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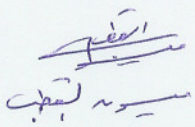
Signature: 

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كلية الدراسات العليا

التاريـــخ: ٢٠١٠/١٢/٢٩

نموذج رقم (١٦)
إقرار والتزام بالمعايير الأخلاقية والأمانة العلمية وقوانين الجامعة الأردنية وأنظمتها وتعليماتها لطلبة الدكتوراه

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عنوان الأطروحة:

Study of the Etiological Factors of Vitamin B_{12} Deficiency in Asymptomatic Volunteers Aged 20-40 Years Visiting the Jordan University Hospital

أعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة بإعداد أطروحات الدكتوراه عندما قمت شخصيا" بإعداد أطروحتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الأطروحات العلمية. كما أنني أعلن بأن أطروحتي هذه غير منقولة أو مستلة من أطاريح أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة إعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بإلغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

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STUDY OF THE ETIOLOGICAL FACTORS OF VITAMIN B_{12} DEFICIENCY IN ASYMPTOMATIC VOLUNTEERS AGED 20-40 YEARS VISITING THE JORDAN UNIVERSITY HOSPITAL

By
Maysoun Subhi Qutob

Supervisor
Dr. Hamed Rabah Takruri, Prof.

Co-Supervisor
Dr. Farihan Fakhri Barghouti, Assoc. Prof.

This Dissertation was Submitted in Partial Fulfillment of the Requirements for the Doctor of Philosophy Degree in Nutrition and Food Technology

Faculty of Graduate Studies
University of Jordan

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COMMITTEE DECISION

This Dissertation (Study of the etiological factors of vitamin B_{12} deficiency in asymptomatic volunteers aged 20-40 years visiting the Jordan University Hospital) was Successfully Defended and Approved on December 9, 2010

Examination Committee	Signature
Dr. Hamed R. Takruri (Supervisor) Prof. of Human Nutrition	
Dr. Farihan F. Barghouti, (Co-Supervisor) Assoc. Prof. of Family Medicine	
Dr. Ahmad M. Faqih (Member) Prof. of Nutrition	
Dr. Yousif Y. Bilto (Member) Prof. of Hematology and Clinical Chemistry	
Dr. Reema F. Tayyem (Member) Assoc. Prof. of Human Nutrition (The Hashemite University)	

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Dedication

To the Soul of my Father
To my Mother and my Brothers

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List of Abbreviations

AI	Adequate Intake
ANOVA	Analysis of Variance
APCA	Anti-parietal cell antibody
BMI	Body mass index
BSTFA	*N,O*-bis[trimethylsilyl]trifluoroacetamide
CDC	Centers for Disease Control and Prevention
DOS	Department of Statistics
DV	Daily Value
ELISA	Enzyme Linked Immunosorbant Assay
EU/ml	ELISA Unit/ milliliter
fl	Femtoliter (10^{-15})
FPIA	Fluorescence Polarization Immunoassay
CI	Confidence interval
GI	Gastrointestinal
H_2SO_4	Sulfuric acid
Hcy	Homocysteine
Hgb	Hemoglobin
HH	Household
Holo TC	Holotranscobalamin
IF	Intrinsic factor
IgA	Immunoglobulin A
L	Liter
MCV	Mean cell volume (Mean RBC volume)
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
ml	Milliliter
MMA	Methylmalonic acid
MO	Microorganisms
MOH	Ministry of Health
MOA	Ministry of Agriculture
MPV	Mean platelet volume
MTBE	Methyl tert-butyrl Ether

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NaN_3	Sodium azide
NIH	National Institute of Health
nm	Nanometer
PBS	Phosphate buffered saline
PCV	Packed cell volume
pg	Picagram
RBC	Red blood cell
RDA	Recommended Dietary Allowances
RDW	Red blood cell distribution width
SEM	Standard Error of Mean
TC	Transcobalamin
tHcy	Total homocysteine
TMB	Tetramethylbenzidine
TMCS	Trimethylchlorosilane
U/ml	Unit/milliliter
WHO	World Health Organization
WBC	White blood cell

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STUDY OF THE ETIOLOGICAL FACTORS OF VITAMIN B_{12} DEFICIENCY IN ASYMPTOMATIC VOLUNTEERS AGED 20-40 YEARS VISITING THE JORDAN UNIVERSITY HOSPITAL

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ABSTRACT

A convenient study was conducted to evaluate vitamin B_{12} status among young healthy adults visiting the Family Medicine Clinic at Jordan University Hospital and to check for the true vitamin B_{12} deficiency, causes of deficiency and factors associated with it. One hundred sixty five subjects were recruited in the study (99 females and 66 males). The subjects were chosen to be healthy aged between 20-40 years. Participants were asked to fill a detailed questionnaire that covers social, educational, medical data on them as well as their dietary intake of the vitamin. Blood tests including CBC and blood film and serum vitamin B_{12} level were measured for all volunteers. For those with serum B_{12} ≤300 pg/ml, other blood tests were done. These tests included plasma methylmalonic acid (MMA), total homocysteine (tHcy), anti-parietal cell antibodies (APCA), *Helicobacter pylori* IgA and anti intrinsic factor (IF) antibodies.

According to our results, 69.1% had serum B_{12} ≤300 pg/ml; 27.3% had vitamin B_{12} deficiency according to standard B_{12} deficiency definition (≤200 pg/ml), and 30.9% had normal B_{12} levels (>300 pg/ml). Among those with B_{12} ≤300 pg/ml, 47.4% had confirmed deficiency, using MMA as an indicator. Vitamin B_{12} status was found to be positively correlated with age. The mean value of serum B_{12} was significantly lower (P=0.047) in the age group 20-29 years (258.78 ± 11.798) than those of age group 30-40 years (293.41 ± 12.191). However, there was no significant difference between the mean values of serum vitamin B_{12} between males (277.85 ± 13.814) and females (270.20 ± 11.093) (P=0.666). No significant associations were found between B_{12} status and BMI, educational level, household size, and family history of vitamin B_{12} deficiency. Mean MCV and homocysteine (Hcy) had inverse correlation with vitamin B_{12} status. The mean values of both MCV and Hcy were significantly higher (P<0.05) in the B_{12} group of ≤200 pg/ml compared to the B_{12} group of B_{12} of 201-300 pg/ml. Our results revealed that 18.52% had APCA, 22.2% having *H. pylori* IgA and 9.3% having anti-IF antibodies. The remaining B_{12} deficient volunteers had unknown cause of low B_{12} status.

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It is concluded that the percentage of true vitamin B_{12} deficiency (with high MMA ≥0.376 µmole/L) is high; (32.7%) in the study group, and that 47.4% of those with serum B_{12} ≤300 pg/ml had confirmed B_{12} deficiency. If we consider the fact that 44.9% of those with serum B_{12} between 201-300 pg/L have true vitamin B_{12} deficiency, it is confirmed that serum B_{12} level is not a specific test for vitamin B_{12} deficiency. Dietary vitamin B_{12} intake in the study sample is higher than RDA and therefore it does not seem to be the cause of B_{12} deficiency. The main cause of B_{12} deficiency in Jordan is not well established, and seems to be related to B_{12} absorption.

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Introduction

Vitamin B_{12} (cobalamin) is an essential nutrient that must be supplied by food (Bor *et al.*, 2006). The usual dietary sources of vitamin B_{12} are animal foods such as meat, dairy products, egg, and fish (Watanabe, 2007). The recommended dietary allowance (RDA) for vitamin B_{12} is 2.4 µg/d for adults (Bor *et al.*, 2006). Therefore, vitamin B_{12} deficiency is most likely to happen with reduced intake of foods of animal source (as in vegetarians) (McLean *et al.*, 2007; Hvas and Nexo, 2006; Dholakia *et al.*, 2005) and those with impaired absorption (Bor *et al.*, 2006).

Unlike other B complex vitamins, vitamin B_{12} is stored in the liver. The body stores around 2-5 mg of cobalamin, which is high amount of the vitamin relative to daily requirements, therefore its deficiency needs several years (2-5 years) before hematological and neurological manifestations appear (Snow, 1999). Vitamin B_{12} deficiency is a worldwide public health problem (Hvas and Nexo, 2006; Kaptan *et al.*, 2006). Prevalence of dietary vitamin B_{12} deficiency was reported to be high in India, Mexico, central and south America, and specific areas of Africa. However, in certain areas of Asia, dietary vitamin B_{12} deficiency is not prevalent except in vegetarians (Stabler and Allen, 2004). In Jordan, results show that there is a high prevalence of vitamin B_{12} deficiency (48.1%) as indicated by suboptimal serum vitamin B_{12} levels (Fora and Mohammad, 2005). This is confirmed by a review of MOH, WHO and MOA (2006) and Barghouti *et al.* (2009).

Vitamin B_{12} deficiency is described as serum cobalamin below 200 pg/ml (Carmel, 2008), although deficiency can be seen in persons with normal serum B_{12} levels (between 200-400 pg/ml) (Oh and Brown, 2003). In addition to low dietary intake, many factors can lead to vitamin B_{12} deficiency. These include: pernicious

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anemia, impaired absorption, certain gastrointestinal (GI) disorders (i.e., Celiac disease, Crohns disease, atrophic gastritis, pancreatic insufficiency) (Hvas and Nexo, 2006) and some medications (i.e., proton pump inhibitors, H_2 receptor blockers and metformin) (NIH, 2010). These factors make vegetarians and elderly to be the most vulnerable to develop vitamin B_{12} deficiency. Recently, it was found that the bacteria *Helicobacter pylori* to be another cause of cobalamin deficiency (Kaptan *et al*., 2000).

Vitamin B_{12} level is usually diagnosed by measuring serum vitamin B_{12} levels. However this test has certain limitations. Carmel (2008) mentioned that 22-30% of persons with serum B_{12} levels <200-250 pg/ml are falsely low by both metabolic and clinical criteria. Measuring serum vitamin B_{12} along with the measurements of metabolites (such as methylmalonic acid and homocysteine) will be more sensitive especially when clinical picture is equivocal (Carmel, 2008; Oh and Brown, 2003). Low serum vitamin B_{12} along with normal values of serum homocysteine and methylmalonic acid will indicate that there is no vitamin B_{12} deficiency These metabolites levels can be early markers for tissue vitamin B_{12} deficiency even before hematologic manifestations occur (Oh and Brown, 2003).

By considering the high prevalence of vitamin B_{12} deficiency in Jordan and the sensitivity of serum B_{12} test as a diagnostic tool for the deficiency of this vitamin, this study was conducted to achieve the following objectives:

(1) determine the true vitamin B_{12} deficiency by measuring serum vitamin B_{12} and MMA in volunteers.

(2) evaluate the eating habits of the subjects enrolled in our study and check for their association with vitamin B_{12} deficiency.

(3) assess the probable causes of low vitamin B_{12} levels in the sample group using different diagnostic tests (e.g., CBC + blood film, serum vitamin B_{12},

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methylmalonic acid, homocysteine, anti-parietal cell antibody and *Helicobacter pylori* IgA tests).

(4) check for the association between vitamin B_{12} status and body mass index (BMI), educational level, household size, family history of vitamin B_{12} deficiency and previous gastrointestinal surgeries (GI) surgeries.

(5) check for possible symptoms of vitamin B_{12} deficiency and their correlation with the serum vitamin B_{12} values.

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Literature review

1. Vitamin B_{12}

1.1. Historical Background

The history of vitamin B_{12} goes back to the year 1822 when pernicious anemia was first described by J.S. Combe. This anemia was invariably fatal until the year 1926 when Minot and Murphy revealed the etiology of pernicious anemia. They found that consuming raw liver was highly effective in treating this disease. Lately in 1928, W.B. Castle postulated that two factors are involved in the control of pernicious anemia, an "extrinsic factor" that comes from food (liver) and an "intrinsic factor" that is found in the gastric juice. In 1948, Folkers at Merck isolated the anti-pernicious anemia (extrinsic) factor in crystalline form. In 1955, D. Hodgkin's finally elucidated the complex chemical structure of vitamin B_{12} using X-ray crystallography. Later in 1970, *de novo* synthesis of vitamin B_{12} was achieved by Woodward and Eshenmoser (Combs, 1998).

1.2. Chemistry of Vitamin B_{12}

Vitamin B_{12} is a water-soluble organo-metallic compound. It is a complex molecule that contains central cobalt atom that is chelated in corrin ring system (Basu and Dickerson, 1996). It is of six coordinations by which four of the coordinations are provided by the corrin ring nitrogens, a fifth by a dimethylbenzimidazole group, and the sixth by one of different ligands being: a cyano group (-CN) (cyanocobalamin), a hydroxyl group (-OH) (hydroxycobalamin), a methyl group ($-CH_3$) (methylcobalamin) or a 5'-deoxyadenosyl group (5'deoxyadenosylcobalamin) (Reidel, 2007; Devlin, 1998 and Basu and Dickerson, 1996) (Figure 1).

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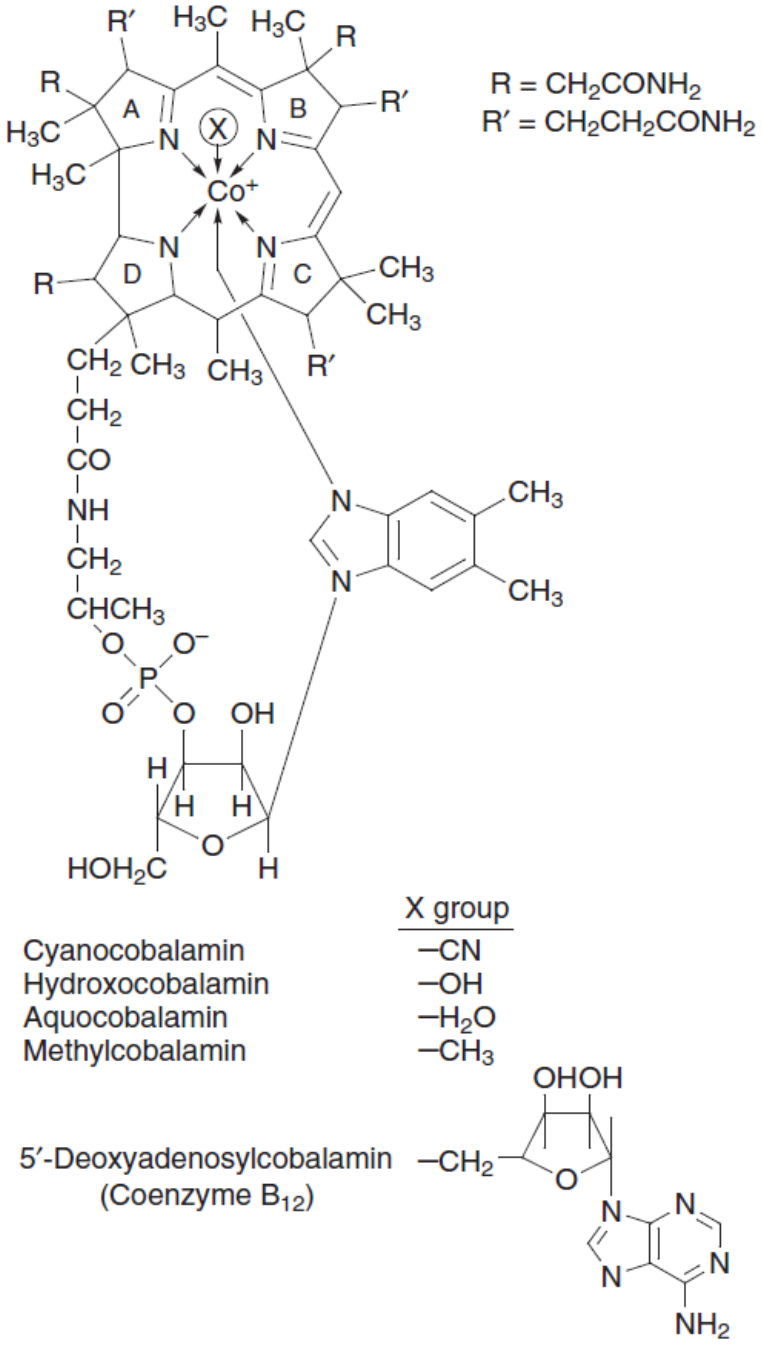

Figure 1: Structure of vitamin B_{12} compounds (Reference: Ball, 2004).

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1.3. Recommended Intake

Vitamin B_{12} is an essential nutrient that must be obtained from foods of animal origin (meat, chicken, fish and eggs) or dairy products (Bor *et al.*, 2006). The Recommended Dietary Allowance (RDA) is 2.4 µg/d for adults (age 14+ years) (Zempleni *et al.*, 2007; Bor *et al.*, 2006; IOM, 1998). No RDA was established for infants <1 year of age, instead adequate intake (AI) has been established (Zempleni *et al.*, 2007). RDAs for other age groups are listed in Table 1.

The average Western diet contains 5-15 µg/d of vitamin B_{12}, which is more than sufficient to meet the recommendations (Snow, 1999) (Several food sources of vitamin B_{12} are listed in Table 2). Therefore, a reduced intake of vitamin B_{12} from food, as in vegetarians and patients with absorptive problems, will induce a negative balance and ultimately lead to severe deficiency when the tissue stores of vitamin B_{12} are depleted (Bor *et al.*, 2006).

Table 1: Recommended dietary allowances (RDAs) for vitamin B_{12}.

Age	Male	Female	Pregnancy	Lactation
0-6 months*	0.4 µg	0.4 µg		
7-12 months*	0.5 µg	0.5 µg		
1-3 years	0.9 µg	0.9 µg		
4-8 years	1.2 µg	1.2 µg		
9-13 years	1.8 µg	1.8 µg		
14+ years	2.4 µg	2.4 µg	2.6 µg	2.8 µg

* Adequate intake (AI) (Reference: IOM, 1998).

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Table 2: Selected food sources of vitamin B_{12}.

Food	µg per serving	Percent DV *
Liver, beef, braised, 1 slice (68 g)	48.0	800
Breakfast cereals, fortified with 100% of the DV for vitamin B_{12}, 1 serving	6.0	100
Salmon, sockeye, cooked, 3 ounces (85 g)	4.9	80
Beef, top sirloin, broiled, 3 ounces (85 g)	2.4	40
Cheeseburger, double patty and bun, 1 sandwich (201 g)	2.05	30
Yogurt, plain, 1 cup (245 g)	1.4	25
Tuna (in water), white, 3 ounces (85 g)	1.0	15
Cheese, Swiss, 1 ounce (28.25 g)	0.95	15
Egg, large, 1 whole	0.6	10
Chicken, roasted, ½ breast (86 g)	0.3	6

*DV= Daily value: DVs were developed by the U.S. Food and Drug Administration (FDA) to help consumers determine the level of various nutrients in a standard serving of food in relation to their approximate requirement for it. The DV for vitamin B_{12} is 6.0 µg. foods providing 20% or more of the DV are considered to be high sources of a nutrient, but foods providing lower percentages of the DV also contribute to a healthful diet (References: NIH, 2010; USDA National Database).

1.4. Digestion and Absorption of Vitamin B_{12}

Vitamin B_{12} absorption occurs by both active and passive mechanisms. Under normal conditions, very small amounts of vitamin B_{12} are absorbed passively by diffusion across the intestinal mucosa. This accounts for less then 1% of large oral doses.

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The major way of absorption is by active mechanism involving specific binding proteins in the digestive tract (Zempleni *et al*., 2007; Bender, 2003).

Vitamin B_{12} is released from dietary proteins by enzymes in gastric juice, aided by the low pH of the stomach. Upon their release, vitamin B_{12} binds to salivary and gastric derived cobalophilin (also known as R-proteins) (Riedel, 2007). After entering the duodenum, R-protein is hydrolyzed by pancreatic trypsin and liberated vitamin B_{12} binds to free intrinsic factors (IF) (Zempleni *et al*., 2007; Ball, 2006; Bender, 2003). IF is an alkali-stable glycoprotein secreted by parietal cells in the stomach and is necessary for vitamin B_{12} absorption in the ileum (Semba, 2007). Binding vitamin B_{12} to IF is favored by the alkaline environment in the jejunum (Zempleni *et al*., 2007; Ball, 2004). Absence of IF or its dysfunction may cause megaloblastic anemia, the classical pernicious anemia and neurological disturbances. If these disorders commence in early childhood, they are accompanied by developmental delay and growth retardation (Riedel, 2007).

By binding the vitamin, IF undergoes a conformational change, producing a more compact form that resists proteolysis (Bender, 2003; Beck *et al.*, 2001). Vitamin B_{12} is absorbed in the distal part of the ileum by receptor-mediated endocytosis, where the IF-vitamin B_{12} complex is internalized into the enterocyte by a Ca^{+2}-dependent process and a pH above 5.5 (Riedel, 2007; Ball, 2004; Bender, 2003). Upon entering the enterocyte, vitamin B_{12} is released by lysosomal proteolysis of the IF (Ball, 2006; Bender, 2003). Vitamin B_{12} is then released in the circulation where it attaches to two binding proteins, transcobalamin II (TC II) and haptocorrin (Mørkbak *et al.*, 2006). Around 70-80% of the total circulating B_{12} binds to haptocorrin, the reminder (20-30%) binds to transcobalamin II, a vitamin B_{12} binding protein synthesized in the enterocytes (Zenpleni *et al*., 2007). Figure 2 shows the steps of absorption of vitamin B_{12}.

