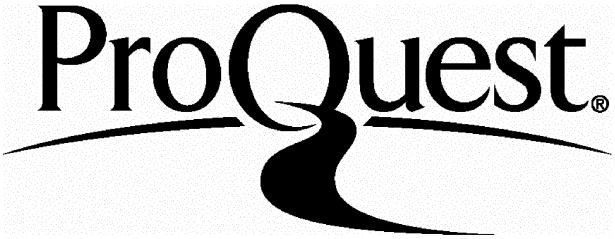
Effects of Acute Exercise, Fitness Training, and Garlic Supplementation on Circulating Markers of Cardiovascular Risk

Thesis submitted to fulfil the requirements for the degree Master of Philosophy of the University of London

Dominic Julian Byrne BSc.

Department of Chemical Pathology, Royal Free Hospital School of Medicine, Rowland Hill Street London NW3 2PF.



.010061

Abstract

The acute effects of a two-day Military Exercise (ME), following a sustained exercise program, on plasma levels of Fibrinogen, serum C-reactive Protein, Lp(a), lipids and glucose were investigated, together with the effects of long term garlic supplementation on plasma lipid profiles and oxidative status.

Male British army recruits (n=318) were sampled at the start of their ten weeks basic training, and again over a five-day period following a strenuous two-day Military Exercise (ME) (n=206). The ME produced a rapid and significant increase in Fibrinogen concentration, lasting three days. CRP and Creatinine were also raised while Albumin was reduced during this period. Lp(a) levels showed little change at any of the sampling points. Cholesterol was reduced during the acute phase, but increased on days 4 and 5 post ME. Triglycerides increased gradually, while glucose concentrations were reduced in all recruits.

Plasma samples were obtained for a second double blind study from general practice, in which garlic or placebo was administered for six months to fifty-one asymptomatic mildly hypercholesterolaemic volunteers. The copper ion induced lag time (oxidative resistance) of isolated LDL was significantly increased in both groups, but was not significantly different between groups, consistent with a 'placebo effect'. Lipid profiles were broadly unchanged. Other markers of oxidative resistance, Vitamin E concentration, LDL subfraction distribution, and levels of autoantibodies to oxidised LDL, did not correlate with the increase in lag time.

Lp(a) concentration was not effected by the increased fitness of the recruits, nor did it respond as an acute phase protein following the ME. Fitness training reduced glucose levels and caused small changes in serum lipids. Dietary garlic supplementation had no effect on LDL oxidation.

Acknowledgements

Having worked long and hard to complete this thesis my deepest gratitude and thanks go to my partner Karen for her love and support. I would also like to thank my mother for her prayers and all my family for their encouragement.

I want to thank Darren, Tim, and Khalid for their help and advice and most of all they're good humour and sanity when these attributes were hard to come by. Special thanks go to Miss Anita Jagroop, Dr. David Vallance, Dr. Hugh Montgomery, Prof. Steve Humphries, Dr. Andrew Neil and Captain Mike Jubb for all their assistance.

Finally I would like to thank Professor Tony Winder and all the staff of the Department of Chemical Pathology, who are too numerous to mention individually, but without whom this project would have been impossible to complete.

Contents **Page**

CHAPTER 2

ARMY RECRUITS STUDY MATERIALS AND METHODS **Page**

CHAPTER 3

GARLIC STUDY MATERIALS AND METHODS

CHAPTER 4

ARMY RECRUITS STUDY RESULTS **Page**

CHAPTER 4 (continued)

ARMY RECRUITS RESULTS **Page**

CHAPTER 5

GARLIC STUDY RESULTS

CHAPTER 5 (continued)

CHAPTER 6

DISCUSSION

CHAPTER 6 (continued)

LIST OF FIGURES **Page**

Army recruits study

Garlic study

LIST OF TABLES **Page**

Army recruits study

LIST OF TABLES (continued) **Page**

Garlic study

ABBREVIATIONS

APP	Acute phase protein
APR	Acute phase response
BFT	Basic fitness test
CHD	Coronary heart disease
CK	Total Creatine Kinase
CRP	C-reactive protein
EDTA	Ethylenediaminetetraacetic acid
HDL	High density lipoprotein cholesterol
HTGL	Hepatic triglyceride lipase
HPLC	High performance liquid chromatography
Iso-LDL	Isolated LDL
LDL	Low density lipoprotein cholesterol
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
ME	Military Exercise
MDA	Malonaldehyde
mins.	Minutes
n	number of subjects or participants
oLAb	Antibodies to oxidised low density lipoprotein

ABBREVIATIONS (continued)

Rf	Rf values represents the distance travelled by the solute, divided by the distance travelled by the solvent front. When applied to non-gradient acrylamide gel rods the solute is LDL and the solvent front is distance from VLDL peak to the HDL peak in mm
S.D.	Standard deviation
Sig.	Level of significance achieved following statistical analysis
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
Tg	Triglyceride, or Triacylglycerol
TRIS-HCL	Trizma Hydrochloride
w/v	weight / volume

CHAPTER 1

INTRODUCTION

1.1.1 Coronary heart disease

Coronary heart disease (CHD) is the major cause of death (and premature death) in the western world. It also leads to substantial morbidity from angina, cardiac failure, and reduced exercise tolerance. Further mortality and morbidity occur from cerebrovascular disease and peripheral vascular disease, which share with CHD the four classic risk factors of hyperlipidaemia, hypertension, diabetes mellitus and cigarette smoking. These risk factors when combined have a cumulative effect on CHD risk (Reckless 1996).

The link between hyperlipidaemia and increased risk of premature CHD has been extensively reported from epidemiological, case control, and prospective studies (Shepherd 1995). Many of these studies have proposed that raised serum concentrations of total cholesterol (TC), LDL cholesterol (LDL) and triglycerides (Tg) and lower HDL cholesterol (HDL) concentrations are associated with increased risk of CHD.

The treatment and prevention of CHD has two main approaches, one using drug treatment and one where patients are encouraged to alter their lifestyle. The pharmacological treatment of hyperlipidaemia using a variety of lipid lowering drugs has proved successful in reducing serum TC and LDL levels while increasing HDL levels and in lowering risk (The Scandinavian Survival Study 1994). Reducing serum lipid concentration by changing lifestyle has also proved successful in reducing CHD risk. The alteration of lifestyle to include taking more exercise, reducing calorific intake, and stopping smoking are all important in reducing risk. Increasing exercise and reducing body weight also have effects on the other markers of risk such as hypertension and blood coagulation.

1.1.2 Exercise training

Increased physical fitness through regular exercise has for many years been advocated as a means of improving health. In western societies participation in regular exercise and its promotion as a means of improving health are multi-million pound industries. Epidemiological studies have demonstrated the association between lack of physical activity and increased risk of premature CHD (Hennekens et al 1977, Oberman et al 1985, Wei et al 1997). The beneficial effects of exercise training relate to an increase in cardiorespiratory fitness and decreased body weight primarily via a reduction in fat mass. These changes coupled together with advantageous modifications in lipid and carbohydrate metabolism both play a central role in the reduction of CHD risk (Despres et al 1994).

Several cross sectional studies have shown that endurance trained athletes have higher levels of HDL and lower levels of Tg and LDL compared with sedentary controls (Wood et al 1976, Herbert et al 1984). Levels of postprandial lipaemia are also reduced in trained athletes (Foger et al 1995). Exercise training has also been shown to alter the composition of LDL by promoting the formation of 'light' less atherogenic particles (Williams et al 1990), and the LDL of trained subjects also shows greater resistance to oxidative modification when compared with sedentary subjects (Sanchez-Quesada et al 1997). Carbohydrate metabolism is also effected by exercise training, trained subjects have lower levels of glucose and insulin and preferentially metabolise free fatty acids as an energy substrate during exercise (Coggan et al 1996).

Longitudinal studies have also reported the effects of exercise on sedentary, obese, dyslipidaemic, and diabetic populations. These studies have shown that increasing levels of exercise can produce beneficial changes in lipid and glucose metabolism similar to those seen in cross sectional studies (Hardman 1996). Less is known about the effects of increased fitness on serum Lipoprotein (a) (Lp(a)) concentration. Conflicting reports into the effect of exercise on Lp(a) concentration have stated that levels are increased after exercise and also show no change in response to exercise (Durstine et al 1996, Hubinger et al 1996). The effect of acute exercise and fitness training on serum Lp(a) were of particular interest in this study

Regular exercise is also associated with a number of other equally important metabolic changes with respect to CHD. These include reduced blood pressure, increased fibrinolytic activity, reduced hypercoagulable states, lower levels of platelet aggregation, and reduced circulating levels of catecholamines. There are also physiological benefits associated with regular exercise, which include a general feeling of well being and reduced levels of depression and anxiety. Participants in regular exercise have also been shown to be more likely to avail themselves of other preventive health services (Chandrashekhar et al 1991). The effects of physical activity and training on lipid and lipoprotein metabolism have produced an overall consensus on the benefits on exercise.

