THE ROLE OF HOMOCYSTEINE, FOLIC ACID, VITAMIN B6, VITAMIN B12 IN DEVELOPMENT OF CORONARY HEART DISEASE IN LEBANON

By

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Submitted in partial fulfillment of the requirements for the degree of pharm.D

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We hereby approve the thesis of

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And
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Candidates for the Pharm D degree.

Dr. Mehmood Marueh
Dr. Gabriel Maliha

Date: June
To the soul of my father who gave me the courage in every difficult situation.

To my lovely mother.

To all who helped me and believed in my capacity.

Souraya
To my parents who supported me.

Fadi
Coronary heart disease (CHD) is one of the leading causes of death worldwide. The well established risk factors which increase the danger of CHD are high blood pressure, cigarette smoking, high blood cholesterol level, diabetes mellitus, obesity, sedentary life style and others.

Recently, it has been found that elevated levels of homocysteine, an amino acid found in all healthy people, is a new independent risk factor for coronary heart disease. It is even more predictive than cholesterol in assessing cardiovascular disease risk.

The connection between elevated homocysteine levels and cardiovascular disease was first noted in 1968-1969 by Kilmer McCully. From then on, several prospective and retrospective studies have been carried to show the correlation.

Like all amino acids, homocysteine is normally used to build many proteins required by the human body. It acts as an intermediary in the conversion of the essential amino acid, methionine, to the non-essential amino acid, cysteine. Because folate, vitamin B₁₂ and vitamin B₆ function as cofactors or cosubstrates in the enzymatic reactions involved in homocysteine metabolism, severe hyperhomocysteinemia may be detected in patients with low level of these vitamins.

In this project, our aim was to study the role of Homocysteine, Folate, Vitamin B₆ and vitamin B₁₂ in the development of cardiovascular disease in the Lebanese population. From January 1, 1998 to May 30, 1998, 148 patients were chosen randomly from several Lebanese countries. Our targeted population was Lebanese persons aged 30 years and above. From those patients, 85 were documented to be cardiac while 63 were not. Homocysteine levels were determined by High Performance Liquid Chromatography, folate and vitamin B₁₂ by Gamma Counter and vitamin B₆ by High Performance Liquid Chromatography (to be determined later on). Results were found to be similar to what was noted in nearly all studies done abroad. Cardiac patients have a higher homocysteine levels (mean 11.46 ± 5.33) than non cardiac patients (mean 9.84 ± 4.8) (p = 0.058). Vitamin B₁₂ correlates negatively with homocysteine (pearson correlation = -0.170 p = 0.039). This applies also to folate levels (pearson correlation = -0.227 p = 0.005). We have also found that only few patients are taking vitamin supplement. Therefore, cardiac patients must be advised of the benefit of vitamin in diminishing the risk factor of cardiovascular disease.
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Overview on the role of homocysteine, folic acid, vitamin B6, vitamin B12 in development of coronary heart disease.

Coronary heart disease (CHD) is one of the leading causes of death worldwide. It accounts for 25% of deaths in Canada, 22% in Norways (1), and over one million people die each year in the United States from this disease. Data reported from Bahrain, Cyprus, Egypt, Iran, Jordan, Kuwait, Qatar, Turkey and United Arab Emirates indicate that CVDs are the leading identifiable cause of deaths, especially among adult population aged 30-64 years.

Coronary heart disease (CHD) is a condition in which fatty deposits accumulate in the cells lining the wall of a coronary artery and obstruct the blood flow. Fatty deposits build up gradually and are scattered in the large branches of the main coronary arteries, which encircle the heart and supply it with blood. Atheromas bulge into the arteries and narrow them. As the atheromas enlarge, portions may rupture and enter the blood stream, or small blood clots may form on their surfaces. For the heart to contract and pump blood normally, the myocardium requires a continuous supply of oxygen-enriched blood from the coronary arteries. But as an obstruction of a coronary artery worsens, ischemia to the heart muscle can develop, causing heart damage. The most common cause of myocardial ischemia is coronary artery disease. The major complications of coronary heart disease are angina, myocardial infarction and sudden death (2). Thus, blockages in the arteries around the heart cause the onset of coronary heart disease. The resulting fall in oxygen supply may bring about a heart attack, due to myocardial infarction (the death of vital heart muscle), or angina (chest pain) resulting from heart muscle being starved of its blood supply and oxygen (3).

Clinical studies and a number of surveys show certain personal characteristics and life-styles pointing to increased danger of coronary heart disease. These danger signs are called "risk factor". The well-established risk factors are high blood pressure, cigarette smoking, high blood cholesterol (TC), high Low Density Lipoprotein (HDL-C), low High Density Lipoprotein (HDL-C), being male and diabetes mellitus. Factors such as obesity, sedentary life-style, aggressive response to stress, family history of premature CHD, certain drugs including estrogen replacement therapy are also considered in defining CHD risk.

Hypertension is defined as an elevation of either the systolic blood pressure, the diastolic blood pressure, or both. Although cardiovascular risk increases linearly with blood pressures greater than 120/80 mmHg, blood
increases linearly with blood pressures greater than 120/80 mmHg, blood pressure is not considered elevated unless the systolic pressure exceeds 130 mmHg or the diastolic pressure exceeds 85 mmHg.

<table>
<thead>
<tr>
<th>Category</th>
<th>Systolic (mmHg)</th>
<th>Diastolic (mmHg)</th>
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<tbody>
<tr>
<td>Optimal</td>
<td>&lt;120</td>
<td>&lt;80</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;130</td>
<td>&lt;85</td>
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<tr>
<td>High normal</td>
<td>130-139</td>
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<td>Hypertension</td>
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<td>140-159</td>
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<td>100-109</td>
</tr>
<tr>
<td>Stage 3</td>
<td>≥180</td>
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Prospective cohort studies from several countries have shown that hypertension (both systolic and diastolic) is independently associated with increased morbidity and mortality from CHD (1). Recent data from clinical trials indicates that lowering blood pressure does in fact decrease CHD risk.

Epidemiologic studies, in cohort and case-controlled populations throughout the world, offer indirect evidence of the link between cholesterol and coronary heart disease. The results of these studies have been very similar. A direct relationship between the total and LDL-cholesterol level in blood and the prevalence of CHD death and disability has been found to be consistent. In general, these studies show that for every 1% increase in blood cholesterol levels, there is a 2% increase in the incidence of coronary heart disease. Additionally, for every 1% decrease in HDL-C levels, there is a 2% to 3% increase in CHD (4).

The death from CHD is higher for men than for women, especially between the ages of 35 and 55. After age 55, the death rate for men declines, and the rate for women continues to climb (2).

Cigarette smoking is a powerful risk factor that probably predisposes the smoker to CHD in several ways. According to autopsy studies, smoking accelerates coronary plaque development. Framingham data further reveal that smoking is a powerful risk factor for myocardial infarction, even stronger than for angina pectoris. Of great importance is the fact that smoking cessation rapidly and markedly reduces risk for myocardial infarction. These 2 findings taken together imply that cigarette smoking probably destabilizes coronary plaques and promotes plaque rupture and
coronary thrombosis (5). Thus, smoking is especially dangerous in patients with advanced coronary atherosclerosis. Those who smoke more than 1 package of cigarettes a day are at extremely high risk for premature CHD.

Patients with diabetes mellitus carry an increased risk for CHD. Framingham data suggest that hyperglycemia as such is an independent risk factor. The mechanisms for this effect are not well understood. Whether improved control of hyperglycemia in diabetic patients reduces risk for CHD remains uncertain. Nonetheless, improved glycemic control apparently does reduce the microvascular complications of diabetes. In addition to the independent risk factor hyperglycemia, patients with diabetes commonly have other risk factors (e.g., hypertension, low serum HDL-C, and hypertriglyceridemia); these additional risk factors accentuate the danger of CHD developing in many diabetic patients (5).

Recently and within the last decade, a new independent risk factor for coronary artery and other vascular diseases has been recognized. It appears that elevated levels of homocysteine, an amino acid found in all healthy people, is an important risk factor for heart disease. It is even more predictive than cholesterol in assessing cardiovascular disease risk. Mildly elevated levels of homocysteine have been identified in 21% of patients with coronary artery disease, in 24% of patients with cerebrovascular disease and in 32% of patients with peripheral vascular disease (6).

