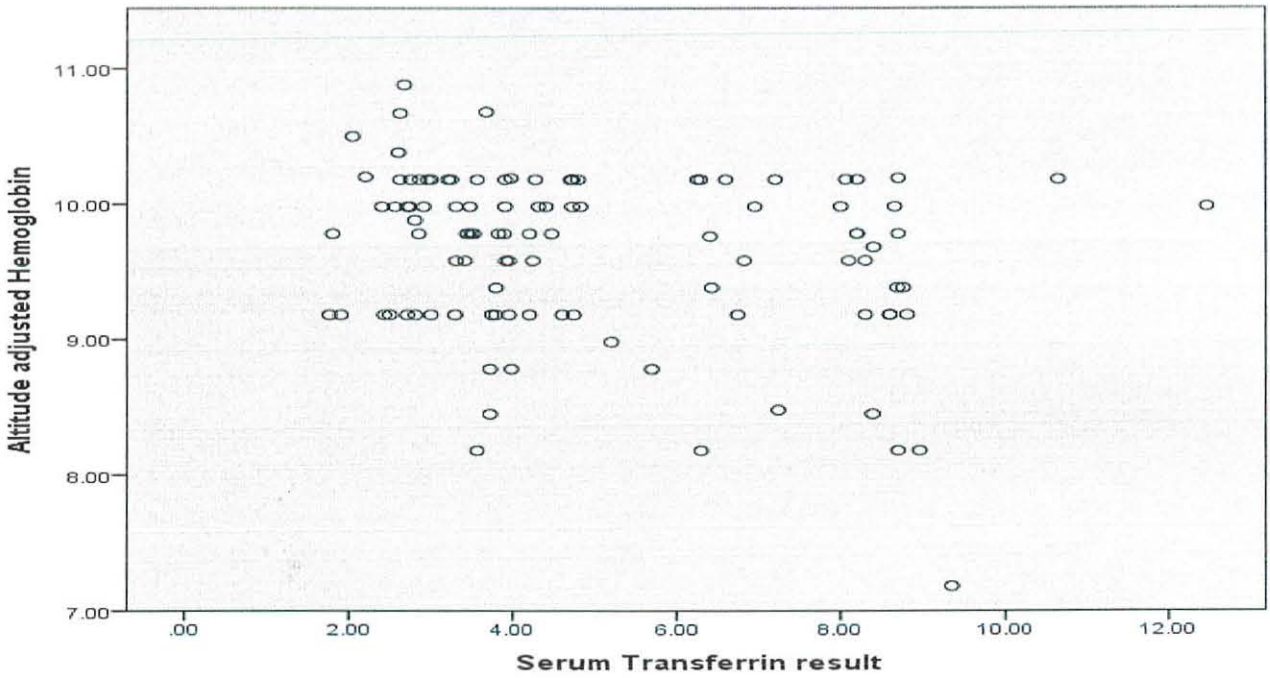
ANOVA[a]

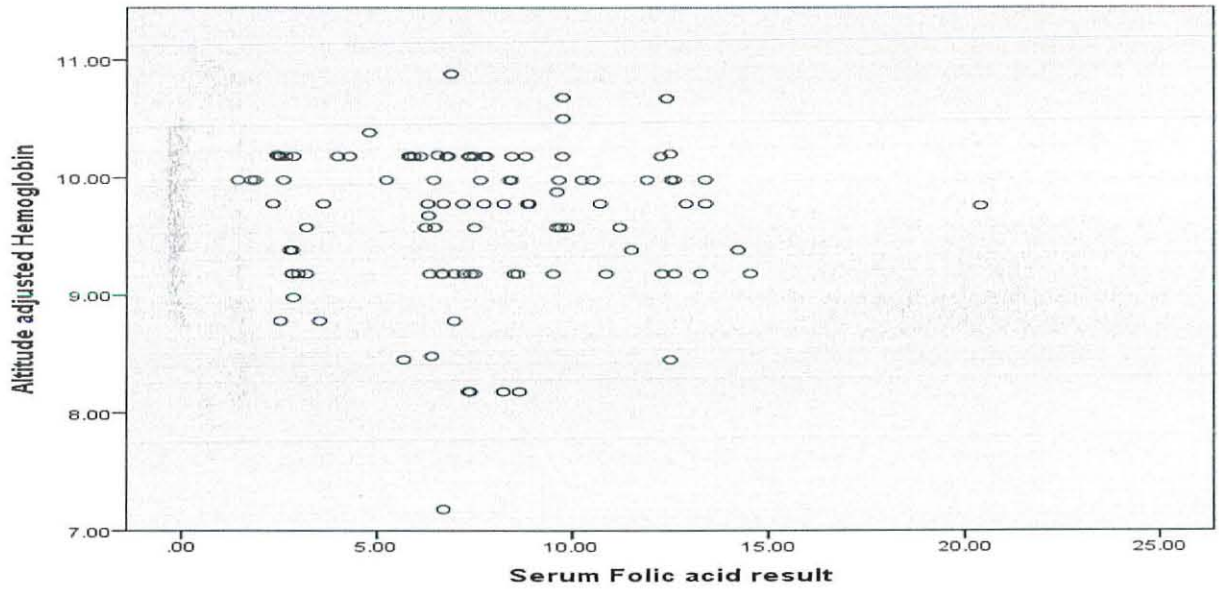
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	6503.037	1	6503.037	1461.679	.000[b]
	Residual	17.796	4	4.449		
	Total	6520.833	5			

a. Dependent Variable: Concentration of STD, b. Predictors: (Constant), Peak Area of STD

Coefficients[a] a. Dependent Variable: Concentration of STD

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-4.424	1.429		-3.096	.036
	Peak Area of STD	.002	.000	.999	38.232	.000





Aneamia base on Hb

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Moderate	69	33.2	33.2	33.2
	Mild	73	35.1	35.1	68.3
	4	66	31.7	31.7	100.0
	Total	208	100.0	100.0	

Coefficients[a]

Hb Description		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
Moderate	(Constant)	.506	1.366		.370	.712	-2.230	3.242
	Serum Vitamin B12 result	.030	.007	.472	4.047	.000	.015	.045
Mild	(Constant)	5.017	1.266		3.964	.000	2.464	7.570
	Serum Vitamin B12 result	-.002	.006	-.040	-.265	.792	-.014	.011

a. Dependent Variable: Serum Transferrin result

		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
Moderate	(Constant)	3.443	1.049		3.281	.002	1.342	5.544
	Serum CRP result	.535	.217	.310	2.460	.017	.099	.970
Mild	(Constant)	6.003	1.117		5.377	.000	3.752	8.255
	Serum CRP result	-.299	.242	-.185	-1.237	.223	-.787	.188

a. Dependent Variable: Serum Transferrin result

Hb Description		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
Moderate	(Constant)	8.625	1.360		6.343	.000	5.902	11.348
	Serum CRP result	-.157	.282	-.074	-.558	.579	-.721	.407
Mild	(Constant)	8.542	1.573		5.429	.000	5.369	11.715
	Serum CRP result	-.311	.341	-.138	-.914	.366	-.999	.376