Both longitudinal and cross sectional studies have produced an overall view of the metabolic changes that can be achieved by exercise, in spite of the difference in the type, intensity and duration of the exercise or training undertaken by the participants,

The follow up sampling period once the exercise has been completed and the way initial and final levels of fitness are assessed also differs between studies. A number of studies also corrected the results for changes in plasma volume or body weight between the start and end of each trial. Despite these differences between studies the overall effects of exercise may be to reduce risk of premature CHD by affecting several potent metabolic variables, and processes.

1.1.3 Lipoprotein (a)

Lp(a) is a lipoprotein particle that is an important risk factor in the development of CHD because it has both atherosclerotic and thrombotic properties (Maher 1995). Lp(a) is an LDL like particle containing one molecule of apoprotein B_{100} joined to one molecule of apo (a) linked by a disulphide bond. Apo (a) can be found bound to two molecules of apo B and a small proportion of apo (a) is unbound and can be found free in serum.

The apo (a) portion of the Lp(a) particle is highly glycosylated and displays considerable size heterogeneity, with a molecular weight varying from 200,000 kD to 800,000 kD. This variation in particle size is due to the number of multiple kringle repeats present in the particle. These kringle structures are triple loops of 114 amino acids stabilised by three internal disulphide bonds. Lp(a) contains varying numbers of repeated kringle IV type 2 motifs, one kringle type V and an inactive serene protease domain. The number of kringle repeats, or length of the apo (a) protein is related to the amount of the protein found in serum.

Low levels of apo (a) in serum are generally associated with long forms of the apo (a) protein and high levels are associated with short forms of apo (a). The amino acid sequence of apo (a) is closely related to that of plasminogen kringle IV, an enzyme involved in clot lysis. Plasminogen and apo (a) are part of a family of similar proteins domain called the plasminogen gene super family. These proteins all contain repeated kringle IV motifs, one kringle V domain and a serine protease domain. This family of proteins includes a number of other enzymes of the clotting cascade including prothrombin, tissue plasminogen activator, coagulation factor XII, and fibronectin. The role and function of apo (a) is unclear but it has been suggested that it may have some inhibitory effects on clot lysis, by competing with plasminogen for binding sites on the fibrin clot. Serum Lp(a) concentrations are mainly under genetic control and are not effected by lipid lowering drug therapy (Kiezovitch et al 1995, Hajjar et al 1996). The effect of lifestyle factors such as exercise and diet on Lp(a) levels have been investigated (Holme et al 1995, Ponjee et al 1995) and it has been proposed that Lp(a) may act as an acute phase protein (APP) (Norma et al 1994)

1.1.4 Acute exercise

Carbohydrates and fats have been studied during and immediately after intense or acute exercise. These studies have investigated the effect of duration and intensity of exercise on a number of markers of lipid and carbohydrate metabolism. A variety of techniques including stable isotope tracer analysis, muscle biopsy and diets containing varying amounts of fat and carbohydrate have been used.

Acute exercise has also been used as a model to reflect the long-term metabolic changes produced by regular exercise. These studies have show how acute periods of exercise can increases the concentration of HDL, and moderately reduce TC and LDL concentrations (Dufaux et al 1986, Thompson et al 1980, Lithell et al 1984). Reduced serum Tg levels have also been reported following acute exercise (Sady et al 1986, Foger et al 1995, Enger et al 1980).

A number of researchers report that Lp(a) levels increase in response to a variety of acute stimuli including infection, post operative trauma (Norma et al 1994), myocardial infarction (Mbewu et al 1993), and angina (Oshima et al 1991). As mentioned earlier a number of studies have found that Lp(a) does not respond as an acute phase reactant in rheumatoid arthritis (Wallberg-Johnson et al 1995) or during acute infection (Paximadas et al 1997). Few of the investigations into the effects of acute exercise on Lp(a) have commented on the possible acute response brought on by the exercise or on the role of Lp(a) as a marker of this acute response.

1.1.5 The Acute Phase Response

The acute phase response (APR) is a specific immune based reaction to a number of non-specific stimuli, including bacterial infection, tissue damage, surgery, and strenuous exercise. Inflammation is the body's response to these forms of tissue damage and the process involves a series of biochemical and cellular processes that result in the removal of tissue debris and foreign organisms by phagocytes. The APR response includes fever, endocrine changes, alterations in immune function, leucocytosis and increased synthesis by the liver of some proteins; the acute phase proteins. Local effects include the activation of phagocytes and the promotion of fibroblast proliferation and healing. These effects are mediated by cytokines released from macrophages accumulating at the site of injury.

The acute phase proteins are a family of approximately 30 plasma proteins produced in increased amounts by the liver following inflammation. These protein markers include C-reactive protein (CRP), fibrinogen, creatinine, serum amyloid A, interleukin 6, and alpha$_1$-antitrypsin. Serum albumin levels are reduced during the APR. Of these proteins a rapid increase in CRP concentration is often used as an indicator of the acute response. CRP can be detected in serum after 6 hours and raised levels can persist for up to forty-eight hours. Erythrocyte sedimentation rate (ESR) and plasma viscosity are also used as markers of the APR and both reflect increases in fibrinogen concentration (Whicher, 1990, Silverman et al 1994). Fibrinogen concentration increases more slowly after inflammation compared to CRP. Raised levels of fibrinogen are also seen in anaemia, microcytosis, and immunoglobulin increases.

1.1.6 Fibrinogen

Fibrinogen has three well accepted roles in hemostasis in vertebrates. First, as a precursor to fibrin, fibrinogen acts to seal blood vessels and to restrain haemorrhage by participating in intra-vascular and extra-vascular coagulation. Second, fibrinogen supplies, as a component of the fibrin structure, a matrix for spatial organisation of cellular participants in wound healing. Third, fibrinogen contributes as a principal participant in the control of blood viscosity. Raised plasma fibrinogen concentration and the associated increased in blood viscosity are not only markers of the APR but are also associated with increased risk of CHD. Studies have demonstrated the presence of raised fibrinogen in individuals with CHD (Handley et al 1997).

Exercise has been shown to enhance clot breakdown (Chandrashekhar et al 1991) and also to reduce circulating levels of fibrinogen (Ernst 1993). The role of different fibrinogen genotypes on plasma fibrinogen levels following acute exercise has been previously published using the same recruit data presented here (Montgomery et al 1996). This study demonstrated that an acute rise in plasma fibrinogen following intensive exercise was influenced by the G-$_{453}$-A polymorphism of the β-fibrinogen gene. The training program followed by these young men allowed further investigation into the effects of increased fitness on plasma fibrinogen levels and the relationship between this marker of premature CHD and Lp(a).

The role of fibrinogen in the APR and the proposed role of Lp (a) are important because there is another connection between these two proteins. Raised levels of both these proteins are independent risk factors for the development of premature CHD.

Statistical analysis has shown that raised Lp(a) concentrations are associated with high fibrinogen levels in older men (Mikhailidis et al 1996) and women (Heinrich et al 1991). Given this association any situation, such as the APR, where both fibrinogen and Lp(a) increase rapidly, even over a period of days, in response to inflammation or injury could contribute to CHD risk. Increased levels of Lp(a) during the acute response could also confound the interpretation of Lp(a) results.

1.1.7 Oxidative Stress

It has been suggested that acute or intensive exercise may lead to the generation of oxygen free radical species. It is thought that during exercise induced oxidative stress, oxygen free radicals are released through mitochondrial oxidative phosphorylation or from inflammatory cells. Other sources of free radicals within the body include cigarette smoke, alcohol, diet, air pollution and ultraviolet radiation (Jacob 1996, Moller et al 1996).

