THE ASSOCIATION OF SERUM COPPER AND PERIPHERAL ARTERIAL OCCLUSIVE DISEASE IN MALES: A CASE CONTROL STUDY

BY

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PREFACE

The work reported here forms part of a project, funded by Health and Welfare Canada. I attached myself to this project when its proposal was just approved and funded. This project is comprised of two different studies, the main study and my thesis project. The two studies were carried out in parallel. The main study, not reported here, provided material for vascular surgery research team to prepare a report on the association of smoking and peripheral arterial occlusive disease (PAOD). I am reporting here on the association of serum copper and PAOD. The methods used in the studies were essentially identical. Using existing survey questionnaires, I developed the questionnaire used for both studies, under supervision of my supervisor, Dr. Gerry Hill. I prepared a manual for the interviewer and also contributed in the development of the consent form. I was independently responsible for the literature review, data entry and management, and statistical analysis of data using SPSS, Epi-Info, and BMDP packages.

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DEDICATION

I dedicate this thesis to the memory of my parents, whose love and blessing will always sustain me.

I also dedicate this thesis to my husband, Hashem Hoda, whose continuous support and encouragement enabled me to accomplish this work, and to my children Shiva and Nima, who were very cooperative and understanding throughout this project.

ABSTRACT

One hundred and two patients with cases of intermittent claudication (IC) from the vascular surgery clinics of the Ottawa General and Ottawa Civic Hospitals along with 99 control patients from different outpatient clinics of these Hospitals were recruited. For each subject information about the background, medical history, and lifestyle were collected by a face to face interview, using a questionnaire. A number of blood tests, and clinical examinations were also done to complete the information about the exposure, outcome, and the potential risk factors. Serum copper status was obtained through atomic absorbtion spectrophotometry and PAOD status was assessed by means of Doppler ultrasound. The univariate analysis of this data exhibited a strong association between serum copper and PAOD. After determining the study confounders by univariate analysis, a logistic regression model was fitted with PAOD as dependent variable and the serum copper along with other covariates as independent variables. The odds ratio for serum copper, [adjusted for the age, SES, smoking group (with 3 category of smoking as non-smokers, ex-smokers, and current smokers), systolic blood pressure, Quetelet group, and other covariates], was 1.40 (95% CI: 0.81, 2.43) for a five unit (μmol/l) increase of copper.

The variable smoking group was shown to be an effect modifier in this data and therefore, the odds ratios for different strata of smoking group were calculated. The association of serum copper and PAOD was statistically significant in ex-smokers with an odds ratio of 2.38 (95% CI: 1.10, 5.15) for a five unit increase of serum copper.

Using the number of cigarettes smoked per lifetime (SMOKLIFE) instead of smoking group in the logistic regression model, demonstrated considerable improvement in the model and

a statistically significant association between serum copper and PAOD was found. The odds ratio, after adjusting for other important covariates, was 1.81 (95% CI: 1.01, 3.22) for a five unit increase of serum copper.

In conclusion, smoking and systolic blood pressure were strongly associated with PAOD. The association of serum copper and PAOD, after adjusting for the variable smoking group, systolic blood pressure, and all other study confounders, was reduced to a non-significant level. However, after considering the variable smoking group as an effect modifier, a statistically significant association between serum copper and PAOD was demonstrated in ex-smokers.

This finding is consistent with a role for copper in the etiology of atherosclerosis, possibly as an oxidant, but further studies are needed to clarify this role before copper can be used as a target for intervention studies.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES:

FIGURES:

1. INTRODUCTION

Arteriosclerosis obliterans (AO) is a peripheral arterial occlusive disease (PAOD), mainly found in the elderly, that involves large and medium size arteries such as: abdominal aorta, common iliac, internal iliac, femoral, popliteal, and tibial arteries. Arteriosclerosis is a term used to describe the thickening and hardening of the arterial walls, a process which progressively narrows the arteries[1].

The most common type of arteriosclerosis is atherosclerosis. The most common symptom of arteriosclerosis obliterans of the arteries of the legs is intermittent claudication (IC) and the walk-pain-rest cycle is the hallmark of this complaint. The progression of the disease leads to ischemic pain at rest, coldness and numbness, ulceration, and necrosis of the foot. The terms intermittent claudication (IC) and peripheral arterial occlusive disease (PAOD) will be used interchangeably in this report.

Diagnosis of PAOD, is based on either X-ray evaluation (angiography), which is usually reserved for surgical cases, or non-invasive methods including the measurement of the ankle to brachial index (ABI) using the Doppler ultrasound. ABI is the ratio of the systolic blood pressure at the ankle to that of the arm. Normally the ABI is equal to one and values less than 0.9 are considered abnormal[1].

The standard treatments to improve walking ability and reduce progression of disease, for the mild and moderate cases of PAOD, are: daily exercise, weight reduction, and smoking cessation. Surgical procedures such as: thromboendarterectomy, transluminal angioplasty, arterial bypass surgery, and amputation are reserved for severe cases. However, the majority of patients

with PAOD will never require reconstructive vascular surgery or amputation, even though they do suffer some disability. In a Danish study, Jelnes (1986)[2] followed 257 newly diagnosed cases of the IC for a mean period of 6.5 years. The results of this follow up showed that 24 (9.3%) of patients developed ischemic rest pain and had reconstructive vascular surgery. 18 (7%) patients developed gangrene that led to leg amputation.

The incidence and prevalence of disease vary with age, sex and geographic location. Kannel[3] (1985) analyzed the 26 years data of the Framingham Study (ages 35-84 years) and found the annual incidence rate to be 3.5/1000 for males and 1.8/1000 for females (a ratio of 2 to 1). Hughson[4] studied 1716 men aged 45-69 years and 1535 women aged 50-69 years in Oxfordshire and found the prevalence of IC to be 2% for men and 1% for women. Reunanen[5] (1982) studied 5733 men and 5224 women aged 30-59 in Finland. He found a similar prevalence of IC for men (2.1%) but a higher value for women (1.8%). The male to female ratio varies in different studies. This might be due to variation in smoking prevalence ratio between population.

The mortality rate among patients with IC is twice the expected rate compared with an age and sex matched population[52]. Mortality among men is almost twice that among women.

The known risk factors for IC are smoking and high blood pressure[1,3] but there has been some interest recently in the possible role of copper[6-8]. Copper is an essential trace element and its main function is its role in the structure of coproenzymes (copper containing enzymes) that catalyse oxido-reduction reactions. The copper content of an adult human is about 50-120 mg with the highest concentration in the liver, kidney, heart, brain and blood. Sixty percent of blood copper is in red blood cells where it is bound to superoxidase dismutase (a copper dependent enzyme) and 40% is in plasma. In plasma 90% of the copper is bound to

ceruloplasmin (a copper containing protein). The remaining 10% is circulating copper which is measured as serum copper. Normal serum copper concentration is 10.8 to 24 μmol/L[9] and it is a specific biochemical index of copper nutritional status[10]. Serum copper concentration is most commonly measured by atomic absorbtion spectrophotometry[11].

The minimum daily requirement of copper for adult humans is 2-3 mg/day[12,13]. The richest sources of copper are; beef, liver, and shellfish. Other sources in descending order of concentration are nuts, high-protein cereals, dried fruits, poultry, fish, other meats, legumes, root vegetables, leafy vegetables, fresh fruits and non-leafy vegetables. Cow's milk is one of the poorest sources of copper[11]. Positive relationship between serum copper and smoking have been reported in a few studies[7,8,14], however a biological explanation for this relationship has not been suggested.

An association between atherosclerosis and the levels of serum copper has been suggested (see below) but the relationship between copper and PAOD has not been examined extensively. This study was undertaken to help filling this gap.

2. LITERATURE REVIEW

As noted above, there is very little information in the literature to show the association of serum copper with PAOD. However, as PAOD is clearly part of the problem of atherosclerosis, other studies of association of serum copper with other atherosclerotic cardiovascular disease are presented here and it is assumed that serum copper imbalance may be a shared risk factor of both PAOD and other atherosclerotic disease. Since hypercholesterolemia is a known risk factor for atherosclerotic disease, studies that show hypercholesterolemic effect of copper imbalance in animal species are also discussed in this report.

2.1 Animal studies:

The association of copper deficiency and cardiovascular disease was hypothesized by Shields[15] in the early 1960's. He showed that copper deficiency in swine could result in death because of rupture of heart, aorta, coronary and pulmonary arteries.

In 1973, Klevay[16] demonstrated in rats that relative deficiency of copper, characterized by a high ratio of zinc to copper in diet, resulted in hypercholesterolemia, which is a known risk factor for atherosclerosis. In this experiment the ratio of zinc to copper was five for the control group and 40 for the experimental group. It is worth mentioning that a diet with such an extremely low amount of copper is not likely to happen in a normal North American diet.

Allen[17] (1978) conducted another experimental study on rats to test the hypothesis of association of copper deficiency with cardiovascular disease and to evaluate the possible involvement of copper in the metabolism of cholesterol. In comparing 10 rats fed a copper

deficient diet with 10 controls on normal diet, he showed that the rats fed a copper deficient diet had a lower plasma concentration of copper (0.06 vs 1.42 μg/ml with P-value of less than 0.001) after 63 days, a lower liver copper concentration (P<0.001), and a higher plasma cholesterol concentration (107 vs 64.8 mg/dl with p-value of less than 0.001) after 61 days.

Another study on rats was performed by Fischer[18] (1980). The objective of this study was to determine whether different levels of dietary copper, together with low to high levels of zinc in diet, at levels likely to occur in a normal mixed diet, have any effect on cholesterol metabolism. Rats were randomized into 21 groups of eight. The animals were fed seven different diets containing various amount of added copper and zinc, with three groups for each diet. After 15 weeks, those animals that were fed diet with low amount of copper (1.5 ppm) and adequate or high amount of zinc had significantly lower serum copper than those with normal diet. However, there were no significant differences in serum or liver cholesterol levels ($p<0.05$) after 15 weeks. They concluded that copper and zinc, at levels likely to be present in normal North American diet, had no effect on cholesterol metabolism. They claimed that the hypercholesterolemic effects of copper deficiency, showed by Klevay[16] and Allen[17], require the consumption of an extremely low copper diet which does not occur in reality.

2.2 Epidemiologic studies:

Most of our knowledge on effects of copper deficiency comes from experiments with animals. Only a limited amount of data on these phenomena have been found in humans. Menkes disease is a rare x-linked recessive disorder caused by a defect in intestinal copper absorption. The prognosis of disease is bad and death generally occurs during infancy. Affected

infants have low serum copper, hypercholesterolemia, glucose intolerance and degenerative arterial disease. The hypercholesterolemic effect of dietary copper deficiency has been reported in only a single human[19]. A young adult man was fed a diet providing 0.8 mg of copper per day for a period of 105 days. Plasma copper concentration dropped from 75 μg/dl to 48 (or 11.6 to 7.4 μmol/lit). Most of decrease in plasma copper occurred late in depletion. Plasma cholesterol increased from 202 to 234 mg/dl. Upon repletion with 4 mg of supplemental copper per day for 39 days, serum copper and cholesterol returned to normal levels.

Vyden[7] (1975) investigated the metabolic and cardiovascular abnormalities in 28 patients with intermittent claudication and 28 controls in California and found that mean serum copper was significantly higher in patients with intermittent claudication than the control subjects (20.6 μmole/l vs 18.2). This difference was statistically significant ($P<0.05$). In this study smoking, serum cholesterol, and serum glucose were not significantly different in the two study groups but, mean SBP, mean diastolic blood pressure, and serum triglyceride were all significantly different in two groups ($p<0.05$).

Klevay[20] in 1975 reviewed previous epidemiologic studies of IHD. This review showed that the data are compatible with the hypothesis that copper deficiency is a major factor in the etiology of IHD. For example: Diets low in fibres and high in fat and sucrose are associated with high risk of coronary disease; such diets are likely to have high ratio of zinc to copper, death due to IHD is less common in patients with cirrhosis; these patients have a low ratio of zinc to copper in the liver, and death due to IHD is more common in patients with chronic renal failure who are under haemodialysis; these patients have a metabolic imbalance of zinc to copper with high ratio of zinc to copper.

Uza[21] (1985) studied the serum zinc and copper levels in 100 cases with hyperlipoproteinemia and 65 controls with normal serum lipoprotein. He found that serum zinc was not significantly different between the two groups but, the mean serum copper level was significantly higher in subjects with hyperlipoproteinemia than in the controls. However, when hyperlipidemic subjects were divided into two groups according to the presence or absence of clinical atherosclerosis, it was noted that in patients with obvious symptoms of vascular disease, serum zinc was significantly lower and serum copper was significantly higher when compared to the control group. The ratio of zinc/copper was also significantly lower in hyperlipidemic subjects when compared to control group.

Kok[14] (1988) determined the association of serum copper and zinc with mortality from cardiovascular disease. The investigator performed a case control study with data obtained in a nine-year prospective follow-up study. Cardiovascular deaths did not differ significantly from controls in mean level of serum copper, serum zinc, or the ratio of copper to zinc. However, the adjusted relative risk of death from cardiovascular disease (adjusted for serum cholesterol, systolic and diastolic blood pressure, body mass index, smoking, and other potential risk factors) was 3.5 (95% confidence interval = 1.4-8.7) for subjects in the highest serum copper quintile (>22.12 μmol/l) compared with those with normal levels. For subjects in the lowest serum copper quintile (<16.24 μmol/l) the adjusted risk was also higher (2.2 with 95% CI=0.8-6.4). These data suggest the possibility of existence of a U-shaped relation.

Uza[6] in 1989, evaluated the serum concentration of zinc and copper in 126 controls and 160 patients with atherosclerosis obliterans (AO) and 53 patients with thromboangiitis obliterans (TO). He found that the values of serum copper were higher in patients with AO or TO than in controls irrespective of the stage of the disease.

Steinberg[22] (1989), by performing in vitro investigations of cultured endothelial cells and LDL, suggested that oxidative modification of low density lipoprotein (LDL) increases its atherogenecity. He showed that an oxidative environment such as high serum copper, enhances the oxidative modification of LDL and consequently increases its atherogenic properties.

Salonen[8] (1991), based on Steinberg's experiments[22], conducted a cohort study to investigate the interactions between serum copper, selenium, and low density lipoprotein cholesterol concentration with regard to the progression of carotid atherosclerosis. The author found that the mean increase in the maximal common carotid intima media thickness after two years was greater in men with serum copper concentrations higher than 17.6 μmol/l as compared with men with concentrations less than 17.6 μmol (0.16 mm vs 0.08 mm; $p = 0.010$).

3. RISK FACTORS OF PAOD

The Framingham study[3] and some other risk factor studies[23-26] of PAOD, suggest a common underlying basis for both intermittent claudication (IC) and ischemic heart disease (IHD)[4-5].

In the Framingham study, where 5203 subjects (ages 35-84) followed for 26 years, 295 developed PAOD manifested as intermittent claudication. Among this cohort, cigarette smoking, impaired glucose tolerance, hypertension and low vital capacity were strong risk factors of PAOD; serum cholesterol, relative weight, and hematocrit were weak risk factors.

A case-control study of 28 cases and 28 controls in California[7] found significantly higher levels of blood pressure, serum copper, serum triglycerides, and a higher prevalence of abnormal ECG and lipoproteins among cases than controls. The prevalence of smoking and abnormal glucose tolerance test was also greater among the cases but the differences were not significant. No differences in the levels of serum cholesterol or hematocrit were observed.

More recently, Skalkidis[26] (1989) reported a larger case-control study in Greece. He evaluated the risk factors of PAOD in 100 cases and 100 controls and found that tobacco smoking, systolic blood pressure, diabetes mellitus, heavy alcohol drinking, and excessive coffee consumption were all strong independent risk factors of PAOD.

Therefore, based on these studies and some other epidemiologic studies all of the following elements were considered as potential risk factors for our study: age, elevated serum cholesterol, socioeconomic status, serum copper imbalance, obesity, high serum low density lipoprotein, smoking, low vital capacity, decreased serum high density lipoprotein, diabetes

mellitus, hypertension, family history of PAOD, coffee consumption, alcohol drinking. It is possible that some of these risk factors may be strongly associated with PAOD whereas others may strongly be associated with IHD or cerebrovascular disease.

4. POSSIBLE BIOCHEMICAL MECHANISMS

Low and high serum copper have both been suggested to be associated with atherosclerotic disease[6-8,14-17,19-22]. Two different mechanisms have been suggested to address these associations.

4.1 Low serum copper mechanism:

It was mentioned earlier that copper has an important role in the structure of coproenzymes that catalyze oxido-reduction reactions. One of these copper-containing enzymes is lysyl oxidase which catalyses the formation of lysyl-derived cross-linkage in both collagen and elastin. Based on experimental studies in rats and other animals, it has been suggested that copper deficiency results in both hypercholesterolemia, an important risk factor in the etiology of cardiovascular disease, and structural impairment of the arterial wall due to the deficiency of lysyl-derived cross-linkages[17], a phenomenon that initiates pathogenesis of atherosclerosis.

