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# PLASMA TOTAL HOMOCYSTEINE AND RISK OF CORONARY ATHEROSCLEROSIS IN PALESTINE

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# PLASMA TOTAL HOMOCYSTEINE AND RISK OF CORONARY ATHEROSCLEROSIS IN PALESTINE

# الهوموسيستين الكلي في البلازما وخطورة الإصابة بتصلب الشرايين التاجية في فلسطين

**BY**

**OSAMA AHMAD NAJJAR**

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# ABBREVIATIONS LIST

| NUMBER | ABBREVIATION | EXPLANATION |
|---|---|---|
| .1 | BUN | Blood Urea Nitrogen |
| .2 | CAD | Coronary Artery Disease |
| .3 | CHOD | Cholesterol Oxidase |
| .4 | CI | Confidence Interval |
| .5 | CVD | Cardio-Vascular Disease |
| .6 | DM | Diabetes Mellitus |
| .7 | EIA | Enzyme Immunoassay |
| .8 | FDA | American Food and Drug Administration |
| .9 | GOD | Glucose Oxidase |
| .10 | GPO | Glycerol-3-Phosphate Oxidase |
| .11 | HCY | Homocysteine |
| .12 | HDL | High Density Lipoprotein |
| .13 | HPLC | High Performance Liquid Chromatography |
| .14 | LDL | Low Density Lipoprotein |
| .15 | MPV | Mean Platelets Volume |
| .16 | MS | Methionine Synthase |
| .17 | MTHFR | Methyl Tetra-Hydrofolate Reductase |
| .18 | OR | Odds Ratio |
| .19 | SD | Standard Deviation |
| .20 | SAH | S-Adenosyl Homocysteine |
| .21 | t-Hcy | Total Homocysteine |
| .22 | TGM | Temporal Gray Matter |

## Objectives:

*** Major:**

- To study the association of increased homocysteine and Coronary Atherosclerosis among Palestinian patients in Palestine.

*** Minor:**

- To correlate between Severity of Atherosclerosis and Homocysteine Concentration.

- To compare Homocysteine as a Risk Factor for Coronary Atherosclerosis and Other Conventional Risk Factors.

ملخص:

العديد من الباحثين ,والعلماء أثبت أن ارتفـاع نسـبة الهوموسيسـتين الكلـي فـي البلازما لديه ارتباط قوي مع أمراض الشرايين التاجية، الأمراض القلبية الوعائيـة، وأمراض القلب الأخرى.

في هذه الدراسة تم البحث في امكانية وجود علاقـة قويـة بيـن الهوموسيسـتين وأمراض الشرايين التاجية في فلسطين( P=0.00 ). وتم أيضا دراسة العلاقة بين الهوموسيستين الكلي وحدة الاصابة بتصلب الشرايين التاجية. وبالاضافة لذلك تم أيضـا قيـاس مسـتويات الكوليسـترول، الـدهنيات الثلاثيـة، حـامض اليوريـك، والكوليسترول عالي الكثافة ومنخفض الكثافة لدراسـتها كعوامـل خطـر تقليديـة لأمراض القلب ومقارنتها مع الهوموسيستين.

الأشخاص في هذه الدراسة تـم تجنيـدهم مـن الـذكور والانـاث بعمـر مـن 21-85سنة والذين خضعوا لعمليات القسطرة القلبية في الفترة مـن شـهر اذار الـى شهر اب من عام 2004 في قسم القسطرة فـي مستشـفى رام الله- الحكـومي في رام الله بفلسطين.

عدد الاشخاص الذين شـاركوا فـي هـذه الدراسـة 388 شـخص مـوزعين علـى النحو التالي:

1) الحـالات المرضـية وعـددهم 189 شـخص: وهـؤلاء هـم الـذين خضعوا لعملية القسطرة وتم تشخيصهم بوجود انسداد بنسبة ≤ 50% في أي من الشرايين التاجية.

2) المجموعة الضابطة السلبية وعددهم 103 اشخاص وهـم هـؤلاء الذين خضعوا لعمليـات القسـطرة وكـانت نتيجـة القسـطرة وجـود انسـداد بنسبة < 50% في اي من الشراين التاجية او عدم وجود اي انسداد.

3) والمجموعة الثالثة وعددها 96 شخص وهم عبارة عـن مجموعـة ضابطة عشوائية تم اختيارها من السكان العاديين بشرط خلـوهم مـن اي من امراض القلب وعدم تعاطيهم اي ادوية لها علاقة بأمراض القلب.

شـوهد فـي هـذه الدراسـة ان الحـالات المرضـية لـديها ارتفـاع فـي معـدل الهوموسيستين بمقدار 31.2% مقارنة بالمجموعة الضابطة السلبية ( P=0.00 )، ولكن بين المجموعتين الضابضطتين لم يكن هناك أي فرق احصائي بينهـم فـي معدل الهوموسيستسن ( P=0.147 ).

النسبة الثنائية بين تصلب الشرايين التاجية وارتفاع نسبة الهوموسيستين في الـدم كانت 3.27 ( 95% CI، 2.14-4.99) وذلك قبل تعديل العمـر والجنـس وبقيـة العوامـل المـؤثرة فـي نسـبة الهوموسيسـتين وذلـك باسـتخدام القيمـة الحديـة

للهوموسيستين 14.7 ± 4.5 ميكرومول/ لتر( هذه القيمة تم تحديدها من هـذه الدراسة وهي تساوي معدل الهيموسيستين لدى المجموعة الضابطة والانحـراف المعياري)، وبعد تعديل العمـر والجنـس وبقيـة العوامـل كـانت النسـبة الثنائيـة= 3.12 (95% CI، 1.95- 4.99).

بالاضافة لذلك كان هناك علاقة احصائية قوية بين حدة ودرجة تصـلب الشـرايين التاجية ( تم قياسها على أسـاس عـدد الشـرايين المغلقـة لـدى المريـض) وبيـن زيادة وارتفاع نسبة الهوموسيستين في الدم ( P=0.00 ).

أما بخصوص عوامل الخطـورة التقليديـة وخاصـة الكوليسـترول عـالي الكثافـة، الكوليسترول الحـر والـدهنيات الثلاثيـة فلـم تظهـر هـذه الدراسـة وجـود علاقـة احصـائية مـع تصـلب الشـرايين التاجيـة مقارنـة بالهوموسيسـتين وكـانت القيـم الاحصائية لها كما يلي على التوالي:

((P= 0.383, P= 0.077, P= 0.507 ، بينما لعوامل الخطورة الأخرى وخاصة حامض اليوريك، والكوليسترول منخفض الكثافـة أظهـرت هـذه الدراسـة وجـود علاقة احصائية قوية مع تصلب الشرايين التاجية وكانت قيمتها الاحصائية كما يلي على التوالي (P= 0.011,

.(P= 0.00

في النتيجة النهائية أظهرت هذه الدراسة أن الهوموسيستين لديه علاقة قوية مـع تصلب الشرايين التاجية وحدة الاصابة بها. ولكن نتائج هذه الدراسـة علـى هـذه الفئة من المرضى في فلسطين يمكنها أن تفتح الطريق وتشجع الباحثين لعمـل دراسـات أشـمل وأعـم لدراسـة الهوموسيسـتين كأحـد عوامـل الاصـابة بتصـلب الشرايين وأمراض القلب المختلفـة وكأحـد عوامـل مراقبـة تطـور الاصـابة بهـذه الأمراض في فلسطين.

## Abstract:

Many researchers have shown that elevated total plasma Homocysteine has a significant association with coronary heart diseases, cardiovascular diseases and other cardiac diseases.

In this present case control study, I investigated whether fasting total Homocysteine has a strong association with coronary artery diseases. Also, correlation between fasting t-Hcy and severity of atherosclerosis was studied. Furthermore, levels of cholesterol, triglycerides, HDL, LDL, and uric acid, were studied as conventional risk factors of atherosclerosis to compare them with total Homocysteine. Subjects were recruited from men and women, aged 21 to 85 years, who underwent coronary angiography (cardiac catheterization) between March 2004 and August 2004 at Ramallah Hospital catheterization laboratory. Cases (n=

189) where defined as those with ≥ 50% occlusion of any coronary artery, while control subjects (n=103) had < 50% occlusion in any coronary artery or had no occlusion at all. In addition a population - based control group free from clinical cardiovascular disease (n= 96) was studied.

Cases had 31.2 % higher mean fasting total homocysteine than both control groups (P=0.00). While between the two control groups the mean total plasma homocysteine is almost the same, and there is no statistical difference between their means (*P*=0.147). The odds ratios (OR) for coronary atherosclerosis and hyperhomocysteinemia was 3.27 (95% CI, 2.14 – 4.99) using Hcy cutoff value 14.7 (Established cutoff value from this study) and before adjusting for age, sex and other confounders. But after adjusting for age, sex and other confounders OR was 3.12(95% CI, 1.95 – 4.99). Furthermore, increasing fasting total homocysteine was associated with severity of atherosclerosis measured as increasing number of occluded arteries (*P*=0.00). For the other conventional risk factors HDL, cholesterol, and triglycerides were statistically insignificant as risk factors for coronary atherosclerosis and their significance was

(*P*=0.383, *P*=0.077, and *P*=0.507 respectively). While for Uric acid and LDL they were statistically significant (*P*= 0.011 and *P*= 0.00 respectively).

In conclusion, the main finding in this population group is that plasma total Homocysteine is strongly related to coronary atherosclerosis and to its severity. These results should encourage further studies of tHcy as a prognostic marker or risk factor for coronary atherosclerosis in Palestine.

# Chapter 1

# INTRODUCTION

# 1.1 Introduction:

Cardiovascular diseases remain the leading causes of mortality in developed countries. Many factors can contribute to the development of atherosclerosis, thrombosis, and subsequent vascular and coronary heart disease[1]. An increase in plasma cholesterol, smoking, and obesity are important risk factors in the development of the atherosclerotic process. Several clinical studies support the link between increased levels of homocysteine and the development of cardiovascular, cerebrovascular, and peripheral vascular disease, and thus the inclusion of plasma homocysteine level as a risk factor.[2]

Several studies have reported that a high plasma homocysteine concentration is a risk factor for vascular diseases. The strongest evidence comes from cross-sectional and case-control studies, which generally support the association between a high plasma homocysteine concentration and a risk of CVD[3, 4]. However, data from prospective cohort studies indicate weaker or no association between the plasma homocysteine concentration and the risk of CVD (Table 1). Five prospective studies have reported that an elevated plasma homocysteine concentration increases the risk of CVD[5,6,7,8,9], whereas an equal number of studies failed to show any association between plasma homocysteine and CVD[10,11,12,13,14] .Thus, data from prospective studies indicate little predictive ability of plasma homocysteine in CVD.

The theory of homocysteine being an independent risk factor for vascular diseases is supported by experimental evidence of mechanisms by which homocysteine might cause vascular damage and disease. The possible mechanisms include endothelial dysfunction and injury, which is followed by platelet activation and thrombus formation. Homocysteine can exert a direct cytotoxic effect on endothelial cells, which is related to

generation of potent reactive oxygen species[15], impaired production of endothelium-derived nitric oxide and endothelial dysfunction[16, 17], and stimulation of smooth-muscle cell proliferation[18]. It has been postulated that homocysteine promotes atherosclerosis by increasing lipid peroxidation and oxidation of LDL, but this has not been confirmed in all studies[19, 20.]Another suggested mechanism of the vascular damage associated with homocysteine relates to formation of oxygen free radicals, which cause vascular damage, proliferation of smooth-muscle cells, alteration of endothelial function and structure, and increased thrombogenicity that leads to atherothrombosis[21]. However, an alternative explanation for an association between homocysteine and vascular damage has been proposed[22]. It has been suggested that plasma homocysteine concentration increases after tissue damage, and the elevated levels of homocysteine further promote the endothelial damage. A high plasma homocysteine level would thus be an indicator of tissue damage and a promoter or enhancer of inflammatory thickening of vascular damage.[22] Findings from a Finnish study support the possibility that homocysteine is a consequence rather than a cause of vascular

damage and disease[14]. In addition, some epidemiological studies have observed that low serum folate and B6 vitamin concentrations increase the risk for vascular diseases, and the elevated plasma tHcy would thus be a marker of the low vitamin concentrations[13, 23, 24.] These findings are strengthened by a recent study showing that folic acid supplementation improves endothelial function in patients with coronary artery disease independently of plasma tHcy reduction[25].