The release of free radicals may trigger and stimulate a host of metabolic events that involve the antioxidant defence system. The main participant in this antioxidant defence mechanism is lipid soluble vitamin E. Plasma also contains other antioxidants including water soluble vitamin C (ascorbate), urate and lipid soluble bilirubin. There is also a belief among athletes that ingestion of antioxidant vitamins, such as vitamin E and C, in the weeks and days before a bout of strenuous exercise can protect against the damaging effects of free radical species.

Less in known about the effects of strenuous exercise on serum levels of these other antioxidants such as bilirubin and urate. Given the intense nature of the military exercise it would seem possible that the recruits could be exposed to some degree of oxidative stress. The data provided on serum levels of urate and bilirubin in these young men may provide some information on the effect of this type of exercise on serum concentrations of these antioxidants.

1.2.1. Garlic

Lifestyle factors such as exercise and diet have been subject to intensive study over the years. Much of the recent research into diet has been based on the differing rates of cardiovascular disease seen in Mediterranean and northern European populations. The dietary intake of caffeine, red wine, olive oil, and garlic of both populations have all been investigated.

Garlic (*Allium sativum L*) and its derivatives have been used for centuries as cures and treatments for a variety of disorders. The Codex Ebers, an Egyptian medical papyrus dating from 1550 BC, contains more than 800 therapeutic formulas, of which 22 mention garlic as an effective remedy for ailments including heart problems, headaches, bites, worms, and tumours. Garlic contains a unique sulphur containing compound, identified by its pungent odour. The odour is produced when the garlic bulb is cut or crushed allowing the amino acid L-alliin and the enzyme alliinase react to form two molecules of allicin, believed to be the active ingredient (Block 1985).

A number of studies have attributed lipid lowering and antioxidant properties to dietary garlic supplementation. Many of these studies have lacked adequate control groups and have also differed in the type and amount of garlic administered. Differences in the duration of the treatment and the degree of compliance also make the results of these trials difficult to compare. This has contributed to the contradictory reports on the effects of dietary garlic supplementation. The associations between elevated serum cholesterol and increased rates of lipoprotein oxidation in atherosclerosis have been widely reported (Strinberg et al 1989).

It has been proposed that garlic may have a variety of cardio-protective properties including, reduction in serum lipids (Silagy et al 1994), lowering of blood pressure (Orekhov et al 1997), enhanced antioxidant activity (Phelps et al 1993) and inhibition of platelet aggregation (Ariga et al 1981).

1.2.2 Oxidised lipoproteins

Evidence has accumulated that the free radical process known as lipid peroxidation plays a crucial and causative role in the pathogenesis of atherosclerosis. It is believed that lipid peroxidation is involved in the oxidative modification of LDL, and this modification results in the formation of atherosclerotic lesions. This hypothesis is supported by data from biochemical studies, experimental animal studies, and from epidemiological investigations (Esterbauer et al 1993). Many studies have investigated the effects of copper ion induced oxidation on isolated LDL as a marker of peroxidation or oxidative status (Chait et al 1992, Jialial et al 1996).

1.2.3 Vitamin E

The lag time of isolated LDL is related to the concentration of antioxidants, such as lipid soluble α- tocopherol (vitamin E), and water soluble ascorbate (vitamin C). Other lipid soluble antioxidants include γ-tocopherol, β-carotene and retinyl stearate. Bilirubin and uric acid (urate) are also potent antioxidants found in plasma. Reduced levels of these antioxidants have been associated with increased levels of atherosclerosis, and it has been suggested that increased intake of antioxidant preparations may reduce the risk of premature CHD (Hoffman et al 1995).

1.2.4 LDL Subfractions

The relationship between increased LDL cholesterol concentration and increased risk of CHD has been established. There is also evidence that subclasses of LDL may have important clinical significance as well. Low density lipoprotein subclasses are characterised by variations in density, size, and chemical composition. These differences in subclasses have been investigated using a number of techniques including density gradient ultracentrifugation, non-denaturing gradient gel electrophoresis (Austin et al 1993), and non-denaturing non-gradient gel electrophoresis (Muniz 1977). The classification of these LDL subfractions depends on the technique used in their isolation therefore a number of different means of classification have developed over the years.

Musliner and Krauss (1988) isolated LDL into six subclasses using gradient gel electrophoresis, and express LDL particle diameter in Angstrom units and in terms of density range in kg/L. The LDL I and II subclasses were referred to as light LDL and the LDL IIIA, IIIB, IVA and IVB as dense LDL. Subclasses of LDL have been reported in terms of LDL score (Campos et al 1991). This method separates LDL using 2-16% gradient gel electrophoresis and then assigns a score to each subclass by multiplying the area under the curve by the subclass number. Those individuals with LDL that is predominantly dense have higher scores than those with a predominantly light LDL. Austin and co-workers (1993) employed the same technique but used a simpler classification by dividing the LDL into phenotype A with a particle diameter >255Å (light) and phenotype B with a particle diameter <255Å (dense).

There is also an intermediate pattern, which is defined as not phenotype A and not phenotype B. These techniques have revealed the relationship between increased amounts of LDL phenotype B or dense LDL and CHD (Austin 1993).

1.2.5 Autoantibodies to oxidised LDL

Oxidative modifications of LDL may play an important role in the development and progression of atherosclerosis. Several studies have demonstrated the immunogenic properties of oxidised LDL, and as a consequence autoantibodies against oxidised LDL (oLAb) have been detected in human plasma. Immunological methods for the measurement of oLAb are based on chemical modifications of LDL with malonaldehyde (MDA) or Cu^{++} oxidised LDL. Oxidatively modified LDL is then bound to solid phase (microtiter plate well) followed by a specific detection of antibodies bound by I^{125} or peroxidase labelled anti human IgG.

Studies using similar kit assays have found higher oLAb ratios in patients with carotid atherosclerosis and essential hypertension. During acute myocardial infraction patients were shown to have low antibodies titres after anti-thrombolytic treatment. Patients undergoing cardiac rehabilitation have significantly higher levels of oLAb when compared to control subjects. On the basis of these and other studies oLAb have been suggested as markers of oxidative status and as possible indicators of CHD risk (Tatzber et al 1995). These and other studies suggest that changes in the concentration of oLAb may be associated with increased risk of premature CHD.

Less is known about the relationship between levels of oLAb and other markers of oxidation or indeed the possible effect of dietary garlic supplementation on the plasma concentration of this marker. Many of the studies into the antioxidant properties of garlic have examined only one marker of oxidative status. By investigating the changes in the concentration or distribution of several markers, such as isolated LDL lag time, vitamin E concentration, LDL subfraction distribution and levels of antibodies to oxidised LDL, determination of the possible antioxidant effects of garlic supplementation may be improved. It may also help to understand the relationship between these markers of antioxidant status.

Garlic supplementation along with other dietary antioxidant preparations offer an inexpensive and easy means of affecting lipoprotein oxidation which in the long term could play an important role in lowering a patients risk of developing CHD. The possible use of naturally occurring and easily available dietary supplements in reducing rates of atherosclerotic disease is of great interest.

1.3.1 Study Aims

The two studies set out in this thesis investigated the effects of acute and prolonged exercise in healthy young men. The antioxidant effects of garlic long term supplementation were also investigated. Both of these studies report the possible impact of changed lifestyle, exercise and dietary supplementation, on a number of risk factors associated with premature CHD.

The army recruit study was designed to observe the effect of ten weeks basic training combined with an acute two day ME on a number of metabolic variables including serum lipids, glucose, fibrinogen and Lp(a). Serum concentrations of total creatine kinase, creatinine, bilirubin, CRP and urate, were also measured in the recruits. The role of Lp(a) as acute phase reactant and the effect of exercise on Lp(a) were of particular interest.

The intake data on these recruits also allowed a number of associations between markers of CHD risk, seen in older patient groups, to be investigated in these younger subjects. These included the association between raised levels of serum TC, LDL, Lp(a) and fibrinogen. The smoking habits of these young men were also recorded.

The dietary garlic supplementation study set out to determine the antioxidant effects of long term garlic supplementation on markers of oxidative status. These markers include isolated low density lipoprotein lag time, vitamin E concentration, LDL subfraction distribution and levels of oLAb. The association between LDL lag time and the other markers of oxidation were also investigated in the pre garlic samples. The effects of Lp(a) concentration on the oxidation characteristics of isolated LDL were also reported.

The studies presented here investigated some possible cardio-protective effects of exercise and dietary supplementation. Both of these lifestyle factors offer a means to reduce CHD risk, which is low in cost and easy to implement. If proven to be effective, these forms of intervention, when used separately or in combination could offer an attractive means of lowering CHD risk.

