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Evaluation of Serum Vitamin B12 Level in Celiac Disease Patients and it's effect on Red Cell Parameters

تقييم مستوى فيتامين ب12 لدى مرضى الداء البطني وتأثيره على معلمات كريات الدم الحمراء

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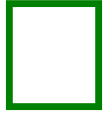
الآية

قال تعالى :

(أَوْلَا يَذْكُرُ الْإِنْسَانُ أَنَّمَا خَلَقْنَاهُ مِنْ قَبْلُ
وَكَمْ يَكُ شَيْبًا)

صدق الله العظيم

سورة مريم الآية (67)



Dedication

To spring kindness my Mother

To my father who learned me that life does no respect the weakness
minds

To my lovely brothers

To my husband for his unwavering support

To our son and our daughter for making it all worthwhile

To my beloved friends

To my colleagues whom help me

To all people whom I love, respect and appreciate

To those I dedicate this research

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Abstract

Celiac disease is one of the most common lifelong disorders. It is genetic autoimmune disorder occur when the ingestion of gluten leads to damage in the small intestine. Vitamin B12 malabsorption are common in celiac disease as the proximal small intestine is predominantly affected. We aimed to study serum vitamin B12 levels in celiac disease patients.

This was a cross sectional study, conducted, to estimate the serum levels of vitamin B12 among 40 Sudanese patients with celiac disease; their age range from 2-43 years .Ten (25%) of the patients were males and 30 (75%) were females; all were diagnosed by serological test and endoscopy and referred at Ibn Sinaa hospital, in the period from April to August 2016. About 3 ml of venous blood sample were collected by venipuncture technique from each patient, and then allowed to clot to obtain serum .The serum levels of vitamin B12 were measured using immunoassay analyzer (Cobas e411).

The result showed that, frequency of B12 deficiency among celiac disease patients were 12.5% (5/40), one (2.5%) was male and four (10%) were females. There was no statistically significant correlation between vitamin B12 deficiency and each of gender, age and duration of disease. There was statistically significant correlation between vitamin B12 deficiency and dietary program commitment (*P value* = 0.017). No significant correlation was found between serum B12 level and RBCs parameters. The results of this study concluded that, 12.5 % of celiac disease patients were deficient for vitamin B12 and there was statistically significant correlation between vitamin B12 deficiency and dietary program commitment.

مستخلص الدراسة

مرض الداء البطني هو واحد من اضطرابات الأكثر شيوعا وهو مرض المناعة الذاتية الوراثي يحدث عند تناول الغلوتين مما يؤدي إلى تلف في الإمعاء الدقيقة. سوء الامتصاص فيتامين ب12 شائع في مرض الداء البطني في الغالب عندما تتأثر الإمعاء الدقيقة. هدف الدراسة قياس مستويات فيتامين ب12 في المرضى الذين يعانون من مرض الداء البطني. أجريت هذه الدراسة المقطعية، لتقدير مستويات المصل من فيتامين ب12 بين 40 من المرضى السودانيين الذين (25%) من المرضى يعانون من مرض الداء البطني في الفئة العمرية من 2-43 سنة. عشرة الذكور و 30 (75%) من الإناث الذين تم تشخيصهم عن طريق الإختبار المصلي والمناظير المحالين إلى مستشفى ابن سينا بولاية الخرطوم، السودان في الفترة من إبريل الي اغسطس 2016.

3مل جمعت من الدم الوريدي بواسطة تقنية بزل الوريد من كل مريض، ثم ترك الدم للتجلط للحصول على مصل لتحديد مستوى قياس فيتامين ب 12 باستخدام جهاز تحليل المناعة كوباس . وأظهرت النتيجة أن التردد في نقص فيتامين ب12 بين المرضى الذين يعانون من مرض الداء البطني بنسبة 12.5% (40/5) ، واحد (2.5%) كان من الذكور و 4 (10%) من الإناث . لم يكن هناك علاقة ذات دلالة إحصائية بين نقص فيتامين ب12 وكل من الجنس والعمر ومدة المرض والدم ومعلومات الخلايا الحمراء . بينما كان هناك علاقة ذات دلالة إحصائية بين نقص فيتامين ب12 والإلتزام ببرنامج غذائي. وخلصت نتائج هذه الدراسة أن 12.5% من المرضى بمرض الداء البطني كان لديهم نقص في ببرنامج فيتامين ب12، وكان هناك إحصائية ارتباط كبير بين نقص فيتامين ب12 والإلتزام غذائي.

List of contents

Content	Page	
الآية	I	
Dedication	II	
Acknowledgment	III	
Abstract	IV	
مستخلص الدراسة	VIVII	
Table of contents	VIVI	
List of tables	VI	
List of figures	VI	
Abbreviations	VI	
Chapter One Introduction and Literature Review		
1.1	Introduction	1
1.2	Literature review	2
1.2.1	Celiac disease	2
1.2.2.1	Signs and symptoms of celiac disease	2
1.2.1.2	Cause of celiac disease	3
1.2.1.2.1	Genetic influence	3
1.2.1.2.2	Immune system abnormalities	4
1.2.1.2.3	Ingestion of gluten	4
1.2.1.3	Diagnosis of celiac disease	5
1.2.1.3.1	Serological test	5
1.2.1.3.2	Intestinal biopsy	5
1.2.1.3.3	Genetic testing	5
1.2.1.3.4	Other diagnostic tests	6

1.2.1.4	Treatment of celiac disease	6
1.2.1.5	Complications of celiac disease	7
1.2.2	Blood	7
1.2.2.1	Blood functions	7
1.2.2.1.1	Transportation	7
1.2.2.1.2	Regulation	8
1.2.2.1.3	Protection	8
1.2.2.3	Blood constituents	8
1.2.2.3.1	Leukocytes	9
1.2.2.3.2	Platelets	9
1.2.2.3.3	Erythrocytes	9
1.2.2.3.3.1	Function of erythrocytes	10
1.2.2.3.3.2	Production of erythrocytes	10
1.2.4	Anemia	10
1.2.4.1	Causes of anemia	11
1.2.4.1.1	Blood loss	11
1.2.4.1.2	Lack of red blood cell production	11
1.2.4.1.3	High rates of red blood cell destruction	12
1.2.4.3	Classification of anemia	12
1.2.4.4	Clinical features of anemia	13
1.2.4.5	Diagnosis	13
1.2.5	Vitamin B12	14
1.2.5.1	Dietary sources and requirements	15
1.2.5.2	Absorption	15
1.2.5.4	Transport	16
1.2.5.5	Role of vitamin B12	17

1.2.5.6	Vitamin B12 deficiency	17
1.3	Previous Studies	17
Chapter Two Objectives and Rationale		
2.1	Rationale	20
2.2	Objectives	20
2.2.1	General objective	20
2.2.2	Specific objectives	20
Chapter Three Materials and Methods		
3.1	Materials	21
3.1.2	Study design	21
3.1.3	Study area	21
3.1.4	Study duration	21
3.1.5	Study population	21
3.1.6	Inclusion criteria	21
3.1.7	Exclusion criteria	21
3.1.8	Sample collection	21
3.1.9	Ethical considerations	22
3.1.10	Data collection and analysis	22
3.1.11	Equipments, disposables and reagents	22
3.2	Methods	23
3.2.1	Estimation of serum B12 level	23
3.2.2	Principle	23

3.2.3	Procedure	23
Chapter Four Results		
Results		24
Chapter Six Discussion, Conclusion and Recommendations		
6.1	Discussion	30
6.2	Conclusion	32
6.3	Recommendations	32
References and Appendixes		
References		33
Appendix 1		37
Appendix 2		38
Appendix 3		39

List of tables

Table	Title	Page
4.1	Red cell parameters, serum B12 level and disease duration in study population	25
4.2	Correlation of serum B12 level with red cell parameters and disease duration in study population	26
4.3	Correlation between gender and serum B12 level in study population	27
4.4	Comparison of age in patients with deficient and normal B12 level	27
4.5	Comparison of red cell parameters and duration of disease in patients with and without B12 deficiency	28
4.6	Comparison of vitamin B12 level and dietary program commitment in study population	29
4.7	Correlation between B12 deficiency and dietary program commitment in study population	29

List of figures

Figure	Title	Page
(4.1)	Gender distribution of study population	24
(4.2)	Distribution of study population according to age group	24
(4.3)	Commitment of study population to gluten free diet	25
(4.4)	The frequency of B12 deficiency in study population.	26

Abbreviation

ACD	Anemia of Chronic Disease
CD	Celiac Disease
ESR	Erythrocyte Sedimentation Rate
GFD	Gluten Free Diet
Hb	Hemoglobin
HLA	Human Leukocyte Antigen
IDA	Iron Deficiency Anemia
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
NK	Natural killer
RBCs	Red Blood Cells
RDW	Red Blood Cells Distribution Width
PCV	Packed Cell Volume
P-value	Probability Value
tTG	Tissue Transglutaminase
SD	Standard Deviation
WBC	White Blood Cell

]

Chapter One

Introduction and Literature Review

1. Introduction

Celiac disease (CD) is a complex autoimmune enteropathy caused by a permanent intolerance to gluten in genetically susceptible individuals. Gluten is the main storage protein of wheat. The alcohol-soluble fraction (prolamin) of gluten, gliadin, is toxic in celiac disease, as are similar proteins in barley (hordein) and rye (secalin) (Vader *et al.*, 2003).