The connection between elevated homocysteine levels and cardiovascular disease was first noted in 1968-1969 by Kilmer McCully, M.D., a Harvard pathologist at the time. While studying the cases of children dying of vascular damage usually reserved for older people, he noticed that children with a unique metabolic defect called homocystinuria had a severe build-up of plaque in their arteries. He theorized a link between the diseased arteries and the high levels of homocysteine that accumulate in the blood of children with homocystinuria. Dr. McCully performed a variety of animal experiments confirming his hypothesis, but it would needed a large scale human epidemiological study before his hypothesis would be accepted (7). That study was finally completed by another Harvard researcher, Dr. Meir Stampfer. (Oddly, Dr. McCully was denied tenure at Harvard decades earlier!)

Several prospective and retrospective studies have shown that homocysteine is an independent risk factor for CHD. Pioneering clinical studies in patients with CAD were performed in 1976 by Wilcken and Wilcken in Australia. These researchers found that patients younger than 50 years of age, who had angiographic evidence of ischemic heart disease but were free of known risk factors for such disease, had elevated homocysteine
levels compared with normal subjects. The Norwegian Tromsø Heart Study showed a 40% increased risk of MI with each 4µmol/L increase in homocysteine levels. In the United States, a substudy of the Physician's Health Study yielded results consistent with the findings of the Norwegian study. Further, in a prospective study of 587 patients with CAD, Nygard et al found that approximately 4% of patients with homocysteine levels below 9 µmol/L died after 4 years, compared with about one quarter of subjects whose levels were 15µmol/L or higher. Elevated homocysteine in this group was strongly related to MI, even after adjustment for confounding factors(8). The European study in 1990 was conducted on 750 cases of atherosclerotic vascular disease and 800 controls. It showed that there is an independent association of homocysteine concentration with all forms of premature vascular disease. In 1991, Clark et al compared homocysteine levels after methionine loading in patients with premature vascular disease to those in normal subjects. Hyperhomocysteinemia was seen in 30% of coronary artery disease but none in control.

Research in this field, however, has not produced uniform results. For example, the Multiple Risk Factor Intervention Trial failed to show a link between elevated homocysteine and CAD. Discrepancies among studies might be explained by the populations under investigation and their nutritional status, the samples and tests used to measure homocysteine, and even the sample sizes. The weight of evidence, however, still supports homocysteine as a vascular risk factor.
Homocysteine is an amino acid found in all people. Human plasma contains both reduced and oxidized species of homocysteine. The sulphydryl or reduced form is called homocysteine and the disulfite or oxidized form is called homocystine. Disulfite forms also exist with cysteine residues (protein-bound homocysteine). The latter oxidized forms are referred to as mixed disulfites. The oxidized forms of homocysteine usually comprise 98-99% of total plasma homocysteine in human plasma, 80-90% of which is protein-bound.

\[
\begin{align*}
\text{HS-CH}_2\text{-CH}_2\text{-CH-COOH} \\
\text{NH}_2 \\
\text{Homocysteine}\end{align*}
\]

\[
\begin{align*}
\text{CH}_2\text{-CH}_2\text{-CH-COOH} \\
\text{S} \\
\text{S} \\
\text{CH}_2\text{-CH}_2\text{-CH-COOH} \\
\text{NH}_2 \\
\text{Homocysteine-homocysteine disulfide} \\
\text{(Homocystine)}\end{align*}
\]

\[
\begin{align*}
\text{CH}_2\text{-CH-COOH} \\
\text{S} \\
\text{S} \\
\text{CH}_2\text{-CH}_2\text{-CH-COOH} \\
\text{NH}_2 \\
\text{Cysteine-homocysteine disulfide}\end{align*}
\]
Total homocysteine (tHcy) is defined as the sum of all homocysteine species in plasma/serum, including free and protein-bound forms. Like all amino acids, homocysteine is normally used to build many proteins required by the human body. It acts as an intermediary in the conversion of the essential amino acid, methionine, to the non-essential amino acid, cysteine. Methionine is a sulfur-containing amino acid that is involved in the synthesis of protein, important in the maintenance of cartilage, and needed for the formation of important amino acids such as taurine and carnitine. Methionine is especially abundant in animal sources (meat, egg, milk). The recommended daily allowance of methionine is 0.9g; however, for example the average American diet contains approximately 2g/d of methionine, the excess of which is converted via enzymatic transmethylation to homocysteine. Homocysteine is converted to cystathionine via a transulfuration pathway that is dependent on the vitamin B6-dependent enzyme cystathionine β-synthase. Cystathionine is then converted into cysteine, which is eventually degraded and excreted in the urine. Homocysteine also may be recycled back into methionine by either of 2 remethylation pathways, the most important of which involves the vitamin B12-dependent enzyme methionine synthase and its cosubstrate, 5-methyltetrahydrofolate. The other remethylation pathway is independent of vitamin B12 and folate but uses betaine as a cofactor.
S-Adenosyl Methionine (AdoMet) formation:

This reaction is catalyzed by MAT (L-methionine S-adenosyltransferase). In this unusual reaction, the adenosyl moiety of ATP is transferred to methionine, forming a sulfonium bond between the 5'-carbon atom of the ribose and the sulfur atom of the amino acid. The triphosphosphate that results from transfer of the adenosyl portion of ATP remains bound to the enzyme that, by virtue of a second catalytic activity, cleaves the triphosphosphate to inorganic phosphate and pyrophosphate. This triphosphatase activity is specially and markedly stimulated by AdoMet. Removal of triphosphosphate assists in making the synthesis of AdoMet essentially irreversible under physiological condition.

Methyl transfer reactions:

Because of its sulfonium bond, AdoMet may be regarded as a "high-energy" compound. Many methyl transfers originate from AdoMet. In addition, this compound, after decarboxylation, is the source of the 3-carbon moieties of the polyamines, spermidine, and spermine. Liver cytosol and mitochondria may contain kinetically distinguishable pools of AdoMet. All these reactions produce a common sulfur-containing product, S-adenosylhomocysteine.

S-Adenosylhomocysteine Hydrolysis:

S-Adenosylhomocysteine is further metabolized by a hydrolase that cleaves the thioether to homocysteine and adenosine. Each subunit of the enzyme contains 1 molecule of tightly bound NAD⁺. During the catalytic cycle the NAD⁺ is reduced to NADH, with concomitant oxidation of the 3'-hydroxyl of S-adenosylhomocysteine to a keto group. After homocysteine is eliminated, the enzyme-bound NADH reduces the keto group back to a hydroxyl before release of adenosine. S-Adenosyl-homocysteine hydrolase activity is widely distributed in mammalian tissues. Although the equilibrium of the reaction favors S-adenosylhomocysteine accumulation, both its products, homocysteine and adenosine, are normally rapidly removed in vivo, so that the hydrolase functions overall in the cleavage direction.

Homocysteine methylation:

Homocysteine lies at an important metabolic branch point. It may be either converted to cystathionine through the transulfuration pathway, thus completing the sulfur conservation cycle. Homocysteine is methylated in mammals by utilization of either betaine or 5-methyltetrahydrofolic acid as methyl donor. In liver, betaine-dependent methylation of homocysteine is catalyzed by two enzymes: betaine-homocysteine methyltransferase and dimethylthetin-homocysteine methyltransferase. The alternative methyl
donor for homocysteine methylation is 5-methyltetrahydrofolic acid. The reaction is catalyzed by a cobalamin (vitamin B12)-containing enzyme, 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase).

Cystathionine synthesis:
In the metabolism of homocysteine, the major alternative to methylation is condensation with serine to form the thioether cystathionine. The reaction is catalyzed by cystathionine-β-synthase (or L-serine hydrolase). The displacement of the OH group of serine by homocysteine proceeds with retention of configuration.

Although the condensation of homocysteine and serine catalyzed by CBS can be reversed if homocysteine is rapidly removed, the equilibrium of the reaction is very much toward cystathionine formation under physiological conditions. Formation of this thioether, therefore, serves to remove sulfur from the homocysteine-methionine cycle.