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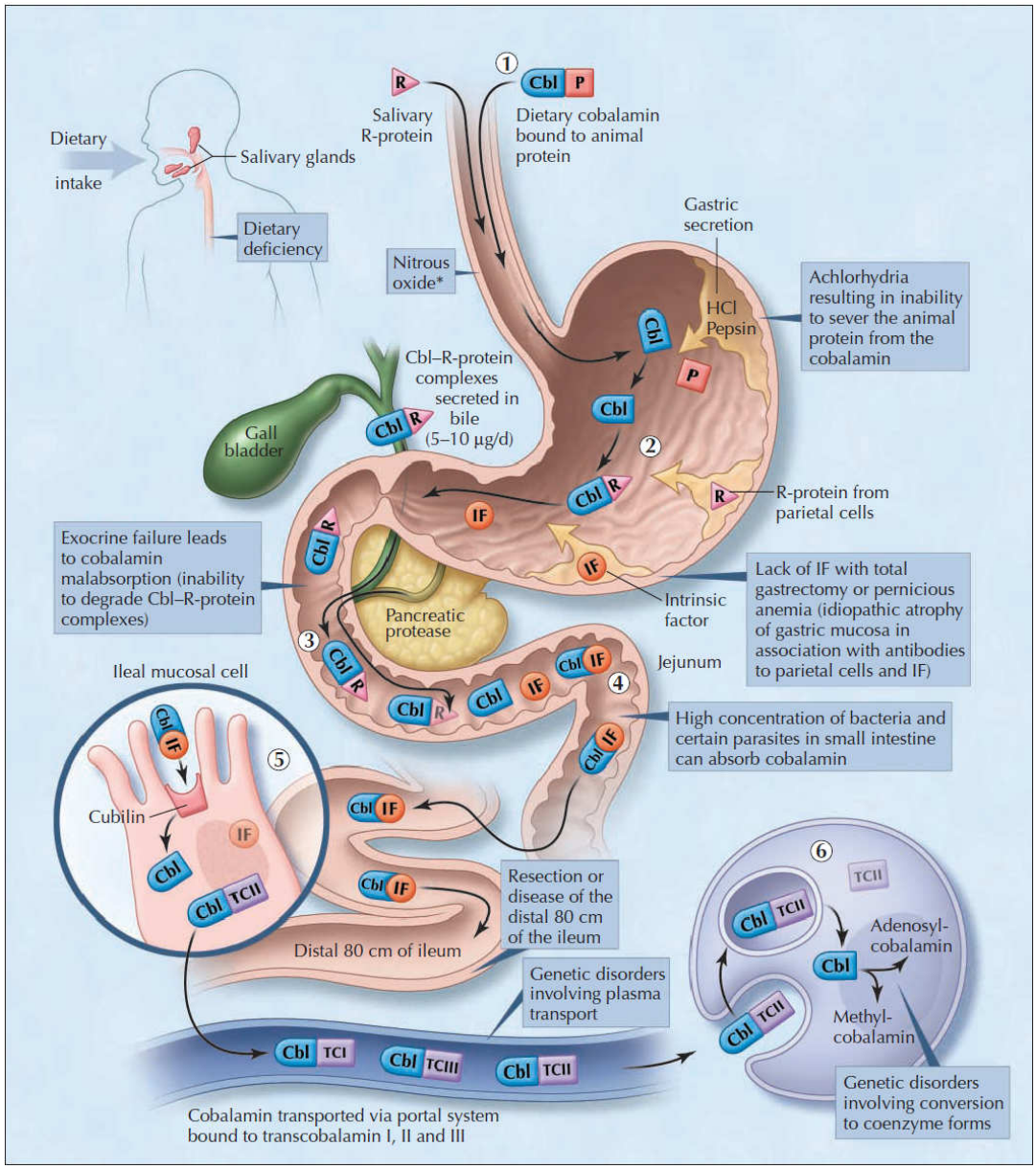

Figure 2: Cobalamin absorption and possible causes of cobalamin deficiency (Reference: Andrès *et al.*, 2004).

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Transcobalamin II is the metabolically important plasma protein. All cells have surface receptors for this protein (Bender, 2003). On the contrary, the role of haptocorrin in vitamin B_{12} metabolism and transport is still unclear (Mørkbak *et al.*, 2006); however it does prevent circulating B_{12} from being filtered by the kidneys (Ball, 2004). Haptocorrin is characterized by its ability to bind both B_{12} and B_{12} analogs (Mørkbak *et al.*, 2006). Uptake of haptocorrins into liver may represent a mechanism by which B_{12} analogs are removed from the circulation and excreted in the bile. These compounds are not reabsorbed by IF-dependent mechanism and thus are excreted in the stool (Zempleni *et al*., 2007).

Transcobalamin II is then taken up by cells in the body by receptor-mediated endocytosis (Semba, 2007; Ball, 2004). Receptors for transcobalamin II are ubiquitously present in tissues (Zempleni *et al.*, 2007). After endocytosis, TC II is degraded in lysosomes releasing B_{12} in the cytoplasm in the form of hydroxocobalamin, which is then either converted directly to methylcobalamin in the cytoplasm, or eventually to adenosylcobalamin in the mitochondria (Ball, 2004).

1.5. The Metabolism and Function of Vitamin B_{12}

In humans, vitamin B_{12} participates as a cofactor in two enzymes, methylmalonyl CoA mutase and methionine synthase. Methylmalonyl CoA mutase requires adenosylcobalamin to catalyze the conversion of methylmalonyl CoA to succinyl CoA. This reaction occurs in the mitochondria (Semba, 2007; Zempleni *et al.*, 2007) and is involved in the degradation of amino acids (valine, isoleucine, methionine and threonine), and odd-numbered fatty acids (Semba, 2007).

Methionine synthase requires methylcobalamin in the folate-dependent methylation of sulfur amino acids homocysteine to form methionine. This reaction

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occurs in the cytosol (Semba, 2007; Zempleni *et al.*, 2007; Ball, 2004). This makes vitamin B_{12} important in nucleic acid metabolism by converting methyl-tetrahydrofolate to tetrahydrofolate. The later is involved in the synthesis of deoxythymidine monophosphate (dTMP) needed for DNA synthesis from deoxyuridine moncophosphate (dUMP) (Semba, 2007; Ball, 2004).

Another enzyme that is mentioned to be vitamin B_{12}-dependent is leucine aminomutase. This enzyme requires adenosylcobalamin to catalyze the isomerization of leucine and β-leucine. Patients with vitamin B_{12} deficiency show an elevation of plasma β-leucine (Bender, 2003).

Vitamin B_{12} is thus an important cofactor in: the maintenance of normal DNA synthesis and the regeneration of methionine needed for maintaining protein synthesis and methylation capacity. It is also needed for the prevention of homocysteine accumulation, an amino acid metabolite that has role in vascular damage. Homocysteine accumulation was further found to cause thrombosis and other degenerative diseases including coronary artery disease, stroke, Alzheimer's disease and osteoporosis (Zempleni *et al.*, 2007).

Vitamin B_{12} has a crucial role in erythropoiesis. Erythroblasts require both folate and vitamin B_{12} for proliferation during their differentiation. Deficiency in one of them will inhibit purine and thymidylate synthesis, impairs DNA synthesis and causes erythroblast apoptosis, resulting in anemia from ineffective erythropoiesis (Koury and Ponka, 2004).

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2. Vitamin B_{12} Deficiency

2.1. Definition of Vitamin B_{12} Deficiency

During the last 10 years, literature has provided several definitions of vitamin B_{12} (cobalamin) deficiency, depending mainly on the population studied and on the particular assay kits (Dali-Youcef and Andrès, 2009; Fernández-Bañares *et al.*, 2009; Klee, 2000; Snow, 1999). It is usually defined in the terms of the serum values of cobalamin and of homocysteine and methylmalonic acid (Andrès *et al.*, 2004). Accordingly, vitamin B_{12} deficiency definitions are: (1) Serum cobalamin levels <150 pmole/L (<200 pg/ml) with clinical features and/or hematological anomalies related to cobalamin deficiency (Oh and Brown, 2003); (2) Serum cobalamin levels <150 pmole/L on two separate occasions; (3) Serum cobalamin levels <150 pmole/L and total serum homocysteine levels >13 μmole/L or methylmalonic acid levels >0.4 μmole/L (Andrès *et al*., 2004); (4) Low serum holotranscobalamin levels <35 pmole/L (Dali-Youcef and Andrès, 2009; Fernández-Bañares *et al.*, 2009).

Till now, there are no generally accepted guidelines for the definition, diagnosis, treatment and follow-up of vitamin B_{12} deficiency. Total serum vitamin B_{12} may not reliably indicate vitamin B_{12} status (Volkov *et al.*, 2006). To increase the sensitivity and specificity of the diagnosis, measurement of the metabolites, methylmalonic acid and homocysteine have been used (Oh and Brown, 2003).

2.2. Stages of Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency can be divided into five stages. These stages include: I: normality; II: negative vitamin B_{12} balance; III: vitamin B_{12} depletion with possible clinical signs and symptoms (reversible neuropsychiatric findings); IV: vitamin B_{12} deficient erythropoiesis with possible clinical signs and symptoms (potentially

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reversible neuropsychologic symptoms); and V: vitamin B_{12} deficiency anemia with probable clinical signs and symptoms (irreversible neuropsychologic symptoms) (Swain, 1995).

2.3. Causes of Vitamin B_{12} Deficiency

Once vitamin B_{12} deficiency is confirmed, search for the etiological factors should be started. Three classes of vitamin B_{12} deficiency are described: nutritional deficiency, malabsorption syndromes, and other gastrointestinal causes (Oh and Brown, 2003).

2.3.1. Nutritional Deficiency: Dietary sources of vitamin B_{12} are primarily meats and dairy products (Oh and Brown, 2003). It is naturally produced by vitamin B_{12} producing microorganisms, thus human must obtain their vitamin B_{12} through diet. Vegetarian diet can be classified as either lactovegetarian, ovovegetarian, lactoovovegetarian and vegan if they include dairy products, eggs, both dairy products and eggs or no animal products at all, respectively (Antony, 2003). Vegans and to a lesser degree lactoovovegetarians and lactovegetarians have biochemical evidence of cobalamin deficiency based on increased blood total MMA and Hcy and low holotranscobalamin II (Herrmann *et al*., 2003).

Nonvegetarians obtain most of their vitamin B_{12} through eating meat. In the developing world, nonvegetarians obtain only marginal amounts of vitamin B_{12} compared to western countries, because meat is expensive in relation to the low income (Antony, 2003). A typical western diet contributes 3-30 µg of vitamin B_{12} per day towards the recommended dietary allowance of 2.4 µg/day for adults (Fernández-Baňares *et al*., 2009). Nonvegetarians in the developing countries have a vitamin B_{12} status that is only marginally better than that of lactoovovegetarians and only daily meat

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eater have vitamin B_{12} status similar to that of nonvegetarians in the west (Antony, 2003).

Nonetheless, vitamin B_{12} deficiency is not common in vegetarians or vegans. This can be due to: they may consume some contaminated food that contain small amount of vitamin B_{12}; their bodies absorb vitamin B_{12} secreted into the bile with high efficiency; their small intestine harbors microflora that may synthesize significant amounts of absorbable vitamin B_{12}.

2.3.2. **Malabsorption Syndromes:** Vitamin B_{12} deficiency caused by dietary deficiency or malabsorption is rare (Dali-Youcef and Andrès, 2009). The classic disorder of malabsorption is pernicious anemia (Biermer's disease), an autoimmune disease that affects the gastric parietal cells and leads to reduction in IF production and secretion (Oh and Brown, 2003). Gastric secretions are neutral to slightly acidic (Fernández-Bañares *et al.*, 2009; Andrès *et al.*, 2004).

2.3.2.1. Food-Cobalamin Malabsorption: Food-bound malabsorption (or food-cobalamin malabsorption) is characterized by the inability to cleave and release vitamin B_{12} bound to food or intestinal transport proteins (Fernández-Bañares *et al.*, 2009; Andrès *et al.*, 2004). This syndrome is defined by cobalamin deficiency in the presence of sufficient food-cobalamin intake and a negative schilling test. This may happen in case of any interference with gastric acid production (hypochlorhydria) where "unbound" cobalamin absorption remains normal through intrinsic factor or passive diffusion mechanism (Fernández-Bañares *et al.*, 2009). Atrophic gastritis is the major cause especially in the elderly. Many other factors that can cause food-bound malabsorption by

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interfering with gastric acid production include subtotal gastrectomy, prolonged use of histamine H_2-receptor blockers and proton pump inhibitors for ulcer disease and long term use of omeprazole (Oh and Brown, 2003). Others include intestinal microbial proliferation that compete with vitamin B_{12} (as in case of the rare Whipple's disease and blind loop) (Andrès *et al*., 2004), chronic alcoholism, partial pancreatic exocrine failure and Sjŏgren syndrome (Fernández-Baňares *et al.*, 2009).

2.3.2.2. Gastrointestinal (GI) Disorders: GI disorders can lead to vitamin B_{12} deficiency by causing food-bound malabsorption due to hypochlorhydria or impaired IF production. They include:

a. GI Surgeries: Gastrectomy is the preferred operation for palliation of gastric cancer. They cause food-bound malabsorption due to hypochlorhydria and impaired IF production. This deficiency will appear 2-4 years or longer after surgery, when the vitamin stores are exhausted (Fernández-Baňares *et al.*, 2009).

b. **Intestinal Surgeries:** Bariatric surgeries are considered an effective treatment of obesity. They include gastric bypass (where distal 24 cm of ileum is removed), laparoscopic adjustable gastric banding, vertical banded gastroplasty, biliopancreatic diversion and biliopancreatic diversion with duodenal switch. Vitamin B_{12} deficiency may appear 1-9 years after gastric surgery with prevalence between 12-33%. Gastric bypass patients have decreased gastric acid production and their duodenum is excluded from digestive continuity (Fernández-Baňares *et al.*, 2009).

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c. **Other GI Disorders:** Other GI disorders can cause vitamin B_{12} deficiency mainly by causing food- bound malabsorption. These include: Crohn's disease: affect ileal mucosa which alters the brush border structure containing the receptors for IF. Atrophic gastritis where patients may have antibodies to parietal cells or antibodies to IF factor, thus affect IF secretion, recurrent peptic ulcer, diarrhea and dyspepsia (Oh and Brown, 2003).

H. pylori can be a probable causative agent in the development of adult vitamin B_{12} deficiency **(**Kaptan *et al*., 2000). These authors detected *H. pylori* in 56% of the patients with vitamin B_{12} deficiency that were recruited in their study, and they found that eradication of this bacteria was improved anemia and serum vitamin B_{12} in 40% of the *H. pylori* infected patients.

d. **Some Medications:** Histamine H_2-receptor blockers and proton pump inhibitors for ulcer disease, anti-acid drugs, psoriasis drugs, colchicine, p-aminosalicylic acid and alcohol. These agents can cause vitamin B_{12} deficiency either by: causing food-bound malabsorption; affecting the intracellular transport of vitamin B_{12}, inhibiting IF secretion; or interfering with the formation of IF-vitamin B_{12} complex (Basu and Dickerson, 1996). Table 3 shows some important vitamin B_{12} /drug interactions.

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Table 3: Important vitamin B_{12}/drug interactions.

Drug	Potential interaction
Proton pump inhibitors (PPIs) used for treating gastroesophageal reflux disease (GERD) and peptic ulcer disease (e.g., Omeprazole and Lansoprazole)	Interfere with vitamin B_{12} absorption from food by slowing the release of HCl into the stomach.
H_2 receptor antagonist used to treat peptic ulcer disease (e.g., Zantac)	Interfere with vitamin B_{12} absorption from food by slowing the release of HCl into the stomach.
Metformin used to treat diabetes	Interfere with calcium metabolism which may indirectly reduce vitamin B_{12} absorption (vitamin B_{12} absorption requires calcium)

(Reference: NIH, 2010).

2.3.2.3. Pernicious Anemia: Pernicious anemia, or Biermer's syndrome, is a classic cause of vitamin B_{12} deficiency (Andrès *et al.*, 2004). It is an autoimmune disease characterized by the destruction of the gastric mucosa, by a primarily cell- mediated process. Gastric secretions in this case, are neutral to slightly acidic even in the presence of gastrin (which normally increases acidity) and contain little or no IF. Two antibodies (anti-IF antibodies and anti- gastric parietal cell antibodies) are present, particularly in plasma and gastric secretions (Fernández-Baňares *et al.*, 2009; Andrès *et al.*, 2004). Pernicious anemia is also associated with many autoimmune disorders, including vitiligo, dysthyroidia, Addison's disease and Sjögren syndrome. It is also associated with an increased

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frequency of gastric neoplasms, adenocarinomas, lymphomas, carcinoid tumors (Andrès *et al*., 2004).

2.3.2.4. Hereditary Diseases: Vitamin B_{12} deficiency can occur in various genetic defects involving deficiency in cubulin (cobalamin-IF complex receptor) (Imerslund syndrome) (Andrès *et al*., 2004), or deletion or defects of methylmalonyl CoA mutase, transcobalamin II and enzymes in the pathway of cobalamin adenosylation (Beck *et al*., 2001). Transcobalamin deficiency and errors in the biosynthesis of cobalamin coenzymes result in severe illness shortly after birth (Gräsbeck, 2006) and thus do not involve elderly (Andrès *et al*., 2004). Other rare genetic disease that causes vitamin B_{12} deficiency is Addisonian pernicious anemia where patients have one or two types of serum IF antibodies (type 1: blocking antibody; type 2: binding antibody) (Basu and Dickerson, 1996).

2.3.2.5. Other Causes: Other factors that were mentioned to cause vitamin B_{12} deficiency include: Zollinger-Ellison syndrome (gastrinoma causing peptic ulcer and diarrhea) (Oh and Brown, 2003), parasites (i.e., fish tapeworm *Diphyllobothrium latum*, which is known to cause vitamin B_{12} deficiency in the children) (Gräsbeck, 2006), and geographical areas (i.e., in Northern China, prevalence of low B_{12} level is 39%, whereas in Southern China, it is 11%) (Hao *et al.*, 2007).

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2.4. People at Risk

By considering the causes of vitamin B_{12} deficiency, any person having one or more of these conditions is at risk of developing vitamin B_{12} deficiency. Elderly people are at risk of vitamin B_{12} deficiency, either because of low intake or malabsorption from atrophic gastritis. Recently, age was reported to be an independent risk factor, irrespective of gastric atrophy (Gümürdülü *et al.*, 2003). Gümürdülü and his colleagues (2003) found that patients with vitamin B_{12} deficiency (<200 pg/ml) (46 ± 12 years) were older than those with normal serum B_{12} levels (>200 pg/ml) (42 ± 12 years).

Obese children and adolescents are another group at risk of developing vitamin B_{12} deficiency. Pinhas-Hamiel *et al.* (2006) reported that obesity was associated with a 4.3 fold risk for low serum B_{12} and an increase of 1 body mass index standard deviation score resulting in 1.24 fold increased risk of vitamin B_{12} deficiency.

Pregnant women are at risk of developing vitamin B_{12} deficiency due to increase requirements especially when fetal demands exceed mother's dietary intake of the vitamin (Beck *et al.*, 2001). Cobalamin deficiency was also observed in babies of nursing mothers that are vegans or those mothers who abuse laughing gas (nitrous oxide) (Gräsbeck, 2006).

Other people who are reported to be at risk of having vitamin B_{12} deficiency are: HIV patients (Nexø *et al.*, 1994), patients with autoimmune disorders (type I diabetes mellitus and thyroid disorders) (Nilsson-Ehie, 1998) and those undergoing nitrous oxide anaesthesia (Carmel, 2000).

2.5. Clinical Manifestations of Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency is characterized by hematopoietic, gastrointestinal and neurological alterations (Semba, 2007). These clinical manifestations vary in severity

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ranging from mild conditions such as fatigue, common sensory neuropathy, atrophic glossitis (Hunter's glossitis), megaloplastic anemia (Lee and Tang, 2004) and impaired bowel function (i.e., constipation) (Lee and Tang, 2004; Dali-Youcef and Andrès, 2009), to severe disorders such as combined sclerosis of the spinal cord (Semba, 2007; Dali-Youcef and Andrès, 2009), hemolytic anemia and even pancytopenia (Dali-Youcef and Andrès, 2009). The neurological syndrome of vitamin B_{12} deficiency may begin with paresthesias in the hands and the feet, loss of vibratory and proprioception sensation, burning sensation in the extremities, and later there may be spastic ataxia (Semba, 2007). Dementia and subtle neuropsychiatric changes may be present (Lee and Tang, 2004).

2.6. Diagnosis of Vitamin B_{12} Deficiency

2.6.1. Serum Vitamin B_{12}: Vitamin B_{12} deficiency is traditionally diagnosed by measuring serum vitamin B_{12} level (usually below 200 pg/ml), along with the clinical evidence of the disease (Oh and Brown, 2003). This test comprises the total amount of circulating cobalamin, either bound to transcobalamin or haptocorrin (Riedel, 2007). Serum vitamin B_{12} test was widely used as the standard screening test for cobalamin deficiency (Green, 2005) since 1950 (Hvas and Nexo, 2006), and is still the standard investigation tools for vitamin B_{12} deficiency worldwide despite its limited specificity (Hvas and Nexo, 2006; Stabler *et al*., 1990).

There are certain limitations concerning measuring serum vitamin B_{12} level (Klee, 2000). The diagnostic efficiency of this test is too low. This can be partially explained by the fact that the major fraction of cobalamin is bound to the protein haptocorrin, which makes it unavailable for uptake by cells (Hølleland *et al*., 1999). Approximately 20% of cobalamin is bound to transcobalamin II forming a complex that can be utilized by peripheral tissue

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through the receptor mediated uptake (Klee, 2000). Cobalamin can also be influenced by changes in the binding protein concentrations (Klee, 2000; Hølleland *et al.*, 1999).

Also, serum total cobalamin is a poor indicator of bioavailable cobalamin because falsely increased values can be seen in myloproliferative disorder. Whereas false low values can be seen with folate deficiency, pregnancy (5%), myelomatosis and transcobalamin deficiency (Hølleland *et al*., 1999). False low values were also seen in 10% of patients after partial gastrectomy and 30% of patients with megaloblastic anemia due to folate deficiency, in some patients with iron deficiency with simple severe atrophic gastritis and in some healthy subjects (Chanarin, 1987).

2.6.2. Serum/Plasma Methylmalonic Acid (MMA) and Total Homocysteine (tHcy): Determination of serum cobalamin as a sole test of vitamin B_{12} deficiency has certain limitations, since it may miss up to one half of patients with actual tissue B_{12} deficiency (Oh and Brown, 2003; Bolann *et al.*, 2000). Moreover, total serum vitamin B_{12} is a late, relatively insensitive and unspecific biomarker of deficiency (Herrmann and Obeid, 2008). Imperfection of using this classical diagnostic tool has lead to development of more reliable tests of functional cobalamin status, including plasma total homocysteine and serum methylmalonic acid (Bolann *et al*., 2000).

Measurements of these metabolites, methylmalonic acid and homocysteine, along with serum vitamin B_{12}, are considered more sensitive indicator than measuring serum vitamin B_{12} levels alone (Hvas and Nexo, 2006;

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Kräutler, 2005). They can be early markers for tissue vitamin B_{12} deficiency, even before hematologic manifestations occur (Oh and Brown, 2003).