CHAPTER 2

ARMY RECRUITS MATERIALS AND METHODS

2.1 Introduction

The techniques, reagents and instrumentation used during the army recruit study are described in this materials and methods chapter. BDH Laboratory Supplies supplied the chemicals and reagents mentioned in the methods below unless stated otherwise. The chemicals used were reagent grade unless stated otherwise in the text. The text contains the names of the suppliers of all other chemicals, reagents and equipment.

2.2 Recruit selection and training protocol

With the approval of the Army Medical Ethical Committee, consecutive groups of male recruits to the Army Training Regiment Bassingbourn were selected to take part in the study. All of these young men were free from disease, were physically fit and all had passed the army medical examination. The majority of the recruits selected for this study would go on to join infantry regiments. Informed written consent was obtained from all the recruits at the start of the study. Information regarding height, weight, medical, drug, and smoking history of the recruits was also obtained.

These new British Army recruits undergo ten weeks of intensive training while at Bassingbourn. During the first five weeks the recruits undergo general fitness training involving short runs (1-5 miles), without full kit, gymnasium exercise and parade ground training. At the end of the first five weeks the recruits were put through a basic fitness test (BFT) in the gym. This involved sit-ups, press-ups, and a short run. The second five weeks of training consists of more exhaustive exercise comprising of more prolonged physical activity in full battle dress and carrying full pack and a weapon.

There were a total of sixty-nine 40-minute periods specifically dedicated to physical training throughout the ten weeks training. The remaining time on the training course involves strenuous physical activity, such as marching on the parade ground and physical labour.

As part of their final assessment the recruits take part in an exhausting two-day ME during the penultimate week of training. Blood samples were taken at intake and at time points after the ME had been completed, in each case following five minutes of supine rest. Groups of recruits were sampled at intake, T1, and again during the penultimate week of training, T2, (Groups G1 to G6). The pre exercise blood samples were not taken from the recruits while they were fasting. The constraints of the training timetable meant that the final recruit assessment was staggered, with the five groups of recruits studied at different time points following the ME. The recruit groups were samples at the following time points:

Recruit group 1 sampled 12 hours post ME.

Recruit group 2 sampled 24 hours post ME.

Recruit group 3 sampled 48 hours post ME.

Recruit group 4 sampled 3 days post ME.

Recruit group 5 sampled 4 days post ME.

Recruit group 6 sampled 5 days post ME.

2.3 Military exercise protocol

During the ninth week of their training, the recruits were put through a strenuous ME as part of their final assessment. The ME comprised prolonged moderate level physical exertion. The exercise was designed to test, under field conditions, the skills the recruits had acquired during training. The military exercise began in the afternoon of day one and continued for a full 48 hours until the early evening on day three. After the ME blood samples were taken from the groups of recruits at the time points indicated above. Again these blood samples were taken in the morning after breakfast and are therefore not fasted.

During the ME recruits wore a pack containing their personal equipment, clothing, food and a weapon. The total weight of the equipment was in the region of 35kg. The distances covered during the ME varied but on average the recruits covered approximately 10 miles over the forty eight hour period. Throughout the ME, the recruits performed tests of map reading, first aid, sentry duty, and field craft. Periods of rest during the ME were often interrupted by short bursts of intensive exercise such as log carrying. The ME is designed to push the recruits to the limit of their physical performance. The army is well aware that dehydration leads to a loss in performance and can be extremely dangerous, with this in mind the recruits are reminded to take on fluids as often as possible during the exercise.

The duration of the ME along with the field conditions resulted in many recruits returning in a state of fatigue, both from prolonged periods of low intense physical activity and lack of sleep. Once the ME had been completed no other fitness tests or strenuous physical activity were undertaken by the recruits during the last week of the training programme.

2.4 Blood collection and transportation

Blood samples were obtained from each recruit group after five minutes of supine rest, at the beginning and end of basic training. Serum samples were collected into 5.5ml Monovet gel tubes (Starsted Ltd. UK), while the plasma samples were taken into tubes containing 3.8% trisodium citrate made up in our laboratory. Serum and plasma samples were stored on ice and then transported by car from Bassingbourn to our laboratory. On arrival, the samples were separated, aliquoted and stored until analysis. The use of serum gel tubes prevented the serum volume of each recruit from being noted prior to storage.

2.5 Serum assays

Whole blood separated by centrifugation on an ICE centrifuge (1000g /10 minutes/4°C). Serum was then stored at 4°C for 24-48 hours prior to analysis, while aliquots for Lp(a) analysis were stored at -70°C. The tests listed below were carried out by on-call staff outside of normal working hours following accredited standard operating procedures. The serum samples were analysed on a Hitachi 747 auto-analyser using commercially available enzymatic test kits. All of the test listed below were determined using reagents supplied by Boeringer Mannheim Ltd UK.

Total cholesterol,	HDL cholesterol,	Triglycerides,	Glucose,
Albumin,	Creatinine,	Bilirubin,	Urate,
Total Creatine Kinase (CK),		C-Reactive Protein.	

Precipitation of Apolipoprotein B containing particles prior to HDL determination was carried out using Phosphotungstic Acid / $MgCl_2$ reagent supplied by Sigma-Aldrich Ltd. UK. Low density lipoprotein cholesterol was calculated using the Friedewald formula (Friedewald et al 1972). The formula for calculating LDL cholesterol is limited to serum samples with triglyceride levels below 4.0mmol/L. All of the pre exercise samples had low triglyceride levels and very few of the post exercise samples in this study presented with levels in this range.

2.6 Lipoprotein (a) assay

Isolated serum was frozen and stored at -70°C until the study was completed, then the paired samples from each recruit were thawed at 37°C and assayed together. The Lp(a) concentration was determined using an ELISA based assay supplied by Immuno Ltd. UK.

A buffer concentrate (0.4mol/L Tris-HCl, pH8.4 Sigma-Aldrich Ltd. UK) was first made up to one litre, and was used to reconstitute the assay calibrators and quality control samples. Serum samples were then diluted 1in 500 in buffer. The diluted serum (100μl) was then added to the ELISA plate wells containing conjugate (100μl) and incubated at room temperature, for two hours. The conjugate consisted of a monospecific anti-apo(a)-Fab-fragment coupled with peroxidase. During the incubation Lp(a) particles and free apo(a) are bound to the plate and simultaneously marked by conjugate. The plate was then washed with buffer (3 x 200μl) to remove all the non-specific serum components and unbound conjugate. Substrate (200μl) was then added to all the wells and incubated at room temperature for thirty minutes.

The substrate consisted of the chromogen, tetra-methyl-benzidine (TMB), in ethanol and dimethyl sulphoxide (DMSO). The reaction was stopped by the addition of sulphuric acid (50μl/1.9mol/L) to each well. The colour produced by the reaction was directly proportional to the Lp(a) concentration of the sample.

Optical density was measured at a wavelength of 450nm using a Multiskan MCC/340 MKII ELISA plate reader (ICN Flow UK). The standard curve for the assay was plotted using the calibrators and the Lp(a) concentration of each sample was determined using Titertek Titersoft II software (ICN Flow UK). The high and low quality control samples provided with the kit ensured the sensitivity of the assay. To improve the range of the Lp(a) assay and to accurately determine the concentration of Lp(a), samples with an Lp(a) concentration >0.70g/L were re-assayed at a higher dilution (1 in 1000).

2.7 Plasma fibrinogen sample collection

Trisodium citrate was made up to a concentration of 3.8% in normal saline. This anticoagulant was pipetted into plastic 10ml tubes (Alpha Labs UK), capped and kept at 4°C. Whole blood (9ml) was added to these tubes by syringe and the sample was mixed by gentle inversion. On arrival in the laboratory, the samples were centrifuged at 300g for 15 minutes at 4°C. Platelet poor plasma was collected from each tube and the samples stored at -20°C until the trial was completed.

2.8 Plasma fibrinogen assay

The fibrinogen concentration of each pair (pre & post) of recruit samples was determined using the Clauss method. This nephelometric assay employs the addition of calcium thromboplastin to citrated plasma samples to simultaneously determine the prothrombin time and fibrinogen concentration. Normal plasma of known fibrinogen concentration was diluted to make a calibration curve. The fibrinogen concentration is determined by comparing the rate of clot formation in the samples with that of the normal plasma. The turbidity of the samples is measured at 660nm during the thrombin enduced clotting and a value for fibrinogen is calculated. This method was adapted for an ACL 300 instrument (IL Laboratories, Warrington UK), and the laboratory reference range for the assay is 200 - 350mg/dl.