4.2 High serum copper mechanism:

On the contrary, high serum copper has also been suggested to be associated with atherosclerosis[6-8,14]. It has been proposed that oxidised LDL is more atherogenic than native LDL[22,27-32]. It was demonstrated by Stienberg et al.[22] that when native LDL was incubated with cultured endothelial cells, smooth-muscle cells, monocytes or macrophages (all major cell types in the artery wall), the native LDL was converted to modified LDL (oxidized LDL). He also showed that exposure to copper ions promotes this cell mediated oxidation of LDL and antioxidants such as vitamin E inhibits this process. Oxidised LDL has different properties that

promote atherosclerosis through the following mechanisms: it facilitates the recruitment of blood monocytes to the artery wall, it is much more susceptible to being taken up by macrophages than native LDL to create foam cells, oxidised LDL is toxic for cells, leading to cell destruction, and finally it promotes platelet aggregation. Oxidative modification of LDL can occur in different situations including exposure to copper ions[22,27,29,30]. Oxidized LDL can be taken up by cultured macrophages 3-10 times more rapidly than native LDL. Therefore high serum copper (oxidative environment) may have an important role in the development and progression of atherosclerosis through oxidizing LDL.

5. RATIONALE FOR THE STUDY

As mentioned earlier, arteriosclerosis obliterans is an atherosclerotic disorder that commonly affects elderly people. Patients with this disease suffer from degrees of pain (at first only when walking, later at rest), and disability which decrease their quality of life. The elderly population in Canada has grown in size and proportion. This trend is expected to continue in the future. It is projected by Statistic Canada that by year 2021, 17.6% of Canadian population will be people 65 years of age or older. Comparing to age distribution of 1993 population, there will be a 45% increase in proportion of elderly population. Therefore, with the increasing elderly population, disability due to the disease has become a public health concern. A better understanding of the risk factors for PAOD is necessary in order to develop preventive strategies.

There are few epidemiologic studies on the association of serum copper and atherosclerosis and the available published material in this area has had contradictory results. The only two epidemiologic studies concentrated on the association of serum copper and PAOD[6,7] suffer from methodological problems such as small sample size and/or lack of control for confounders in their statistical analyses.

Therefore, on account of the important public health relevance of the issue, rarity of research in this area, and methodological problems with the available published studies of PAOD and serum copper, further epidemiological investigations with a better design and methodology are required to increase our knowledge about the risk factors of PAOD.

The present thesis provides further information on the association of serum copper and risk of PAOD from a case control study of peripheral arterial occlusive disease conducted at the University of Ottawa.

6. OBJECTIVES OF THE STUDY

The primary objective of this study is to determine whether elevated serum copper levels are associated with peripheral arterial occlusive disease (PAOD).

The secondary objective of the study is to investigate the possible association of PAOD with the standard risk factors of atherosclerosis such as: smoking, hypercholesterolemia, high blood pressure, obesity, diabetes mellitus, low physical activity, socioeconomic status, caffeine consumption , and alcohol intake.

7. METHODS

7.1 General design of the study:

Since PAOD is a relatively rare disease, we chose the case-control design as the most appropriate method to investigate the risk factors of interest. At the same time this method satisfied the issues of time limitation and financial barrier, which were a concern. Also the case-control design allows for the assessment of potential risk factors other than the risk factor of interest (serum copper) as well as the interrelationship among these factors.

We restricted our study to males because of the low prevalence of PAOD in females. We selected hospital cases because, based on time and financial limitations, this was the only feasible method to apply. On the other hand, hospital cases may be more severe and not representative of all the cases in the population (selection bias).

In this study, as usual in a case-control study, we measured serum copper after the cases were diagnosed. However, as serum copper level is stable and does not easily fluctuate by day to day changes of dietetic copper, we assumed that serum copper measurements in this study referred to the levels before diagnosis. In addition, the standard treatments of PAOD, except for smoking cessation, do not seem to have any effect on the levels of serum copper. Smoking cessation would most likely decrease the serum copper level and therefore, would decrease the measure of association of serum copper and PAOD rather than introducing a bias by producing a spurious result.

We selected hospital controls for the following reasons. First of all, they were easily identified in sufficient numbers. Second, they were more likely to be cooperative and participate in the study than healthy persons, thus minimizing non-response bias. Finally, using patients with other diseases as controls from the same hospital as the cases means that they are likely to have been exposed to the same selection factors that persuaded the cases to come to the hospital. Therefore, by reducing non-response and selection bias, we hoped to satisfy comparability of cases and controls and to assure internal validity of the results.

Approval of the project was obtained from the Ottawa General Hospital and Ottawa Civic Hospital ethics committees. Using existing Canadian survey questionnaires, a questionnaire was developed to collect information from cases and controls [appendix A]. The questionnaire consisted of about 150 questions regarding subjects' background, medical history, and their present and past experience with different risk factors of PAOD.

7.2 Hypothesis:

The hypothesis of the study is that the mean serum copper value is higher in cases than in controls and this difference is statistically significant.

7.3 Sample size and power of the study:

The sample size for this study was pre-determined to be 100 cases and 100 controls. The power of the study, based on comparison of two means in two groups, to detect a difference in serum copper at least half of standard deviation was calculated from the following formula[33]:

$$n = \frac{(Z_{\alpha/2}+Z_{\beta})^2\ \sigma^2\ (r+1)}{(\delta^*)^2\ r}$$

$Z_{\alpha/2}$ = 1.96 (The cut off point for type I error is set at 5%)

Z_{β} = Cut off point for type II error (β)

σ = 2.16 μmol/l (The standard deviation of serum copper in population[34])

δ^* = 1.08 μmol/l (The magnitude of differences in serum copper means)

r = 1 (The ratio of the number of controls to the number of cases in the study)

n = 100 (The number of cases)

Based on the above equation Z_{β} is equal to 1.57. This figure corresponds to "β" value of 0.058 (5.8%). Power of the study is equal to 1 minus β which is 94.2%. As the selection of value for δ was arbitrary, we calculated power for different ratios of δ/σ and the results are presented as a curve in appendix D.

7.4 Case definition and selection:

Cases were defined as male patients who were referred to the vascular surgery clinic of Ottawa General Hospital (OGH) and Ottawa Civic Hospital (OCH) during September 1990 to October 1991. Diagnosis of arteriosclerosis obliterans was verified by means of a Doppler ultrasound (see below). Cases were selected if they had fulfilled the following eligibility criteria:

1- Intermittent claudication with walk-pain-rest cycle,

2- The ankle systolic blood pressure of equal or less than 80% of

the brachial systolic blood pressure (that is ABI $\leq$ 0.8) in one or both legs.

3- Ability to answer questions in English or French and,

4- Ability and willingness to give consent.

Cases were excluded from the study if they had experienced the following situations:

1- Pain at rest,

2- Ulcer in feet because of insufficient blood circulation,

3- Previous surgical therapy for correction of arterial occlusion,

4- Copper related metabolic disorders

The first three criteria are related to the very severe and long standing cases. We exclude these cases to reduce problem of the temporal relationship between exposure and disease. The fourth criterion involves subjects with diseases known to be related to the exposure variable (serum copper) such as Wilson's disease. This is a very rare condition and patients with this disease have high level of liver copper, high level of urinary copper, low plasma ceruloplasmin, and low level of serum copper. There is no evidence that the prevalence of PAOD in these patients are different from general population.

7.5 Control definition and selection:

Controls were elderly male patients who attended outpatient clinics of OGH and OCH during September 1990 to October 1991. All outpatient clinics were asked to participate in the study with the exception of vascular, cancer, cardiology, and diabetes mellitus clinics. We excluded cancer, cardiac, and vascular clinics because of the existing evidence of possible

association of serum copper with cancer and cardiovascular disease[14]. We also excluded patients from diabetes mellitus clinic because diabetics often have significantly elevated ankle systolic blood pressure due to medial calcinosis of peripheral vessels. Therefore, they tend to have artificially elevated levels of ABI[35]. Controls were selected if they had normal ABI in both legs, were able to sign the consent form and answer the questions in English or French. As PAOD is usually a disease of the older population, the controls were also selected amongst the older men. However, individual matching for age was not attempted. The ABI for controls was measured by means of Doppler ultrasound to avoid problems of misclassification.

7.6 Contact with cases and controls:

A trained bilingual nurse identified and approached the cases with confirmed diagnosis of arteriosclerosis obliterans, as well as the controls from the outpatient clinics of OGH and OCH. She asked them to read a one page general information sheet about the study of PAOD. Once a subject's eligibility, based on inclusion and exclusion criteria of the study protocol, was verified, he was asked by the nurse if he wished to take part in the study. Subjects who agreed to participate in the study were asked to sign the consent form [appendix B]. Then each participant was given an appointment to come back to the hospital for an interview, blood tests, and a physical examination. In order to measure fasting blood glucose and lipids, participants were asked to be fasting at the day of interview. If any participant was fasting while they were visiting the clinic; the interview, blood test, and physical examination were done the same day, if the participant preferred.

7.7 Data collection:

7.7.1 Questionnaire

Information about each case and control was collected by a face-to-face interview using a structured questionnaire [appendix A], blood tests, and physical examinations. The same nurse who identified study subjects interviewed them later. Cases and controls were interviewed with the same questionnaire and at the hospital. The interviewer was also asked to spend the same amount of time for cases and controls to reduce information bias. The questionnaire consisted of seven sections including:

1- Personal background: education, employment, ethnicity, and marital status.

2- Family history of PAOD

3- Medical history: high blood pressure, diabetes, heart diseases, pulmonary diseases, and the use of acetylsalicylic acid.

4- Physical activity

5- Past and present status of smoking

6- Past and present history of alcohol intake

7- Caffeine consumption

7.7.2 Physical examination

Physical examinations were done to assess disease status, weight, height, skin-fold thickness, vital capacity, and forced expiratory volume.

The presence or absence of peripheral arterial occlusive disease was evaluated by noninvasive techniques for both cases and controls. A Doppler blood-flow detector was used for

this purpose. First, systolic blood pressure value in both arms and ankles was obtained by means of Doppler blood-flow detector with use of an occluding pneumatic cuff. Then, ankle-to-arm systolic blood pressure ratios were calculated, for both right and left side, for all study subjects. This measurement is the most widely used objective measurement of PAOD. The reproducibility and validity of this noninvasive method was confirmed by different studies[36,37].

Skin-fold thickness was measured by Lange skinfold calipers. This is a precision instrument designed for measurement of subcutaneous tissue. It is calibrated to accuracy of ± 1 millimetre.

Vital capacity and forced expiratory volume in one second, were measured by means of a Vitalograph-compact spirometer to evaluate pulmonary function. Vital capacity is the volume change between maximum inspiration and full expiration. A reduction in the vital capacity indicates a pattern of restriction. Forced expiratory volume is the volume of air expelled from the lungs over a timed period from a position of maximum inspiration with the subject making a maximum effort. The usual time interval is one second (FEV_1). A reduction in the measurement indicates airway obstruction.

7.7.3 Laboratory tests

Finally to complete data collection, blood samples were drawn to measure the following blood tests: serum copper, fasting serum glucose, cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), copper, zinc, and cotinine.

Serum copper concentration was determined by atomic absorbtion spectrophotometry which is the most common analytic technique for copper determination in serum or plasma[11].

A Varian 1475 series atomic absorbtion spectrometer was used with a flame technique with standards made in 5% glycerol. The between batch coefficient of variation was 4.5% (n=20). Copper free tubes were used for storing blood. Serum copper was determined by flame atomic absorbtion spectroscopy following a 1:10 dilution with distilled water. The results were expressed as micromoles per litre (μmol/l).

The serum level of cotinine, a metabolite of nicotine, was measured to validate subjects' answers on their history of smoking.

7.8 Analysis:

7.8.1 Selection of potential confounders

Information on attributes of the factors that might associate both with serum copper and with PAOD were assessed from data obtained by the interviewer through the questionnaire [appendix A], physical examination, and blood tests. Sixteen potential confounders were considered. Many of these items have been regarded as established or suspected risk factors of PAOD in previous studies[3,4,25,26,38]. Cigarette smoking was considered as a confounder due to its known association with PAOD and also its relationship with serum copper[7,8,11,14]. SMOKLIFE, the number of cigarettes smoked per lifetime, was also used as a measure of smoking. Systolic blood pressure, diabetes mellitus, alcohol drinking, and coffee consumption all were identified as independent risk factors of PAOD in Skalkidis study[26]. Low vital capacity was a predictor of intermittent claudication in Framingham study[3] but, the scientific mechanism for this finding is not clear. Hypercholesterolemia, and low serum HDL, were significantly associated with PAOD[38]. Triglycerides were also significantly higher in these patients[4]. Obesity has been considered as a risk factor for PAOD. However, in some studies, it did not appear to be an

independent risk factor of PAOD[26,38]. On the contrary, relative weight was inversely related to PAOD in Framingham study[3]. This study restricted only to elderly male subjects, therefore gender is not a relevant confounder in this study. Other established or potential confounding factors examined included: age, physical activity, socioeconomic status (SES), family history of PAOD, history of heart disease, ethnicity, and education.

7.8.2 Data preparation

Based on their smoking history, subjects were assigned to one of three categories: non-smokers, ex-regular cigarette smokers, and current regular cigarette smokers (current smokers are those who smoke every day). Category of pipe or cigar smokers, and occasional cigarette smokers were too small to assure a meaningful interpretation of results and consequently were excluded (three subjects were pipe or cigar smokers and one was an occasional smoker).

SMOKLIFE was calculated by multiplying the number of cigarettes smoked per day, number of days in a year (365), and number of years smoked. SMOKLIFE was categorized into six groups with cutpoints of 0, 99999, 199999, 299999, 399999. These cutpoints were also used in the main study of smoking and PAOD. Obesity has been evaluated using the quetelet index, that is weight in kilogram (kg) divided by square of height in metres.

Socioeconomic status of participants was calculated from information in the questionnaire about their jobs. The job information was first coded, using job classification codes[39], and these codes were later translated to socioeconomic score (ranging from 20.0 to 70.0), based on the Blishen scale[40]. These socioeconomic scores were then grouped into three socioeconomic status

(SES). These groups were low SES (socioeconomic score of 20 to 40), medium SES (socioeconomic score of 40 to 60), and high SES (socioeconomic score of > 60).

The amount of caffeine consumption was calculated in mg/day. This was measured based on available literature information on the caffeine content of different foods and beverages[41-43] and answers to questions of caffeine consumption. The answers to questions about physical activity were used to calculate a physical activity score and physical activity group. The score is a summation of frequency of each activity reported in the period of two weeks multiplied by the average duration in minutes of each activity and by the average metabolic cost of that activity (adapted from Canada health survey)[44]. These scores were then grouped into quintile with four categories of physical activity namely, active, occasionally active, infrequently active, and inactive.

The total skin-fold measure is the sum of four skin-fold sites; triceps, biceps, subscapular, and suprailiac. The total skin-fold measurement was converted into percentage of body fat using tables adapted from the Mayo Clinic Diet Manual.

7.8.3 Statistical analysis

In order to use and manage the data in a computerized format, all collected information was converted to proper names, labels, and codes (appendix C). The SPSS data entry II program was used to enter the data into the computer.

Univariate analyses were performed using the SPSS-PC and Epi-Info programs. The BMDP statistical package was used for multiple logistic regression analyses. The 5% level ($\alpha = 0.05$) for statistical significance was used.

Descriptive statistics and the distribution of different characteristics between cases of peripheral arterial occlusive disease and controls were assessed. The comparison of serum copper means among the cases and controls was assessed with a two-sample t-test. Further, to evaluate a possible dose response relationship for the risk factor of interest, serum copper, the relative risk (estimated by the odds ratio), overall chi-square, and the chi-square for trend were calculated with the lowest serum copper category as the reference group. To enable a straightforward interpretation of the magnitude of risk and its 95% confidence interval (CI), a two by two contingency table was formed by using the serum copper median value as the cutoff value.

Since one of the epidemiologic studies[14] raised the possibility of a U-shaped relationship between serum copper and cardiovascular disease, we explored this condition by comparing the cumulative probability distributions of cases and controls.

To control for the effect of confounding and effect modification on the association of serum copper and PAOD, multiple logistic regression analysis was used. In order to fit the logistic regression model to the data, first the existence of confounders in the data was examined. We explored the association of potential and known risk factors of PAOD with both the outcome variable (PAOD) and the primary independent risk factor of interest, serum copper, by using univariate analyses and the p-value considered to be 0.05 or less. The investigation began with a univariate analysis of each variable with PAOD. For nominal and ordinal variables this was done with a contingency table of PAOD versus the k levels of the covariate. The overall association was measured by the overall chi-square and chi-square for trend. For those variables, exhibiting an association, individual odds ratios were estimated, using one of the levels as the

reference group. For interval variables the univariate analysis based on two sample t-tests was used.

The next step was to identify the association of serum copper with other covariates. For the categorical variables an analysis of variance was done with the serum copper as dependent and each of the covariates as independent variables. Finally a correlation analysis was used to examine the relationship of serum copper and continuous potential confounders. All the identified confounding variables were then controlled simultaneously by means of a logistic regression model.

To assess the validity of the fitted model, the possibility of existence of effect modification (interaction) between covariates was explored next. Stratified analyses were done for study confounders (SBP, SMKGRUP, FEV1/VC, and CAFEINE) and for the variables AGE, SES, and QUETGRUP. However, the sample size was not large enough to stratify data for more than one cofactor at a time. Woolf's method was used to evaluate the homogeneity of odds ratios across different strata, when warranted.

Due to the problem of small sample size, we then explored the existence of interaction by the logistic regression method. The interactions between COPPER and other risk factors of PAOD in the model were examined. Each interaction term was added, one at a time, to the previously developed model, containing COPPER and the study confounders, one at a time to see if any of them would significantly contribute to the model. Statistically significant interaction terms were then added to the main effects model. The odds ratios for the association of serum copper and PAOD were calculated for one and five units increase of serum copper.