Table (1): Major prospective studies on plasma tHcy concentration and CVD[26].

| Study ((Reference | Follow-up ((years | Out-come | Study population | Cases / controls | Sex | Age | Plasma tHcy cases / others ((µmol/l | Adjusted relative (risk (95% CI |
|---|---|---|---|---|---|---|---|---|
| Physicians' Health Study (Stampfer *et* (*al*. 1992 | 5 | MI, CHD death | 14916 | / 271 271 | M | 84–40 | 10.5 / 11.1 | [a] (8.8–1.3) 3.4 |
| North Karelia Study(Alfthan *et al*. (1994 | 9 | MI, stroke | 7424 | / 265 265 | M, F | 64–40 | ) 9.8 / 10.0 (M | [b] (1.77–0.64) 1.06 |
| Tromso Study (Arnesen *et al*. (1995 | 3.5 | CHD | 21826 | / 122 478 | M, F | 61–12 | 11.3 / 12.7 | [c] (1.65–1.05) 1.32 |
| Physicians' Health Study (Chasan-(Taber *et al*. 1996 | 7.5 | MI | 14916 | / 333 333 | M | 84–40 | not reported | [a] (3.3–0.9) 1.7 |
| Multiple Risk Factor Intervention Trial (MRFIT) (Evans *et* (*al*. 1997 | 11> | MI | 12866 | 186 / 93 | M | 57–35 | 13.1 / 12.6 | [d] (1.54–0.55) 0.82 |
| | 11> | CHD death | | / 147 286 | | | 12.7 / 12.8 | |
| Atherosclerosis Risk is Communities | 3.3 | CHD | 15792 | / 232 527 | M, F | 64–45 | 8.5 / 8.9 | [e] (3.2–0.5) 1.28 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (ARIC) (Folsom *et* (*al*. 1998 | | | | | | | | |
| British United Provident Association Study (BUPA) (Wald *et al*. (1998 | 8.7 | Fatal CHD | 21250 | / 229 1126 | M | 64–35 | 11.8 / 13.1 | [d] (4.12–2.04) 2.9 |
| Framingham Study (Bostom *et al*. (1999 | 10 | CVD mortality | 1933 | 244 cases | M, F | 91–59 | not reported | [f] (1.98–1.16) 1.52 |
| Women's Health Study (Ridker *et al*. (1999 | 3 | CVD | 28263 | / 122 244 | F | postm .enop | 12.4 / 14.1 | [d] (4.3–1.2) 2.3 |
| Finnish Mobile Clinic Health Examination Survey ((Knekt *et al*. 2001 | 13 | MI, CHD death | 3471 | / 272 524 | M | 64–45 | 11.2 / 10.8 | [e] (1.60–0.51) 0.90 |
| MI, myocardial infarction; CHD, coronary heart disease; IHD, ischaemic heart disease[a] top 5 % compared with lowest 10 % of total homocysteine levels, [b] highest 10 % compared with lower 90 % of total homocysteine levels, [c] per 4 µmol/l increase in total homocysteine level, [d] highest compared with lowest quartiles of total homocysteine levels, [e] highest compared with lowest quintile of total homocysteine levels, f highest compared with three lower quartiles of total homocysteine levels | | | | | | | | |

Unlike some independent risk factors of cardiovascular disease, i.e., lipoprotein (a), plasma homocysteine levels can often be therapeutically altered by vitamin supplementation. Consequently, several trials are underway in the United States and Europe[26] are conducted to prove that reducing plasma total homocysteine will reduce the risk of atherosclerosis; these ongoing trials are mentioned in Table (2).

Table (2): Ongoing clinical trials of Homocysteine-lowering vitamin therapy[26].

| **Study** | **Sample Size** | **Folic acid (mg)** |
|---|---|---|
| Vitamin Intervention for Stroke Prevention(VISP),USA | 3600 | 2.5 vs 0.2 |
| The Women's Antioxidant Cardiovascular Study(WACS), USA | 6000-8000 | 2.5 |

| | | |
|---|---|---|
| The Study of Effectiveness of Additional Reduction in Cholesterol and Homocysteine(SEARCH), UK | 12000 | 2 |
| Cambridge Heart Antioxidant Study(CHAOS), UK | 4000 | 5 |
| Norwegian Vitamin Interventional Trail(NORVIT), Norway | 3000 | 0.8 |
| Western-Norway B-Vitamin Intervention Trial (WENBIT), Norway | 2000 | 0.8 |
| The Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease( PACIFIC), Australia | 10000 | 2 |
| Heart Outcome Prevention Evaluation-2(HOPE-2) | 5000 | 2.5 |

All these ongoing trials concentrate on homocysteine-lowering vitamin therapy. Most of them did not issue their results till now. But, the most common target for all of them is to reduce risk of coronary atherosclerosis, CAD, CVD, and stroke by lowering homocysteine through vitamin therapy.

Epidemiological studies have shown that too much homocysteine in the blood is related to a higher risk of coronary heart disease, stroke and peripheral vascular disease. Those epidemiological studies are listed chronologically as follow:

- 1993- Prospective studies demonstrating that tHcy within the normal range is related to myocardial infarction and to stroke [6], [27].
- 1994- Demonstrating that children of vegan mothers had elevated Methylmaonic acid and tHcy levels consistent with a functional vitamin

B12 deficiency, predisposing to neurological damage in infancy and even in adolescence[28, 29].

- 1995- Demonstrating that several life-style factors such as smoking, coffee drinking, intake of fruits and vegetables and physical activity are among the important determinants of tHcy (the Hordaland Homocysteine Study) [30].
- 1996- Showing that markedly elevated tHcy (>40 μM) in the general population is usually due to a combination of a genetic disposition (MTHFR 677C->T mutation) and life-style factors predisposing to high tHcy levels. The majority of these individuals obtained a normal tHcy level by increasing their folate intake (the Hordaland Homocysteine Study) [31].
- 1996- tHcy in children was found to be associated with cardiovascular mortality of their male relatives, suggesting a strong genetic predisposition of high tHcy levels [32].
- 1997- Results from the European concerted action (COMAC) project "Homocysteine and Vascular Disease", demonstrated for the first

time that tHcy strongly interacts with established risk factors for vascular disease, especially hypertension and smoking [33].

- 1997- tHcy was reported to be a strong predictor of mortality in patients with confirmed coronary artery disease. The mortality risk within 5 years was 25 % in patients with tHcy >20 μM, vs 4 % in patients with tHcy <9 μM. The finding was published in the NEJM [34].
- 1997- Finding that the inborn error homocysteinuria is probably much more common (>1/20,000) than previously anticipated (1/300,000). Moreover, in Norway, homocysteinuria is most often related to one particular 'Norwegian mutation'. Patients with this mutation respond to treatment with pyridoxine [35]. This finding motivates new-born screening for homocysteinuria in Norway (pilot study starting in 1998/99).
- 1998- The first report on a substantial increase in tHcy in subjects consuming large amount of filtered coffee [34].
- 2000- Reporting that plasma tHcy was associated with pregnancy complications and adverse outcomes in the large Hordaland homocysteine cohort [36].

- 2001-Reporting that Plasma tHcy is a strong predictor of both cardiovascular and noncardiovascular mortality in a general population of 65–72-y-olds [36].
- 2003- Reporting that hyperhomocysteinemia and the MTHFR 677TT genotype are related to depression in 6000 subjects from the Hordaland homocysteine cohort [37].
- 2004- Demonstration that changes in lifestyle has a modest (compared to folate supplementation) but significant effect on tHcy in 7000 subjects over 6 years period [38].
- 2004-

The largest study to date (2168 case-control pairs) on the MTHFR 677C->T polymorphism and (colorectal ) cancer, demonstrating reduced risk of 27% and 35%, respectively, of colorectal cancer in subjects with the MTHFR 677 TT and MTR 2756 GG genotypes [39].

Two reports have strengthened the evidence for this relationship:

1. A large multi-center European trial, published in the issue of the Journal of the American Medical Association, found that among men and women younger than age 60, the overall risk of coronary and other

vascular disease was 2.2 times higher in those with plasma total homocysteine levels in the top fifth of the normal range compared with those in the bottom four-fifths[4]. The risk was independent of other risk factors, but was notably higher in smokers and persons with high blood pressure.[33]

2. A Norwegian study, published in the issue of the New England Journal of Medicine[34], found that among 587 patients with coronary heart disease, the risk of death after four to five years was proportional to plasma total homocysteine levels. The risk rose from 3.8% in those with the lowest levels (below 9umol/L) to 24.7% with the highest levels (greater than 15 umol/L). Further, in this study Nygard et.al.[34] found that approximately 4% of patients with homocysteine levels below 9 umol/L died after 4 years, compared with about one quarter of subjects whose levels were 15umol/L or higher. Elevated homocysteine in this group was strongly related to MI, even after adjusting for confounding factors. These investigators calculated an adjusted mortality ratio of 1.6:1 for patients with total homocysteine levels of 15umol/L[34].

Also, pioneering clinical studies in patients with CAD were performed by Wilcken and Wilcken in Australia[40]. Those researchers found that patients younger than 50 years of age with CAD had elevated homocysteine levels compared with normal subjects. The Norwegian Tromoso Heart Study showed a 40% increased risk of MI with each 4 umol/L increase in homocysteine levels[41].

In the United States, a sub study of the Physicians' Health Study[5,11] yielded results consistent with the findings of the Norwegian study. A meta-analysis of studies of patients with coronary heart disease demonstrated a similar increase in risk for each 5umol/L increase in homocysteine[42,43]. 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. The populations under investigation and their nutritional status, the samples and tests used to measure homocysteine, and even the sample sizes, might explain discrepancies among studies[3,42]. The weight of evidence, however, still supports homocysteine as a vascular risk factor[44].

## 1.2 Review of Homocysteine Metabolism

### 1.2.1 Background:

In the late 1960's Dr. Kilmer McCully, a Harvard Professor, had been reviewing cases of the rare condition - homocysteinuria in children. He noted that all of the cases showed marked development of atherosclerotic plaques. From this observation, he postulated that elevation of blood homocysteine caused arterial lesions by a direct effect on the cells and tissues of the arteries[45, 46].

Homocysteine is a naturally occurring, sulfur-containing amino acid formed from the metabolism of methionine, an essential amino acid, derived from the diet. Sources of methionine include meats and fish (highest content), vegetables, fruits, nuts, and cereal grains. Methionine is the only known source of homocysteine. Normally, homocysteine is rapidly metabolized to prevent its concentration from increasing in the circulation[47,48].

Homocysteine metabolism is controlled by one of two biochemical pathways: Transsulfuration and remethylation (Fig 1)[49]

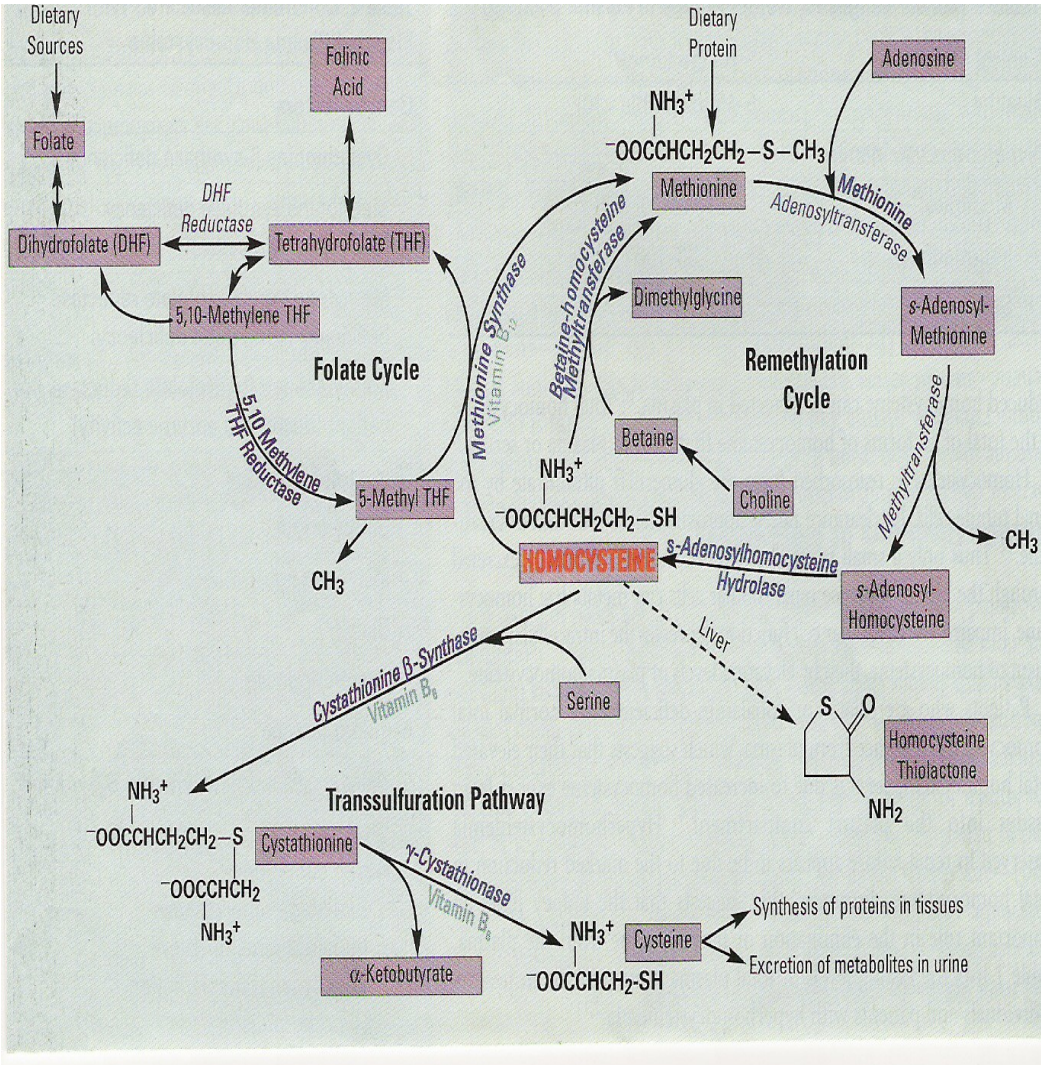



Fig 1. Methionoine cycle and homocysteine metabolism.

