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Measurement of Serum Vitamin B12 by Different Machines: A Comparative Study in West Bank / Palestine

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Dedication

This work is dedicated to my husband, Jamal Nasser, for his caring support, and to my great parents, for their love and encourage.

To all my compassionate teachers who have never failed to give me advises and moral support.

Finally, to my sweet children; Mohammad and Juman, “your smiles are the way of my happiness”.

Suhair Fayez Mahmoud Al-Baraghithy

Declaration:

I Certify that this thesis submitted for the degree of Master in Medical Laboratory Sciences, Hematology Track, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for higher degree to any other university or institution.

Signed

Suhair Fayez Mahmoud Al-Baraghithy

Date: ……..…/……/……..

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Abstract

Vitamin B12 or cobalamin, a water soluble coenzyme, plays major roles in several biochemical pathways resulting in the production of other intermediate substance important in DNA replication as well as several neurological activities. Deficiency of vitamin B12 can lead to a wide spectrum of hematological and neuropsychiatric disorders that can often be reversed by early diagnosis and prompt treatment.

Our study aimed to compare the results of the four widely used automated analyzers for vitamin B12 assay in the West Bank / Palestine. The machines compared were; Abbott AxSYM®, ADVIA Centaur®, Immulite®2000, and TOSOH®. Thirty adult participants have been chosen. Serum samples have been run in three different clinical laboratories in Ramallah city on four different automated analyzers.

A Pearson's correlation coefficient (r) was computed to assess the correlation between the four machines. There was a positive significant correlation between each pair of the machines. With varying degrees of correlation between the comparative pairs; r ranged from 0.67 to 0.90. However, significant correlation does not mean agreement between machines or methods. The result of the slope also varies, with slopes ranging from 0.69 to 0.81. The y-intercept which reflects the constant bias (difference) between the methods also varies widely, with values ranging from 32.2 to 134.6. Despite the presence of a significant positive correlation between all comparative pairs, none of them met the conditions for methods agreement. Conditions for methods agreement are: $r > 0.95$, y- intercept close to zero and slope of the best –fit- line must be between 0.9 and 1.1.

The precision of the compared analyzers (reflected by the coefficient of variation (CV)) matches with that claimed by the manufactures. Relatively good precision has been obtained from the four methods. However, only one sample agreed upon; this sample was low in vitamin B12 concentration when measured by each of the four machines. Otherwise, variability of results was inconsistent and very high among the four machines. Some machines gave double the result of the others. This inconsistency in results can be attributed to a sample factor that requires further investigations.

This study confirms the presence of variations in the serum vitamin B12 results between different analyzers/ methods. Physician and lab-technicians should start including the metabolic products tests (tHcy and MMA) in their routine work when serum vitamin B12 result is in the gray area.

In conclusion, an isolated abnormal result of serum vitamin B12 should not be the sole criterion on which treatment decisions are made. A repeat assay, other confirmatory tests, and the clinical evaluation of the patient are necessary prior to a diagnostic conclusion.

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List of Abbreviations

Abbreviations	word
5,6 dimethylbenzim- idazole	DMB
5-deoxyadenosylcobalamin	Ado-Cbl
Atomic absorption spectrometry	AAS
Azidothymidine	AZT
Capillary electrophoresis	CE
Chemiluminescence	CL
Coefficient of variation	CV
Complete blood count	CBC
Cyanocobalamin	CN-Cbl
Deoxyribonucleic acid	DNA
Dithiothreitol	DTT
Enzyme protein binding assay	EPBA
Enzyme-linked immunosorbent assay	ELISA
Gas–liquid chromatography	GLC
Haptocorrin	HC
Hexose monophosphate shunt	HMPS
High-performance liquid chromatography	HPLC
Holo-transcobalamin	holoTC
Horseradish peroxidase	HRP
Hydroxylcobalamin	OH-Cbl
Intrinsic factor	IF
Matrix-assisted laser desorption/ionization	MALDI
Mean corpuscular volume	MCV
Methylcobalamin	Me-Cbl
Methylmalonic Acid	MMA
Microparticle Enzyme Immunoassay	MEIA
National Health and Nutrition Examination Survey	NHANES
Pearson's correlation coefficients	R
Platelet distribution width	PDW
Polymorphonuclear	PMN

Radioimmunoassay	RIA
Radioisotope dilution	RID
Radiolabeled protein binding assay	RPBA
Red blood cells	RBCs
S-adenosylhomocysteine	SAH
S-adenosylmethionine	SAM
Tetrahydrofolate	THF
Thin layer chromatography	TLC
Total homocysteine	tHcy

Chapter One:

1.1. Introduction.

Vitamin B12 or cobalamin, a water soluble coenzyme, plays major roles in several biochemical pathways that resulting in the production of other intermediate substances important in DNA replication as well as several neurological activities (Rodgers and Young, 2005). It is one of the complexes containing cobalt as central atom attached to different groups or ligands (Vogeser and Lorenzl, 2007).

Deficiency of vitamin B12 can lead to a wide spectrum of hematological and neuropsychiatric disorders. Studies mention that 15% of the individuals above 65 years old have laboratory evidence of vitamin B12 deficiency (Oh and Brown, 2003). Vitamin B12 deficiency is associated with macrocytic (megaloblastic) anemia and pancytopenia. Neurologic sequencing from vitamin B12 deficiency includes paresthesias, peripheral neuropathy, irritability, personality change, mild memory impairment and depression (Oh and Brown, 2003). When listing the consequences of vitamin B12 deficiency, we cannot ignore the effect of increased hemocystein level as a result of both folate and vitamin B12 deficiency in increasing the risk for thrombosis (Lewis et al., 2006).

Although the body stores of vitamin B12 are sufficient for at least two years the deficiency features still appear. The hematological abnormalities can be reversed by either vitamin B12 supplements or folate supplement. Folate can overcome or mask B12 deficiency since vitamin B12 itself is not involved in DNA synthesis by itself; while it is so important in recycling of folate. Folate in turn is an essential cofactor in thymidylate synthesis which is a rate limiting step in DNA synthesis, that's how you can by increasing the folate intakes overcome megaloblastic anemia (Rodgers and Young, 2005). Another important role of

B12 is its role in S-adenosyl-methionine synthesis pathway, the only way to donate methyl group essential in several reactions in brain proteins, membrane phospholipids and neurotransmitters production (Rodgers and Young, 2005).

Causes of vitamin B12 deficiency can be divided into three classes: nutritional deficiency, malabsorption syndromes and other gastrointestinal causes. Nutritional deficiency is common especially in old age people and in vegetarians. A malabsorption syndrome is another cause of deficiency. It is characterized in pernicious anemia, an autoimmune disease that affects the gastric parietal cells and so impaired the production of intrinsic factor and subsequently limits vitamin B12 absorption. Other causes of vitamin B12 deficiency include; chronic gastrointestinal symptoms, Zollinger-Ellison syndrome, Crohn's disease, patients with a history of intestinal surgery, the bacterial overgrowth that can compete for dietary vitamin B12 in the small bowel, the infestation with tapeworms or other intestinal parasites, and congenital transport-protein deficiencies, including transcobalamin II deficiency (Oh and Brown, 2003). Genetic factors may also play a role in B12 deficiency and the case of methylene tetrahydrofolate reductase polymorphisms is an example (Lewis et al., 2006).

As mentioned above, a chronic gastrointestinal disease is a major cause for B12 deficiency. This fact could be explained by understanding the vitamin B12 absorption cycle, where the acidic environment of the stomach facilitates the breakdown of vitamin B12 that is bound to food. The intrinsic factor, released by parietal cells in the stomach and binds to vitamin B12 in the duodenum. This complex subsequently aids in the absorption of vitamin B12 in the terminal ileum. Once absorbed, vitamin B12 binds to transcobalamin II and is transported throughout the body (Oh and Brown, 2003). Nearly 99% of serum B12 is bound to transcobalamin so the releasing step in the assay steps is so important (Lewis et al., 2006).

Different methods for vitamin B12 determination are now present; some of the methods have been mentioned in our previous work (Karmi et al., 2011). The microbiological method was considered as the "reference" and a traditional method. Actually, there is no

internationally reference method for B12 determination but the British standers chose the microbiological method as a reference one. It is minimally used now-a-day in the clinical laboratories but may be used to evaluate the automated methods (Lewis et al., 2006). The microbiological method first described by Ross (1950) using *Euglena gracilis* as test organism (Karmi et al., 2011). Other methods include the radioassay method, immunoassay, nonisotopic competietive protein binding assay, and mass spectrometry (Lewis et al., 2006). Because those methods are not all available for the routine clinical use for total serum cobalamin assay, the use of ligand binding methods at present as a screening test in suspected cobalamin deficiency cases are getting wider (Vogeser and Lorenzl, 2007) despite the limitations of this assay. The specificity of the total vitamin B12 assay is about 50%; this means that only 50% of the patients will have low level of B12 and clinical evidence of deficiency. While the sensitivity of it is about 95%, that is, 5% of the patients are clinically deficient with normal level of serum vitamin B12 (Lewis et al., 2006). So, one cannot rely solely on the serum vitamin B12 result to diagnose vitamin B12 deficiency; because of these limitations mentioned, the importance of other tests appears. Such diagnostic and confirmatory tests are; Methylmalonic acids (MMA), plasma hemocystin, and other vitamin B12 derivatives. (Vogeser and Lorenzl, 2007; Lewis et al., 2006).

Our study aims to compare the results of the four widely used automated analyzers for vitamin B12 assay in the West Bank of Palestine. The machines to be compared are; Abbott AxSYM®, ADVIA Centaur®, Immulite®2000, and TOSOH®. This will be the first study to be done in Palestine for this purpose.

1.2. Vitamin B12; the history, structure, and bioavailability.

Physicians were always fascinated about the causes of diseases. In 1897 the term "deficiency diseases "appeared to include all the diseases that are not caused by germs or toxics. Since - in that time - physicians were not able to isolate all of the diseases causative agents, they call some of the diseases causative agents as "unknown substances or factors". Vitamins were some of this "unknown causes"; their deficiency was a cause for different diseases that time. Later and in 1905, Cornelius Adrianus Pekelharing found that animals

fed with purified proteins, carbohydrates, fats, inorganic salts, and water would thrive only if small amounts of milk were added to the diet. So they thought that milk contains "unrecognized agents" that is essential for growth and needed in small quantities. One of the" deficiency diseases" that attracts the physicians interests that time is the disease known as polyneuritis in animals and beriberi in humans. The secret cause of this disease have been resolved after a Dutch physician Christiaan Eijkman noticed that the disease appeared only in chickens restricted to polished rice diet, and so, changing the animals diet to unpolished rice or rice polishing will cure it. In 1911, Casimir Funk isolated a concentrate from rice polishing that cured polyneuritis in pigeons, he named the concentrate "vitamine" because it appeared to be vital to life and because it was probably an amine. That was the story of the word "vitamin". From that time the name stuck to what was previously called "accessory food substances". It was not before 1920 when Jack Cecil Drummond suggested that, since there was no evidence to support the original idea that these constituents were amines, the final "e" have been dropped (Rosenfeld, 1997).

This year 2014, marks the 80th anniversary of the Noble Prize shared by Whipple, Minot, and Murphy for their discovery that raw liver contained a substance, the "anti-pernicious anemia" factor, which reversed this anemia in dogs and humans. It also marks the 50th anniversary of Dorothy Crowfoot Hodgkin's Noble prize for her X-ray diffraction work on numerous important biomolecules, including, of course, the landmark crystal structure of vitamin B12, and is also the 18th anniversary of her death (Froese and Gravel, 2010). Martens (2002) actually presents a good historical overview of vitamin B12 including its discovery, the isolation, the crystallization, and even the resolution of the crystal structures of B12 dependent enzymes and the different forms discovered. (Martens et al., 2002).

Vitamin B12 belongs to corrinoids family which represents the tetrapyrrole components (Kumar et al., 2010). As mentioned above, the structure of vitamin B12 was first solved by Hodgkin in 1964, using x-ray crystallography (Froese and Gravel, 2010). The structure of vitamin B12 is very complicated, actually the most chemically complex vitamin known (Froese and Gravel, 2010; Kumar et al., 2010). It is large organometallic molecule, range approximately form 1300–1500 Dalton in size (Kumar et al., 2010; Martens et al., 2002).

Vitamin B12 was very attractive material to study by chemists may be due to its unique ligands bound to it. Four of the ligands are the nitrogen atoms of the planar corrin ring that surround the cobalt atom. The α-axial ligand, extending below the corrin ring, is nitrogen of the 5,6 dimethylbenzimdazole (DMB), phosphoribosyl moiety that also attaches back to the corrin ring through one of its propionamide side chains. The upper or β-axial ligand varies, depending on the modification state of cobalamin (R-group), (Froese and Gravel, 2010; Kumar et al., 2010). This R group could be either an adenosyl group, cyano group, or a methyl group. Corrinoids also include vitamin B12 analogs as members in this wide family. The natural forms of vitamin B12 are hydroxylcobalamin (OH-Cbl), methylcobalamin (Me-Cbl) and 5-deoxyadenosylcobalamin (Ado-Cbl) which considered as the biological active form. Those forms are the final products for B12-biosynthesis in bacteria, while cyanocobalamin (CNCbl), is the industrial form and the most stable one which are similar in structure to cobalamin, but they are inactive or functionless in human systems, and are not bound by the protein intrinsic factor (IF).Whereas, natural forms of vitamin B12 have a strong affinity toward IF for complete absorption to those forms. As examples on vitamin B12 analogs: Cobyrinic acid hexaamide, Cobinamide (factor B), Cobamide, and Pseudo-vitamin B12 (Kumar et al., 2010).

Those derivatives or what as they called “analogs” are the results of changing of the R group in some anaerobic bacteria, or even when the R group is not presents at all as in pseudo-vitamin B12 (Martens et al., 2002). The Me-Cbl, Adl-Cbl, and OH-Cbl compounds are known to be sensitive to light, and once they are isolates they have to be transformed into the more stable form CN-Cbl by the addition of cyanide (Kumar et al., 2010).

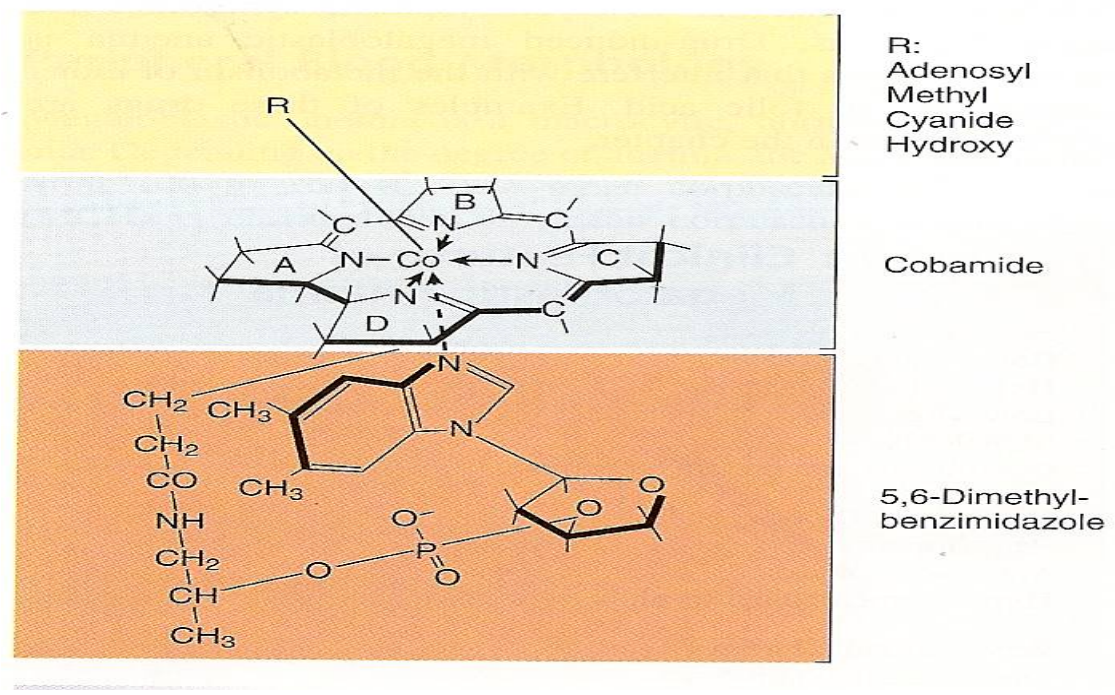

Figure 1.1: Vitamin B12 structure. Adapted from: (Kumar et al., 2010).

This unique structure gives vitamin B12 unique biochemical proprieties as well, and so this unique structure also contributes to the reactivity and function of this molecular. The reactivity is not the function of the different R groups attached to the corrin ring only, but there is another two important factors contribute to that; whether the DMB is coordinated to cobalt in the lower axial position, and the oxidation state of the central cobalt atom. The cobalt atom of cobalamin may exist in three different oxidation states which are; the +3 [cob (III) alamin], +2 [cob (II) alamin] or +1 [cob (I) alamin] oxidation state. AdoCbl, MeCbl, CNCbl and OHCbl, all of which are cob (III) alamins with the DMB nitrogen base coordinated to the cobalt in the lower axial position (Froese and Gravel, 2010).When bind to MeCbl and AdoCbl, methylmalonyl- CoA mutase and methionine synthase enzymes replaced the DMB nitrogen by a histidine of the enzyme (Froese and Gravel, 2010).

Despite their great dependence on vitamin B12, animals including humans, do not synthesize it, otherwise they depends on some bacteria and archaea which solely synthesize it, whereas, plants and fungi are thought to neither synthesize nor use it. Reports mention that if vitamin B12 presents in plants, fungi or yeast it may be due to bacterial

contamination (Martens et al., 2002). It has been suggested that vitamin B12 is an extremely old molecule in evolution (Froese and Gravel, 2010).

1.3. Vitamin B12; the dependent reactions in bacteria.

Unlike mammals and other higher eukaryotes which have only two cobalamin-dependent enzymes, methylmalonyl- CoA mutase and methionine synthase, prokaryotes depends on cobalamin as cofactor for different enzymes. These enzymes include three classes of AdoCbl-dependent mutases, the isomerases (e.g. MCM, ribonucleotide reductase, glutamate mutase), the eliminases (e.g. diol dehydratase) and the aminomutases (e.g. Dlysine- 5, 6-aminomutase), as well as the MeCbl-dependent methyltransferases (e.g. MS) and the vitamin B12-dependent reductive dehalogenases (e.g: 3–6chloro-4-hydroxybenzoate dehalogenase). (Martens et al., 2002). Other detailed information about the vitamin B12 biosynthesis and function in bacteria could be found in J. H. Marten's mini review (Martens et al., 2002).