In order to explore further the effect of smoking on the association of serum copper and PAOD, we also examined the impact of variable SMOKLIFE as a confounding variable. A logistic regression model was fitted using PAOD as the dependent variable and COPPER, SMOKLIFE, and other confounders as independent variables. The existence of interaction between COPPER and the confounders was also explored for this model.

Finally, a forward stepwise logistic regression analysis for the association of serum copper and PAOD, using all the important covariates and the interaction terms, was performed to evaluate the importance of each variable in the model and to see if the results were similar to our previous approach (and thus to validate the logistic regression model building strategy). The significance of each variable was assessed by the likelihood ratio chi-square test (improvement chi-square) and its corresponding p-value.

8. RESULTS

From the total of 107 cases and 103 controls identified and approached for the study, 95% of the cases and 96% of the controls accepted to participate (refusal rates of 5% and 4% for cases and controls respectively). The reasons for refusal were either that they were not interested or did not have time. The cases of PAOD and controls were recruited and interviewed by a trained, bilingual nurse. The clinics from which the controls were recruited and their contribution were as follows: Orthopaedic (29%), Ophthalmology (23%), Urology (18%), General surgery (16%), and the other clinics (14%).

We excluded one control because he failed to meet the inclusion criteria (he was inadvertently selected from vascular clinic). Another seven subjects were excluded because their serum copper results were not available. We further excluded another control whose ABI was abnormal. We ended up with 97 subjects in the case series with ages ranging from 36 to 83 and a mean of 65 years. There were also 95 controls with ages ranging from 44 to 80 and a mean of 62 years.

In order to make it easier to follow the results of the study, an abbreviated code sheet is given in table 1 which describes the variables and provides the code names for the variables often used in this study. A complete code sheet is given in appendix C.

The serum copper levels in the study population ranged from 9.7 to 27 with a mean of 16.2 μmole/l and a standard deviation of 3.6. The descriptive statistics and the distribution of the study variables among cases and controls are shown in table 2. The results of univariate descriptive analysis of data by two-sample t-test demonstrated that cases had a statistically

significantly higher mean serum copper level than controls, 17.24 ± 3.64 μmol/l vs 15.19 ± 3.24 ($p<0.001$).

Table 3 provides the distribution and univariate risk estimates for various serum copper levels for 97 cases of PAOD and 95 controls. Serum copper, measured on a continuous scale, was grouped into quintiles with cut points based on the entire study sample. There were more cases in the higher serum copper categories and more controls in the lower serum copper categories. Considering the lowest serum copper category (<13.1) as the reference category, odds ratios generally increased as serum copper increased with an odds ratio of 6.46 for the highest quintile. The overall chi-square was 19.5 ($p<0.001$) and the chi-square for trend was 17.5 ($p< 0.0001$). Figure 1 shows the distribution of serum copper among cases and controls and the measure of the association between PAOD and serum copper. Dichotomizing serum copper on its median value (15.9 μmol/l) as the cut off point, gave an odds ratio of 3.04 with 95% confidence interval of 1.69 and 5.47.

Figure 3 shows the histogram of the frequency distribution of cases and controls for different serum copper levels. The case-control ratios increases uniformly with the level of serum copper and there is no evidence of a U-shaped relationship. Figure 4 displays the cumulative probability distribution of cases and controls. For both groups there was only one point of inflection, again pointing to the absence of a U-shaped relationship.

The results of the analysis of the association of PAOD and the potential categorical confounders are presented in table 4. Risk of having PAOD was larger in higher age groups. In particular, age categories of 60 to 69 and over 70 seemed to be at higher risk than the younger groups. The overall chi-square was 8.18 ($p < 0.05$) and the chi-square for linear association was 5 ($p<0.05$). More controls achieved post secondary education than cases (28.3%

vs 20.6%), and more cases were in lower education levels. However the differences were not statistically significant. There were no significant differences between cases and controls for place of birth, ethnicity, or martial status.

More cases belonged to lower socioeconomic status (76.3% of cases in the middle and lower categories vs 57.9% of controls). The overall chi-square was 7.67 ($p<0.05$) the chi-square for trend was 6.87 ($p<0.01$). Controls tended to be heavier than cases (70.5% of controls in the middle and higher levels vs 57.7% of cases) but these differences were not statistically significant.

Personal history of hypertension and heart disease was significantly more frequent among cases than controls with p-values of 0.01 and 0.001 respectively. Personal history of diabetes, pulmonary disease, and family history of PAOD did not differ significantly ($p>0.05$).

Cases tended to be more often in the ex-regular smoking and current regular smoking groups than the controls. The overall chi-square was equal to 18.4 ($p<0.001$) and chi-square for trend was equal to 17.6 ($p<0.001$). Furthermore, the number of cigarettes smoked in lifetime were also significantly higher in cases than in controls with chi-square for trend of 27.6 and p-value of less than 0.00001.

In these data, drinking alcoholic beverages had no particular trend and did not show a significant overall difference among cases and controls. There were more cases in higher caffeine group (25.8% vs 14.7%) but the difference was not statistically significant. Finally, cases were less frequent in very active (15.5% vs 34.7%) and active groups (23.7% vs 25.3%) but more frequent in inactive (30.9% vs 20.0%) and sedentary groups (29.9% vs 20.0%). The overall chi-square was 11.3 ($p<0.05$) and chi-square for trend was 9.5 ($p<0.005$).

The results of the analysis of the comparison of the continuous potential confounders are shown in table 5. The average age was higher in case group comparing to the control group (65.23 vs 62.11) and the difference was statistically significant ($p<0.05$). The case group had a higher average of body fat percentage and a somewhat lower average of the Quetelet index but, neither were statistically significant. Average SBP was significantly higher in cases than in controls ($p<0.001$) The mean value for vital capacity (VC) and forced expiratory volume for one second (FEV_1) were both significantly higher in control group ($p<0.001$). The ratio of FEV_1 to VC has been considered as a measure of obstructive pulmonary disease. Its average was higher in the case group ($p<0.05$). The case group tended to have a higher average of glucose, cholesterol, and LDL than control group but the differences were not significant.

Mean triglyceride was slightly higher in case group compared to the control group ($p<0.05$) and the average for HDL was significantly higher in the control group ($p<0.01$). The means for zinc were not significantly different in the two study groups, but the ratio of zinc to copper (ZN/CU), was significantly different in the two study groups ($p<0.05$). Both the socioeconomic score and the activity score had a significantly higher average in the control group than the case group ($p<0.01$). Finally, caffeine consumption had a higher average in the case group than the control group ($p<0.05$).

The results of the analyses on the relationship of serum copper and the potential confounding variables are shown in table 6. For those variables where the assumption of homogeneity of variances was confirmed, the levels of significance were assessed by the standard "F" test. For those variables where the hypothesis of homogeneity of variances was rejected by

Leven's test, the results of the significance level were assessed by Welch's test that does not assume the variances to be equal.

Mean serum copper levels in different age groups (less that 60, 60-70, and >70) were not significantly different. The "F" ratio is equal to 1.23 and the "F" probability is greater than 0.10. There were no significant differences among groups of low, medium, and high socioeconomic status regarding mean serum copper. Mean serum copper levels were significantly different among different Quetelet index categories (<25.0, 25.0-29.9, >29.9). The group with the lowest quetelet index (<25.0) had the highest serum copper level (17.2 μmol/l), and the group with the highest index (>29.9) had the lowest serum copper concentration (15.5 μmol/l). The "F" ratio is equal to 4.36 and the "F" probability is smaller than 0.05.

Mean serum copper levels were significantly different between non-smokers and current smokers (14.8 μmol/l vs 17.6 μmol/l), and between ex-smokers and current-smokers (15.7 vs 17.6) but were not significantly different between non-smokers and ex-smokers. The group with caffeine consumption of less than 500 mg/day had significantly lower mean serum copper levels than groups with caffeine consumption of more than 500 mg/day (15.8 vs 17.7). No statistically significant differences of mean serum copper levels between any two groups of physical activity groups were noticed.

Mean serum copper concentration was significantly higher in group with systolic blood pressure of greater than 140 mm/Hg compared to group with systolic blood pressure of less than 140 mm/Hg (17.3 μmol/l vs 15.5 μmol/l). For the group with high density lipoprotein (HDL) greater than 1.0 mmol/l, mean serum copper level was nonsignificantly lower than the group with HDL equal or less than 1.0 mmol/l (16.0 μmol/l vs 16.5 μmol/l). The group for which the proportion of their forced expiratory volume in one second was equal or less than 80% for their

vital capacity ($FEV_1/VC \leq 0.8$) had higher mean serum copper level than group with $FEV_1/VC > 0.8$ (16.5 μmol/l vs 15.4). This difference was statistically significant ("F" ratio = 4.03 "F" probability = 0.04).

For the variables that were measured on a continuous scale, the association of serum copper and different risk factors were also explored using the correlation coefficients. The results of these analysis were the same as the results of the analysis of variance.

Based on the above investigations, the variables AGE, SES, HDL, SBP, VC, FEV1, FEV1/VC, SMKGRUP, CAFFEINE, and ACTYGRUP were all significantly associated with PAOD. Variables QUETGRUP, CAFFEINE, SBP, VC, FEV1, FEV1/VC, and SMKGRUP were all associated with serum copper.

Variables VC, FEV1 and FEV1/VC were all highly correlated. Therefore, to select one of them for the logistic regression model, their associations with serum copper were examined by means of univariate regression analysis using COPPER as dependent and each of these variables as independent variables one at a time. FEV1/VC had the highest R^2 (0.12) versus VC (0.08), and FEV1 (0.10). We selected FEV1/VC for the rest of analysis because it could explain the variation of copper the best and also from the clinical point of view it is an important measure of pulmonary obstructive disease.

The variables which showed statistically significant association ($p \leq 0.05$) with both PAOD (outcome) and serum copper (study risk factor) were SBP, SMKGRUP, FEV1/VC, and CAFFEINE. These variables together with AGE and SES were considered for multivariate logistic regression model. Variable QUETGRUP was not associated with PAOD (P=0.15) but

was inversely related to copper. To be on the conservative side this variable was also added to the model.

The results of fitting a multivariate logistic regression model to these data are given in table 7. For each variable the following information is given: (1) the estimated slope coefficient, (2) the estimated standard error of the coefficient, (3) the estimated odds ratio (obtained by exponentiating the estimated coefficient), and (4) the 95% confidence interval (CI) for the odds ratio. The value of the log-likelihood for the model is -98.54. From this analysis, it appears that smoking was the most important risk factor of PAOD. The odds ratio for ex-smokers, comparing to non-smokers as the reference group, were 4.20 (95% CI 1.04, 16.9) and for current-smokers were 8.10 (95% CI: 1.76, 37.4).

The odds ratio for one unit (μmole/l) increase of serum copper was 1.07 (95% CI: 0.96, 1.20). This means that the risk of having PAOD increases about 7% with each unit increase of serum copper. The odds ratio for five unit increase of serum copper was 1.40 (95% CI: 0.81, 2.43). The odds ratio for five unit increase of serum copper was calculated, since a one unit change would not be clinically meaningful.

Systolic blood pressure was also important with odds ratio of 1.04 (95% CI 1.02, 1.05) for each mm/Hg increase. The odds ratio for age was 1.03 (95% CI: 0.99, 1.08) for one year increase of age. The odds ratio for 1 mg/day increase of caffeine consumption is shown in table 7. However, as one mg/day increase of caffeine is not biologically important, the odds ratio was also calculated for 100 mg/day increase:1.10 (95% CI: 0.98, 1.24). The rest of the variables did not show to be important in this model, because the 95% CI for their odds ratios were either too wide to warrant precision or their lower confidence limits were much less than unity.

The results of the stratified analysis for the study variables are shown in table 8. Woolf's statistical method[45] for the evaluation of homogeneity of chi-square across different strata showed that none of the variables used in the logistic regression model was a significant effect modifier. However, we observed sufficient variation between stratum specific odds ratios for the variables SES and SMKGRUP. It is worth mentioning, that for non-smoker stratum there were three cells with less than 5 subjects in them, and the assumption of an underlying chi-square distribution may be invalid.

The results of investigating effect modification, using the logistic regression, are presented in table 9. In this table we display information for main effect only model and also for main effect plus one interaction model. This information includes the log-likelihood for each model, and the improvement chi-square (the likelihood ratio test) along with its degrees of freedom and the level of significance. The likelihood ratio test was obtained by multiplying the difference between log-likelihood for main effect only model and log-likelihood for the model with each interaction term by -2.

From this table we observed that only two interaction terms appeared important to pursue. These were the COPPER*SMKGRUP and COPPER*SES interactions with the improvement chi-square equal to 6 and p-value of <0.05. Three additional models containing main effect variables plus one or both of the significant interaction terms were fitted.

Table 10 shows the results of fitting the model containing main effect and the interaction term COPPER*SMKGRUP. This model provided a moderate improvement over the main effect only model in table 7 (log-likelihood of -95.54 vs -98.54). Based on this analysis, the odds ratios were calculated for different smoking status group and the results are shown in table 11.

For one μmol/l increase of copper the odds ratios were 1.28 (95% CI: 0.71, 2.29) for non-smokers, 1.19 (95%CI: 1.02, 1.39) for ex-smokers, and 0.91 (95% CI: 0.89, 1.07) for current-smokers. For 5 unit increase of serum copper, odds ratios were 3.37 (95% CI: 0.18, 62.57) for non-smokers, and 2.38 (95% CI: 1.10, 5.15) for ex-smokers, and 0.62 (95% CI: 0.36, 1.43) for current-smokers.

Table 12 shows the results of the logistic regression model with main effect and COPPER*SES as interaction term. The estimated odds ratios for different changes of serum copper level are demonstrated in table 13. For one unit increase of copper odds ratios are 1.06 (95% CI: 0.85, 1.31) for high SES, 1.38 (95% CI: 1.06, 1.78) for medium SES, and 0.98 (95% CI: 0.86, 1.12) for low SES. For 5 units increase of copper, odds ratios were 1.32 (95% CI: 0.45, 3.89) for high SES, 4.93 (95% CI: 1.36, 17.8) for medium SES group, and 0.92 (95% CI: 0.47, 1.79) for low SES group.

Table 14 shows the model with both of the interaction terms, COPPER*SMKGRUP and COPPER*SES. However, in the computer output of this analysis, the values of correlation of coefficients and covariance of coefficients for some of the variables were so small that they came out as zeros. Therefore, due to the insufficient information in the output, the calculation of odds ratios for different sub-categories of SMKGRUP and SES was not possible. In these cases a univariate analysis of sub-groups of SMKGRUP, and SES was done. Figure 2 shows the comparisons of serum copper means in cases of PAOD and the controls. For all sub-groups except the current-smokers/low-SES category, the mean serum copper were higher in cases than in controls. The differences were statistically significant in the ex-smokers/medium-SES and the current-smokers/medium-SES categories. Due to the lack of the cases of PAOD in the category

of non-smokers/medium-SES, the comparison of group means was not possible for this sub-group.

In order to further explore the effect of smoking on the relationship between copper and PAOD, we also examined the impact of the variable SMOKLIFE which is the number of cigarettes smoked in lifetime. This variable was grouped in six categories and cutpoints were 0, 99999, 199000, 299999, 399999. SMOKLIFE variable was one of the principal variables used in the main study of smoking and PAOD. The results of fitting this model are given in table 15. This model seems to provide a considerable improvement over the model with SMKGRUP (log-likelihood -92.03 vs -98.54). The odds ratio for each unit increase of copper was 1.13 (95% CI: 1.00, 1.26), for 5 unit change of copper was 1.81 (95% CI: 1.01, 3.22), and for ten unit increase was 3.26 (95% CI: 1.02, 10.38).

To explore the validity of above model we then repeated the procedures for investigating the existence of interaction the same way as we did for the model in table 7 (the model with variable SMKGRUP instead of SMOKLIFE). The results of this investigations confirmed that no interaction terms significantly contributed to the model.

The results of stepwise logistic regression analysis are shown in tables 16, 17, 18, and 19. Table 16 shows the summary of results of stepwise logistic regression analysis using the variable SMKGRUP. For each step the name of the variable that entered at that stage, degrees of freedom, log-likelihood value, improvement chi-square and its p-value, and the goodness of fit chi-square and its p-value are demonstrated. As is shown, at steps one and two variables SBP and SMKGRUP produced the greatest changes in log-likelihood values relative to the previous step with significant improvement of chi-square and p-values of <0.001. At the third step,

variable COPPER entered in the model with a relatively moderate improvement of the model. The value of likelihood ratio chi-square for improvement at this step was 3.63 (p = 0.057).

Table 17 shows the estimates for slope coefficient, standard error of coefficient, and odds ratios and their 95% CI. The odds ratio for 1 mm/Hg increase of SBP was 1.04 (95% CI: 1.02, 1.06) and the odds ratio for 20 mm/Hg increase of SBP was 2.16 (95% CI: 1.54, 3.02). The odds ratio for ex-smokers (comparing to non-smokers) was 5.34 (95% CI: 1.35, 21.1) and for current-smokers was 9.96 (95% CI: 2.28, 43.5). The odds ratio for one unit increase of serum copper was 1.10 (95% CI: 0.99, 1.22) , for five unit change was 1.58 (95% CI: 0.94, 2.65) and for 10 unit change was 2.48 (95% CI: 0.88, 7.02).