In conditions in which methionine is in excess or cysteine synthesis is required, homocysteine enters the transsulfuration pathway. The reaction is catalyzed by the enzyme cystathionine B-synthase, the activity of which depends on the cofactor vitamin B6. During periods of methionine

deficiency, homocysteine enters the remethylation pathway. In most tissues, remethylation occurs via the folate cycle, using the enzymes N5N10-methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase (MS); methylated folate serves as the methyl donor[49].

An alternative pathway occurs primarily in the liver via the enzyme betaine-homocysteine methyl-transferase; this pathway is independent of vitamin B12 and folate, and the cofactor betaine donates the methyl group[49].

The intracellular levels of homocysteine are kept within a narrow range. Increase of 1 level of intracellular homocysteine caused by augmented production or a reduction in its metabolism results in corresponding increases in blood levels. These changes therefore reflect intracellular homeostasis of those pathways involved with homocysteine metabolism. Although plasma concentrations of homocysteine have low short-term and long-term, within-individual variation, high methionine, low folate, or low cobalamin (vitamin B12) levels, as well as agents that interfere with homocysteine metabolism, cause an increase in homocysteine

export from cells, thus increasing the concentration of plasma homocysteine[50].

Human plasma contains both the reduced and oxidized species of homocysteine. The reduced or sulfhydryl (-SH) form is called homocysteine; the oxidized or disulfide (-S-S-) form is composed of homocysteine and mixed disulfides (Fig 2)[51].

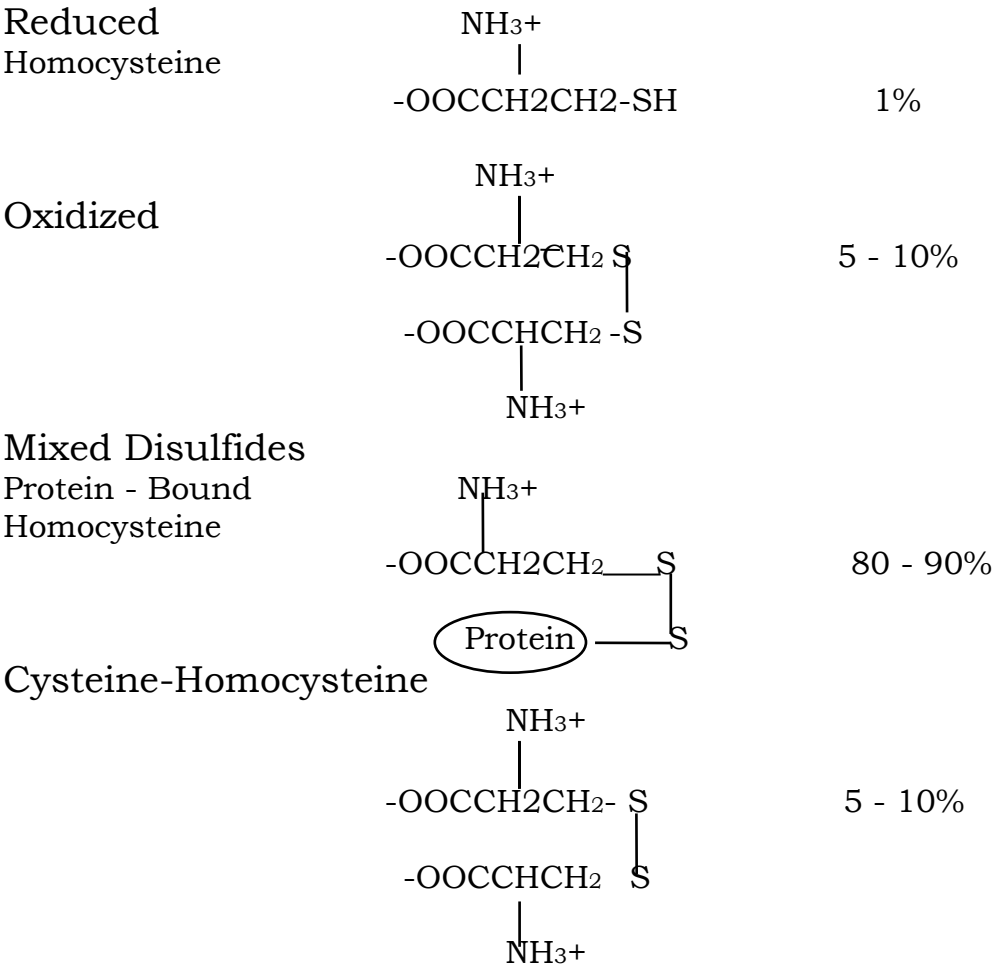



Fig 2: Constituents of total homocysteine and percentage of each in plasma.

The mixed disulfides of plasma homocysteine occur by binding with other thiols, i.e., compounds containing —SH groups; they are primarily homocysteine-cysteine residues or proteins that contain reactive cysteine groups that form protein-bound homocysteine.

Albumin binds approximately 70% to 80% of the plasma homocysteine; it is the major binding protein for thiols in human plasma. Most (98%-99%) of the plasma homocysteine is in the oxidized form, i.e., protein-bound and "free." Healthy individuals have free homocysteine (reduced and mixed disulfides) levels of approximately 2 to 3 (umol/L or 20% to 30% of the total." Only a trace amount (<0.3 umol/L) of free reduced homocysteine can be detected in plasma. Total homocysteine is the total of all forms of homocysteine that exist in plasma or serum[51].

Homocysteine is reabsorbed from the glomerular ultra filtrate by the renal tubule cells. Its clearance rate is approximately 1% of that of creatinine. Thus only a small portion of homocysteine is normally excreted through the kidneys. Because renal tubular cells can metabolize homocysteine, impairment of tubular cell function depresses the intracellular catabolism of homocysteine, thereby elevating levels of plasma homocysteine[51].

Patients who are folate - or cobalamin-deficient have normal total homocysteine clearance from plasma, which suggests that their elevated

total homocysteine level is due to increased homocysteine export from tissues into plasma compartment. Hyperhomocysteinemia observed in renal failure appears to be due to the marked reduction in total homocysteine clearance; this suggests that the kidney play an important role in the elimination of homocysteine from the plasma. Table (3) lists the fasting levels of total plasma homocysteine in healthy individuals and patients with hyperhomocysteinemia[51].

Table (3). Clinical Ranges for Homocysteine in plasma (Fasting) [51]

| **Clinical Condition** | **(Range (umol/L** |
|---|---|
| Healthy | 5-15 ( desirable > 10 ) |
| Hyperhomocysteinemia | |
| Moderate | 15 – 30 |
| Intermediate | 30 – 100 |
| Severe | < 100 |

### 1.2.2 Factors Affecting Homocysteine Level:

Because homocysteine metabolism is complex involving several enzymatic reactions and cofactors (i.e., vitamin B6, vitamin B12, folic acid) any inhibition of its metabolism or depletion of cofactors can lead to hyperhomocysteinemia and homocysteinuria[52]. A variety of factors are known to affect plasma levels of homocysteine, including enzyme

deficiencies (genetic disorders), liver and renal disease, hypothyroidism, pharmacological agents, age, and possibly gender[53]. Although males tend to have slightly higher levels than females, a recent study suggests that gender does not significantly influence homocysteine levels. Conditions associated with elevated levels of homocysteine are listed in Table (4)[33, 51].

Table 4. Conditions Associated With Elevated Plasma Homocysteine[51]

| |
|---|
| **Genetic factors** |
| Cystathionine B- synthase deficiency. |
| Methionine synthase deficiency ( Cobalamin C disease ) |
| Methylene tetrahydrofolate reductase deficiency ( congenital deficiency ) |
| Methylene tetrahydrofolate reductase variant (decreased enzyme activity). |
| **Demographic factors** |
| Ethnic origin |
| Increased age |
| Male (?) |
| Postmenopausal |
| **Acquired factors** |
| Deficiencies in folate, vitamin B6, vitamin B12 |
| Health conditions : |
| End-stage renal disease |
| Impaired renal function |
| Post organ transplantation. |
| Hypothyroidism |
| Malignant diseases |
| Hypertension |
| Therapeutic drugs |
| **Lifestyle factors** |
| Chronic alcohol consumption |
| Chronic illicit substance use |
| Excessive coffee consumption |
| Lack of exercise |
| Smoking |
| **Preanalytical variables** |
| Delay in separating plasma from cells |
| Nonfasting sample |

## 1.3 Genetic Disorders of Metabolism:

Genetic defects leading to deficiencies in the enzymes involved in homocysteine metabolism result in marked elevations in the concentration of homocysteine in plasma and in urine. The most common genetic cause of severe hyperhomocysteinemia is cystathionine B-synthase deficiency[13,54]. The homozygous form of this disease - congenital homocysteinuria - is associated with fasting plasma homocysteine levels up to 40 times the upper limit of normal (400 umol/L); methionine concentration in plasma is also elevated. The homozygous trait is rare, occurring in only 1 in 200,000 births[55]. Its clinical manifestations include skeletal deformities, mental retardation, venous thrombosis, and severe, premature atherosclerosis[19]. Homozygous persons frequently develop atherosclerotic complications in early adulthood that are often fatal. In the USA, heterozygous deficiency of this enzyme occurs in 1 of 300 births. Heterozygous persons typically have plasma homocysteine levels in the range of 20 to 40 umol/L; patients are usually treated with vitamin B12, betaine, or both[13,54,55].

Homozygous deficiency in N5, N10-methylene tetrahydrofolate reductase (MTHFR) may also lead to severe hyperhomocysteinemia. Patients with severe deficiencies of this enzyme may have variable phenotypes, such as developmental delay, motor and gait abnormalities, seizures; and psychiatric disturbances[13]. Pathologic changes observed in the severe form include the vascular changes found in conditions with elevated homocysteine and reduced neurotransmitter and methionine levels in the cerebrospinal fluid. A variant of this enzyme (decreased activity) is the most common genetic cause of mild hyperhomocysteinemia. Homozygosity of this mutant enzyme occurs in 9% to 17% of the population, and this percent varies among different populations, and heterozygosity can be detected in 30% to 41% of the general population. Treatment of both disorders involves administration of vitamin B12, folic acid (Leucovorin), and betaine[13,54].

A much more rare disorder (cobalamin C disease) is caused by a congenital deficiency of the liver enzyme metyltetrahydrofolate homocysteine methyltransferase (methionine synthase) that uses folic acid and vitamin B12 to convert homocysteine to methionine. Clinical

manifestations include arteriolar damage and accumulation of homocysteine and cystathionine in the urine. Treatment consists of administration of vitamin B12[13,54].

## 1.4 Acquired Disorders of Hyperhomocysteinemia:

Inadequate plasma concentrations of vitamin B6, and vitamin B12, and/or folate, contribute to nearly two thirds of all cases of hyperhomocysteinemia. Thus vitamin supplementation in these patients has been shown to effectively reduce elevated plasma homocysteine levels and may improve cardiovascular morbidity and mortality. In addition to vitamin deficiencies, several therapeutic drugs have been shown to affect homocysteine levels[11,55]. These compounds, along with their proposed mechanisms of action, are listed in Table (5) [5,11].