2.9 Quality Assurance

All of the routine chemical pathology assays described in the materials and methods section above are part of an external quality assurance scheme. This national scheme is operated from Bristol Royal Infirmary and involves the analysis of three monthly samples for each test. The Lp(a) and fibrinogen assays also take part in this scheme. The results of the assays are reported to the centre at Bristol. The coefficients of variance (CV) for all the results from all the participating centres are published on a monthly basis. Throughout this study the performance of the laboratory was in the top ten percent of all the participating centres.

CHAPTER 3

GARLIC STUDY MATERIALS AND METHODS

3.1 Subject selection

The investigation into the antioxidant effects of garlic was set up as part of a larger study into the effects of dietary garlic supplementation (Neil 1996). Participants were recruited through surveys in general practice. Men and women of European origin, aged 35-64 years, were eligible for inclusion in the trial given they met certain criteria. The selection procedure ensured that patients and controls were included in the trial after repeated cholesterol measurement, and a research nurse advised participants about their diet before the study started. The advice related the amount of saturated and unsaturated fat in the diet along with the recommended amounts of carbohydrate, protein and fibre. Finally, a research nurse reviewed all of the participants eight weeks after randomisation.

Study subjects were included in the trial if they had a TC concentration of 6.5-9.0mmol/L on screening and a repeat fasting concentration of 6.0-8.5mmol/L, and an LDL ≥3.5mmol/L. Subjects were excluded from the trial if their fasting Tg were ≥5.6mmol/L, or their HDL concentration was ≥2.0mmol/L. Further details of the exclusion criteria are set out in the paper by Neil et al 1996.

Participants in this double blind, randomised, six month parallel trial, took 900mg/day of Kwai™ dried garlic tablets (standardised to 0.6% Allicin, 1.3% Allinin) or placebo. The placebo tablets were lactose and were coated with a small amount of garlic (<0.01% Allicin) to give them a slight odour and so to make them indistinguishable from the garlic tablets. The larger trial into the effects of garlic on serum lipids was completed by 115 subjects.

3.2 Sample collection and storage

The parent study reported final data on 57 garlic and 58 placebo volunteers. Additional EDTA plasma samples were collected from these participants and sent on ice by courier to our laboratory. Samples were separated by centrifugation at 600g for 10 minutes at 4°C. The isolated plasma was stabilised by the addition of a 600g/L sucrose solution (1%w/v) (Rumsey et al 1992 modified by Klienveld et al 1992). The sucrose was added to the plasma to prevent LDL aggregation during storage at -70°C. The samples were stored for a maximum of twelve months. The analysis presented here was restricted to the 20 subjects receiving garlic and 11 control subjects receiving placebo after a compliance level of ≥75% had been applied to all the participants.

3.3 Lipid and lipoprotein analysis

The lipid and lipoprotein, excluding Lp(a), data presented here was first published by Neil et al in 1996. The number of subjects in the garlic and placebo groups in this presentation is smaller than in the original study because the results were based on the number of paired samples obtained for LDL oxidation analysis.

Total cholesterol and triglycerides in plasma were determined by standard enzymatic methods. HDL was determined after precipitation of the Apo B fractions by phosphotungstate, and LDL was calculated using the Friedewald formula (Friedewald et al 1972). Apoproteins A-I and B were determined by immunoturbidimetry. All of these assays used reagents supplied by Boehringer Mannheim UK.

Plasma levels of Lp(a) were determined using the Immuno ELISA based assay described in Chapter 2.6. The total cholesterol, apolipoprotein B and the Lp(a) concentration of isolated LDL were measured using the same enzymatic, immunoturbidometric and ELISA based techniques outlined above. Again all of these assays were supported by participation in quality assurance schemes.

3.4 LDL Isolation and copper induced oxidation

The original method of Esterbauer et al. (1989) modified by McDowell et al (1995) was used to determine the copper induced oxidation of isolated LDL. Briefly, the density of isolated plasma was increased to 1.30kg/L by dissolving potassium bromide (KBr) in the plasma (0.4946gKBr/ml plasma). The density adjusted plasma (0.9ml) was then underlayered into a 'Quickseal' 3ml polyallomer tube (Beckman Instruments UK) containing 2.1ml. 0.15M saline (d=1.006g/ml). Using a Beckman TL-100 bench-top ultracentrifuge and a fixed angle rotor the density adjusted plasma was centrifuged at 541,000g (100,000rpm) for 60 minutes at 4°C. The isolated LDL fraction was clearly identifiable as a single orange coloured band in the middle of the tube. The isolated LDL was removed from the tube by careful tube piercing with a needle and syringe.

Between 0.7 and 1ml of LDL was isolated as an orange coloured band in the centre of the 'Quickseal' tube. The KBr was then removed by ion exchange chromatography using a PD-10 column containing Sephadex G-25 in 0.15M saline (Pharmacia Biotech UK). Albumin was removed by filtering the desalted LDL through a 0.45μm filter (Sigma-Aldrich UK).

The protein concentration of the desalted LDL was determined using Pierce Coomassie Plus reagent (Pierce & Warriner UK Ltd), in a microtiter plate assay (Redinbaugh 1985).

The LDL was then further diluted in 0.15M saline to a final protein concentration of 50mg/L. Aliquots of 950μl were then incubated in the presence of 50μl of a solution of cuprous chloride (40μmol/L) at 37°C. The optical density (OD) of the reaction was measured at 234nm every 3 minutes using a Kontron 932 spectrophotometer (Kontron Instruments UK). The remaining desalted LDL was stored in aliquots containing sucrose at -70°C. The TC, apoprotein B and Lp(a) concentration of these aliquots were analysed using the methods outlined earlier. From the optical density (OD) data an oxidation profile of the LDL was drawn and the lag time for the reaction calculated.

3.5 LDL Lag Time Calculation

The lag time of each curve was calculated by plotting the lines of intercept between the slope of the initial phase and the slope of the propagation phase for the oxidation reaction. The point where these two lines intersect gives a position for a third vertical line that was drawn onto the x-axis (time) of the graph. The point at which the third line intercepts the x-axis denotes the lag time for the isolated LDL. Two methods were used to calculate the lag time of each curve. The first method plotted the OD data against time on graph paper and lines of intercept between the initial and propagation slopes were drawn by hand using a pencil and ruler.

The second method calculated the point of intercept between the initial and propagation slopes using a computer programme. Mr. P Butowski, (Anthra Pharmaceuticals Inc. UK), set up the computer programme in Visual Basic 5.0 to perform the calculations. The programme determined the line of best fit between the slope of the initial phase and the slope of the propagation phase and thus calculated the LDL lag time for the reaction from the raw data.

3.6 Determination of Vitamin E

The vitamin E (α-tocopherol) content of the plasma samples (n=110) was determined using a high-performance liquid chromatography (HPLC) procedure (Rice-Evans, Diplock and Symonds 1991). There were several stages involved in the vitamin E assay, which are outlined below.

Firstly, a stock solution of δ-tocopherol (Sigma Aldrich Ltd. UK) was made up in HPLC grade hexane. The molecular weight of δ-Tocopherol = 402.7, therefore 21mg of δ-tocopherol in 25ml Hexane = 2.08mmol/L (2080μmol/L). This δ-tocopherol stock solution was then added to all the plasma samples before extraction. Plasma (1ml) plus δ-tocopherol sock (20μl) gave a final δ-tocopherol concentration of 40.78μmol/L.

20μ stock + 1000μ Plasma = 1020 ÷20 = 51(dilution factor)

2080μmol/L stock ÷51(dilution factor) = 40.78μmol/L δ-tocopherol final plasma concentration. The δ-tocopherol was added to all the plasma samples before the extraction and acted as an internal standard for the efficiency of extraction procedure.

The α-tocopherol standard curve was then set up as follows. The α-tocopherol was supplied by Sigma Aldrich Ltd. UK, had a molecular weight of 430.7, and was 95% pure. A stock α-tocopherol solution was made up as by dissolving 10.767mg in 25ml of HPLC grade hexane to give a concentration of 250μmol/L α-tocopherol.

A standard curve was made up by diluting the stock α-tocopherol solution in distilled water and had a range from 0-100μmol/L. The standards did not contain the δ-tocopherol internal standard.