Cystathionine cleavage:
The transsulfuration sequence is completed by cleavage of cystathionine to cysteine and α-ketobutyrate, catalyzed by γ-cystathionase (L-cystathionine cysteine-lyase) (9).

Decreased rates of metabolism of homocysteine by impairment of the activity of either CBS or 5-methyltetrahydrofolate-homocysteine methyltransferase lead to accumulation of homocysteine with resultant hyperhomocysteinemia and homocystinuria. Impairment of CBS activity may be genetically determined or be brought about by administration of 6-azauridine triacetate or isonicotinic acid hydrazide. One of six subjects given 300mg of the latter drug daily for 1 month developed mild homocystinuria. This was attributed to interference with pyridoxine metabolism, an interpretation supported by the fact that all six patients developed symptoms of peripheral neuritis and increased cystathionine excretions which, after methionine loads, attained statistical significance. In these sorts of homocystinurias, methionine concentrations will tend also to be elevated.

Hyperhomocysteinemia may result from abnormalities in the function of any of the enzymes involved in homocysteine metabolism or from deficiencies of the enzyme cofactors or cosubstrates such as folate, vitamin B6, and/or vitamin B12. Because these vitamins function as cofactors or cosubstrates in the enzymatic reactions involved in homocysteine metabolism, severe hyperhomocysteinemia may be detected in patients with low levels of folate or vitamin B12, even when serum levels of these vitamins are in the low-normal range. The association between vitamin B6
deficiency and hyperhomocysteinemia is less clear; however, vitamin B6 and homocysteine levels seem to be inversely related.

Clinically, tHcy levels tend to increase with older age and tobacco use. Men tend to have higher tHcy levels than women, and tHcy levels tend to be elevated in individuals with renal dysfunction, unexplained deep venous thrombosis, systemic lupus erythematosus, malignant neoplasms, psoriasis, and solid organ transplantation. After a myocardial infarction or a cerebrovascular accident, tHcy levels are subject to an acute-phase response, characterized by an initial reduction of approximately 25%, followed by a convalescent increase of approximately 20% to 22%, that can interfere with interpretation of laboratory values obtained up to 3 months after these events. The effect of surgery and other systemic illnesses on tHcy levels has not been well characterized. Finally, several commonly used medications, including methotrexate, nitrous oxide, phenytoin, carbamazepine, nicotinic acid, colestipol, and thiazide diuretics, increase tHcy levels.

High homocysteine level in the blood lead to vascular and hematologic abnormalities. Hypotheses on the atherogenic role of homocysteine have been generated by experimental and in vitro studies mostly in animals. Eventhough this is true, no one is sure how homocysteine causes CHD. Several hypotheses have been postulated:
A buildup of homocysteine in the body leads to overproduction of a highly reactive form of homocysteine called homocysteine thiolactone causing plaques in the arteries. This reactive form is made from methionine in the liver where it becomes aggregated with LDL cholesterol. The LDL-homocysteine thiolactone aggregates are then, released into the blood and taken up by macrophages of the artery wall to form foam cells of early atherosclerotic plaques. These foam cells degrade the LDL-homocysteine thiolactone aggregates and release fat and cholesterol into developing plaques. The foam cells also release homocysteine thiolactone into surrounding cells of the artery wall, affecting the way cells handle oxygen. As a result, highly reactive oxygen radicals accumulate within cells damaging the lining cells of arteries, promoting blood clot formation, and stimulating growth of arterial muscle cells which form fibrous tissue, mucoid matrix, and degenerative elastic tissue (11).

- High level of homocysteine causes endothelial cell injury, the initial event in the development of atherosclerosis, manifested as impaired
endothelium-dependent vasodilation and impaired endogenous tissue-type plasminogen activator activity (10).

- Hyperhomocysteinemia causes increased platelet aggregation, related to increased synthesis of thromboxane A2 and decreased synthesis of prostacyclin (10).
- Hyperhomocysteinemia causes abnormalities of the clotting cascade, such as activation of factors V, X, and XII, and inhibition of natural anticoagulants, such as antithrombin III and factor C. Homocysteine promotes the binding of lipoprotein (a) to fibrin and the growth of smooth muscle cells, and tHcy levels correlate with levels of fibrinogen, an independent risk factor for atherosclerotic vascular disease (10).

A positive correlation between plasma levels of homocysteine and cholesterol has been found in patients with homocysteinemia as well as in experimental animals. Based on epidemiological studies, the elevation of plasma homocysteine appears to correlate well with increased levels of cholesterol and triglycerides in homocysteinemic patients. In another study, the supplement of six nutrients (pyridoxine, folate, cobalamin, choline, riboflavin and troxerutin) in the diet to patients after acute myocardial infarction was found to produce a decrease in plasma homocysteine levels, with corresponding decreases in plasma cholesterol and triglycerides. The biochemical events leading to elevated plasma cholesterol in homocysteinemic patients are largely unknown. It is possible that homocysteine would produce an elevated cholesterol level in the hepatic cell and stimulate the secretion of cholesterol level by the cells. It appears that the stimulatory effect of cholesterol synthesis was mediated via the enhancement of HMG-CoA reductase, which catalyzes the rate-limiting step in the cholesterol biosynthetic pathway.

The good news is that elevated homocysteine levels, whether due to nutrient deficiencies or defective genes or any other reason, can easily be normalized in virtually all cases, simply and inexpensively, using a combination of nutritional supplements. Many studies have shown that homocysteine levels are influenced by dietary intakes of folate, vitamin B6 and vitamin B12.

In fact, evidence from the Framingham Heart Study, on going analysis of the risk factors for heart disease which began almost 50 years ago and involves over 1000 men and women, supports the links between folate, vitamin B6, vitamin B12, homocysteine and heart disease. During the study, researchers examined the relationship between intake of folate from foods and supplements with blood plasma folate and homocysteine concentrations among 885 elderly people. The results showed that plasma folate was
significantly greater and homocysteine lower in women than in men. Users of supplements, breakfast cereals, or green leafy vegetables has significantly greater plasma folate and lower homocysteine levels than non-users. Plasma folate concentration was also greater in those who drank orange juice. Similar results have been noticed for the B vitamins.

A 1998 study reported in the New England Journal of Medicine provides further support for the possibility of reducing homocysteine levels by fortifying foods with folic acid. Researchers assessed the effects of breakfast cereals fortified with three levels of folic acid in a randomized double blind, placebo-control, crossover trial in 75 men and women with coronary artery disease. The results showed that folic acid increased and plasma homocysteine decreased in proportion to the folic acid content of the cereal. Cereal providing 127 mcg of folic acid daily, (which is about the amount that would result from the FDA's enriched policy) decreased plasma homocysteine by only 3.7 percent. However, cereals providing 499 and 665 mcg of folic acid daily decreased plasma homocysteine by 11 percent and 14 percent respectively. These results suggest that folic acid fortification at levels higher than that recommended by the FDA may be necessary to effectively reduce homocysteine levels and reduce the risk of cardiovascular disease.

Another study published in 1998 in the American Heart Association journal Circulation provides further evidence of the importance of B vitamins and folic acid in preventing heart disease. Researchers involved in a study done in several centers in Europe compared 750 patients with vascular disease and 800 control subjects of the same ages and sex. They measured blood levels of homocysteine, folate, vitamin B12, and vitamin B6. The results showed that those with high blood homocysteine concentrations had a high risk of vascular disease. In addition, low concentrations of folate, vitamin B6, and vitamin B12 were also associated with increased risk.

Those results were supported by another study where Irish researchers screened a group of clinically healthy working men aged 30 to 49 years and selected 132 with mildly raised homocysteine concentrations. They then assessed the effect of eight weeks of supplementation with B group vitamins and antioxidant vitamins on homocysteine concentrations. The men were randomly assigned to one of four groups: supplementation with B group vitamins alone (1 mg folic acid, 7.2 mg pyridoxine, and 0.02 mg vitamin B12), antioxidant vitamins alone, B-group vitamins with antioxidant vitamins, or placebo. The results showed significant decreases in both groups receiving B group vitamins either with or without antioxidants. The
effect of the B group vitamins alone was a reduction in homocysteine concentration of almost 30 percent.