Almost about 10 years was the time interval has been taken by Woodward and Eschenmoser to illustrate vitamin B12 chemical production. With the participation of more than 100 researcher, their work has been crowned in 1973 by the illustration of the biochemical pathway for cobalamin synthesis. Nevertheless their illustrated way is not the way followed now a day in vitamin B12 commercial production, since it is very complicated, involving more than 70 synthesis step, which make it also an expensive process. In contrast to the chemical way of synthesis, the biosynthesis of vitamin B12 using genetically optimal microorganisms considers the commercially accepted and the preferred way to produce vitamin B12 supplements now days (Martens et al., 2002).

Among the B12-producing species are the following genera: *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micromonospora*, *Mycobacterium*, *Norcardia*, *Propionibacterium*, *Protaminobacter*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus* and *Xanthomonas* (Martens et al., 2002). Those are some of the common microorganism species that have been used in vitamin B12 production. Logically, naturally high vitamin

B12 producers and rapid growth species are the optimal for the industrial production, since the two characteristics shared by the two strains *Propionibacterium shermanii* and *Pseudomonas denitrificans,* one may think that genus *Propionibacterium* have been preferred in this industry, because this genus have obtained the GRAS (generally recognized as safe) status from the United States Food and Drug Administration, but the non expected truth is that *P. denitrificans* is the most one has been used by the main B12-producing company, which cover more than 80% of the world production of vitamin B12, "Aventis"(a company appears from the fusion of two companies; Rhône-Poulenc Rorer "RPR" (France) with Hoechst AG (Germany)), this genetically engineered *P. denitrificans* used by Rhône-Poulenc Rorer supposed to reach a productivity of 100–300 mg/L. The optimal culture conditions for the production of vitamin B12 using *Pseudomonas denitrificans* could be summarized in the needs for the aeration during the whole fermentation process of about 2–3 days at 30 °C and pH values are maintained at 6–7 with the presence of sucrose as a main component in the culture medium (Martens et al., 2002).

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In contrast to *Pseudomonas denitrificans, Propionibacterium* strains dependent method is divided into two stages; in the first 3 days of fermentation, the bacteria are grown anaerobically to produce the vitamin B12 precursor cobamide, (a vitamin B12 intermediate missing the DMBI moiety), then, vitamin B12 formation is completed by gentle aeration of the whole culture for 1–3 days, allowing the bacteria to undertake the oxygen-dependent synthesis of the DMBI. The final steps in vitamin B12 production are the extraction and purification steps: the harvested cells suspension is heated at 80–120 °C for 10–30 min at pH 6.5–8.5 in order to extract the vitamin B12. While the next step is the conversion to cyanocobalamin (the most stable form) this is done by treating the heated broth or cell suspension with cyanide or thiocyanate. Then filtration or precipitated with auxiliaries tannic acid or cresol produce 80% pure vitamin B12, which is suitable for animal feed. Further purification and crystallization need for the production of cyanocobalamine to be used as food supplements (Martens et al., 2002).

1.4. Vitamin B12 absorption and malabsorption:

One of the well known facts about vitamin B12 is the non ability of mammalian synthesis for this vital organo-molecule. The question that may arise from this fact is: if the microorganisms in the mammalian large intestine are able for vitamin B12 synthesis, then why this great dependence on the acquired dietary intake of it? The answer for this logic question is actually simple when remembering that the absorption of vitamin B12 could not take place in the large intestine, while only the upper small intestine is the absorption site. So that, human depends on the natural sources such as; liver, kidney, meat, fish, dairy products, eggs, and shellfish. The average daily intake is about 4 μg, whereas the physiological needs are only 0.5-1.0 μg (Schjonsby, 1989; Froese and Gravel, 2010).

The absorption of vitamin B12 starts in stomach where it has to be released from dietary proteins, and then binds to R-protein or haptocorrin (Schjonsby, 1989; Froese and Gravel, 2010).The acidity of the stomach accompanied to pepsin actually facilitates the releasing process for dietary protein, but it is not the only factor play a role; food sources, and cooking and food preparation may also play a role. It has been proved that gastric juice R-proteins are found in many body fluids rather than gastric fluid, including; saliva, bile, intestinal juice, and serum (Schjonsby, 1989).

Vitamin B12 remains bound to R-protein until pancreatic proteases in upper small intestine partially degrade R-protein and enable vitamin B12 to bind to intrinsic factors ”IF”. The journey of vitamin B12 absorption continues in the ileum, where the vitamin B12-intrinsic factor complex attaches to specific membrane receptors which are located at the bottom of the pits between the microvilli, in the entire distal of the human small intestine (Schjonsby, 1989; Froese and Gravel, 2010). The attachment is highly specific, means that the receptor does not take up vitamin B12 bound to binders other than intrinsic factor nor does it take up vitamin B12 analogues. Have to know also that the binding site on vitamin B12 for intrinsic factor is separate from that which attaches to the receptor, means that the abnormal intrinsic factors bind vitamin B12 normally and also react normally with antibody to intrinsic factor, but it cannot attach to the receptor (Schjonsby, 1989). This

highly specific attachment considered as the first screening mechanism to prevent degraded cobalamins from being intracellular taken (Froese and Gravel, 2010). The receptor is composed of a heterodimer of amnionless and cubilin, called cubam (Froese and Gravel, 2010) and megalin, an associated membrane transport protein (Koury and Ponka, 2004).

Although the receptor has not been fully characterized, the attachment optimal conditions have been well studied and summarized in the need for the presence of calcium-ions and a neutral pH (Schjonsby, 1989). The next event in the vitamin B12 absorption series occurs in inside the cell, where IFs are degraded in the lysosomes and vitamin B12 is released into the cytosol, from where it has to be transported and released into the bloodstream (Froese and Gravel, 2010). The fate of intrinsic factor is unclear; since it is not absorbed into the blood, so, it has to be transported into the cell along with vitamin B12, or it may be released at the cell surface (Schjonsby, 1989).

Our vital element “vitamin B12” is now in the bloodstream where it has to be bind to either haptocorrin (HC) or transcobalamin II (TC). Around 75–90% of plasma cobalamin binds HC, this amount binds HC is not involved in cellular cobalamin uptake, thats why individuals who have deficient or absent HC have serum cobalamin values in the deficient range, but show no sign of cobalamin deficiency. Only 10–25% of plasma cobalamin binds to transcobalamin (that is produced by the microvascular endothelium of ileal villi (Koury and Ponka, 2004)), and this is the part of cobalamin actually uptakes by the cells (Froese and Gravel, 2010).

Congenital abnormalities such that mutations occurs in the gene encoding TC (TCN1) result in severe tissue cobalamin insufficiency, megaloblastic anemia, failure to thrive or even neurological complications, despite normal plasma cobalamin level. This feature of cobalamin deficiency could be reversed by raising the plasma level to very high levels of cobalamin so that some tissue may take up unbound cobalamin (Froese and Gravel, 2010). This treatment strategy resembles some how the oral administration of high dose of cyanocobalamin, which according to Alfred Doscherholmen and Paul S.Hagen

experiments may increase the plasma level of cobalamin in pernicious anemia patient (Doscherholmen and Hagen, 1957). It is also important to mention that TC, like IF, is very specific for cobalamin rather than its analogous (Froese and Gravel, 2010).

Of course, vitamin B12 absorption journey could not be finished elsewhere, it is finished here in liver, the place where vitamin B12 has to be conserved and stored, and where the cobalamin analogous released through bile to outside the body (Schjonsby, 1989).

The malabsorption of vitamin B12 has different causes, and so, different pathophysiological basics. Any abnormality in the gastric function or total gastrectomy cause deficiency of intrinsic factor, and so, malabsorption of vitamin B12. While the partial gastrectomy decreases the plasma level of vitamin B12 without significant hematological or neurological abnormalities. The deficiency or the insufficient production of intrinsic factors called “pernicious anemia”. Pernicious anemia could also be congenital occurs before the age of two, where there is no production to IFs at all, but the gastric structure and function is normal (Schjonsby, 1989).

Another cause for vitamin B12 malabsorption is the pancreatic insufficiency. The secretion of pancreas -which includes sodium bicarbonate - is required for optimizing the pH for the absorption process of the vitamin B12-intrinsic factor complex. Not only for pH but also it is believed that the pancreatic secretion contains enzymes that play role in appropriate absorption (Schjonsby, 1989).

Actually, vitamin B12 in some cases may be absorbed normally, till it reaches the intestinal lumen, where the bacterial over growth may take up and consume the intrinsic factor bound vitamin B12, and that cause is another suspected one that may results in vitamin B12 malabsorption. This condition is treated by antibiotics to reduce the over growth of normal flora (Schjonsby, 1989). Ileal diseases such as coeliac disease, tropical sprue and Crohn's disease of the ileum, all result in vitamin B12 malabsorption which is due to the loss of absorptive surface (Schjonsby, 1989).

1.5. Vitamin B12 deficiency; the prevalence, causes and symptoms:

Our search for sources that may provide us with complete information about the exact prevalence of vitamin B12 deficiency here in Palestine was failed. The only source was the thesis of Amani Ghaleb Mahmoud Yasin submitted for Master Degree in An-Najah University. This thesis showed that 43.3% of boys and 44.8% of girls among the age of 10-18 years have vitamin B12 level of < 200 pg/mL. However, only 10 of those have MCV level > 92 fl indicating that 94.4% of those with vitamin B12 level < 200 pg/mL cannot be considered having vitamin B12 deficiency, especially since the analysis of the attached questionnaire showed that they had no symptoms associated with vitamin B12 deficiency. The results of this study spot light the importance for a wide investigation of normal vitamin B12 level among Palestinian adolescents (Yasin, 2009). This thesis actually could be misleading somehow; because the presence of a relation between hematological abnormalities and neurological disease in vitamin B12 deficiency is not must, meaning that more than one quarter of patients with neurological manifestations of vitamin B12 deficiency have either a normal hematocrit or a normal mean corpuscular volume (MCV), or even the both may be normal. Also, mean corpuscular volume (MCV) as a diagnostic tool for vitamin B12 deficiency lacks specificity since other causes for megaloblastic disorder rather than vitamin B12 deficiency presents. Also, in patients with either vitamin B12 or folate deficiency, the MCV tends to increase. In addition, if concurrent iron deficiency or thalassemia presents the MCV remains within the reference (Snow, 1999).

The true prevalence of vitamin B12 deficiency in the general population is unknown. The incidence appears to increase with age. Some mentions that 15 % of adults older than 65 years had laboratory evidence of vitamin B12 deficiency which could be due to the use of gastric acid–blocking agents (Oh and Brown, 2003). Several studies aimed to estimate the prevalence of vitamin B12 deficiency in specific groups of people. Pflipsen et al. *(2004)* studied the prevalence of vitamin B12 deficiency in type 2 diabetic patients and found that 22% prevalence of metabolically confirmed B12 deficiency presents in the primary care type 2 diabetic population. Pflipsen et al.*(2004)* re-back their study results to this specific group of patients taking of Metformin which may contribute to vitamin B12 deficiency . Another specific group of patients was those patients under a continuously treatment for

Phenylketonuria (PKU). The work of studying the prevalence of functional vitamin B12 deficiency in PKU patients has been taken by Vugteveen et al. They found that vitamin B12 concentration within the reference range does not automatically imply a sufficient vitamin B12 status in their study group (Vugteveen et al., 2010).

As previously mentioned, the incidence of vitamin B12 deficiency appears to increase with age. It has been shown in elderly people, the prevalence of atrophic gastritis ranges from 20% to 50%. Gastric atrophy is a possible cause, but only in the most severe cases of gastric atrophy the intrinsic factor secretion decreases. Nevertheless, atrophic gastritis has been reported to cause impaired release of vitamin B12 from food proteins and peptides due to impaired acid secretion and reduced digestion by pepsin. Keeping in mind, the effect of atrophic gastritis on bacterial overgrowth in the stomach and proximal small bowel, which may also reduce the available amount of vitamin B12 via consumption (Selhub et al., 2000).

Another cause for vitamin B12 deficiency is the nutritional deficiency. Normally, human storage of vitamin B12 is sufficient for at least two years, but in the case of severe malabsorption especially in elderly, vitamin B12 deficiency could be progressed. Other causes include chronic alcoholics, the strict vegetarians (Oh and Brown, 2003), or even some medications that interfere with gastric acid secretion such as H2-histamine receptor blockers or proton pump inhibitors (Koury and Ponka, 2004).

Deficiency of vitamin B12 can lead to a wide spectrum of hematological and neuropsychiatric disorders that can often be reversed by early diagnosis and prompt treatment. Vitamin B12 deficiency is associated with macrocytic (megaloblastic) anemia, and in advanced cases of deficiency, pancytopenia. Neurologic sequencing from vitamin B12 deficiency includes paresthesias, peripheral neuropathy, irritability, personality change, mild memory impairment and depression (Oh and Brown, 2003). When listing the consequences of vitamin B12 deficiency, one cannot ignore the effect of increase hemocystein level as a result of both folate and vitamin B12 deficiency which increase the risk for thrombosis (Lewis et al., 2006).

Functional vitamin B12 deficiency defined as high methylmalonic acid [MMA] values despite normal serum vitamin B12 levels. In other words, functional vitamin B12 deficiency was defined as MMA values >250 nmol/L with vitamin B12 levels >400 pg/mL. This is common condition, has to be remembered when talking about vitamin B12 deficiency (Vugteveen et al., 2010).

1. 6. Vitamin B12; the physiology.

Animals and humans both requires vitamin B12 as an essential element in their diet, although it is required in small quantities, vitamin B12 is essential for DNA synthesis, RNA synthesis ,for fatty acids metabolism, and for other metabolic path ways. It is needed as cofactor for only two enzymes in animals and humans; methylmalonyl- CoA mutase (adenosylcobalamin- dependent) and methionine synthase (Methylcobalamin-dependent enzyme), the crystal structures of them have been solved in 1996 and 1994, respectively (Martens et al., 2002).

Methylmalonyl-CoA mutase is involved in the metabolism of propionyl-CoA, which in turn derived from the degradation of compounds like thymine, valine, methionine and odd-chain fatty acids. Propionyl-CoA is carboxylated to form (S) - methylmalonyl-CoA. Here the turn of methylmalonyl-CoA mutase starts, this enzyme is responsible about the rearrangement of (S) - methylmalonyl-CoA to succinyl-CoA, which ends up in the tricarboxylic acid cycle. In vitamin B12-deficiency cases, the methylmalonyl-CoA accumulates and then cleaved to coenzyme A and methylmalonic acid, which in turn leads to acidosis. Propionyl-CoA also accumulates until citrate synthase condenses it with oxaloacetic acid to 2-methylcitric acid (Martens et al., 2002).

The other cobalamin / folate dependent enzyme is methionine synthase, which methylates homocysteine to form methionine, which in turn utilizing 5-methyltetrahydrofolate as methyl donor. Despite the needs of this enzyme to cobalamin as essential cofactor, normal

methionine concentrations are maintained in vitamin B12 deficient patients because high folic acid level masks low vitamin B12 level (Martens et al., 2002).

As previously mentioned, cobalamin and folate deficiency are mainly associated with hematological abnormalities; one of the hematological abnormality features is anemia. The question is how the cobalamin and folate deficiency leads to anemia? Erythropoiesis is the process by which the hematopoietic tissue of the bone marrow produces red blood cells (Koury and Ponka, 2004).

Erythropoiesis required the presences of several nutrients such as; folate, vitamin B12, and iron. This process is forced by many bio-signals, in order to produce sufficient and normal red blood cells (RBCs) capable to appropriately do their functions. RBCs functions could be summarized in transporting carbon dioxide and nitric oxide, and to deliver oxygen from the lungs to the other tissues of the body. Sufficient and normally function red blood cells is as important as having normal lifespan (about 120 day) according to RBCs, otherwise hemolytic anemia will results. Hemolysis or bleeding makes a pressure on hematopoietic stem cells to exceed the rate of erythropoiesis from 200 billion cells per day to about one trillion per day (Koury and Ponka, 2004). The production of such huge number of cells requires the maintenance of adequate supplies of the nutrients needed. Our interest "vitamin B12" delivered to the cells in his active form; holotranscobalamin II (vitamin B12–transcobalamin II complex) and enters to the cells via specific receptor proteins that are displayed on the surface of many different types of cells, including, of course, the hematopoietic tissue (Koury and Ponka, 2004).

An inadequate supply with vitamin B12 to the hematopoietic tissue is responsible about the appearance of megaloblastic anemia features. This type of anemia; megaloblastic anemia, although affects all hematopoietic lineages, but seems to be most prominent in the erythroid lineage. Megaloblastic anemia has common characteristics and features such; pancytopenia with macrocytic erythrocytes, hypersegmented neutrophilic granulocytes (even with increased lobe size as well as number of nuclear segments (Lewis et al., 2006), and reticulocytopenia (Koury and Ponka, 2004).

The bone marrow - and occasionally the blood stream (Lewis et al., 2006), has increased numbers of large immature-appearing erythroblasts and myeloblasts, which are called megaloblasts (Koury and Ponka, 2004). Megaloblasts have a characteristic chromatin pattern with immature nucleus for the degree of cytoplasmic haemoglobinization (Lewis et al., 2006).

Other hematological and chemical parameters appear include; elevated serum bilirubin, lactate dehydrogenase, myeloperoxidase, and increased iron turnover, as result of hemolysis (Koury, Ponka, 2004). Mean corpuscular volume (MCV) may increase up to 130 fl, while the platelet distribution width (PDW) decreases. Blood film preparation is important to investigate the following abnormalities; oval macrocytes, poikilocytes, and hypersegmentation of neutrophils, Howell–Jolly bodies, basophilic stippling, and platelet anisocytosis (Lewis et al., 2006).

Impaired DNA synthesis is the responsible element in the increased hematopoietic cell death that characterizes the megaloblastic anemia, especially in such high proliferation rate where the erythroid progenitor cells becomes more susceptible to the impaired DNA synthesis. In S-phase of the cell cycle, erythroblasts in patients with folate or vitamin B12 deficiency anemia had no active incorporation of 3H-thymidine into DNA, despite the increase in the total DNA content (between 2N and 4N) (Koury and Ponka 2004). Experiments aimed to study the percentages of cells in S-phase in bone morrow of vitamin B12 or folate deficient patients shows increased percentages of cells in S-phase compared to controls, with relatively decreased in the rate of DNA synthesis (Koury and Ponka 2004). Impaired DNA synthesis result in chromosomal breakage which appears as increased in Howell-Jolly bodies (Lewis et al., 2006).