It is multisystem autoimmune disorder that can cause symptoms involving the gastrointestinal tract and other organ system such as the skin and bone. Beside gastrointestinal symptoms, celiac disease is associated with a variety of diseases, including dermatitis herpetiformis, malabsorption of several nutrients (potentially leading to osteoporosis, iron deficiency anemia and other disorders). The vitamin deficiency is due to the damage to the intestinal makes it hard for the body to absorb nutrients (Kochhar, 2016).

Celiac disease (CD) may be considered as an archetypal malabsorption syndrome, and it is a frequent cause of anemia without associated intestinal symptoms indeed, several studies demonstrate these deficiencies with varying results. Microcytic or macrocytic anemia or folate deficiency may occasionally be the only clinical symptom to suggest CD. Deficiencies of water-soluble vitamins, like B-vitamins, would be expected since they are absorbed in the proximal small bowel, which is the most prominent site affected in CD-patients. However, available data, in particular regarding vitamin B 12 and B 6 deficiencies do not support this in untreated CD-patients (Dickey *et al.*, 2008).

1.2 Literature review

1.2.1 Celiac disease

Celiac disease also known as celiac sprue or gluten sensitive enteropathy is a systemic disorder with protean manifestations. It is a common disease, previously described mainly in children but is now increasingly being diagnosed in persons of all ages. Celiac disease is becoming an increasingly recognized disorder (Green and Jabri, 2003).

Celiac disease is due to immune-mediated gluten intolerance and may present in a number of ways. It has become more frequently diagnosed due to the recognition of the atypical presentation. In recent years, more sensitive and specific serological markers have been developed but the gold standard of diagnosis remains duodenal biopsy. Complication with a strict, lifelong gluten-free diet is the cornerstone of management, improving symptoms and reducing complication of the disease (Leeds *et al.*, 2008).

Celiac disease is caused by a reaction to gluten when people with celiac disease eat gluten. Their body mounts an immune response that attacks the small intestine. These attacks lead to damage to villi, small finger-like projections that line the small intestine, that promote nutrient absorption. When the villi get damaged, nutrients cannot be absorbed properly into the body (Di Sabatino and Corazza, 2009).

1.2.1.1 Signs and symptoms of celiac disease

The clinical presentation of celiac disease varies greatly, depending on a patient's age, the duration and extent of the disease, and presence of extraintestinal manifestation. The classification of celiac disease regarding symptoms are classical celiac disease is associated with gastrointestinal

symptoms such as abdominal bloating and discomfort, diarrhea and weight loss. Atypical celiac disease has few, if any, gastrointestinal symptoms, with non gastrointestinal symptoms present due to complication from celiac disease being the predominant features and silent celiac disease and latent celiac disease do not cause symptoms (Green and Jabri, 2003).

Atypical clinical manifestations of celiac disease are characterized by few or no gastrointestinal symptoms, instead, extraintestinal symptoms such as iron deficiency anemia, reduced bone mineral density, chronic fatigue, irritable bowel, dyspepsia, infertility, miscarriage, coagulopathy hypertransaminemia, short stature, pubertal delay, arthralgia, aphthous stomatitis, folate/zinc deficiency, hypoplasia, and otherwise unexplained neurological disorders predominate (Dewar and Ciclitira, 2005).

1.2.1.2 Cause of celiac disease

In order to develop celiac disease, several factors must be present. Some of these factors are present from the time of conception and several play roles later on in the process. Here are those key factors:

1.2.1.2.1 Genetic influence

Celiac disease represents a unique model of autoimmunity because of the identification of a close genetic association with HLA-DQ2 and HLA-DQ8 and a highly specific humoral autoimmune response (autoantibodies against the autoantigen, tTG) and, most important the external trigger, gluten peptides (Fanso *et al.*, 2003).

Because of the genetic predisposition to celiac disease, an individual's intolerance to gluten is lifelong and self-perpetuating. The amount of gluten ingested, the gene dose of HLA-DQ2 and HLA-DQ8 (homozygous individuals appear to be at highest risk of celiac disease), and the local

expression of tTG appear to be important determinants of celiac disease manifestation and severity (Sollid *et al.*, 2001).

1.2.1.2.2 Immune system abnormalities

To get celiac disease, a specific problem within the immune system has to develop in which, after person ingest certain type of grain proteins (gluten), the immune system behaves abnormally, including making antibodies against some of certain tissue (Blumer and Crowe, 2010). Adaptive immune response involves T cells in the lamina propria that recognize specific immunogenic gluten peptides processed and presented by antigen-presenting cells (Sollid, 2000).

Gluten derived peptides can also activate an innate response. The innate response is typified by increased expression of interleukin-15 by enterocytes, which drives the activation of populations of intraepithelial lymphocytes that express the NK marker (Hue *et al.*, 2004).

Gluten antigens are modified enzymatically by tissue transglutaminase (tTG). Tissue transglutaminase deaminates gliadin and increases its immunogenicity by causing it to bind to receptors on antigen-presenting cells with stronger affinity. Furthermore, gliadin-tissue transglutaminase complexes formed by protein cross-linkages generate an autoantibody response (predominantly immunoglobulin A type) that can exacerbate the inflammatory process (Caputo *et al.*, 2009).

1.2.1.2.3 Ingestion of gluten

Celiac disease is caused by a reaction to gliadin, a prolamins (gluten protein) found in wheat and similar proteins found in crops of the tribe triticales (which include other common grains such as barley and rye). When ingested, trigger the abnormal immune response present in celiac

disease. If a person has never eaten gluten, they could never get celiac disease (Blumer and Crowe, 2010).

1.2.1.3 Diagnosis of celiac disease

Recognizing celiac disease can be difficult because some of its symptoms are similar to those of other diseases. No single test exists that can definitively diagnose or exclude celiac disease in every individual (Rubio *et al.*, 2013).

1.2.1.3.1 Serological test

Serological testing, used as an initial noninvasive screen, is the first step in pursuing a diagnosis of celiac disease. Widely available serological tests used for detecting celiac disease include anti-gliadin antibodies, anti-endomysium antibodies and anti-tTG antibodies. The most sensitive and specific tests are anti-endomysium and anti-tTG (Green *et al.*, 2005).

The IgA anti-tTG has 95% to 97% specificity and approximately 90% to 96% sensitivity. If the patient has an IgA deficiency, screening should be done by checking the level of IgG antibodies to tissue transglutaminase (Rubio *et al.*, 2013).

1.2.1.3.2 Intestinal biopsy

If blood test and symptoms suggest celiac disease, a biopsy of small intestine is performed to confirm the diagnosis. An upper endoscopy with biopsy of duodenum (beyond the duodenal bulb) or jejunum is performed. Ideally, one to two samples should be taken from the duodenal bulb and at least four samples from the rest of the duodenum, preferably from two different locations (Rubio *et al.*, 2013).

1.2.1.3.3 Genetic testing

Although the combination of positive serologic tests and pathologic changes confirms the diagnosis of celiac disease, in some cases one type

of test is positive and the other is negative. In this situation, genetic testing for *HLA-DQ2* and *HLA-DQ8* can help rule out the diagnosis, as a negative genetic test rules out celiac disease in more than 99% of cases. Genetic testing is also useful in patients who are already adhering to a gluten-free diet at the time of presentation to the clinic and who have had no testing done for celiac disease in the past. If the test is positive, further testing needs to be done, as a positive genetic test cannot differentiate celiac disease from non celiac gluten sensitivity (Hadithi *et al.*, 2007).