In a study done in 1993 and published in the American Journal of Clinical Nutrition, South African researchers measured vitamin B6, vitamin B12, and folic acid levels in a group of healthy men with moderately high homocysteine levels. They found these levels to be low. In a placebo-controlled follow-up study, they found that a daily vitamin supplement containing 10 mg vitamin B6, 1.0 mg folic acid and 0.4 mg of vitamin B12 normalized elevated plasma homocysteine concentrations within six weeks. Thus, the most effective defense against homocysteine buildup is a combination of vitamin B-6, vitamin B-12, and folic acid.

Vitamin B12, which is also known as cobalamin, was the last B vitamin to be identified. It is water soluble, bright red in color and has an atom of cobalt at its center. The average adult body contains 2 to 5 mg of vitamin B12, with 80 per cent of this stored in the liver.

Vitamin B12 is essential for metabolism of fats and carbohydrates and the synthesis of proteins. Vitamin B12 is also essential for the transport and storage of folate in cells and for conversion to its active form. Rapidly dividing cells, such as those in the epithelium and bone marrow, have the greatest need for vitamin B12. Vitamin B12 is involved in the manufacture of the myelin sheath, a fatty layer that insulates nerves. It is also essential in the formation of neurotransmitters. The manufacture and normal functioning of blood cells requires vitamin B12. Vitamin B12 is necessary for the production of nucleic acids, which make up DNA, the genetic material of the cell.

A compound known as intrinsic factor, which is secreted by the cells lining the stomach, is necessary for absorption of vitamin B12 from the small intestine. Those with malabsorption problems; such as celiac disease, low stomach acid, or who have had stomach or intestinal surgery; may have problems absorbing vitamin B12. Calcium and iron inhere vitamin B12 absorption.

As the body stores vitamin B12, symptoms of deficiency can take up to four to five years of poor dietary intake or lack of intrinsic factor production to appear. Deficiency is more commonly linked to the inability to absorb the vitamin due to lack of intrinsic factor than to insufficient dietary intake. In fact, any inherited disorder in the transport and/or metabolism of cobalamine manifest itself in most of the cases as hyperhomocystenemia. The general pathway of the cellular uptake and subcellular compartmentation of cobalamins is summarized in figure 1.
Vitamin B12 deficiency is more common in the elderly than in younger people. This is usually because of decreased absorption due to reduced production of intrinsic factor or to a stomach disorder known as atrophic gastritis. Supplementation can prevent irreversible neurological damage if started early. Vitamin B12 deficiency causes pernicious anemia. It also leads to reduced numbers of white blood cells, which causes increased susceptibility to infection. It leads to a loss of nerve-insulating myelin. Vitamin B12 deficiency causes poor cell formation in the digestive tract and leads to nausea, vomiting, loss of appetite, poor absorption of food, soreness of the mouth and tongue, and diarrhea.

Good sources of vitamin B12 include liver and organ meats, muscle meats, fish, eggs, shellfish, milk and most dairy products. Sea vegetables and fermented soybean products also contain forms of vitamin B12, although some research suggests that the human body may not be able to absorb these forms and they may even block true vitamin B12 absorption. Cooking has little effect on vitamin B12 although some may be lost when food is cooked to temperatures above 212 degrees F.

**Sources of vitamin B12:**

- Beef liver, cooked 85g 95.0 mg
- Beef kidney, cooked 85g 43.6 mg
- Tuna, canned, 1 cup 4.38 mg
- Pink salmon, cooked ½ fillet 4.29 mg
- Beef steak, grilled 100g 2.11 mg
- Cottage cheese 1 cup 1.36 mg
- Oysters 6 oysters 1.02 mg
- Cheeseburger 1 serve 0.97 mg
- Skim milk 1 cup 0.88 mg
- Whole milk plain yogurt 1 cup 0.86 mg
- Whole milk 1 cup 0.83 mg
- Feta cheese 1 wedge 0.64 mg
- Eggs, hard boiled 1 large 0.56 mg
- Eggs, scrambled 1 large 0.47 mg
- Chicken, roast 1 cup, chopped 0.44 mg
- Eggs, omelette 1 large 0.43 mg
- Breaded fried chicken 6 pieces 0.31 mg
- Cheddar cheese 1 slice 0.23 mg
- Ham 1 slice 0.23 mg

**Recommended dietary allowances (RDA):**

The RDAs for vitamin B12 have recently been raised in the United States of America.
### USA
- Men: 2.4 mcg
- Women: 2.4 mcg
- Pregnancy: 2.6 mcg
- Lactation: 2.8 mcg

### UK
- Men: 1.5 mcg
- Women: 1.5 mcg
- Pregnancy: 2.0 mcg

### Australia
- Men: 2.0 mcg
- Women: 2.0 mcg
- Pregnancy: 3.0 mcg

Vitamin B12 is available in several supplemental forms, both oral and injectable. Cyanocobalamin is the main synthetic form and has a cyanide molecule attached. Methylcobalamin is one of two active forms of vitamin B12 and may be a more effective supplement. Vitamin B12 tablets should be taken one hour before food for optimal absorption.

Vitamin B6 is a family of chemically related compounds including pyridoxamine and pyridoxal which are found in animal products, and pyridoxine which is found in plants. The form most commonly used in fortified food and supplements is pyridoxine.

Like the other B complex vitamins, vitamin B6 is involved in the functioning of enzymes involved in the release of energy from food. The coenzyme forms of vitamin B6 are pyridoxal 5' phosphate and pyridoxamine 5' phosphate, and these are necessary for nearly 100 enzymatic reactions. These include the synthesis and breakdown of amino acids, the conversion of amino acids to carbohydrate or fat, and the conversion of one type of fat to another. Thus, it is involved in the manufacture of most protein-related compounds and plays a role in almost all bodily processes. Vitamin B6 is essential for the manufacture of fat-derived substances known as prostaglandins which are involved in processes such as blood pressure regulation and heart function. Vitamin B6 is also necessary for red blood cell formation. Vitamin B6 plays a role in maintaining a healthy immune system by affecting functions such as cell multiplication and antibody production. Adequate vitamin B6 is vital to the healthy development and function of the nervous system. Vitamin B6 is important in maintaining healthy hair and skin. Vitamin B6 plays a role in modulating the effects of hormones, including male and female sex hormones and adrenal hormones. Vitamin B6 is also involved in the manufacture of the genetic material of the cell,
sodium-potassium balance, histamine metabolism, the conversion of tryptophan to niacin, absorption of vitamin B12 and the production of hydrochloric acid.

Vitamin B6 is readily absorbed in the small intestine. Excess vitamin B6 is excreted in the urine so adequate daily intake is essential. The forms of vitamin B6 found in food are converted to active forms in the liver. Zinc and riboflavin are necessary for this process.

Adolescents, the elderly, people with heart disease, those on restricted diets and alcoholics are at risk of vitamin B6 deficiency. Others at risk include women on oral contraceptives, those under stress, those whose diets are high in sugar and fat as well as those taking certain medications. People who exercise heavily and athletes often have low vitamin B6 levels. Exercise causes vitamin B6 blood levels to increase during an exercise session possibly because of the release of vitamin B6-dependent enzymes from muscle storage or the transfer of the vitamin from the liver to the muscles. As vitamin B6 is involved in a wide range of body functions, the symptoms of deficiency are widespread. They include mental symptoms, skin problems quality and quantity of antibodies...low levels of vitamin B6 in pregnant women can affect the development of a baby's nervous system...

The richest sources of vitamin B6 are chicken, fish, liver, kidney, pork, eggs, milk, wheatgerm and brewer's yeast. Other good sources include brown rice, soybeans, oats, whole-wheat products, peanuts and walnuts. Long-term storage, canning, roasting or stewing of meat and food processing techniques can destroy vitamin B6 content of food because of losses into the water.