MMA is a functional vitamin B_{12} marker that will increase when vitamin B_{12} status is depleted (Herrmann and Obeid, 2008). Plasma total homocysteine (tHcy) is mentioned to be a sensitive marker of folate and vitamin B_{12} status and an increase in its level occurs long before classic deficiency of folate and vitamin B_{12} becomes evident (Fakhrazadeh *et al*., 2006). Thus, abnormal MMA or tHcy levels suggest a latent or overt tissue deficiency of vitamin B_{12} or folate (Bjőrkegren and Svärdsudd, 2001; Nexø *et al*., 1994).

However, by considering the reactions that use vitamin B_{12}, an elevated MMA level is considered more specific for vitamin B_{12} deficiency than an elevated tHcy level (Oh and Brown, 2003).

Although these tests are considered more sensitive to vitamin B_{12} deficiency, they have certain limitations. The major limitation of tHcy is that it also increases in patients with folate deficiency (Hvas and Nexo, 2006; Kräutler, 2005; Snow, 1999), vitamin B_6 deficiency, renal failure, proliferative disorders, in response to certain drugs and in some inborn errors of metabolism (Bolann *et al*., 2000), hyperhomocysteinemia, and hypothyroidism (Carmel *et al*., 2003). On the other hand, MMA increases in several cases including renal failure (Hvas and Nexo, 2006; Kräutler, 2005; Oh and Brown, 2003; Snow, 1999), dehydration, inherited methylmalonic aciduria (Bolann *et al*., 2000). Other limitations for using serum MMA test is the complexity of the assay and the high cost. By considering these limitations, there has been a continuous search for new biochemical markers (Hvas and Nexo, 2006).

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2.6.3. Mean Cell Volume (MCV): Mean cell volume was reported to be a good indicator of vitamin B_{12} deficiency (Galloway and Hamilton, 2007). It was mentioned that as MCV increases to more than 100 fl, the probability of vitamin B_{12} and folate deficiency increases (Galloway and Hamilton, 2007; Snow, 1999), although values of 100-110 fl are more likely to be associated with other causes of macrocytosis, such as alcohol abuse, liver disease and hypothyroidism (Galloway and Hamilton, 2007).

2.6.4. Holotranscobalamin: Holotranscobalamin (holoTC) is vitamin B_{12} associated with the transport protein transcoblamin and it represents the functionally important fraction of plasma vitamin B_{12} (Miller *et al.*, 2006). Testing holoTC measures the amount of cobalamin available for the cells (Hvas and Nexo, 2006). By comparing the sensitivity of holoTC, MMA and total serum $B_{12,}$ Herrmann *et al.* (2003) found that holoTC is the most sensitive marker for vitamin B_{12} deficiency followed by MMA, and that both holoTC and MMA provide a better index of vitamin B_{12} status than measurement of total serum B_{12}. HoloTC is considered as an early marker of vitamin B_{12} deficiency (Hvas and Nexo, 2006), and is claimed to be more sensitive than methylmalonic acid (Devalia, 2006).

2.6.5. Anti-parietal Cell Antibody and Anti-Intrinsic Factor Antibody: Pernicious anemia is a disorder characterized by megaloblastic hemopoiesis and/or a neuropathy due to vitamin B_{12} deficiency resulting from severe atrophic gastritis (Chanarin, 1987). A variety of autoantibodies can be found in serum of patients with pernicious anemia. These include antibodies to gastric parietal cells and to intrinsic factor (IF). Anti-parietal cell antibody can be found in 85% of

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patients affected with autoimmune gastritis, often found in patients with autoimmune endocrinopathy and are also present in 3-10% of healthy persons. This makes it a nonspecific test. On the other hand, anti-IF antibody is relatively insensitive (only 50% sensitive) (Carmel *et al.*, 2003; Oh and Brown, 2003; Bolann *et al.*, 2000). It is an autoantibody that is detectable in about 50% of the patients with pernicious anemia (Snow, 1999; Chanarin, 1987). However, it is highly specific (near 100% specificity) (Carmel *et al*., 2003). It is rarely found in healthy persons (Bolann *et al.*, 2000; Snow, 1999; Chanarin, 1987).

2.6.6. Schilling Test: The Schilling test has been the gold standard method for detecting vitamin B_{12} malabsorption (Zempleni *et al.*, 2007). This test is based on the fact that free cobalamin does not occur in plasma unless all binding proteins are saturated. Once this is achieved, the free cobalamin is then filtered through the glomerulus and excreted in the urine (Ball, 2004). This test, which measures 24- hour urine excretion of radiolabeled B_{12} given orally, distinguishes pernicious anemia from bacterial overgrowth and other absorption problems (Lee and Tang, 2004). Specifically, Schilling test results were once used to determine whether a patient required parenteral or oral vitamin B_{12} supplementation (Oh and Brown, 2003). The test, which consists of three steps, is performed as shown in Table 4 and the interpretation of results is shown in Table 5

Schilling test is no longer readily available, due to increasing difficulties in obtaining labeled vitamin B_{12} and IF (Zempleni *et al.*, 2007; Hvas and Nexo, 2006), complexity and lengthy of the procedure (up to 1 week to carry out all three steps), cost (Zempleni *et al.*, 2007).

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Although Schilling test provides evidence of vitamin B_{12} malabsorption, it does not provide evidence of deficiency. For example, vegetarians show normal results of Schilling test although they show low serum B_{12} levels, and patients with pernicious anemia will give abnormal result, even though their serum B_{12} levels are normal from treatment with vitamin (Ball, 2004).

3. Prevalence of Vitamin B_{12} Deficiency

3.1. Vitamin B_{12} Deficiency Worldwide

Vitamin B_{12} deficiency is a worldwide public health problem (Hvas and Nexo, 2006; Kaptan *et al*., 2006). Prevalence of dietary vitamin B_{12} deficiency was reported to be high in India, Mexico, central and south America, and specific areas of Africa. However, in certain areas of Asia, dietary vitamin B_{12} deficiency is not prevalent except in vegetarians (Stabler and Allen, 2004).

In a study done on 1214 participants on the Cardiovascular Risk Factors Survey in Tehran, Iran, including 428 men and 786 women, Fakhrazadeh *et al.* (2006) found that the age-adjusted prevalence of low serum vitamin B_{12} level was 26.32% in men and 27.2% in women.

In China, Hao *et al.* (2007) found that prevalence of low serum vitamin B_{12} level is high and it was higher in northern Chinese participants than in the southerns. In a cross-sectional study, plasma vitamin B_{12} was measured in 2407 apparently healthy Chinese men and women, 35-64 years old, living in the south and the north of China. They found that prevalence of low vitamin B_{12} (<185 pmole/L) was 11% in the southerns and 39% in the northerns. Within each region, men were found to have lower plasma vitamin B_{12} concentrations than women (15 vs. 8% in the south and 47 vs. 34% in the north) (Hao *et al.*, 2007).

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Table 4: Steps of the Schilling test.

Step 1	Co labeled vitamin B_{12} (1µg) is given orally to fasting patients + 1 mg of non-radioactive vitamin B_{12} is given intramusculary (to saturate the protein binding site in the blood to vitamins thus ensure that any radioactivity absorbed is readily excreted in the urine and not taken up by tissues)	24 hr urine collection is taken Radioactivity is measured using a gamma counter
Step 2	Performed 72 hrs later: Co labeled vitamin B_{12} with commercial preparation of IF capsule are given orally to fasting patients + 1 mg of non-radioactive vitamin B_{12} is given intramusculary	24 hr urine collection is taken Radioactivity is measured using a gamma counter
Step 3	Rarely used patients are treated with GI active antibiotics (i.e., tetracycline) for 5 days before repeating step 2 of test (to eliminate intestinal vitamin B_{12} utilizing MO)	24 hr urine collection is taken Radioactivity is measured using a gamma counter

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Table 5: Interpretation of the Schilling test results.

Results	Possible interpretation
Step 1, Normal	Dietary Deficiency Food-bound malabsorption Partial gastrectomy
Step 1, abnormal Step 2, normal	Pernicious anemia Gastrectomy Inadequate urine collection in step 1
Steps 1 and 2, abnormal	Ileal disease or resection Renal insufficiency Inadequate urine collection Bacterial overgrowth syndrome Tapeworm infestation

(Reference: Oh and Brown, 2003; Snow, 1999).

In the USA, prevalence of low vitamin B_{12} was found to be 17% according to the Framingham Offspring Study (Tucker *et al*., 2000). This study was done on 2999 subjects, vitamin B_{12} levels (<148, <185, <258 pmole/L) were assessed for 3 different age groups of subjects (26-49, 50-64, 65-83 years). It was found that 17% of these subjects had vitamin B_{12} concentration <185 pmole/L (with little difference between age groups) (Tucker *et al*., 2000).

In a study in Bangladesh of 1650 adults, the prevalence of cobalamin deficiency (≤205 pg/ml) was 8% for men and 13% for women (Gamble *et al*., 2005). In a study in India on 441 men (age 30-50 years) from different areas (rural, slum and urban), Yajnik *et al*. (2006) reported that 67% have B_{12} levels <150 pmole/L (<200 pg/ml) where 68% were in rural area, 51% in slums and 81% in urban area. In Brazil, a study done on 500 individuals divided into 2 groups (30-59 and >60 years), it was reported that 7.2% of those >60 years and 6.4% of those between 30-59 years had B_{12} levels <200 pmole/L

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(<270 pg/ml) (Xavier *et al.*, 2010). Table 6 shows the prevalence of vitamin B_{12} deficiency in several selected countries of the world.

Very few studies were done in the Arab countries for evaluating the prevalence of vitamin B_{12} deficiency. In a study conducted in Lebanon on women, it was found that 39.4% of Lebanese women at childbearing age had vitamin B_{12} deficiency (n= 470 non-pregnant women aged 15-45 years) (Al Khatib *et al.*, 2006). Whereas in Bahrain, Madan *et al.* (2009) reported that only 4.9% of their study group living in Bahrain had vitamin B_{12} deficiency, 88% of them were Bahraini and the rest (12%) were from other nationalities. Women accounted for the majority (85%) of the deficient group (Table 6).

3.2. Vitamin B_{12} Deficiency in Jordan

Vitamin B_{12} deficiency in Jordanians has increased in an alarming rate in the last few years (Abu-Samak *et al.*, 2008). Prevalence of vitamin B_{12} deficiency was reported to be high among Jordanians (MOH, WHO and MOA, 2006). In a study done on 216 healthy subjects (124 males and 92 females, aged 19-50 years) in north Jordan, Fora and Mohammad (2005) reported a high frequencies of vitamin B_{12} deficiency (48.1%) of suboptimal serum vitamin B_{12} levels (<222 pg/ml) (Table 7). Other study done by Abu-Samak *et al.* (2008) on 120 male students from Applied Science University (aged 18-24 years) from Amman City showed a 16% prevalence of vitamin B_{12} deficiency (<200 pg/ml), where 65% of them were overweight (Table 7).

In a more recent study done at Jordan University Hospital on 838 volunteers (aged 18-78) visiting family medicine clinic and diabetic foot clinic at Jordan University Hospital, a high prevalence of vitamin B_{12} deficiency was found (44.7%) (cutoff level is 180 pg/ml) (Barghouti *et al.*, 2009). They also found that around 50% of those having low serum level of vitamin B_{12} were between 18-24 years (Table 7).

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Table 6: Prevalence of vitamin B_{12} deficiency in selected countries of the world.

Country	Cutoff point of vitamin B_{12} deficiency	n	Age (years)	Prevalence (%)
Iran (Fakhrazadeh *et al.*, 2006)	185 pmole/L (250 pg/ml)	1214 (786 ♀ & 428 ♂)	25-64	♀ 27.2% ♂ 26.32%
China (Hao *et al.*, 2007)	185 pmole/L (250 pg/ml)	2407 Northern 1216 Southern 1191	35-64	N 39% S 11%
USA (Tucker *et al.*, 2000)	185 pmole/L (250 pg/ml)	2999 (♀ & ♂)	26-83	17%
Kenya (McLean *et al.*, 2007)	148 pmole/L (200 pg/ml)	503 (♀ & ♂)	5-14	40%
Nigeria (McLean *et al.*, 2007)	134 pmole/L (181 pg/ml)	162 ♀	12-16	9%
Caribbean (Kwan *et al.*, 2002)	185 pmole/L (250 pg/ml)	603 Hispanic 449 Non-Hispanic 154	60-93	Hispanic 17% Non-Hispanic 10%
Lebanon (Al Khatib *et al.*, 2006)	300 pg/ml	470 ♀	15-45	39.4%
Bahrain (Madan *et al.*, 2009)	132 pmole/L (180 pg/ml)	4180 (♀ & ♂)	-	4.9% from them: 88% were Bahrainis 12% were other nationalities

(♀= females, ♂= males, N=northern, S=southern).

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Table 7: Prevalence of vitamin B_{12} deficiency in Jordan.

	Cutoff point of vitamin B_{12} deficiency	n	Age (years)	Percentage (%)
Jordan (Barghouti *et al.*, 2009)	180 pg/ml	837 (♀ & ♂)	18-78	44.7%
Jordan (Abu-Samak *et al.*, 2008)	200 pg/ml	120 ♂	18-24	16%
Jordan (Hakooz *et al.*, 2006)	200 pg/ml	290 118 Arabs 172 Circassians	16-72	50.8% in Arabs 46.9% in Circassians
Jordan (Fora and Mohammad, 2005)	222 pg/ml	216 ♀ 92 ♂ 124	19-50	48.1%

(♀= females, ♂= males).

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Methods

1. Preparation of Client Assessment Questionnaire

A client assessment questionnaire considering demographic, medical and drug history, and a semi-quantitative food frequency questionnaire was prepared according to Harvard University School of Public Health Food Frequency Questionnaire (Lee and Nieman, 2007) (Appendix 1). The questionnaire was tested for its validity and reliability using the Cronbach's alpha test (Cronbach's alpha is a numerical coefficient of reliability (Santos, 1999)). The test was done for 25 questionnaires and it gives a Cronbach's alpha coefficient of 0.6964 (Appendix 2). The food frequency questionnaire (FFQ) is an acceptable method for assessing vitamin intake in different studies (Henríquez-Sánchez *et al*, 2009; Wakai, 2009)

2. Sample Selection

A total of more than 300 apparently healthy persons visiting the Family Medicine Clinic at Jordan University Hospital were interviewed for their acceptance to participate in this study. Only 165 of them aged 20-40 years (males=66, females=99) were recruited in the study. The study was approved by the Research Ethics Committee of the Jordan University Hospital and a written informed consent was obtained from all volunteers (Appendix 3). The selected volunteers were apparently healthy with no previous diagnosis of vitamin B_{12} deficiency or not treated with vitamin B_{12} supplementation for at least one year. Persons with current diagnosis of liver disease, kidney problems, folate deficiency, pregnancy and untreated hypothyroidism were excluded from the study. The protocol that was used in this study is shown in Figure 3.

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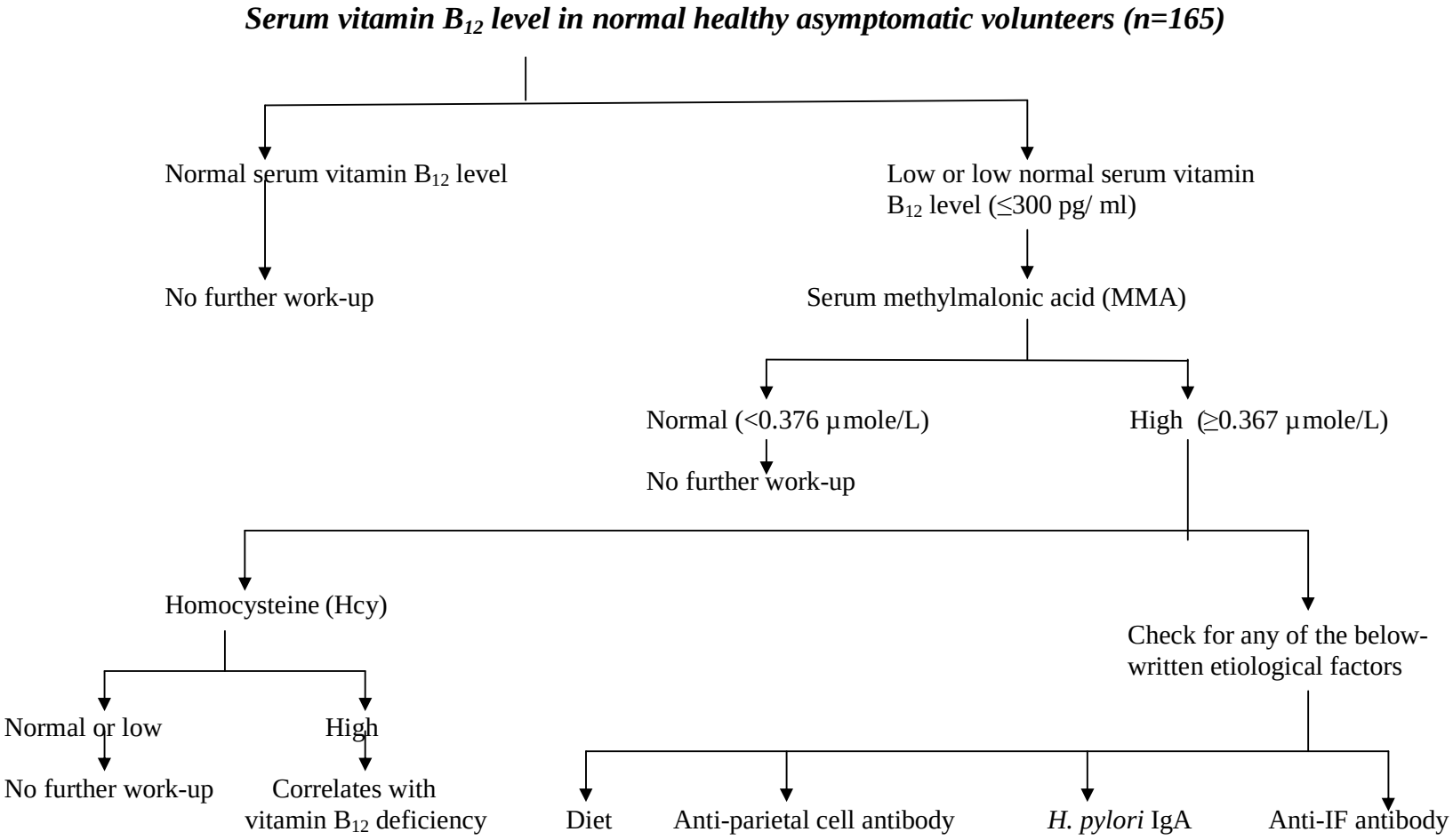

Figure 3: Flow diagram of the diagnosis methodology used in this study.

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3. Anthropometric Measurements

Weight and height were measured for all volunteers, and body mass index (BMI) values were calculated for them. Body weight was measured by using digital weighing scale (Tanita, United Kingdom), which measures body weight to the nearest 0.1 kg. Height was measured by asking subjects to take off their shoes and to stand straight, feet together, kees straight and heels, buttocks and shoulder blades were in contact with the stadiometer (Gibson, 1993). Subjects were then grouped according to BMI into underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2).

4. Medical and Nutritional History

Informed consent was obtained from each participant (Appendix 3) and all volunteers were asked to fill the previously prepared and tested questionnaire (Cronbach's alpha coefficient= 0.6964) (Appendix 2) that summarizes their medical history, social and educational information. Eating habits regarding the consumption of foods of animal sources were determined by semi-quantitative food frequency questionnaire (Appendix 1). The subjects were asked to give an approximate amount of food items eaten and the frequency of its consumption.

5. Blood Sample Collection and Biochemical Tests

Blood samples were collected for all volunteers. Automated complete blood count (CBC) using Cell-DYN37300 (USA/ Germany) and blood film reading using the Wright stain were performed. Serum vitamin B_{12} level was determined using automated microparticle enzyme intrinsic factor assay (AxSYM B_{12} Kit, Abbott, USA) at Al-Khaldi Medical Center Laboratory, Amman.

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From all the volunteers, those with serum vitamin B_{12} level ≤300 pg/ml (n= 114) were tested for plasma methylmalonic acid (MMA). MMA test was done at Jordan University Hospital Laboratories, Amman. This test was done to confirm vitamin B_{12} deficiency as MMA value of ≥ 0.376 µmole/L indicates deficiency (CDC, 2009).

MMA Test: MMA test were done according to the procedure described by Kushnir and Komaromy-Hiller (2000) with some modifications. In this test, sample and control were prepared by taking 1 ml of sample or control and adding to it 10 ml of d_3-MMA and 1 ml of acetonitrile. The mixture is then centrifuged for 5 minutes at 4000 g. The supernatant of both sample and control were then transferred into separate clean extract tubes, previously conditioned with 2 ml methanol and 3 ml distilled water. The tubes were then washed by sequential addition of 10 ml distilled water, 5 ml of methanol and 2 ml of methyl tert-butyrl ether (MTBE). The analytes were eluted with 5 ml of 3% formic acid in MTBE. The eluates were evaporated at 35˚C. Derivatization of the residues were then done by adding BSTFA and TMCS for 30 minutes.

6. Possible Causes of Vitamin B_{12} Deficiency

As vitamin B_{12} deficiency was confirmed (MMA ≥0.376 µmol/L) several biochemical tests; including total serum homocysteine (tHcy), anti-parietal cell antibody (APCA), *Helicobacter pylori* IgA and anti-intrinsic factor antibodies (anti-IF antibody) tests; were done to determine the possible causes of vitamin B_{12} deficiency. These tests were done at Al-Khaldi Medical Center Laboratory, Amman.

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6.1. Homocysteine Test:

A fully automated serum total homocysteine test (tHcy) was done using fluorescence polarization immunoassay (FPIA) (AxSYM Homocysteine Kit, Abbott, USA). Homocysteine value that contributes to vitamin B_{12} deficiency is >13 µmole/L (Andrès *et al.*, 2004).