1.1.3.4 Other diagnostic tests

There are further investigations may be performed to identify complication, such as iron deficiency (by full blood count and iron studies), folic acid and vitamin B12 deficiency and hypocalcaemia due to decrease vitamin D level . Thyroid function test may be requested during blood test to identify hypothyroidism, which is more common in people with celiac disease. Investigations to measure bone density may be performed at diagnosis to identify risk of fracture (Presutti *et al.*, 2007).

1.2.1.4 Treatment of celiac disease

The only treatment for celiac disease is a gluten-free diet. For most people, following this diet will stop symptoms, heal existing intestinal damage and prevent further damage. The small intestine usually heals in 3 to 6 months in children but may take several years in adults. A healed intestine means a person now has villi that can absorb nutrients from food into the bloodstream (Murray *et al*, 2004).

Patients who present with nutrient deficiencies may require temporary or long-term nutrient supplementation with gluten-free vitamins, minerals and protein to correct deficiencies and replenish nutrient stores. Anemia may be treated with iron, folate, or vitamin B12, depending on the origin

of the anemia. If the patient is found to have a deficiency of vitamin D, then a vitamin D supplement should be given (Caruso *et al.*, 2013).

1.2.1.5 Complications of celiac disease

After years of being undiagnosed, some adults may experience “refractory” celiac disease, which means that the body does not respond to a gluten-free diet and that symptoms continue and can lead to intestinal damage. Celiac disease is associated with various other autoimmune diseases including Hashimoto thyroiditis, type1 diabetes mellitus, primary biliary cirrhosis, primary sclerosing cholangitis, Addison disease and dermatitis herpetiformis (Elfstrom *et al.*, 2007).

Patients with celiac disease have a higher risk of developing enteric malignancies, particularly intestinal T-cell lymphoma, and they have smaller increased risk of colon, oropharyngeal, esophageal, pancreatic, and hepatobiliary cancer (Askling *et al.*, 2002).

1.2.1.2 Blood

The blood is a vitally important fluid for the body. It is thicker than water, and feels a bit sticky. Blood is made up of many parts, including red blood cell, white blood cell, platelet and plasma. Approximately 50 to 60% of the blood volume is liquid; the remainder is cell. The average person has approximately 70 ml of blood per kilogram body weight (70 ml/kg), or 5 L total for a 70-kg man (Kern, 2002) .

1.2.2.1 Blood functions

Blood has three important functions

1.2.2.1.1 Transportation

The blood transports oxygen from the lungs to the cells of the body, where it is needed for metabolism. The carbon dioxide produced during metabolism is carried back to the lungs by the blood, where it is then

exhaled. Blood also provides the cells with nutrients, transports hormones and removes waste products, which the liver, the kidneys or the intestine, for example, then get rid of (Schmidt *et al.*, 2011).

1.2.2.1.2 Regulation

The blood helps to keep certain values of the body in balance. For instance, it makes sure that the right body temperature is maintained. This is done both through blood plasma, which can absorb or give off heat, as well as through the speed at which the blood is flowing. When the blood vessels expand, the blood flows more slowly and this causes heat to be lost. When the environmental temperature is low the blood vessels can contract, so that as little heat as possible is lost. Even the so-called pH value of the blood is kept at a level ideal for the body. A constant pH value is very important for bodily functions (Schmidt *et al.*, 2011).

1.2.2.1.3 Protection

If a blood vessel is damaged, certain parts of the blood clot together very quickly and makes sure that a scrape, for instance, stops bleeding. This is how the body is protected against losing blood. White blood cells and other messenger substances also play an important role in the immune system (Schmidt *et al.*, 2011).

1.2.2.3 Blood constituents

The liquid component, called plasma, is nearly 90% water. The remaining 10% includes ions, glucose, amino acids and other metabolites, hormones, and various proteins. The proteins of interest are the coagulation proteins. Serum is the liquid remaining after blood clots; it is essentially the same as plasma, except that the clotting factors and fibrinogen have been removed. The cells of the blood can be divided in to erythrocytes, leukocytes of various types, and platelets (Kern, 2002) .

1.2.2.3.1 Leukocytes

Leucocytes may be divided into two broad groups the phagocytes and the immunocytes. Granulocytes, which include three types of cell neutrophils (polymorphs), eosinophils and basophils together with monocytes comprise the phagocytes. Only mature phagocytic cells and lymphocytes are found in normal peripheral blood (Hoffbrand *et al.*, 2006) .

The normal white blood cell count is 4,000 to 10,000/ uL. Leukocytes predominantly function in tissues. They are only in the blood transiently, while they travel to their site of action (Kern , 2002) .

1.2.3.2 Platelets

Platelets are anucleate discs with a diameter of ~1 to 4 μ m. They have pale blue cytoplasm with reddish-purple granules. Platelets are produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes, one of the largest cells in the body (Hoffbrand *et al.*, 2006).

The normal platelet number is ~150,000 to 350,000 cells/ L. Platelets have different types of granules, designated alpha granules and dense bodies. Platelets have a life span of approximately 10 days. They are removed by the spleen. Platelets are involved in hemostasis. They adhere to tears in the endothelial lining of blood vessels, forming a platelet plug. (Kern, 2002) .

1.2.3.3 Erythrocytes

Erythrocytes are shaped like biconcave disks approximately 7 to 8 μ m in diameter. The biconcave disk shape gives red blood cells the flexibility to pass repeatedly through the microcirculation whose minimum diameter is 3.5 μ m, to maintain hemoglobin in a reduced (ferrous) state and to maintain osmotic equilibrium despite the high concentration of protein

(hemoglobin) in the cell. Its total journey throughout its 120-day lifespan has been estimated to be 480 km (Hoffbrand *et al.*, 2006).

The normal RBC count is approximately 4.5 to 6 million cells per microliter. The parameters by which erythrocytes are usually measured are the blood hemoglobin in grams per deciliter (g/dL), the hematocrit (Hct) or packed cell volume (volume of RBCs as a percent of total blood volume) (Kern, 2002).

1.2.3.3.2 Function of erythrocytes

The primary function of erythrocytes is gas exchange. They carry oxygen from the lungs to the tissues and return carbon dioxide (CO₂) from the tissues to the lungs to be exhaled. They are anucleate cells containing few organelles; a large proportion of their cytoplasm consists of the iron containing oxygen transport molecule hemoglobin (Kern, 2002).

1.2.3.3.3 Production of erythrocytes

Erythrocytes and the digestive system are linked closely from the very beginning of life. The yolk sack is the origin of first generation of erythrocyte precursors. Yolk sac derived progenitor cell may seed the developing liver via the circulation and produce mature red blood cells that are required to meet the metabolic needs of the fetus. By week 8, liver-derived red cell evident and the liver is the only source of the erythrocyte until the 18th week of gestation. Afterwards, the spleen and bone marrow take over (Palis and Segel, 1998).

1.2.4 Anemia

This is defined as a reduction in the hemoglobin concentration of the blood. Although normal values can vary between laboratories, typical values would be less than 13.5 g/dL in adult males and less than 11.5 g/dL in adult females. From the age of 2 years to puberty, less than 11.0 g/dL

indicates anemia. As newborn infants have a high hemoglobin level, 14.0 g/dL is taken as the lower limit at birth (Hoffbrand *et al.*, 2006).

The normal hemoglobin concentration is somewhat lower (between 11 and 15 g/dL). A reduction of the hemoglobin value is often accompanied by changes in other red cell parameters (e.g., microcytic and hypochromic in iron deficiency anemia, macrocytic in vitamin B 12 deficiency and other conditions) (Munker *et al.*, 2007).

1.2.4.1 Causes of anemia

There are many causes of anemia

1.2.4.1.1 Blood loss

It is the most common cause of anemia, especially iron deficiency anemia. Blood loss can be short term or persist over time. Heavy menstrual period or bleeding in the digestive or urinary tract can cause blood loss. Surgery, trauma, or cancer also cause blood loss. If a lot of blood is lost, the body may be loss enough red blood cell to cause anemia (Peter, 2016).

1.2.4.1.2 Lack of red blood cell production

Both acquired and inherited condition and factors can prevent your body from making enough red blood cells. Acquired condition and factors that can lead to anemia include poor diet, abnormal hormone level, some chronic disease and pregnancy. Aplastic anemia can prevent your body from making enough red blood cell .It can be acquired and inherited condition. A diet that lack iron , folic acid or vitamin B12 cell and any condition that make it hard the body to absorb nutrients can prevent your body from making enough red blood cell (Peter, 2016).