**Sources of vitamin B6:**
- Wheatgerm 1 cup 1.42 mg
- Beef liver, fried 85g 1.22 mg
- Wheat bran 1 cup 0.72 mg
- Bananas 1 medium 0.68 mg
- Chicken, roast 1 cup, chopped 0.63 mg
- Avocado 1 medium 0.56 mg
- Ham, cooked 1 cup 0.51 mg
- Tuna, canned in water 1 cup 0.51 mg
- Spinach, cooked 1 cup 0.42 mg
- Kidneys, cooked 85g 0.44 mg
- Soybeans, cooked 1 cup 0.38 mg
- Raisins 1 cup 0.34 mg
- Beef, grilled 85g 0.35 mg
- Green peas, cooked 1 cup 0.33 mg
Pink salmon, cooked ½ fillet 0.29 mg
Brown rice, cooked 1 cup 0.27 mg
Peanuts ½ cup 0.25 mg
Potatoes, flesh, baked ½ cup 0.17 mg
White rice, cooked 1 cup 0.14 mg
Brussels sprouts, cooked ½ cup 0.13 mg

**Recommended dietary allowances:**

**USA**
- Men 1.3 mg
- (Over 50) 1.7 mg
- Women 1.3 mg
- (Over 50) 1.5 mg
- Pregnancy 1.9 mg
- Lactation 2.0 mg

**UK**
- Men 1.4 mg
- Women 1.6 mg

**Australia**
- Men 1.3-1.9 mg
- Women 0.9-1.4 mg
- Pregnancy +0.7-0.8 mg

The tolerable upper intake limit has been set at 100 mg per day.

Vitamin B6 is available as pyridoxine hydrochloride and pyridoxal-5'-phosphate. The latter is the more active form and may be best for those with liver disease who cannot convert pyridoxine to pyridoxal-5-phosphate.

Folate is the name used for any compound which has vitamin-like activity similar to that of folic acid, the form of this vitamin most commonly used in supplements and fortified foods. Folic acid takes its name from the Latin word for foliage as it was originally isolated from leafy green vegetables. The terms folic acid and folate are generally used to refer to the same substance.

Folic acid is essential for the synthesis of DNA and RNA, the genetic material of cells. It plays a vital role in the growth and reproduction of all body cells, maintaining the genetic code, regulating cell division and transferring inherited characteristics from one cell to another. Folic acid is essential for protein metabolism. As part of its role in protein metabolism, folate converts the amino acid known as homocysteine to methionine as mentioned earlier. The formation of healthy red and white blood cells requires folic acid. Folic acid is involved in the production of neurotransmitters such as serotonin and dopamine, which regulate brain
functions including mood, sleep and appetite. Folic acid is essential for the development of the brain, spinal cord and skeleton in the fetus.

Folic acid is absorbed from the small intestine. The amount of folic acid absorbed from food depends on the source but the average is around 50 percent. Research shows that synthetic forms of folic acid are absorbed better than natural food forms with around 85 percent of supplemental folic acid being absorbed if it is taken with small amount of food. Around 50 percent of body stores are in the liver. The amount stored may last for about four months before symptoms of deficiency develop.

Folate deficiency is the most common nutritional deficiency in the world. Diets low in vegetables, frequent alcohol and prescription drug use and the sensitivity of folate to light and heat contribute to this widespread deficiency. The elderly, alcoholics, psychiatric patients, people taking certain medications and women taking the contraceptive pill may be at greater risk of folate deficiency. Prolonged stress, viral infections and chronic liver disease are also risk factors. When folate intake is inadequate, levels in serum fall, levels in red blood cells also fall, homocysteine concentration rises and finally, changes in the blood cell producing bone marrow and other rapidly dividing cells occurs. Ultimately, folate deficiency affects the growth and repair of all the cells and tissues of the body. Because red blood cells have a life span of 120 days, folate levels in the blood can be lowered for many weeks before symptoms of anemia become apparent. Folic acid deficiency may affect up to a third of all pregnant women and is associated with neural birth defect.

The best sources of folate are liver, brewer’s yeast and dark green leafy vegetables such as spinach. Dried beans, green vegetables, oranges, avocados and whole-wheat products are also good sources. Food processing such as boiling and heating can destroy folic acid. It can also be destroyed by being stored unprotected at room temperature for long periods.

Sources of Folic Acid:
Chicken liver, cooked ½ cup, chopped 512
Lentils, cooked 1 cup 340
Beef liver, fried 85g 187
Spinach, cooked 1 cup 242
Peanuts ½ cup 218
Avocado 1 fruit 124
Peas, cooked 1 cup 96.0
Asparagus, cooked 4 spears 87.6
Yellow corn 1 cup 72.3
Orange juice 1 cup 71.3
Brussels sprouts ½ cup 43.5
Oranges 1 medium 39.7
Walnuts ½ cup, chopped 37.6
Broccoli ½ cup, chopped 37

**Recommended dietary allowances:**

**USA**
- Men 400 mcg
- Women 400 mcg
- Pregnancy 600 mcg
- Lactation 500 mcg

**UK**
- Men 200 mcg
- Women 200 mcg
- Pregnancy 300 mcg
- Lactation 260 mcg

**Australia**
- Men 200 mcg
- Women 200 mcg
- Pregnancy 400 mcg
- Lactation 350 mcg

Folic acid is the type of folate usually found in supplements and fortified foods, as it is the most stable.

In fact not all vitamin products found in the market are effective. The reasons are:

1. **Products delivering the B vitamin mixture in tightly compressed caplets, tablets or cheap-grade gelatin capsules do not dissolve in time to free folic acid into the first third of the small intestine.** Thus far, one study done at University of Maryland School of Pharmacy revealed that two thirds of the folate preparations on the market are so cheaply prepared that they do not dissolve in time to release their contents in the critical folate absorption area (first third of the jejunum). The United States Pharmacopoeia Convention's (USP) dissolution standard requires that a tablet release 75% of the labeled folic acid amount within one hour.

2. **The presence of any amount of ascorbic acid in a mixture of folic acid and vitamin B12 will destroy and deplete the content of vitamin B12 by 50% and of folic acid by 25% while the vitamin mixture remains in the stomach thus robbing the patient of folate and B12 (12).**
Additionally, in the presence of vitamin C there is rapid generation of dangerous "B vitamin analogues" from B12 (within minutes of ingestion) which may cause toxic reactions and act to further reduce B12 efficacy (12).

3. Most products deliver too much vitamin B12. High dose of B12 given to those with severe B12 deficiency may cause a rapid lowering of blood potassium levels that has been associated with sudden death due to cardiac arrhythmia and heart attack.

4. Many products contain too much vitamin B6. Too much vitamin B6 is associated with severe neurological degeneration that is often times irreversible and has led to total paralysis in some unaware of this danger.

5. Some products deliver folic acid without enough vitamin B12. This will cause rapid onset of neuropsychiatric problems including a) memory loss b) depression c) numbness and tingling of the hands and feet d) severe weakness, unsteady gait and finally, paralysis.
Method

Setting
From January 1, 1998 to May 30, 1998, 150 patients were selected randomly from the American University of Beirut (infirmary center), Dr. Jaber Sawaya cardiology clinic, Beirut, and from Dr Wassim Younes medical clinic, Nabatieh.

People involved in the study were Lebanese aged 30 years and above. Persons who had a major systemic illness including Myocardial infraction within the last 3 months were excluded.

All included persons gave informed consent and were asked for the following information (refer to table 1):

- Age
- Town of origin
- Address
- Eating habits
- Intake of vitamins
- Cardiac risk factors such as:
  1. Smoking
  2. Positive family history of coronary heart disease
  3. Obesity
  4. Hypertension: the patient is considered hypertensive if he or she is taking antihypertensive medications or systolic blood pressure is more than 140 mmHg or diastolic blood pressure is more than 90 mmHg.
  5. Hyperlipidemia
  6. Diabetes
- Intake of medications
- Other medical diseases
- Alcohol intake
- Coffee intake
- Exercise
- If he/she is known case of CHD

The 150 patients are divided into case and control groups. Cases are defined as those taking cardiac medications and/or those who have definite myocardial infarction and/or those having angina.
Laboratory measurements

- **Cholesterol levels determination**

  Blood was collected in redtop vacutainer tubes (no additive) and serum separated within 15 minutes and stored at -83°C.

  **Total cholesterol**

  Total cholesterol is determined using the Human kit. It is done by the CHOD-PAP-method, enzymatic colorimetric test with lipid clearing factor.