The results of the stepwise logistic regression analysis using variable SMOKLIFE are given in tables 18 and 19. From table 18, it is demonstrated that this model is a better fit compared to the model in table 16 (log-likelihood -94.66 vs -102.3). Variables SBP and SMOKLIFE are again important factors in this model. Variable COPPER which entered the model at third step provided a significant contribution to the model. The value of the likelihood ratio test of the model at step 3 versus previous step was 8.1 with the corresponding p-value of 0.004.

Table 19 shows the odds ratio and 95% CI for each variable in the model. The odds ratio for 1 unit increase of copper was 1.14 (95% CI: 1.02, 1.28), for 5 unit increase was 1.93 (95% CI: 1.10,3.38), and for 10 unit change of copper was 3.73 (95% CI: 1.22, 11.42). The results of these analyses confirm that the association of serum copper and PAOD, after controlling for important confounders, is statistically significant.

9. DISCUSSION OF RESULTS

The null hypothesis for this study was that mean serum copper would be the same among patients with diagnosis of peripheral arterial occlusive disease and among the control group. This null hypothesis was rejected using univariate analysis (two sample t-test). Mean serum copper was higher in cases than in controls and the observed excess was highly significant ($p<0.001$). Therefore the results supported our alternate hypothesis of the association of high serum copper with PAOD. These results were consistent with the reports from earlier California case-control study[7]. Kok[14] also concluded that high serum copper were significantly associated with cardiovascular disease.

Furthermore, the results of chi-square analysis for different levels of copper (serum copper levels were grouped in quintile) also supported the hypothesis of the association of high serum copper and PAOD. We also repeated this analysis using the serum copper level cut points from another study[14] and the results were almost the same as the first one.

The excess of mean serum copper in cases might be a true difference of mean serum copper between two study groups but, the other possibilities due to chance and biases must be considered as well. The chance of erroneously rejecting the null hypothesis (type I error) with the t-test and chi-square analyses of data was smaller than 0.1%.

The biases we are concerned with for this case control study are selection bias, measurement bias (information bias), and confounding bias. This case control study is not likely to suffer from selection bias because cases and controls seem to have equal chance of being exposed to copper (through diet or environment) and furthermore we are selecting both of the

cases and controls from the same hospital. Therefore, it seems that controls will represent the population of non-diseased persons who would have been selected as cases if they had developed the disease. Therefore, the cases and the controls are comparable with each other and such a case control study will have a high internal validity and low selection bias. Non-response bias, which is another source of selection bias, is again very unlikely to occur in this study since, response rates were very high for both cases and controls (95% of cases and 96% of controls). Detection bias is also unlikely to be a source of selection bias, as high serum copper does not cause symptoms leading patients to seek medical attention.

Observation or information biases regarding the exposure or disease are again very unlikely to happen in this study. The exposure variable, serum copper, was measured by the laboratory test and therefore is not expected to produce either interviewer bias or recall bias. Additionally, all of the serum copper measurement were performed in one biochemistry lab and the technician was blind to the subjects' diagnostic category. The inter-observer variability of serum copper measurements was also absent, because only one technician accomplished all of the measurements.

Misclassification of disease was also unlikely to happen as the prevalence of disease is relatively low, and diagnostic test (blood flow detection by Doppler ultrasound) has been performed for both cases and controls. Even if there is a small chance of misclassification of disease, it will be independent of exposure status, so a random or nondifferential misclassification of disease status will occur. This situation introduce a bias toward the null value[46].

Finally, the third group of bias (confounding) is a major concern for this study. To assess this source of bias in our data, we first verified the study confounders by univariate analyses and then we built a model with logistic regression analysis including all known and potential risk factors of PAOD. As we observed in tables 7, SBP and smoking were strongly associated with PAOD. The distribution of cases and controls according to cigarettes smoking, is in broad agreement with the general evidence on the epidemiology of peripheral arterial occlusive disease. After adjusting for all confounders, copper was not significantly associated with PAOD. These results need further interpretation.

First, it is possible that the observed univariate association of serum copper and PAOD was confounded by variables SPB and SMKGRUP. Controlling for these confounders has reduced the magnitude of the association to a nonsignificant level.

The second possibility is that if high serum copper can cause people to smoke, then smoking would be an explanatory variable in the causal pathway and therefore, adjusting for that would delete the hypothesized association of serum copper and PAOD. However, this hypothesis does not seem to be biologically plausible.

Moreover, the wide confidence interval for odds ratio of copper suggests that the sample size may have been too small to estimate the magnitude of the association with precision. The power of this study were calculated based on the comparison of two means and a predetermined sample size. It is quite possible that this sample size may not be large enough for more detailed analyses such as logistic regression.

In addition, cigarette smoking in this study population was positively associated with serum copper. These results were consistent with some other reports[7,8,11,14]. In this study mean

serum copper is highest in current-smokers, lower in ex-smokers, and lowest in non-smokers. A plausible biological explanation for this association has not been published. However, a possible mechanism might be as follows: cadmium that exists in cigarettes smoke, induce the production of protein methalothionine in the liver. This protein is able to sequestrate heavy metals. This means that methalothionine may take copper from tissues, such as liver, to the plasma. As a consequence, the serum copper level increases. We mentioned earlier that Stienberg[22] has shown that high serum copper levels, through the mechanism of oxidative modification, increase the atherogenic properties of LDL. We may then postulate that cigarette smoking promotes atherosclerosis partly through increased serum copper but mainly through its direct effect on the artery walls (that is by the constriction and injury of arteries and arterioles due to the generation of free radicals by cigarettes smoking). If this is true, then allowing for smoking in the statistical analysis would represent an overadjustment on the association of serum copper and PAOD. As a result, controlling for the smoking status would dominate, and the effect of copper would be washed out.

To further investigate the effect of smoking on the serum copper in two study groups, we calculated the mean values of serum copper in cases and controls for different categories of smoking status. The mean serum copper was lower in controls than in cases in non-smokers (14.59 μmol/l vs 16.17) and in ex-smokers (14.70 μmole/l vs 16.80) but were almost the same in current-smokers (17.13 μmole/l vs 17.80). This means that current-smokers have high serum copper (possibly at a saturated level) regardless of their disease status and therefore, the association of serum copper and PAOD can not be shown in that subgroup. Based on this discussion, the smoking status should be considered as an effect modifier and we would then expect to see a stronger relationship of copper and PAOD in non-smokers and ex-smokers than

in current-smokers. The data from this study support this suggestion. As we observed earlier in the results of logistic regression analysis for the association of serum copper and PAOD, the odds ratios for five unit change of copper, were 3.37 for non-smokers, 2.38 for ex-smokers, and 0.62 for current-smokers. However, due to small number of subjects in the non-smoker group, the 95% CI was wide and included unity but the association were statistically significant in ex-smokers.

The results of logistic regression analysis with variable SMOKLIFE in the model suggest that it was a better fit to the data and provided a significant improvement over the model with variable SMKGRUP. As we observed in table 15 the association of serum copper and PAOD, after controlling for all important confounders, were statistically significant. The odds ratio for 5 unit change of copper was 1.80 (95% CI: 1.01, 3.22).

This study has some shortcomings. First of all, the incubation period for PAOD is long and therefore, patients may have the disease long before the clinical symptoms are evident. In addition, there might be a time lag between the onset of symptoms and their referral to the hospital. In this study the average gap between beginning of symptoms and entry to the study was about 12 months. Therefore, the temporal relationship between serum copper and PAOD is difficult to establish.

Finally as we are selecting our cases and controls from the hospitals, they might be a selected group, not comparable to the population (more severe cases come to the hospital). This process may reduce the external validity (generalisability) of the results. However, we decided not to compromise internal validity in order to achieve generalisability.

10. CONCLUSION

Serum copper showed a strong relationship with PAOD by univariate analysis but, controlling for smoking (as smoking status categories), systolic blood pressure, and other risk factors of PAOD by logistic regression analysis reduced the magnitude of association to a non-significant level. However, we concluded that because of the strong association of smoking with serum copper, the association of copper and PAOD was modified by smoking group. The association of serum copper and PAOD was not clearly evident in current-smokers, but was demonstrated in the other two categories, most strongly in ex-smokers. We also concluded that possibly the sample size and consequently the power of the study might not be sufficient to detect statistically significant association in all of the analyses. In addition, using variable SMOKLIFE (amount smoked per lifetime), instead of smoking group, in the model did reduce the magnitude of association between serum copper and PAOD but copper still shows a statistically significant association with PAOD. We also concluded that smoking and high systolic blood pressure were strongly associated with PAOD.

11. SUGGESTION FOR FUTURE RESEARCH

Although, this study provided further epidemiological evidence of an association of high serum copper and PAOD, larger prospective studies are needed to confirm this association. We mentioned earlier that copper is an oxidant and that high level of serum copper might promote oxidative modification of LDL and consequently initiate or cause progression of atherosclerosis. Use of antioxidants such as vitamin E and C in the diet[47,48], or other dietary intervention[49] such as diet rich in oleic acid versus linoleic acid may prevent or slow the progression of atherosclerosis by generating LDL that is highly resistant to copper-induced oxidative modification.

Recently the results of two well conducted non-randomized follow up studies[50,51] have reported that the use of vitamine E was associated with a reduced risk of coronary heart disease in both men and women.

A large randomized clinical trial would be required to confirm the role of natural or synthetic antioxidants in protecting LDL against oxidation in human. However, randomized clinical trials should be conducted only when there is sufficient evidence on the role of copper and other oxidants in oxidative modification of LDL and on the atherogenic role of oxidized LDL in human.

The findings of these future investigations will be useful in designing intervention strategies to slow the progression of atherosclerosis in patients with PAOD. This will result in a better quality of life and a reduction of the health care cost due to PAOD and other atherosclerotic disease in elderly people.

TABLES AND FIGURES

TABLE 1
Code Sheet for Variables for Study of Serum Copper and PAOD

Variables Name	Variables labels and values
PAOD	Peripheral Arterial Occlusive Disease (1=control, 2=case)
COPPER	Serum Copper (μmol/l)
AGE	Age of the Study Subjects (year)
SES	Socioeconomic Status (1=high, 2=medium, 3=low)
SESSCORE	Socioeconomic score
QUETELET	Quetelet Index [Weight in Kg/Height in m^2]
QUETGRUP	Quetelet Index Category (1=Quetelet<25.1, 2=Quetelet 25.1-29.9, 3=Quetelet>29.9)
SBP	Systolic Blood Pressure (mm/Hg)
SMKGRUP	Smoking Status (1=non-smoker, 2=ex-smoker, 3=current-smoker)
SMOKLIFE	Number of Cigarettes Smoked in Lifetime (1=0, 2=1-99999, 3=100000-199999, 4=200000-299999, 5=300000-399999, 6 is $\geq$ 400000
CAFEINE	Caffeine Consumption (mg/day)
DRKGRUP	Drinking Status Categories
VC	Vital Capacity (l)
FEV1	Forced Expiratory Volume in One Second (l)
FEV1/VC	Ratio of VC to FEV1
ACTYGRUP	Physical Activity Group (1=active, 2=occasionally active, 3=infrequently active, 4=inactive)
ACTYSCOR	Physical Activity Score
HISOFHBP	History of High Blood Pressure
HISOFHRT	History of Heart Disease
LDL	Low Density Lipoprotein (mmol/l)
HDL	High Density Lipoprotein (mmol/l)
CHOLSTRL	Serum Cholesterol (mmol/l)
TRGLYCRD	Serum Triglyceride (mmol/l)

TABLE 1.....continued Code Sheet for Variables for Study of Serum Copper and PAOD	
Variables Name	Variables labels and values
ZINC	Serum Zinc (μmol/l)
GLUCOSE	Serum Glucose (mmol/l)

μmol/l = Micromole per litre

Kg = Kilogram

m = Metre

mm/Hg = Millimetre of mercury

l = litre

mmol/l = Millimole per litre

mg/day = Milligram per day

Table 2
Distribution of study variables among cases and controls

Variables	Cases			Controls			Significance level
	Count	Mean	SD	Count	Mean	SD	
COPPER	97	17.24	3.64	95	15.19	3.24	0.000
AGE	97	65.22	9.81	95	62.11	8.75	0.022
SESSCOR	96	45.34	14.77	95	51.33	15.77	0.007
QUETELET	97	26.27	3.48	95	26.97	3.32	0.152
SBP	94	151.89	24.92	95	133.81	19.53	0.000
GLUCOSE	96	6.21	2.16	95	5.94	1.41	0.291
CHOLSTRL	97	5.91	1.28	95	5.71	1.13	0.254
TRGLYCRD	97	2.34	1.30	95	2.01	0.96	0.048
HDL	97	1.00	0.26	95	1.12	0.25	0.001
LDL	97	3.83	1.18	95	3.66	1.03	0.280
ZN	97	10.09	1.58	95	9.89	1.54	0.371
CAFEINE	97	380	368	95	277	241	0.023
SMOKLIFE	95	390415	253318	95	209786	230051	0.000
VC	97	3.23	0.70	94	3.95	0.91	0.000
FEV1	97	2.32	0.62	94	2.98	0.78	0.000
FEV1/VC	97	0.71	0.117	94	0.75	0.09	0.011

TABLE 3

Univariate risk estimates of serum copper among males with PAOD and controls

Serum Copper (μmol/l)	Cases N(%)	Controls N(%)	Odds Ratio
< 13.1	13 (13.4)	27 (28.4)	1
13.1-14.9	13 (13.4)	25 (26.3)	1.08
15.0-16.9	22 (22.7)	17 (17.9)	2.69
17.0-18.7	21 (21.6)	17 (17.9)	2.57
> 18.7	28 (28.9)	9 (9.5)	6.46

Overall chi-square: 19.5 $p < 0.001$
Chi-square for trend: 17.5 $p < 0.0001$

TABLE 4

Univariate association of potential categorical confounding variables among males with PAOD and controls

Characteristics	Cases N(%)	Controls N(%)	OR	Overall X^2 (p-value)	X^2 for trend (p-value)
AGE				8.18 (<0.05)	5.00 (<0.05)
<50	9(9.3)	10(10.5)	1.00		
50-59	15(15.5)	30(31.6)	0.56		
60-69	40(41.2)	34(35.8)	1.31		
70+	33(34.0)	21(22.1)	1.75		
SES				7.67 (<0.05)	6.87 (<0.05)
High	23(23.7)	40(42.1)	1.00		
Medium	34(35.1)	28(29.5)	2.11		
Low	40(41.2)	27(28.4)	2.58		
QUETELET				3.67 (>0.10)	3.40 (>0.05)
<25	41(42.3)	28(29.5)	1.00		
25-29.9	40(41.2)	45(47.4)	0.61		
> 29.9	16(16.5)	22(23.1)	0.50		
HISOFHBP				7.45 (<0.05)	
No	52(53.6)	69(72.6)	1.00		
Yes	45(46.4)	26(27.4)	2.30		
HISOFHRT				11.0 (<0.001)	
No	56(57.7)	76(80.0)	1.00		
Yes	41(42.3)	19(20.0)	2.93		
SMKGRUP				18.4 (<.001)	17.6 (<0.0001)
Non smokers	3(3.1)	18(18.9)	1.00		
Ex-smokers	53(55.2)	58(61.1)	5.48		
Current-Smokers	40(41.7)	19(20.0)	12.63		
SMOKLIFE				30.1 (<.0001)	28.4 (<0.0001)
<1	3(3.1)	18(18.9)	1.00		
1-99999	9(9.3)	23(24.2)	2.34		
100000-199999	11(11.3)	16(16.8)	4.12		
200000-299999	16(16.5)	10(10.5)	9.60		
300000-399999	16(16.5)	10(10.5)	9.60		
>399999	42(43.3)	18(18.9)	14.0		

TABLE 4.....continued

Univariate association of potential categorical confounding variables among males with PAOD and controls

Characteristic	Cases N(%)	Controls N(%)	OR	Overall X^2 (p-value)	X^2 for trend (p-value)
DRKGRUP				7.72 (>0.05)	2.35 (>0.05)
Never drank	2(2.1)	4(4.2)	1.00		
former drinker	11(11.6)	3(3.2)	7.33		
occasional drinker	15(15.8)	9(9.4)	3.33		
current drinker	67(70.5)	79(83.2)	1.70		
CAFEINE				3.61 (>0.05)	
<500	72(74.2)	81(85.3)	1.00		
>500	25(25.8)	14(14.7)	2.01		
ACTYGRUP				11.3 (<0.05)	9.50 (<0.005)
Very active	15(15.7)	33(34.7)	1.00		
Active	23(23.7)	24(25.3)	2.11		
Inactive	30(30.9)	19(20.0)	3.47		
Sedentary	29(29.9)	19(20.0)	3.36		

TABLE 5
Association of PAOD and potential interval confounding variables among males with PAOD and controls

Variables	Mean (Cases)	Mean (Controls)	t-value	p-value
AGE	65.23	62.11	-2.32	*
QUETELET	26.26	26.97	1.44	NS
SBP	151.89	133.81	-5.55	***
SMOKLIFE	398811	258827	-3.81	***
VC	3.23	3.95	6.10	***
FEV1	2.32	2.98	6.55	***
FEV1/VC	0.75	0.71	-2.58	*
GLUCOSE	6.21	5.94	-1.06	NS
CHOLSTRL	5.91	5.71	-1.14	NS
LDL	3.83	3.66	-1.08	NS
TRGLYCRD	2.34	2.01	-1.99	*
HDL	1.00	1.12	3.35	**
ZINC	10.09	9.84	-0.90	NS
ZN/CU	0.616	0.681	2.41	*
SES	45.34	51.33	2.71	**
ACTYSCOR	5235	6576	3.24	**
CAFEINE	381	277	-2.30	*