Table (5). Drugs or Agents Known to Affect Plasma Homocysteine Level[5, 11]

| Drug or Agent | Proposed Mechanism |
|---|---|
| Increases plasma homocysteine | |
| Anticonvulsant : Carbamazepine, phenobarbital, phenytoin, primidone, valporic acid | Interfere with folate metabolism by impairing intestinal absorption, inducing hepatic enzymes that require and deplete folate, and interfering with metabolism of folate coenzymes. |
| 6-Azauridine | Vitamin B6 antagonist |
| Cyclosporine | Possible folate antagonist; interferes with folate remethylations of homocysteine. |
| Metformin | Affects homocysteine homeostasis |
| Methotrexate | Blocks folate cycle; dihydrofolate reductase inhibitor; reduces 5-methyltetrahydrofolate . |
| Nitrous oxide | Methionine synthase inhibitor; inactivates vitamin B12 cofactor |
| Theophylline | Vitamin B6 antagonist ( phosphodiesterase inhibitor of pyridoxal phosphate ) |
| Decreases plasma homocysteine | |
| D-penicillamine | Forms mixed disulfides |
| Folic acid | Enhances remethylation |
| Oral contraceptives | Unknown |
| Tamoxifen | Unknown |

## 1.5 Homocysteine and Atherosclerosis

The “prothrombotic state” (any condition associated with a high frequency of thrombosis) due to hyperhomocysteinemia arises from minor changes in homeostatic mechanisms. For example, homocysteine

increases platelet synthesis of thromboxane A2 and enhances the expression of selectin, the platelet adhesion molecule; both conditions cause an increase in platelet adhesiveness and aggregation[52,53,56]. Homocysteine may also produce cytotoxic endothelial cell injury, abnormalities in the blood clotting factors and fibrinolysis, and alteration of cholesterol and lipoprotein metabolism. Endothelial cell injury with subsequent platelet hyperactivity and the presence of abnormal clotting factors may contribute directly to formation of thromboembolic obstructions of the coronary, carotid, and peripheral arteries. These obstructions can lead directly to transient ischemic attacks and eventually coronary heart disease, ischemic stroke, and peripheral arterial disease, especially in elderly persons[18,57].

The development of atherosclerosis and cardiovascular disease is a continuous process that occurs at several stages, beginning with endothelial injury and leading to smooth muscle proliferation. When homocysteine accumulates in the blood, its metabolism becomes altered with the formation of a major by-product called homocysteine thiolactone. It has been proposed that homocysteine thiolactone reacts

with LDL to form so-called homocysteine thiolactone aggregates. These aggregates are sequestered by macrophages, and the resulting foam cells become prevalent in atherosclerotic plaques. Within these plaques, homocysteine thiolactone can react with the various proteins, modifying the oxidative process of the blood vessels. Auto-oxidation of homocysteine results in the formation of superoxide and hydrogen peroxide, which may contribute to the oxidation of LDL, endothelial dysfunction, and proliferation of vascular smooth muscle[18, 58].

## 1.6 Homocysteine and Osteoporosis:

Osteoporosis is a disease of bone matrix. The process of normal bone formation is dependent on the collagen matrix, and requires the synthesis of collagen cross-links. The sulfhydryl group (- SH) of homocysteine interferes with the formation of these cross-link precursors of collagen, and thus the normal synthesis of collagen and bone formation. Patients with hyperhomocysteinemia and homocysteinuria develop osteoporosis at a higher rate than normohomocyteinemia subjects. The overall risk of developing osteoporosis in the presence of hyperhomocysteinemia has

been reported to be 50% by age 16 years among homocysteinuria patients[58,59].

## 1.7 Homocysteine and Other Degenerative Diseases

Elevated homocysteine can be a sign of a methylation deficiency throughout the body. Methylation is fundamental to DNA repaired. If DNA is not adequately repaired, mutations and strand breaks will result. This will lead to accelerated aging, as greater amounts of faulty proteins are synthesized from the damaged DNA. The liver depends on methylation to perform the numerous enzymatic reactions required to detoxify every drug and foreign substance that the body is exposed to. Methylation is also required for the growth of new cells. Without it, new cells cannot be made[58].

A study published in the Journal Medical Hypothesis provides evidence that aging may be exclusively a result of cellular "demethylation," or, said differently, the aging process is caused by the depletion of enzymatic "remethylation" activity that is required to maintain and repair cellular DNA. This study suggests that aging may be

reversible if aged cells could be programmed to remethylate rather than demethylate[60].

Homocysteine induces cellular damage by interfering with the methylation process. Methylation will be compromised if homocysteine is elevated, and elevated homocysteine is a warning sign that the methylation cycle is not functioning properly. Homocysteine may also damage cells directly by promoting oxidative stress[49, 60].

There is a growing consensus that deficient methylation is the major cause of the degenerative diseases of aging. The consumption of methylation-enhancing nutrients like (Temporal Gray Matter) TGM, choline, folic acid, and vitaminB12 may be one of the most readily available and effective anti-aging therapies presently known. However, it is important to tailor the intake of methylation-enhancing nutrients to one's individual biochemistry. The best way of assessing one's body rate of methylation is to measure blood level of homocysteine[49].

## 1.8 Homocysteine and Alzheimer's disease

Recent studies show that patients with dementia of the Alzheimer's type have elevated levels of homocysteine in their blood.

While scientists speculated that Alzheimer's disease could be avoided if people reduced their homocysteine levels, it has not yet been determined whether homocysteine itself contributes to Alzheimer's disease[48]. A more likely explanation is that elevated homocysteine is an indication of the severe disruption in the methylation pathway that occurs in the brains of Alzheimer's patients. It has been reported that people with Alzheimer's disease have virtually no S-adenosylmethionine (SAMe) in their brains. SAMe is required for DNA methylation (maintenance and repair) of brain cells. Thus, while homocysteine itself may not cause Alzheimer's disease, it appears to represent an important measurable biomarker of a methylation deficit that could cause Alzheimer's and a host of other degenerative diseases[48,49,61].

## 1.9 Treatment of Hyperhomocysteinemia

Elevated levels of homocysteine can be reduced or normalized with dietary modifications and vitamin therapy, except in those patients with severe hyperhomocysteinemia. Dietary modifications include minimizing methionine intake. Processed and refined foods should be kept to a minimum, as should foods high in saturated fats. The US

Department of Agriculture recommends 2 to 4 servings of fruit, 3 to 5 servings of vegetables, and 6 to 11 servings of breads, grains, and pastas. Studies have shown that folic acid given at concentrations of 400ug or more daily can lower total homocysteine levels by 30% to 40%; vitamin B12 appears to be less effective, only lowering the total homocysteine level by 15%; pyridoxine supplementation is least effective, in the absence of vitamin B6 deficiency[42,51].

Initial vitamin therapy includes folic acid and a multivitamin rich in vitamin B6 and B12; supplemental therapy usually includes higher doses of vitamin B6 and B12.

For resistant cases of hyperhomocysteinemia, betaine and choline therapy is added. Plasma levels of vitamin B12 and folic acid are often measured along with plasma concentrations of homocysteine to monitor the effects of patient therapy. Vitamin B6 however, is not routinely measured[3,51].

# Chapter 2

# LABORATORY ASSESSSMENT OF HOMOCYSTEINE

## 2.1 Measurement Methods:

Plasma homocysteine levels can be measured by several techniques[62]: acid hydrolysis followed by amino acid analysis, high performance liquid chromatography (HPLC), gas chromatography- mass spectrometry and most recently, enzyme immunoassay (EIA) and chemiluminescence's competitive immunoassay (Bayer Method). Until the development of enzyme immunoassays, HPLC was the most common approach to homocysteine analysis[62]. Almost all homocysteine procedures are set up to measure total homocysteine. Only a few HPLC procedures have been developed to measure "free" homocysteine (reduced or oxidized forms or both); these require lowering the pH (Acidification) as a pretreatment step. Most of HPLC procedures require (1) pretreatment of plasma with a reducing agent to convert disulfide forms such as homocysteine or cysteine-homocysteine to homocysteine and other thiols, (2) a derivatization step to form a fluorescent derivative with the - SH or amino (- NH2) group of homocysteine, (3) analysis with fluorometric detection. HPLC methods that use electrochemical detection do not require derivatization step[63, 64, 65].

A major disadvantage of HPLC analysis of homocysteine is that the methods are not standardized. This is not the case for the new EIA procedures. In fact, the recent approval of two EIA methods by FDA will make the test available to many clinical laboratories. The Bio-Rad homocysteine EIA (Hercules, Calif), a solid phase immunoassay for the determination of total homocysteine in plasma, uses the Axis Biochemical's ASA (Oslo, Norway) technique in which protein-bound homocysteine is reduced by dithiothreitol to free homocysteine, which is converted to S-adenosyl-L-homocysteine (SAH) by the enzyme SAH-hydrolase and excess adenosine. Competition for the binding sites on an anti-SAH antibody takes place between SAH in the sample and SAH immobilized on the microtiter plate walls. After removal of unbound anti-SAH antibody, a secondary antibody labeled with horse-raddish peroxidase is added. The peroxidase activity is measured spectrophotometrically after addition of substrate. The absorbance is inversely proportional to the concentration of total homocysteine in the sample[66].

The homocysteine method by Abbot Laboratories (Abbott Park III) [67, 68] uses fluorescence polarization immunoassay in conjunction with the Abbott IMX; it also employs the patented Axis enzymatic conversion of homocysteine to SAH. The three-step assay includes (1) reduction (with dithiothreitol) and enzymatic conversion (SAH-hydrolase) to produce SAH, (2) addition of the anti-SAH antibody, and (3) addition of the fluorescein tracer. The Bayer Chemiluminescence's immunoassay is a competitive immunoassay using direct chemiluminescent technology. In this case control study, total homocysteine was measured by a chemiluminescence's method (Bayer Health care Diagnostics Company) using the ACS-180 fully automated chemiluminescence's system. The ACS-180 fully automated chemiluminescence's assay is a competitive immunoassay using direct, chemiluminescent technology[67,68]. The different forms of homocysteine in the patient samples were reduced to free homocysteine by the reducing agent dithiothreitol. Free homocysteine is then converted to S-adenosylhomocysteine (SAH) by the enzyme SAH-hydrolase. Converted SAH from the patient sample competes with SAH covalently coupled to paramagnetic particles in the

solid phase for a limited amount of acridinium ester- labeled monoclonal mouse anti-SAH antibody in phosphate buffer with bovine serum albumin and preservatives. Commonly used methods of homocysteine analysis, their characteristics, and their advantages and disadvantages are given in Table (6)[63,64,65,66,67,68,69].

Table (6). Characteristics of common Methods for Plasma Homocysteine Analysis

| Method / Reference | Principle of Method | Plasma Volume ( uL) | Preperation Time * | Analysis Time (10 samples) * | Reportable Range (umol/L ) | Comments + |
|---|---|---|---|---|---|---|
| **HPLC** | | | | | | |
| Araki & Sako | TBP reduction , SBD-F derivatization | 500 | 90-120 min | 3-4 h | 2 -65 | A , D |
| Fermo et al | $NaBH_4$ reduction , opA derivatization | 200 | 60 -90 min | 5 h | 0 - 320 | A , B , D |
| Fiskerstrand et al | $NaBH_4$ reduction , mBrB derivatization | 30 | 1 - 2 h | 4 h | 0.5 - 100 | A , B , D |
| Dias et al ( Bio-Rad ) | TBP reduction, ABD-F Derivatization | 50 | 1 h | 30 - 45 min | 0.5 - 100 | B , C , D |
| Solomon & Duda ( BAS ) | Proprietary reduction and electrochemical detection | 200 | 15 - 20 min | 100 min | 2 - 60 | E |
| EIA | | | | | | |
| Frantzen et al ( Bio-Rad ) | Reduction with DTT , homocysteine enzymatically converted to SAH, mesured by ELISA | 50 | 2 h | 30 - 45 min | 2 - 50 | C , F |
| Alfheim et al (Abbott Imx ) Bayer Diagnostics. | Reduction with DTT , homocysteine enzymatically converted to SAH, mesured by FPIA,CHL. | 50 | 5 min | 60 min | 0 - 50 | C , F |

ABD-F indicates 4(aminosulfonyl)-7-fluoro-benzo-2-oxa-1,3-diazole; DTT, dithiothreitol; EIA, enzyme immunoassay; ELISA, enzyme - linked Immunosorbent assay; FPIA, fluorescence polarization immunoassay; HPLC, high - performance liquid chromatography; mBrB, monobromobiman; NaBH4, sodium borohydride; opA, o-phthaldialdehyde; SAH, s-adenosylhomocysteine; SBD-F, ammonium-7- fluorobenzo-2-oxa-1,3-diazole-4-sulphonate; TBP, tri-n-butylphosphine.