The samples and standards were then extracted according to the procedure set out below. Plasma or standard solution (1ml) was pipetted into a glass tube with a screw cap. The stock δ-tocopherol solution (20μl) was then added to all the plasma samples and they were mixed. Hexane (4ml) and HPLC grade ethanol (2ml) were then added to the each tube. The tubes were then capped and vortex mixed for twenty seconds. The tubes were then centrifuged for five minutes at 2000rpm at 0°C. After centrifugation the upper 2ml of the organic phase containing the vitamin E was removed. The vitamin E containing extract (1ml) was then transferred into a auto-sampler tube and placed into an auto-sampler rack.

The standards and the extracted plasma samples were separated in the normal phase by size exclusion chromatography using a Beckman System Gold HPLC set up (Beckman Instruments UK). This set up included a Micropack Si5 column (150 x 5.4mm), HPLC grade hexane mobile phase, and a flow rate of 2.0ml/minute.

The assay was run overnight using a programmable auto-sampler. As the α, and δ-tocopherol were eluted off the column both forms were detected in the ultraviolet region at 294nm. As the samples were analysed and the retention time (RT) and peak area (PA) of both forms of tocopherol were printed out. The retention time was used to identify each peak as α, or δ-tocopherol, while the peak area was used to determine the concentration of each tocopherol.

A standard curve was constructed using the peak areas of the standard solutions. Using the Graphpad Prism software package (USA) the peak area produced by each of the extracted plasma samples was used to determine the concentration of α-tocopherol and δ-tocopherol present in each sample.

3.7 Determination of antibodies to oxidised LDL

Antibodies to oxidised LDL were determined using the oLAb microtiter plate assay following the manufacturers instructions (Biomedica, Austria). The kit employs copper oxidised LDL as an antigen coated onto microtiter plate wells.

Firstly the assay buffer concentrate is diluted 1:10. This buffer was used to wash the plate and to pre-dilute (1:50) the calibrators, controls and serum samples. The pre diluted calibrators, control and serum samples (220μl) were then pipetted into the wells of the coated microtiter plate. The plate was incubated at room temperature for 90 minutes and the autoantibodies if present bind specifically to the oxidised LDL antigen. The plate was washed in buffer (4 X 300μl). After the wash an anti-human IgG antibody conjugated to a specific peroxidase conjugate (100μl) was added to each well and incubated at room temperature for a further 30 minutes. The plate was then washed in buffer (4 X 300μl) for a second time to remove any unbound conjugate.

After the final washing step 100μl of the chromogenic substrate tetramethylbenzidine was added to each well. The plate was incubated for a further 30 minutes at room temperature in the dark and the colour reaction was stopped by the addition of 50μl of stop solution (1.9mol/L Sulphuric Acid). The colour change of the reaction was directly related to the presence of bound IgG autoantibodies. The reaction was measured at 450nm against a standard curve derived from the calibrators provided.

3.8 Determination LDL subfractions

This was carried out using 3% nongradient polyacrylamide gel electrophoresis rods (Quantimetrics Corp. USA). The technique as modified by Muniz separates LDL subfractions from whole serum or plasma on the basis of size and charge and defines mobility relative to that of VLDL and HDL (Muniz 1977, Rajman et al. 1996).

Briefly, 25μl of plasma was mixed with 200μl of stacking gel and applied to the top of the gel rod containing 3% polyacrylamide. The stacking gel contained acrylamide (0.24g/L), N, N, methylenebisacrylamide 0.02g/L and Sudan Black B (0.36g/L). The stacking gel containing the sample was then photopolymerised for 30 minutes. The electrophoresis was carried out for 60 minutes at 3mA per gel rod. Up to seven LDL subfractions have been defined using this nongradient technique.

The individual and combined area of each subfraction was calculated by gel densitometery. Gels were scanned using a Bio-Rad GS-700 imaging densitometer set to 84μm (300dpi). The integrated area of each LDL subfraction was then quantified using the Molecular Analysis software (Bio-Rad UK).

The subfractions were expressed according to the manufacturer's instructions and by the notation adopted by Musliner and Krauss 1988 which defined the combined LDL subfraction areas are as;

Large or Light LDL = LDL 1 + 2 (d <1.038g/ml)

Small or Dense LDL = LDL 3 + 4 + 5 (d >1.038g/ml)

The changes in LDL subfraction areas of placebo and garlic groups were then compared using both notations.

CHAPTER 4

ARMY RECRUIT RESULTS

4.1 Army recruits definitions

Initially 318 recruits were studied and of these 206 recruits completed the training and provided pre and post serum samples. A smaller number of the recruits provided pre (n=243) and post (n=151) training plasma samples needed for the fibrinogen assay. The missing results are due to the constraints of the training programme, incomplete recruit data pairs and in some cases small serum sample volumes.

Each recruit group represents the time after the ME when the blood samples were taken. These groups contain the maximal number of paired results available. This use of all the data has given rise to different numbers of recruits being present for some tests in each group. Group 6 for example contains 67 pairs of cholesterol results while there are only 54 pairs of Lp(a) results available for this group.

Recruit group 1 (12 hours) n=41.

Recruit group 2 (24 hours) n=13.

Recruit group 3 (2 days) n=31.

Recruit group 4 (3 days) n=15.

Recruit group 5 (4 days) n=39.

Recruit group 6 (5 days) n=67 (max), 27 (min)

4.2 Statistical analysis

All of the pre and post exercise results were compared using a Students paired t test. The parametrically distributed data is expressed as n, mean, standard deviation (S.D.), and p value. The triglyceride and lipoprotein (a) data were non-parametrically distributed and were log transformed prior to the t test. These results are expressed as n, median, minimum, maximum, and p value. The degree of statistical significance (Sig), and the p value, achieved by each two tailed paired t tests is expressed as follows:

$p>0.0500$ is not significant = ns

$p<0.0500$ is significant = *

$p<0.0050$ is significant = **

$p<0.0001$ is significant = ***

All transformations and statistical analysis were performed using the Graphpad Prism software package (USA). The results of each test for each group of recruits are shown as a series of tables and as a graphs or figures of the change (post – pre = change) for each test.

4.3.1 Baseline Results

The table below shows the mean and standard deviation for those variables measured in each recruit at intake, including smoking and non-smoking data. Blood pressure readings were obtained before and after five minutes of supine rest.

Table 4.3.1	Mean	± S.D.
Age (Years)	18.8	0.11
Height (Centimetres)	175.4	0.5
Weight (Kilograms)	67.9	0.6
BMI (kg/M)	22.2	0.12
Initial Blood Pressure (mm Hg)	119 / 72	0.85 / 0.74
Final Blood Pressure	120 / 70	0.84 / 0.85
Smoking / Non-smoking (n)	178 / 140	

All of the recruits were free of clinically evident cardiovascular disease and in order to be entered into the training programme all had a high level of cardiovascular fitness. The initial sample (T1) showed no significant differences between groups for any of the analytes measured.

Table 4.3.2 Total cholesterol results mmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	3.74 (0.71)	4.07 (0.82)	0.33	0.0014	**
G2	13	4.24 (0.58)	4.18 (0.54)	-0.06	0.7052	ns
G3	31	3.91 (0.73)	3.64 (0.72)	-0.27	0.0180	*
G4	15	3.95 (0.56)	4.13 (0.47)	0.18	0.1558	ns
G5	39	3.49 (0.49)	3.88 (0.60)	0.39	<0.0001	***
G6	67	3.95 (0.73)	4.23 (0.72)	0.28	0.0004	***

Table 4.3.2 shows the change in total cholesterol for each recruit group after the military exercise. Total cholesterol levels increased significantly 12 hours after the ME. Reductions occurred after 1 and 2 days followed by significant increases on days 4 and 5.

Table 4.3.3 LDL cholesterol results mmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	2.03 (0.71)	2.06 (0.68)	0.03	0.6951	ns
G2	13	2.18 (0.65)	1.94 (0.61)	-0.24	0.1976	ns
G3	31	2.19 (0.71)	2.00 (0.61)	-0.19	0.0726	ns
G4	15	2.13 (0.56)	1.77 (0.51)	-0.36	0.0487	*
G5	37	1.83 (0.55)	1.52 (0.52)	-0.31	<0.0001	***
G6	67	2.09 (0.70)	2.13 (0.65)	0.04	0.5867	ns

Table 4.3.3 shows the change in LDL cholesterol for each recruit group after the military exercise. LDL concentrations were increased 12 hours after the ME. All of the other recruit groups sampled displayed reductions in LDL that reached significance on days 3 and 4.