  Principle of the test:
  Total cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminophenazone in the presence of phenol and peroxidase.

  Reaction principle:

  \[
  \text{cholesterolesterase} \\
  \text{Cholesterol + H}_2\text{O} \rightarrow \text{cholesterol + fatty acid} \\
  \text{cholesteroloxidase} \\
  \text{Cholesterol + O}_2 \rightarrow \text{cholestene-3-one + H}_2\text{O}_2 \\
  \text{peroxidase} \\
  2\text{H}_2\text{O}_2 + 4\text{-aminophenazone + phenol} \rightarrow \text{quinoneimine + 4H}_2\text{O}
  \]

  Procedure:

<table>
<thead>
<tr>
<th>Pipette into cuvettes</th>
<th>Reagent blank</th>
<th>Sample or standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/standard Reagent</td>
<td>1000µL</td>
<td>10µL</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000µL</td>
<td>1000µl</td>
</tr>
</tbody>
</table>

  Mix, incubate 5 minutes at 37°C. Measure the absorbance of the sample/standard against the reagent blank (ΔA) within 60 min.

  Calculation:

  \[
  \Delta A \text{ (sample)} \\
  C = 200 \times \text{[mg/dl]}
  \]
ΔA (standard)
Clinical interpretation:
Suspect over: 220 mg/dl
Elevated over: 260 mg/dl

Triglycerides

Triglyceride is determined using the GPO-PAP method, enzymatic colorimetric test with lipid clearing factor.

Principle of the test:
The triglycerides are determined after enzymatic hydrolysis with lipases. Indicator is quinoneimine formed from hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase.

Reaction principle:

Lipases
Triglycerides \rightarrow glycerol + fatty acids

Glycerol kinase
Glycerol + ATP \rightarrow glycerol-3-phosphate + ADP

Glycerol-3-phosphate oxidase
Glycerol-3-phosphate + O_2 \rightarrow dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-aminoantipyrine + 4-chlorophenol \rightarrow quinoneimine + HCL + H_2O

Procedure:

<table>
<thead>
<tr>
<th>Pipette into cuvettes</th>
<th>Rb</th>
<th>Sample/standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/standard</td>
<td>10μl</td>
<td>1000μl</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1000μl</td>
<td>1000μl</td>
</tr>
</tbody>
</table>

Mix and incubate for 5min. at 37°C. Measure the absorbance of the sample (ΔA sample) and the standard (ΔA standard) against the reagent blank within 60 min.
Calculation:
\[ \Delta A_{\text{sample}} \\ C = 200 \times \frac{\Delta A_{\text{standard}}}{[\text{mg/dl}]} \]

Clinical interpretation:
Suspect over: 150 mg/dl
Increased over: 200 mg/dl

**HDL-cholesterol**

Principle of the test:
The chylomicrons, VLDL (very low density lipoproteins) and LDL (low density lipoproteins) are precipitated by addition of phosphotungstic acid and magnesium chloride. After centrifugation the supernatant fluid contains the HDL (high density lipoproteins) fraction, which is assayed for HDL cholesterol with the HUMAN Cholesterol liquicolor test kit.

Procedure:
1. Precipitation:
Pipette into centrifuge tubes 500\(\mu\)l of sample and 1000\(\mu\)l of precipitant. Mix well; incubate for 10 minutes at room temperature. Centrifuge for at least 2 minutes at 10000 g. After centrifugation separate the clear supernatant from the precipitate within 1 hour and determine the cholesterol concentration using the HUMAN Cholesterol liquicolor reagent.

2. Cholesterol determination:

<table>
<thead>
<tr>
<th>Pipette into cuvettes</th>
<th>Reagent blank [(\mu)l]</th>
<th>Standard [(\mu)l]</th>
<th>Sample [(\mu)l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dist. water</td>
<td>100</td>
<td>(\text{-----})</td>
<td>(\text{-----})</td>
</tr>
<tr>
<td>Standard</td>
<td>(\text{-----})</td>
<td>100</td>
<td>(\text{-----})</td>
</tr>
<tr>
<td>HDL supernatant</td>
<td>(\text{-----})</td>
<td>(\text{-----})</td>
<td>100</td>
</tr>
<tr>
<td>Reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Mix, incubate for 20 minutes at 20-25°C. Measure the absorbance of the sample and the standard, respectively, against the reagent blank within 60 minutes (\(\Delta A\)).
Calculation:

\[ \Delta A \text{ (Sample)} \]

\[ C = 150 \times \frac{\Delta A \text{ (Standard)}}{\Delta A \text{ (Sample)}} \text{ mg/dl} \]

Clinical interpretation:

<table>
<thead>
<tr>
<th></th>
<th>Men [mg/dl]</th>
<th>Women [mg/dl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prognostically favorable</td>
<td>&gt; 55</td>
<td>&gt; 65</td>
</tr>
<tr>
<td>Standard risk level</td>
<td>35-55</td>
<td>45-65</td>
</tr>
<tr>
<td>Risk indicator</td>
<td>&lt; 35</td>
<td>&lt; 45</td>
</tr>
</tbody>
</table>

**LDL-Cholesterol**

The LDL Cholesterol concentration is determined by simple computation. It is calculated from the total cholesterol concentration (TC), the HDL Cholesterol concentration (HDL-C) and the triglycerides concentration (TG) according to Friedewald et al.

\[ \text{LDL-C} = \text{TC - HDL-C} - \frac{\text{TG}}{5}. \text{ [mg/dl]} \]

Clinical interpretation:

Suspicious: 150 mg/dl
Elevated: 190 mg/dl

**Vitamin B12 and folic acid**

Blood was collected in redtop vacutainer tubes (no additive), serum separated within 15 minutes and stored at -83°C.

Vitamin B12 and folic acid serum levels were determined by the Solid Phase No Boil Dualcount kit, radioassay designed for the simultaneous measurement of vitamin B12 and folic acid.

Principle of the test:

Vitamin B12 and folic acid in patient sample are released from carrier proteins by incubation at an elevated pH, above 12, in the presence of dithiothreitol and potassium cyanide. This technique is able to inactivate intrinsic factor antibodies and even the most extreme levels of vitamin B12 transport proteins.
Purified hog intrinsic factor and folate binding protein are employed as the binders for vitamin B12 and folic acid, respectively. B12 analogs do not interfere, since the binder is free of R protein. Moreover, the reaction takes place at a pH where intrinsic factor is fully active and where the folic acid binder has equal affinity for MTHF (the predominant form of folic acid in circulation) and PGA (the stable form used in the DPC calibrators).

With the binders immobilized on microcrystalline cellulose particles, isolation of the bound fraction becomes a simple matter of centrifuging and decanting. Counts in the precipitate are then transformed by comparison with a calibration curve into vitamin B12 and folic acid concentrations. The protein-based calibrators and solid-phase separation are significant factors contributing to the system's freedom from patient blank problems.

Procedure:

1. Label seventeen tubes in duplicate: T (total counts), A (maximum binding) and B through G. Label additional tubes, also in duplicate, for controls and patient samples.

<table>
<thead>
<tr>
<th>Vitamin B12</th>
<th>Approximate pg./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>300</td>
</tr>
<tr>
<td>E</td>
<td>600</td>
</tr>
<tr>
<td>F</td>
<td>1,200</td>
</tr>
<tr>
<td>G</td>
<td>2,400</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Folic acid</th>
<th>Approximate ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
</tr>
<tr>
<td>F</td>
<td>12</td>
</tr>
<tr>
<td>G</td>
<td>24</td>
</tr>
</tbody>
</table>

2. Add 200μL of the zero calibrator A into A tubes, and 200μL of the remaining calibrators B through G into
correspondingly labeled tubes. Pipet 200\(\mu\)L of each serum sample into the tubes prepared.