As mentioned above, methylcobalamin is the coenzyme for the vital enzyme methionin synthase. This enzyme transfers the methyl group from 5-methyl-tetrahydrofolate (THF) to homocysteine, thereby regenerating methionine and tetrahydrofolate (THF). In the deficiency cases, where vitamin B12 is not provided in sufficient amounts; there will be an accumulation in homocysteine and decrease in the availability of methionine and

tetrahydrofolate (THF) (there will be trapping of intracellular folate in the 5-methyl-THF form). THF an especially its form (10-formyl-THF) is involving in the synthesis of purines. The other important form of THF that involving in the synthesis of thymidylate is the 5, 10-methylene-THF. Both purines and thymidylate are important in the synthesis of deoxynucleotides. So, the deficiency in them as a result of vitamin B12 or folate deficiency will be reflected as decrease in the deoxynucleotide nuclic acid (DNA). This impaired DNA synthesis and repair is the cause of cell death. According to erythropoiesis, ineffective erythropoiesis results, where erythroid cells cannot reach maturity and undergo apoptosis causing anemia and other hematological abnormalities (Koury and Ponka, 2004).

In one experiment aimed to study the correlation between the impaired DNA synthesis and the cellular events ends with apoptosis, it has been noted that proerythroblasts freshly isolated from folate-deficient mice and when cultured with erythropoietin under folate-deficient conditions accumulate in S-phase of the cell cycle, till they will undergo apoptosis eventually (Koury and Ponka, 2004). Those cells may be saved from apoptosis if sufficient amounts of both thymidine and a purine add to the culture medium in-vitro. In conclusion, there are two main causes that contribute to the features of megaloblastic anemia; the shift to left occurs in erythroid differentiation stages leading to the accumulation of cells in S-phase, and the increased size compared with less immature appearance of erythroid cells in the bone marrow (Koury and Ponka, 2004).

Although the erythroid linage is the most affected one, it is not the only affected linage in the hematopiosis process in vitamin B12 deficiency. Megaloblastic anemia is also associated with qualitative and quantitative abnormalities of developing and mature polymorphonuclear (PMN) leukocytes. Such deficiency usually is associated with macrocytic PMN precursors and reduced numbers of circulating PMN leukocytes with hypersegmented nuclei (Kaplan and Basford, 1976). It has been believed that those abnormalities may also be a result of impaired DNA synthesis (Kaplan and Basford, 1976).

Kaplan and Basford, in their experiment tried to find an answer about the presence of any relation between this abnormal PMN cells and the function of them. To study the presence

of such relation they investigated seven patients; three with folic acid deficiency and four with vitamin B12 deficiency. They evaluated the efficiency of phagocytosis, the phagocytosis associated activation of the hexose monophosphate shunt (HMPS) and microbicidal capacity against *Staphylococcus aureus* (Kaplan and Basford, 1976). Before they illustrate their findings, Kaplan and Basford re-back to the previous work had been done. It had been previously noted that leukocyte in vitamin B12 deficiency had impaired propionate oxidation to CO_2 due to the decrease in methylmalonyl CoA mutase activity; even this finding could not investigate the effect of that on the leukocyte function. Another work had been demonstrated that animals newborn feed on folate deficient diet were susceptible to infections. Kaplan and Basford, had been concluded that" no impairment of phagocytosis-associated metabolism or microbicidal activity in the leukocytes of patients with folic acid deficiency. Only in four patients with vitamin B12 deficiency a marked decrease in the phagocytosis-associated HMPS activation occurs (Kaplan and Basford, 1976). They also found that this defect has been corrected with therapy (Kaplan and Basford, 1976).

There is a certain relation between vitamin B12 deficiency and some neurological dysfunction. It has also been reported that in case of low vitamin B12 and other vitamins level, healthy elderly subjects scored poorly on tests of memory and nonverbal abstract thinking (Selhub et al., 2000).The possible interpretation of how vitamin B12 deficiency left that dangerous effect on the neurological function could be easily understood when reviewing the biochemical pathway involved vitamin B12 as a cofactor. 5, 10-methylenetetrahydrofolate generated mainly from serine used in several reactions leads to the production of thymidylate and purines that's used for nucleic acid synthesis, and also of methionine. Methionine used for protein synthesis and biological methylations, and it is so important for the brain function. Methionine synthesis by the reduction of 5, 10-methylenetetrahydrofolate to 5-Methyltetrahydrofolate, this reaction catalyzed by the methylenetetrahydrofolate reductase. 5-methyltetrahydrofolate, in turn, act as a substrate to methylate homocysteine. This reaction is catalyzed by vitamin B12 dependent enzymes. Methionine is also converted to *S*-adenosylmethionine (SAM) (a universal methyl donor), SAM-dependent methylations in the brain results in the production of neurotransmitters,

phospholipids, and myelin Such deficiency usually is associated with macrocytic PMN precursors and reduced numbers of circulating PMN leukocytes with hypersegmented nuclei no impairment of phagocytosis-associated metabolism or microbicidal activity in the leukocytes of patients with folic acid deficiency. That could explain the loss of the neurocognitive functions in vitamin B12 and folate deficiency. The final thing have to be added according to how low vitamin B12 affects the neurocognitive functions is the observation that SAM has to be converted to *S*-adenosylhomocysteine (SAH) in this methylation reactions, which in turn hydrolyzed to homocysteine and adenosine. So, in the state of folate or vitamin B12 deficiency, there will be a decrease in methylation of homocysteine, which leads to SAH accumulation, which acts as inhibitor for other vital SAM- dependent reactions (Selhub et al., 2000).

1. 7. Vitamin B12 deficiency; diagnosis and treatment.

In megaloblastic anemia, the impaired production of tetrahydrofolate causes a defect in DNA synthesis acompanied with normal cytoplasmic component production. This defect results in abnormal large cells that characterized this anemia. The presence of unexplained anemia, macrocytosis, or neurological disease, turns the eyes toward vitamin B12 deficiency (Snow, 1999). Other conditions where vitamin B12 deficiency should be suspected include; sore tongue, anorexia, and diarrhea. Attention should also be paid to patients at risk of developing vitamin B12 deficiency including; elderly people, vegetarians , patients with intestinal diseases, patients with autoimmune disorders such as Graves' disease, thyroiditis, patients receiving proton pump inhibitors, and histamine receptor antagonists (Hvas and Nexo, 2006).

The presence of a relation between hematological abnormalities and neurological disease in vitamin B12 deficiency is not a must, meaning that more than one quarter of patients with neurological manifestations of vitamin B12 deficiency have either a normal hematocrit or a normal mean corpuscular volume (MCV), or even the both may be normal (Snow, 1999).

Also, the presence of macrocytic red cells is not an exclusive feature for vitamin B12 deficiency. Macrocytosis is also seen in myelodysplasia (could be confirmed by the presence of hypogranular neutrophils or monocytosis), excess alcohol consumption, hypothyroidism, liver disease, aplastic anemia, rare inherited orotic aciduria, or Lesch-Nyhan syndrome also increased the MCV. In addition, some drugs that interfere with DNA synthesis (e.g.; azathioprine, azidothymidine [AZT], or hydroxy-carbamide) may lead to macrocytosis and megaloblastic (Lewis et al., 2006). From here, it is become clear that accurate and reliable diagnosis for vitamin B12 deficiency is a base where the proper treatment lay on.

Complete blood count (CBC), blood film, vitamin B12 assay, antiparietal cell antibodies, anti-intrinsic factor antibodies, Schilling test, and the assay of other biomarkers, all of these have been considered as diagnostic tools in the journey of vitamin B12 deficiency diagnosis. All have drawbacks and benefits, and that's what will be discussed in the following few lines.

Macrocytosis is a term that has been used to describe the condition when MCV is above 100 fl. This condition may or may not associate with anemia. Megaloblastic disorder is a term that has been used to define the presence of morphologic changes in the bone marrow that are caused by impaired DNA synthesis. It is usually implies a diagnosis of vitamin B12 or folate deficiency. Note that, the term "usually" has been used that's because in some cases the presence of macrocytosis may also be cause by nonmegaloblastic disorder, i.e. due to the direct effect of alcohol and chemotherapeutic agents on the bone marrow, or due to the effect of some drugs on the synthesis of DNA, or due to the acceleration of erythropoiesis caused by hemolysis or hemorrhage (both conditions increase the reticulocytes count which in turn will be reflected as elevated MCV), even it could be artificial; due to cold agglutination (causes RBCs clumps) or severe hyperglycemia (that causes RBCs swelling) (Snow, 1999).

The fact that mean corpuscular volume (MCV) as a diagnostic tool for vitamin B12 deficiency lacks specificity becomes clear when reviewing the presence of other causes for

megaloblastic disorder rather than vitamin B12 deficiency. It also lacks sensitivity; that is, in patients with either vitamin B12 or folate deficiency, the MCV tends to increase before the hemoglobin level decreases. In addition, if concurrent iron deficiency or thalassemia presents the MCV remains within the reference range even when there is biochemical evidence of vitamin deficiency (Snow, 1999).

When talking about vitamin B12 diagnosis, examination of the peripheral smear seems a helpful tool. Well examination of well prepared smear may provide diagnostic clues as the presence of oval macrocytes suggesting a megaloblastic disorder, whereas the presence of stomatocytes is significantly more common in patients with macrocytosis caused by alcoholism. The presence of hypersegmented neutrophils has been found to be highly sensitive and specific for the diagnosis of megaloblastic anemia but not in the mild deficiency cases where it becomes neither sensitive nor specific (Snow, 1999).

Serum vitamin B12 level measurement has been used worldwide since the 1950s (Hvas and Nexo, 2006). It is the most fabulous test. Varying sensitivity and specificity have been reported. Also, different methods have been established. The sensitivity and specificity of vitamin B12 level assays will be discussed in chapter 4.

The two metabolic markers methylmalonic Acid (MMA) and homocysteine (tHcy) are generally considered more sensitive indicators of vitamin B12 status than are plasma vitamin B12 levels (Hvas and Nexo, 2006). They have been used as confirmatory test for vitamin B12 status in addition to their role in differentiation of vitamin B12 and folate deficiencies. Both metabolite levels increase in vitamin B12 deficiency, whereas, only tHcy level would be expected to increase in patients with folate deficiency (Snow, 1999). Factors other than vitamin B12 deficiency that may elevate methylmalonic acid include; renal insufficiency (modest elevation compared with vitamin B12 deficiency), hypovolemia, and inherited metabolic defects. While, antibiotic-related reductions in bowel flora may reduce methylmalonic acid. According to homocysteine, folate deficiency and pyridoxine deficiency, in addition to renal insufficiency and hypovolemia may elevate

it. Because of that, it seems difficult to estimate the specificity of those markers (Snow, 1999).

The disadvantages of those bio-markers assay have been noticed. A study mention that normal plasma vitamin B12 levels as well as normal MMA and tHcy levels were found in patients with clinical signs of vitamin B12 deficiency improving after treatment with the vitamin. This note should be added to the fact that MMA and tHcy assays are complex, and expensive (Hvas and Nexo, 2006).

When vitamin B12 deficiency found to be a result of inadequate production of intrinsic factor (IF), as in pernicious anemia patients, the assay of different antibodies may be helpful in diagnosis. Antiparietal cell and Anti–intrinsic factor antibodies are two examples; antiparietal cell antibodies as a cause of autoimmune gastritis found in about 85% of pernicious anemia patients. It seems to be nonspecific in pernicious anemia diagnosis. In contrast, the anti–IF antibody test is considered a relatively specific one, despite having problem concerning its sensitivity; where only half of the patients with pernicious anemia have detectable anti-IF antibody (Snow, 1999).

To overcome the limitations of the tests listed above, eyes have to go toward the measurement of that fraction of vitamin that could enter the cell (via specific receptors) and do the function, rather than the measurement of total plasma vitamin B12 level. This active form of vitamin B12 attached to transcobalamin and called holo-transcobalamin (holoTC) may be a sensitive marker (Hvas and Nexo, 2006) (Woo et al., 2010). One-quarter of circulating vitamin B12 binds to transcobalamin (holoTC) and is thereby available for the cells of the body while the larger fraction of it binds to haptocorrin (Nexo and Hoffmann-Lucke, 2011). In 1980s holo-transcobalamin could only be measured by calculation of the difference between vitamin B12 and the fraction of vitamin B12 that was not attached to transcobalamin, so, the results was not accurate enough to evaluate this test and to compare it with other assays such vitamin B12 plasma level and other biomarkers (Nexo and Hoffmann-Lucke, 2011). Now a day, methods for measuring holoTC have been introduced. Actually, three methods for its measurement are present; first, the direct

measurement of the complex between transcobalamin and vitamin B12. Second, is measurement of vitamin B12 attached to transcobalamin, and the final is the measurement of the amount of transcobalamin saturated with vitamin B12. Although these three methods give similar results, direct measurement of holoTC complex is preferable since it is more practical than the others (Nexo and Hoffmann-Lucke, 2011). It has been shown from the evaluation of the available methods that AxSYM holoTC assay is a reliable and reproducible technique for the measurement of holoTC levels (Woo et al., 2010). In conclusion, if the serum vitamin B12 level is between 151-300 pmol/L, holoTC alone or in combination with the serum vitamin B12 is likely to be more useful than serum vitamin B12 levels alone to indicate vitamin B12 status (Woo et al., 2010). HoloTC is an early marker of vitamin B12 deficiency, but further studies are also needed in order to evaluate it (Snow, 1999).

The speech about holoTC never stopped; because of the risky inputs of vitamin B12 deficiency in old age people. Finding a reliable screening test should take greater interests. This facet attracts Clarke group attention. They design a study in order to compare between the usage of conventional vitamin B12 assay and the new interest holoTC. Their population was composed of 2403 of older people with normal and abnormal renal function. Modest superior diagnostic utility of holoTC compared with conventional vitamin B12 testing for the detection of vitamin B12 deficiency has been demonstrated. This superior diagnostic accuracy of holoTC was also confirmed in two independent populations in Oxford, using slightly different assay methods to measure holoTC concentrations. They also recommend that neither test can be used to screen asymptomatic populations (Clarke et al., 2007)

In its roundtable which took place in July 2010 the National Health and Nutrition Examination Survey (NHANES) discussed the potential utility of measuring holoTC for future NHANES. In summary, the roundtable experts (23 experts in folate and vitamin B12 assessment, clinical laboratory science, and biostatistics and 10 scientists from US government agencies that generate or use NHANES data and develop reference methods and materials for these measures) supported reinstating vitamin B12 related biomarkers in

future NHANES. The roundtable also agreed that future NHANES should include at least one biomarker of the two circulating vitamin B12 concentrations (they prefer vitamin B12 rather then holoTC) and one metabolic indicator of vitamin B12 insufficiency (they prefer MMA on tHcy) (Yetley et al., 2011).

In order to diagnose whether the vitamin deficiency is caused by lack of intrinsic factor, Shilling's test has been introduced. In this test labeled vitamin B12 has to be administered orally alone (Shilling's test I) or together with intrinsic factor (Shilling's test II).With the development of advanced techniques such as acceleration mass spectrometry, theoretically feasible vitamin B12 absorption test is recently described. Here, acceleration mass spectrometry is used to assess the absorption and kinetics of carbon-14-labeled vitamin B12 (Hvas and Nexo, 2006). One of the disadvantages of Shilling's test is the difficulties in its procedure concerning the use of radioactives. This encourages Anne-Mette Hvas and Ebba Nexo to develop an alternative approach to measuring vitamin B12 absorption, named CobaSorb. This test depends on the oral administration of non-radioactive vitamin B12, followed by the measuring of active vitamin B12 (holoTC) absorption. When no increase in active vitamin B12 detected this indicates the presence of Imerslund Grasbecks syndrome or inherited lack of intrinsic factor. For wider applications, this CobaSorb needs wider evaluation (Hvas and Nexo, 2006).

From the above discussion, one can notice the difficulties in diagnosis of vitamin B12 deficiency. When it is being diagnosed, vitamin B12 deficiency, have to be probably treated to overcome the very bad inputs of deficiency. When malabsorption has not been the ultimate cause for the deficiency, the case easily treated by the daily supplement in the form of a vitamin pills. Forms of vitamin B12 supplements includes; cyanocobalamin, hydroxycobalamin, and methylcobalamin. Cyanocobalamin is the only form available in the USA, although hydroxycobalaminmay has advantages due to a slower metabolism. Another route for vitamin B12 administration, rather than the oral route, also presents. Intramuscular injections in the form of cyanocobalamin or hydroxycobalamin vary according to the countries strategies. In the USA the usual treatment regime is injections with a daily dose of 1000 μg cyanocobalamin for the first week followed by weekly

injections for the next month and after that monthly injections used (Hvas and Nexo, 2006).

According to the oral administration, a daily oral dose of at least 1000 µg of vitamin B12 should be sufficient to maintain a normal vitamin B12 level to overcome the hematological manifestations of vitamin B12 deficiency. But, these doses are not so effective in giving that desirable benefit in case of severe neurological manifestations. Assistant treatments includes; iron supplements (because when red cell production increase the demand on iron stores also increase) and folate supplements (to correct the situation where folate deficiency has been masked by vitamin B12 deficiency) (Hvas and Nexo, 2006). Monitoring the treatment divided into two branches; the first, hematological monitoring includes; reticulocytes (after 1 week) and hemoglobin (after 1 to 2 months). The second monitoring branch is the biochemical monitoring which is not needed in patients receiving intramuscular injection treatment, only in patients given oral vitamin B12, plasma cobalamins, MMA or tHcy should be assayed to monitor the response to the treatment (Hvas and Nexo, 2006).

1.8. Vitamin B12 deficiency; the available methods for analysis.

Accurately quantifying vitamin B12 has been considered a challenge. Because the daily requirement of vitamin B12 is as low as 1 to 3 micrograms, the deficiency may be at the nanogram to picogram level; this fact is what makes the quantification process as difficult as it (Kumar et al., 2010). Different methods have been established to measure vitamin B12 in different components such as; serum, food and supplements, and microbial broth. Methods used frequently for vitamin B12 determination include microbiological assay that uses *Lactobacillus leichmannii* as the test organism, radioisotopic assay, high-performance liquid chromatography (HPLC), chemiluminescence (CL) assay, fluorimetric assay, capillary electrophoresis (CE), matrix-assisted laser desorption/ionization (MALDI), time-of-flight mass spectrometry (TOFMS), and atomic absorption spectrometry (AAS) (Kumar et al., 2010). Not all of those methods could be used for the determination of the clinical samples. In addition, not all are practical for the routine analysis. In the following few lines

the available methods for determination of vitamin B12 in the clinical samples will be discussed, and the advantages and disadvantages of each method will be highlighted.

Prior to any analysis procedure, the extraction step is important in order to extract vitamin B12 from its binding proteins. This extraction step could easily be done by denaturing the protein either by ultraviolet (UV)–visible light, heat, and/ or in alkaline conditions. It is also important to convert the light sensitive forms into more stable CN-Cbl form by exposing it to dilute cyanide solutions (Kumar et al., 2010). Measuring the activity of vitamin B12 differs from the quantification of it. The activity has been measured through the assessment of macrocytic anemia improvements, Hcy reduction, and MMA reduction (Kumar et al., 2010).