NS = $p > 0.05$
* = $p < 0.05$
** = $0.001 < p < 0.01$
*** = $p < 0.001$

TABLE 6

Analyses of variance for association of serum copper and potential categorical confounding variables among study subjects

Variables	Count	Mean	SD	Significance level "F"	Significance level Welch's
AGE				>0.10	
<60	64	15.7	3.2		
60-70	74	16.7	3.9		
>70	54	16.2	3.5		
SES					>0.10
High	63	15.8	2.9		
Medium	62	16.2	3.1		
Low	67	16.6	4.5		
QUETELET				<0.05	
<25	69	17.2	3.7		
25-29.9	85	15.7	3.5		
>29.9	38	15.5	3.2		
SMKGRUP				<0.001	
Non-smokers	21	14.8	2.3		
Ex-smokers	111	15.7	3.4		
Current-smokers	59	17.6	3.9		
SMOKLIFE				<0.05	
<1	21	14.8	2.3		
1-99999	32	16.2	3.7		
100000-199999	27	17.6	3.6		
200000-299999	26	16.3	3.2		
300000-399999	26	14.9	3.6		
≥400000	58	16.6	3.9		
CAFEINE				<0.01	
<500	153	15.8	3.3		
>500	39	17.7	4.2		

TABLE 6.....continued

Analyses of variance for association of serum copper and potential categorical confounding variables mong cases and controls

Variables	Count	Mean	SD	Significance level "F"	Significance level Welch's
ACTYGRUP					>0.10
Group 1	48	15.6	3.5		
Group 2	47	16.3	3.0		
Group 3	49	16.1	3.3		
Group 4	48	16.9	4.4		
SBP				<0.001	
<140	109	15.5	3.2		
>140	80	17.3	3.8		
HDL				>0.10	
<1.0	86	16.5	3.5		
>1.0	106	16.0	3.6		
FEV1/VC				<0.05	
<0.8	137	16.5	3.7		
>0.8	54	15.4	3.1		

TABLE 7

Multivariate Logistic Regression Model for the association of serum copper and PAOD (using variable SMKGRUP)

Variables	Coefficient	Standard Error coefficient	OR(95% CI)
AGE	0.0327	0.0212	1.03 (0.99, 1.08)
SES(1)	0.5525	0.4480	1.74 (0.72, 4.20)
SES(2)	0.5357	0.4570	1.71 (0.69, 4.21)
SBP	0.0345	0.0093	1.04 (1.02, 1.05)
SMKGRUP(1)	1.434	0.7060	4.20 (1.04, 16.9)
SMKGRUP(2)	2.092	0.7750	8.10 (1.76, 37.4)
COPPER*	0.0683	0.0560	1.07 (0.96, 1.20)
CAFEINE	0.0010	0.0006	1.00 (1.00, 1.00)
FEV1/VC	-0.5189	1.5000	0.60 (0.03, 11.5)
QUETGRUP(1)	-0.5616	0.4020	0.57 (0.26, 1.26)
QUETGRUP(2)	-0.7038	0.4940	0.49 (0.19, 1.31)
CONSTANT	-9.597	2.2600	

Log-likelihood = -98.54

* Odds ratio for 5 unit increase of copper = 1.40 (95% CI: 0.81, 2.43)

SMKGRUP(1) = Ex-smokers
SMKGRUP(2) = Current-smokers
Reference = Non-smokers

SES(1) = Medium SES
SES(2) = Low SES
Reference = High SES

QUETELET(1) = Quetelet index = 25.1-29.9
QUETELET(2) = Quetelet index > 29.9
Reference = Quetelet index < 25.1

TABLE 8

Association of serum copper and PAOD, stratified by study covariates

Covariates	Copper μmol/l	Cases (N)	Controls (N)	Odds ratios		
				Stratum (95% CI)	Crude	MH
AGE						
<60	>16.2	12	14	1.86		
	<16.2	12	26	(0.59, 5.93)		
60-70	>16.2	26	12	3.40		
	<16.2	14	22	(1.18, 10.0)		
>70	>16.2	20	6	3.85	2.93	2.86
	<16.2	13	15	(1.03, 14.91)		
SES						
High	>16.2	12	15	1.82		
	<16.2	11	25	(0.64, 5.14)		
Medium	>16.2	23	7	6.27		
	<16.2	11	21	(2.05, 19.17)		
Low	>16.2	23	10	2.30	2.93	2.87
	<16.2	17	17	(0.84, 6.26)		
QUETGRUP						
<25.1	>16.2	29	11	3.73		
	<16.2	12	17	(1.21, 11.77)		
25.1-29.9	>16.2	22	14	2.71		
	<16.2	18	31	(1.02, 7.27)		
>29.9	>16.2	7	7	1.67	2.93	2.74
	<16.2	9	15	(0.36, 7.84)		

TABLE 8.....continued

Association of serum copper and PAOD, stratified by study covariates

Covariates	Copper μmol/l	Cases (N)	Controls (N)	Odds ratios		
				Stratum (95% CI)	Crude	MH
SMKGRUP						
Non-smokers	>16.2	2	4	7		
	<16.2	1	14	(0.50,98.60)		
Ex-smokers	>16.2	28	18	2.49		
	<16.2	25	40	(1.15, 5.40)		
Current-smokers	>16.2	27	10	1.87	2.88	2.41
	<16.2	13	9	(0.61, 5.71)		
SBP						
<140	>16.2	21	22	3.25		
	<16.2	15	51	(1.31, 8.12)		
>140	>16.2	36	10	1.96	3.03	2.64
	<16.2	22	12	(0.65, 5.96)		
CAFEIN						
<500	>16.2	42	24	3.33		
	<16.2	30	57	(1.62, 6.88)		
>500	>16.2	16	8	1.33	2.93	2.76
	<16.2	9	6	(0.29, 6.23)		
FEV1/VC						
>0.8	>16.2	11	12	1.67		
	<16.2	11	20	(1.75, 8.30)		
<0.8	>16.2	47	19	3.80	3.02	2.98
	<16.2	28	43	(0.48, 5.81)		

TABLE 9

The contribution of possible interaction terms in the multivariate logistic regression model (association of serum copper and PAOD)

Interaction	Log-likelihood	Improvement chi-square	df	p-value
Main effect only*	-98.54			
COPPER*AGE	-97.10	2.88	1	>0.05
COPPER*SES	-95.53	6.02	2	<0.05
COPPER*SBP	-97.90	1.28	1	>0.20
COPPER*QUETGRUP	-98.32	0.44	2	>0.50
COPPER*SMKGRUP	-95.54	6.00	2	<0.05
COPPER*CAFEINE	-97.79	1.50	1	>0.20
COPPER*FEV1/VC	-98.53	0.02	1	>0.50
COPPER*SMKGRUP+COPPER*SES	-93.26	10.56	4	<0.05

* Main effect model was consisted of PAOD as dependent variable with COPPER, AGE, SES, SMKGRUP, SBP, CAFEINE, FEV1/VC, and QUETGRUP as INDEPENDENT variables

TABLE 10

Association of serum copper and PAOD, multivariate logistic regression model controlling for smoking as effect modifier

Variables	Coefficient	Standard Error coefficient	OR(95% CI)
AGE	0.0383	0.0220	1.04 (0.99, 1.09)
SES(1)	0.7033	0.4630	2.02 (0.81, 5.04)
SES(2)	0.6529	0.4730	1.92 (0.75, 4.89)
SBP	0.0360	0.0095	1.04 (1.02, 1.06)
SMKGRUP(1)	2.4730	4.9300	
SMKGRUP(2)	7.5760	5.0000	
COPPER	0.2432	0.2980	1.28 (0.71, 2.29)
CAFEINE	0.0011	0.0006	1.00 (1.00, 1.00)
FEV1/VC	-0.9321	1.5100	0.39 (0.02, 7.69)
QUETGRUP(1)	-0.6124	0.4120	0.54 (0.24, 1.22)
QUETGRUP(2)	-0.6054	0.5000	0.55 (0.20, 1.46)
COPPER*SMKGRUP(1)	-0.0695	0.3070	0.93 (0.51, 1.71)
COPPER*SMKGRUP(2)	-0.3388	0.3090	0.71 (0.39, 1.31)
CONSTANT	-12.670	5.2200	

Log-likelihood = -95.54

Note: The odds ratios for smoking are not reported since they are not valid in presence of interactin The odds ratio for copper is valid only for non-smokers

TABLE 11

Estimated odds ratios and 95% CI for the association of serum copper and PAOD by smoking status

Unit Increase of serum copper	Smoking status		
	Non-smokers	Ex-smokers	Current-smokers
	OR (95% CI)	OR (95% CI)	OR (95% CI)
1	1.28 (0.71, 2.29)	1.19 (1.02, 1.39)	0.91 (0.89, 1.07)
5	3.37 (0.18, 62.6)	2.38 (1.10, 5.15)	0.62 (0.36, 1.43)
10	11.4 (0.03, 3912)	5.68 (1.21, 26.6)	0.38 (0.07, 2.05)

TABLE 12

Multivariate logistic regression model for the association of serum copper and PAOD, controlling for SES as effect modifier

Variables	Coefficient	Standard Error coefficient	OR(95% CI)
AGE	0.0318	0.0217	1.03 (0.99, 1.08)
SES(1)	-3.6230	2.7800	
SES(2)	1.7250	2.1000	
SBP	0.0357	0.0095	1.04 (1.02, 1.06)
SMKGRUP(1)	1.5150	0.7140	4.55 (1.11, 18.6)
SMKGRUP(2)	2.0510	0.7740	7.78 (1.69, 35.8)
COPPER	0.0561	0.1100	1.06 (0.85, 1.31)
CAFEINE	0.0010	0.0006	1.00 (1.00, 1.00)
FEV1/VC	-0.6029	1.5100	0.54 (0.03, 10.7)
QUETGRUP(1)	-0.5895	0.4090	0.55 (0.25, 1.24)
QUETGRUP(2)	-0.6267	0.5070	0.53 (0.20, 1.45)
COPPER*SES(1)	0.2630	0.1710	1.30 (0.93, 1.82)
COPPER*SES(2)	-0.0730	0.1270	0.93 (0.72, 1.19)
CONSTANT	-9.4680	2.7600	

Log-likelihood = -95.53

Note: The odds ratios for SES is not reported since they are not valid in presence of interaction
The odds ratios for copper is valid only for high SES group

TABLE 13

Estimated odds ratios and 95% CI for the association of serum copper and PAOD by SES

Unit increase of serum copper	SES status		
	High SES	Medium SES	Low SES
	OR (95% CI)	OR (95% CI)	OR (95% CI)
1	1.06 (0.85, 1.31)	1.38 (1.06, 1.78)	0.98 (0.86, 1.12)
5	1.32 (0.45, 3.89)	4.93 (1.36, 17.8)	0.92 (0.47, 1.79)
10	1.75 (0.02, 15.1)	24.3 (1.86, 316.)	0.84 (0.22, 3.21)

TABLE 14

Multivariate logistic regression model for the accociation of copper and PAOD, controlling for smoking group and SES as effect modifiers

Variables	Coefficient	Standard Error coefficient	OR(95% CI)
AGE	0.0372	0.0223	1.04 (0.99, 1.08)
SES(1)	-3.578	2.8400	
SES(2)	1.107	2.2200	
SBP	0.0370	0.0097	1.04 (1.02, 1.06)
SMKGRUP(1)	1.1300	4.7300	
SMKGRUP(2)	5.6800	4.8600	
COPPER	0.1049	0.2990	1.11 (0.61, 2.00)
CAFEINE	0.0011	0.0006	1.00 (1.00, 1.00)
FEV1/VC	-0.9513	1.5300	0.39 (0.02, 7.85)
QUETGRUP(1)	-0.6304	0.4180	0.53 (0.23, 1.22)
QUETGRUP(2)	-0.5310	0.5100	0.59 (0.21, 1.61)
COPPER*SMKGRUP(1)	0.0220	0.2950	1.02 (0.57, 1.83)
COPPER*SMKGRUP(2)	-0.2200	0.3010	0.80 (0.44, 1.45)
COPPER*SES(1)	0.2663	0.175	1.31 (0.92, 1.84)
COPPER*SES(2	-0.0279	0.136	0.97 (0.74, 1.27)
CONSTANT	-10.560	5.2100	

Log-likelihood = -93.26

Note: The odds ratios for smoking group and SES are not reported since they are not valid in the presence of interaction
The odds ratio for copper is valid only for non-smokers/high SES subgroup

TABLE 15

Multivariate logistic regression model for the association of serum copper and PAOD (using variable SMOKLIFE)

Variables	Coefficient	Standard Error coefficient	OR(95% CI)
AGE	0.0216	0.0224	1.02 (0.98, 1.07)
SES(1)	0.6566	0.4740	1.93 (0.76, 4.91)
SES(2)	0.4358	0.4820	1.55 (0.60, 4.00)
SBP	0.0354	0.0101	1.04 (1.02, 1.06)
SMOKLIFE(1)	0.4507	0.8200	1.57 (0.31, 7.93)
SMOKLIFE(2)	0.9353	0.8490	2.55 (0.48, 13.6)
SMOKLIFE(3)	2.2170	0.8530	9.18 (1.71, 49.4)
SMOKLIFE(4)	2.4500	0.8310	11.6 (2.25, 59.8)
SMOKLIFE(5)	1.9410	0.7760	6.97 (1.51, 32.2)
COPPER*	0.1182	0.0591	1.80 (1.01, 3.22)
CAFEINE	0.0010	0.0006	1.00 (1.00, 1.00)
FEV1/VC	-0.6429	1.6400	0.53 (0.02, 13.3)
QUETGRUP(1)	-0.6932	0.4330	0.50 (0.21, 1.18)
QUETGRUP(2)	-1.1410	0.5150	0.32 (0.12, 0.88)
CONSTANT	-9.542	2.4400	

Log-likelihood = -92.03

* OR for copper is calculated for five unit change of copper

TABLE 16

The summary of stepwise logistic regression analysis for the association of serum copper and PAOD in males (using variable SMKGRUP)

Step #	df	Log-likelihood	Improvement chi-square(p-value)	Goodness of fit chi-square(p-value)
0)		-130.3		260.6 (0.000)
1) SBP	1	-115.6	29.4 (0.000)	231.2 (0.014)
2) SMKGRUP	2	-105.6	19.9 (0.000)	211.3 (0.082)
3) COPPER	1	-103.8	3.63 (0.057)	207.7 (0.102)
4) CAFEINE	1	-102.3	3.04 (0.081)	204.6 (0.120)

TABLE 17

The results of stepwise logistic regression analysis for the association of serum copper and PAOD (using variable SMKGRUP)

Variables	Coefficient	SE of coeff.	OR (95% CI)
SBP	0.038	0.860E-02	1.04 (1.02, 1.06)
SMKGRUP(1)*	1.675	0.697	5.34 (1.35, 21.1)
SMKGRUP(2)*	2.299	0.747	9.96 (2.28, 43.5)
COPPER	0.091	0.053	1.10 (0.99, 1.22)
CAFEINE	0.99E-03	0.59E-03	1.00 (1.00, 1.00)
CONSTANT	-8.992		

Log-likelihood = -102.32

* SMKGRUP(1) is the comparison of ex-smokers to non-smokers

* SMKGRUP(2) is the comparison of current smokers to non-smokers

TABLE 18

The summary of stepwise logistic regression analysis for the association of serum copper and PAOD in males (using variable SMOKLIFE)

Step #	df	Log-likelihood	Improvement chi-square(p-value)	Goodness of fit chi-square(p-value)
0)		-129.6		259.2 (0.000)
1) SBP	1	-114.8	29.6 (0.000)	229.6 (0.014)
2) SMOKLIFE	2	-101.5	26.5 (0.000)	203.1 (0.115)
3) COPPER	1	-97.48	8.11 (0.004)	194.9 (0.196)
4) QUETGRUP	2	-94.66	5.64 (0.060)	189.3 (0.250)