6.2. *Helicobacter pylori* IgA Test:

H. pylori IgA was tested using enzyme immunoassay (ELISA) (IBL International, Germany). In this test, serum samples were diluted to 1:101 using a diluent buffer (containing PBS buffer, BSA, < 0.1% NaN_3). 100 µL of both standard and diluted serum were pipetted into Microliter Plate, covered and incubated for 60 minutes at 18-25°C. The cover was removed, incubation solution was discarded and the plate was washed 3 times with 300 µl of diluted wash buffer (containing PBS buffer, Tween 20). Then 100 µL of enzyme conjugate (anti-human IgA) was added, the plate was covered and incubated for 30 minutes at 18-25°C. The cover was removed, incubation solution was discarded and the plate was washed 3 times with 300 µl of diluted wash buffer. 100 µl of TMB substrate solution was added to the wells of the plate, and was incubated uncovered for 20 minutes in the dark. The substrate reaction was stopped using 100 µL of TMB stop solution (0.5M H_2SO_4) (color changed from blue to yellow). Optical density was measured within 60 minutes after adding the stop solution using a photometer at 450 nm (reference wavelength: 600-650 nm). The results were interpreted according to this table (Table 8):

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Table 8: Interpretation of results of serum *H. pylori* IgA.

U/ml	Interpretation
< 8	Negative
8-12	Equivocal
> 12	Positive

6.3. Anti-parietal Cell Antibody Test:

Anti-parietal cell antibody (APCA) was tested using indirect immunofluorescence assay (Anti-smooth muscle antibodies test) (ASMA, BioSystems Reagents and Instruments, Spain). In this test, serum was diluted with PBS to a ratio of 1:20. One drop of the diluted serum was fixed on a slide then placed in a moist chamber immediately and was incubated for 30 minutes at room temperature (15-30°C). Sample drops were then drained using inclinated slide and were rinsed briefly with PBS. Slides were then washed thoroughly in a staining dish containing PBS for 5 minutes (this step is repeated by changing the PBS solution). The slide was then blotted carefully and a drop of the conjugate was added and the slide was incubated for 30 minutes at room temperature. The slide was then washed and blotted and several drops of mounting medium were added. The slide was then covered and examined under fluorescence microscope. The results were interpreted as follows (Table 9):

Table 9: Interpretation of results of serum anti-parietal cell antibodies.

Ratio	Interpretation
< 1:10	Negative
> 1:10	Positive

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6.4. Anti-Intrinsic Factor (IF) Antibody Test:

Anti intrinsic factor antibodies were tested using enzyme linked immunosorbent assay (ELISA) (IMMCO Diagnostic, USA). In this test, blood sample is diluted to the ratio 1:101 by mixing 5 µl of serum with 500 µl of ready to use serum diluent (contains < 0.1% NaN_3). 100 µl of ready to use calibrators, positive and negative controls and the diluted blood samples were pipetted to the appropriate microwells as directed by the protocol sheet provided with the kit. The samples were then incubated for 30 minutes at room temperature, washed with 4x with wash buffer, then 100 µl of conjugate were added into microwells and was incubated for 30 minutes at room temperature. The samples were washed again with the wash buffer. Enzyme substrate (100 µl) were then added into each microwell, and samples were again incubated for 30 minutes at room temperature. To stop the reaction, 100 µl of stop solution were added to the microwells. Absorbance of each well were read at 405 nm using a single or at 405/630 nm using a dual wavelength microplate reader against the reagent blank set at zero absorbance. The results were interpreted as follows (Table 10):

Table 10: Interpretation of results of serum anti-intrinsic factor antibodies.

Anti-IF value	Interpretation
<20 EU/ml	Negative
20-25 EU/ml	Intermediate (Borderline)
>25 EU/ml	Positive

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7. Dietary Vitamin B_{12} Content Calculations

Vitamin B_{12} content for the selected food items included in the questionnaire was calculated using ESHA Food Processor program, version 7.71 (2001) (Salem, OR, USA). To convert the frequency of eaten items to daily basis with once per week equals to one, we divide the frequency of consumption per week or month over the number of days in the week or month respectively (e.g., item consumed 2 times per week is equivalent to 2/7, item consumed 3 times per month is equivalent to 3/30). Then we converted the frequency per day to quantity (weight in grams) by multiplying the frequency by usual amount consumed as reported by participant (Gibson, 1993). The amount in grams consumed per day were then entered to the ESHA Food Processor program, where the average amount of vitamin B_{12} consumed per day was calculated for each item.

8. Statistical Analysis

Participants were divided into three groups depending on serum vitamin B_{12} level: Group 1 (Low) with serum vitamin B_{12} level $\leq$ 200 pg/ml; Group 2 (Low normal) with serum vitamin B_{12} level between 201-300 pg/ml; and Group 3 (Normal) with serum vitamin B_{12} level >300 pg/ml.

Statistical analysis was performed using SPSS software, version 17.0 (Chicago, IL, USA). The association between both serum vitamin B_{12} level and plasma MMA level with other parameters were assessed using Pearson correlation coefficient. The results of each variable were subjected to cross-tabulation and Chi-square test to evaluate the variables that can influence vitamin B_{12} level. The difference in means between different variable groups and vitamin B_{12} level were subjected to analysis of variance

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(ANOVA). Differences in mean variables for different B_{12} groups and for different MMA groups were also done using ANOVA. The association between serum vitamin B_{12} and vitamin B_{12} intake for the two MMA groups (≥0.376 µmole/L and <0.376 µmole/L) were assessed using the T-test. Results were expressed as mean ± standard error of mean (SEM). $P \leq 0.05$ was considered significant.

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Results

1. Description of the Study Sample

Table 11 shows that the mean age of the study group was 28.22 years with mean serum vitamin B_{12} level of 273.26 pg/ml, hemoglobin 13.79 g/dl and BMI of 24.07 kg/m^2. Other characteristics are also shown in Table 11. All subjects were educated with minimum of school certificate (20%), 10.9% had college degree, 57.0% had a bachelor degree and 12.1% continued their graduate studies (Appendix 4). Table 12 shows the distribution of subjects according to the educational levels.

1.1. Body Mass Index (BMI)

Table 13 and Figure 4 show the distribution of subjects according to BMI. The majority of the study group had normal BMI (60.0%), while those who were underweight were very few (4.2%).

1.2. Eating Habits and Nutrient Intake

Table 14 shows the total vitamin B_{12} intake and the contribution of different food items to intake by the study subjects. As it is shown, organ meats (liver and spleen) accounts for the majority of vitamin B_{12} intake (38.49%), 18.84% comes from red meat, 17.82% from dairy product, 12.18% from fortified bread, 5.54% from fish, 3.58% from chicken whereas eggs comes the last in its contribution of total vitamin B_{12} intake (3.51%) (Figure 5).

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Table 11: Characteristics of the study group.

	Mean ± SEM*	n
Age (years)	28.22 ± 0.476	165
BMI (kg/m^2)	24.07 ± 0.292	165
Family size (person)	5.9 ± 0.186	165
Serum B_{12} (pg/ml)	273.26 ± 8.629	165
Hemoglobin (g/dl)	13.79 ± 0.131	164
Erythrocytes ($x10^{12}/L$)	4.87 ± 0.0369	164
MCV (fl)	84.99 ± 0.379	164
PCV (L/L)	0.41 ± 0.004	164
MCH (pg)	28.30 ± 0.174	164
MCHC (g/L)	332.94 ± 0.789	164
RDW (%)	16.47 ± 0.149	164
Platelets ($x10^9/L$)	259.90 ± 4.404	164
MPV (fl)	9.23 ± 0.117	162
WBCs ($x10^9/L$)	6.83 ± 0.124	164
Neutrophils ($x10^9/L$)	3.85 ± 0.103	164
Lymphocyres ($x10^9/L$)	2.09 ± 0.047	164
Monocytes ($x10^9/L$)	0.47 ± 0.012	164
Eosinophils ($x10^9/L$)	0.14 ± 0.009	164
Basophils ($x10^9/L$)	0.06 ± 0.002	164
MMA (µmole/L)	0.44 ± 0.063	114
Homocysteine (µmole/L)	12.69 ± 0.816	54

*SEM= Standard error of mean

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Table 12: Distribution of subjects according to educational level.

Educational level	Total n	Percentage
School	33	20.0
Junior college (diploma)	18	10.9
BSc	94	57.0
Higher studies	20	12.1
Total	165	100

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Table 13: Distribution of subjects according to BMI.

BMI (kg/m^2)	Frequency	Percentage
<18.5	7	4.2
18.5-24.9	99	60.0
25-29.9	48	29.1
≥30	11	6.7
Total	165	100

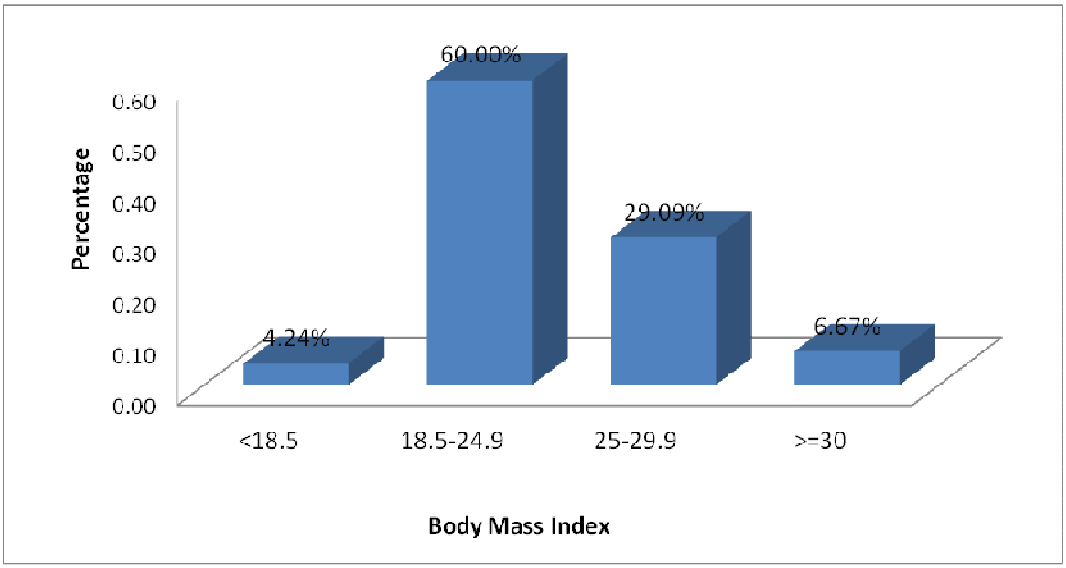

Figure 4: A bar diagram representing the percentage distribution of the study sample according to BMI.

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Table 14: Contribution of different food items to vitamin B_{12} intake (µg/day).

Food Item	Mean intake ± SEM	% of contribution of total B_{12} intake	% of contribution from RDA
Red meat	1.103 ± 0.078	18.84%	45.83%
Chicken	0.210 ± 0.010	3.58%	8.75%
Fish	0.324 ± 0.037	5.54%	13.33%
Organ meats (Liver and spleen)	2.254 ± 0.302	38.49%	93.75%
Dairy products	1.044 ± 0.058	17.82%	43.33%
Fortified bread	0.713 ± 0.046	12.18%	29.58%
Eggs	0.205 ± 0.021	3.51%	8.75%
Total intake	5.857 ± 0.370	100%	244.17%

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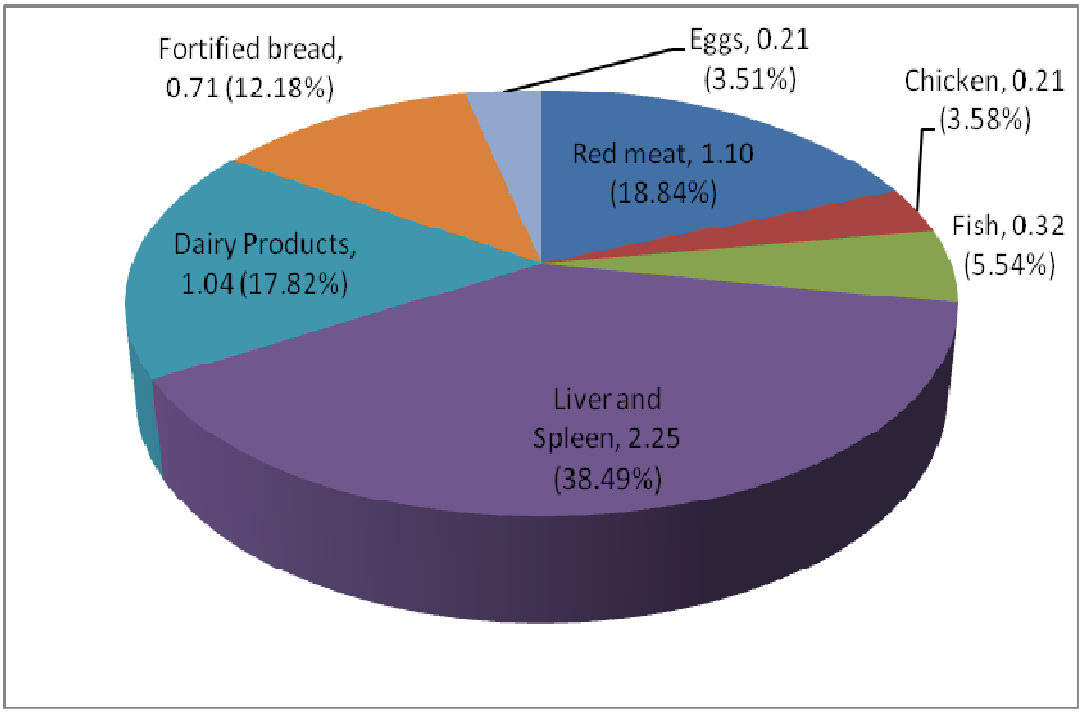

Figure 5: Contribution of different food items to vitamin B_{12} intake.

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1.3. Hematological Tests

1.3.1. CBC and Blood Film

From all patients, 86% had normal hemoglobin (Hgb) (Table 15), 94.5% had normal MCV (Table 16). By blood film test, no subject showed macrocytic erythrocytes, 77.4% of subjects showed normal blood film with normocytic normochromic red blood cells (Table 17). Normal values of blood indices are listed in Appendix 5.

1.3.2. Serum Vitamin B_{12} Level

The mean serum vitamin B_{12} level was 273.26 pg/ml (Table 11). Serum B_{12} levels were divided into 3 categories (Table 18 and Figure 6):

≤ 200 pg/ml	deficient (low)
201-300 pg/ml	low normal
> 300 pg/ml	normal

This table shows the percentage distribution of subjects according to serum B_{12} categories. It can be noted that 27.3% (n=45) had vitamin B_{12} deficiency (defined as serum vitamin B_{12} ≤200 pg/ml); 41.8% (n= 69) had low-normal serum B_{12} level (defined as serum vitamin B_{12} level between 201-300 pg/ml); and 30.9% (n=51) had normal serum B_{12} levels (defined as serum vitamin B_{12} >300 pg/ml).

1.3.3. Plasma MMA Level

Plasma MMA concentrations were measured in those with serum B_{12} level ≤300 pg/ml. These subjects accounted for 69.1% of the total study group (Table 18). This test was done to confirm deficiency for those with serum B_{12} level ≤200 pg/ml, and to check for possible hidden deficiency for those having serum B_{12} levels between 201-300 pg/ml. The cutoff point of MMA used to confirm deficiency was 0.376 µmole/L.

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Table 15: Distribution of subjects according to hemoglobin value*.

		Frequency	Percentage
Hemoglobin (g/dl)	Normal	141	86.0
	Abnormal (low)	23	14.0
n		164	100

* Normal range of Hgb for males is 14-18 g/dl, and for females 12-16 g/dl; the mean Hgb of the males in the noraml group (n=62) was 15.39 ± 0.86 g/dl; for the females in this group (n=79), it was 13.28 ± 0.058 g/dl. In the low Hgb group, the mean of Hgb in males (n=3) was 13.0 ± 0.82 g/dl, and that of the females (n=20) was 10.93 ± 0.252 g/dl.

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Table 16: Distribution of subjects according to MCV.

		Frequency	Percentage
MCV (fl)*	Normal	155	94.5
	Abnormal	9	5.5
n		164	100

* Normal range of MCV for males: 76-94 fl, and for females: 76-99 fl; the mean MCV of the males in the noraml group (n=63) was 85.59 ± 3.62 fl; for the females in this group (n=92) it was 85.46 ± 3.60 fl. Low MCV values were seen only in females (n=7) with mean value of 70.57 ± 4.84 fl, whereas high MCV values were seen only in males (n=2) with a mean of 94.95 ± 1.06 fl.

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Table 17: Distribution of subjects according to RBCs characteristics by blood film test.

		Frequency	Percentage
RBC	Abnormal RBCs (hypochromic microcytic and hypochromic anisocytic)	20	12.1
	normochromic anisocytic RBCs	17	10.4
	normochromic normocytic RBCs	127	77.4
n	Total	164	100.0

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Table 18: Distribution of subjects according to serum B_{12} levels.

Serum B_{12} (pg/ml)	Frequency	Percentage (%)	Cumulative percentage (%)
<200	45	27.3	27.3
201-300	69	41.8	69.1
>300	51	30.9	100.0
Total	165	100.0	

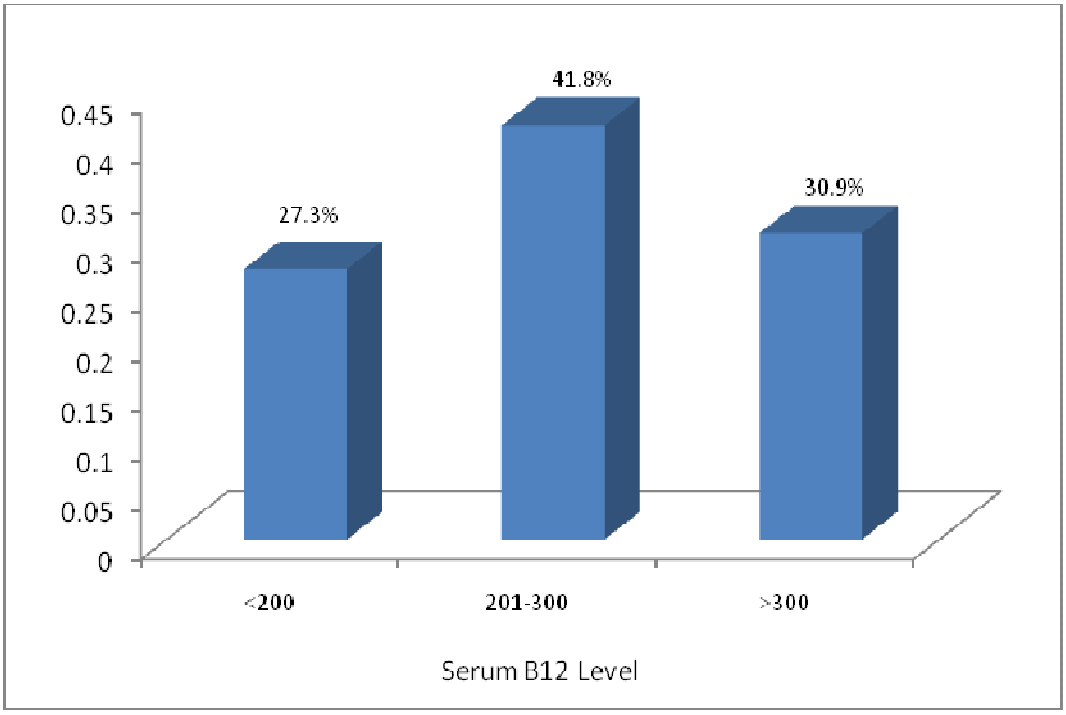

Figure 6: The percentage distribution of subjects according to their serum vitamin B_{12} level.

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Table 19 shows the percentage of subjects in which vitamin B_{12} deficiency is confirmed with plasma MMA.

2. Age and Gender

The percentage distribution of vitamin B_{12} status according to different age groups is also depicted in Table 20. As shown in Appendix 6, age was significantly correlated with vitamin B_{12} status (r=0.178; P=0.022). However, the mean vitamin B_{12} level for the age group 30-40 years was significantly higher than that of the age group 20-29 years (P=0.047) (Table 21). Moreover, there was no significant difference in the means of serum vitamin B_{12} level between the age groups in males as well as in females (Tables 22).

According to Table 21, there was no significant difference in the means of vitamin B_{12} levels among males and females (P=0.666).

3. Educational Level

Distribution of the study group according to their educational levels are listed in Appendix 4. Table 21 shows no significant difference in serum B_{12} level between different educational levels (P=0.205).

4. Family Size

Household (family) size represents the number of people living in the same house. Our results showed no significant difference between different household groups (≤4 persons, 4-7 and >7 persons) in regard to serum vitamin B_{12} level (P=0.916) (Table 21).

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Table 19: Distribution of subjects according to plasma MMA level *.

	Frequency	Percentage
MMA < 0.376 µmole/L	60	52.6
MMA ≥ 0.376 µmole/L	54	47.4
n	114	100

*The cutoff point of MMA is 0.376 µmole/L.

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Table 20: Vitamin B_{12} status distribution according to age groups.

Age	Serum B_{12} level			Total
	≤200 pg/ml	200-300 pg/ml	>300 pg/ml	
20-29 years	31 (32.3%)	41 (42.7%)	24 (25%)	96 (100%)
30-40 years	14 (20.3%)	28 (40.6&)	27 (39.1%)	69 (100%)
Total	45 (27.3%)	69 (41.8%)	51 (39.9%)	165 (100%)

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Table 21: Serum vitamin B_{12} levels of the study group according to different variables studied.

Variable		n	Mean serum B_{12} (pg/ml) ± SEM	P-value
Age (years)	20-29	96	258.78 ± 11.798	0.047*
	30-40	69	293.41 ± 12.191	
Gender	Females	99	270.20 ± 11.093	0.666
	Males	66	277.85 ± 13.814	
Educational level	School	33	309.33 ± 18.981	0.205
	Junior college	18	253.56 ± 19.268	
	BSc	94	266.72 ± 12.453	
	Higher studies	20	262.20 ± 16.916	
Family size (persons)	≤4	54	277.81 ± 17.575	0.916
	4-7	71	269.41 ± 11.339	
	>7	40	273.95 ± 17.714	
BMI (kg/m^2)	Underweight	7	253.86 ± 30.866	0.696
	Normal weight	99	272.11 ± 11.658	
	Overweight	48	270.10 ± 15.944	
	Obese	11	309.73 ± 24.252	
Total B_{12} intake (µg/day)	<2.4	19	265.68 ± 24.019	0.752
	≥2.4	146	274.25 ± 9.262	

* Significant at P ≤0.05.