1.2.4.1.3 High rates of red blood cell destruction

Both acquired and inherited condition and factors can cause destroy red blood cell. One example of acquired condition is an enlarged or diseased spleen; it may remove more red blood cell than normal, causing anemia. Example of inherited condition including sickle cell anemia, thalassemias and lack of certain enzymes. Acquired and inherited condition can cause hemolytic anemia include immune disorder, infection, certain medicines or reaction of blood transfusion (Peter, 2016).

1.2.4.3 Classification of anemia

Anemia can be classified from three points of view pathogenesis, red cell morphology and clinical presentation. Pathogenic mechanisms involved inadequate production and loss of erythrocytes as a result of bleeding or hemolysis. Based on these pathogenic mechanisms anemia can divided into two types hyporegenerative when bone marrow production is decrease as a result of impaired function, decreased number of precursor cell, reduced bone marrow infiltration or lack of nutrients. Regenerative when bone marrow responds appropriately to a low erythrocyte mass by increasing production of erythrocyte (Sabrafen *et al.*, 2006).

Classification based on basic parameters of red cell morphology such as the mean corpuscular volume (MCV) and other red cell parameters (e.g., microcytic and hypochromic in iron deficiency anemia, macrocytic in vitamin B 12 deficiency and other conditions) (Sabrafen *et al.*, 2006).

Anemia also can be classified according to form of clinical presentation as acute (usually bleeding or hemolysis) or chronic (Sabrafen *et al.*, 2006).

1.2.4.4 Clinical features of anemia

The major adaptations to anemia are in the cardio-vascular system (with increased stroke volume and tachycardia) and in the hemoglobin oxygen dissociation curve. In some patients with quite severe anemia there may be no symptoms or signs, whereas others with mild anemia may be severely incapacitated (Hoffbrand *et al.*, 2006).

Symptoms are usually shortness of breath particularly on exercise, weakness, lethargy, palpitation and headaches. In older subjects, symptoms of cardiac failure, angina pectoris or intermittent claudication or confusion may be present. Visual disturbances may complicate severe anemia, particularly of rapid onset (Hoffbrand *et al.*, 2006).

Signs may be divided into general and specific. General signs include pallor of mucous membranes which occurs if the hemoglobin level is less than 9-10 g/dL. Conversely, skin color is not a reliable sign. A hyperdynamic circulation may be present with tachycardia, a bounding pulse, cardiomegaly and a systolic flow murmur especially at the apex. Particularly in the elderly, features of congestive heart failure may be present. Specific signs are associated with particular types of anemia (e.g. koilonychia 'spoon nails' with iron deficiency, jaundice with hemolytic or megaloblastic anemia, leg ulcers with sickle cell and other hemolytic anemia, bone deformities with thalassaemia major and other severe congenital hemolytic anemia) (Hoffbrand *et al.*, 2006)

1.2.4.5 Diagnosis

The clinical history should cause blood loss and recent history of gastrointestinal symptom. All symptoms should be recorded. A single blood sample can give information on the concentration of hemoglobin, red cell indices MCV and MCH, RDW (estimate of anisocytosis) and

ESR. Blood smear to show if abnormal erythrocyte have been detected, routine biochemical test may help to guide the diagnosis but in some cases, erythropoietin serum level and bone marrow examination are necessary. In cases of macrocytic anemia serum levels of vitamin B12 and folate will guide the diagnosis to deficiency of one of those (Gasche *et al.*, 2007).

A single blood sample can give information on the concentration of hemoglobin), the erythrocyte indices MCV and MCH (useful for the morphological classification of anemia), RDW (estimate of anisocytosis) and ESR, which reports on possible ACD (Gasche *et al.*, 2007).

In cases of suspected IDA, the concentration of soluble transferrin receptor should be included as a key parameter in differentiation between IDA and ACD. The most likely causes of normocytic anemia are ACD, renal failure and primitive hematological diseases (least frequently). Iron metabolism and routine biochemical tests may help to guide the diagnosis, but in some cases, erythropoietin serum levels and bone marrow examination are necessary. In cases of macrocytic anemia, serum levels of vitamin B12 and serum and/or erythrocyte levels of folate will guide the diagnosis to deficiency of one of these. In the absence of gastric disease, the most likely cause of malabsorption of vitamin B12 is Crohn's disease, with involvement of the terminal ileum (Gasche *et al.*, 2007).

1.2.5 Vitamin B12

Vitamin B12 (cobalamin) exists in a number of different chemical forms. In nature, the vitamin is mainly in the 5'-deoxyadenosyl (ado) form. This is the main form in human tissues and is located in the mitochondria. It serves as the cofactor for the enzyme methylmalonyl CoA mutase. The other major natural cobalamin is methylcobalamin, the main form in

human plasma, as well as the cytosolic form in cells. It serves as the cofactor for the enzyme methionine synthase (Hoffbrand and Catovsky, 2005).

1.2.5.1 Dietary sources and requirements

Vitamin B12 is synthesized solely by micro-organisms. The only source for humans is food of animal origin. The highest amounts are found in liver and kidney (up to 100 μ g/100 g) but it is also present in shellfish, organ and muscle meats, fish, chicken and dairy products like eggs, cheese and milk which contain small amounts (6 μ g/L). Vegetables, fruits and all other foods of non-animal origin are free from vitamin B12 unless they are contaminated by bacteria. A normal Western diet contains between 5 and 30 μ g of cobalamin daily. Adult daily losses (mainly in the urine and faeces) are between 1 and 3 μ g (about 0.1% of body stores) and daily requirements are also about 1–3 μ g. Body stores are of the order of 2–3 mg and are sufficient for 3–4 years if supplies are completely cut off (Hoffbrand and Catovsky, 2005).

1.2.5.2 Absorption

Two mechanisms exist for cobalamin absorption. One is passive, occurring equally through the duodenum and the ileum; it is rapid but extremely inefficient as less than 1% of an oral dose can be absorbed by this process. The other mechanism is active; it occurs through the ileum in human and is efficient for small (a few micrograms) oral doses of vitamin B12. This is the normal mechanism by which the body acquires vitamin B12 and is mediated by gastric intrinsic factor (IF). Dietary vitamin B12 is released from protein complexes by enzymes in the stomach, duodenum and jejunum; it combines rapidly with a salivary glycoprotein (R binder) related to plasma transcobalamin I (TCI). Subsequently, the R binder is

digested by pancreatic trypsin and cobalamin transferred to IF. Binding of vitaminB12 to IF is favoured by an alkaline pH. The IF– vitaminB12 complex passes to the ileum, where IF attaches to a specific receptor (cubilin) on the microvillus membrane of the brush border surface of the ileal absorptive cells. VitaminB12 then enters the ileal cell. Intrinsic factor does not enter the bloodstream as such, as after a delay of about 6 h, absorbed vitaminB12 appears in portal blood, attached to transcobalamin(II) which is probably synthesized in the ileum. The ileum has a restricted capacity to absorb vitaminB12 because of limited receptor sites and, although 50% or more of a single dose of 1 μ g of vitaminB12 may be absorbed, with doses above 2 μ g the proportion absorbed falls rapidly (Hoffbrand and Catovsky, 2005).

1.2.5.4 Transport

Two main vitaminB12 transport proteins exist in human plasma; they both bind vitaminB12 one molecule for one molecule one heptocorrin, also known as TCI, is a glycoprotein. TCIII was a name used to describe a minor isoprotein of TCI in plasma, which differs from TCI by its composition of sugars and vitaminB12 content. TCI and TCIII are derived primarily from the specific granules in neutrophils. Glycoprotein receptors on liver cells are concerned in the removal of heptocorrins from plasma, and TCI may have a role in the transport of vitaminB12 analogues to the liver for excretion in bile. The other major vitaminB12 transport protein in plasma is transcobalamin (TC). TC is β -globulin synthesized by liver, and by other tissues including macrophages, ileum and endothelium (Hoffbrand and Catovsky, 2005).

1.2.5.5 Role of vitamin B 12

Vitamin B 12 is essential for several enzyme systems include Adenosylcobalamin is a coenzyme involved in the conversion of methylmalonyl-coenzyme A (CoA) to succinyl CoA, the synthesis of methionine from homocysteine, and The synthesis of S-adenosyl methionine (Munker *et al.*, 2007).

1.2.5.6 Vitamin B12 deficiency

The body's requirement for vitamin B 12 is about 1 μ g daily. The underlying mechanism is an autoimmune gastritis that results in achlorhydria and the absence of intrinsic factor in pernicious anemia Veganism is an unusual cause of severe deficiency, almost vegetarians and vegans include some vitamin B 12 in their diet. Gastric resection and intestinal causes of malabsorption of vitamin B 12 for example, ileal resection or the intestinal stagnant loop syndrome are less common now that abdominal tuberculosis is infrequent and H₂ -antagonists have been introduced for treating peptic ulceration, thus reducing the need for gastrectomy (Munker *et al.*, 2007).