The microbiological assay is the first traditional method that has been used to quantify vitamin B12. It is based on Shorb observation in 1947; he noted that *Lactobacillus lactis* would not grow in vitamin B12 free medium. The method found to be unreliable for measuring the vitamin B12 content of plants which often contains vitamin B12 analogs. In addition, it has been considered as a time-consuming, nonspecific, and gives low values in the presence of antibiotics or cytotoxic drugs (Kumar et al., 2010; Ruff, 1979). Hence, the search for another method to overcome the disadvantages of the microbiological assay has been succeeded in the development of a method that has been considered as; simple, readily available in the form of commercial kits, specific, sensitive, and not influenced by antibiotics. It is the radioisotopic dilution assay which is based on the competition between vitamin B12 in the solution being assayed and a known amount of 57Co-labeled vitamin B12 for the vitamin B12 binding sites on a protein such as IF or R-protein. One cannot ignore the false normal values have been taken using the radioisotopic dilution assay, which was due to the non pure IFs have been incorporated in the commercial kits. The presence of R- proteins in those kits also binds the vitamin B12 analogs and falsely elevates the results (Kumar et al., 2010; Ruff, 1979).

In 1965, relatively similar method with some developments has been described. In this method, unlabelled vitamin B12 released from serum and diluted with a known quantity of

57CoB12, then bound to intrinsic factor which, having a B12-binding capacity less than the amount of added 57CoB12. Intrinsic factor becomes saturated with the B12 mixture. Unbound B12 is removed by absorption onto protein-coated charcoal and the degree of dilution of the radioactive B12 is proportional to the amount of unlabelled B12 (Raven et al., 1966). Raven has compared the results of microbiological procedure (*L. leichmannii*) with the new method; he found that this method appears to be as sensitive and reliable as microbiological assays for measuring vitamin B12 (Raven et al., 1966).

Chromatographic methods is not a determination method by itself, rather, it is a separation method that must be combined to another detection method in order to quantify the separated molecules. So, the precision and accuracy of the methods depend on the detection method. Thin layer chromatography (TLC), gas–liquid chromatography (GLC), HPLC, or Gas chromatography, all may combined to mass spectrometry, fluorescence and electrochemical detection or other detectors. Even UV visible light may be the detection method such in isocratic or gradient elution UV–visible reverse phase HPLC which has been used for determination of vitamin B12. UV detection is not sensitive and the use mass spectrometry method or other detection methods is expensive and time consuming (Kumar et al., 2010).

Chemiluminescence (CL) is defined as processes that result in the emission of light due to chemical reactions. A fully automated CL analyzer (Chiron Diagnostics, Erst Walpole, MA, USA) for the determination of vitamin B12 in serum has been used. Good linearity and sensitivity for this method has been reported, whereas specificity is still questionable (Kumar et al., 2010).

Enzyme-linked immunosorbent assay is the new track that has been developed in order to overcome the drawbacks of the other methods and as achievement for the continuous researches aimed to develop rapid and specific methods for detection of total vitamin B12. Immuno-based methods could be divided into two procedures; those using antibodies antigen reaction such as radioimmunoassay [RIA] and enzyme-linked immunosorbent assay ELISA), and those using natural vitamin binding proteins with either radiolabels (as

in radiolabeled protein binding assay [RPBA]) or enzyme labels (as in enzyme protein binding assay EPBA) (Kumar et al., 2010).

The indirect type of ELISA is the type commonly used for vitamins; where vitamin (from the sample) and protein conjugate (IF or R-protein) are immobilized on the surface. Labeled binding proteins have to be added; which may be conjugated to horseradish peroxidase (HRP). R-protein was labeled with HRP. Another procedure has been described by Tsalta and coworkers in 1989 based on the competition between the analyte and glucose-6-phosphate dehydrogenase– vitamin B12 conjugate for a limited number of R-protein binding sites immobilized on Sepharose particles. The procedure farther improved and sample pretreatment with denaturing agents was described by Watkins and coworkers in 1992 (Kumar et al., 2010).

The competitive binding assay is the method on which the four automated analyzers used in this study rely. The general assay steps and the differences between the analyzers will be discussed in the following chapter.

1.9. Literature review for vitamin B12 measurement:

As the first line screening test for vitamin B12 deficiency, attention has been paid to serum vitamin B12 assay. Because there are different methods for analysis, different pretreatment steps, and different automated analyzers have been developed. The investigation of the agreement between these widely used fully automated vitamin B12 assays is must. The previous work of Michael Vogeser and Stefan Lorenzl in 2007 aimed to investigate the agreement between three of these widely used fully automated vitamin B12 assays. For this purpose; aliquots of 100 samples which were left after requested cobalamin determination have been frozen, then analyzed using the three investigated systems. Data has been collected and regression analysis has been performed according to Passing and Bablock (Vogeser and Lorenzl, 2007).

Here is the difference; Michael Vogeser and Stefan Lorenzl compared the systems of; Abbott Architect B12 (Abbott, Abbott Park, USA), ADVIA Centaur VB12 (Bayer

Healthcare, Fernwald, Germany) and Roche Cobas Vitamin B12 (Roche Diagnsotics GmbH, Mannheim, Germany) (Vogeser and Lorenzl, 2007). Since those are not the commonly used analyzers in our country, different systems "analyzers" would be included in our study.

Vogeser and Lorenzl results showed a rather good numeric correlation between serum cobalamin results obtained with three fully automated methods, but a difference of 16% in the mean concentration of the lowest reading, and the highest reading. Vogeser and Lorenzl also paid some attention to number of samples analyzed in two analytical batches in one laboratory. They noticed that among-laboratory differences may be even more pronounced (Vogeser and Lorenzl, 2007).

A wider study also has been done by Hendriks et al. in 2000. They evaluated the analytical performance of the new generation immunoassay analyzers (ACS:Centaur®, Architect TM*i*2000, Elecsys®2010, Immulite®2000 and Vitros ECi) for the following analytes: TSH, FT4, vitamin B12, ferritin, folate, CEA, HCG, PSA, estradiol, LH, FSH, prolactin, and progesterone. Their results showed that the within-run precision of the test results for most assays was good, and for some assays on the Vitros ECi (HCG, PSA, FSH and prolactin) and Elecsys 2010 (FT4, FSH and prolactin) even very good precision (< 2%) has been measured. They also observed a relatively higher CV's for the anemia assays (ferritin, vitamin B12 and folate), on the ACS:Centaur and the Immulite 2000. They also found no sample carry-over for any of the analyzers. Most methods showed good correlation ($r>0.97$). Significant intercept differences were measured for the prolactin assay on the Vitros ECi and the vitamin B12 assay on the Elecsys 2010 (Hendriks et al., 2000).

In 2010, another work for Badiou S. et al. compared the radioimmunoassay (RIA) (Simultrac, Diasorin, Italy) method for vitamin B12 assay with the immunoenzymatic method (IEA) using Access 2 (Beckman Coulter, France). They found relatively good performance for the automated immunoenzymatic machines. Comparison of RIA and IEA on the Access2 using EDTA samples showed a discrepancy for B12 ($y = 0.67x + 211$; $r = 0.37$). This discordance for vitamin B12 was not observed when comparing

RIA and IEA on the Architect i2000 (y = 0.74x + 40; r = 0.92) and disappeared when using heparinized samples for the Access2 (y = 0.85x + 7.05; r = 0.90).They concluded that automated IEA may be a good alternative to RIA for the determination of folate and vitamin B12 (Badiou et al., 2010).

Steijns et al. in 1996 evaluated of the microparticle enzyme immunoassay for estimating vitamin B12 in serum. The assays have been performed with the Abbott IMx analyzer. Intra-assay coefficients of variation have been found to be from 3.4% to 8.0% for vitamin B12. Linearity has been considered satisfactory, with an analytical recovery of 119 +/- 4% for vitamin B12. The detection limit was 56.2 pmol/L for vitamin B12 (Steijns et al., 1996).

Assay for vitamin B12 on the Abbott AxSYM Analyzer first developed and evaluated in 1999. For method comparison, this assay has been compared with three existent methods; IMx B12 (Abbott Laboratories), Quantaphase B12 (Bio-Rad Laboratories), and ACS: 180 B12 (Chiron). They concluded that the AxSYM B12 assay is sensitive and precise, and its results correlate well with that of a manual isotopic method also (Wilson et al., 1999).

Actually, there is no reference method for vitamin B12 determination. For many years the microbiological assay using *Euglena gracilis* or *Lactobacillus leichmannii*, were used in both routine and reference laboratories (Mollin et al., 1980). The complexity of the microbiological assay encourages the use of the newly introduced, that time, radioisotope dilution (RID) assays, not only because it is available in the form of commercial kits, but also because its providing rapid results, and, in contrast to the microbiological assay, and not being affected by antibiotic or other drugs in serum (Mollin et al., 1980). Mollin et al reported in their paper the results of interlaboratory trials of serum and plasma vitamin B12 assay carried out in the United Kingdom by participants in the National Haematology Quality Control Scheme (Mollin et al., 1980). The interlaboratory trials results have been compared of microbiological and radioisotope dilution (RID) assay of serum vitamin B12 in Britain. Wide variation between the *L. leichmannii* microbiological assay and the RID methods has been observed, RID gave higher results than microbiological assay (Mollin et al., 1980). In addition, the development of a (mass spectrometric) reference method for

vitamin B12 is hardly possible due to its complexity, cost, and its need for trained technicians (Vogeser and Lorenzl, 2007).

1.10. Problem statement.

Although there is some limitations according to its sensitivity and its specificity (Lewis et al., 2006), total serum vitamin B12 assay still the first test to be done in vitamin B12 status investigation in many laboratories. It is considered as a screening test not only to investigate megaloblastic anemia but also because neuropathy and neuropsychiatric changes may occur also in vitamin B_{12} deficiency in the absence of macrocytosis or anemia (Lewis et al., 2006). Many reports have discussed the role of increased plasma homocysteine and serum methylmalonic acid (MMA) levels as indicators for vitamin B12 deficiency. Although they are helpful in vitamin B12 status investigation, those biomarkers are technically difficult in measurement and so are not routine in many laboratories, especially here in Palestine where possibilities are limited.

Starting from the point that total serum B12 assay is an important screening tool, and from our knowledge about the huge number of vitamin B12 requests received yearly, and from the notice that the number of requests are consequently increased (in some countries there is up to 72% increased in vitamin B12 requests between 2003-2008 (McHugh et al., 2012)), one can understand the importance of obtaining an accurate results considering vitamin B12 tests. In addition, during the last several years the number of vitamin B12 requests increased progressively in the West Bank (personal communication) and vitamin B12 deficiency become a dogma among Palestinian.

1. 11. Justifications.

This study is designed to compare the results of total serum vitamin B12 obtained from four different widely used automated analyzers in West Bank, this will be the first such study done in Palestine and the first comparison between those four automated analyzers. When comparing the results of those analyzers we actually compare between the different methods, although all are based on immunoassay method but some differences in the assay

steps are present. This study will also help in establishing a clear relation between the different methods used and the results obtained, which in turn will help in answering the questions about the relation between the method used in the assay and the results obtained. In other words; the presence or absence of a difference between the results obtained will illustrates the differences exist in the patients results. Never forget that the results of this study will be helpful in assessing the agreement and harmony between laboratories concerning serum vitamin B12 results.

1.12. Goals.

Two main factors contribute to the progressing of immunoassay testing over years. One is the introduction of several alternatives to the radioimmunoassay which are less hazardous detecting labels, with the improvement in antibody production methods have been taken place. The other is, the improvement in the automation which further increase the reproducibility and sensitivity and shorting the reaction times. (Hendriks et al., 2000)

The purpose of this study is to compare the results of total serum B12 obtained from four different widely used automated analyzers in West Bank, the repeating of a serum sample assay 10 times will be helpful in the assessment of the within-run precision for those analyzers. Another important part and the core of this study is the method comparison part, where we will calculate the correlation coefficient for this purpose and investigate the agreement level between the methods. If these methods do not match or agree, this will support the claim of Younis (Younis, 2010) that vitamin B12 assay is highly variable among laboratories in the West Bank.

Chapter Two: Materials and Methods

2.1. Materials used in this study.

The materials that were used in phlebotomy and in aliquots preparation are shown in Table (2.1).

Table 2.1: Materials used in the study.

Item	Trade mark
23G Needles	BD Microlance
Tourniquet	----------
7 ml plain tubes "vacuum tubes"	AFCO
70 % methyl alcohol swabs	----------
Sterile gauze swabs and Adhesive dressings	----------
Rack	----------
1000 µL automatic pipette	----------
5 mL syringes	MEDI-PLUS
Eppendorf tubes	----------
Permanent marker	----------
Centrifuge (Centurion Scientific)	Ford Airfeild industrial estate, ford , UK

2.2. The questionnaire.

A questionnaire has been designed purposefully for this study. It includes personal questions about the volunteers participating; such as names, age, and sex, address and phone numbers. The volunteers' health status was also investigated; in terms of previous testing for vitamin B12 levels, any current diseases, and any medications administered at

the time of the study; including vitamins. The questionnaire has been filled by all the participants and each participant signed a consent form.

2.3. Sample collection.

Thirty adult participants aging 17 – 53 years have been randomly chosen. Individuals with systemic diseases such as diabetes, hypertension or any cardiovascular diseases were excluded. Also, individuals taking any vitamin B12 supplements of any type were also excluded. We aimed at choosing donors so that our group of samples may contain different levels of vitamin B12; low, normal, and high. This was accomplished by asking the participants about any previous results for vitamin B12.

Samples have been collected through vein puncture. Five mL of venous blood has been collected from each participant in 7 mL plain tubes. Each sample has taken a number from 1 to 30.

2.4. Sample preparation.

Samples have been centrifuged at 3000 rpm for 3 minutes within less than one hour of phlebotomy and serum has been obtained. Each serum sample was divided (using 1000 µL automated pipette) into four aliquots (300 µL each). Serum aliquots were transferred to plastic Eppendorf tubes (1 mL volume). Each set of aliquots was given the same number that correlates with that given to each sample during collection (from 1 to 30). Aliquots were stored at -18°C until analysis. All aliquots were subjected exactly to the same handling procedures before analysis by each method. In the analysis day, samples have been transported to each laboratory on ice.

2.5. Sample assay.

Serum samples have been run in three different clinical laboratories in Ramallah city on four different automated analyzers. The clinical laboratories participated in the study are; Horus Specialized Medical Laboratories (assay vitamin B12 on IMMULITE ®1000),

Specialized Medical Laboratories (assay vitamin B12 on TOSOH® and ADVIA Centaur®), and Ramallah Specialty laboratory (assay vitamin B12 on AxSYM system®).

The laboratories received 30 frozen coded serum samples and a labeled control. Samples have been left at room temperature until complete thawing and then well mixed. Laboratories were asked to perform vitamin B12 assay on their usual methodology within 3 hours of samples' thawing. All handling instructions for the tests given by the respective manufacturer have also been followed. We did not repeat any reading and took the first measurement by each method to be used in the methods comparison study.

2.6. Repeated measurements.

In order to assess the reproducibility of the analyzers, 15 mL of venous whole blood has been collected from three normal individuals using 21 G Becton-Dickinson Vacutainer® multiple sample needle. The samples were centrifuged at 3000 rpm for 3 minutes after complete clotting (after 15 minutes of drawing) and then, serum was separated and pooled. After that, 3 aliquots (2.0 mL each) were prepared from the pooled sample. Aliquots were transferred on ice within less than half an hour to the intended laboratories. Aliquots were kept refrigerated at 4 -6 C^{o} until analysis. The serum aliquots were assayed 10 times on each analyzer in one run and within 3 hours of drawing and preparation.

Assay has been performed after the acceptance of the control results. All handling instructions for the tests given by the respective manufacturer have also been followed. Finally, results have been collected and kept for the statistical analyses.

2.7. Assay methodology and principle of determination.

There are currently no internationally recognized reference methods for serum vitamin B12 measurement. However, the four analyzers to be compared for vitamin B12 assay rely on the competitive immunoassay technology. This method involves common steps despite the automated analyzer to be used, but different signal detection techniques and pre-treatment steps.

The 1st step in the assay is the releasing step. In this step vitamin B12 has to be released from its endogenous binding proteins (transcobalamins) before measurement. The releasing is accomplished by alkaline hydrolysis (NaOH at pH 12–13) or by heat in the presence of potassium cyanide (KCN) which converts the cobalamin (vitamin B12) forms into the more stable cyancobalamin form. The releasing step reagents also must contain dithiothreitol (DTT) which prevents the rebinding of the released vitamin B12.

The 2nd step is the competitive step of the assay in which serum-derived cyancobalamin competes with labelled cobalamin (which is usually attached to a chemiluminescent, fluorescent substrate or an enzyme) for limited binding sites on porcine intrinsic factor.

The 3rd step is the separation of bound and unbound vitamin B12 done by a number of electro- or physico-chemical and immunological methods. Other differences between the four analyzers will be highlighted in the following illustration of the principle of the assay for each analyzer.

2.7.1. Principle of the procedure in IMMULITE ®1000.

The vitamin B12 determination using IMMULITE ® 1000 analyzer (Seimens Healthcare Diagnostics Products Ltd; Llanberis,UK) is a solid-phase, competitive chemiluminescent enzyme immunoassay procedure. IMMULITE vitamin B12 involves a preliminary heat denaturation step. Vitamin B12 in the patient sample is released from carrier proteins by incubation at 100°C in the presence of dithiothreitol (DTT) and potassium cyanide to inactivate vitamin B12-binding proteins, as well as antibodies to intrinsic factor. After the heat denaturation step, the treated patient sample and the hog intrinsic factor are simultaneously introduced into an IMMULITE test unit containing a polystyrene bead coated with a B12 analog, and incubated for approximately 30 minutes at 37°C with intermittent agitation. During this incubation, vitamin B12 in the treated sample competes with the B12 analog on the solid phase for a limited number of vitamin B12 binding sites on the purified intrinsic factor. (Endogenous vitamin B12 analogs do not interfere, because the binder is free of R-protein.) Alkaline phosphatase-labeled anti-hog intrinsic factor is

introduced, and the test unit is incubated for another 30-minutes cycle. The unbound enzyme conjugate is removed by a centrifugal wash, then, a chemiluminescent substrate added which produce a detectable signal (product insert).

2.7.2. Principle of the procedure in ADVIA Centaur® System.

In ADVIA Centaur® (Seimens Medical Solutions Diagnostics; Tarrytown, USA) vitamin B12 assay is a competitive immunoassay using direct chemiluminescent technology in which vitamin B12 from the patient sample competes with vitamin B12 labeled analog (labeled with acridinium ester), for a limited amount of purified intrinsic factor. The purified intrinsic factors are covalently coupled to paramagnetic particles in the Solid Phase. The assay uses releasing reagent which contains sodium hydroxide and DTT to release the vitamin B12 from the endogenous binding proteins in the sample. It also contains cobinamide to prevent rebinding of vitamin B12 to the binding proteins after the addition of the solid phase to the reaction matrix. An inverse relationship exists between the vitamin B12 in the sample and the relative light units (RLUs) detected by the system (product insert).