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Table 22: Serum vitamin B_{12} levels for males and females according to age groups.

	Age Group	n	Mean serum B_{12} level (pg/ml) ± SEM	P-value
Males	20-29 years	31	259.58 ± 23.615	0.216
	30-40 years	35	294.03 ± 15.353	
Females	20-29 years	65	258.40 ± 13.433	0.142
	30-40 years	34	292.76 ± 19.272	

* Significant at P ≤0.05.

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5. Body Mass Index (BMI)

Appendix 7 gives the distribution of subjects according to BMI classifications. Table 21 shows no significant difference in mean serum B_{12} levels among different BMI groups (P=0.696).

6. Total Vitamin B_{12} Intake

The mean total vitamin B_{12} intake was 5.86 µg/day (Table 14). As shown in Appendix 6, this intake had no significant correlation with vitamin B_{12} status (r = 0.075; P=0.342). The mean serum B_{12} level was not different in subjects with total B_{12} intake ≥2.4 µg/day and those with total B_{12} intake < 2.4 µg/day (P=0.752) (Table 21).

7. Hematological Parameters

Different hematological parameters were compared for the three B_{12} groups. There was an inverse correlation between vitamin B_{12} status and MCV (r=-0.170; P=0.03); platelets (r=-0.158, P=0.044) and MPV (r=0.163, P=0.038), whereas it showed marginal correlation with MCH (r= -0.131, P=0.095) (Appendix 6). On the other hand, as it is noticed from Table 23, there was a trend of having higher MCV values in the group of serum vitamin B_{12} ≤200 pg/ml compared to the other groups (P=0.065). No difference in the mean of other blood values were found when compared for the B_{12} groups (Table 23).

Vitamin B_{12} status was found to have no relation with hemoglobin level (r = -0.048; P=0.545) (Appendix 6). By comparing the different B_{12} groups, no significant difference was found in hemoglobin level among them (Table 23). No correlations were found between vitamin B_{12} status and other blood count values (P >0.05) (Appendix 6).

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Table 23: Mean CBC values for subjects with different serum B_{12} levels.

Variable	B_{12} Group	n	Mean Variable ± SEM	P-value
Hemoglobin (g/dl)	≤200 pg/ml	45	13.91 ± 0.223	0.777
	201-300 pg/ml	68	13.80 ± 0.214	
	>300 pg/ml	51	13.67 ± 0.244	
MCV (fl)	≤200 pg/ml	45	86.22 ± 0.653	0.065
	201-300 pg/ml	68	84.99 ± 0.617	
	>300 pg/ml	51	83.91 ± 0.665	
Erythrocytes ($x10^{12}$/L)	≤200 pg/ml	45	4.83 ± 0.060	0.708
	201-300 pg/ml	68	4.88 ± 0.062	
	>300 pg/ml	51	4.90 ± 0.068	
PCV (L/L)	≤200 pg/ml	45	0.416 ± 0.006	0.803
	201-300 pg/ml	68	0.414 ± 0.006	
	>300 pg/ml	51	0.410 ± 0.006	
MCH (pg)	≤200 pg/ml	45	28.79 ± 0.296	0.141
	201-300 pg/ml	68	28.28 ± 0.267	
	>300 pg/ml	51	27.89 ± 0.336	
MCHC (g/L)	≤200 pg/ml	45	333.78 ± 1.381	0.775
	201-300 pg/ml	68	332.38 ± 1.146	
	>300 pg/ml	51	332.94 ± 1.638	
RDW (%)	≤200 pg/ml	45	16.17 ± 0.212	0.257
	201-300 pg/ml	68	16.425 ± 0.222	
	>300 pg/ml	51	16.81 ± 0.327	
Platelets ($x10^{9}$/L)	≤200 pg/ml	45	265.89 ± 6.735	0.605
	201-300 pg/ml	68	260.15 ± 6.989	
	>300 pg/ml	51	254.28 ± 8.825	
MPV (fl)	≤200 pg/ml	45	8.99 ± 0.182	0.317
	201-300 pg/ml	67	9.22 ± 0.183	
	>300 pg/ml	50	9.45 ± 0.237	

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Table 23 (cont'd): Mean CBC values for subjects with different serum B_{12} levels.

Variable	B_{12} Group	n	Mean Variable ± SEM	P-value
WBCs ($x10^9$/L)	≤200 pg/ml	45	6.48 ± 0.212	0.210
	201-300 pg/ml	68	6.98 ± 0.204	
	>300 pg/ml	51	6.94 ± 0.220	
Neutrophils ($x10^9$/L)	≤200 pg/ml	45	3.56 ± 0.200	0.208
	201-300 pg/ml	68	3.93 ± 0.166	
	>300 pg/ml	51	4.01 ± 0.168	
Lymphocytes ($x10^9$/L)	≤200 pg/ml	45	2.04 ± 0.094	0.765
	201-300 pg/ml	68	2.12 ± 0.072	
	>300 pg/ml	51	2.11 ± 0.074	
Monocytes ($x10^9$/L)	≤200 pg/ml	45	0.46 ± 0.022	0.785
	201-300 pg/ml	68	0.47 ± 0.017	
	>300 pg/ml	51	0.48 ± 0.023	
Eosinophils ($x10^9$/L)	≤200 pg/ml	45	0.137 ± 0.018	0.847
	201-300 pg/ml	68	0.135 ± 0.013	
	>300 pg/ml	51	0.148 ± 0.018	
Basophils ($x10^9$/L)	≤200 pg/ml	45	0.062 ± 0.004	0.864
	201-300 pg/ml	68	0.064 ± 0.003	
	>300 pg/ml	51	0.061 ± 0.003	

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As discussed in the methodology section, MMA was measured for all subjects with serum vitamin B_{12} ≤300 pg/ml. Mean MMA levels were not significantly different in both B_{12} groups as shown in Table 24.

Homocysteine was also measured for only those with confirmed vitamin B_{12} deficiency (n=54). Homocysteine, in contrast to MMA, showed an inverse correlation with vitamin B_{12} status (r = -0.423; P=0.001) (Appendix 6). It was found to be significantly higher in B_{12} group of serum B_{12} ≤200 pg/ml than those between 201-300 pg/ml (P=0.019) (Table 24).

8. Medical History

Medical history includes medicine consumption, family history of vitamin B_{12} deficiency and the history of undergoing gastrointestinal (GI) surgeries. According to the questionnaire filled by the participants, they were found to use the listed medications in Appendix 2 very rarely or even not at all; so it was ignored from being statistically analyzed. Total number of those having a family history of vitamin B_{12} deficiency was 50 persons (30.3% (50 from 165) of the study group). However, no association was found between having family history of vitamin B_{12} deficiency and vitamin B_{12} status (P=0.212) (Table 25). Moreover, only four patients from 165 had undergone gastrointestinal (GI) surgeries. It was found that there was no association between having a previous GI surgery and vitamin B_{12} status (P=0.942) (Table 25).

9. Deficiency Symptoms

Several symptoms were studied for their associations with vitamin B_{12} status. These symptoms included headache, weight loss, numbness, general weakness, fatigue, gait loss, visual impairment, focal impairment, glossitis, joint pain and diarrhea. There were no significant associations between serum vitamin B_{12} level and the clinical

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Table 24: Mean plasma MMA and total homocysteine for subjects with different serum B_{12} levels.

Variable	B_{12} Group	n	Mean Variable ± SEM	P-value
MMA	≤200 pg/ml	45	0.400 ± 0.025	0.615
	201-300 pg/ml	69	0.466 ± 0.103	
Homocysteine	≤200 pg/ml	23	14.90 ± 1.688	0.019*
	201-300 pg/ml	31	11.06 ± 0.539	

* Significant at P ≤0.05.

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Table 25: Distribution of subjects in the different serum B_{12} categories according to their medical history.

Medical History		Serum B_{12} level			P-value
		≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	
Family history of vitamin B_{12} deficiency	Don't know	2 (4.4%)	2 (2.9%)	0 (0%)	0.212
	No	25 (55.6%)	47 (68.1%)	39 (76.5%)	
	Yes	18 (40.0%)	20 (29.0%)	12 (23.5%)	
History of having GI surgeries	No	44 (97.8%)	67 (97.1%)	50 (98.0%)	0.942
	Yes	1 (2.2%)	2 (2.9%)	1 (2.0%)	

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symptoms of vitamin B_{12} deficiency except for visual impairment which was found in significantly more patients with vitamin B_{12} than other patients (P=0.012) (Table 26).

10. Determination of True Vitamin B_{12} Deficiency

True vitamin B_{12} deficiency was determined for those with serum vitamin B_{12} level ≤ 300 pg/ml (n = 114). We measured plasma MMA levels for those subjects. Those having MMA level ≥ 0.376 µmole/L were confirmed of having true vitamin B_{12} deficiency, whereas the others were considered normal.

10.1. Characteristics of the studied group

Table 27 shows the demographic, social and hematological characteristics of the studied group (with B_{12} level ≤ 300 pg/ml). it showed that mean age was 27.67 years, BMI was within normal range (23.95 kg/m^2) and family size was 5.8 persons.

10.2. Age and Gender

Comparison between those with true vitamin B_{12} deficiency (MMA ≥0.376 µmole/L) and those without (MMA <0.376 µmole/L) were done. No significant difference in the age was found between the two groups (Table 28). Moreover, age was not correlated with plasma MMA level (r = 0.056, P=0.553) (Appendix 8). No significant difference in plasma MMA values was found between males and females (Table 28).

10.3. . Body Mass Index (BMI) and Family Size

Results showed no significant difference in the mean BMI between the two groups (P=0.618) (Table 28). Furthermore, no significant difference in plasma MMA values was found between different BMI categories (Table 29). Although there was no

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Table 26: Distribution of subjects in the different serum B_{12} categories according to having clinical symptoms related to vitamin B_{12} deficiency.

Symptom	Occurrence	Total No. (165) n	Serum B_{12} level			P-value
			≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	
Headache	No	113	32 (71.1%)	47 (68.1%)	34 (66.7%)	0.893
	Yes	52	13 (28.9%)	22 (31.9%)	17 (33.3%)	
Weight loss	No	155	43 (95.6%)	65 (94.2%)	47 (92.2%)	0.779
	Yes	10	2 (4.4%)	4 (5.8%)	4 (7.8%)	
Numbness	No	141	39 (86.7%)	61 (88.4%)	41 (80.4%)	0.452
	Yes	24	6 (13.3%)	8 (11.6%)	10 (19.6%)	
Weakness	No	85	27 (60.0%)	36 (52.2%)	22 (43.1%)	0.245
	Yes	80	18 (40.0%)	33 (47.8%)	29 (56.9%)	
Fatigue	No	101	27 (60.0%)	42 (60.9%)	32 (62.7%)	0.960
	Yes	64	18 (40.0%)	27 (39.1%)	19 (37.3%)	

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Table 26 (cont'd): Distribution of subjects in the different serum B_{12} categories according to having clinical symptoms related to vitamin B_{12} deficiency.

Symptom	Occurrence	Total No. (165) n	Serum B_{12} level			P-value
			≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	
Gait loss	No	146	40 (88.9%)	61 (88.4%)	45 (88.2%)	0.995
	Yes	19	5 (11.1%)	8 (11.6%)	6 (11.8%)	
Visual impairment	No	131	29 (64.4%)	60 (87.0%)	42 (82.4%)	0.012*
	Yes	34	16 (35.6%)	9 (13.0%)	9 (17.6%)	
Focal impairment	No	122	29 (64.4%)	56 (81.2%)	37 (72.5%)	0.134
	Yes	43	16 (35.6%)	13 (18.8%)	14 (27.5%)	
Glossitis	No	150	42 (93.3%)	62 (89.9%)	46 (90.2%)	0.801
	Yes	15	3 (6.7%)	7 (10.1%)	5 (9.8%)	
Joint pain	No	133	39 (86.7%)	54 (78.3%)	40 (78.4%)	0.483
	Yes	32	6 (13.3%)	15 (21.7%)	11 (21.6%)	
diarrhea	No	160	44 (97.8%)	65 (94.2%)	51 (100.0%)	0.175
	Yes	5	1 (2.2%)	4 (5.8%)	0 (0%)	

* Significant at P ≤0.05.

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Table 27: Characteristics of the subjects with vitamin $B_{12} \leq 300$ pg/ml.

	Mean ± SEM	n
Age (years)	27.67 ± 0.558	114
BMI (kg/m^2)	23.95 ± 0.340	114
Family size (persons)	5.8 ± 0.220	114
Serum B_{12} (pg/ml)	214.68 ± 5.029	114
Hemoglobin (g/dl)	13.842 ± 0.156	113
Erythrocytes ($x10^{12}$/L)	4.858± 0.044	113
MCV (fl)	85.482 ± 0.455	113
PCV (L/L)	0.415 ± 0.004	113
MCH (pg)	28.483 ± 0.200	113
MCHC (g/L)	332.938 ± 0.881	113
RDW (%)	16.324 ± 0.158	113
Platelets ($x10^9$/L)	262.434 ± 4.9747	113
MPV (fl)	9.125 ± 0.132	112
WBCs ($x10^9$/L)	6.782 ± 0.150	113
Neutrophils ($x10^9$/L)	3.785 ± 0.128	113
Lymphocyres ($x10^9$/L)	2.084 ± 0.057	113
Monocytes ($x10^9$/L)	0.464 ± 0.013	113
Eosinophils ($x10^9$/L)	0.136 ± 0.106	113
Basophils ($x10^9$/L)	0.063 ± 0.003	113
MMA (µmole/L)	0.440 ± 0.063	114
Homocysteine (µmole/L)	12.69 ± 0.816	54

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Table 28: Comparison of different variables studied between groups with confirmed and non-confirmed vitamin B_{12} deficiency.

Variable	MMA Group	n	Mean Variable ± SEM	P-value
Age (years)	<0.376	60	27.92 ± 0.778	0.639
	≥0.376	51	27.39 ± 0.807	
Serum B_{12} (pg/ml)	<0.376	60	218.85 ± 7.131	0.384
	≥0.376	54	210.04 ± 7.083	
BMI (kg/m^2)	<0.376	60	23.79 ± 0.455	0.618
	≥0.376	54	24.13 ± 0.513	
Family size (persons)	<0.376	60	5.43 ± 0.287	0.081
	≥0.376	54	6.2 ± 0.333	
Total B_{12} intake (µg/day)	<0.376	60	6.21 ± 0.658	0.335
	≥0.376	54	5.35 ± 0.575	

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Table 29: Plasma MMA levels according to different variables studied.

Variable		n	Plasma MMA level (µmole/L) ± SEM	P-value
Gender	Females	68	0.473±0.105	0.52
	Males	46	0.390±0.021	
BMI (kg/m^2)	Underweight	5	0.465±0.104	0.96
	Normal weight	69	0.464±0.103	
	Overweight	34	0.402±0.024	
	Obese	6	0.355±0.080	
Educational level	School	18	0.769±0.389	0.15
	Junior college	14	0.365±0.034	
	BSc	67	0.391±0.020	
	Higher studies	15	0.331±0.036	
Total B_{12} intake (µg/day)	<2.4	10	0.449±0.052	0.97
	≥2.4	104	0.439±0.069	

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significant correlation between MMA level and family size (r = -0.031, P=0.744) (Appendix 8), there was a marginal significance in the mean family size between the two groups (P=0.081). There was a trend in having true vitamin B_{12} deficiency in those having larger family size (Table 28).

10.4. Educational Level

Table 29 shows no significant difference in mean plasma MMA levels when compared for different educational levels (P=0.15).

10.5. Total Vitamin B_{12} Intake

No significant difference was found between total B_{12} intakes in the two groups. Moreover, there was no significant difference in MMA for those obtaining vitamin B_{12} above the RDA (2.4 µg/day) and those obtaining less vitamin B_{12} less than the RDA (P=0.97) (Table 29).

10.6. Hematological Parameters

By comparing the means of different CBC values between the two groups, no significant differences were found in the mean values of these blood components. Similar results were observed for serum B_{12} level in the two groups (Table 30).

In contrast, MCV and MCH were found to have significant inverse correlations with plasma MMA level (r = -0.2, P=0.034; r = -0.201, P=0.032, respectively) (Appendix 8).

Serum homocysteine (Hcy) level was measured for those with true vitamin B_{12} deficiency (n=54). It was found that only 25.9% (n=14) had high Hcy level (≥13 µmole/L) (Table 31).

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Table 30: Mean CBC values for subjects with different plasma MMA levels.

Variable	MMA Group	n	Mean Variable ± SEM	P-value
Hemoglobin (g/dl)	<0.376	60	13.81 ± 0.205	0.817
	≥0.376	53	13.88 ± 0.239	
MCV (fl)	<0.376	60	85.958 ± 0.556	0.267
	≥0.376	53	84.943 ± 0.737	
Erythrocytes ($x10^{12}$/L)	<0.376	60	4.83 ± 0.061	0.473
	≥0.376	53	4.89 ± 0.064	
PCV (L/L)	<0.376	59	0.414 ± 0.006	0.952
	≥0.376	53	0.415 ± 0.006	
MCH (pg)	<0.376	60	28.60 ± 0.248	0.537
	≥0.376	53	28.35 ± 0.322	
MCHC (g/L)	<0.376	60	332.3 ± 1.076	0.443
	≥0.376	53	333.7 ± 1.434	
RDW (%)	<0.376	60	16.218 ± 0.233	0.479
	≥0.376	53	16.443 ± 0.210	
Platelets ($x10^9$/L)	<0.376	60	259.6 ± 6.56	0.552
	≥0.376	53	265.4 ± 7.62	
MPV (fl)	<0.376	60	9.21 ± 0.203	0.499
	≥0.376	53	9.03 ± 0.163	

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Table 30 (cont'd): Mean CBC values for subjects with different plasma MMA levels.

Variable	MMA Group	n	Mean Variable ± SEM	P-value
WBCs ($x10^9$/L)	<0.376	60	6.86 ± 0.223	0.565
	≥0.376	53	6.69 ± 0.198	
Neutrophils ($x10^9$/L)	<0.376	60	3.58 ± 0.191	0.585
	≥0.376	53	3.71 ± 0.169	
Lymphocytes ($x10^9$/L)	<0.376	60	2.01 ± 0.076	0.146
	≥0.376	53	2.17 ± 0.085	
Monocytes ($x10^9$/L)	<0.376	60	0.456 ± 0.0197	0.531
	≥0.376	53	0.473 ± 0.0177	
Eosinophils ($x10^9$/L)	<0.376	60	0.134 ± 0.0143	0.799
	≥0.376	53	0.139 ± 0.016	
Basophils ($x10^9$/L)	<0.376	60	0.064 ± 0.004	0.791
	≥0.376	53	0.062 ± 0.003	

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Table 31: Distribution of subjects with true vitamin B_{12} deficiency according to their serum homocysteine levels.

	Homocysteine level	Frequency	Percentage (%)
Homocysteine (µmole/L)	<13	40	74.1%
	≥13	14	25.9%
Total		54	100%

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10.7. Medical History

Medical history including family history of vitamin B_{12} deficiency and history of having GI surgeries was evaluated for both groups. No association between being true deficient and having family history of B_{12} deficiency was found (Table 32). In contrast, there was a trend of having previous GI surgeries and incidence of being a true deficient (Table 32).

10.8. Clinical Symptoms

Clinical symptoms were studied for their association with true vitamin B_{12} deficiency. There were no significant associations between true B_{12} deficiency and clinical symptoms except for visual impairment. However, a trend of having weakness was seen in true deficient subjects (Table 33).

11. Distribution of True Deficient Subjects According to their Serum B_{12} Level

Table 34 showed that 51.1% of those with serum B_{12} level $\leq$ 200 pg/ml were true deficient while the percentage of true deficiency for those with low-normal vitamin B_{12} levels was 44.9% (as indicated by elevated MMA ($\geq$ 0.376 µmole/L) which confirmed their subclinical vitamin B_{12} deficiency).

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Table 32: Distribution of subjects in the different MMA categories according to their medical history.

Medical history		Total No. (114) n	Plasma MMA level		P-value
			≥ 0.376 µmole/L n (%)	< 0.376 µmole/L n (%)	
Family history of vitamin B_{12} deficiency	Don't know	3	2 (3.7%)	1 (1.7%)	0.790
	No	72	34 (63.3%)	38 (63.0%)	
	Yes	39	18 (33.3%)	21 (35.0%)	
History of having GI surgeries	No	111	51 (94.4%)	60 (100%)	0.064
	Yes	3	3 (5.6%)	0 (0%)	

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Table 33: Distribution of subjects in the different MMA categories according to having clinical symptoms related to vitamin B_{12} deficiency.

Symptom	Occurrence	Total No. (114) n	Plasma MMA level		P-value
			≥ 0.376 µmole/L n (%)	< 0.376 µmole/L n (%)	
Headache	No	79	39 (72.2%)	40 (66.7%)	0.521
	Yes	35	15 (27.8%)	20 (33.3%)	
Weight loss	No	108	50 (92.6%)	58 (96.7%)	0.331
	Yes	6	4 (7.4%)	2 (3.3%)	
Numbness	No	100	47 (87.0%)	53 (88.3%)	0.833
	Yes	14	7 (13.0%)	7 (11.7%)	
Weakness	No	63	25 (46.3%)	38 (63.3%)	0.068
	Yes	51	29 (53.7%)	22 (36.7%)	
Fatigue	No	69	32 (59.3%)	37 (61.7%)	0.793
	Yes	45	22 (40.7%)	23 (38.3%)	

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Table 33(cont'd): Distribution of subjects in the different MMA categories according to having clinical symptoms related to vitamin B_{12} deficiency.