1.3 Previous Studies

Many studies were done concerning serum vitamin B12 in celiac disease; one of these studies is a prospective study of 39 consecutive biopsy proven celiac disease patients (32 women, seven men; median age 48 years, range 22-77 years) between September 1997 and February 1999. The serum vitamin B12 was measured before and after a median of 4 months (range 2-13 months) of treatment with a gluten-free diet. The result of these study is total of 16 (41%) patients were vitamin B12 deficient (<220 ng/L) and 16 (41%) patients (11 women and five men) were anemic. The Schilling test, performed in 10 of the vitamin B12-

deficient patients, showed five low and five normal results. Although only five patients received parenteral vitamin B12, at follow-up the vitamin B12 results had normalized in all patients (Dahele *et al.*, 2001).

Another study was collected hematologic parameters from a cohort of patients seen at a tertiary care center for CD to assess the characteristics of anemia in this population. Hematological parameters measured ≤ 3 months of diagnosis and degree of villous atrophy from 405 patients diagnosed was analyzed. Vitamin B12 deficiency was found in approximately 5%. Macrocytic anemia with concurrent B12 was rare (3%) (Harper *et al.*, 2007).

In Danish patient cohort study included all patients aged 15+ years, who were diagnosed with CD between January 2008 and August 2013. A total of 93 patients with a valid CD diagnosis were identified. In total, vitamin B12 deficiency was found in 17% (Schøsler *et al.*, 2015).

In this study done by Hjelt *et al.*, were studied longitudinally in 20 coeliac children aged 1.2-16.6 years (mean 7.5 years) during periods of gluten-free and gluten containing diets. The absorption methods were specially adapted to use in children, and age-related reference limits were established. Also, dietary intakes of B12 were registered. Plasma (P)-B12 concentrations demonstrated a wide range of values above the lower normal limit, whereas the level in a single patient was within the "intermediate range" of B12 insufficiency (150-200 pmol/l). Bacterial overgrowth of the small intestinal tract was not found to be a plausible cause of the B12 malabsorption in the case of 5 patients observed.

Another a prospective study involved 19 children with potential disease, 67 with partial or subtotal villous atrophy (P/SVA) and 16 with total villous atrophy (TVA). Twenty-three healthy children comprised the

control group. The prevalence of abnormal parameters was as follows (controls, potential celiac disease, P/SVA and TVA, respectively): anemia 0%, 15%, 22% and 63%; One subject had low folate and none had low vitamin B12 (Repo *et al.*, 2016).

Chapter Two

Objectives and Rationale

2.1 Rationale

Celiac disease lead to truncating of the villi lining of the small intestine, this interfere with the absorption of nutrients like vitamin B12 and lead to deficiency of it. Vitamin B12 deficiency can cause anemia because this vitamin is essential for the formation and growth of red blood cells. Testing of vitamin B12 is not carried out routinely for patients with celiac disease. To our knowledge there is no study in Sudan addressing vitamin B12 deficiency as a cause of anemia in patients with celiac disease. Identification of hematological abnormalities will improve patients management protocols and thus improve patients quality of life.

2.2 Objectives

2.2.1 General objective

To study serum vitamin B12 levels in celiac disease patients and study its effect on RBCs parameters.

2.2.2 Specific objectives

- To estimate serum vitamin B12 level in celiac disease patients using cobas immunoassay analyzer.
- To correlate vitamin B12 level to celiac disease patients age and gender.
- To correlate dietary program of celiac disease patients, the effect of disease duration, and RBCs parameters with vitamin B12 deficiency.

Chapter Three

Materials and methods

3.1 Materials

3.1.1 Study design

Hospital based descriptive cross-sectional study.

3.1.2 Study area

This study was conducted at Ibn Sinaa hospital, Khartoum state, Sudan.

3.1.3 Study duration

The study was carried during the period from April to August 2016.

3.1.4 Study population

This study was conducted among 40 Sudanese patients with celiac disease referred to Ibn Sinaa hospital and diagnosed by serological test and endoscopy.

3.1.5 Inclusion criteria

Sudanese patients with Celiac disease.

3.1.6 Exclusion criteria

Patients with other gastrointestinal disorders and conditions known to have effect on haematological parameters- such as pregnancy, chronic blood loss, malaria, and renal failure were excluded from the study.

3.1.7 Sample collection

Three milliliters of venous blood were collected by venipuncture technique from each patient. The samples collected under aseptic conditions then allowed to clot, centrifuged at 3000 rpm for 5 minutes to obtain serum for estimation of vitamin B12 level.

3.1.8 Ethical considerations

This study was approved by Ministry of health, Khartoum, Sudan and by Ibn Sinaa hospital management. Informed consent was taken from all participants or parents in case of children before sample collection. No risk is known as a result of sample collection. Patient with abnormal hematological parameters was advised to visit their physician.

3.1.9 Data collection and analysis

Patients' data will be collected using structured questionnaire and hematological parameters currently from patient's record and analyzed using statistical package for social science software (SPSS).

3.1.10 Equipments, disposables and reagents

- Centrifuge
- Sterile plain containers
- Disposable syringes
- 70% alcohol
- Tourniquets
- Cotton
- Micropipettes
- Reagents working solutions include:
 - The rackpack (kit placed on instrument)
 - Streptavidin coated microparticles
 - Reagent 1 (ruthenium labeled intrinsic factor)
 - Reagent 2 (vitamin B12 labeled biotin).
 - Pretreatment 1 (Dithiothreitol) .
 - Pretreatment 2 (sodium hydroxide, sodium cyanide).
- Calibrator (Cal 1and Cal 2)

3.2 Methods

3.2.1 Estimation of serum B12 level

Serum was collected from clotted samples and used for measurement of serum vitamin B12 by electrochemiluminescence immunoassay “ECLIA” (Cobas e411, Germany).

1.2.2 Principle

Vitamin B12 II assays employs a competitive test principle using intrinsic factor specific for vitamin B12. Vitamin B12 in the sample competes with the added vitamin B12 labeled with biotin for the binding sites on the ruthenium-labeled intrinsic factor complex. Binding assay for the in vitro quantitative determination of vitamin B12 in human serum and plasma. Measurements obtained are used in the diagnosis and treatment of anemias of gastrointestinal malabsorption. The electrochemiluminescence immunoassay “ECLIA” is intended for use on cobas e411 immunoassay analyzer

3.2.3 Procedure

Measurement of serum vitamin B12 level carried out using full automated cobas e411 immunoassay analyzer (appendix no.3)

Chapter Four

Results

The level of serum vitamin B12 was measured in 40 Sudanese patients with celiac disease who diagnosed by serological test and endoscopy. The study was conducted at Ibn Sinaa Hospital in the period from April to August 2016. Ten (25%) of the patients were males and 30 (75%) were females (Figure 4.1).

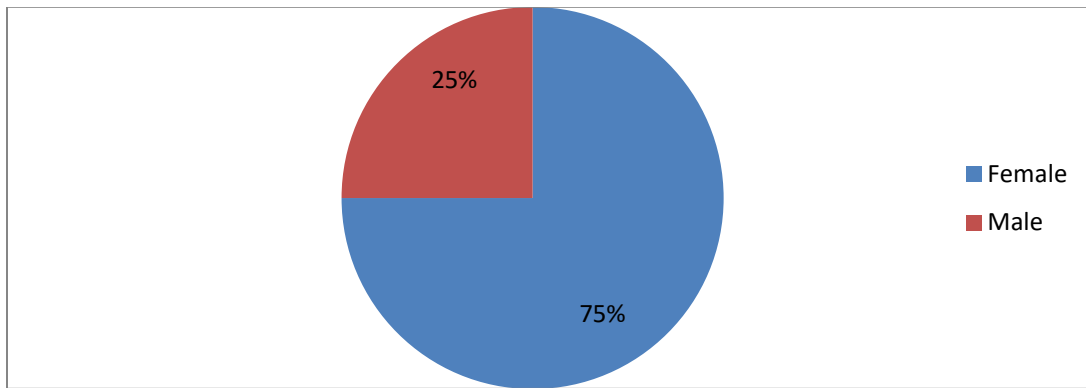


Figure (4.1): Gender distribution of study population.

Patients age group was ranged from 2-43 years (Mean \pm SD:16.59 \pm 10.284); 17 (42.5%) of patients were children (<13years) and 23(57.5%) of them were adults (\geq 13 years) (Figure4.2).