3. Prepare the working solution no more than 30 minutes before use. The volumes required, in milliliters per assay tube, are tabulated below:

<table>
<thead>
<tr>
<th>Milliliters per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>tracer</td>
</tr>
</tbody>
</table>

4. Add 1,000\(\mu\)L of freshly prepared working solution to all tubes. Vortex

5. Incubate for 30 minutes at room temperature (15-28°C).

6. Add 50\(\mu\)L of NaOH/KCN to all tubes. Vortex.

7. Incubate for 30 minutes at 37°C.

8. Add 1,000\(\mu\)L of Vitamin B12/Folic Acid Binder to all tubes. Vortex vigorously.

9. Incubate for 60 minutes at room temperature (15-28°C)

10. Centrifuge for at least 15 minutes at 2000xg or higher.

11. Decant the supernatants and retain the precipitates for counting.

12. Count the precipitate for 1 minute in a gamma counter.

❖ Vitamin B6

Vitamin B6 was planned to be determined using the technique published in the journal of chromatography by Mieko Kimura at al. Due to the lack of time vitamin B6 range (high or low) was detected from the level of cysteine since homocysteine need vitamin B6 to be metabolized to cysteine through several steps (refer to metabolism of homocysteine).

❖ Homocysteine and cysteine:

The levels of homocysteine and cysteine were determined by high performance liquid chromatography (HPLC). The method was developed by the analytic technical laboratory (ATL), Beirut, Achrafieh.

Principle of the test

Determination of total Hcy in plasma requires the reduction of disulfite bond between Hcy and other thiols or albumin. Sulfhydryl-containing reducing agent such as mercaptoethanol liberate Hcy from various disulfites. A precipitating agent such as methanol is necessary to
remove as much as possible interfering agents, proteins. Precolumn derivatization with fluorogenic reagents for thiols followed by HPLC has become increasingly popular. Useful reagents must form Hey adducts with sufficient fluorescent yield to measure Hey at picomolar concentration or less. In addition to the double bond formed that will be detected by UV, the Hey molecule will be transformed from polar compound to non polar that can stick to the column. For, two steps are required: blocking free thiol group using iodoacetic acid followed by derivatization with O-phthaldialdehyde in the presence of excess thiol:

**Chemicals**

All chemicals were from Sigma except the plasma homocysteine controls which were from Bio-Rad. Deionized double sterile water is used to prepare all reagents.

**Equipment**

- High-performance liquid chromatography (HPLC):
  - Two Shimadzu LC-10Advp pumps
  - Shimadzu high pressure mixer
  - System controller with computer SCL-10Avp
  - Rheodyne manual injector (7125-093)
  - Waters fluorescence detector (474)
  - Waters Precolumn LC-18
  - Waters Column LC-18 (10cm x 4.6m I.D.)
  - Shimadzu integrator (data processor) C-R6A chromatopac

**Sample collection**

Blood was collected in green top vacutainer tubes (sodium heparin), left in ice-water bath for 15 min then plasma was separated and stored at -83°C.

**OPA reagent (o-phthaldialdehyde)**

Six mg OPA was dissolved in 4ml methanol in a foil-wrapped autosampler tube. To this were added 1ml 0.5 Molar boric acid and 100μl β-mercaptoethanol.

**Sample preparation**

To 250μl plasma were added 50μl of 50mmole/l in 0.1 Hcl homocysteic acid (internal standard) and 50μl 5% β-mercaptoethanol (reducing agent). The sample was then vortexed and left for 1 minute. After, a 500μl methanol (precipitating agent) was added. The mixture was vortexed and centrifuged for around 5 minutes. To 20μl supernatant was added 100μl iodoacetic acid (17mg/ml) and 20μl OPA. The reaction was left 1 minute to
occur. Then, 20µl 1.2N HCl was added to stop the reaction. After 30 seconds 10µl was injected into the apparatus.

**Chromatography**
Excitation and emission wavelengths were set at 330 and 450 nm, respectively.

**Mobile phase**
Mobile phase A was 50mM sodium acetate buffered to pH 6.8 and mobile phase B was acetonitrile. The mobile phases were degassed with vacuum before use.

**Separation**
The flow-rate was 1.75ml/min. Separation of internal standard, cysteine and homocysteine was accomplished isocratically using 92% of the mobile phase A and 8% of the B. after 10 min a gradient run starts to wash out the other amino acids.

Run time for each sample takes 20 minutes.

<table>
<thead>
<tr>
<th>Time Range</th>
<th>Mobile Phase A</th>
<th>Mobile Phase B</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10 minutes</td>
<td>92%</td>
<td>8%</td>
<td>Analysis time</td>
</tr>
<tr>
<td>10-15 minutes</td>
<td>60%</td>
<td>40%</td>
<td>Washing time</td>
</tr>
<tr>
<td>15-16 minutes</td>
<td>92%</td>
<td>8%</td>
<td>Washing time</td>
</tr>
<tr>
<td>16-20 minutes</td>
<td>92%</td>
<td>8%</td>
<td>Equilibration time</td>
</tr>
</tbody>
</table>

**Reference range**
Standardized reference ranges do not exist and there is no consensus between the laboratories on the use of a standardized calibrator. The difference seen between laboratories may be because of efficiency of disulfide bond reduction and use of different internal standards and calibrators. Also differences may be seen between two different population. This may be due to genetic factors.

According to Jacobsen the working ranges of homocysteine are:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range</td>
<td>5-15 µmol/L</td>
</tr>
<tr>
<td>Desirable (?)</td>
<td>&lt;10 µmol/L</td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>15-25 µmol/L</td>
</tr>
<tr>
<td>Intermediate</td>
<td>25-50 µmol/L</td>
</tr>
<tr>
<td>Severe</td>
<td>50-500 µmol/L</td>
</tr>
</tbody>
</table>
Data analysis
Data was entered on a computerized statistic program: SPSS
Tests used:
- Descriptive percentages
- Fisher's exact test
- Chisquare
Results and Discussion

In our descriptive pilot study, accessibility and availability of patients have played an important role, and this limited our study to a total of 148 patients chosen from different Lebanese's areas.

The regional distribution was as follows: Eleven patients are from the "Jabal", 11 from the North, 12 from the Bekaa, 45 from Beirut and finally 69 from the south. Thus, our population is formed from patients that come from all over Lebanon, from the 5 districts. (fig.1)

From the 148 patients 101 were males and 47 were females. (fig.2)

All eligible persons are divided into case and control groups. Cases are defined as those taking cardiac medications and/or those having angina. According to the WHO criteria, myocardial infarction is present if there is fulfillment of at least 2 of the following:

1. Clinical symptoms suggestive of MI
2. EKG changes (ST elevation>1mV)
3. Increase of serum biochemical markers.

Angina is considered present if there is documentation of clinical symptoms and/or EKG changes and/or there is evidence of stenosis of at least 50% of one major artery diameter, which is equivalent to 75% of lumen reduction, by angiography. In the absence of all those criteria, patients were considered as control group. This resulted in 57.4% (85) cardiac and 42.6% (63) non-cardiac patients. (fig.3).

Ages range from 32 years to 77 years with a mean of 55.34 ± 10.78 years. (fig.4).

Elevated plasma concentrations of homocysteine are common in patients with coronary artery diseases and confer an independent risk of atherosclerosis. In the present study, links between homocysteine, low vitamin concentrations, and vascular disease risk were seen. In fact, 20.9% of cardiac patients have elevated homocysteine levels. This finding is very similar to the literature results, which state that mildly elevated levels of homocysteine are identified in 21% of patients with coronary artery disease. We can note also that among patients, who have homocysteine levels between 15 and 24 nmol/l, 88.2% are cardiac (table1). Only one non-cardiac patient was detected to have high homocysteine levels of 38.4μmol/l. The patient has a strong family history of coronary heart disease. The father had a heart attack in his early 30th without having any detected risk factor such as high cholesterol. This fact highlight two main issues:

1. high level of homocysteine may be a risk factor for coronary heart disease and
2. Homocysteine is an independent risk factor.

In fact, no correlation is detected between high cholesterol levels and homocysteine levels. For example, between patients, who have a homocysteine level between 15 and 25 nmol/l, 52.9% are hyperlipidemic and 47.1% are not with a non significant p value (table 2).