Symptom	Occurrence	Total No. (114) n	Plasma MMA level		P-value
			≥ 0.376 µmole/L n (%)	< 0.376 µmole/L n (%)	
Gait loss	No	101	46 (85.2%)	55 (91.7%)	0.277
	Yes	13	8 (14.8%)	5 (8.3%)	
Visual impairment	No	89	37 (68.5%)	52 (86.7%)	0.019*
	Yes	25	17 (31.5%)	8 (13.3%)	
Focal impairment	No	85	36 (66.7%)	49 (81.7%)	0.066
	Yes	29	18 (33.3%)	11 (18.3%)	
Glossitis	No	104	49 (90.7%)	55 (91.7%)	0.861
	Yes	10	5 (9.3%)	5 (8.3%)	
Joint pain	No	93	41 (75.9%)	52 (86.7 %)	0.140
	Yes	21	13 (24.1%)	8 (13.3%)	
diarrhea	No	109	52 (96.3%)	57 (95.0%)	0.736
	Yes	5	2 (3.7%)	3 (5.0%)	

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Table 34: Percentage of subjects whom vitamin B_{12} deficiency is confirmed using plasma MMA levels.

Serum vitamin B_{12} (pg/ml)	% of patients with MMA ≥0.376 µmol/L	% of patients with MMA <0.376 µmol/L
≤200	23 (51.1%)	22 (48.9%)
201-300	31 (44.9%)	38 (55.1%)
Total	54 (47.4%)	60 (52.6%)

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12. Possible Causes of Vitamin B_{12} Deficiency

As shown in Table 35, 1.9% of these subjects have anti-parietal cell antibodies (APCA), 7.4% have *Helicobacter pylori* IgA positive test and 3.7% have anti-intrinsic factor (IF) antibodies. Those with daily B_{12} intake less than the recommended daily allowances (<2.4 µg/day) accounts for 13.0% (Table 35). If the subjects on borderline values were considered positive, this will increase the percentages to 18.52% having APCA, 22.2% having *H. pylori* IgA and 9.3% having anti-IF antibodies (Figure 7). As a result, 27.8% had diagnosed pernicious anemia, and 22.2% had *H. pylori* infection.

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Table 35: Numbers and percentages of subjects with confirmed vitamin B_{12} deficiency according to different possible causes of B_{12} deficiency.

Test		n	Percentage
APCA	-ve	44	81.48%
	+ve	1	1.85%
	Borderline	9	16.67%
Anti-If antibodies	-ve	49	90.7%
	+ve	2	3.7%
	Borderline	3	5.6%
H. pylori IgA	-ve	42	77.8%
	+ve	4	7.4%
	Borderline	8	14.8%
Daily B_{12} intake	<2.4 µg/day	7	13.0%
	≥2.4 µg/day	47	87.0%

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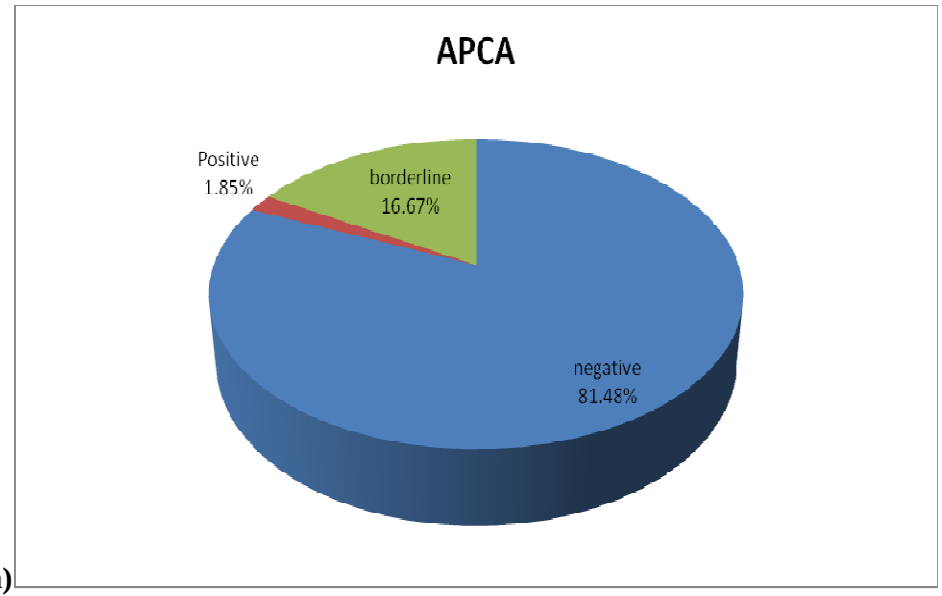

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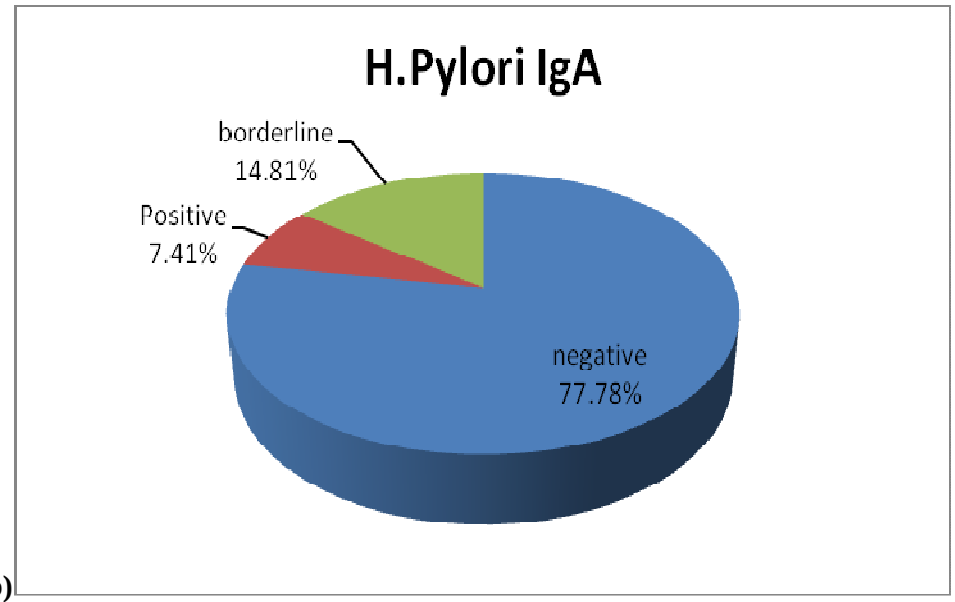

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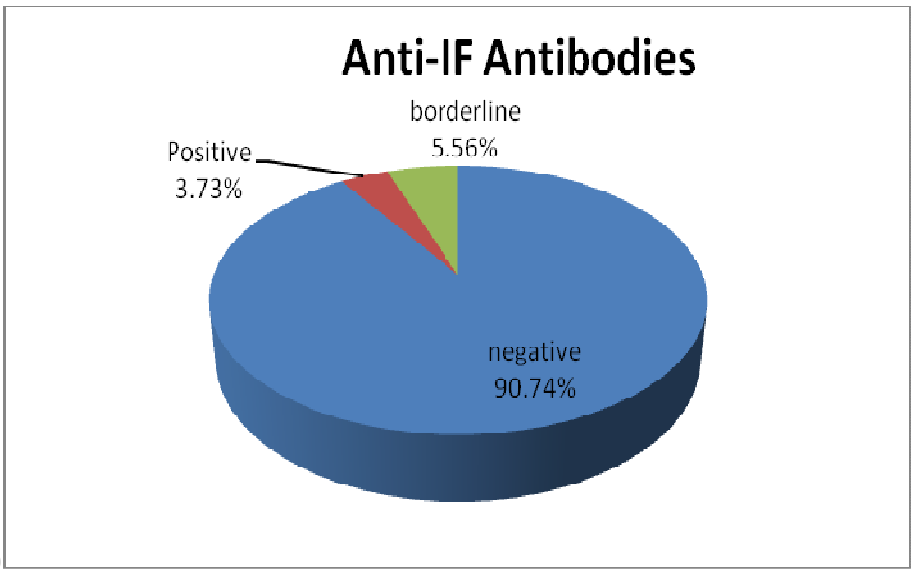

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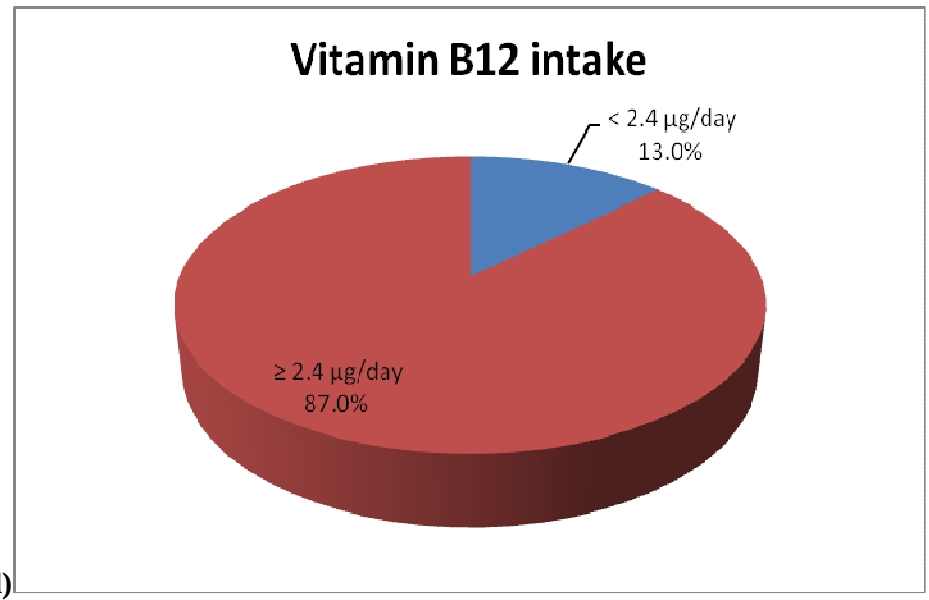

(d)

Figure 7: A pie chart representing percentages of patients with confirmed B_{12} deficiency: (a) according to anti-parietal cell antibodies (APCA) test, (b) according to *H. pylori* IgA test, (c) according to anti-IF antibodies test, (d) according to vitamin B_{12} intake

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Discussion

Vitamin B_{12} deficiency in Jordan has increased to an alerting rate in the last few years (MOH, WHO and MOA, 2006). Several studies were conducted to evaluate the B_{12} status in Jordan, and they showed that there is high prevalence of vitamin B_{12} deficiency among Jordanians. Serum vitamin B_{12} level was used as a tool for determining the prevalence of deficiency; however, the exact etiology of deficiency and other diagnostic tools were not explored. In this study, we used other diagnostic tools to confirm the deficiency, including the MMA test, which is a confirmatory test for B_{12} deficiency if plasma methylmalonic acid (MMA) level was greater than 0.376 µmole/L (CDC, 2003). MMA being a metabolite in the B_{12} metabolic pathway, is considered an early marker for tissue vitamin B_{12} deficiency, even before hematologic manifestations occur (Oh and Brown, 2003). Other indicators of vitamin B_{12} deficiency were also used; they include serum homocysteine (Hcy), anti-parietal cell antibody (APCA), anti intrinsic factor antibody and *Helicobacter pylori* IgA, as these determine the hidden cause of vitamin B_{12} deficiency.

1. Age and Gender

The present study focused on the young adults (age 20-40 years) and it excluded all those older than 40 years. The reason was to limit the effect of age on increasing the prevalence of low serum vitamin B_{12} levels. Elderly patients may increase the prevalence of vitamin B_{12}, either because of low intake, or malabsorption by atrophic gastritis, or other chronic diseases such as diabetes mellitus. Age was reported to be an independent risk factor for developing vitamin B_{12} deficiency, irrespective to gastric atrophy (Gümürdülü *et al.,* 2003). In a study done in Spain, serum vitamin B_{12} was

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reported to be significantly higher in younger age (25-39 years) than in older age groups (40-49 years) (Planells *et al.*, 2003). However, Barghouti *et al.* (2009) found that high frequency of vitamin B_{12} was observed in the age group between 18-24 years (51.8%), while B_{12} hypovitaminosis (serum B_{12} level between 180-300 pg/ml) was mostly in patients aged >64 years (44.4%). The present study confirmed the results of Barghouti *et al.* (2009). The mean serum B_{12} level in the younger age group (20-29 years) was significantly lower than the older group (30-40 years) (P=0.047) (Table 21). Moreover, high frequency of low and low normal vitamin B_{12} were seen in the age group between 20-24 years (P=0.038) (Appendix 9). This can be explained by their eating habits that are usually highly dependent on consumption of junk foods by the younger group.

However, by considering the age group and cutoff point of vitamin B_{12} level used, we found that only 18.8% had vitamin B_{12} <180 pg/ml as compared to 47.1% of the same age group (18-39 years, n=408) in Barghouti *et al.* (2009) study, and 35.2% had vitamin B_{12} level <222 pg/ml compared to 48.1% in Fora and Mohammad (age 19-50 years, n=216) (Table 36).

High frequencies of vitamin B_{12} deficiency among young adults were reported in several countries. In India, Yajnik *et al.* (2006) found that 67% of the participating healthy men (aged 30-50 years) had low vitamin B_{12} levels (<150 pmole/L). In Iran, Shams *et al.* (2003) studied vitamin B_{12} deficiency in 984 healthy subjects aged 20-80 years. From them, 408 subjects were between 20-40 years. Shams and his colleagues found that among this young age group, 28.3% (156 of 408) had serum B_{12} level less than 200 pg/ml, and this percentage increased to reach 53.9% having serum B_{12} <250 pg/ml (220 of 408). This comes in consistence with our results presented previously (Table 36).

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Table 36: Vitamin B_{12} deficiency in Jordan

Jordan	Cutoff point of vitamin B_{12} deficiency	n	Age (years)	Prevalence (%)
Fora and Mohammad (2005)	222 pg/ml	216	19-50	48.1%
Abu-Samak *et al.* (2008)	200 pg/ml	120	18-24	16%
Habib Allah (2008)	210 pg/ml	460	20-24	18.0%
Barghouti *et al.* (2009)	180 pg/ml	837 403	18-79 18-39	44.7% 47.1%*
The present study	180 pg/ml 200 pg/ml 222 pg/ml	165	20-40	≤180 pg/ml 18.8% ≤200 pg/ml 27.3% ≤222 pg/ml 36.4% ≤300 pg/ml 69.1%†

* In Barghouti *et al*. (2009) study, the total number of patients recruited in the study were 837 (aged 18-79 years); of them only 403 persons who matched with the age range of our study group (18-39 years).

† Prevalence of low vitamin B_{12} status (≤300 pg/ml) was 69.1%; 47.4% of them had confirmed vitamin B_{12} deficiency (according to high MMA level (≥0.376 µmole/L)); this makes the prevalence of true vitamin B_{12} deficiency 32.7%.

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Gender was shown to have no effect on the occurrence of low vitamin B_{12} level. There was no significant difference in the mean serum vitamin B_{12} level between males and females (P=0.666). Similar results were found in Fora and Mohammad study (2005), Planells *et al.*, 2003; Carmel *et al.*, 1999). These authors reported no significant differences in the mean serum B_{12} between both sexes. However, Barghouti *et al*. (2009) and Hakooz *et al*. (2006) found that males had significantly lower vitamin B_{12} level than females (P<0.05). In contrast, Habib Allah (2008) found that males had marginally higher serum B_{12} concentration than females (P=0.72).

2. Educational Level and Household Size

It was postulated that with higher educational levels, more awareness about B_{12} sources and complications is expected, and thus better B_{12} status can be expected. In the present study group, the minimum educational level was school level. The majority of them (94 of 165; 57.0%) hold Bachelor's degree. However, no association was found between educational level of participants and vitamin B_{12} status.

The average household size in Jordan according to Department of Statistics (DOS, 2008) is 5.2 persons. It was expected that upon increasing household size, the total intake of food will decrease. Moreover, bigger household size is usually associated with lower economic status; this also will contribute to lower dietary intake of food. In our study, household size had no association with vitamin B_{12} status of the volunteers (P>0.05), and the mean values of serum vitamin B_{12} level of the three groups of family size categories were not significantly different.

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3. Body Mass Index (BMI)

Body mass index (BMI) is an anthropometric index of weight and height that is defined as body weight in kilograms divided by height in meters squared **(BMI = weight (kg) / height (m)^2)**. BMI is the commonly accepted and used index for classifying adiposity in adults (CDC, 2008). Obesity was reported to be associated with increasing risk of vitamin B_{12} deficiency (Pinhas-Hamiel *et al.*, 2006). Abu Samak *et al.* (2008) found that 50% of those having vitamin B_{12} deficiency (<200 pg/ml) (16% of their study group), were overweight. However, in this study no association was found between BMI and vitamin B_{12} status (r = 0.067; P=0.424). Moreover, there was no difference in the mean serum B_{12} levels among the different BMI categories (P=0.696).

4. Hematological Parameters

Similar to the results reported by Fora and Mohammad (2005) and Barghouti *et al.* (2009), the values of several hematological parameters (MCV, Hemoglobin and peripheral blood smear) were normal in most patients, and there were no obvious clinical manifestations of vitamin B_{12} deficiency in this study. This could be explained by mild and early form of B_{12} deficiency (Herbert, 1987).

High mean cell volume (MCV) (above 100 fl) was reported to be an indicator of vitamin B_{12} deficiency (Galloway and Hamilton, 2007). In the present study, although MCV was within normal range, it showed an inverse correlation with vitamin B_{12} level (r= -0.170; P=0.03). Moreover, there was a marginal higher MCV values among those with vitamin B_{12} <200 pg/ml (Table 23). In spite of this finding, we cannot conclude that MCV alone can give an idea of having risk of vitamin B_{12} deficiency. Other blood indices showed no association with total serum B_{12} level. When correlated with MMA values, MCV and MCH showed a significant inverse correlation with MMA, although

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no significant differences were found in the mean values of these indices between the two MMA groups (<0.376 µmole/L vs. ≥0.376 µmole/L) (Table 30).

Serum vitamin B_{12} level is widely used as the standard method for diagnosing vitamin B_{12} deficiency despite its limited specificity and controversy about its sensitivity (Hvas and Nexo, 2006). Previous studies done in Jordan relay only on serum vitamin B_{12} level to determine the prevalence of vitamin B_{12} deficiency (Table 7). To check for the true prevalence, MMA test was used as a confirmatory test for vitamin B_{12} deficiency using the cutoff point defined by CDC (CDC, 2009) (which is 0.376 µmole). Any value above this level is considered a positive result and it will confirm deficiency.

Homocysteine is another metabolite of B_{12} metabolism. It was reported that values of serum vitamin B_{12} concentration were significantly and negatively correlated with plasma Hcy concentrations (Ardawi *et al.,* 2002; Fakhrazaddeh *et al.,* 2006). In this study, similar result was obtained and it was found that tHcy concentration is inversely correlated with vitamin B_{12} status (r= -0.423; P=0.001). Our results showed that those with B_{12} ≤200 pg/ml had significantly higher tHcy value than those having B_{12} between 201-300 pg/ml. However, a strict conclusion about tHcy could not be given since the test was only done for those with confirmed B_{12} deficiency (who had MMA ≥0.376 µmole/L). Nevertheless, as it can be observed, only 25.9% of those with high MMA had elevated tHcy. This makes tHcy less sensitive to B_{12} deficiency as compared with MMA.

It was previously reported that approximately 50% of patients with serum vitamin B_{12} levels >200 pg/ml have elevated tHcy or MMA. This explain that using low serum vitamin B_{12} level as the sole means of diagnosis may miss up to one half of patients with actual tissue B_{12} deficiency (Oh and Brown, 2003). In our study, we found that 44.9% of those with low-normal vitamin B_{12} levels have elevated MMA confirming their subclinical vitamin B_{12} deficiency (Table 34). Widespread metabolic testing found

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elevated MMA and/or tHcy in a considerable number of apparently healthy volunteers who had low-normal cobalamin level (up to 300 or 350 pg/ml). This represents subclinical deficiency. Moreover, in the Framingham study, it was found that 32% of those with cobalamin levels up to 350 pg/ml had abnormal tHcy and/or MMA levels (Carmel *et al*., 2003).

In the present study, 51.1% of those with low serum B_{12} level (≤200 pg/ml) had elevated MMA (≥0.376 µmole/L) (Table 34). It was reported that at least 25% of low serum B_{12} levels are not associated with elevated metabolite levels and may not indicate B_{12} deficiency. Some of these are caused by partial deficiency of transcobalamin (Wickramasinghe, 2006).

The recommendation was made to raise the cobalamin cutpoint to 300 or 350 pg/ml in order to capture these unrecognized cases of presumptive deficiency. However, this recommendation was opposed because: the gain in new cases will be almost entirely in subclinical cobalamin deficiency which predominates among persons with low-normal cobalamin level (between 200 to 300 or 350 pg/ml); and thus relatively few additional cases of clinical deficiency will be found. Also, 58-78% of persons with cobalamin level <200 pg/ml have metabolic evidence for deficiency, whereas only 32-35% of those low-normal cobalamin levels have such metabolic evidence (65-68% of persons with low-normal levels have normal cobalamin status by metabolic criteria) (Carmel *et al*., 2003).

5. Medical History

Participants in this study were chosen to be apparently healthy, with no previous diagnosis of vitamin B_{12} deficiency nor treated with vitamin B_{12} supplementation for at least one year. Participants were also free of any disease that affect B_{12} level such as:

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liver diseases, kidney problems, folate deficiency, pregnancy and untreated hypothyroidism. Any subject had any of the above conditions were excluded from the study. This helped avoiding any falsely negative or falsely positive B_{12} levels.

Subjects were checked for their family history of vitamin B_{12} deficiency. Nevertheless, no association was found between having family history of B_{12} deficiency and risk of B_{12} deficiency.

GI surgeries may have a role in increasing risk of vitamin B_{12} deficiency (Fernández-Bañares *et al*., 2009). In this study, only four subjects reported having done a GI surgery. GI surgeries in this study were found not to be associated with vitamin B_{12} deficiency. This can be related to the kind of surgery performed. In our study, two subjects had appendicitis surgery, one spleen removal surgery and one hernia rupture fixing surgery. These surgeries were not reported to increase risk of vitamin B_{12} deficiency. In contrast, there was a trend of increasing incidence of true deficiency with having previous GI surgery (Table 32).

6. Symptoms of Vitamin B_{12} Deficiency

Signs and symptoms of vitamin B_{12} deficiency show no significant difference between the different B_{12} groups except for visual impairment which was the only symptom correlated with vitamin B_{12} deficiency (P=0.012). However, these symptoms were reported by the patients and were not diagnosed by a specialized physician during the study, thus it is subject to patient recording error. Moreover, most of these symptoms are very general and can result from a variety of medical conditions other than vitamin B_{12} deficiency (NIH, 2010).