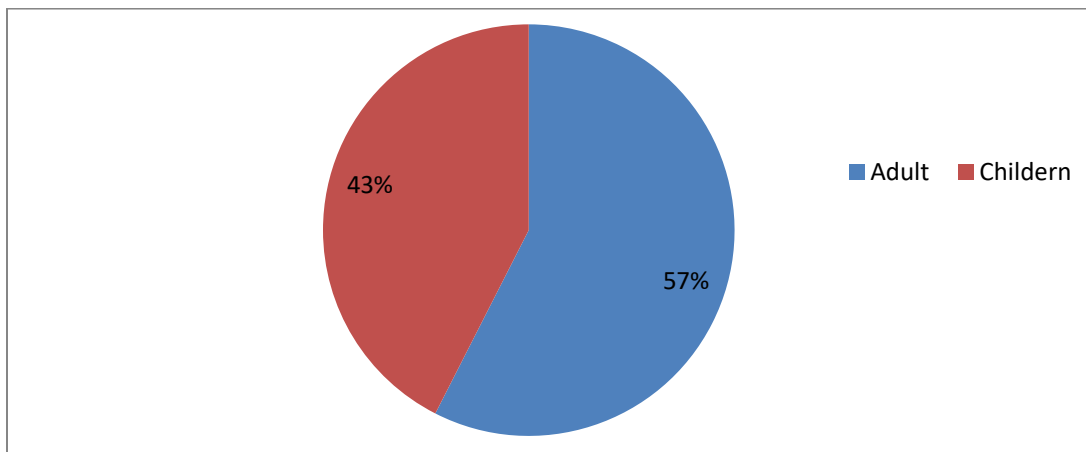


Figure (4.2): Distribution of study population according to age group

Four (10%) of the patients were not committed to gluten free diet program, while 36(90%) were committed (Figure 4.3).

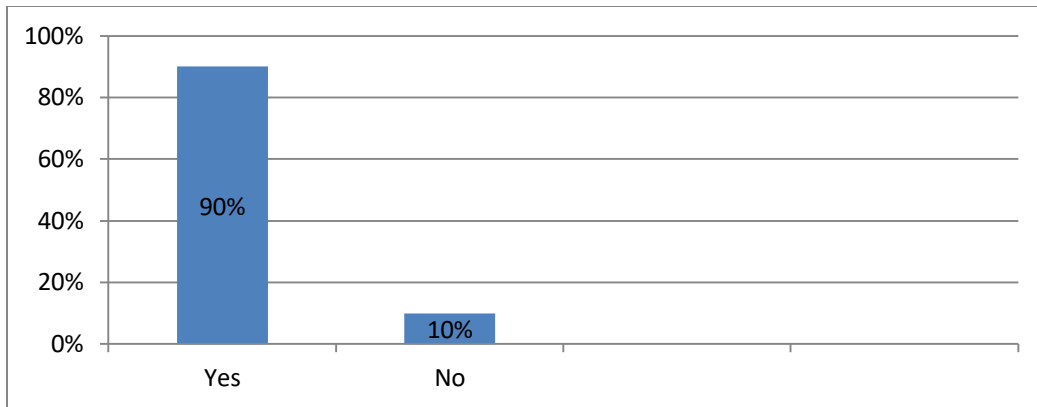


Figure (4.3): Commitment of study population to gluten free diet

Duration of disease was range from one month to 240 months (Mean±SD:38.92±47.02).

Red cell parameters, serum B12 level and duration of the disease in study population are shown in table (4.1).

Table (4.1): Red cell parameters, serum B12 level and disease duration in study population

Parameters	Mean	SD
RBCs (u/l)	4.77	0.51
Hb (g/dl)	10.6	2.2
PCV (%)	35.9	4.6
MCV(fl)	75.7	9.2
MCV(fl)	75.7	9.2
MCH (pg)	23.2	4.0
MCHC(g/dl)	30.7	2.4
Serum B12	417.9	183.5
Duration (month)	38.9	47.0

The result showed that, frequency of B12 deficiency (normal range 211-950 ng/L) among patients with celiac disease was 12.5% (5/40), 1(2.5%) was male and 4 (10%) were female, while the serum B12 level was normal in 35(87.5%), 9(22.5%) were male and 26(65%) were female (Figure 4.4).

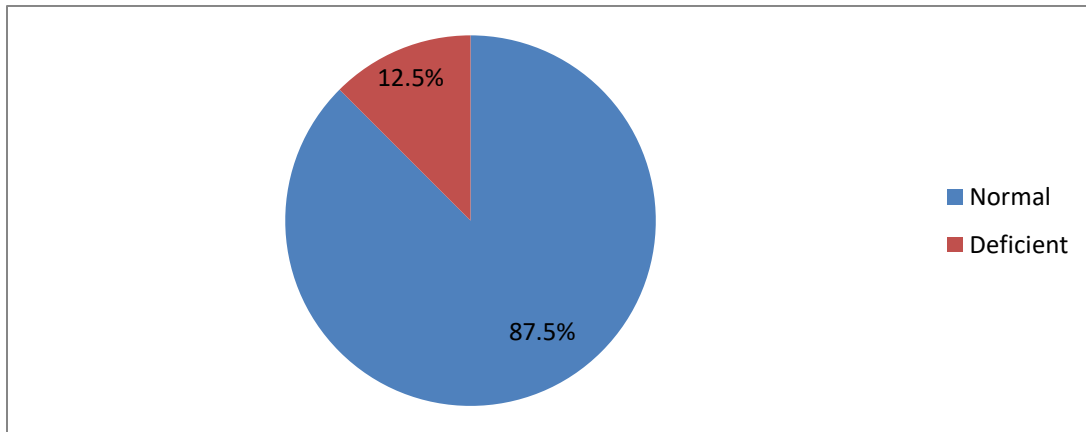


Figure (4.4): The frequency of B12 deficiency in study population.

There was no statistically significant correlation between serum B12 level and red cell parameters (RBCs count, Hb, PCV, MCV, MCH, and MCHC). Also there was no significant correlation between serum B12 level and duration of disease.

Table (4.2): Correlation of serum B12 level with red cell parameters and disease duration in study population

Parameters	Person correlation	P.value
RBCs(u/l)	0.060	0.71
PCV (%)	0.082	0.61
MCV(fl)	0.045	0.78
MCH(pg)	0.015	0.92
MCHC(g/dl)	0.083	0.60
Duration	-0.010	0.95

When correlate gender of the patients with normal and low level of serum B12 there was no statistically significant correlation ($P.value= 0.78$) (Table4.3).

Table (4.3): Correlation between gender and serum B12 level in study population

B12 level	Gender				P. value
	Male		Female		
	N	%	N	%	
Normal	9	22.5	26	65	0.78
Deficient	1	2.5	4	10	

The mean of age was no statistically significant in patients with normal level of serum B12 compared to those with low level of serum B12 ($P.value= 0.64$) (Table4.4).

Table (4.4): Comparison of age in patients with deficient and normal B12 level.

Age	Patients with normal B12level		Patients with B12 deficiency		P .value
	Mean	SD	Mean	SD	
	16.30	10.38	18.60	10.45	

In comparison of red cells parameters in patients with normal serum B12 and those with low serum B12 showed no statistically significant correlation with RBCs parameters (RBCs count, Hb, PCV, MCV, MCH and MCHC) and duration of disease.

Table (4.5): Comparison of red cell parameters and duration of disease in patients with and without B12 deficiency.

Red cell parameters	Patients with normal B12 level		Patients with B12 deficiency		P .value
	Mean	SD	Mean	SD	
RBCs(u/l)	4.8	0.52	4.6	0.41	0.46
Hb(g/dl)	10.6	2.3	11.3	1.1	0.49
PCV (%)	35.8	4.8	36.2	3.1	0.85
MCV(fl)	74.8	8.9	81.6	9.8	0.20
MCH(pg)	23.0	4.1	24.8	3.5	0.37
MCHC (g/dl)	30.6	2.6	31.2	0.79	0.63
Duration(months)	42.2	49.1	15.4	14.2	0.23

Regarding the effect of dietary program on serum B12 level no significant difference was found (Table 4.6)

Table (4.6): Comparison of B12 level according to dietary program commitment in study population.