2.7.3. Principle of the procedure in Abbott AxSYM system®.

In AxSYM ® (Abbott Ireland Diagnostics Division; Longford CO, Longford, Ireland) vitamin B12 determination is based on the Microparticle Enzyme Immunoassay (MEIA) technology. The first step in the assay is the pretreatment step in which the denaturant (0.8 N Sodium Hydroxide with 0.005% Potassium Cyanide) envelopes the vitamin B12.

Then, the anti-intrinsic factor antibodies complexed with intrinsic factor coated microparticles are added to the reaction mixture. Then, B12 present in the sample binds to the intrinsic factor coated microparticles forming a B12-intrinsic factor-microparticle complex. The aliquot of the reaction mixture is then transferred to the matrix cell. The microparticles bind irreversibly to the glass fiber matrix. After that, the matrix cell is washed to remove materials not bound to the microparticles. The B12- Alkaline Phosphatase Conjugate is dispensed onto the matrix cell forming a B12-intrinsic factor microparticle conjugate complex. Then the matrix cell is washed to remove unbound

conjugate. Finally, the substrate, 4-Methylumb-elliferyl Phosphate is added to the matrix cell and the fluorescent product is measured by the MEIA optical assembly (product insert).

2.7.4. Principle of the procedure in AIA-PACK B12 (TOSOH®).

The AIA-PACK B12 (TOSHO Corporation; Tokyo, Japan) vitamin B12 assay is a competitive enzyme immunoassay, which needs a B12 releasing step. The sample pretreatment reagents contain potassium cyanide, sodium hydroxide, and dithiothreitol. Vitamin B12 present in the pretreated test sample competes with enzyme- labeled vitamin B12 for a limited number of binding sites on a fluorescein labeled porcine intrinsic factor, which then binds to anti-FITC (fluorescein isothiocyanate) antibody immobilized on magnetic beads. The beads are washed to remove the unbound enzyme labeled vitamin B12 and are then incubated with a fluorogenic substrate, 4-methylumb-ellifeeryl phosphate (4MUP). The amount of enzyme labeled vitamin B12 that binds to the beads is inversely proportional to the vitamin B12 concentration in the test sample. A standard curve using a range of known standard concentrations is constructed to calculate the unknown concentrations (product inserts).

The main points of difference between the four analyzers are; the main principle, the releasing step, and the detection principle. Other differences such as; the sample type, the sample volume, and the detection limit, the normal range, the sensitivity of the method and the effect of the interference in the sample, if present, are listed in Table 2.2.

Table 2.2: Comparison of the four analyzers.

Type of analyzer	Main principle	Sample type	Sample volume (µL)	Detecti-on limit (pg/ml)	The sensitivity (pg/mL)	The normal range (pg/mL)	Interference of cobinamide (vitamin B12 analogs)
IMMULITE 1000®.	Solid-phase, Competitive Chemilumine-scent enzyme Immunoassay.	Serum	200 µL	125-1200	125	174-878	No cross reactivity with cobinamide even at concentration as high as 1.800 ng/mL and 50.000 ng/mL
ADVIA® Centaur System	Competitive Chemilumine-scent Immunoassay	Serum / Plasma	100 µL	45-2000	45	156-672	No interference seen when 20 ng of cobinamide added
AxSYM®	Microparticle Enzyme Immunoassay	Serum / Plasma	210 µL	84-1200	84	208-963	cross reactivity was 0.1% when 9 ng/mL cobinamide added
AIA-PACK B12 (TOSOH ®).	Competitive Enzyme Immunoassay	Serum	100 µL	84-2100	84	100-700	Cross reactivity was < 0.1% with 200 ng/mL cobinamide.

2.8. The statistical analysis.

In order to assess the agreement between the results of the four methods, the automated machines were divided into 6 pairs and data analysis was done on those pairs. Data were analyzed by correlation and regression.

The null hypothesis established in this study suspects the absence of any correlation or difference between the values of each pair of the automated analyzers. In other words, the study has been designed to test the hypothesis that there is no significant difference or correlation between the results of each pair of the automated analyzers at the 0.05 level of significance.

Based on Bland and Altman, statistical methods for assessing agreement between two methods in clinical measurement starts with plotting data and drawing the regression line,

which helps the eye in gauging the degree of agreement between measurements (Bland and Altman, 1986). The regression line of two agreed methods should be a straight line, non-significantly different from the equality line (in the equality line: slope =1, the intercept =0 and r =1) (Magari, 2002). Accordingly, the next statistical step is the calculation of the slope, Y- intercept and the correlation coefficient (r). A slope deviation from 1 indicates the presence of proportional difference between the two methods. The value of Y-intercept indicates the presence of constant bias (difference between the two methods). The correlation coefficient (r) will be helpful in estimating the strength of a relation between two methods, and not the agreement between them.

For a method or a machine to replace another, three conditions should be met: r >0.95, slope of the regression line should be between 0.9 and 1.1, and Y- intercept should be close to Zero (Snyder and Larsen, 1983). Depending on that, the comparative pairs' results were assessed and the agreement between them was checked according to this criteria.

To assess the significant of the difference between the methods at the predetermined significant level (α=0.05) data was analyzed using SPSS. The probability (*P*- values) has been calculated. The null hypothesis will be rejected if the value of the probability (*P*-value) for each pair equals or is less than 0.05. A larger *P*-value suggests that there is no significant difference between the results of the two comparative analyzers. In other words, if the *P*- value is greater than 0.05 then the null hypothesis would be accepted.

To examine the reproducibility or the precision of the methods, the same pooled serum sample was repeated 10 times on the four machines. The mean and standard deviation (SD) were calculated for each method. On the other hand, the following formula was used to calculate the coefficient of variation (CV) for each machine, and compared with that claimed by the manufacturer; evaluating thereby the precision of the machines:

$$\text{Coefficient of variation (CV)} = \frac{\text{Standard deviation}}{\text{Mean}} * 100\%$$

Chapter Three: Results

3.1. Questionnaire results.

This study was designed to assess the agreement in the results of the four commonly used analyzers for serum vitamin B12 assay in Palestine. For this purpose, 30 participants donated their blood samples and 30 serum samples were prepared; each serum sample belongs to one participant and took a serial number from 1 to 30. Samples had been tested using the four analyzers on the same day (see appendix A).

The participants were 11 males and 19 females. They were considered as the donors for the 30 blood samples. But only the results of 29 samples have been included in the statistical analysis; since one serum sample result was >2000 pg/mL and was therefore excluded, because it seems that the relevant participant was taking vitamin B12 supplements.

All the participants were asked to complete the questionnaire prior to blood sampling. The questionnaire was designed to collect general information about each one of the participants, concerning the age, gender and the general health status of each of them. The detailed information about each participant is shown in appendix B.

The percentage of the male participants in the study was 33.3 % compared to 66.7 % females. The age of the participants ranged from 17 to 53 years, and the age range of males was 23- 53 years, while, the ages range of females were 17- 50 years (Table 3.1).

Table 3.1: The average age of the participants according to gender.

Gender	Number	Age range (years)
Female	18	17-50
Male	11	23-53
Total	29	17-53

Two of the participants were suffering form joint pain and arthritis and were under medications at the time of blood sampling. A third participant was under medication for *H.pylori* at the blood sampling time. Also, one of the participants was suffering from personal schizophrenia and has been taking antidepressant drugs for a long period. The rest of the participants (87%) did not report taking drugs nor suffering from any mentionable diseases.

The participants were asked to mention if they had any of the general symptoms of vitamin B12 deficiency at the time of blood sampling. They have been informed about the general symptoms of vitamin B12 deficiency which include anemia, paresthesias, peripheral neuropathy, irritability, personality change, mild memory impairment and depression. The answers came as follows: 22 of the participants (approximately 76%) answered "yes" meaning that they had some of these symptoms. Only 7 of the participants were not suffering from any of those symptoms. None of the participants in the study were taking any form of vitamin B12 supplements during the last three months prior to blood sampling.

3.2. Serum vitamin B12 results.

Thirty samples had been run in three different clinical laboratories in Ramallah city on four different automated analyzers; IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System. Vitamin B12 assays have been performed according to the usual methodology, and after the acceptance of the routine quality control procedures. All handling instructions for the tests given by the respective manufacturer had also been followed.

The first measurement has been taken and used in the methods' comparison study. One sample has been excluded from the data analysis study because its result was very high (>2000 pg/mL) and exceeded the upper limit of some machines (participant might be taking vitamin supplements). The results of the participants varied widely and included the three vitamin B12 concentration levels; low, medium and high (Table 3.2).

Eye balling the data, one can see the differences in the serum vitamin B12 results obtained from the assay of the same sample on four different machines. As shown in Table 3.2, the mean of measurement of vitamin B12 and the CV% for the four machines (ADVIA, TOSOH, IMMULITE, and AxSYM) were 313.9 (40.7), 292.9 (46.7), 351.4 (37.3%), and 317.7 (37.4%) respectively. Sample number 11 was the only sample with low level of vitamin B 12 to agree on by the four machines and samples number 4 and 6 may to some extent hold the same impression. Samples number 1, 3, 25, 26, and 27 are low by some of the machines and normal by other. Thus, there was no consistency or harmony in vitamin B12 results among the four machines. The question now is; to what extent does the results of those machines vary? i.e., is the difference between the results of the machines significant in the clinical terms to cause a problem in patient's diagnosis and results interpretation?

Table 3.2: The results of serum vitamin B12 analysis on IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System. (Results less than 200 pg/mL were bolded).

Sample number	ADVIA ® pg/mL	TOSOH® pg/mL	IMMULITE ®1000 pg/mL	AxSYM® pg/mL
1	**191**	221	266	253
2	606	490	546	524
3	300	**196**	213	226
4	**160**	208	**150**	207
5	363	244	352	332
6	**186**	**196**	212	**153**
7	312	254	295	314
8	304	257	373	317
9	356	426	507	419
10	635	847	672	701
11	**144**	**173**	**150**	**168**
12	398	485	517	399
13	340	237	345	319
14	519	215	273	235
15	225	206	397	240
16	225	212	315	287
17	370	254	439	379
18	392	283	378	378
19	252	236	323	269
20	227	305	349	288
21	515	415	533	515
22	260	360	321	324
23	241	232	268	316
24	261	301	534	338
25	381	302	**192**	**175**
26	241	**185**	207	225
27	**141**	212	269	228
28	309	289	460	398
29	249	252	335	286
Mean	**313.9**	**292.9**	**351.4**	**317.7**

3.3 Comparative study.

Results of 29 serum samples for vitamin B12 were available for the method comparison. A Pearson's correlation coefficient (r) was computed to assess the relationship between the four machines. There was a positive significant correlation between each pair of the machines. With varying degrees of correlation between the comparative pairs, r ranges from 0.67 to 0.90. The highest correlation was seen when comparing the results of IMMULITE vs. AxSYM results (0.90). Overall, there was a moderately strong, positive correlation between each pair of the machines. The *P*- value was less than the predetermined significant level (0.05), and less than 0.001 for all comparative pairs (Table 3.3). One can conclude that there is a statistically significant positive correlation between the methods/machines. However, significant correlation does not mean agreement between machines according to Bland and Altman 1986.

The result of the slope also varies, with slopes ranging from 0.69 to 0.81. The y-intercept which reflects the constant bias (difference) between the methods also varies widely, with values ranging from 32.2 to 134.6. Comparing of ADVIA with IMMULITE yields the highest y-intercept value =134.6 (Table 3.3).

Table 3.3: Comparison of 29 samples results for vitamin B12 measured with 4 different machines (IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System).

Comparative pairs		N	slope	Y-intercept	Correlation coefficient (r)	*P*-value
Pair 1	ADVIA vs. TOSOH	29	0.78	47.6	0.73	<.001
Pair 2	ADVIA vs. IMMULITE	29	0.69	134.6	0.67	<.001
Pair 3	ADVIA vs. AxSYM	29	0.71	93.8	0.77	<.001
Pair 4	TOSOH vs. IMMULITE	29	0.76	129.9	0.79	<.001
Pair 5	TOSOH vs. AxSYM	29	0.76	96.2	0.88	<.001
Pair 6	IMMULITE vs. AxSYM	29	0.81	32.2	0.90	<.001

Despite the presence of a significant correlation between all the comparative pairs, none of them met the conditions for methods agreement suggested by Snyder and Larsen. The three conditions can be summarized as: the correlation coefficient (r) should be > 0.95, the slope of the regression line should be between 0.9 and 1.1, and Y intercept should be close to Zero. The three conditions should be met in order for a method to replace another (Snyder and Larsen, 1983).

3.3.1 ADVIA vs. TOSOH.

The ADVIA method had a slope of 0.78 when compared with the TOSOH method. Also, comparing of ADVIA with TOSOH yields a y-intercept value equals to 47.6 and r of 0.73. The *P*-value for this comparative pair was less than the predetermined significant level (0.05) which suggests the presence of a statistically significant correlation between the two methods.

The presence of a correlation between the methods does not mean that they can be used interchangeably. In fact, these two methods failed to pass the criteria for agreement between methods suggested by Snyder and Larsen. When comparing ADVIA method vs. TOSOH the resulting r of 0.73 is not high enough for the methods to replace one another, despite the presence of a significant correlation between them.

The two types of errors; proportional and constant, could be detected when comparing the results of ADVIA vs. TOSOH method and are reflected by the results of the slope and y – intercept respectively. (Fig 3.1)

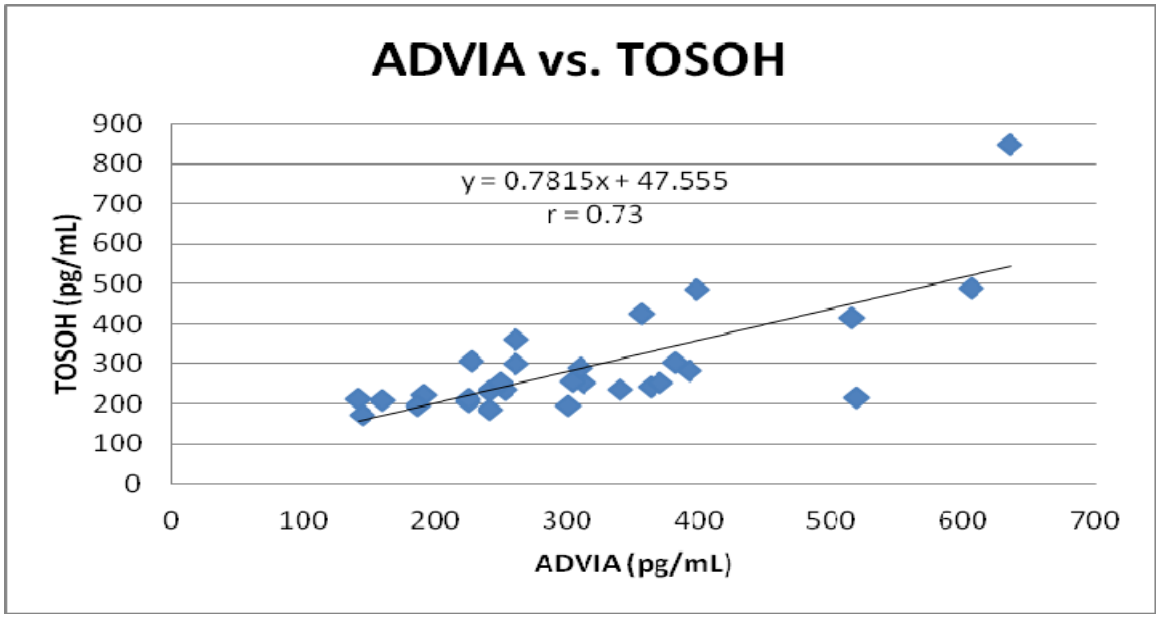

Figure 3.1: Comparison of automated serum vitamin B12 methods: **ADVIA vs. TOSOH.** The solid line is the regression line.

3.3.2 ADVIA vs. IMMULITE.

Comparing the results of ADVIA with that of IMMULITE method for vitamin B12 determination yields the lowest slope (0.69) between all of the comparative pairs and the highest y-intercept value (y- intercept =134.6), and -as a logical consequence of that- the lowest correlation coefficient (r =0.67). This comparative pair actually has the highest constant and proportional error among the other comparative pairs (Fig 3.2).

With the low *P*-value (<0.001), one can infer the existence of a significant correlation between ADVIA and IMMULITE methods. But, again this correlation is not enough for the ADVIA to replace the IMMULITE according to Snyder criteria (Snyder and Larsen, 1983).

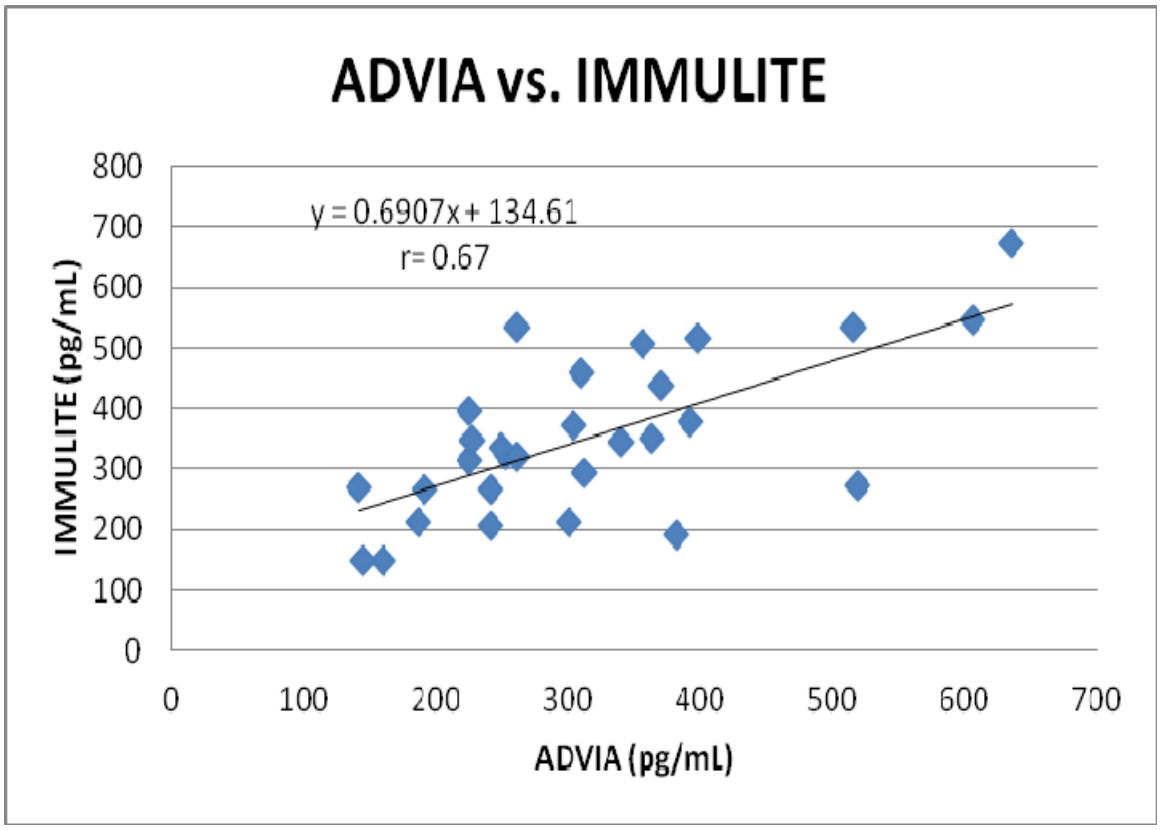

Figure 3.2: Comparison of automated serum vitamin B12 methods: ADVIA vs.IMMULITE**.** The solid line is the regression line.