TABLE 19

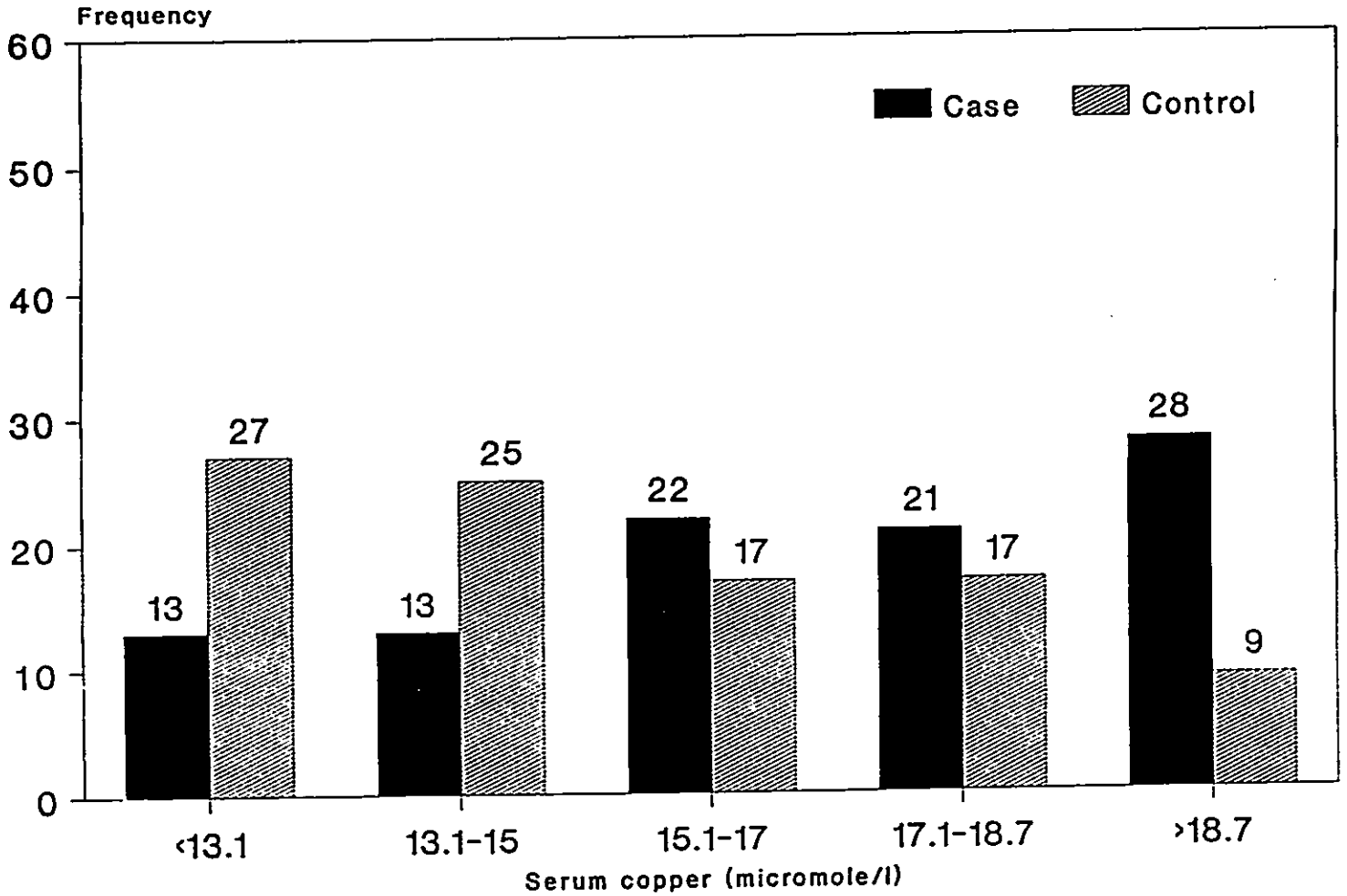
The results of stepwise logistic regression analysis for the association of of serum copper and PAOD (using variable SMOKLIFE)

Variables	Coefficient	SE of coeff.	OR (95% CI)
SBP	0.035	0.009	1.04 (1.02, 1.05)
SMOKLIFE(1)	0.696	0.802	2.01 (0.41, 9.77)
SMOKLIFE(2)	1.234	0.821	3.43 (0.68, 17.3)
SMOKLIFE(3)	2.633	0.831	13.9 (2.70, 71.8)
SMOKLIFE(4)	2.617	0.820	13.7 (2.71, 69.1)
SMOKLIFE(5)	2.473	0.742	11.9 (2.75, 51.3)
COPPER	0.132	0.057	1.14 (1.02 1.28)
QUETELET(1)	-0.703	0.422	0.49 (0.21, 1.14)
QUETELET(2)	-1.122	0.507	0.33 (0.12, 0.89)
CONSTANT	-8.479		

Log-likelihood = -94.66

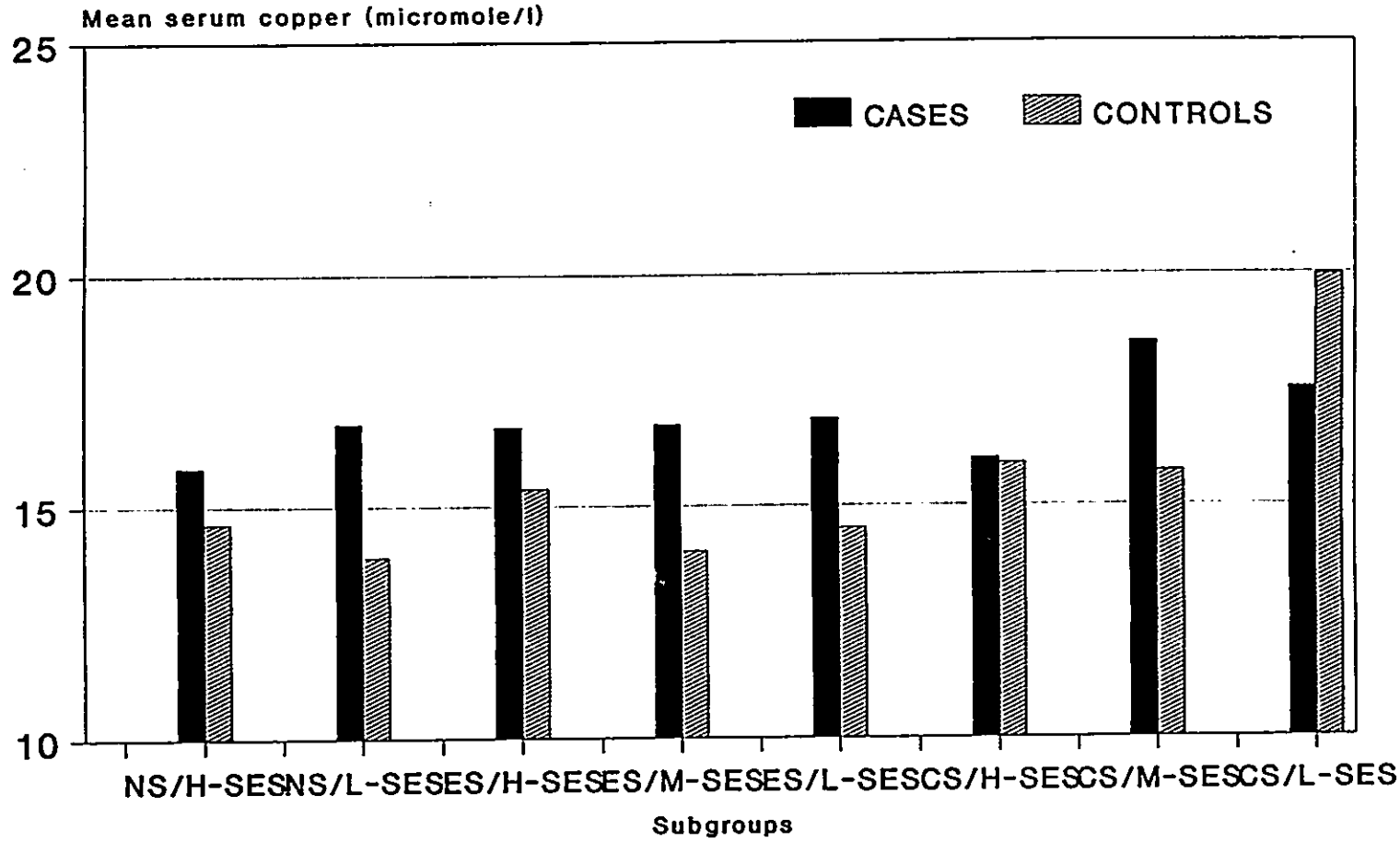
The odds ratio for 5 unit increase of copper = 1.93 (95% CI:1.10,3.38)
The odds ratio for 10 unit increase of copper = 3.73 (95% CI:1.22,11.42)

Fig.1: Distribution of different levels of serum copper among cases and controls



Overall Chi-Square = 19.5 $p<0.001$
Chi-Square for trend = 17.5 $p<0.00005$

Fig 2: Distribution of serum copper among cases and controls in different subgroups of smoking status and SES



NS = Non-smokers H-SES = High SES
ES = Ex-smokers M-SES = Medium SES
CS = Current-smokers L-SES = Low SES

Fig. 3: Frequency distributions of cases and controls for different levels of serum copper

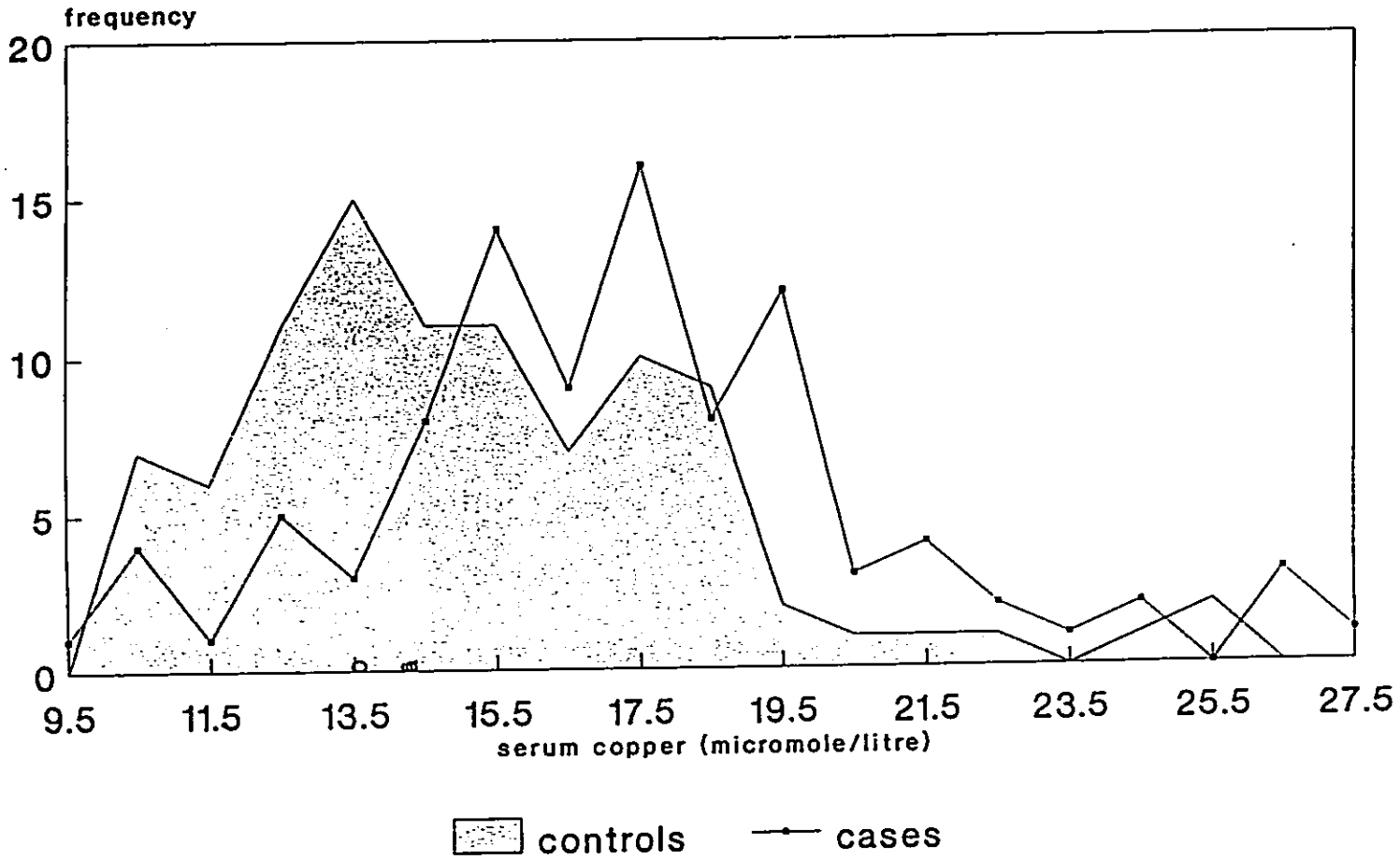
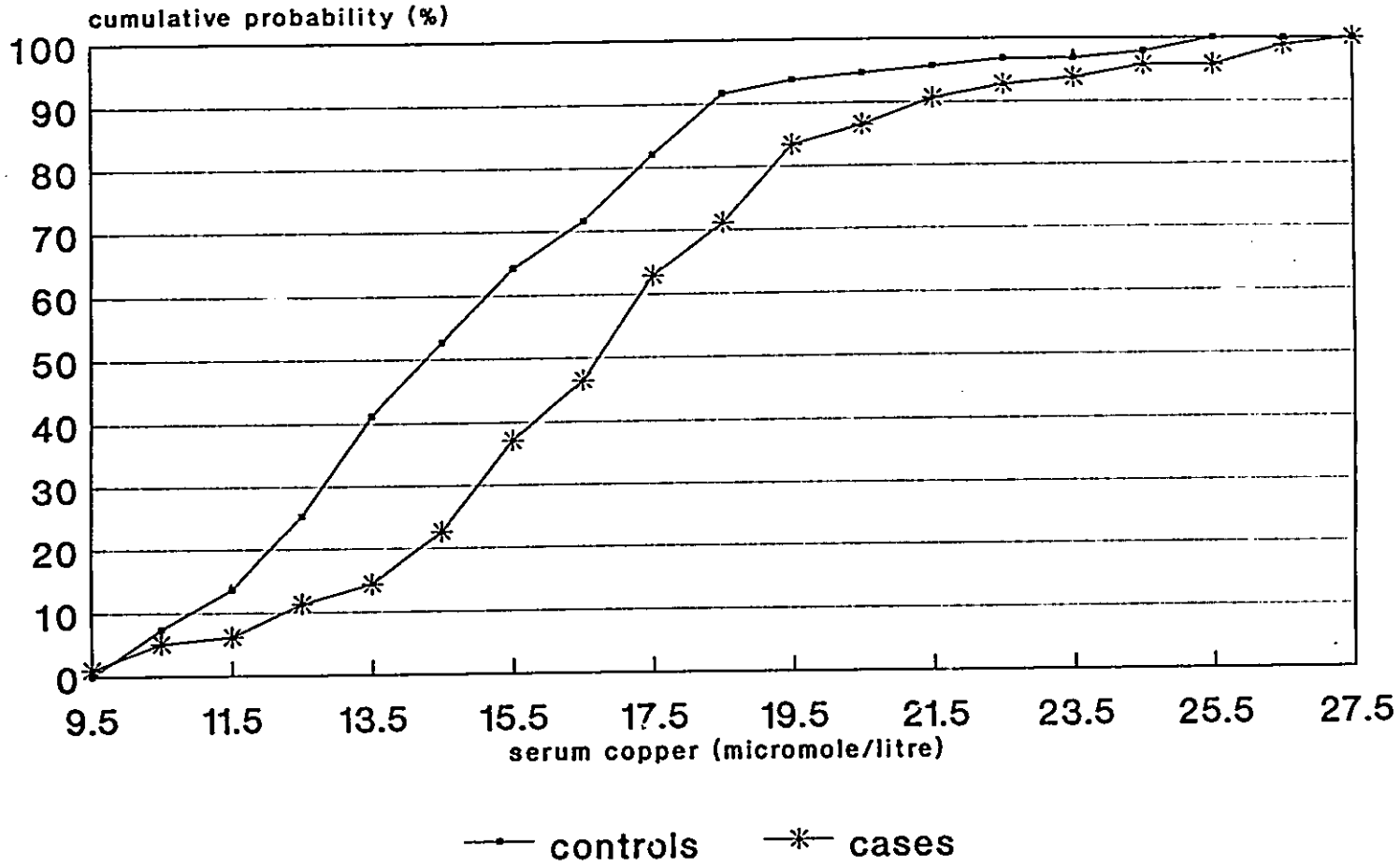


Fig 4: Cumulative probability distribution of cases and controls for different levels of serum copper



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APPENDIX A

QUESTIONNAIRE FOR THE STUDY OF SERUM COPPER AND PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

SOURCES:

CANADA HEALTH SURVEY

GENERAL SOCIAL SURVEY

HEALTH PROMOTION SURVEY

SMOKING BEHAVIOUR OF CANADIAN

1 I would like to ask you some questions about your background, medical history , family history , and life style. This interview is completely confidential and your name will not appear on any reports related to the study. If there are any questions that you would rather not answer we will move onto the next one .

First I would like to ask you some background information.

1- how many years of elementary or secondary education have you completed?

No schooling	____	
One	____	
Two	____	
Three	____	
Four	____	
Five	____	GO TO 3
Six	____	
Seven	____	
Eight	____	
Nine	____	
Ten	____	
Eleven	____	
Twelve	____	
Thirteen	____	

2- Have you graduated from secondary school?

No	____	
Yes	____	

3- Have you had any further schooling beyond elementary/secondary school?

No	____	GO TO 5
Yes	____	

4- What is the highest level? (accept multiple response)

Some community college , CEGEP , or nursing school

_____

Diploma or certificate from community college , CEGEP , or nursing school

_____

Some university or some teacher's college

_____

Bachelor or undergraduate degree from university or teacher's college

_____

Master or doctorate

_____

Other (please specify)

_____

5- Where were you born?

Newfoundland	_____	
Prince Edward Island	_____	
Nova Scotia	_____	
New Brunswick	_____	
Quebec	_____	
Ontario	_____	GO TO 7
Manitoba	_____	
Saskatchewan	_____	
Alberta	_____	
British columbia	_____	
Yukon	_____	
North West Territories	_____	

Country outside Canada (please specify)

6- In what year did you immigrate to Canada?

19___

7- To which ethnic or cultural group do you or did your ancestors belong? (accept multiple response)

French	____	Polish	____
English	____	Jewish	____
Irish	____	Chinese	____
Scottish	____	Japanese	____
German	____	Native Indians	____
Italian	____	and Inuits	____
Ukrainian	____	Other (please	
Dutch	____	specify)	_____

8- What is your current employment status?