Table 4.3.4 HDL cholesterol results mmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	1.37 (0.23)	1.43 (0.24)	0.06	0.0666	ns
G2	13	1.25 (0.25)	1.17 (0.21)	-0.08	0.1801	ns
G3	31	1.35 (0.29)	1.11 (0.23)	-0.24	0.0007	***
G4	15	1.39 (0.21)	1.32 (0.21)	-0.07	0.2338	ns
G5	37	1.35 (0.26)	1.25 (0.24)	-0.10	0.0078	**
G6	67	1.29 (0.27)	1.25 (0.27)	-0.04	0.0877	ns

Table 4.3.4 shows the change in HDL cholesterol for each recruit group after the military exercise. HDL levels showed a similar pattern to that of LDL, with a small increase after 12 hours and reductions in concentration on all the subsequent days.

Table 4.3.5 Triglyceride results mmol/L

	n	Pre (Range)	Post (Range)	Δ	p	Sig.
G1	41	0.72 (0.23-1.75)	1.10 (0.40-2.72)	0.38	<0.0001	***
G2	13	1.56 (0.73-4.03)	2.18 (0.78-5.28)	0.59	0.2343	ns
G3	31	0.86 (0.35-2.02)	1.16 (0.45-3.69)	0.30	0.0069	**
G4	15	0.93 (0.45-1.74)	1.70 (0.71-6.60)	0.77	0.0007	***
G5	39	0.64 (0.33-1.69)	2.14 (0.96-5.95)	1.50	<0.0001	***
G6	67	1.08 (0.42-2.47)	1.73(0.51-4.45)	0.65	<0.0002	***

Table 4.3.5 shows the serum concentrations of triglyceride increasing gradually in all the recruit groups tested. The increase in triglyceride reached statistical significance 12 hours and 5 days after the military exercise. This pattern of increased serum triglyceride was matched by reduction in the concentration in serum glucose in all the recruits.

Table 4.3.6 Glucose results mmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	4.7 (0.92)	4.3 (0.90)	-0.4	0.0300	*
G2	9	4.6 (1.11)	4.0 (0.96)	-0.6	0.0184	*
G3	12	4.9 (0.97)	4.4 (1.02)	-0.5	0.3128	ns
G4	15	5.1 (1.02)	4.4 (0.75)	-0.7	0.0354	*
G5	37	4.2 (0.96)	4.0 (0.69)	-0.2	0.2010	ns
G6	41	4.4 (1.01)	4.1 (0.74)	-0.3	0.1650	ns

All of the recruits tested displayed reduced glucose levels after the ME, Table 4.3.6. These reductions were significant in the recruits tested 12 hours, 1day and 4 days after the ME.

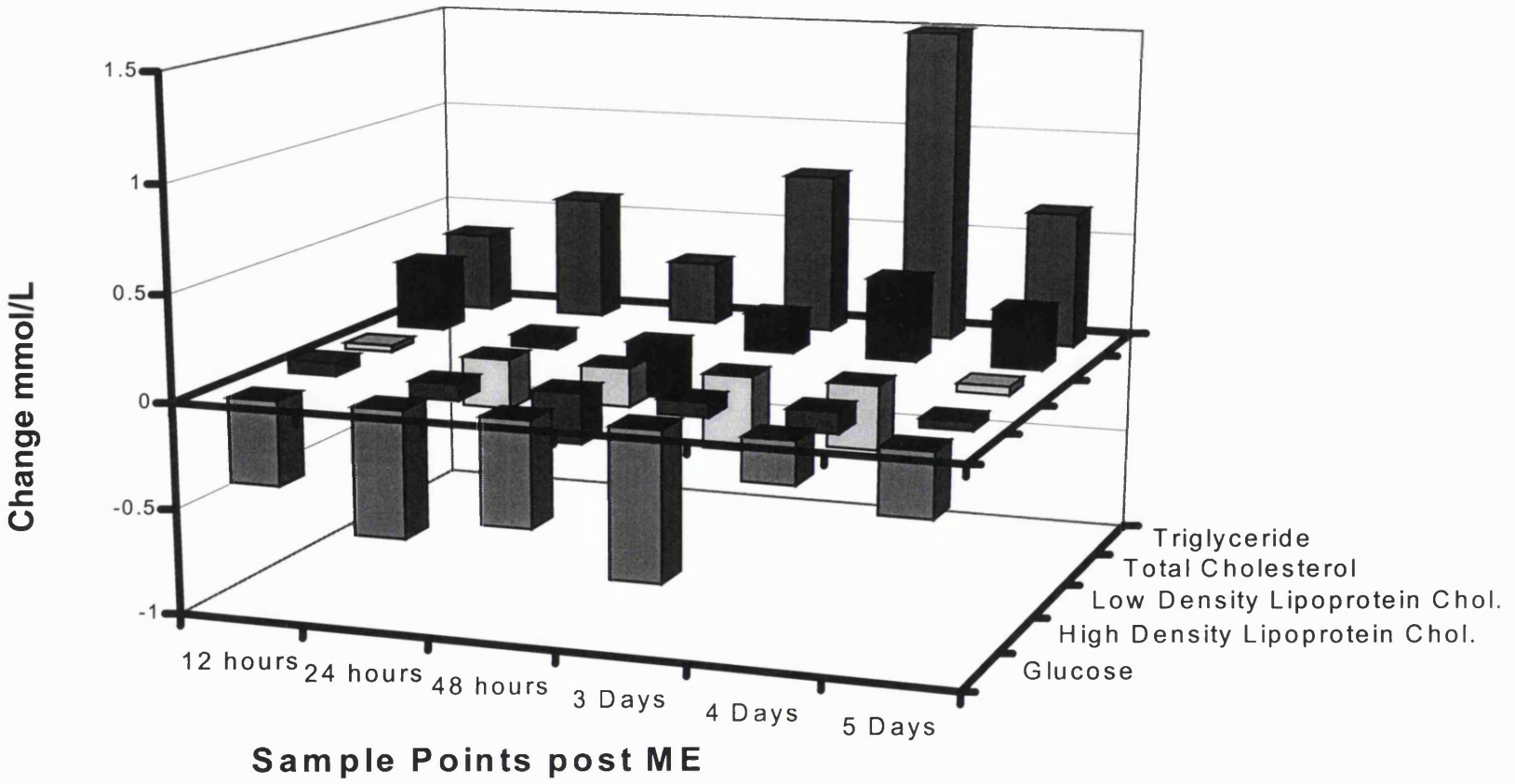


Figure 4.3a Combined lipid and glucose data. This figure shows the mean or median change serum lipids and glucose for each group of recruits.

Table 4.3.7 Lipoprotein (a) results g/L

	n	Pre (Range)	Post (Range)	Δ	p	Sig.
G1	39	0.09 (0.00-0.91)	0.10 (0.00-0.89)	0.01	0.0599	ns
G2	13	0.12 (0.01-1.00)	0.10 (0.01-0.86)	-0.02	0.3384	ns
G3	31	0.15 (0.00-0.68)	0.14 (0.00-0.59)	-0.01	0.5243	ns
G4	15	0.06 (0.00-1.05)	0.06 (0.00-0.93)	0.00	0.6266	ns
G5	35	0.13 (0.04-0.66)	0.11 (0.04-0.66)	-0.02	0.2216	ns
G6	54	0.13 (0.05-0.94)	0.12 (0.00-0.98)	-0.01	0.1317	ns

The changes that occurred in lipoprotein (a) levels are displayed in table 4.3.7. These results show no significant changes in Lp(a) concentration either during the acute phase response or by fitness training. Figure 4.3b shows the changes in Lipoprotein (a) that did occur in each recruit group

Figure 4.3b Combined Lp(a) data.