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7. Vitamin B_{12} Intake

The only source of vitamin B_{12} is food from animal origin like meats, organ meats, dairy products, fish and poultry. The average daily intake of vitamin B_{12} in the study group was 5.857 µg/day, which is much higher than the daily requirement (2.4 µg/day) (244.17% of the RDA) (Table 14). This high amount of vitamin B_{12} in diet can be related to the consumption of liver and spleen, which account for 38.49% of total intake. High amounts of vitamin B_{12} in organ meats result in the highest percentage contribution among the meat products. Red meat and dairy products comes next to liver and spleen in percentage of daily intake. Although chicken is the most frequently used animal product by participants in their meals, but it accounts for only 3.58% (0.210 µg/day) of the daily vitamin B_{12} intake. This can be explained by the low content of vitamin B_{12} in chicken compared to other meats and organ meats. Moreover, fortified bread, though consumed daily by participants, accounts for only 12.18% (0.713 µg/day) of total intake. Table 37 lists the content of vitamin B_{12} in different food items commonly consumed in Jordan.

Regarding bread fortification with vitamin B_{12}, it was found that bread accounts for only 12.18% of total vitamin B_{12} intake (Table 14). Even when we exclude it from diet, total intake will be reduced to 5.144 µg/day; which is still much higher than the recommended daily allowances (RDA). Moreover, no correlation was found between fortified bread and vitamin B_{12} status ($r= -0.045$; $P=0.561$) (Appendix 6).

Mean vitamin B_{12} intake was calculated for Jordanians based on the report by DOS (2008) (Table 38). It was found that on average, a Jordanian adult consumes a total daily amount of vitamin B_{12} of 4.62 µg/day (which equals to 192.5% of the RDA). Fortified bread was the major item to provide vitamin B_{12} and contributes to 33.12% of total daily intake and 63.75% of RDA. Fortification of bread with vitamin B_{12} in Jordan

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Table 37: Vitamin B_{12} content in food items commonly consumed in Jordan

Food items	Vitamin B_{12} µg/ 100g
Lamb liver	90.05
Chicken liver	22.98
Sardines in oil, canned	8.94
Lamb spleen	5.34
Salmon	3.18
Red meat	2.56
Catfish fillet	2.33
Tuna in oil, canned	2.2
Feta, Nabulsi cheese	1.75
Shrimps	1.16
Processed cheese	1.12
Eggs, raw fresh whole, medium	1
Monkfish (Armouti)	0.9
Yogurt cheese (Labaneh)	0.61
Grouper (Hammour) fish	0.6
Fortified bread (Jordan)*	0.51
Spread cheese	0.4
Yogurt, plain	0.37
Whole milk	0.36
Chicken thigh with bones	0.35
Chicken breast with bones	0.34

Source: ESHA, 2001

* Bread is enriched with 9 minerals and vitamins, including B_{12}; the vitamin B_{12} in bread is calculated to be 5.1 µg/kg bread (MOH, 2008).

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Table 38: Mean intake of vitamin B_{12} in Jordan as calculated from DOS (2008).

Food Item	Mean B_{12} intake ± SEM	% of contribution of daily intake	% contribution of RDA
Red meat	0.98	21.21%	40.83%
Chicken	0.4	8.66%	16.67%
Fish	0.22	4.76%	9.17%
Organ meats (Liver and spleen)	0.62	13.42%	25.83%
Dairy products	0.67	14.50%	27.92%
Fortified bread	1.53	33.12%	63.75%
Eggs	0.2	4.33%	8.33%
Total B_{12} intake	4.62	100%	192.5%

(Reference: DOS, 2008).

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aimed to cover 50% of the RDA. However, contribution of bread to vitamin B_{12} intake was half this amount (29.58% of RDA) in our study.

In contrast to what was reported before by Fora and Mohammad (2005) and Barghouti *et al.* (2009) that low vitamin B_{12} intake is associated with vitamin B_{12} deficiency, we found no significant association between total vitamin B_{12} intake and vitamin B_{12} deficiency. No differences were also found between those subjects with MMA $\geq$0.376 µmole/L and those with <0.376 µmole/L regarding vitamin B_{12} intake (Appendix 10). In the previous studies, the average daily amount of vitamin B_{12} consumed was not calculated, thus it would be difficult to conclude that Jordanians have low consumption of meat products in their daily meals. Low dietary intake appears to be uncommon as a cause of vitamin B_{12} deficiency in Jordan. Furthermore, it was also reported that low intake of vitamin B_{12} was not common in Europe and was reported to only be seen in one small Greek study. Vitamin B_{12} intake was not associated with vitamin B_{12} inadequate status in Netherlands and Germany; this explains the inadequate vitamin B_{12} status despite sufficient intake levels in these countries (Dhonukshe-Rutten, 2009).

8. Vitamin B_{12} Deficiency in Jordan

Vitamin B_{12} deficiency in most references is defined as serum vitamin B_{12} less than 200 pg/ml (Oh and Brown, 2003). Our study demonstrates that 27.3% of the subjects had vitamin B_{12} levels of less than 200 pg/ml (vitamin B_{12} deficient). Similar or higher frequencies of vitamin B_{12} deficiency were previously reported in Jordan. Fora and Mohammad (2005) showed that 48.1% of their studied population (n=216) have low vitamin B_{12} status (<222 pg/ml), while Barghouti *et al.* (2009) reported a 44.7% of subjects (total n=837) had vitamin B_{12} deficiency (<180 pg/ml). In another study done

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by Hakooz *et al.* (2006) who used cutoff point of 200 pg/ml, percentage of vitamin B_{12} deficient subjects in their study group was 50.8% in Arabs and 46.9% in Circassians living in Amman. Results of high frequency of vitamin B_{12} deficiency were reported worldwide. The prevalence of vitamin B_{12} deficiency was reported to be 26.7% in Iran (Fakhrazadeh *et al.*, 2006), 39% in North China (Hao *et al.*, 2007), and 46.8% in Turkey (Gümürdülü *et al.*, 2003). These frequencies are higher than the reported global prevalence (range between 3-40% (Carmel, 2000; Stabler and Allen, 2004). This variation may be related to ethnic and geographical variations, different lifestyle and dietary habits, different age or to different cutoff points and laboratory procedures used for measuring serum vitamin B_{12} levels.

It is clear from Table 7 that using different cutoff points for diagnosing vitamin B_{12} deficiency gave different percentages of deficiency. In this study, it was found that by increasing the cutoff level, the percentage gets higher. However, by comparing these results with those of the previous studies, percentage was found to be lower: 18.8% compared to 44.7% in Barghouti *et al.* (2009) and 36.4% compared to 48.1% in Fora and Mohammad (2005). These differences can be primarily explained by using different age groups. In the study of Barghouti *et al.* (2009), they used a wide range of age (18-78 years), having elderly present in their study. Elderly are at higher risk of having vitamin B_{12} deficiency as they may have more chronic diseases and malabsorption syndrome. Moreover, in Barghouti *et al*. (2009) study, some patients were diabetic and others have peptic ulcers. Although these conditions may cause vitamin B_{12} deficiency which in turn can give higher prevalence, Barghouti *et al*. (2009) found no significant relation between them and occurrence of vitamin B_{12} deficiency.

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9. Causes of Vitamin B_{12} Deficiency

Although diet was not found to be associated with low vitamin B_{12} status in our study, this high percentage of vitamin B_{12} deficiency may be related to changes in dietary habits by people from being dependent on homemade to manufactured food (Fora and Mohammad, 2005), or due to low bioavailability of vitamin B_{12} in food due to certain cooking procedures (Madan *et al*, 2009) or due to impaired malabsorption (Bor *et al*., 2006).

Helicobacter pylori infection was reported to be more than 60% in subjects with normal gastric mucosa (Hussain Latif *et al*., 1991). It was reported to be a probable causative agent in developing vitamin B_{12} deficiency. In one study, it was found that 56% of the patients with vitamin B_{12} deficiency had *H. pylori* infection (Kaptan *et al.*, 2000). In the present study, only 22.2% had positive *H. pylori* test. Antibodies to check for pernicious anemia were examined. It was found that 18.52% had anti-parietal cell antibodies and 9.3% had anti-intrinsic factor antibodies. The remaining subjects had no identified cause for deficiency.

Malabsorption of vitamin B_{12} may be a main contributing factor to vitamin B_{12} deficiency; any person with clinical cobalamin deficiency has a >90% likelihood of having GI diseases with malabsorption of free cobalamin. Approximately 30-40% of those patients will have food-cobalamin malabsorption. Transcobalamin I (TCI) deficiency may account for 15% of unexplained low cobalamin levels, especially those with no metabolic evidence of cobalamin deficiency at all. The causes of the remaining patients with low cobalamin levels (about 50% of all cases), whether accompanied by metabolic abnormalities or not, have not been identified (Carmel *et al*., 2003).

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Conclusions and Recommendations

Conclusions:

From the results of the present investigation, the following were concluded:

- It was found that there is high prevalence of true vitamin B_{12} deficiency in our study among the young adults (age between 20-40 years), and its higher in younger age (20-29 years) than in older age (30-40 years).
- Body mass index (BMI), educational level and household size were not found to be related to vitamin B_{12} deficiency.
- Family history of vitamin B_{12} deficiency and history of GI surgeries also were not correlated to vitamin B_{12} deficiency.
- MCV and Homocysteine levels were found to be to be higher among vitamin B_{12} deficient subjects compared to those with low normal serum B_{12} levels.
- Low dietary vitamin B_{12} intake is not related to vitamin B_{12} deficiency in Jordan and is not the main cause of deficiency as it was previously reported.
- Pernicious anemia and *H. pylori* infections account for only part of deficiency causes, and the major cause, which could be the poor absorption, is not yet determined.
- Clinical symptoms that is usually reported by people may not be related to vitamin B_{12} deficiency but to other conditions.

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Recommendations

Based on the obtained findings, the following recommendations were suggested:

- Awareness about vitamin B_{12} deficiency and other nutritional aspects should be increased at school level.
- Early diagnosis of vitamin B_{12} deficiency is recommended to prevent its clinical complications.
- For patients diagnosed for vitamin B_{12} deficiency, it is recommended that they check for the possible medical cause for deficiency in order to get the best treatment for them.
- MMA and homocysteine tests should be introduced in the medical laboratories in Jordan.
- The holotranscobalamin II (holoTC II) test should also be introduced in the medical laboratories (since it is not available in Jordan).
- MMA and homocysteine levels for those with serum B_{12} level between 200-400 pg/ml should be checked in order to check for hidden deficiency.
- The Ministry of Health is to be addressed for reducing the prices of MMA and homocysteine tests and to cover them under medical insurance as one of the routine blood tests.
- There is a need for a national study to determine the true prevalence of vitamin B_{12} deficiency by using MMA test along with total serum B_{12} test, and to determine the cutoff point of vitamin B_{12} deficiency in Jordan.
- There is a need for a national study to determine the major causes of vitamin B_{12} deficiency and to set policies to prevent the problem.

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Appendix 1

استبانة شخصية، صحية وتغذوية

الرقم المتسلسل: التاريخ:
رقم الملف:

معلومات شخصية واجتماعية

الاسم:
العنوان: تلفون:
فاكس: إيميل:
الجنس: □ ذكر □ إنثى تاريخ الميلاد: الطول: الوزن:
الجنسية:

- ضع دائرة حول السنة الدراسية التي أنهيتها
١ ٢ ٣ ٤ ٥ ٦ ٧ ٨ ٩ ١٠ ١١ ١٢ كلية مجتمع ١ ٢ ٣ ٤ ماجستير دكتوراه
المرحلة الأساسية الثانوية البكالوريوس

- ما عدد الأشخاص الذين يعيشون معك في المنزل؟

السيرة المرضية

١. هل تعاني من أي مرض؟
□ نعم حدد:
□ لا

٢. هل تعاني أي من الأمور التالية؟
□ أمراض الكبد □ الحمل
□ نقص فيتامين حمض الفوليك
□ تتعاطى أقراص أو إبر فيتامين ب١٢ □ ألم مزمن في البطن
□ أمراض الكلى □ الجفاف
□ كسل في الغدة الدرقية □ الصدفية
□ قرحة معدية أو معوية □ قرحة معدية نازفة

٣. هل تعاني من أي من الأعراض التالية؟
□ صداع □ نقصان الوزن
□ تنميل □ إرهاق
□ تعب عام □ فقدان التوازن
□ ضعف في النظر □ ضعف التركيز في النظر
□ إسهال □ ألم مفاصل
□ تقرحات في الفم

٤. هل أجريت عملية في البطن (استئصال جزئي أو كلي للمعدة أو الأمعاء) من قبل؟
□ نعم متى؟ حدد
□ لا

٥. هل يوجد تاريخ عائلي بالإصابة بأمراض الدم أو نقص فيتامين ب١٢؟
□ نعم حدد:
□ لا

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التاريخ الدوائي

• أذكر جميع الأدوية التي تتناولها ***حاليا*** بما فيها الأدوية التي لا تحتاج لوصفة طبية أو مكملات غذائية:

□ **مضادات الحساسية**
الكمية:__ حبة/اليوم
ما المدة (منذ متى)؟
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **مضادات الحموضة**
الكمية:__ حبة (أو ملعقة)/اليوم
ما المدة (منذ متى)؟
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **أدوية القرحة المعدية**
الكمية:__ حبة/اليوم
ما المدة (منذ متى)؟
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **أدوية الصدفية**
الكمية:__ حبة/اليوم
ما المدة (منذ متى)؟
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **أدوية الصداع**
الكمية:__ حبة/الأسبوع
ما المدة (منذ متى)؟
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **مكملات غذائية**: حدد النوع (مثال، Centrum)
الكمية:__ حبة/اليوم
ما المدة (منذ متى)؟ حدد النوع:
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

□ **أدوية أخرى**: أذكرها:
الكمية:__ حبة/اليوم
ما المدة (منذ متى)؟ *(لكل دواء)*
□ سنة واحدة أو أقل □ ٢-٤ سنوات □ ٥-٩ سنوات □ ١٠ سنوات أو أكثر

معلومات تغذوية

١. هل أنت نباتي؟
□ نعم □ لا

٢. هل اتبعت أية حمية غذائية خاصة سابقا؟
□ نعم: حدد نوعها □ لا

٣. هل يوجد أطعمة معينة لا تتناولها؟
أذكرها:

٤. هل تتناول المشروبات الكحولية؟
ما نوعها؟
كم مرة في الأسبوع؟
ما الكمية؟

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٥. هل تتناول أي من الأتي؟

اللحوم (اللحوم الحمراء)

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما نوعها (خاروف، بقر،...إلخ)
ما الكمية (في اليوم الواحد)؟ _____وقية
(أو)_____قطعة رأس عصفور
(أو)___قطعة (لحم المنسف)

□ لا

السمك (سمك فيليه، هامور، لقس، سلمون، جمبري، ،...)

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما نوعها؟
ما الكمية (في اليوم الواحد)؟

□ لا

الدواجن (دجاج، ديك رومي)

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما نوعها؟
ما الكمية (في اليوم الواحد)؟ _____صدر دجاج/ديك رومي
(و/أو) _____فخذ دجاج/ ديك رومي

□ لا

كبد، طحال، فوارغ،... إلخ

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما نوعها؟ (كبد (دجاج أو خاروف)، طحال (دجاج أو خاروف)، فوارغ)

ما الكمية(في اليوم الواحد)؟

□ لا

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منتجات الألبان (حليب، لبن، لبنة، أجبان)

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما نوعها؟

ما الكمية (في اليوم الواحد)؟ حليب:___كوب (كم مرة في الأسبوع؟)
لبن:____كوب أو _____ملعقة طعام (كم مرة في الأسبوع؟)
لبنة:____ملعقة طعام (كم مرة في الأسبوع؟)
جبنة:___قطعة (مثال: قطعة جبنة صفراء بحجم قطعة الجبن النابلسي)
(كم مرة في الأسبوع؟)
جبنة نابلسية:_____قطعة (كم مرة في الأسبوع؟)
جبنة قابلة للدهن:___ملعقة طعام أو___مثلث (كم مرة في الأسبوع؟)

(إذا كنت تستهلك أكثر من نوع في اليوم الواحد, الرجاء تحديدهم):

□ لا

البيض

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما الكمية (في اليوم الواحد)؟

□ لا

الخبز العربي الأبيض

□ نعم كم مرة؟

□ يوميا
□ ٥-٦ مرات في الأسبوع
□ ٣-٤ مرات في الأسبوع
□ ١-٢ مرات في الأسبوع
□ أقل من ذلك (حدد كم مرة في الشهر)

ما الكمية (في اليوم الواحد)؟ _____رغيف صغير أو_____رغيف كبير

□ لا

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Appendix 2

Reliability Test

Reliability in the general use means “dependable” or “trustworthy”. In research, the term reliability means “repeatability” or “consistency”. A measure is considered reliable if it would give us the same result over and over again. There are four general classes of reliability estimates:

- **Inter-Rater or Inter-Observer Reliability**

  Used to assess the degree to which different raters/observers give consistent estimates of the same phenomenon.

- **Test-Retest Reliability**

  Used to assess the consistency of a measure from one time to another.

- **Parallel-Forms Reliability**

  Used to assess the consistency of the results of two tests constructed in the same way from the same content domain.

- **Internal Consistency Reliability**

  Used to assess the consistency of results across items within a test (Trochim, 2006).

Cronbach’s alpha

Cronbach’s alpha is a test reliability technique that requires only a single test administration to provide a unique estimate of the reliability for a given test. It is the average value of the reliability coefficients one would obtain for all possible estimations of the items when split into two half-tests (Gliem and Gliem, 2003).

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Cronbach's alpha reliability coefficient normally ranges between 0 and 1, and may be used to describe the reliability if factors extracted from dichiotomous (questions with two possible answers) and/or multi-point formatted questionnaire or scales (i.e., rating scale: 1 = poor, 5 = excellent) (Santos, 1999). The closer the Cronbach's alpha is to 1.0, the greater the internal consistency (reliability) of the items in the scale as shown in the following table:

Cronbach's alpha	Reliability
> 0.9	Excellent
> 0.8	Good
> 0.7	Acceptable
> 0.6	Questionable
> 0.5	Poor
< 0.5	Unacceptable

(Reference: Gliem and Gliem, 2003).

Thus alpha equals to 0.7 is considered acceptable reliability coefficient but lower thresholds are sometimes used in the literature (Santos, 1999).

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R E L I A B I L I T Y A N A L Y S I S - S C A L E (A L P H A)

Statistics for	Mean	Variance	Std Dev	N of Variables
SCALE	7.8800	13.1933	3.6323	41

Item-total Statistics

	Scale Mean if Item Deleted	Scale Variance if Item Deleted	Corrected Item-Total Correlation	Alpha if Item Deleted
Q1	7.6800	12.7267	.1030	.6982
Q21	7.8800	13.1933	.0000	.6968
Q22	7.8800	13.1933	.0000	.6968
Q23	7.6800	11.6433	.4965	.6677
Q24	7.8800	13.1933	.0000	.6968
Q25	7.8400	13.2233	-.0481	.7007
Q26	7.8800	13.1933	.0000	.6968
Q27	7.8400	13.3900	-.1617	.7047
Q28	7.8800	13.1933	.0000	.6968
Q29	7.8800	13.1933	.0000	.6968
Q210	7.8800	13.1933	.0000	.6968
Q211	7.8800	13.1933	.0000	.6968
Q31	7.5600	11.3400	.5073	.6636
Q32	7.8400	13.0567	.0669	.6965
Q33	7.5200	10.6767	.7111	.6423
Q35	7.8800	13.1933	.0000	.6968
Q36	7.8000	12.7500	.1854	.6915
Q37	7.7600	12.1067	.4232	.6766
Q38	7.6000	11.8333	.3648	.6772
Q39	7.7600	12.1900	.3857	.6790
Q310	7.7200	12.4600	.2246	.6890
Q311	7.7200	13.0433	.0037	.7040
Q4	7.8000	13.4167	-.1479	.7081
M1P	7.7200	12.2100	.2301	.6891
M2P	7.8400	13.1400	.0092	.6986
M3P	7.8800	13.1933	.0000	.6968
M4P	7.8800	13.1933	.0000	.6968
M5P	7.6800	11.4767	.4329	.6700
M6P	7.6000	10.8333	.5797	.6529
M7P	7.8400	12.8067	.2422	.6901
D1	7.8000	13.0833	.0166	.7000
D2	7.7200	13.4600	-.1481	.7140
D3	7.7600	12.1067	.4232	.6766
D4	7.8400	13.1400	.0092	.6986
MEATG	7.0800	13.9100	-.2901	.7260
FISHG	7.0000	13.0000	.0348	.7006
POLTRYG	7.0000	13.0833	.0000	.7027
INTERNLG	7.4000	12.0833	.2398	.6887
MILKG	7.4400	11.6733	.3649	.6765
EGGG	7.0400	12.3733	.2583	.6866
BREADG	7.6400	11.4067	.5423	.6624

Reliability Coefficients

N of Cases = 25.0 N of Items = 41

Alpha = .6964

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Appendix 3

نموذج موافقة واطلاع

عنوان الدراسة: دراسة العوامل المسببة لنقص فيتامين ب ١٢ في عينة من المتطوعين بعمر ٢٠-٤٠ عاما من مراجعي مستشفى الجامعة الأردنية..

١. بعد قراءة المعلومات في الأسفل، يمكنك الموافقة بالمشاركة في هذا المشروع الدراسي بالتوقيع على هذا النموذج.

٢. ***جملة الأهداف والخطوات***: شكرا لاهتمامك في مشروع الدراسة والذي سيتم بالتعاون بين قسم التغذية والتصنيع الغذائي/ كلية الزراعة في الجامعة الأردنية ومستشفى الجامعة الأردنية. سيتم الحصول على معظم المعلومات المجموعة لهذا المشروع عن طريق إجراء بعض الفحوصات المخبرية ومن المعلومات الصحية والتغذوية والاجتماعية. سوف يتم التعامل مع جميع المعلومات بسرية تامة ولن يتم ذكرك بالاسم أو بأي صفات معروفة في أي كتابات أو منشورات مستقبلية.
الرجاء العلم أنه:

١. أن هذه الدراسة لن تؤثر سلبيا على صحتك ولن تكلفك أي أعباء مالية لإجراء الفحوص المطلوبة للدراسة.
٢. يمكن مشاركة نتائج الدراسة على شكل ورقة بحث أو مقال صحفي أو محاضرة في مؤتمر.
٣. لن يتم نشر اسمك في أي من المنشورات أو المحاضرات، وخلال جمع المعلومات سوف يتم استبداله برمز وستتعرف الباحثة فقط على الاسم الحقيقي.