* Preperation and analysis time are estimates, and may vary with degree of laboratory automation.

+ Advantages/disadvantages : A : indicates may be used to analyze urine and other biological samples; B : higher assay linearity ; C : standardization of calibrators; D: labor-intensive sample preperation; E: no sample derivatization required; F : automated analysis, CHL: Chemilumeniscence's Assay.

## 2.2 Preanalytical Variables That May Affect Homocysteine Measurement:

### 2.2.1 Sample Transport and Processing

Homocysteine is highly unstable at room temperature because RBCs produce and release homocysteine into the plasma compartment. Blood samples collected for homocysteine analysis should be kept cold and protected from light, and serum or plasma should be separated from cells as soon as possible. Any delay in transport or processing of the sample will cause a spurious increase in plasma homocysteine. Plasma homocysteine increases by 10% within 1 hour and up to 75% in 24 hours if blood is stored at room temperature after collection[62,70].

### 2.2.2 Collection Tubes:

Various anticoagulants - sodium heparin, sodium fluoride, EDTA- have been proposed to yield the blood specimen of choice for homocysteine determinations. Although EDTA is most commonly used, most of the aforementioned anticoagulants do not inhibit the cellular production of homocysteine. Sodium fluoride has been shown to partially inhibit the accumulation of homocysteine in blood. Recent

studies, however, have shown that this anticoagulant has limited usefulness in preventing the export of homocysteine from cells[69, 70].

When the collection tube contains acidic sodium citrate (pH 4.5) as the anticoagulant, however, the level of homocysteine appears to be more effectively maintained after blood collection. Although acidic sodium citrate has been used in coagulation testing for many years, it is not FDA approved for homocysteine determination[70, 71]

## 2.3 Population Screening

Screening for hyperhomocysteinemia should be considered for persons with a strong family history of atherosclerotic disease, as well as those with early symptoms of coronary heart disease, cerebrovascular disease, or peripheral vascular disease. Those patients are considered as high risk groups for CAD. Patients with poor nutritional status (including elderly persons and chronic abusers of alcohol and illicit drugs) should likewise be tested for plasma homocysteine, because dietary deficiencies of the essential micronutrients of homocysteine are common findings in these groups[70,71,72].

# Chapter 3

# METHODOLOGIES AND TECHNIQUES:

## 3.1 Study population:

A case control study was conducted from March 2004 to August 2004 at Ramallah hospital catheterization laboratory. Cases and one control group were selected from patients aged 21 to 85 years who underwent coronary catheterization in Ramallah hospital catheterization laboratory. Patients with coronary occlusions (referred to as cases) or without medically significant coronary occlusions (referred to as negative control subjects) were included. A second control group was drawn from the general population from subjects without history of cardiovascular diseases (referred to as population-based control subjects).Exclusion criteria from all groups were cancer, alcohol or drug abuse, B vitamin supplementation and psychiatric illness.

During the above mentioned period 318 patients attend the Catheterization unit at Ramallah hospital for cardiac catheterization. From those 318 patients 292 were accepted to this case control study, the other 16 patients were rejected due to such conditions like intake of

psychiatric drugs, vitamins, and nonfasting. All the 292 patients were catheterized.

At catheterization, projections were made of the major coronary vessels, which are the left main coronary artery, the left circumflex, the left anterior descending, the right coronary artery and the aorta.

A cardiologist reviewed the projections and prepared the catheterization report. Cases were defined as those having ≥ 50% occlusion in any coronary artery and those were 189 patients. Coronary control subjects who were defined as those having <50% occlusion in any coronary artery were 103 patients.

The conditions that led to catheterization were mainly angina pectoris and a known cardiac or valve disease in some patients. One hundred and two cases (51.1%) and thirteen negative control subjects (7.7%) had a history of myocardial infarction before catheterization. In the coronary control subjects, myocardial infarctions were due to coronary spasms or other nonatherosclerotic causes. Since atherosclerosis was the end point of interest, these control subjects were not excluded from the study.

The population-based control group was obtained from different places like Ministry of Labor, Ministry of Finance, colleges and universities. Before the day of blood collection all those who agreed to participate in the study were informed that they should fast completely for at least 12 hours before withdrawal of blood, and they should fast for 24 hours the day before blood phlebotomy from lipids. All of them were not suffering or have been treated for cardiac problems, diabetes, or any other major disease that may affect the study. Participants, males and females age from 21 to 85 years of old are only accepted to participate. Thus, a total of 126 population-control subjects were accepted to share, from them only 96 subjects were included in the study. The reminder 30 subject were refused because they inform us that they are taken medications for some cardiac problems.

All participants gave their written informed consent as medical ethics protocols emphasize.

## 3.2 Data Collection:

History of angina, hypertension, diabetes, family history of premature CAD, other medical problems, medications, lipid lowering

diets, smoking history and history of vascular events (myocardial infarction, cerebrovascular disease, peripheral vascular disease) was collected through a questionnaire to the three groups of cases.

## 3.3 Blood sampling and examination:

At the day of catheterization, venous blood samples were obtained from all subjects between 8:30 AM and 9:30 AM after 10-12hours fasting. Two types of samples were obtained one was EDTA blood to measure tHcys, the other was plain sample to measure total Cholesterol, HDL-C, Triglycerides, and LDL-C.

EDTA blood for measurement of tHcy was placed on ice and in the dark immediately and centrifuged at 4℃ within 1 hour to avoid false elevation caused by the release of homocysteine from RBCs, a process that continues at room temperature. Once the plasma was separated from cells, it was stored refrigerated at -20˚ C for several weeks before analysis.

## 3.4 Biochemical Analysis:

Cholesterol, HDL-C, LDL and Triglycerides was measured spectrophotometrically using commercial kits. The kits were from

Diasys Company (Germany). Cholesterol was measured using the cholesterol oxidase method (CHOD). HDL and LDL were measured by the direct precipitation techniques then LDL-cholesterol, HDL-cholesterol were measured. Triglycerides were measured by colorimetric enzymatic test using glycerol-3-phosaphate oxidase (GPO). The reference intervals for these biochemical markers were considered according to WHO standards.

Platelets counts, MPV, BUN, and Creatinine were registered from the patients' medical file, all of which were done the day before the day of the catheterization.

Total homocysteine was measured by a chemiluminescence's method (Bayer Health care Diagnostics Company) using the ACS-180 fully automated chemiluminescence's system. The ACS-180 fully automated chemiluminescence's assay is a competitive immunoassay using direct, chemiluminescent technology. The different forms of homocysteine in the patient samples were reduced to free homocysteine by the reducing agent dithiothreitol. Free homocysteine is then converted to S-adenosylhomocysteine (SAH) by the enzyme SAH-hydrolase. Converted

SAH from the patient sample competes with SAH covalently coupled to paramagnetic particles in the solid phase for a limited amount of acridinium ester- labeled monoclonal mouse anti-SAH antibody in phosphate buffer with bovine serum albumin and preservatives.

An inverse relationship exists between the amount of homocysteine present in the patient sample and the amount of relative light units detected by the system.

For homocysteine, studies have not yet definitively established reference standards by age category and gender, although these variables are known to affect homocysteine levels. Men have higher concentrations of homocysteine than women, and amounts of homocysteine tend to increase with age.

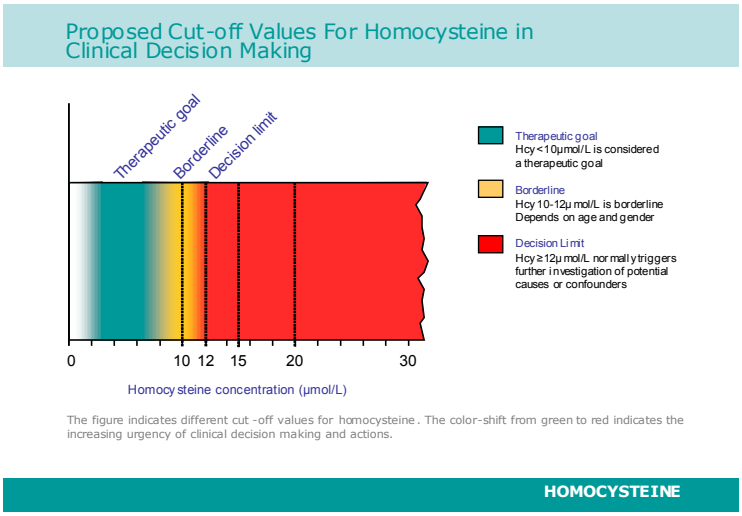



Fig.3. Proposed cutoff values for homocysteine in clinical use[70].

As shown in Figure 3 above it may be more clinically relevant to identify a "relative risk range "for homocysteine concentrations, rather than precise reference intervals (analogous to the manner in which serum cholesterol values are now interpreted). In general, a plasma homocysteine concentration of 10umol/L or less is considered optimal. A plasma homocysteine level of 10 to 12 umol/L is considered borderline, while a concentration above 12 umol/L is associated with high risk of occlusive vascular disease.

# Chapter 4

# DATA ANALYSIS

## Data Analysis:

Smoking status, hypertension, cholesterol, triglycerides, HDL, and LDL levels were determined at the time of the investigation. Current smoking was defined as the use of any tobacco. In addition, duration of smoking per years and amount of cigarettes smoked (packs per day) was calculated. Subjects were diagnosed as hypertensive at a systolic blood pressure ≥ 160mmHg or diastolic blood pressure ≥90 mm Hg or when they were using antihypertensive drugs.

Hypercholesterolemia was defined as serum cholesterol ≥ 200 mg% or use of cholesterol lowering drugs.

Differences in mean values of cardiovascular risk factor levels between cases and control subjects were tested with Students t-test for continuous variables and Chi-Square test for frequency measures. To evaluate potential confounding, associations of the cardiovascular risk factors were studied with plasma total homocysteine levels among the combined control groups by calculating Chi-square.

Means and Standard Deviations (SDs) of age, total homocysteine, cholesterol, and triglycerides, HDL, LDL, BUN and Creatinine were calculated for all three studied groups.

By means of logistic regression analysis, the (Odd Ratios) ORs for severe coronary atherosclerosis (case status) were calculated for those with elevated total homocysteine levels of the combined control groups. ORs were considered to estimate relative risks of subjects. To evaluate a possible graded association of plasma total homocysteine with coronary atherosclerosis, the ORs were computed for different results of cardiac catheterization. Also, the ORs were calculated for elevated fasting total

homocysteine, based on previously established cutoff points (14.0 umol/L, cutoff points of Stampfer, and 15.8 umol/L, cutoff points of Selhub)[5,11,42].

To study whether total homocysteine was related to the number of significantly occluded coronary arteries, all the 292 catheterized patients were grouped into five groups (1,2,3,4,5) according to number of arteries occluded. All reported probability values are two tailed.

# Chapter 5

# RESULTS

## 5.1 Characteristics of Study Groups:

Tables (7) and (8) show characteristics of the cases and the two control groups at the time of catheterization. The percentage of males and mean age were higher in cases than in both control groups. The majority of subjects in all groups were aged 21 to 85 years. Mean total cholesterol levels, HDL cholesterol levels, BUN, creatinine and LDL cholesterol levels were similar among the groups. The proportion of hypertension among subjects was higher among population control subjects than the other two groups. Mean serum level of triglycerides was highest in population control subjects, but this could be explained in that those

subjects were not strictly committed to the test instructions concerning fasting hours and nutrition habits before the test. So, if this were the case, mean serum triglycerides in cases will be the highest. At the time of examination, a lower percentage of cases were smoking, but mean years of smoking was highest in cases. Uric acid concentrations were highest in cases in comparison to the other groups of subjects.