3.3.3 ADVIA vs. AxSYM.

This comparative pair of ADVIA and AxSYM yields a moderately strong significant correlation. With a slope equals to 0.71, a y- intercept equals to 93.8 and r value equal to 0.77. However, this comparative pair did not meet the three conditions for methods agreement. Although ADVIA and AxSYM has significant correlation between them but that was not sufficient for one method to replace another (Fig 3.3).

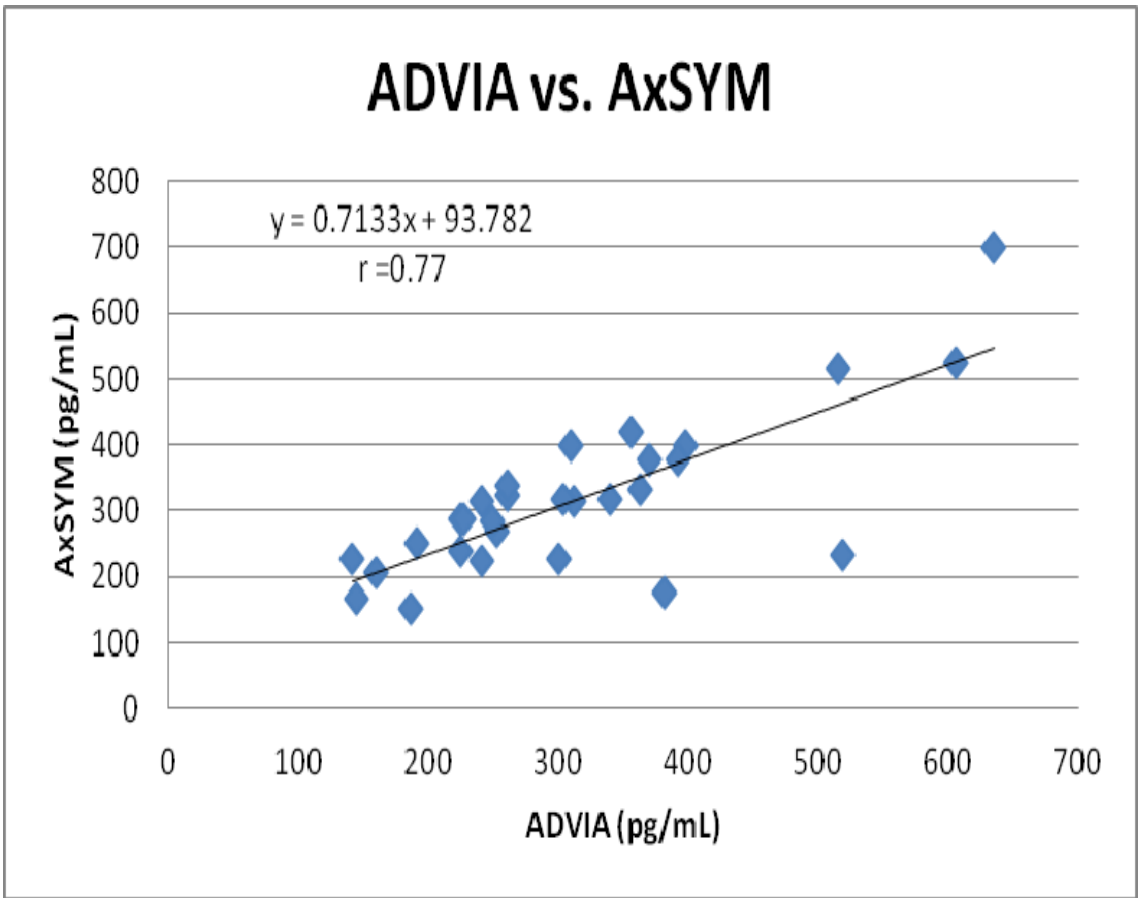

Figure 3.3: Comparison of automated serum vitamin B12 methods: ADVIA vs. TOSOH**.** The solid line is the regression line.

3.3.4 TOSOH vs. IMMULITE.

Vitamin B12 results of TOSOH compared with that of IMMULITE, the results of this comparison did not widely differ from that of the other pair's comparison. The results of this comparison were as follow: the slope of the regression line in this comparison equals to 0.76, y-intercept was 129.9 and r was 0.79.

Actually, this comparison has achieved the 2nd highest y- intercept value after ADVIA vs. IMMULITE comparison; this high y-intercept result confirms the presence of a relatively high constant error between the two methods. The low *P*-value emphasized the significant of this correlation, but, again the strength of this correlation is not sufficient for those methods to agree (Fig 3.4).

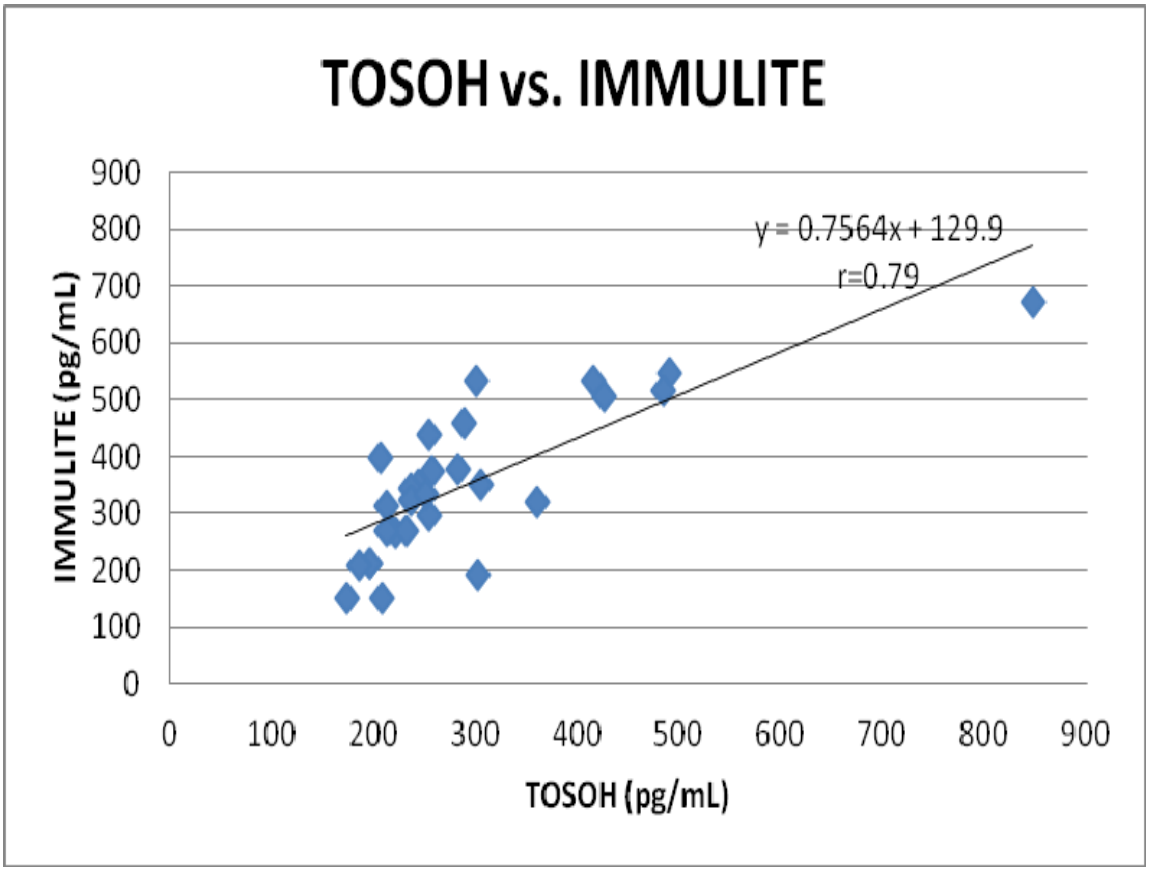

Figure 3.4: Comparison of automated serum vitamin B12 methods: TOSOH vs. IMMULITE**.** The solid line is the regression line.

3.3.5 TOSOH vs. AxSYM.

The next comparison is the comparison of vitamin B12 results for TOSOH and AxSYM. The slope of the regression line of this comparison was 0.76, y- intercept was 96.2 and r was 0.88.

The value of r in this correlation was the 2nd highest one. Despite the relatively high correlation coefficient value (r), the value of r still not high enough for TOSOH to replace AxSYM (Fig 3.5).

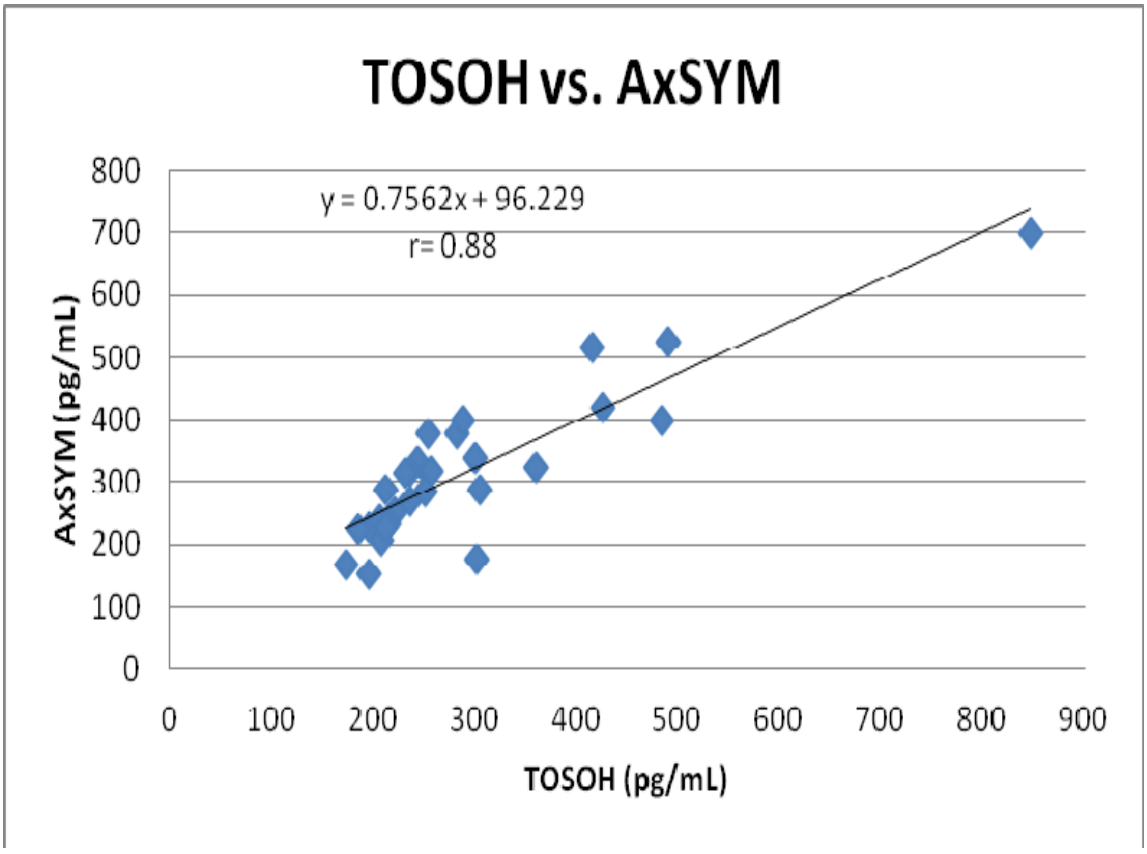

Figure 3.5: Comparison of automated serum vitamin B12 methods: TOSOH vs. AxSYM**.** The solid line is the regression line.

3.3.6 IMMULITE vs. AxSYM.

IMMULITE vs. AxSYM results comparison yields the best results between all of the comparative pairs for the slope, y- intercept and correlation coefficient (r). The result of the slope was 0.81 which was the closer slope result to 1 -between the other pairs- and reflects the lowest proportional error between them.

The y- intercept result was also the best among the other pairs, with a y-intercept result equals to 32.2 (the closer one to zero among the other pairs). This comparative pair scored the lowest constant error.

The r value was also good and equals to 0.90 (the highest r value among the other pairs) which represents the strangest correlation. Nevertheless, the relatively good results of this comparison did not meet the Snyder and Larsen criteria for those methods to replace one another (Fig 3.6).

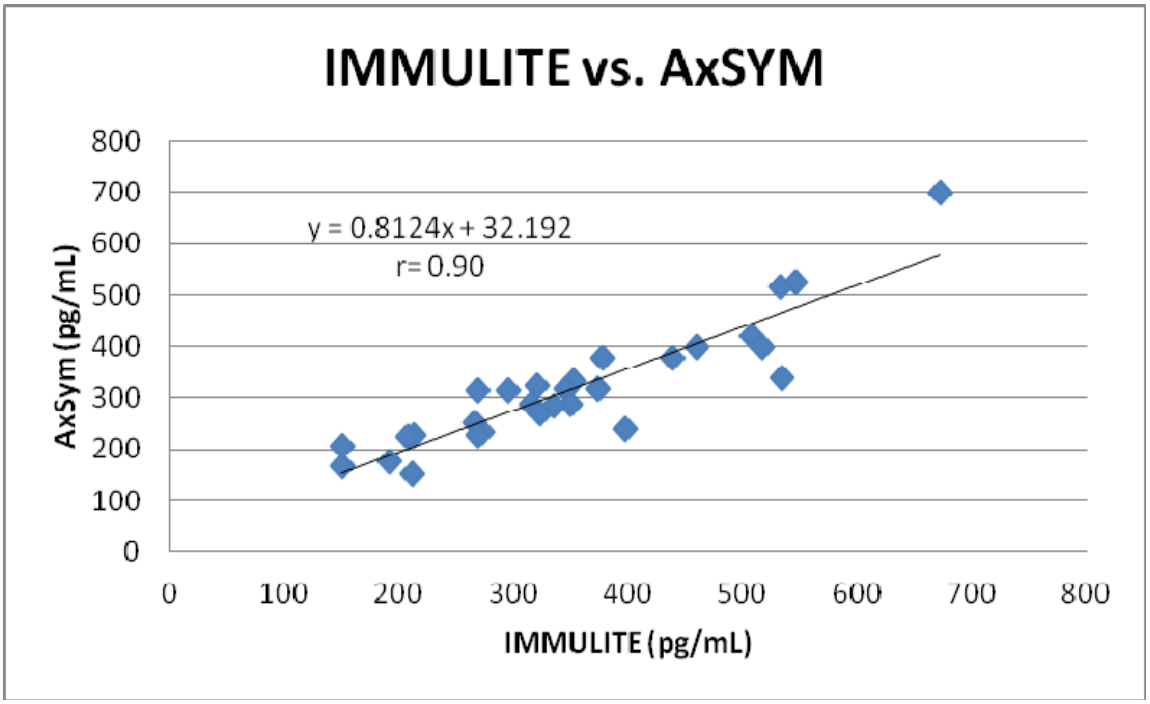

Figure 3.6: Comparison of automated serum vitamin B12 methods: IMMULITE vs. AxSYM**.** The solid line is the regression line.

3.5 Normal vs. abnormal results.

In order to classify the participants in the study as vitamin B12 deficient or normal, according to the results obtained in the study, and considering the lower limit of normal vitamin B12 as 200 pg/mL (personal communication with local physicians), five results were abnormal with the ADVIA®, three abnormal results with IMMULITE®1000 assay, four with the TOSOH® assay, and three with the AxSYM® assay (Table 3.4). However, only sample number 11 gave low results of vitamin B12 with the four machines (Table 3.2).

Table 3.4: Number of abnormal vitamin B12 results in respect to the four different machines.

Machine	No. of results <200 pg/mL	No. of normal results
ADVIA ®	**5**	**24**
TOSOH®	**4**	**25**
IMMULITE ®1000	**3**	**26**
AxSYM®	**3**	**26**

The difference is non-significant between the methods when it is due only to the amount of measurement imprecision. That's why imprecision needs to be assessed before any agreement testing (Bland and Altman, 1986).

3.6 Repeated measurements.

Repeatability is relevant to the study of method comparison because the repeatability of the methods limit the amount of agreement possible. i.e. if one method has poor repeatability, there is considerable variation in repeated measurements on the same subject, then the agreement between the two methods is bound to be poor (Bland and Altman, 1986).

Four serum aliquots (2.0 ml each) have been prepared and transferred to the intended laboratories. Serum aliquots were assayed 10 times on ADVIA, TOSOH, IMMULITE,

and AxSYM analyzers in one run and within 3 hours of sample drawing and serum preparation. Assay was performed after the acceptance of the control results. All handling instructions for the tests given by the respective manufacturer have also been followed. The results of the ten repeated measures are shown in Table 3.5.

Table 3.5: Repeated measures of vitamin B12 (pg/mL) assayed by the four machines.

	R 1	**R 2**	**R 3**	**R 4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
ADVIA	367	362	387	363	343	370	352	365	380	369
TOSOH	262	283	285	259	242	265	292	279	261	285
IMMULITE	328	338	312	336	327	319	312	351	330	309
AxSYM	382	374	386	390	371	387	372	408	391	368

A precision study evaluated the random error by investigating intra-assay repeatability in terms of coefficient of variation (CV). The repeatability of a 10-run intra-assay was assessed by repeated measurements of one serum sample with 4 different machines. For each machine, standard deviation SD and CV was calculated to evaluate precision of the machine. Standard deviation for the repeated measures ranged from 12.2 to 15.8, with the lowest value for the AxSYM system®, followed by ADVIA ®, followed by IMMULITE ®1000 System, the highest value was for TOSOH® (Table 3.6).