٣. ***الفوائد المتوقعة لهذه الدراسة:*** النتائج المستخلصة من هذه الدراسة سوف يكون لها أثر ايجابي كبير في المجال الصحي خصوصا في الوقاية وعلاج نقص فيتامين ب١٢.

٤. ***جملة الموافقة***: لقد قرأت وفهمت بشكل كامل المعلومات في الأعلى، وأوافق على المشاركة تطوعا في هذه الدراسة.

اسم المشارك:_____

توقيع المشارك:_____ التاريخ:_____

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Appendix 4

Distribution of the study group according to both serum vitamin B_{12} and educational level.

Educational level	Serum B_{12} level			Total n (%)
	≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	
School	6 (13.3%)	12 (17.4%)	15 (29.4%)	33 (20.0%)
Junior college (diploma)	4 (8.4%)	10 (14.5%)	4 (7.8%)	18 (10.9)
BSc	30 (66.7%)	37 (53.6%)	27 (59.2%)	94 (57.0)
Graduate (higher) studies	5 (11.1%)	10 (14.5%)	5 (9.8%)	20 (12.1%)
Total	45 (100%)	69 (100%)	51 (100%)	165 (100%)

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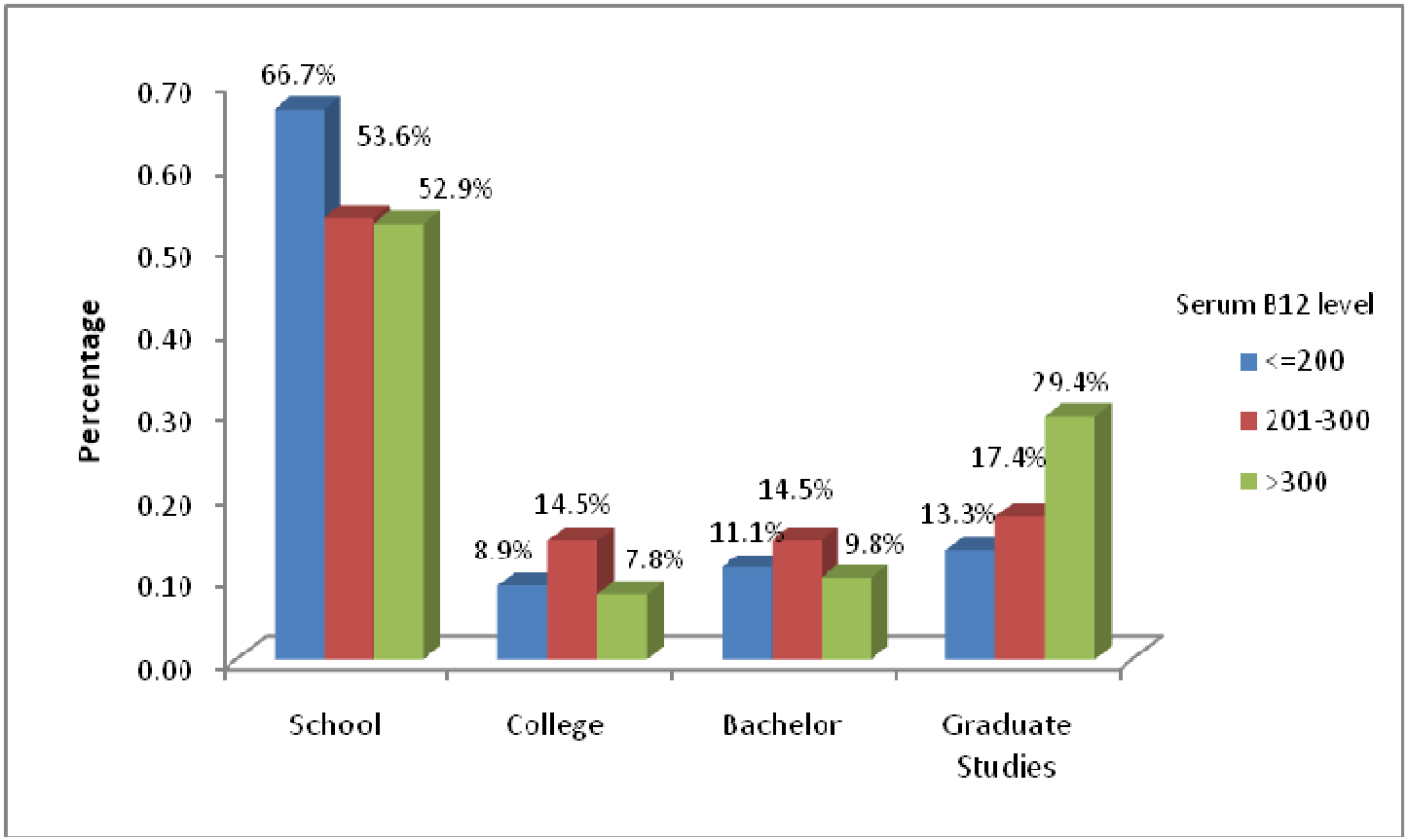

A bar diagram representing the distribution of the study group according to both serum vitamin B_{12} and educational level.

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Appendix 5

Normal values of blood indices.

Variable		Low	Normal range	High
Hemoglobin (g/dl)	Male	<140	140-180	>180
	Female	<120	120-160	>160
PCV (L/L)	Male	<0.42	0.42-0.52	>0.52
	Female	<0.37	0.37-0.47	>0.47
Erythrocytes ($x10^{12}$/L)	Males	<4.7	4.7-6.1	>6.1
	Females	<4.2	4.2-5.4	>5.4
MCV (fl)	Males	<76	76-94	>94
	Females	<76	76-99	>99
MCH (pg)	Both	<27	27-31	>31
MCHC (g/L)	Both	<320	320-360	>360
RDW (%)	Both	<14.5	14.5-19.5	>19.5
Leucocytes (WBC)	Both	<4.5	4.5-10	>10
Neutrophils ($x10^9$/L)	Both	<2.5	2.5-7.5	>7.5
Lymphocytes ($x10^9$/L)	Both	<1.5	1.5-3.5	>3.5
Monocytes ($x10^9$/L)	Both	<0.04	0.04-0.8	>0.8
Eosinophils ($x10^9$/L)	Both	<0.04	0.04-0.44	>0.44
Basophils ($x10^9$/L)	Both	<0.015	0.015-0.1	>0.1
Platelets ($x10^9$/L)	Both	<140	140-400	>400
MPV (fl)	Both	<6.3	6.3-10.3	>10.3

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Appendix 6

Correlation between selected independent variables and serum vitamin B_{12} level.

Independent variable	Serum vitamin B_{12} level (pg/ml)	P-value
	Pearson correlation (r)	
Age (years)	0.178	0.022*
BMI (kg/m^2)	0.063	0.424
Household size (persons)	-0.001	0.994
Hemoglobin (g/dl)	-0.048	0.545
MCV (fl)	-0.170	0.030*
MMA (µmole/L)	0.068	0.475
Homocysteine (µmole/L)	-0.423	0.001**
Total B_{12} intake (µg/day)	0.075	0.342
B_{12} intake from red meat (µg/day)	0.052	0.505
B_{12} intake from chicken (µg/day)	0.082	0.296
B_{12} intake from fish (µg/day)	0.034	0.661
B_{12} intake from organ meats (µg/day)	0.063	0.423
B_{12} intake from dairy products (µg/day)	0.050	0.525
B_{12} intake from fortified bread (µg/day)	-0.045	0.561
B_{12} intake from eggs (µg/day)	0.100	0.202

* Correlation is significant at P ≤0.05.

** Correlation is significant at P ≤0.01.

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Correlation between selected independent variables and serum vitamin B_{12} level (cont'd).

Independent variable	Serum vitamin B_{12} level (pg/ml)	P-value
	Pearson correlation (r)	
Erythrocytes ($x10^{12}$/L)	0.049	0.535
PCV (L/L)	-0.050	0.523
MCH (pg)	-0.131	0.095
MCHC (g/L)	-0.004	0.957
RDW (%)	0.118	0.131
Platelets ($x10^9$/L)	-0.158	0.044*
MPV (fl)	0.163	0.038*
WBC ($x10^9$/L)	0.082	0.294
Neutrophils ($x10^9$/L)	0.113	0.149
Lymphocytes ($x10^9$/L)	0.010	0.900
Monocytes ($x10^9$/L)	0.072	0.360
Eosinophils ($x10^9$/L)	0.057	0.467
Basophils ($x10^9$/L)	-0.027	0.734

* Correlation is significant at $P \leq 0.05$.

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Appendix 7

Distribution of the study group according to both serum vitamin B_{12} and body mass index classification.

BMI [1]	Serum B_{12} level			Total
	≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	N (%)
Underweight	2 (4.4%)	3 (4.3%)	3 (3.9%)	7 (4.2%)
Normal weight	29 (64.4%)	40 (58.0%)	30 (58.8%)	99 (60.0%)
Overweight	14 (31.1%)	20 (29.0%)	14 (27.5%)	48 (29.1%)
Obese	0 (0%)	6 (8.7%)	5 (9.8%)	11 (6.7%)
Total	45 (100%)	69 (100%)	51 (100%)	165 (100%)

[1] BMI= Body mass index.

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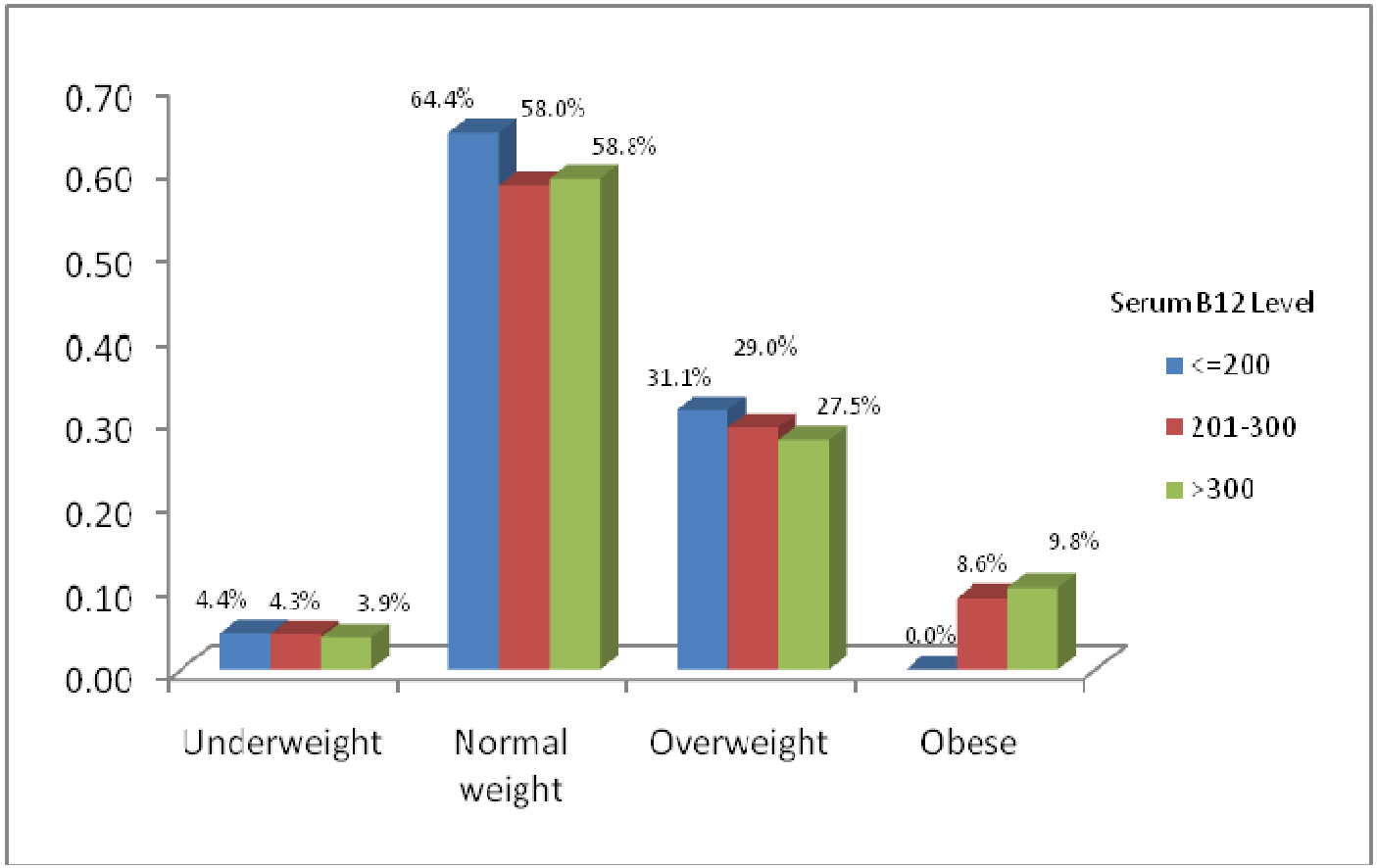

A bar diagram representing the distribution of the study groups according to both serum vitamin B_{12} and body mass index classification.

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Appendix 8

Correlation between selected independent variables and plasma MMA level.

Independent variable	Plasma MMA level (µmole/L) Pearson correlation (r)	P-value
Age (years)	0.056	0.553
BMI (kg/m^2)	0.005	0.955
Family size (persons)	-0.031	0.747
Serum B_{12} level (pg/ml)	0.068	0.475
Homocysteine (µmole/L)	-0.088	0.527
Hemoglobin (g/dl)	-0.135	0.155
Erythrocytes (x10^{12}/L)	-0.023	0.811
PCV (L/L)	-0.120	0.205
MCV (fl)	-0.200	0.034*
MCH (pg)	-0.201	0.032*
MCHC (g/L)	-0.138	0.144
RDW (%)	0.002	0.979
Platelets (x10^9/L)	0.043	0.648
MPV (fl)	0.049	0.611
WBC (x10^9/L)	0.020	0.833
Neutrophils (x10^9/L)	0.029	0.758
Lymphocytes (x10^9/L)	0.048	0.612
Monocytes (x10^9/L)	-0.091	0.335
Eosinophils (x10^9/L)	0.142	0.133
Basophils (x10^9/L)	-0.081	0.393
Total B_{12} intake (µg/day)	-0.070	0.460

***** Correlation is significant at P ≤0.05.

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Appendix 9

Distribution of the study group according to both serum vitamin B_{12} and age.

Age (years)	Total No. (165) n	Serum B_{12} level			P-value
		≤200 pg/ml n (%)	201-300 pg/ml n (%)	>300 pg/ml n (%)	
20-24	60	22 (48.9%)	22 (31.9%)	16 (31.4%)	0.038*
25-29	36	9 (%20.0)	19 (27.5%)	8 (15.763%)	
30-34	36	11 (24.4%)	11 (15.9%)	14 (27.5%)	
35-40	33	3 (6.7%)	17 (24.6%)	13 (25.5%)	

* Significant at P ≤0.05.

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Appendix 10

B_{12} intake for the MMA groups (<0.376 µmole/L and ≥0.376 µmole/L).

Food item	MMA group	N	Mean intake (µg/day) ± SEM	P-value
Red meat	<0.376 µmole/L	60	1.22 ± 0.115	0.150
	≥0.376 µmole/L	54	0.97 ± 0.129	
Chicken	<0.376 µmole/L	60	0.22 ± 0.017	0.279
	≥0.376 µmole/L	54	0.19 ± 0.017	
Fish	<0.376 µmole/L	60	0.25 ± 0.042	0.035*
	≥0.376 µmole/L	54	0.44 ± 0.082	
Liver & spleen	<0.376 µmole/L	60	2.62 ± 0.562	0.240
	≥0.376 µmole/L	54	1.75 ± 0.459	
Dairy products	<0.376 µmole/L	60	1.01 ± 0.105	0.561
	≥0.376 µmole/L	54	1.09 ± 0.101	
Fortified bread	<0.376 µmole/L	60	0.72 ± 0.066	0.970
	≥0.376 µmole/L	54	0.71 ± 0.092	
Eggs	<0.376 µmole/L	60	0.17 ± 0.022	0.457
	≥0.376 µmole/L	54	0.19 ± 0.032	
Total B_{12} intake	<0.376 µmole/L	60	6.21 ± 0.658	0.335
	≥0.376 µmole/L	54	5.35± 0.575	

* Significant at P ≤0.05.

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دراسة العوامل المسببة لنقص فيتامين ب ١٢ في عينة من المتطوعين بعمر ٢٠-٤٠ عاماً من مراجعي مستشفى الجامعة الأردنية.

إعداد

ميسون صبحي القطب

المشرف

الأستاذ الدكتور حامد رباح تكروري

المشرف المشارك

الدكتورة فريهان فخري البرغوثي

ملخص

أجريت هذه الدراسة بهدف معرفة وضع فيتامين ب١٢ في عينة من الأصحاء بعمر ٢٠-٤٠ عاماً ممن يراجعون عيادة طب الأسرة في مستشفى الجامعة الأردنية، ولمعرفة نسبة النقص الحقيقي للفيتامين لديهم، ولدراسة الأسباب والعوامل المرتبطة بالنقص. تكونت عينة الدراسة من ١٦٥ شخصاً (منهم ٩٩ أنثى و ٦٦ ذكراً) بعمر ٢٠-٤٠ عاماً، وقد قاموا بتعبئة الاستبانة المقدمة لهم والتي تحتوي معلومات اجتماعية وتعليمية وصحية وتغذوية. تم عمل فحص الدم الروتيني (CBC) وفحص فيتامين ب١٢ لجميع عينة الدراسة. بعد ذلك تم اختيار الأشخاص الذين وجد مستوى ب١٢ لديهم أقل من ٣٠٠ بيكاغرام لكل مل من مصل الدم، حيث تم إجراء فحوص مخبرية أخرى لهم. شملت هذه الفحوص فحص حمض المثيل مالونيك (Methylmalonic acid (MMA))، الهوموسيستيين (Homocysteine) ، فحص أضداد الخلايا الجدارية للمعدة Anti-parietal cell antibodies (APCA)، وفحص مضاد هيليكوبكتيريا بيولوري *Helicobacter pylori* IgA، وفحص أضداد العامل الذاتي Anti-intrinsic factor (IF) antibodies.

أظهرت النتائج أن ٦٩,١% لديهم مستوى ب١٢ أقل من ٣٠٠ بيكاغرام لكل مل، وأن ٢٧,٣% فقط لديهم نقص فيتامين ب١٢ حسب التعريف المتعارف عليه للنقص (وهو أقل من ٢٠٠ بيكاغرام لكل مل)، وأن ٣٠,٩% لديهم مستوى طبيعي من الفيتامين. وقد وجد أيضاً أن ما نسبته ٤٧,٤% من الذين لديهم مستوى ب١٢ أقل من ٣٠٠ بيكاغرام لكل مل كان لديهم نقص حقيقي للفيتامين (وذلك باستخدام مستوى MMA كمعيار مؤكِّد للنقص). كذلك أظهرت النتائج وجود علاقة طردية بين فيتامين ب١٢ والعمر؛ حيث وجد أن مستوى ب١٢ لدى الأشخاص بعمر ٢٠-٢٩ عاماً (٢٥٨,٧٨±١١,٧٩٨) كان أقل معنوياً لدى الأشخاص بعمر ٣٠-٤٠ عاماً (٢٩٣,٤١±١٢,١٩١) (P=0.047). كذلك وجد أنه لا يوجد فرق معنوي في مستوى فيتامين ب١٢ بين الذكور (٢٧٧,٨٥±١٣,٨١٤) والإناث (٢٧٠,٢٠±١١,٠٩٣) (P>0.05). كما أظهرت النتائج عدم وجود علاقة معنوية بين وضع فيتامين ب١٢ ومؤشر كتلة الجسم والمستوى التعليمي وعدد أفراد الأسرة أو إصابة أحد أفراد الأسرة بنقص فيتامين ب١٢. بالإضافة إلى ذلك، وجدت

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علاقة عكسية بين مستوى ب١٢ ومتوسط حجم كرية الدم (MCV) والهوموسيستيين؛ إذ إن معدل MCV والهوموسيستيين كان أعلى معنوياً لدى الأشخاص الذين يعانون من نقص فيتامين ب١٢ (أقل من ٢٠٠ بيكاغرام لكل مل) منه لدى الأشخاص الذين لديهم مستوى ب١٢ بين ٢٠١-٣٠٠ بيكاغرام لكل مل ($P<0.05$). أما بالنسبة لأسباب النقص، فقد أظهرت الدراسة أن ١٨,٥٢% كان لديهم أجسام مضادة للخلايا الجدارية في المعدة (APCA)، ٢٢,٢% كان لديهم *H. pylori* IgA، وأن ٩,٣% كان لديهم Anti-IF antibodies. أما بقية الأشخاص، فلم يتم التعرف على السبب الرئيسي للنقص.

يستنتج من هذه الدراسة وجود نسبة مرتفعة للنقص الحقيقي لفيتامين ب١٢ لدى عينة الدراسة باستخدام مؤشر حمض المثيل مالونيك (MMA)، وهي ٣٢,٧%؛ وأن ٤٧,٤% ممن لديهم مستوى ب١٢ أقل من ٣٠٠ بيكاغرام لكل مل لديهم نقص حقيقي للفيتامين. كما أن هذه النسبة كانت ٤٤,٩% عند من لديهم نقص حدّي من فيتامين ب١٢ (٢٠١-٣٠٠ بيكاغرام لكل مل)، مما يشير لعدم دقة فحص مستوى فيتامين ب١٢ في مصل الدم. كما وجد أن نسبة المتناول من فيتامين ب١٢ لدى عينة الدراسة كانت أعلى من الموصى بها (RDA)، وأن الغذاء ليس السبب في نقص الفيتامين. ولم يتم التعرف على الأسباب الرئيسية المسببة لنقص فيتامين ب١٢، لكنه من المرجح أن يكون ضعف امتصاص الفيتامين في الجسم هو السبب.

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