Table 7. Characteristics of Cases and two groups of control subjects for .continuous variables

| | **Cases (N=(189 X±SD** | **Negative Control Subjects N=(103) X±SD** | **Population Control Subjects (N=(96 X±SD** |
|---|---|---|---|
| **,Age** | 56.3±12.0 | 47.8±8.4 | 43.9±9.9 |
| **%Total Cholesterol,mg** | 195.6±52.6 | 185±36.8 | 188.9±51.3 |
| **%HDL-Cholesterol,mg** | 44.4±18.4 | 42.3±13.1 | 43.5±16.7 |
| **%LDL-Cholesterol,mg** | 129.6±50.9 | 117.3±33.9 | 117.9±47.7 |
| **%Triglycerides,mg** | 137.8±83.6 | 121.1±68.7 | 146.3±84.5 |
| **%BUN, mg** | 20.5±8.2 | 19.3±4.6 | 20.0±7.0 |
| **%Creatinine, mg** | 0.91±0.28 | 0.94±0.26 | 0.95±0.25 |
| **%Uric acid,mg** | 6.8±1.9 | 6.0±1.8 | 5.9±1.3 |
| **Platelets /thousand/uL** | 255±78 | 251±54 | 253±57 |
| **MPV** | 9.8±1.6 | 9.7±1.0 | 8.2±1.0 |

Table 8: Characteristics of Cases and two groups of control subjects for .other variables

| | Cases (N=(189 | Negative Control (Subjects N=(103 | Population Control Subjects (N=(96 |
|---|---|---|---|
| %Male | 75.1% | 79.6% | 77.1% |
| %Female | 24.9% | 20.4% | 22.9% |
| % Hypertension | 44.4% | 37.9% | 0.6% |
| %Currently Smoking | 36.4% | 35.0% | 38.5% |
| %Ex-smoking | 36.2% | 14.9% | 14.6% |

## 5.2 Plasma Total Homocysteine Concentration of Study Groups:

### 5.2.1 Patients versus Negative Control Group:

To study the effect of sex on Hcy concentration between these two groups t-test was calculated and it was found that there is no statistical significance between males and females in Hcy concentration as shown in Table(9), *P*=0.945.

Table (9): Hcy concentration among males and females in patients and negative control groups.

| | Males | Females |
|---|---|---|
| Patients<br>Hcy (Mean ± SD) umol/L | 142<br>20.8±8.8 | 47<br>20.7±10.6 |
| Negative Control Group<br>Hcy (Mean ± SD) umol/L | 82<br>14.4±4.4 | 21<br>14.0±4.4 |
| Hcy (Mean ± SD) umol/L | 18.5±8.2 | 18.6±9.6 |

Also, when results of cardiac catheterization (number of occluded arteries were studied among these two groups by using t-test, it was found that there is a statistical significance between those who have any coronary artery disease( occlusion of any coronary artery) and those who are normal, P= 0.00.But it was calculated that there was no statistical significance between those who have single artery disease and double artery disease, P=0.816, and between those who have double artery disease and triple artery disease, P= 0.996.These results imply that there is no strong association between Hcy concentration and number of occluded arteries, the association is variable.

To study the risk estimate of CAD among patients and negative control subjects, all patients were grouped into two groups according to Selhub Hcy cutoff value, Stampfer Hcy cutoff value and my established cutoff value, then multinomial logistic regression was calculated to obtain the ORs before age, sex and other confounders adjustments and after adjustments. The results are shown below in the Tables 10 and 11.

Table 10: ORs among patients and Negative Control subjects for Hyperhomocysteinemia using different cutoffs for Hcy before adjustment for age, sex, Hypertension, cholesterol, triglycerides, HDL and LDL.

| **By using Selhub Cutoff of Hcy** | | | | | | |
|---|---|---|---|---|---|---|
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **42** | **147** | | | | |
| **Negative Controls** | **61** | **42** | **40.0** | **0.00** | **5.08** | **3.02-8.56** |
| **By Using Stampfer Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **68** | **121** | | | | |
| **Negative Controls** | **72** | **31** | **30.7** | **0.00** | **4.13** | **2.47-6.92** |
| **By Using established Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **52** | **137** | | | | |
| **Negative Controls** | **65** | **38** | **35.2** | **0.00** | **4.51** | **2.70-7.52** |

Table 11: ORs among patients and Negative Control subjects for Hyperhomocysteinemia using different cutoffs for Hcy after adjustment for age, sex, Hypertension, cholesterol, triglycerides, HDL and LDL.

| **By using Selhub Cutoff of Hcy** | | | | | | |
|---|---|---|---|---|---|---|
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **42** | **147** | **49.0** | **0.00** | **5.26** | **3.01-9.21** |
| **Negative Controls** | **61** | **42** | | | | |
| **By Using Stampfer Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **68** | **121** | **46.0** | **0.00** | **4.65** | **2.64-8.19** |
| **Negative Controls** | **72** | **31** | | | | |
| **By Using established Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **52** | **137** | **35.2** | **0.00** | **4.84** | **2.77-8.44** |
| **Negative Controls** | **65** | **38** | | | | |

### 5.2.2 Patients versus Population (Random) Control Group:

To study the effect of sex on Hcy concentration between these two groups t-test was calculated and it was found that there is no statistical significance between males and females in Hcy concentration as shown in Table(12), *P*=0.553.

Table(12): Hcy concentration among males and females in patients and Population control groups.

| | Males | Females |
|---|---|---|
| Patients<br>Hcy (Mean ± SD) umol/L | 142<br>20.8±8.8 | 47<br>20.7±10.6 |
| Population Control Group<br>Hcy (Mean ± SD) umol/L | 74<br>15.6±5.0 | 22<br>13.5±3.0 |
| Hcy (Mean ± SD) umol/L | 19.1±8.1 | 18.4±9.5 |

Also, when results of cardiac catheterization (number of occluded arteries were studied among these two groups by using t-test, it was found that there is a statistical significance between those who have any coronary artery disease( occlusion of any coronary artery) and those who are normal, P= 0.00.But it was calculated that there was no statistical significance between those who have single artery disease and double artery disease, P=0.816, and between those who have double artery disease and triple artery disease, P= 0.996.These results also imply that the association between Hcy concentration and number of occluded arteries, is variable, and this is the same for negative control subjects. All these results increase the assumption that these two control groups should be treated as one single control group which was done in the forward studies and calculations.

To study the risk estimate of CAD among patients and population control subjects, all patients were grouped into two groups according to Selhub Hcy cutoff value, Stampfer Hcy cutoff value and my established cutoff value, then multinomial logistic regression was calculated to obtain the ORs before age, sex, cholesterol, triglycerides, HDL and LDL adjustments and after adjustments. The results are shown below in the Tables 13 and 14.

Table 13: ORs among Patients and Population Control subjects for Hyperhomocysteinemia using different cutoffs for Hcy before adjustment for age, sex, cholesterol, triglycerides, HDL and LDL.

| **By using Selhub Cutoff of Hcy** | | | | | | |
|---|---|---|---|---|---|---|
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **42** | **147** | | | | |
| **Population Controls** | **39** | **57** | **10.6** | **0.001** | **2.40** | **1.41-4.10** |
| **By Using Stampfer Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Patients** | **68** | **121** | | | | |
| **Population** | **62** | **34** | **21.2** | **0.00** | **3.25** | **1.94-5.42** |
| **Controls** | | | | | | |
| **By Using established Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **52** | **137** | | | | |
| **Population** | **47** | **49** | **12.9** | **0.00** | **2.53** | **1.52-4.22** |
| **Controls** | | | | | | |

Table 14: ORs among Patients and Population Control subjects for Hyperhomocysteinemia using different cutoffs for Hcy after adjustment for age, sex, cholesterol, triglycerides, HDL and LDL.

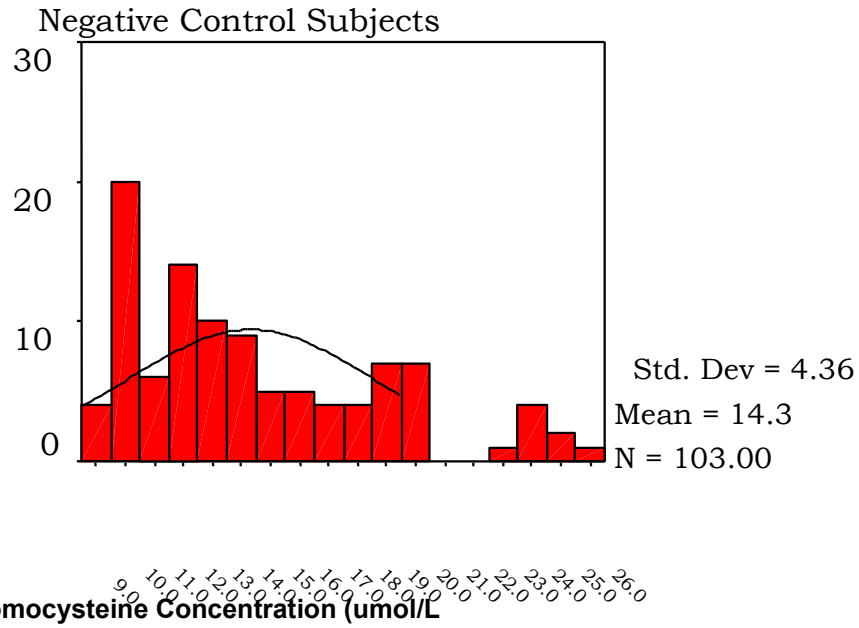
| **By using Selhub Cutoff of Hcy** | | | | | | |
|---|---|---|---|---|---|---|
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **42** | **147** | | | | |
| **Population** | **39** | **57** | **37.9** | **0.001** | **2.25** | **1.24-4.08** |
| **Controls** | | | | | | |
| **By Using Stampfer Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **68** | **121** | | | | |
| **Population** | **62** | **34** | **45.7** | **0.00** | **2.95** | **1.65-5.28** |
| **Controls** | | | | | | |
| **By Using established Cutoff of Hcy** | | | | | | |
| | **Normal Hcy** | **Hyper. Hcy** | **$X^2$** | **P** | **OR** | **CI 95% Lower-Upper** |
| **Patients** | **52** | **137** | | | | |
| **Population** | **47** | **49** | **40.0** | **0.00** | **2.39** | **1.33-4.28** |
| **Controls** | | | | | | |

For all study groups, mean levels of plasma total homocysteine concentration and standard deviation were calculated. The mean levels of plasma total homocysteine were higher in cases compared to the other two groups, (*P*=0.00). While for the two control groups there were no statistical significance in mean homocysteine concentrations between them,(P=0.728).Also distributions of total Homocysteine levels were very similar among negative control patients and population control subjects as shown in Figures 4 and 5. Therefore, these two groups were combined in all subsequent analysis as one negative control group. So, when dealing with these two groups there is a statistically significant difference between them in the mean homocysteine concentration, (P=0.00). Cases had statistically higher mean levels of fasting total homocysteine as shown in Table (15).

Table 15: Plasma Total Homocysteine among cases and two control groups.

| | **Cases N=(189)** | **Negative Control Subjects N=(103)** | **Population Control Subjects N=(96)** |
|---|---|---|---|
| | Mean±SD | Mean±SD | Mean±SD |
| Fasting t-Hcy,umol/L | 20.8±9.3 | 14.3±4.4 | 15.2±4.7 |

Also, as Figure (6) shows, the higher mean levels of fasting total homocysteine in cases were a consequence shift toward the left of the cases frequency distribution. For fasting total homocysteine, this was slightly more consistent throughout the entire distribution.



Figur4. Frequency distribution of plasma total Homocysteine among Negative Control Patients.

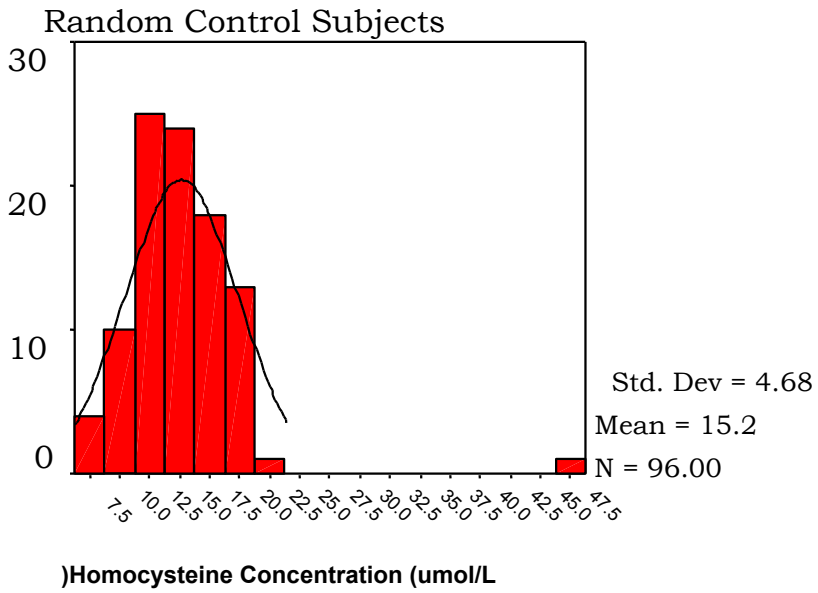



Figure 5. Frequency distribution of plasma total Homocysteine among Population Control Patients.