Table 3.6: Mean, median, standard deviation, and coefficient of variation for the repeated measures assay by: IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System. And the coefficient of variation claimed by the manufactures at three vitamin B12 concentration levels; low, medium and high.

	Mean (pg/mL)	**Median (pg/mL)**	**S.D.**	**CV %**	**Manufacturer CV%**		
					At low Vit. B12 conc.	**At medium vit. B12 conc.**	**At high vit. B12 conc.**
ADVIA	366	463	12.5	3.4	5.0	4.0	2.7
TOSOH	271	272	15.8	5.8	18.4	9.1	7.0
IMMULITE	326	328	13.4	4.1	10.3-11.3	5.3 – 6.7	5.9
AxSYM	382	384	12.2	3.2	6.8- 10.2	3.0 – 5.1	2.9 – 4.9

Relatively good precision has been obtained for the four machines that have been evaluated. For the repeated measures, CVs have been compared with that claimed by the manufacture at the medium vitamin B12 level since the mean of the repeated measures occurs at this range of concentration. As Table 3.6 shows, the CVs of the methods were in the range that claimed by the manufacturer or even less than the CV that claimed by the manufacturer at the medium vitamin B12 concentration.

CVs of machines ranged from 3.2 to 5.8, where the lowest value was for AxSYM. TOSOH® has the lowest precision among the four machines (CV = 5.8 %), nevertheless, its high CV% value still lower than that reported in the manufacture insert.

In conclusion, all the machines have excellent within- run precision for the same sample, but results vary widely when measuring the same sample on different machines for vitamin B12.

Chapter four: Discussion.

In an assay or instrument-validation process, evaluation of the agreement of two or more quantitative assays or methods is of essential importance; in order to show that the assays/ methods give "equivalent' results. Also, in order to exclude the presence of any systematic difference between them. The disagreement between methods will cause a problem in results interpretation and may be a reason for searching another or a confirmatory test that may reflect the patient status best.

For vitamin B12 assay, it is recognized that there is a continuously increase in vitamin B12 assay requests by physicians. Also physician's interest in vitamin B12 deficiency status increased in the past few years; physicians believed that a low vitamin B12 result is responsible for many hematological and neuropsychiatric disorders. They do rely on it in the illustration of many patients symptoms (Oh and Brown, 2003). For that reasons, the assessment of the four commonly used automated analyzers for vitamin B12 assay via assessing the agreement between their results is so important. However, there are currently no internationally recognized reference methods for vitamin B12 measurement.

In this study, 29 different serum sample have been done on the IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System, results have been analyzed and compared as mentioned previously.

4.1. Method correlation.

This study found a relatively moderate to good numeric correlation between serum vitamin B12 results obtained with the four fully automated machines. A positive significant correlation (*P*-value <0.001) with varying degrees of (r) ranging from 0.67 to 0.90 was found between each of the comparative pairs.

Overall, the dispersion of data around the regression line was less than desirable. The comparison of IMMULITE vs. AxSYM method demonstrated the best overall correlation with the comparison method, with the lowest y- intercept (32), and the highest slope and correlation coefficient (0.81 and 0.90, respectively). The other comparative pairs showed poorer agreement than that seen with IMMULITE vs. AxSYM comparison, with different results for slope, y- intercept and correlation coefficient (r). On method comparison studies, low correlation coefficient represents random error, differences in slopes are suggestive of proportional error, and substantial y-intercepts can be attributed to constant error between methods. The comparison of ADVIA vs. IMMULITE shows the highest y-intercept (134.6) that reflects the highest constant error, also, the same comparative pair shows the highest proportional error and random error (slope=0.69 and r=0.67). The correlation between TOSOH and AxSYM shows mainly a constant error with a y-intercept equals to 129.9. While the comparison of ADVIA vs. AxSYM shows mainly proportional error with slope equals to 0.71.

4.2. Methods agreement.

Despite the presence of a significant correlation between all the comparative pairs, none of them meet the conditions for methods agreement suggested by Snyder and Larsen, the three conditions can be summarized as: the correlation coefficient (r) should be > 0.95, the slope of the regression line should be between 0.9 and 1.1, and y- intercept should be close to Zero (Snyder and Larsen, 1983).

Thus; there is no agreement between the methods. The presence of differences between IMMULITE and AXSYM assay results could be due to the differences in the pretreatment

step (heat vs. alkaline hydrolysis, respectively) and in the assay principle between the two assays. Also, the TOSOH method envelope an alkaline hydrolysis step vs. heat in the IMMULITE method, this also could be a reason for the significant difference found in the results of those two methods.

The variations in vitamin B12 results have been mentioned in many other studies; Michael Vogeser and Stefan Lorenzl in their study in 2007 found the same discrepancies in vitamin B12 results between the assessed methods. Vogeser and Lorenzl assessed the agreement between Abbott Architect Vitamin B12, ADVIA Centaur Vitamin B12, and Roche Cobas Vitamin B12. When they applied the expected values of the respective assay, six results were sub-normal with the Abbott assay, three with the Bayer assay, and seven with the Roche assay. Despite this result variations and diagnosis discrepancy; Vogeser and Lorenzl study confirms the close numeric correlation between the vitamin B12 results obtained with the three tests studied with Pearson's correlation coefficients between 0.95 and 0.98. However, they also observed a relevant systematic bias between the assays (Vogeser and Lorenzl, 2007).

4.3. The results discrepancy.

Serum vitamin B12 concentration used to be the first line screening test for the detection of vitamin B12 deficiency subjects, but, could the depending on serum vitamin B12 concentration assay be a problem considering patients diagnosis? The limitation of total serum vitamin B12 measurment have been highlighted by studies that showed poor specificity of only 50% (i.e. healthy persons with a low level of vitamin B12 with no evidence of deficiency) and sensitivity of 95% (i.e. 5% clinically deficient with normal level) (Stabler et al., 1990). Some authors have advocated measurement of MMA and tHcy to assist in the assessment of vitamin B12 status (Green, 1995).

This study shows that the diagnosis of the patient as vitamin B12 deficient or normal may differ for the same individual depending on the vitamin B12 assay method used. The discrepancy in the patients' classification as vitamin B12 deficient or normal was obvious when the number of the deficient vs. normal individual was counted. Considering the

lower limit of normal vitamin B12 as 200 pg/mL (personal communication with local physicians, 5 results were abnormal when assayed by the ADVIA ® method, 3 with IMMULITE ®1000 assay, whereas, 4 with the TOSOH® assay, and 3 with the AxSYM® assay. Meaning that, at least five patients whom diagnosed as vitamin B12 deficient by ADVIA® assay were misdiagnosed with the other assays.

This discrepancy in the assay results and in patients' diagnosis may leave its serious impacts on patients' situation causing serious clinical complication. The discrepancy may confuse both physician and patient. In view of the lack of specificity and sensitivity of vitamin B12 assay and basing the diagnosis of its deficiency solely on laboratory results is not straight forward.

4.4. Reasons for results discrepancy.

There are several potential reasons for disagreement between serum vitamin B12 results obtained with different methods; differences in the sample pre-treatment steps is one of those causes of the results discrepancy (Vogeser and Lorenzl, 2007). Nearly 99% of serum B12 is bound to endogenous binding proteins (transcobalamins) and must be released from these before measurement (Lewis et al., 2006). Two releasing ways are available: alkaline hydrolysis and heat. Usually, in the alkaline hydrolysis NaOH (pH 12–13) used to remove vitamin B12 from its binning proteins, the process also required the presence of potassium cyanide (KCN) which converts cobalamin to the more stable cyancobalamin and dithiothreitol (DTT) to prevent rebinding of released B12 (Lewis et al., 2006). In the four automated analyzers used in the study only IMMULITE depends on high temperature (100 C^{o}) in proteins denaturation.

The second potential reason for the results discrepancy is the alkaline reaction medium result after the first assay step (the alkaline hydrolysis) the alkaline pH may not be optimal for the second step with enveloped the binding of the treated vitamin B12 in the serum sample with the purified intrinsic factors, so the pH has to be subsequently adjusted to be optimal for the binding process (Lewis et al., 2006).

Another source of error could be the different binding characteristics of the intrinsic factor used for ligand binding, which potentially resulting in different cross reactivity of the assays with vitamin B12 analogues formed by the bacterial gut flora (Vogeser and Lorenzl 2007). Also, vitamin B12 analogues could bind to R proteins (Transcobalamin I or rapid protein) if impure sources of intrinsic factor are used in competitive binding assays cause a falsely increased vitamin B12 measurements (Klee, 2000).

Since using purified IF enhances the specificity of vitamin B12 immunoassays, purification of the IF is important and could be accomplished by: chemical purification of porcine IF or by blocking contaminating R proteins by addition of excess blocking cobinamide (vitamin B12 analogues). Using Recombinant IF may also be another choice and offer enhanced specificity over chemically purified porcine IF. Other assays rely on an alkaline denaturation step to inactivate the R proteins. Specificity of pure and blocked IF can be demonstrated by the addition of cobinamide to serum. There should be no increase in assay value (Lewis et al., 2006).

For the four analyzers used in the study, specificity has been determined and data has been given in the product insert of each test. In IMMULITE the manufacturers claimed the absence of any crossreactivity with cobinamide even at concentration as high as 1,800,000 pg/mL and 50,000,000 pg/mL. Also, the manufacturers of ADVIA mention the absence of any crossreactivity with cobinamide when 20ng/mL of cobinamide added to the sera. Whereas, AxSYM manufacturers can report a 0.1% crossreactivity when 9 ng/mL cobinamide added. Less crossreactivity (<0.1 %) reported in TOSOH with 200 ng/mL cobinamide. (Product inserts)

Another potential discrepancy source can be the difference in the signal detection method. Chemiluminescent substrate, fluorescent substrate, or enzymes are the different complexes generates during the assay process and usually used to produce different signals which in turn can be detected and concentration can be calculated (Lewis et al., 2006). In IMMULITE, the substrate adamantyl dioxatane phosphate cleaved by alkaline phosphatase–labelled B12-IF complex, resulting in generation of a plateau

chemiluminescent signal. In ADVIA Centaur direct chemiluminescent technology is used in which vitamin B12 from the patient sample competes with vitamin B12 labeled analog, analogs are labeled with acridinium ester and bound to IF coupled to paramagnetic particles, photons are emitted in response to pH change. In the Abbott AxSYM the substrates 4-methylumbelliferyl phosphate are cleaved by an alkaline phosphatase enzyme – vitamin B12 analog complex, and the resulting reaction generates fluorescence. In TOSOH method B12 present in the sample competes with enzyme- labeled vitamin B12 analog for a limited number of binding sites and then incubated with a fluorogenic substrate, 4-methylumb-ellifeeryl phosphate (4MUP) (Lewis et al., 2006).

The National Health and Nutrition Examination Survey (NHANES) is a program of studies designed to assess the health and nutritional status of adults and children in the United States. This organization interested in vitamin B12 levels in populations as an informative source about general health status. It is depending on the interpretation of the population data after the determination of vitamin B12 levels using different analytical procedures. NHANES reported great variability among procedures which was a problem in results interpretation. They mention that the absence of a reliable reference material (calibrator) and reference analytical method in addition to, the lack of full realization to the modern concepts of measurement accuracy and standardization by many health scientists, are possible source for the variability (Bock and Eckfeldt, 2011).

For vitamin B12, the methodology of establishing a reference material (based on mass spectrometric) is challenging because of the lower concentrations and the complex and heterogenous molecular structure of vitamin B12 (Vogeser and Lorenzl, 2007) (Bock and Eckfeldt, 2011). The WHO International Reference Reagent 03/178 seems to be the only serum derived calibrator in use with a vitamin B12 value assigned by a consensus approach (Bock and Eckfeldt, 2011). Lyophelized standards, also, are available from the National Institute for Biological Standards and Control (Lewis et al., 2006). The absence of such reference material makes it hard to establish a reference method as well (Vogeser and Lorenzl, 2007).

4.5. The precision.

Pooled serum sample aliquots were assayed 10 times on the four analyzers, and results of the 10 repeated measures were collected and analyzed to evaluate the precision of each method. The standard deviation of the repeated measures was calculated and ranged from 12.2 to 15.8. The lowest value was for AxSYM system® followed by IMMULITE ®1000 System, followed by ADVIA ®, and the highest value was for TOSOH®.

From the standard deviation and the mean results; the coefficient of variation (CV) for each method has been calculated. The coefficient of variation (CV) for the methods matches with that claimed by the manufactures. Relatively good precision has been obtained from the four methods. TOSOH® has the lowest precision (CV = 5.8 %) among the four machines. This high CV% for TOSOH® method has been reported in the manufacture insert and proven by the repeated measures data analysis in the study. AxSYM system® has the lowest CV% value (CV = 3.2%), followed by ADVIA Centaur® (CV = 3.4 %) and IMMULITE ®1000 (CV= 4.1 %). In general, the four methods CV% was in that range claimed by the manufacturers.

A previous comparative performance assessment of specified methods have shown serum B12 intra-method and overall assay coefficients of variation (CV) of 4%–12% and up to 20% at clinically relevant levels (Lewis et al., 2006).This high CV% was also reported by the College of American Pathologist's Survey K-A summary report in 1999, this survey assessed the CVs for six commercial methods for erythrocyte folate and serum vitamin B12. Serum vitamin B12 had CVs of 4.4–10.0%; the lowest CV% was obtained by Abbott AxSYM and the highest by IMMULITE (Klee, 2000). The mentioned CV obtained by AxSYM method (4.4 %) is close to that detected by this study (3.2 %). For IMMULITE the situation is different; the College of American Pathologist's Survey recorded higher CV% (10.0%) results for serum vitamin B12 than that obtained by this study (4.1 %)

4.6. Vitamin B12 as diagnostic tool.

Early diagnosis of vitamin B12 deficiency is important to prevent progressive, irreversible neurological impairment. Delayed diagnosis- and in turn treatment- may lead to undesirable consequences. Thus, testing of serum vitamin B12 earned a consequently increased interest. With the development of many assays, the need for evaluation of the newly introduced and the widely used automated assays was appeared (Solomon, 2005).

Serum vitamin B12 was considered as the first screening test to evaluate the vitamin B12 status in individuals. Questions about this test as an effective tool in diagnosis have been raised. The great variations founded in this and other studies in vitamin B12 results among methods and intra laboratories, in addition to, the published researches that discussed the analytical issues and limitations of serum vitamin B12 assay, make vitamin B12 assay as a diagnostic tool questionable.

Measuring serum vitamin B12 level has been used worldwide since the 1950s (Hvas and Nexo 2006). It is the most fabulous test. Varying sensitivity and specificity have been reported. The specificity of vitamin B12 assay in general, seems to be limited. It is obvious that many patients with low serum B12 levels do not have clinical or subclinical cobalamin deficiency (Lewis et al., 2006). In one study, specificity of 60% has been reported when considering vitamin B12 level below 200 pg/mL as a predictive level for the deficiency status, and 90% specificity when predictive level has been decreased to100 pg/mL (Snow, 1999).

According to its sensitivity; vitamin B12 assay sensitivity varies between methods. The lower limit of detection usually set at about 200 pg/mL (Snow, 1999), whereas, lower detection level will increase sensitivity (Lewis et al., 2006). Few factors contribute to this variation in the sensitivity of this assay; first, the use of different methods and techniques and the absent of reference one and standards, second, the different definition to the deficient patients.

Sensitivity of 90% to 95% (i.e. 90-95 % of patients have clinical vitamin B12 deficiency symptoms with vitamin level below 200 pg/mL) had been estimated when patients considered vitamin B12 deficient if they have characteristic clinical features that responded to therapy (Snow, 1999). But, when defined the deficient patients as; those having an increased in the level of vitamin B12 metabolites, only 50% of the patients have serum vitamin B12 level below 200 pg/ml accompanied with increase in the metabolite level (Snow, 1999).

Despite its cheapness and the advantage of being an easy performed test (Hvas and Nexo, 2006), there are several causes for misleading serum vitamin B12 levels; myeloproliferative disorders, polycythemia vera, chronic myelogenous leukemia and liver disease, all may increase haptocorrin and in turn cause falsely elevated vitamin B12 level. In addition, congenital transcobalamin II deficiency and Intestinal bacterial overgrowth (due to the production of vitamin analogous) both are additional causes for falsely elevated vitamin B12 level. On the other hand, folate deficiency (the relation is not well understood), pregnancy, the use of oral contraceptives, congenital deficiency of serum haptocorrins and multiple myeloma, all have been considered as falsely lowered vitamin B12 level causes (Snow, 1999).

The two metabolic markers methylmalonic Acid (MMA) and homocysteine (tHcy) are generally considered more sensitive indicators of vitamin B12 status than are plasma vitamin B12 levels (Hvas and Nexo, 2006). They have been used as confirmatory test for vitamin B12 status in addition to their role in differentiation of vitamin B12 and folate deficiencies (Hvas and Nexo, 2006).

Although there are other causes for their elevation rather than vitamin B12 or folate deficiency (Table 4.1) (Snow, 1999), serum MMA and tHcy measurement seems to be sensitive for the diagnosis of vitamin B12 deficiency (Snow, 1999). When using a response to vitamin B12 therapy as evidence of deficiency, 86% of vitamin B12 responsive patients had elevated levels of MMA and 85% had elevated levels of tHcy, whereas, 94% had elevated levels of at least one serum metabolite (usually MMA) (Snow, 1999). What also

makes those metabolites helpful in vitamin B12 status investigation, is that, the elevation in their levels in serum becomes obvious even before the development of hematological abnormalities and before the reductions in the serum vitamin B12 level (Snow, 1999).

Table 4.1: Causes for elevation of serum MMA and tHcy.

MMA	tHcy
* Vitamin B12 deficiency * Hypovolemia * Renal insufficiency * Inherited metabolic defects	* Vitamin B12 deficiency * Hypovolemia * Renal insufficiency * Folate deficiency * Hopothyroidism * Pyridoxine deficiency * Inherited metabolic defects * Psoriasis

.

Snow (Snow 1999) also summaries the performance of the serum metabolites assay in patients with clinical defined vitamin B12 or folate deficiency (Table 4.2).

Table 4.2: Performance of the serum metabolites assay in patients with clinical defined vitamin B12 or folate deficiency.

Metabolites levels	% Performance in patients with,	
	Vitamin B12 deficiency	Folate deficiency
Elevated serum MMA	98	12
Elevated serum Hcy	96	91
Elevated serum MMA with normal Hcy	4	2
Elevated serum Hcy with normal MMA	1	80
Normal MMA and Hcy	0.2	7

Vitamin B12 deficiency diagnosis is not easy and must not rely on serum vitamin B12 level alone; many strategies and approaches were established to diagnose vitamin B12 deficiency. Hvas and Nexo confirms vitamin B12 deficiency if vitamin B12 level was 25% lower than the bottom reference interval suggested by the manufacturer. They also suggest measuring MMA if serum vitamin B12 level was in the gray area (Hvas and Nexo 2006). The suggested gray area for serum vitamin B12 level suggested by Hvas and Nexo was 170-330 pg/mL, in contrast to Snow diagnostic approaches, they do not differentiate between patients with hematological abnormalities and those with neurological abnormalities.