Health behavior is very important in improving the quality of life. Alcohol and coffee consumption, smoking, and lack of exercise are known risk factors for coronary heart disease. It is important to note that our population is aware of those risk factors. In fact a good percentage, 44.7%, of cardiac patients quitted smoking compared to 14.3% of non-cardiac, 44.7% exercise daily versus 38.1%, and 7.1% stopped alcohol consumption versus 3.1% (table 3). Even though, this is true, vitamin importance among this group of patients in specific and most patients in general is not known. Only few patients are taking vitamin supplement (table 4). As we have mentioned earlier, vitamin B6, B12 and folate play a major role in lowering homocysteine level. In the present study, we can note that the three vitamins decrease when homocysteine level increases (tables 5, 6, 7, 8). Thus, physicians must be aware of the fact that a highly killer risk factor can be eliminated easily and cheaply, by simply prescribing more vitamins and of course the proper ones. In fact, physicians must be knowledgeable about the marketed vitamins. The combination of vitamins in one tablet must be an important concern since for example in the presence of ascorbic acid there is rapid generation of dangerous B vitamin analogues from B12.
Conclusion

The association between elevated homocysteine levels, low vitamin B_{12}, folate and vitamin B_{6}, and coronary heart disease is well established; however, data proving the causal relationship are conflicting. In fact many hypothesis have been postulated but no one is sure how homocysteine causes CHD. There is also a lack of agreement as to what constitute a high homocysteine level or even a desirable one.

Elevated homocysteine levels can be easily and cheaply lowered with vitamin supplementation. Nevertheless, we have no evidence that lowering homocysteine levels will decrease cardiovascular risk. This require data from large scale, randomized, controlled clinical trials. Until data are available it is wise to screen homocysteine levels in patients at risk of coronary heart disease and to recommend that all persons maintain a balanced diet with adequate dietary intake of vitamins. If the patient is unable to adhere to this, then a multiple vitamin containing vitamin B_{12}, folate and vitamin B_{6} must be given. Patients detected with a high homocysteine levels should have vitamin levels measured and be treated with the vitamins in question.

Pharmacist have the opportunity here to encourage patients to maintain a balanced diet along with appropriate vitamin supplementation. By appropriate we mean not any multiple vitamin but the ones containing the necessary dose of vitamin B_{12} and vitamin B_{6}, not a high dose, and not having vitamin C in combination.
Figure 1

Regions:
- Jabal: 11 (7.4%)
- Beirut: 45 (30.4%)
- South: 69 (46.6%)
- Bekaa: 12 (8.1%)
- North: 11 (7.4%)

Region Pie Chart
Figure 2

Male 101 (68.2%) ; female 47 (31.8%)
Figure 3

Cardiac 85 (57.4%) ; non cardiac 63 (42.6%)
Figure 4

Age range 32-77 years; mean: 55.34 ± 10.78 years

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac (57.4%)</th>
<th>Non cardiac (42.6%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>57.41 (10.58)</td>
<td>52.54 (10.48)</td>
<td></td>
</tr>
</tbody>
</table>

SD: standard deviation
Table 1

<table>
<thead>
<tr>
<th></th>
<th>4-15&lt;sup&gt;1&lt;/sup&gt;</th>
<th>15-25&lt;sup&gt;1&lt;/sup&gt;</th>
<th>25-50&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac</td>
<td>53.1%</td>
<td>88.2%</td>
<td>66.7%</td>
</tr>
<tr>
<td>Non Cardiac</td>
<td>46.9%</td>
<td>11.8%</td>
<td>33.3%</td>
</tr>
</tbody>
</table>

<sup>1</sup> Ranges of homocysteine in μmol/l
p value is equal to 0.022
Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac (57.4%)</th>
<th>Non cardiac (42.6%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>15.3% (13)</td>
<td>11.1% (7)</td>
<td>0.462</td>
</tr>
<tr>
<td>Hyperlipidemic</td>
<td>49.4% (42)</td>
<td>57.1% (36)</td>
<td>0.352</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>37.6% (32)</td>
<td>34.9% (22)</td>
<td>0.733</td>
</tr>
<tr>
<td>FHCHD</td>
<td>55.3% (47)</td>
<td>41.3% (26)</td>
<td>0.092</td>
</tr>
</tbody>
</table>

FHCHD: family history of coronary heart disease
Table 3

Healthy behavior:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac (57.4%)</th>
<th>Non cardiac (42.6%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage (no.)</td>
<td>Percentage (no.)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes</td>
<td>23.5% (20)</td>
<td>36.5% (23)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>31.8% (27)</td>
<td>49.2% (31)</td>
</tr>
<tr>
<td></td>
<td>Used to</td>
<td>44.7% (38)</td>
<td>14.3% (9)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Yes</td>
<td>10.6% (9)</td>
<td>17.5% (11)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>82.4% (70)</td>
<td>79.4% (50)</td>
</tr>
<tr>
<td></td>
<td>Used to</td>
<td>7.1% (6)</td>
<td>3.2% (2)</td>
</tr>
<tr>
<td>Exercise</td>
<td>Yes</td>
<td>44.7% (38)</td>
<td>38.1% (24)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>51.8% (44)</td>
<td>58.7% (37)</td>
</tr>
<tr>
<td></td>
<td>Used to</td>
<td>3.5% (3)</td>
<td>3.2% (2)</td>
</tr>
<tr>
<td>Coffee</td>
<td>Yes</td>
<td>83.5% (71)</td>
<td>88.9% (56)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>16.3% (14)</td>
<td>9.5% (6)</td>
</tr>
<tr>
<td></td>
<td>Used to</td>
<td>0% (0)</td>
<td>1.6% (1)</td>
</tr>
</tbody>
</table>
Table 4

Vitamins supplementation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac (57.4%)</th>
<th>Non cardiac (42.6%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivitamin</td>
<td>12.9% (11)</td>
<td>15.9% (10)</td>
<td>0.437</td>
</tr>
<tr>
<td>Folate</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td></td>
</tr>
<tr>
<td>B12</td>
<td>1.2% (1)</td>
<td>0% (0)</td>
<td>0.388</td>
</tr>
<tr>
<td>B6</td>
<td>2.4% (2)</td>
<td>1.6% (1)</td>
<td>0.744</td>
</tr>
</tbody>
</table>
Table 5

Homocysteine, vitamin B12 and folate levels in cardiac patients versus non cardiac.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cardiac Mean (SD)</th>
<th>Non cardiac Mean (SD)</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>11.464 (5.330)</td>
<td>9.8423 (4.798)</td>
<td>1.908</td>
<td>0.058</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>567.915 (318.852)</td>
<td>651.698 (383.567)</td>
<td>-1.449</td>
<td>0.149</td>
</tr>
<tr>
<td>Folate</td>
<td>4.755 (2.967)</td>
<td>4.696 (2.906)</td>
<td>0.120</td>
<td>0.905</td>
</tr>
</tbody>
</table>
Table 6

Homocysteine in correlation with vitamin B12 and folate in all patients (cardiac and non cardiac).

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of cases</th>
<th>Pearson correlation (r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td>148</td>
<td>-0.170</td>
<td>0.039</td>
</tr>
<tr>
<td>Folate</td>
<td>148</td>
<td>-0.227</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Table 7

Homocysteine in correlation with vitamin B12 and folate in cardiac patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of cases</th>
<th>Pearson correlation (r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td>85</td>
<td>-0.075</td>
<td>0.493</td>
</tr>
<tr>
<td>Folate</td>
<td>85</td>
<td>-0.254</td>
<td>0.019</td>
</tr>
</tbody>
</table>
Table 8

- Homocysteine in correlation with vitamin B12 and folate in non cardiac patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of cases</th>
<th>Pearson correlation (r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td>63</td>
<td>- 0.256</td>
<td>0.043</td>
</tr>
<tr>
<td>Folate</td>
<td>63</td>
<td>- 0.198</td>
<td>0.120</td>
</tr>
</tbody>
</table>
References

1. Eivind Meland. "CHD Risk In General Practice". Internet site. Eivind. Meland@isf.vib.no.1998


5. Internet. "Primary Prevention Of Coronary Heart Disease: Guidance From Framingham". http://www.amhrt


27. Jacob Selhub and Armando D' Angelo. " Relationship between Homocysteine and Thrombotic Disease". the american journal of the medical sciences. 1998;316(2):129-141.