Serum B12level	Committed		Not committed		p.value
	Mean	SD	Mean	SD	0.22
	429.8	184.3	311.1	155.5	

There was statistically significant correlation between B12 deficiency and commitment to dietary program (Table4.7)

Table (4.7): Correlation between deficiency B12 and dietary program commitment in study population

B12 level	Diet free gluten				P. value
	Committed		Not committed		
	N	%	N	%	
Normal	33	82.5	2	5	0.017
Deficient	3	7.5	2	5	

Chapter Five

Discussion, Conclusion and Recommendations

6.1 Discussion

Haematological abnormalities are considered as a hallmark of celiac disease, and reported to be most pronounced in patient with digestive symptoms as result of the malabsorption of several nutrients. The vitamin deficiency is due to the damage to the intestinal makes, it hard for the body to absorb nutrients, although they were controlled by GFD

This cross-sectional study was conducted among 40 Sudanese patients with celiac disease their age ranged from 2-43 years to examine the correlation between serum B12 level and celiac disease.

In this study B12 deficiency was present in 5 (12.5 %) patients, 1 (2.5 %) was male and 4 (10%) were females. Presence of vitamin B12 in small proportion of patients may be due to the fact that, vitamin B12 stores in the body about 2-3 mg, it is sufficient for 2-4 years (Hoffbrand *et al.*, 2006).

This finding agrees with many previous studies reported that vitamin B 12 deficiency was found in 19%, 17% and 9.1% of celiac patients (Wierdsma *et al.*, 2013, Schøsler *et al.*, 2015, Abbas *et al.*, 2013).

Our finding is disagree with previous study done by Dahele *et al.*, who found that B12 deficiency was present in 41% of patients and other study done by Hjelt *et al.*, who reported that, B12 concentrations demonstrated a wide range of values above the lower normal limit. In contrast another study found B12 deficiency in 5% of celiac disease patients (Harper *et al.* 2007). In another study done by Repo *et al.*, who reported that, no one of their celiac disease patients had vitamin B12 deficiency.

In this study ten (25%) of the patients were males and 30 (75%) were females, when we correlate gender of the patients with normal and low level of serum B12 there was no statistically significant correlation, (p . *value* >0.05).

The results of this study revealed that, the mean age was not significantly different in patients with normal level of serum B12 compared to those with low level of serum B12.

In the present study no statistically significant correlation between vitamin B12 deficiency and duration of disease. A previous study done by hallert *et al*, reported that, half of the adult celiac patients carefully treated with a gluten-free diet for several years showed signs of a poor vitamin status. This can be explained by short mean duration in our study group (3years).

In our study B12 deficiency showed statistically significant correlation, (p . *value* <0.05) with commitment to dietary program , as 3(7.5%) of patients with B12 deficiency were committed to dietary program that may be due to gluten free diet is to be poor in micronutrients like vitamin B12 (Vici *et al.*,2016).

The presence of vitamin B12 in patients who were not committed to gluten free diet 2(5%) is probably related to the loss of proximal small intestine villi resulting in malabsorption of micronutrients in untreated patients, vitamin B12 concentrations normalize on a gluten free diet alone due to the expected histological recovery of the intestinal mucosa is within 6 to 12 months after the onset of gluten free diet (Wahab *et al.*, 2002). Furthermore, other study found that B12 status showed significant variations related to dietary changes (Valente *et al.*, 2015).

Another study done by Hjelt *et al*, conducted on 20 celiac disease patients during periods of gluten-free and gluten containing diets and reported no significant change in B12 level related to shifts in diet.

In this study, no statistically significant correlation was found between serum B12 and RBCs parameters (RBCs count, Hb, PCV, MCV, MCH and MCHC) ; and no macrocytosis reported, this agrees with study by Harper *et al*, who reported that, macrocytic anemia with concurrent B12 deficiency was rare.

6.1 Conclusion

The results of this study it is concluded twelve and half percent of patients were found to have vitamin B12 deficiency. There was no statistically significant correlation between vitamin B12 deficiency and each of RBCs parameters, duration, age and gender. When correlate serum vitamin B12 low level with dietary program commitment it was statistically significant correlation.

6.3 Recommendations

- Further studies on large sample size should be performed.
- Determination of vitamin B12 level should be one of the basic parts of monitoring of celiac disease patient
- Health education and more attention should be given to the quality of the nutrients offered by gluten free diet because this constitutes a treatment for life.

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Sudan University of Science and Technology

**Evaluation of Serum Vitamin B12 Level in Celiac Disease Patients
and it's effect on Red Cell Parameters**

Appendix (1)

Questionnaire

(a) General Information:

Serial No:.....

Age:

Sex: Male () Female ().

(b) Clinical Information

Duration of the disease:.....years.

Any chronic disease:

Dietary program commitment: Yes () No ().

(c) Laboratory investigation:

Serum vitamin B12..... μ g /dl

HB :g/dl RBCs:.....u/l

PCV:..... % MCV :.....fl

MCH pg MCHC:.....g/d

Date:.....

Signature:.....

Appendix (2)

Cobas e411 immunoassay analyzer



Appendix (3)

04761707001V4

Vitamin B12

Vitamin B₁₂

04745736 190

100 tests

• Indicates analyzers on which the kit can be used

Elecsys 2010	MODULAR ANALYTICS E170	cobas e 411	cobas e 601
•	•	•	•

English

Intended use

Binding assay for the in vitro quantitative determination of vitamin B₁₂ in human serum and plasma.

The electrochemiluminescence immunoassay "ECLIA" is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Summary

Nutritional and macrocytic anemias can be caused by a deficiency of vitamin B₁₂. This deficiency can result from diets devoid of meat and bacterial products, from alcoholism, or from structural/functional damage to digestive or absorptive processes (forms of pernicious anemia). Malabsorption is the major cause of this deficiency through pancreatic deficiency, gastric atrophy or gastrectomy, intestinal damage, loss of intestinal vitamin B₁₂ binding protein (intrinsic factor), production of autoantibodies directed against intrinsic factor, or related causes.¹ This vitamin is necessary for normal metabolism, DNA synthesis and red blood cell regeneration. Untreated deficiencies will lead to megaloblastic anemia, and vitamin B₁₂ deficiency results in irreversible central nervous system degeneration. Vitamin B₁₂ or folate are both of diagnostic importance for the recognition of vitamin B₁₂ or folate deficiency, especially in the context of the differential diagnosis of megaloblastic anemia.

Radioassays were first reported for vitamin B₁₂ in 1961.^{3,4} All utilize ⁵⁷Co-cyanocobalamin radiolabeled tracers and intrinsic factor for binding vitamin B₁₂. The various commercial assays differ in their free versus bound separation techniques and choice of specimen pretreatment. The presence of endogenous serum binding proteins for cyanocobalamin (transcobalamins including R-protein) and of immunoglobulins directed against intrinsic factor require that specimens are either boiled or treated at an alkaline pH to release the vitamin B₁₂ and destroy the binding proteins. In the late 1970's, radioassays using serum binding proteins or partially purified intrinsic factor measured levels of vitamin B₁₂ which exceeded those determined by microbiological methods. This was caused by the presence of the serum binding protein or R-proteins in the assay. R-protein specificity is poor compared to that of intrinsic factor and vitamin B₁₂ analogs were being measured in addition to vitamin B₁₂ itself.^{5,6,7,8} Since that time, recommendations have been established for the use of highly purified intrinsic factor throughout the industry. The Elecsys Vitamin B₁₂ assay employs a competitive test principle using intrinsic factor specific for vitamin B₁₂. Vitamin B₁₂ in the sample competes with the added vitamin B₁₂ labeled with biotin for the binding sites on the ruthenium-labeled intrinsic factor complex^a.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex (Ru(bpy)₃²⁺)

Test principle

Competition principle. Total duration of assay: 27 minutes.

- 1st incubation: By incubating the sample (15 µL) with the vitamin B₁₂ pretreatment 1 and pretreatment 2, bound vitamin B₁₂ is released.
- 2nd incubation: By incubating the pretreated sample with the ruthenium labeled intrinsic factor, a vitamin B₁₂-binding protein complex is formed, the amount of which is dependent upon the analyte concentration in the sample.
- 3rd incubation: After addition of streptavidin-coated microparticles and vitamin B₁₂ labeled with biotin, the still-vacant sites of the ruthenium labeled intrinsic factor become occupied, with formation of a ruthenium labeled intrinsic factor-vitamin B₁₂ biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

cobas[®]

- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents - working solutions

- PT1 Pretreatment reagent 1 (white cap), 1 bottle, 4 mL:
Dithiothreitol 1.028 g/L; stabilizer, pH 5.5.
- PT2 Pretreatment reagent 2 (gray cap), 1 bottle, 4 mL:
Sodium hydroxide 40 g/L; sodium cyanide 2.205 g/L.
- M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.
- R1 Intrinsic factor-Ru(bpy)₃²⁺ (gray cap), 1 bottle, 10 mL:
Ruthenium labeled porcine intrinsic factor 4 µg/L; cobinamide dicyanide 15 µg/L; stabilizer; human serum albumin; phosphate buffer, pH 5.5; preservative.
- R2 Vitamin B₁₂-biotin (black cap), 1 bottle, 8.5 mL:
Biotinylated vitamin B₁₂ 25 µg/L; biotin 3 µg/L; phosphate buffer, pH 7.0; preservative.