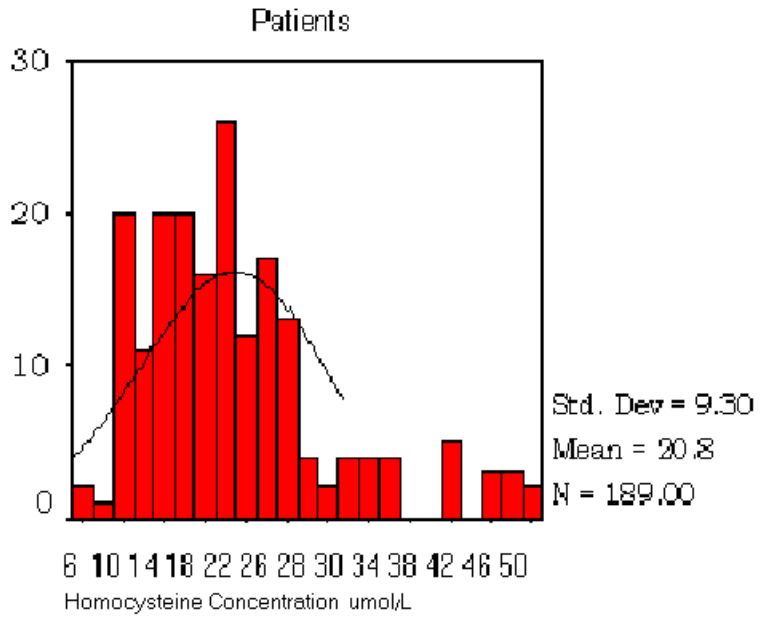



Figure 6. Frequency distribution of plasma total Homocysteine among Cases.

Also it's worth here to mention that if we consider the negative controls (n=103) as cases (n=189) since those negative controls are considered atherosclerotic patients regardless of the degree of stenosis in their arteries, we will find that there is a statistically significant difference between this new grouping of cases and population control group regarding the mean Hcy concentration as shown in Table (16), P=0.000.

Table (16): Mean Plasma Total Hcy among new formulated cases (cases + negative controls) and population control groups.

| | Cases<br><br>( n=292) | Population Control<br><br>Group ( n=96) |
|---|---|---|
| | Mean±SD | Mean±SD |
| Fasting t-Hcy,umol/L | 18.5±8.5 | 15.2±4.7 |

Furthermore, by using the new arrangement of the cases as mentioned in table (16) if we calculate the ORs and $X^2$ test for these two groups using the three cutoff values for Hcy( Selhub, Stampfer,and my cutoff values), the following results were obtained.

From Table(17) it's clear that there is only statistical significance in hyperhomocysteinemia when using Stampfer cutoff, $P$=0.005, but for the other cutoffs there is no statistical significance.

When ORs for these two groups were calculated by logistic regression, the following results where obtained.

Table (17): Comparison of Hyperhomocysteinemia between Cases and Negative Controls.

| Risk Factors | Case | Groups | | Patients vs. Controls ($x^2$) | *P* Value |
|---|---|---|---|---|---|
| | | Patients (n=292) | Controls (n=96) | | |
| Hyperhomocysteinemia ( Stampfer Cutoff) | Absent | 140 | 62 | 8.00 | 0.005 |
| | Present | 152 | 34 | | |
| Hyperhomocysteinemia ( Selhub Cutoff) | Absent | 103 | 39 | 0.892 | 0.393 |
| | Present | 189 | 57 | | |
| Hyperhomocysteinemia ( Established Cutoff) | Absent | 117 | 47 | 2.34 | 0.153 |
| | Present | 175 | 49 | | |

Table (18): ORs for Cases and negative controls according to Hcy mean concentration using different cutoff values.

| Homocysteine Result | Cutoff used | OR | 95% CI Lower - Upper |
|---|---|---|---|
| Normal Hcy -Hyper Hcy | Stampfer | 0.505 | 0.313 - 0.814 |
| | Selhub | 0.796 | 0.496 - 1.278 |
| | Established | 0.697 | 0.438 – 1.11 |

## 5.3 Plasma Total Homocysteine and Risk of Coronary Artery Occlusion:

The mean and standard deviation of total homocysteine were calculated for each result of catheterization obtained from the different study groups. Results are shown in Table (19) below.

Table19: Mean and SD of Total Plasma Homocysteine according to catheterization results.

| **Catheterization Result** | **Number of Patients** | **Fasting Total Hcy, umol/L Mean ± SD** |
|---|---|---|
| Normal | 105 | 14.40 ± 4.4 |
| Occlusion of 1 Artery | 45 | 19.01 ± 7.6 |
| Occlusion of 2 Arteries | 60 | 19.30 ± 6.6 |
| Occlusion of 3 Arteries | 57 | 19.30 ± 7.1 |
| Occlusion of 4 Arteries | 19 | 33.5 ± 15.4 |
| Other CVD | 6 | 24.5 ± 11.1 |
| Unknown | 96 | 15.20 ± 4.7 |

From these results it was shown that for those patients who have 1, 2, or 3 occluded arteries there is no significant difference in the mean of total homocysteine (P=0.816). Whereas, in those who have 4 occluded arteries there is a significant rise in the mean compared to the other groups or to the normal level of homocysteine, (P=0.00). Also, the other two groups of this case controlled study, the population controlled group and the negative one; there is no significant difference between the

means of the total homocysteine concentration among both of these groups, (P= 0.728).

Table (20) shows ORs for severe coronary atherosclerosis for patients and controls with total homocysteine using the three mentioned cutoff values for homocysteine before adjustment for age, sex, and other confounders. These odd ratios mentioned in the table below show the increased risk of having a coronary artery occlusion with hyperhomocysteinemia in all the proposed cutoffs for homocysteine. Also, these ORs show that increase in homocysteine concentrations increases the number of occluded arteries.

Considering the coronary artery disease as the net outcome of occluded arteries, this outcome when its association with hyperhomocysteinemia was measured statistically by logistic regression using the established cutoff from this study (Homocysteine=14.7 umol/L ± 4.52), the OR was 3.267(95% CI, 2.1 – 5.0). The OR for an increase of 1SD in plasma total homocysteine was 6.70, (95%CI, 4.10 – 10.86) before adjustment for age, sex and other confounders, and OR was 8.21 (95% CI, 4.14 – 16.3) after adjustment for age, sex, cholesterol, triglycerides, HDL and LDL.

Also, as shown in Table (21), there were no statistical association after adjusting for age, sex, cholesterol, triglycerides, HDL and LDL.

Table 20. Odd Ratios of severe coronary atherosclerosis for patients and controls using Selhub cutoff, Stampfer Cutoff, and established cutoff values for Homocysteine concentrations, before adjustment for age, sex, cholesterol, triglycerides, HDL and LDL.

| Catheterization Result | Cutoff used | OR | 95% CI |
|---|---|---|---|

| | | | Lower - Upper |
|---|---|---|---|
| Normal-Single artery Disease | Selhub | 3.06 | 1.47 - 6.38 |
| | Stampfer | 4.00 | 2.02 - 7.94 |
| | Established | 3.10 | 1.54 – 6.25 |
| Normal-Double artery Disease | Selhub | 3.96 | 1.99 – 7.90 |
| | Stampfer | 3.46 | 1.89 – 6.30 |
| | Established | 3.46 | 1.83 – 6.54 |
| Normal- Triple artery Disease | Selhub | 2.77 | 1.45 – 5.32 |
| | Stampfer | 2.96 | 1.62 – 5.41 |
| | Established | 2.52 | 1.36 – 4.67 |
| Normal – Four artery disease | Selhub | 3.71 | 1.19 – 11.58 |
| | Stampfer | 4.33 | 1.58 – 11.91 |
| | Established | 4.72 | 1.51 – 14.72 |

Table 21. Odd Ratios of severe coronary atherosclerosis for patients and controls using Selhub cutoff, Stampfer Cutoff, and established cutoff values for Homocysteine concentrations, after adjustment for age, sex, cholesterol, triglycerides, HDL and LDL.

| Catheterization Result | Cutoff used | OR | 95% CI<br>Lower - Upper |
|---|---|---|---|
| Normal-Single artery Disease | Selhub | 2.22 | 0.846 – 5.84 |
| | Stampfer | 2.31 | 0.915 – 5.85 |
| | Established | 2.05 | 0.809 – 5.2 |
| Normal-Double artery Disease | Selhub | 2.92 | 1.30 – 6.54 |
| | Stampfer | 2.13 | 1.01 – 4.53 |
| | Established | 2.35 | 1.09 – 5.05 |
| Normal- Triple artery Disease | Selhub | 2.22 | 0.950 – 5.21 |
| | Stampfer | 2.24 | 0.990 – 5.08 |
| | Established | 2.17 | 0.850 – 12.9 |
| Normal – Four artery disease | Selhub | 3.31 | 1.34 – 4.02 |
| | Stampfer | 4.33 | 1.58 – 11.9 |
| | Established | 5.09 | 1.16 – 22.21 |

## 5.4 Catheterization Results among Different groups According to Sex:

By using the Chi-square test there was no statistically significant difference between males and females in all the studied groups in the number of arteries occluded or the catheterization results as shown in Table(22) below, ($x^2$ =4.1, P=0.536).

Table (22): Results of Catheterization among males and females in all groups.

| Catheterization Results | | | | | | |
|---|---|---|---|---|---|---|
| | Normal | Single Artery Disease | Double Artery Disease | Triple Artery Disease | Four Artery Disease | Other Cardiac Disease |
| Males | 156 | 36 | 46 | 41 | 16 | 3 |
| Hcy. Mean ±SD | 15.0±4.7 | 19.8±7.8 | 20.2±6.5 | 18.8±6.2 | 30.9±15.5 | 19.0±5.5 |
| Females | 45 | 9 | 14 | 16 | 3 | 3 |
| Hcy. Mean ±SD | 13.9±3.7 | 15.9±5.9 | 16.5±6.1 | 20.7±9.0 | 46.9±2.6 | 30.0±13.7 |
| *P* | 0.146 | 0.173 | 0.067 | 0.368 | 0.099 | 0.265 |

Also, T-test shows no significant difference between males and females in homocysteine concentration in all studied groups as shown in Table (22). Furthermore, statistically it was found that there is statistical significance between the concentration of homocysteine in normal

patients and those who have any cardiac problem (P=0.008) which increases the evidence that homocysteine may be a marker worth considering in coronary artery and other vascular diseases. Also, we can find that there is no statistical significance between the results of catheterization and homocysteine concentration in the patients who didn't undergo catheterization (population control group) and those who have normal results in catheterization ( P= 0.139). This evidence increases my assumption that those two populations of patients (Population control group and Negative control group) should be treated as one group (Negative control group).

## 5.5 Comparison of Risk Factors between Patients and Controls:

### 5.5.1 Comparison of Risk Factors Between Patients and Negative Control Subjects.

Table (23) Comparison of risk factors between patients and negative control subjects.

| Risk Factors | Case | Groups | | Patients vs. | P Value |
|---|---|---|---|---|---|
| | | Patients | Controls | | |

| | | (n=189) | (n=103) | Controls ($x^2$) | |
|---|---|---|---|---|---|
| Hyperhomocysteinemia ( Stampfer Cutoff) | Present | 121 | 31 | 30.7 | 0.000 |
| | Absent | 68 | 122 | | |
| Hyperhomocysteinemia ( Selhub Cutoff) | Present | 147 | 62 | 39.9 | 0.001 |
| | Absent | 42 | 42 | | |
| Hyperhomocysteinemia ( Established Cutoff) | Present | 137 | 38 | 35.2 | 0.000 |
| | Absent | 52 | 65 | | |
| Hypercholesteremia | Present | 80 | 41 | 0.378 | 0.617 |
| | Absent | 102 | 61 | | |
| Smoking | Present | 68 | 36 | 0.058 | 0.898 |
| | Absent | 119 | 67 | | |
| Ex-Smoking | Present | 67 | 15 | 14.6 | 0.000 |
| | Absent | 118 | 86 | | |
| Hypertension | Present | 84 | 39 | 2016 | 0.177 |
| | Absent | 68 | 47 | | |
| Diabetes Mellitus | Present | 56 | 39 | 1.86 | 0.214 |
| | Absent | 96 | 46 | | |

From this comparison it was clear that for the three cutoffs used for homocysteinemia there is statistical significance between patients and negative controls.

But for the other risk factors there was no significant difference between the two groups except for ex-smokers which was statistically significant.