Full-time (35 hours or more)	____	
Part-time (less than 35 hours)	____	GO TO 10
Unemployed	____	
Laid-off	____	GO TO 9
Retired	____	
Student full-time	____	
Homemaker	____	GO TO 12
Other (please specify)	_____	

9- Did you have a job at anytime in the past?

No ____ GO TO 12

Yes ____

10- What kind of business, industry, or service is/was this? (give full description: e.g. paper-box manufacturing, retail shoe store, municipal board of education)

11- What kind of work do/did you do?

12- What is your current marital status?

Never married ____

Divorced ____

Married/common law ____

Widowed/widower ____

Separated ____

****

The next few questions are about your medical history

1- Have you ever had high blood pressure?

NO ____ **GO TO 6**

Yes ____

2- What was the age of onset of high blood pressure?

____

3- Are you currently taking medication for high blood pressure?

No ____ **GO TO 6**

Yes ____

4- What is the medication you are currently taking for high blood pressure?

_____

_____

_____

5- How long have you been taking medication for high blood pressure?

_____ years

6- Have you ever had diabetes?

No _____ GO TO 11

Yes _____

7- What was the age of onset of diabetes?

_____

8- Are you currently under treatment for diabetes (including diet)?

No _____ GO TO 11

Yes _____

9- How long have you been under treatment for diabetes?

_____ years

10- What kind of medication are you taking?

Insulin _____

Oral medication _____

Diet only _____

11- Have you ever had heart disease such as angina, arrythmia, or myocardial infarction (heart attack)?

No ____ GO TO 16

Yes ____ PLEASE SPECIFY _____

12- What was the age of onset of heart disease?

____

13- Are you currently taking medication for heart disease?

No ____ GO TO 16

Yes ____

14- What kind of medication are you taking for heart disease?

_____

_____

_____

15- How long have you been taking this treatment?

____ years

16- Have you ever had an heart operation?

No ____

Yes ____ PLEASE SPECIFY _____

17- Have you ever had emphysema, chronic bronchitis, or asthma?

No ____ GO TO 22

Yes ____ PLEASE SPECIFY _____

18- What was the age of onset?

____

19- Are you currently taking medication for this(these) conditions?

No _____ GO TO 22

yes _____

20- How long have you been taking medication for this(these) problem(s)?

_____ years

21- What kind of medication(s) are you taking for this(these) condition(s)?

_____

_____

22- Are you currently taking aspirin or ASA?

No _____ GO TO 25

Yes _____

23- How often do you take this medicine?

Every day or less _____

Once a week or less _____

Once a month or less _____

24- For how long have you been taking this medicine?

_____ years

25- Have you ever had an operation?

No _____ GO TO 27

Yes _____ PLEASE SPECIFY _____

26- When did you have the operation?

19____

27- Have you ever had any other serious health problem? (please specify)

_____

****

Now I would like to ask you few questions about your family.

1- How many brothers or sisters do you have? (please include any who have died)

_____ brothers

_____ sisters

2- How many children do you have? (please include any who have died)

_____ children

3- Has any of your immediate family (that is your parents, children , sisters or brothers) ever had operation or medical treatment because of bad circulation in the legs(excluding varicose veins)?

No ____ **GO TO 5**

Yes ____

4- What is his/her relationship to you?

_____

5-Has any of your immediate family ever lost a leg because of a circulation problem in the legs?

No ____ **GO TO NEXT SECTION**

Yes ____

6- What is his/her relationship to you?

The next few questions concern your physical activity .

1- Thinking back when you were 35 years old did you participate in any of the following exercises, sports, or recreational activities? How frequently? **(show respondent table 1 and read the following definitions of frequency)**

Often is equal to twice a week and more.

Sometimes is equal to twice a month and more.

Occasionally is equal to once a month .

Never is equal to less than once a month.

PHYSICAL ACTIVITY	FREQUENCY				AVERAGE DURATION IN MINUTES			
	OFTEN	SOMETIMES	OCCASIONALLY	NEVER	1-15	16-30	31-60	> 60
WALKING A MILE	___	___	___	___	___	___	___	___
JOGGING/RUNNING	___	___	___	___	___	___	___	___
CALISTHENICS	___	___	___	___	___	___	___	___
BICYCLING	___	___	___	___	___	___	___	___
BOWLING	___	___	___	___	___	___	___	___
VIGOROUS DANCING	___	___	___	___	___	___	___	___
SKATING	___	___	___	___	___	___	___	___
CURLING	___	___	___	___	___	___	___	___
SKIING (DOWN HILL, CROSSCOUNTRY)	___	___	___	___	___	___	___	___
RACQUET SPORTS (SQUASH, RACQUETBALL, TENNIS, BADMINTON)	___	___	___	___	___	___	___	___
BASEBALL/SOFTBALL	___	___	___	___	___	___	___	___
OTHER TEAM SPORTS (HOCKEY, BASKETBALL FOOTBALL, SOCCER, VOLLEYBALL)	___	___	___	___	___	___	___	___
GOLF	___	___	___	___	___	___	___	___
SWIMMING	___	___	___	___	___	___	___	___
OTHER (PLEASE SPECIFY)								
_____	___	___	___	___	___	___	___	___

2- Thinking back when you were 35 years old did you do the following tasks around your home ? How frequently ? (show respondent table 2 and read the following definitions)

Often is equal to twice a week and more.

Sometimes is equal to twice a month and more.

Occasionally is equal to once a month .

Never is equal to less than once a month.

	FREQUENCY				AVERAGE DURATION IN MINUTES			
PHYSICAL ACTIVITY	OFTEN	SOMETIMES	OCCASIONALLY	NEVER	1-15	16-30	31-60	> 60
MOWING THE GRASS	___	___	___	___	___	___	___	___
SHOVELLING SNOW	___	___	___	___	___	___	___	___
CLEANING FLOORS	___	___	___	___	___	___	___	___
RAKING LEAVES	___	___	___	___	___	___	___	___
GARDENING	___	___	___	___	___	___	___	___
CARPENTRY	___	___	___	___	___	___	___	___
HANDYMAN WORK, PAINTING	___	___	___	___	___	___	___	___
IRONING	___	___	___	___	___	___	___	___
MAKING BEDS	___	___	___	___	___	___	___	___
OTHER (PLEASE SPECIFY)								
_____	___	___	___	___	___	___	___	___

3- Would you say when you were 35 years old, you were physically more active, about the same, or less active than other persons your age?

More active ____ About the same ____

Less active ____ Don't know ____

4- Which of the following choices best describes the work or other activity you usually did?

I was usually sitting during the day and did not walk about very much .

____

I stood or walked about quite a lot during my day ,but I did not have to carry or lift things very often .

____

I usually lifted or carried light loads, or I had to climb stairs or hills often .

____

I did heavy work or carried very heavy loads .

____

****

Now I would like to ask you a few questions about smoking.

1- In any time in your life, have you ever smoked cigarettes, cigars or a pipe?

No ____ **GO TO 21**

Yes ____

2- At the present time, do you smoke a pipe?

No ____ **GO TO 4**

Yes ____

3- At the present time, do you smoke a pipe regularly (usually every day) or occasionally (not everyday)?

Regularly ____ Occasionally ____

4- At the present time, do you smoke cigars?

No _____ GO TO 6

Yes _____

5- At the present time, do you smoke cigars regularly (usually every day) or occasionally (not every day)?

Regularly _____ Occasionally _____

6- At the present time, do you smoke cigarettes?

No _____ GO TO 12

Yes _____

7- At the present time, do you smoke cigarettes regularly (usually very day) or occasionally (not every day)?

Regularly _____ Occasionally_____

8- How many cigarettes do you usually smoke per day?

_____ cigarettes

9- At what age did you start smoking cigarettes daily?

At age _____

10- Do you usually inhale the smoke ?

No _____

Yes _____

Don't no _____

11- What kind of cigarettes do you usually smoke?

Filter _____

Non-filter _____

Roll my own cigarettes _____

GO TO 18

12- Have you ever smoked cigarettes regularly?

No _____ GO TO 18

Yes _____

13- About how many cigarettes did you usually smoke daily?

_____ cigarettes

14- At what age did you start smoking daily?

At age _____

15- At what age did you stop smoking daily?

At age _____

16- Did you usually inhale the smoke?

No _____

Yes _____

Don't know _____

17- What kind of cigarettes did you usually smoke?

Filter _____

Non- filter _____

Rolled my own cigarettes _____

18- Are you exposed to other people's tobacco smoke at home?

No _____ GO TO 20

Yes _____

19- How many people in your house,excluding yourself, smoke daily?

Enter the number of people _____

20- Are you exposed by someone else's smoking at work?

No _____

Yes occasionally _____

Yes daily or almost daily _____

21- At the present time do you chew tobacco?

No _____

Yes _____

22- At the present time,do you use snuff? (i.e. a kind of fine cut, moist, tobacco generally sniffed or held in the mouth)

No _____

Yes _____

****

The next few questions are about alcohol consumption .but before starting questions, I would like to show you the following table . It might help you answer some of the questions. **(show the respondent table 3 and read the following descriptions)**

One drink equals ...

- One pint bottle of beer (12 ounces)
- One small glass of wine (4-5 ounces)
- One shot of liquor or spirits (1-1.5 ounces) with or without mix.
- One small sherry, port, or vermouth (3 ounces)

A shot with a beer chaser or a double should be counted as two drinks

1- In the last twelve months, about how often have you taken at Least one drink of beer, wine, liquor or any other alcoholic beverage?

Two or more times a day	_____	
Once a day	_____	
4 to 6 times a week	_____	
2 or 3 times a week	_____	**GO TO 2**
About once a week	_____	
2 or 3 times a month	_____	
About once a month	_____	
Less often than once a month	_____	
Not at all in the last twelve months	_____	**GO TO 7**

2- Not counting small sips, at what age did you start drinking alcoholic beverages?

At age _____

3- Beginning with yesterday, how many drinks did you have on each of the last 7 days?

a) Yesterday

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

b) 2 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

c) 3 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

d) 4 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

e) 5 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

f) 6 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

g) 7 days ago

No drinks	____	4 to 7	____
1	____	8 to 11	____
2 or 3	____	12 or more	____

4- Would you say that this is more, less or about the same amount that you usually consume during a week?

More ____

Less ____

Same ____

5- Has your drinking changed over the last 12 months?

Drinking more now ____

Drinking less now ____

No change over last 12 months ____

6- What do you usually drink? (Choose one only)

Beer ____

Wine ____

Liquor or mix drinks ____

Other ____

Or it varies ____

GO TO NEXT SECTION

7- In the past, What experience with alcohol have you had?

Drink occasionally
(less than once a month) ____

Never drink ____ **GO TO NEXT SECTION**

Or used to drink ____ **GO TO 8**

8- a) At what age did you start?

At age ____

b) At what age did you have your last drink?

At age _____

9- About how often did you usually drink?

2 or more times a day _____

Once a day _____

4 to 6 times a week _____

2 or 3 times a week _____

About once a week _____

2 or 3 times a month _____

Less often than once a month _____

10- About how many drinks did you have at a time?

One _____

2 or 3 _____

4 or 5 _____

6 or 7 _____

More than that _____

11- What did you usually drink? (choose one only)

Beer _____

Wine _____

Liquor or mixed drinks _____

Other _____

Or it varied _____

****

Finally I would like to ask you a set of questions about the amount of caffeine you drink from coffee and other beverages.

1- Do you drink coffee?

No _____ GO TO 5

Yes occasionally (not every day) _____

Yes regularly (every day) _____

2- What type of coffee do you drink?

Instant _____

Non instant (ground coffee) _____

Instant decaffeinated _____

Non instant decaffeinated (ground) _____

Don't know _____

3- How many cups of coffee do you drink on average each day?

_____ cups

4- How strong is the coffee that you drink?

Weak _____

Medium _____

Strong _____

Don't know _____

5- Do you drink tea?

No _____ GO TO 10

Yes _____

6- What type of tea do you drink?

Canadian brand _____

Imported _____

Herbal, Decaffeinated _____ GO TO 10

Not sure _____

7- How many cups of tea do you drink on average each day?

_____ Cups

8- How strong is the tea that you drink?

Weak _____

Medium _____

Strong _____

9- How long do you let the tea steep before removing the tea bags or pouring it?

Less than one minute _____

More than one minute and less than 3 minutes _____

3 minutes _____

More than 3 minutes _____

10- Do you drink cola type drinks?

No ____ GO TO 13

Yes ____

11- Do you ever drink regular colas, that is cola with caffeine?

No ____ GO TO 13

Yes ____

12-a) How many cola type drinks do you drink on average each day?

_____ Cups

12-b) On average how big is each of your drinks?

Small (less than 8 oz) _____

Medium (8-12 oz) _____

Large (more than 12 oz) _____

13- Do you drink chocolate drinks?

Yes ____

No ____ **END**

14- How many chocolate drinks do you drink on average each day?

____ drinks

APPENDIX B

CONSENT FORM FOR THE STUDY OF SERUM COPPER AND PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

Informed consent to participate in the
peripheral arterial disease study

THE IMPORTANCE OF LIFE STYLE FACTORS IN THE ETIOLOGY OF PERIPHERAL ARTERIAL DISEASE (PAD) AMONG CANADIAN MALES: A CASE CONTROL STUDY

I, _____, understand that I have been requested to participate in a study to determine the effect of life style factors on peripheral arterial disease. By signing, I agree to be interviewed (about 30 minutes), and to have following determinations carried out: blood pressure, weight, hight, forced expiratory volume (breathing into a machine), and also to have approximately one tablespoon of blood drawn for the purpose of measuring sugar, lipids (eg. cholesterol), and copper.

I may refuse to participate now, or withdraw at any time, without it affecting my treatment.

the information gained from this study may be used in publications, but my name will be kept confidential.

_____ _____

WITNESS PATIENT/GUARDIAN

_____

DATE

APPENDIX C

CODEBOOK FOR THE STUDY OF SERUM COPPER AND PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

VARIABLE NAME	VARIABLE LABEL AND VALUE
SECTION 1: Physical examination	
1- ID	Study number
2- PAOD	Peripheral arterial occlusive disease 1 = Control 2 = Case
3- AGE	Age of the subjects (year)
4- HEIGHT	Height of the subjects (centimetre)
5- WEIGHT	Weight of the subjects (kilogram)
6- QUETELET	Quetelet index [weight in kg/(Height in m)^2]
7- SKINTHIC	Skinfolds thickness (millimetre)
8- FATPRCTG	Body fat percentage
9- BPRA	Right arm systolic blood pressure (mm/Hg)
10- BPLA	Left arm systolic blood pressure (mm/Hg)
11- BPRANK	Right ankle systolic blood pressure (mm/Hg)
12- BPLANK	Left ankle systolic blood pressure (mm/Hg)
13- ABIRT	Right ankle brachial index
14- ABILT	Left ankle brachial index
15- VC	Vital capacity (litre)
16- FEV1	Forced expiratory volume in 1 second

****

SECTION 2: Laboratory tests

17- GLUCOSE	Fasting serum glucose (mmol/litre)
18- CHOLSTRL	Fasting serum cholesterol (mmol/litre)
19- TRGLYCRD	Fasting serum triglyceride (mmol/litre)
20- HDL	High density lipoprotein (mmol/litre)
21- LDL	Low density lipoprotein (mmol/litre)
22- COPPER	Serum copper (umol/litre)
23- ZINC	Serum zinc (umol/litre)
24- COTININE	Serum cotinine (ug/litre)

****

SECTION 3: Background

25- ELSECEDU	Years of elementary or secondary education
26- SECGRADU	Graduation from secondary school 1 = No 2 = Yes
27- BEYELSEC	Beyond elementary or secondary school 1 = No 2 = Yes
28- HIESTEDU	Highest level of education 1 = Master or doctorate 2 = Bachelor or undergraduate degree from

university or teacher's college

3 = Some university or some teacher's college

4 = Diploma or certificate from community college, CEGEP, or nursing school

5 = Some community college, CEGEP. or nursing school

29- EDUCLASS — Educational class

1 = University degree
2 = Non- university post secondary degree
3 = More than grade 9 without post secondary degree
4 = Grade 9 or less

30- PLCEBIRT — Place of birth

1 = Ontario
2 = Quebec
3 = Rest of Canada
4 = Outside Canada

31- IMGRYEAR — Year of immigration

32- IMGRDUR — Duration of immigration

33- ETHNCULT — Ethnic or cultural group

1 = French
2 = English
3 = Irish
4 = Others

34- EMPLRECN — Recent employment status

1 = Full time
2 = Part time
3 = Unemployed
4 = Laid-off
5 = Retired
6 = Student
7 = Homemaker

8 = Sick leave
9 = Disability

35- EMPLPAST — Past employment status

1 = No
2 = Yes

36- JOBCLASS — Job classification code

37- SESSCORE — Socioeconomic score (index)

38- SES — Socioeconomic status categories

1 = High
2 = Medium
3 = Low

39- MARISTAT — Marital status

1 = Never married
2 = Divorced
3 = Married/Common law
4= Widowed/Widower
5 = Separated

****

Section 4: Family history

40- SIBLING — Number of brothers and sisters

41- CHILDREN — Number of children

42- FMLHIPVD — Family history of PVD

1 = No
2 = Yes

43- RELATON1 — Relationship for family history of PVD

1 = Mother

2 = Father
3 = Child
4 = Sister or brother
5 = One parent and one sibling
6 = Sister&brother