Precautions and warnings

For in vitro diagnostic use.

Exercise the normal precautions required for handling all laboratory reagents.

Disposal of all waste material should be in accordance with local guidelines.

Safety data sheet available for professional user on request.

This kit contains components classified as follows according to the European directive 88/379/EEC:



PT2: C - CORROSIVE, R 34, S 26, S 37/39 (sodium hydroxide)
Causes burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective gloves and eye/face protection.



PT2: Xn - HARMFUL, R 20/21/22, S 45 (sodium cyanide)
Harmful by inhalation, in contact with skin and if swallowed. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Contact phone: all countries: +49-621-7590, USA: +1-800-428-2336

All human material should be considered potentially infectious.

All products derived from human blood are prepared exclusively from the blood of donors tested individually and shown to be free from HBsAg and antibodies to HCV and HIV.

The testing methods applied were FDA-approved or cleared in compliance with the European Directive 98/79/EC, Annex II, List A.

However, as no testing method can rule out the potential risk of infection with absolute certainty, the material should be treated just as carefully as a patient specimen. In the event of exposure the directives of the responsible health authorities should be followed.^{9,10}

Avoid the formation of foam with all reagents and sample types (specimens, calibrators, and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is read in via the respective reagent barcodes.

Storage and stability

Store at 2-8°C.

Store the Elecsys Vitamin B₁₂ reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:

unopened at 2-8°C	up to the stated expiration date
after opening at 2-8°C	12 weeks
on Elecsys 2010 and cobas e 411	5 weeks
on MODULAR ANALYTICS E170 and cobas e 601	5 weeks



Vitamin B12

Vitamin B₁₂

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable. Serum collected using standard sampling tubes or tubes containing separating gel.

Na-heparin and K₃-EDTA plasma. When sodium citrate, sodium fluoride/potassium oxalate are used, the values obtained are by 23% lower as compared to serum.

Criterion: Recovery within 90-110% of serum value or slope 0.9-1.1 + intercept within $\pm 2 \times$ analytical sensitivity (LDL) + coefficient of correlation > 0.95. Stable for 2 days at 2-8°C, 2 months at -20°C. Freeze once only. Protect from light.

Stability of serum obtained with separating tubes: 24 hours at 2-8°C (note the data provided by the tube manufacturer).

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay. Do not use heat-inactivated samples. Do not use samples and controls stabilized with azide.

Vitamin B₁₂ determinations should be performed on serum or plasma samples from fasting patients.

Note: Samples with extremely high total protein concentrations (e.g. patients suffering from Waldenström's macroglobulinemia) are not suitable for use in this assay, since they may lead to the formation of protein gel in the assay cup. Processing protein gel may cause a run abort. The critical protein concentration is dependent upon the individual sample composition. The formation of protein gel was seen in samples (spiked with human IgG or human serum albumin) having a total protein concentration > 160 g/L.

Ensure the patients' samples, calibrators, and controls are at ambient temperature (20-25°C) before measurement.

Because of possible evaporation effects, samples, calibrators, and controls on the analyzers should be measured within 2 hours.

Materials provided

See "Reagents - working solutions" section for reagents.

Materials required (but not provided)

- Cat. No. 04572459, Vitamin B₁₂ CalSet II, for 4 x 1 mL
- Cat. No. 04415299, PreciControl Anemia, for 2 x 2 mL each of PreciControl Anemia 1, 2 and 3
- Cat. No. 11732277, Diluent Universal, 2 x 16 mL sample diluent or Cat. No. 03183971, Diluent Universal, 2 x 36 mL sample diluent
- General laboratory equipment
- Elecsys 2010, MODULAR ANALYTICS E170 or **cobas e** analyzer

Accessories for Elecsys 2010 and **cobas e** 411 analyzers:

- Cat. No. 11662988, ProCell, 6 x 380 mL system buffer
- Cat. No. 11662970, CleanCell, 6 x 380 mL measuring cell cleaning solution
- Cat. No. 11930346, Elecsys SysWash, 1 x 500 mL washwater additive
- Cat. No. 11933159, Adapter for SysClean
- Cat. No. 11706802, Elecsys 2010 AssayCup, 60 x 60 reaction vessels
- Cat. No. 11706799, Elecsys 2010 AssayTip, 30 x 120 pipette tips

Accessories for MODULAR ANALYTICS E170 and **cobas e** 601 analyzers:

- Cat. No. 04880340, ProCell M, 2 x 2 L system buffer
- Cat. No. 04880293, CleanCell M, 2 x 2 L measuring cell cleaning solution
- Cat. No. 12135027, CleanCell M, 1 x 2 L measuring cell cleaning solution (for USA)
- Cat. No. 03023141, PC/CC-Cups, 12 cups to prewarm ProCell M and CleanCell M before use
- Cat. No. 03005712, ProbeWash M, 12 x 70 mL cleaning solution for run finalization and rinsing during reagent change
- Cat. No. 03004899, PreClean M, 5 x 600 mL detection cleaning solution
- Cat. No. 12102137, AssayTip/AssayCup Combimagazine M, 48 magazines x 84 reaction vessels or pipette tips, waste bags

- Cat. No. 03023150, WasteLiner, waste bags
- Cat. No. 03027651, SysClean Adapter M

Accessories for all analyzers:

- Cat. No. 11298500, Elecsys SysClean, 5 x 100 mL system cleaning solution

Only available in the USA:

- Cat. No. 04836693, Elecsys Vitamin B₁₂ CalCheck, 3 concentration ranges

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator manual for analyzer-specific assay instructions. Resuspension of the microparticles takes place automatically before use. Read in the test-specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read, enter the 15-digit sequence of numbers.

MODULAR ANALYTICS E170, Elecsys 2010 and **cobas e** analyzers: Bring the cooled reagent to approx. 20°C and place on the reagent disk (20°C) of the analyzer. Avoid the formation of foam. The system **automatically** regulates the temperature of the reagents and the opening/closing of the bottles.

Calibration

Traceability: This method has been standardized against the Elecsys Vitamin B₁₂ assay (Cat. No. 11820753).

Every Elecsys Vitamin B₁₂ reagent set has a barcoded label containing the specific information for calibration of the particular reagent lot.

The predefined master curve is adapted to the analyzer by the use of Elecsys Vitamin B₁₂ CalSet II.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer). Renewed calibration is recommended as follows:

MODULAR ANALYTICS E170, Elecsys 2010 and **cobas e** analyzers:

- after 1 month (28 days) when using the same reagent lot
- after 7 days (when using the same reagent kit on the analyzer)
- as required: e.g. if quality control findings are outside the specified limits

Quality control

For quality control, use Elecsys PreciControl Anemia 1, 2 and 3. Other suitable control material can be used in addition.

Controls for the various concentration ranges should be run as single determinations at least once every 24 hours when the test is in use, once per reagent kit, and after every calibration. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits.

Each laboratory should establish corrective measures to be taken if values fall outside the limits.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in pmol/L or pg/mL).

Conversion factors: $\text{pmol/L} \times 1.36 = \text{pg/mL}$
 $\text{pg/mL} \times 0.738 = \text{pmol/L}$

Limitations - interference

The assay is unaffected by icterus (bilirubin < 1112 $\mu\text{mol/L}$ or < 65 mg/dL), hemolysis (Hb < 0.621 mmol/L or < 1.0 g/dL), lipemia (triglycerides < 17.1 mmol/L or < 1500 mg/dL), and biotin < 205 nmol/L or < 50 ng/mL. Criterion: Recovery within $\pm 10\%$ of initial value.

In patients receiving therapy with high biotin doses (i.e. > 5 mg/day), no sample should be taken until at least 8 hours after the last biotin administration.

No interference was observed from rheumatoid factors up to a concentration of 1500 IU/mL.

In vitro tests were performed on 54 commonly used pharmaceuticals.

No interference with the assay was found.

In rare cases, interference due to extremely high titers of antibodies to streptavidin and ruthenium can occur.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.