**5.5.2 Comparison of risk factors between Patients and Population Control Subjects.**

Risk factors between population control groups and patients was studied for statistical significance to compare their significance with the negative control group. Results of this comparison and statistical significance are mentioned in Table (24).

From this Table it was seen that the similar exists between risk factors in the two groups compared to the other two groups in Table (23). These results mean that there is no statistical difference between the two control groups which increases my assumption to study them as one control group. But its worth to mention here that significance between these two groups exists only for DM where there is a difference.

Table (24) Comparison of risk factors between patients and population control subjects.

| Risk Factors | Case | Groups | | Patients vs. Controls ($x^2$) | P Value |
|---|---|---|---|---|---|
| | | Patients (n=189) | Controls (n=96) | | |
| Hyperhomocysteinemia ( Stampfer Cutoff) | Present | 121 | 34 | 21.0 | 0.000 |
| | Absent | 68 | 62 | | |
| Hyperhomocysteinemia ( Selhub Cutoff) | Present | 147 | 39 | 10.6 | 0.001 |
| | Absent | 42 | 57 | | |
| Hyperhomocysteinemia | Present | 137 | 49 | 12.9 | 0.001 |

| | | | | | |
|---|---|---|---|---|---|
| ( Established Cutoff) | Absent | 52 | 47 | | |
| Hypercholesteremia | Present | 80 | 42 | 0.001 | 1.00 |
| | Absent | 102 | 54 | | |
| Smoking | Present | 68 | 37 | 0.129 | 0.795 |
| | Absent | 119 | 59 | | |
| Ex-Smoking | Present | 67 | 14 | 14.4 | 0.000 |
| | Absent | 118 | 82 | | |
| Hypertension | Present | 84 | 6 | 4.52 | 0.056 |
| | Absent | 68 | 14 | | |
| Diabetes Mellitus | Present | 56 | 14 | 13.1 | 0.000 |
| | Absent | 96 | 3 | | |

Table (25) shows that there is a statistical significant association between the two control groups in homocysteine mean concentration using Selhub cutoff value for Hcy (P=0.011), and my established cutoff value (P=0.047). While it was insignificant when using Stampfer cutoff value (P=0.452).

For the other risk factors and confounders there is no statistical significance between them except for DM (P=0.007).

Table (25) Comparison of risk factors between negative and population control subjects.

| Risk Factors | Case | Controls | | Patients vs. Controls ($x^2$) | P Value |
|---|---|---|---|---|---|
| | | Négative (n=103) | Population (n=96) | | |
| **Hyperhomocysteinemia** ( Stampfer Cutoff) | Present | 31 | 34 | 0.639 | 0.452 |
| | Absent | 72 | 62 | | |
| **Hyperhomocysteinemia** ( Selhub Cutoff) | Present | 42 | 57 | 6.88 | 0.011 |
| | Absent | 61 | 39 | | |
| **Hyperhomocysteinemia** ( Established Cutoff) | Present | 38 | 49 | 4.04 | 0.047 |
| | Absent | 65 | 47 | | |
| Hypercholesteremia | Present | 41 | 42 | 0.257 | 0.666 |
| | Absent | 61 | 54 | | |
| Smoking | Present | 36 | 37 | 0.276 | 0.660 |
| | Absent | 67 | 59 | | |
| Ex-Smoking | Present | 15 | 14 | 0.003 | 1.00 |
| | Absent | 86 | 82 | | |
| Hypertension | Present | 39 | 6 | 1.57 | 0.315 |

| | | | | | |
|---|---|---|---|---|---|
| | Absent | 47 | 14 | | |
| Diabetes Mellitus | Present | 39 | 14 | 7.55 | 0.007 |
| | Absent | 46 | 3 | | |

### 5.5.3 Comparison of Risk Factors between Patients and Control Subjects.

From all the previous analysis of data and results, and since there were no significant differences in most of the factors and characteristics between the two control groups, the two groups were merged in one control group. All the statistical analysis will consider one patients group (Cases) and one control group (Controls).

The statistical comparison between patients and controls are summarized in Table (26), that shows statistically significant difference between the two groups for hyperhomocysteinemia using Stampfer cutoff (p=0.00) and Selhub cutoff (p=0.00).A statistically significant difference was not noted between patients and controls for smoking (p=1.00) while the significance exists for ex-smokers (p=0.00).

Table 26. Comparison of Risk Factors between Patients and Controls:

| Risk Factors | Case | Groups | | Patients vs. Controls ($x^2$) | P Value |
|---|---|---|---|---|---|
| | | Patients (n=189) | Controls (n=199) | | |
| Hyperhomocysteinemia ( Stampfer Cutoff) | Present | 121 | 65 | 38.2 | 0.000 |
| | Absent | 68 | 134 | | |
| Hyperhomocysteinemia ( Selhub Cutoff) | Present | 147 | 99 | 32.8 | 0.000 |
| | Absent | 42 | 100 | | |
| Hypercholestermia | Present | 80 | 83 | 0.161 | 0.756 |
| | Absent | 102 | 115 | | |
| Smoking | Present | 68 | 73 | 0.004 | 1.00 |
| | Absent | 119 | 126 | | |
| Ex-Smoking | Present | 67 | 29 | 23.4 | 0.00 |
| | Absent | 118 | 168 | | |
| Hypertension | Present | 84 | 45 | 4.1 | 0.053 |
| | Absent | 68 | 57 | | |
| Diabetes Mellitus | Present | 56 | 53 | 5.7 | 0.020 |
| | Absent | 96 | 49 | | |

However, statistically significant difference was observed between patients and controls for diabetes mellitus (p=0.020).But for hypertension and hypercholesteremia there was no statistical significance between patients and controls,( p=0.756) and( p=0,053) respectively.

Table (27) shows the association of hyperhmoocysteinemia with other risk factors among patients, using established cutoff value for homocysteine from this study. To evaluate the statistical significance of hyperhomocysteinemia with these risk factors, patients were grouped into two groups, group A represents those patients who have Hcy > 14.7umol/L, while group B represents those who have Hcy<14.7 umol/L then Chi-square test was calculated.

Table (27): Association of hyperhomocysteinemia with other risk factors among patients (n=207), using my cutoff for homocysteine (14.7±4.5 umol/L).

| Risk factors | Group A (n=137) Hcy>14.7 umol/L | Group B (n=70) Hcy<14.7 umol/L | Group A Vs. Group B $X^2$ | p-Value |
|---|---|---|---|---|
| Hypercholesteremia | 46 | 34 | 14.84 | 0.000 |
| Hypertension | 54 | 51 | 2.02 | 0.164 |
| Diabetes Mellitus | 33 | 23 | 4.77 | 0.046 |
| Smoking | 46 | 22 | 1.10 | 0.312 |
| Ex-Smoking | 52 | 15 | 1.15 | 0.307 |
| Sex: Males | 107 | 53 | 2.35 | 0.135 |
| Females | 30 | 17 | | |

Hypercholesteremia and Diabetes Mellitus were statistically significant with hyperhomocysteinemia in all the proposed cutoff values used for homocysteine; p-values for them were mentioned in tables 15,and 16. However, there is no statistical difference between males and females in association with hyperhomocysteinemia (P=0.051, P=0.164, P=0.135) respectively in the three cutoff values used, Selhub, Stampfer, and my case control study cutoffs. Although smoking is one of the risk factors associated with hyperhomocysteinemia, according to my study, there is no statistical significance between smokers, nonsmokers, and ex-

smokers. Hypertension was statistically significant when using Stampfer cutoff (p=0.019), and my study cutoff (p=0.00), while it was insignificant when using Selhub cutoff (p=0.190).

# Chapter 6

# DISCUSION

## Discussion:

My study findings support the hypothesis that elevated plasma total homocysteine is a risk factor for severe coronary atherosclerosis, independent of other risk factors for coronary artery disease. The relation was apparent over a wide range of total homocysteine levels.

Several other retrospective case control studies on the association of plasma total homocysteine with angiographically defined coronary atherosclerosis have demonstrated positive associations, with fasting total homocysteine levels[73, 74, 75, 76,77,78,79]. This study is the first one done in Palestine with this number of cases and control subjects to demonstrate that fasting total plasma homocysteine levels are positively related to risk of coronary atherosclerosis. My data concur with a retrospective case control study[80] of coronary artery diseases showing that frequency distribution of fasting total homocysteine were displaced to the right relative to control subjects. A clear advantage of using angiographically defined coronary atherosclerosis as a disease end point is the possibility to grade the vascular disease. Others have shown that fasting total homocysteine increased with increasing number of occluded coronary arteries[75,77]. In my study, I had the same observation, which reinforces the hypothesis that elevated total homocysteine plays a role in the atherosclerosis process; although a clear cause-effect relationship cannot be derived from this type of study.

The results of more than 75 clinical and epidemiological studies have indicated a positive correlation between plasma total homocysteine levels and CAD, peripheral arterial disease, stroke and venous thrombus[34, 81].

The results of these clinical studies are consistent and agree with my findings that total plasma homocysteine has a strong positive association with coronary atherosclerosis. Also, my finding that increased plasma total homocysteine is correlated with atherosclerosis.

A meta-analysis of 27 studies relating homocysteine levels to CAD and 11 studies of the effects of folic acid on total homocysteine levels suggested that an increase of 5 umol/L in the level of homocysteine (normal level, 5-15 umol/L) [82] increased the risk for CAD as much as increase of 0.52 mmol/L (20 mg/dl) in the level of plasma cholesterol does[3]. The results of these meta-analysis studies agree with my finding that the estimated risk of atherosclerosis increases from 3.267(95% CI, 2.1 – 5.0) to 8.21(95% CI, 4.1 – 10.86) for increase of homocysteine of 1SD, using this study cutoff value which is 14.7±4.72 umol/L.

With few exceptions[83, 84], most other retrospective epidemiological studies of coronary heart disease showed a positive relation with plasma total homocysteine[85,86,87,88,89]. However, data from prospective studies offer weaker support than case –control studies for an association between homocysteine concentration and cardiovascular disease. In the Physicians' Health Study, with a 5 year follow up, plasma total homocysteine levels above the 95$^{th}$ percentile of the control distribution were associated with a 3.4 fold increased risk of myocardial infarction[5]. However, the relative risk was 1.7 with 7.5 years of follow up, and this was no longer statistically significant. Two other prospective studies clearly failed to show an association whereas another showed no association with incident coronary heart disease and an increased risk of coronary heart disease mortality only during the first 1.5 years of follow up[5]. Two other studies found positive associations, but these were generally smaller than those observed in retrospective studies, especially when subjects with preexisting cardiovascular disease were excluded from analyses. Reasons for weaker associations in prospective studies may include deterioration of total homocysteine in blood samples or

variations in subjects over time, leading to effect attenuation. Of course, another possibility may be that elevated total homocysteine is not a cause but merely a consequence of cardiovascular disease.

Also, the accumulating epidemiological evidence linking high plasma levels of homocysteine to increased rates of heart disease is impressive. Researchers have postulated an even greater role for homocysteine than for cholesterol in atherogenesis[75]. This finding is also compatible with my findings that homocysteine is stronger as a risk factor for atherosclerosis compared to cholesterol. But, still research is yet to definitively establish how an elevated level of homocysteine ranks as a marker for heart disease among the traditionally accepted risk factors. It appears reasonable to conclude that future outcome studies may release homocysteine from being a nontraditional risk factor for CAD to a wide acceptance as an independent risk factor for heart disease[90,91].

Although my data are compatible with a positive association between plasma total homocysteine and risk of coronary atherosclerosis, part of the findings did not reach statistical significance, possibly due to relatively small numbers of subjects, especially when using cutoff points

for elevated total homocysteine. Like other studies, this study found that elevated total homocysteine is a graded risk factor, but my ORs for increased plasma total homocysteine were higher. My ORs compared to the summary of the ORs mentioned in the latest meta-analysis[82] were higher also, and this could be explained that this is the case in our population, since there was no statistical difference between the cutoffs used in the study and my cutoff. So, to prove that, a large clinical trial should be done in Palestine on a large number of people to assess the exact relation between Hcy. and CAD, and to establish a more reliable cutoff value for Hcy.

# Chapter 7

# CONCLUSION

## Conclusion:

In conclusion, my data suggest that elevated fasting total homocysteine is an independent risk factor for severe coronary atherosclerosis. The association exists over a wide range of total homocysteine levels.

Further studies are necessary to complement the growing evidence of the important role of elevated total homocysteine in cardiovascular disease etiology and to further elucidate the role of genetic, nutritional, and lifestyle factors. In my opinion, reduction of total homocysteine levels in the general population, and not only in those with clearly abnormal total homocysteine levels, may protect against cardiovascular disease.

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# APPENDICIES