Two different diagnosis approaches were suggested by Snow; the two approaches differ depending on the patient's signs and symptoms, in other words, they differ if hematological or neurological abnormalities appear on the patient. The following flowcharts summarize the diagnosis strategies suggested by Snow (Snow 1999).

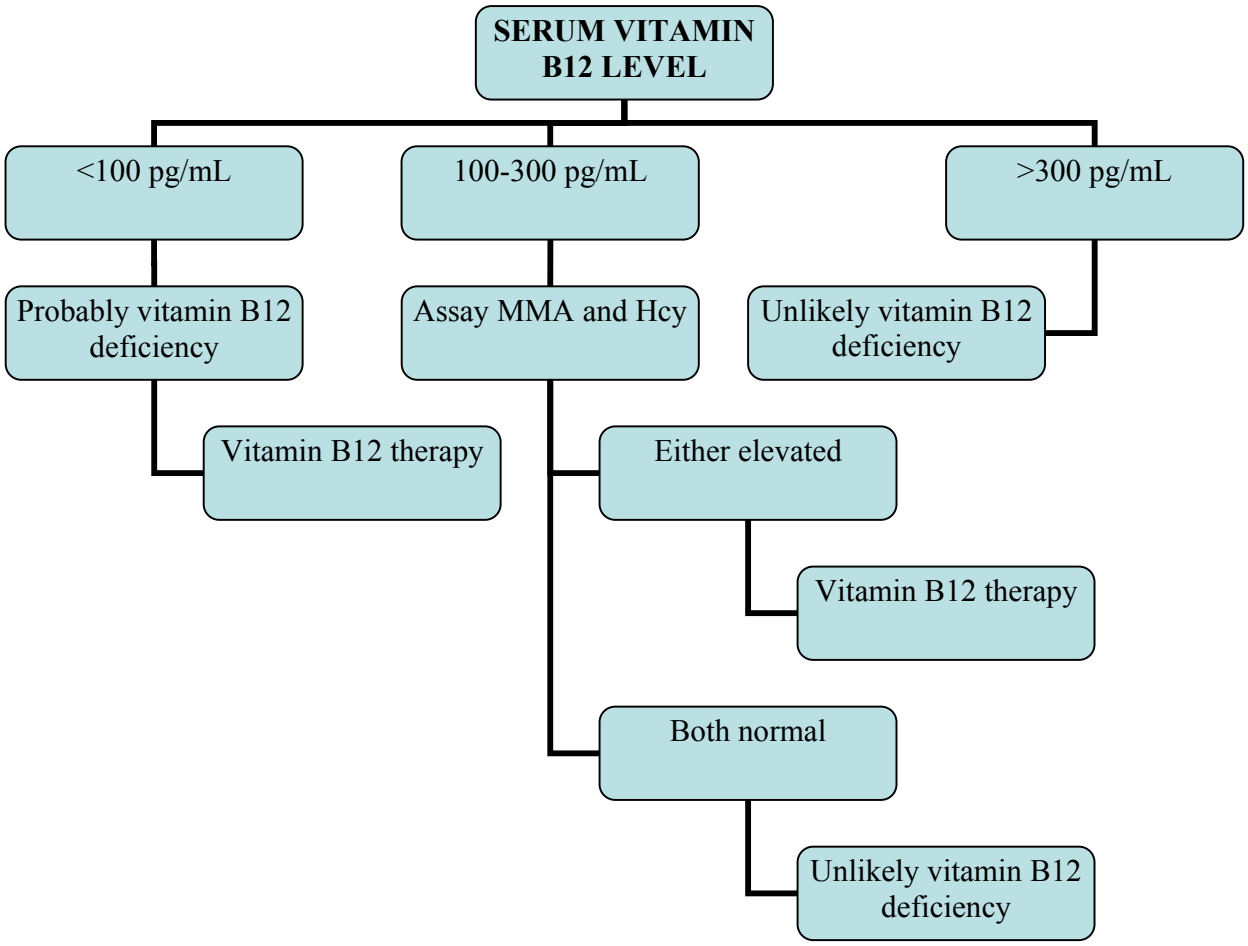

Figure 4.1: Evaluation of the hematological abnormalities suggestive for possible vitamin B12 deficiency. MMA indicates methylmalonic acid and Hcy indicates homocysteine. Modified from Snow (Snow, 1999).

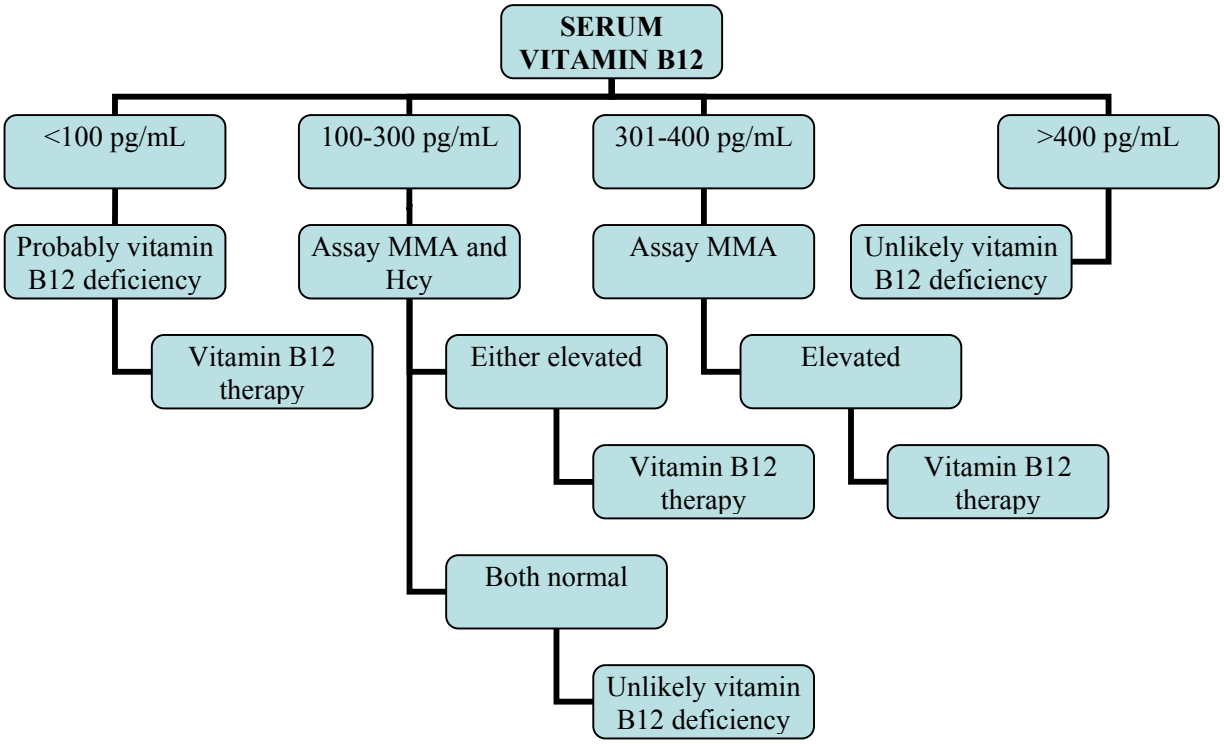

Figure 4.2: Evaluation of the neurological abnormalities suggestive for possible vitamin B12 deficiency. MMA indicates methylmalonic acid and Hcy indicates homocysteine. Modified from Snow (Snow, 1999).

Again and again, it seems very important to us to focus on the absent of gold standards in vitamin B12 deficiency assessment. Ralph Green comments on Lawrence R. Solomon published article (Solomon, 2005) concerns measurement of vitamin B12, homocysteine, and methylmalonic acid. Green has focused on the shocking observation that vitamin B12, homocysteine, and methylmalonic acid assay may not be the ultimate "gold standard" for diagnosis of vitamin B12 deficiency. He reports that the over-diagnosis of cobalamin deficiency is one of the problems, carrying with it the negative consequences of unnecessary treatment as well as the cost. Missed diagnosis is the second one, with its risky consequences on the neurological functions. Green finally concluded that" the currently available assays for identifying or excluding vitamin B12 deficiency, though potentially useful, should be used with full awareness of their possible limitations", and so "Nothing gold can stay" (Green, 2005). Since it is difficult to diagnose vitamin B12 deficiency, researchers recommended clinical evaluation of the treatment response. In patients with anemia they recommend monitoring reticulocytes after 1 week of treatment

administration and hemoglobin after 1 to 2 months (Hvas and Nexo, 2006). On the other hand, patients with neurological abnormalities due to vitamin B12 deficiency should recheck for elevated metabolites levels after 14 day of treatment; if level retain to normal then vitamin B12 therapy will be continued, if not, therapy should be discontinued and patient should examine for other possible causes for his neurological finding (Snow, 1999).

4.7. Conclusion.

In this study, 29 different serum sample have been tested on the IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System, results have been analyzed and compared.

This study found that the results of vitamin B12 were highly variable among the four machines with a relatively moderate to good numeric correlation between serum vitamin B12 results obtained with the four fully automated methods. Despite the presence of a significant correlation between all the comparative analyzers, none of them meet the conditions for methods agreement.

The precision of the compared analyzers (reflected by the coefficient of variation (CV)) matches with that claimed by the manufactures. Relatively good precision has been obtained from the four methods. TOSOH® has the lowest precision among the four machines.

In conclusion, medical technologists need to be aware of the presence of variations in the serum vitamin B12 results between different analyzers/ methods. They should start measuring the metabolic products tests (mainly MMA) in their routine work and encourage physician to request it when serum vitamin B12 result is in the gray area. The second message is for physician whom their increased number of requests for serum vitamin B12 measurement seems to be built on a convenient and traditional work rather than scientific evidence. Physician should be familiar with the analytical and technical issues about serum vitamin B12 assay. They should request a repeat assay in conjunction with the clinical evaluation of the patient and other metabolites assays, if necessary, to make a decision

wither to start vitamin B12 treatment or not. An isolated abnormal result of vitamin B12 should not be the sole criterion on which treatment decisions are made.

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Appendix A: The study questionnaire and consent form.

دراسة بحثية بعنوان

Measurement of Vitamin B12 by Different Methods: A Comparison Study West Bank / Palestine

تهدف الدراسة إلى معرفة مدى التوافق في نتائج فحص فيتامين ب12 باستخدام الأجهزة المختلفة والأكثر استعمالا في الضفة الغربية/ فلسطين, إن مشاركتك بهذه الدراسة اختيارية ولكنها تعود بالنفع على الباحث وتساهم في تطوير أداء المختبرات الطبية .
إن سرية المعلومات الشخصية محفوظة كما نضمن لك عدم استعمالها لأي أغراض أخرى.

الاسم:_____
رقم الهاتف:_____
مكان السكن:_____
الجنس:_____
العمر:_____

هل تعاني من أي مرض: (نعم) (لا)

إذا كانت إجابتك نعم الرجاء توضيح مرضك:_____

هل سبق إن أجريت فحص فيتامين B12 : (نعم) (لا)

ماذا كانت النتيجة_____

هل لديك أحد هذه الأعراض:فقر دم ،ضعف بالذاكرة،تنميل بالأطراف،كسل وضعف عام ؟؟ (نعم) (لا)

هل تتعاطى أي أدوية؟؟ (نعم) (لا)

ما نوع الدواء؟؟_____

هل تتعاطى حبوب أو حقن فيتامين ب 12 ؟؟ (نعم) (لا)
موافقة :أنا الموقع أدناه_____ أوافق على إعطاء عينة من دمي لغرض البحث العلمي على أن لا تستعمل لأي أغراض أخرى.

التوقيع:_____

Appendix B: Age, gender, general health status, and average serum vitamin B12 results* of each participant.

Sample number	Age (year)	Gender	Mentionable diseases	Have any of vitamin B12 deficiency symptoms	Taking vitamin B12 supplements	Average serum vitamin B12 results* (pg/mL)
1	25	M	NO	NO	NO	233
2	29	F	NO	NO	NO	542
3	23	M	NO	NO	NO	234
4	23	M	NO	NO	NO	192
5	40	M	Personal schizophrenia	YES	NO	323
6	42	M	NO	YES	NO	187
7	36	M	NO	NO	NO	294
8	35	M	NO	YES	NO	313
9	50	F	NO	YES	NO	427
10	50	F	NO	YES	NO	714
11	50	F	NO	YES	NO	162
12	42	F	Arthritis	YES	NO	450
13	40	M	NO	NO	NO	310
14	30	F	NO	YES	NO	311
15	45	M	NO	YES	NO	267
16	25	F	NO	YES	NO	260
17	26	F	NO	YES	NO	361
18	26	F	NO	YES	NO	358
19	22	F	NO	YES	NO	270
20	23	M	NO	NO	NO	292
21	28	F	NO	YES	NO	495
22	47	F	*H.pylori*	YES	NO	316
23	17	F	NO	YES	NO	264
24	53	M	NO	NO	NO	359
25	24	F	NO	YES	NO	263
26	22	F	NO	YES	NO	215
27	28	F	NO	YES	NO	213
28	23	F	NO	YES	NO	364
29	27	F	NO	YES	NO	281

***** The average of the four results obtained after the analysis of each sample on; IMMULITE ®1000, TOSOH®, AxSYM system®, and ADVIA Centaur® System.

Appendix C: Serum vitamin B12 results (pg/mL) for 29 patients assayed by different machines. The highest and the lowest results for the same sample applied.

Sample no.	ADVIA ®	TOSOH®	IMMULITE ®1000	AxSYM®	Highest-lowest result*
1	191**	221	266	253	75
2	606***	490	546	524	116
3	300	196	213	226	104
4	160	208	150	207	58
5	363	244	352	332	119
6	186	196	212	153	59
7	312	254	295	314	58
8	304	257	373	317	116
9	356	426	507	419	151
10	635	847	672	701	**212**
11	144	173	150	168	29
12	398	485	517	399	119
13	340	237	345	319	108
14	519	215	273	235	**304**
15	225	206	397	240	191
16	225	212	315	287	103
17	370	254	439	379	185
18	392	283	378	378	109
19	252	236	323	269	87
20	227	305	349	288	122
21	515	415	533	515	118
22	260	360	321	324	100
23	241	232	268	316	75
24	261	301	534	338	**273**
25	381	302	192	175	189
26	241	185	207	225	70
27	141	212	269	228	128
28	309	289	460	398	**171**
29	249	252	335	286	86

* The highest result – the lowest one for the same patient.

** The blue color indicates the lowest result "for the same patient" between the four results.The red color indicates the highest result "for the same patient" between the four results.

*** The average of (the highest result – the lowest) = 125 pg/mL.

قياس فيتامين B12 باستخدام ألأجهزة المختلفة /دراسة مقارنة
الضفة الغربية/ فلسطين

إعداد: سهير فايز محمود البراغيثي
إشراف الدكتور: د. خالد يونس

الملخص

يعتبر فيتامين B12 أحد أهم العناصر للجسم ، إذ يلعب أدوارا رئيسية في عدة مسارات بيوكيميائية تهدف إلى إنتاج مواد أخرى وسيطة مهمة في تكرار الحمض النووي وكذلك تلعب هذه المواد دورا مهما لضمان صحة الجهاز العصبي. إن نقص فيتامين B12 يمكن أن يؤدي إلى مجموعة واسعة من الاضطرابات العصبية والنفسية و الدموية و التي يمكن تفاديها عن طريق التشخيص المبكر والعلاج الفوري.

تهدف هذه الدراسة إلى مقارنة نتائج التحليل الآلي لأربعة أجهزة مستخدمة على نطاق واسع لفحص فيتامين B12 في الضفة الغربية / فلسطين. إن الآلات التي تم مقارنتها كانت ؛® Axsym ، ® ADVIA ، Immulite ® 2000 ، و TOSOH®.

تم في هذه الدراسة أخذ عينات مصل من 30 مشاركا و فحص فيتامين B12 لكل عينة مصل على أربعة أجهزة تحليل آلي مختلفة في ثلاثة مختبرات مختلفة في مدينة رام الله.

أظهر حساب معامل الارتباط بيرسون "r " وجود علاقة إيجابية هامة بين الآلات مع درجات ارتباط متفاوتة، تراوحت من 0.67 الى 0.90 . وكانت كذلك نتيجة الميل مختلفة و متفاوتة أيضا ،إذ تراوحت من 0،69 الى0،81 . كما تراوح التقاطع مع المحور الصادي من 32،2 حتى 134،6 وهو ما يعكس وجود تحيز ثابت وفرق واضح بين الأجهزة.

على الرغم من وجود ارتباط كبير بين الأجهزة المقارنة ، فإن أيا منها لم يستوفي شروط " اتفاق الأساليب" التي يمكن تلخيصها بأن تكون نتيجة معامل الارتباط بيرسون "r " اكبر من 0.97 وأن تكون نقطة التقاطع مع المحور الصادي قريبة للصفر وأن يكون الميل ما بين 0.90 و 1.1. أي إن من الأجهزة المقارنة هي غير متماثلة النتائج .

أظهرت النتائج أيضا وجود دقة في التحليل والتي عكسها معامل الاختلاف (CV) الذي تم الحصول عليه من تكرار العينة الواحدة عشر مرات على الجهاز الواحد .لقد تم الحصول على دقة جيدة نسبيا من الأجهزة الأربعة ويكمن اعتبارها مقبولة حيث أنها تماشت الدقة التي ادعاها المصنع لكل من الأجهزة.

إلا أن العينة الوحيدة التي أعطت نتائج اقل من 200 pg/mL لفيتامين B12 على الأجهزة الأربعة هي عينة رقم11, فيما عدا ذلك كان هناك إخلاف كبير بين نتائج الأجهزة الأربعة للعينة الواحدة حيث تضاعفت بعض النتائج عند قياسها على الأجهزة المختلفة.

تؤكد الدراسة وجود اختلافات في النتائج مصل فيتامين B12 بين مختلف أجهزة تحليل / الأساليب وعليه أوصت الدراسة بعدم الاعتماد على قياس فيتامين B12 في المصل لوحده في التشخيص بل

ينبغي على الطبيب المعالج الأخذ بعين الاعتبار الفحص ألسريري للمريض و على فنيين المختبرات البدء بإدخال اختبارات أخرى تأكيدية مثل فحص المنتجات الأيضية (بشكل رئيسي MMA) في عملهم الروتيني خاصة عندما تكون نتيجة فيتامين B12 في الدم هي في المنطقة الرمادية التي لا تؤكد أو تنفي وجود نقص في الفيتامين بشكل قاطع.