44- FMLHIAMP — Family history of amputation

1 = No
2 = Yes

45- RELATON2 — Relationship for family history of amputation

1 = Mother
2 = Father
3 = Child
4 = Sister
5 = Brothe

****

Section 5: Medical history

46- HISOFHBP — History of high blood pressure
1 = No
2 = Yes

47- AGOFOHBP — Age of onset of high blood pressure

48- YRSOFHBP — Years of having high blood pressure

49- RXFORHBP — Medication for high blood pressure

1 = No
2 = Yes

50- TYPRXHBP — Type of medications are used for high blood pressure

1 = One antihypertensive
2 = Two antihypertensive

3 = Diuretics
4 = 1 and 3
5 = 2 and 3
6 = Diuretic/antihypertensive
7 = Beta adrenergic receptor blocking agent
8 = Antianginal/antihypertensive
9 = 6 and 8
10 = 1 and 6
11 = 7 and 8
12 = 1 and 7
13 = 1 and 8

51- NORXHBP — Number of medication for high blood pressure

52- YRSRXHBP — Years of treatment for high blood pressure

53- HISOFDIB — History of diabetes

1 = No
2 = Yes

54- AGOFODIB — Age of onset of diabetes

55- YRSOFDIB — Years of having diabetes

56- TYPOFDIB — Type of diabetes

1 = Non-insulin-dependent diabetes (NIDDM)
2 = Insulin dependent diabetes (IDDM)

57- RXFORDIB — Current treatment for diabetes

1 = No
2 = Yes

58- YRSRXDIB — Years of treatment for diabetes

59- TYPRXDIB — Type of medications are used for diabetes

1 = Insulin
2 = Oral medication
3 = Diet only

60- HISOFHRT

History of heart disease

1 = No
2 = Yes

61- TYPOFHRT

Type of heart disease

1 = Angina
2 = Heart attack
3 = Heart insufficiency
4 = Arrythmia
5 = Angina + heart attack
6 = Angina + heart insufficiency
7 = Mitral regurgitation
8 = Mitral valve prolapse
9 = 1, 2, and 4

62- AGOFOHRT

Age of onset of heart disease

63- YRSOFHRT

Years of having heart disease

64- RXFORHRT

Medication for heart disease

1 = No
2 = yes

65- TYPRXHRT

Type of medication for heart disease

1 = Cardiotonic glycoside
2 = Anti-arrythmia
3 = Coronary vasodilator
4 = Antianginal
5 = Anti- hypertensive
6 = Beta blocker
7 = Antianginal/antihypertensive.
8 = Diuretics
9 = 1 and 10
10 = 3 and 4
11 = 1 and 4
12 = 3, 4, and 7
13 = 3 and 7
14 = 1, 2, and 3
15 = 3 and 5
16 = 3, 5, and 7

66- RXNOHRT — Number of medication for heart disease

67- YRSRXHRT — Years of treatment for heart disease

68- HISHRTOP — History of heart operation

1 = No
2 = Yes

69- TYPHRTOP — Type of heart operation

1 = CABG
2 = Angioplasty

70- HISOFPUL — History of pulmonary disease

1 = No
2 = Yes

71- TYPOFPUL — Type of pulmonary disease

1 = Emphysema
2 = Chronic bronchitis
3 = Asthma
4 = Others

72- AGOFOPUL — Age of onset of pulmonary disease

73- YRSOFPUL — Years of having pulmonary disease

74- RXFORPUL — Medication for pulmonary disease

1 = No
2 = Yes

75- YRSRXPUL — Years of treatment for pulmonary disease

76- TYPRXPUL — Type of medication for pulmonary disease

1 = One bronchodilator
2 = Two bronchodilators
3 = Steroid
4 = 1 + 3
5 = 2 + 3

77- HISRXASA

History of current use of aspirin

1 = No
2 = Yes

78- FRQRXASA

Frequency of use of aspirin

1 = Every day or less
2 = Once a week or less
3 = Once a month or less

79- YRSRXASA

Years of treatment with aspirin

80- HISOFOPR

History of operation

1 = No
2 = Yes

81- TYPOFOPR

Type of operation

01 = Hernia
02 = Gallbladder
03 = Prostate
04 = Diverticulum
*05= angioplasty
*06= Angiobypass
07 = Tonsillectomy
08 = Knee surgery
09 = Elbow surgery
10 = Appendectomy
11 = Arthroscopy
12 = Back surgery
13 = Hydrocele
14 = Lung surgery
15 = Hip surgery
16 = Peptic ulcer
17 = Cataract
18 = Joint operation
19 = Diskectomy
*20= 1, 2, 3, 4, and 5
*21= Septoplasty
22 = Hemorrhoidectomy
23 = Bowel resection
24 = Femur surgery

25 = Varicose vein
26 = Throat surgery
27 = Rectal fissure
28 = Osteomyelitis
29 = Eye surgery
*30= ABG (Aorto bifemural graft)

82- YROFOPR — Year of operation

83- HISOFOTH — History of other serious disease

01 = NO
02 = Pneumonia
*03 = Stroke
04 = Arthritis
05 = Hiatus hernia
06 = Hepatitis
*07 = Abdominal aortic aneurism (AAA)
08 = Nephritis
09 = Depression
10 = Tuberculosis
11 = Kidney failure
12 = Cancer
13 = Diverticulitis
14 = Tyroid problem
15 = Poliomyelitis
16 = Pulmonary embolism
17 = Peptic ulcer
*18 = Carotid stenosis
19 = Allergy
20 = Glaucoma
21 = Ulcerative colitis

****

section 6: Physical activity

84- ACTYSCOR — Total physical activity index score for household chores, sports, and exercises

85- ACTYPERC — Activity perception

1 = More active
2 = About the same
3 = Less active

86- ACTYDALY — Daily activity

1 = Heavy load carrying or lifting
2 = Light load carrying or lifting, climbing
3 = Standing, walking
4 = Sitting

87- ACTYGRUP — Physical activity index categories

1 = Active (5500+ activity score)
2 = Moderately active (3125-5499 activity score)
3 = Moderately inactive (750-3124 activity score)
4 = Inactive (0-749 activity score)

****

Section 7: Smoking

88- SMOKEVER — History of ever smoking cigarettes, cigars, or pipe

1 = No
2 = Yes

89- SMPIPNOW — Smoking pipe presently

1 = Yes
2 = No

90- SMPIPREG — Smoking pipe regularly

1 = Occasionally
2 = Regularly

91- SMCGRNOW — Smoking cigars presently

1 = No
2 = Yes

92- SMCGRREG — Smoking cigars regularly

1 = Occasionally
2 = Regularly

93- SMCGTNOW — Smoking cigarettes presently

1 = No
2 = Yes

94- SMCGTRG1 — Smoking cigarettes regularly at the present time

1 = Occasionally
2 = Regularly

95- SMCGTDY1 — Number of cigarettes per day at the present time

96- SMSTRAG1 — Age of start of smoking cigarettes regularly for present smokers

97- SMOKDUR1 — Duration of smoking for present smokers

98- SMKINHL1 — Inhaling smoke for present smokers

1 = No
2 = Yes

99- TYPCGRT1 — Type of cigarettes for present smokers

1 = Filter
2 = Non-filter
3 = Roll my own cigarettes
4 = 1 + 2
5 = 2 + 3
6 = 1 + 3

100-SMCGTRG2 — Smoking cigarettes regularly in the past

1 = No
2 = Yes

101-SMCGTDY2 — Number of cigarettes per day in the past

102-SMSTRAG2	Age of start of smoking cigarettes regularly for previous smokers
103-SMSTOPAG	Age of stop of smoking cigarettes regularly
104-SMOKDUR2	Duration of smoking for previous smokers
105-YRSQUIT	Years after stop smoking
106-SMKINHL2	Inhaling smoke for previous smokers 1 = No 2 = Yes
107-TYPCGRT2	Type of cigarettes for previous smokers 1 = Filter 2 = Non-filter 3 = Roll my own cigarettes 4 = 1 + 2 5 = 3 + 2 6 = 1 + 3 7 = 1+ 2 + 3
108-SMEXPHOM	Expose to smoke at home 1 = No 2 = Yes
109-NOSMKER1	Number of regular smokers at home (except the subject)
110-SMEXPWRK	Expose to smoke at work 1 = No 2 = Yes occasionally 3 = Daily
111-CHWTOBAC	Chewing tobacco at the present 1 = No 2 = Yes

112-SNFTOBAC

Snuffing tobacco at the present

1 = No
2 = Yes

113-SMKGRUP

Smoking status categories

1 = Non-smokers (refer to respondents who never smoked or who used to smoke cigarettes, pipes or cigars occasionally but are presently not smoking)

2 = Smoke only pipe or/and cigar (refers to respondents who smoke a pipe and/or cigars but do not smoke cigarettes)

3 = Ex-regular cigarette smokers (are those who reported that they smoked cigarettes regularly in the past but do not at present smoke a pipe, cigars or cigarettes)

4 = Occasional cigarette smokers (smoke cigarettes occasionally, not every day)

5 = Regular smokers (are those who reported smoking cigarettes daily)

6 = Smoke only pipe now but used to smoke cigarettes regularly

****

Section 8: Alcohol consumption

114-DRKFRQ

Frequency of taking alcoholic drinks in the past 12 months

01 = Not at all in the last twelve months
02 = Less often than once a month
03 = About once a month
04 = 2 or 3 times a month
05 = About once a week
06 = 2 or 3 times a week
07 = 4 to 6 times a week
08 = Once a day

09 = 2 or more times a day

115-DRKSRAG1 — Age of start of drinking alcoholic beverages for present drinkers

116-DRKDUR1 — duration of drinking for the present drinkers

117-DRKNOWEK — Number of drinks taken a week before interview

118-DRKPRCPT — Drinking perception per week

1 = More
2 = Same
3 = Less

119-DRKCHNG — Change of drinking habit over the last 12 months

1 = Drinking less now
2 = Drinking the same
3 = Drinking more now

120-DRNKTYP1 — Type of drink for current drinkers

1 = Beer
2 = Wine
3 = Liquor or mixed drink
4 = other
5 = It varies

121-DRKHSPST — History of drinking in the past

1 = Never drink
2 = Drink occasionally (less than once a month)
3 = Used to drink

122-DRKSRAG2 — Age of start of drinking alcoholic beverages for the previous drinkers

123-DRKSTPAG — Age of stop drinking alcoholic beverages for the previous drinkers

124-DRKDUR2 Duration of drinking in previous drinkers

125-DRKFQPST Frequency of drinking for previous drinkers

01 = Once a month
02 = 2 or 3 times a month
03 = about once a week
04 = 2 or 3 times a week
05 = 4 to 6 times a week
06 = Once a day
07 = 2 or more times a day

126-DRKNOPST Number of drinks at a time for previous drinkers

1 = One
2 = 2 Or 3
3 = 4 Or 5
4 = 6 Or 7
5 = More than that

127-DRNKTYP2 Type of drink for previous drinkers

1 = Beer
2 = Wine
3 = Liquor or mixed drinks
4 = Other
5 = It varied

128-DRKGRUP Drinking status categories

1 = Never drank (those who never drank)
2 = Former drinker (those who used to drink at least once a month but has had no drinks in the last 12 months)
3 = Occasional drinker (those who drinks less often than once a month, but has had drinks in last 12 months)
4 = Current drinker (those who drinks at least once a month)

129-CURDKGRP Current drinker categories (according to the number of drinks per week)

1 = No drink
2 = 1-6 drinks
3 = 7-13 drinks

4 = 14 or more drinks

****

Section 9: Caffeine consumption

130-COFEDRNK — Drink coffee

1 = No
2 = Yes occasionally (not every day)
3 = Yes regularly (every day)

131-COFETYPE — Type of coffee

1 = Instant decaffeinated
2 = Non instant decaffeinated (ground)
3 = Instant
4 = Non instant (ground coffee)

132-COFECUPS — Number of cups of coffee per day

133-COFESTRG — Strength of coffee

1 = Weak
2 = Medium
3 = Strong

134-TEADRNK — Drink tea

1 = No
2 = Yes

135-TEATYPE — Type of tea

1 = Herbal, decaffeinated
2 = Canadian brand
3 = Imported
4 = 1 and 2
5 = 1 and 3

136-TEACUPS — Number of cups of tea per day

137-TEASTRG

Strength of tea

1 = Weak
2 = Medium
3 = Strong

138-TEASTEP

Steeping time for tea making

1 = Less than one minute
2 = 1 or 2 minutes
3 = 3 minutes
4 = More than 3 minutes

139-COLADRNK

Drinking cola drinks

1 = No
2 = Yes

140-COLAREG

Drinking regular cola

1 = No
2 = Yes

141-COLANODY

Number of cola drinks per week

142-COLASIZE

Average size of cola drinks

1 = Small
2 = Medium
3 = Large

143-CHOCDRNK

Drink chocolate drink

1 = No
2 = Yes

144-CHOCNO

Number of chocolate drinks per week

145-CAFEINE

Amount of caffeine (mg/day)

146-CAFNGRUP

Cafeine consumption categories

1 = <500
2 = >500

147-QUETGRUP

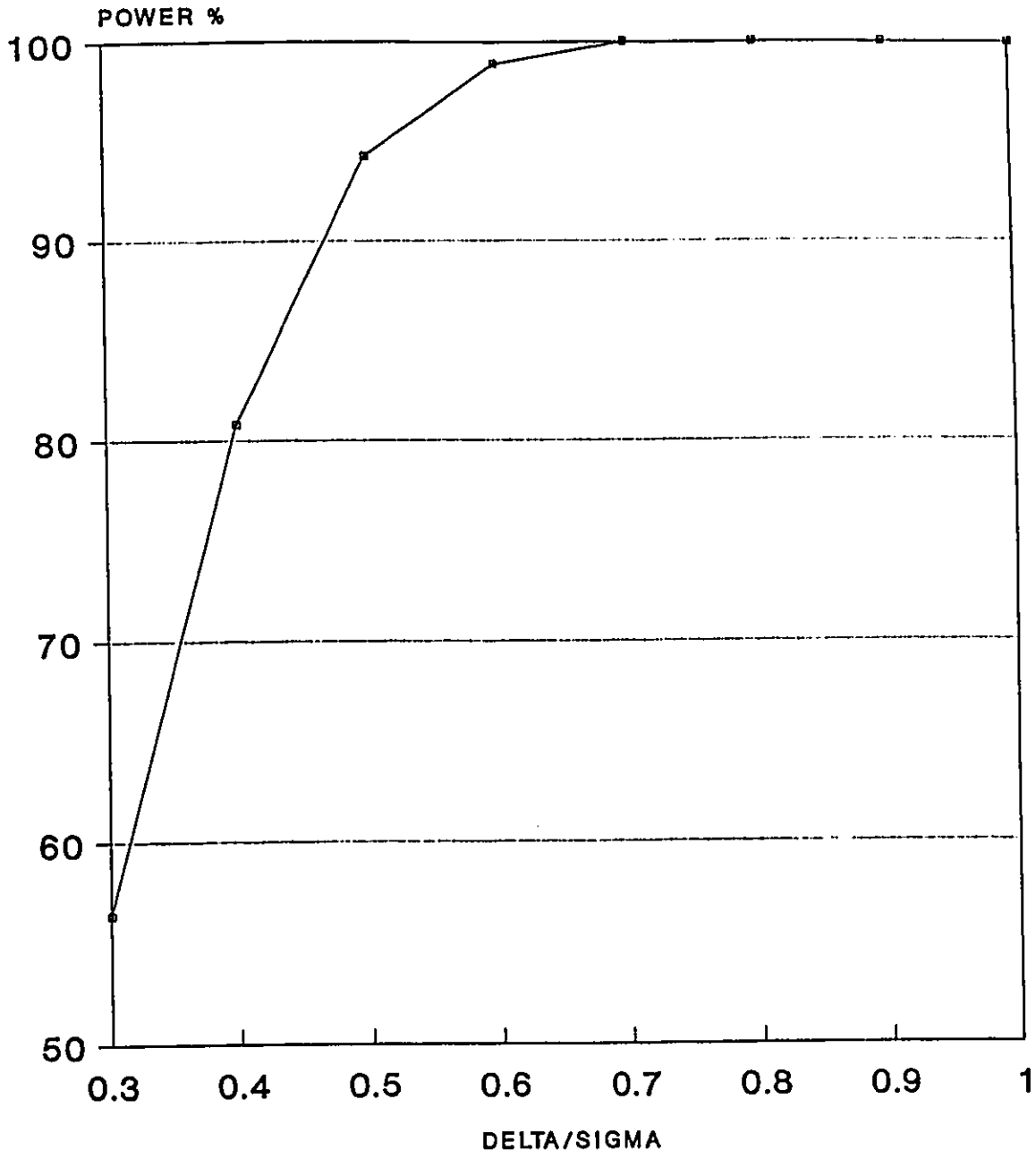
Quetelet index category

1 = <25.1
2 = 25.1-29.9
3 = >29.9

APPENDIX D

ESTIMATION OF POWER FOR THE STUDY OF SERUM COPPER AND PERIPHERAL ARTERIAL OCCLUSIVE DISEASE FOR DIFFERENT RATIOS OF δ TO σ

ESTIMATES OF POWER FOR THE STUDY OF SERUM COPPER AND PAOD



DELTA = DIFFERENCE BETWEEN MEANS OF COPPER IN TWO STUDY GROUPS
SIGMA = STANDARD DEVIATION OF COPPER