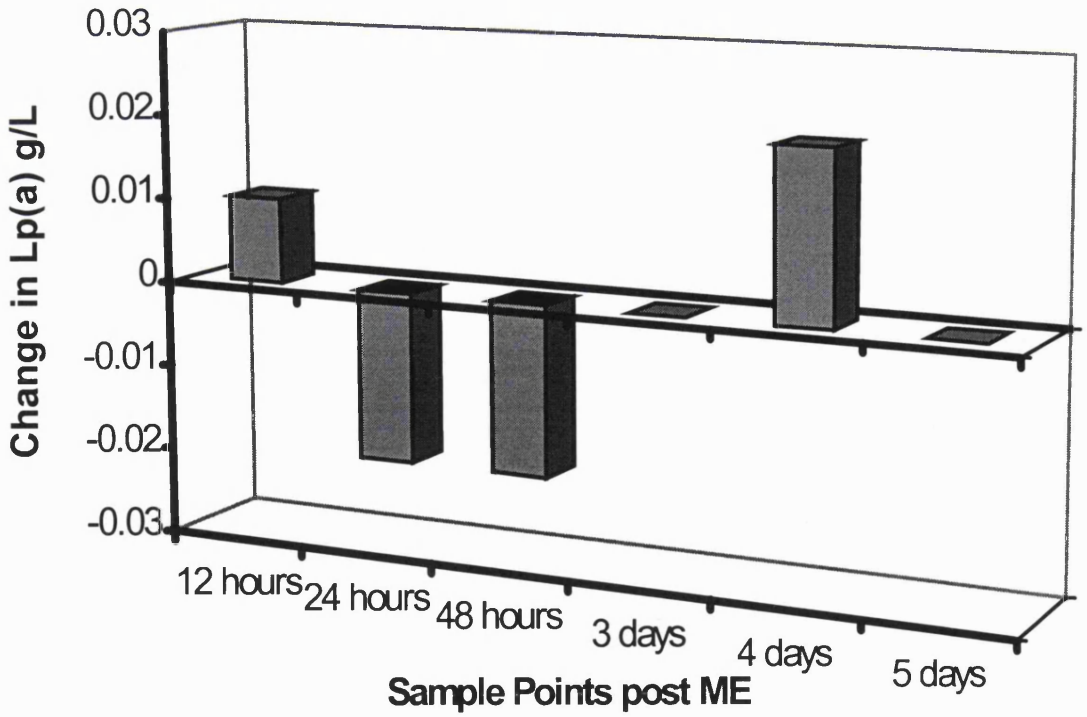


Table 4.3.8 Fibrinogen results mg/dl

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	263 (44.8)	266 (57.0)	3	0.7112	ns
G2	13	279 (39.8)	356 (71.6)	77	0.0011	**
G3	34	253 (48.4)	339 (85.5)	86	<0.0001	***
G4	19	270 (49.2)	311 (52.8)	41	0.0433	*
G5	33	245 (46.2)	247 (60.1)	2	0.9139	ns
G6	27	267 (46.5)	295 (57.4)	28	0.0298	*

The results in table 4.3.8 show the changes in plasma fibrinogen concentration for each recruit group. Fibrinogen was unchanged 12 hours after the ME, then levels increased significantly on days 1, 2, and 3, suggesting a strong acute phase response to the ME. The plasma concentration of fibrinogen was unchanged in the recruits tested 4 days post ME, those recruits sampled 5 days after the ME displayed a small but significant increase.

Table 4.3.9 C-reactive protein results g/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	5.4 (4.8)	8.2 (11.6)	2.8	0.1533	ns
G2	13	2.6 (3.2)	4.9 (6.0)	2.3	0.1105	ns
G3	13	4.3 (1.3)	14.0 (17.1)	9.7	0.0636	ns
G4	15	5.4 (4.0)	6.7 (1.5)	1.3	0.1388	ns
G5	34	6.5 (6.3)	4.9 (1.4)	-1.6	0.1705	ns
G6	25	7.0 (7.4)	6.3 (5.7)	-0.7	0.6962	ns

Table 4.3.9 shows increased serum CRP concentrations in the recruits sampled up to 3 days after the ME. The recruits sampled on days 4, and 5 display reduced levels of CRP. None of the changes in CRP were statistically significant.

Table 4.3.10 Albumin results g/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	49.3 (2.3)	48.7 (2.7)	-0.6	0.0160	*
G2	9	49.1 (3.6)	46.7 (2.5)	-2.4	0.0076	**
G3	12	48.2 (2.8)	46.6 (2.8)	-1.6	0.0487	*
G4	15	48.5 (2.0)	48.5 (2.1)	0.0	0.9439	ns
G5	39	50.6 (2.4)	48.5 (3.6)	-2.1	0.0014	**
G6	41	49.5 (2.6)	48.0 (3.3)	-1.5	0.0016	**

Table 4.3.10 shows the changes in serum albumin levels that occurred in each recruit group after the ME. Apart from recruits tested on day 3 all of the other recruit groups displayed significant reductions in albumin after the ME.

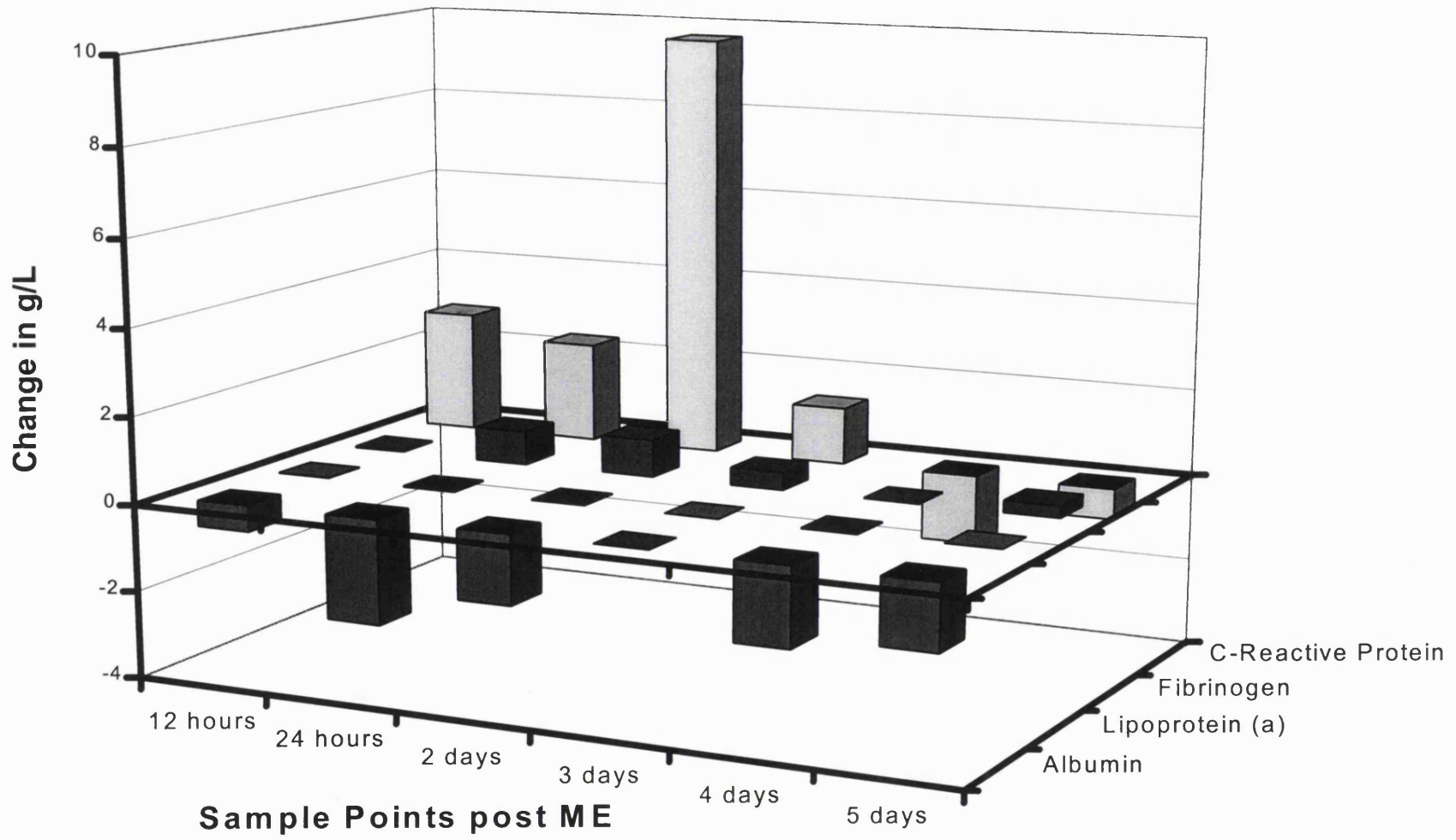


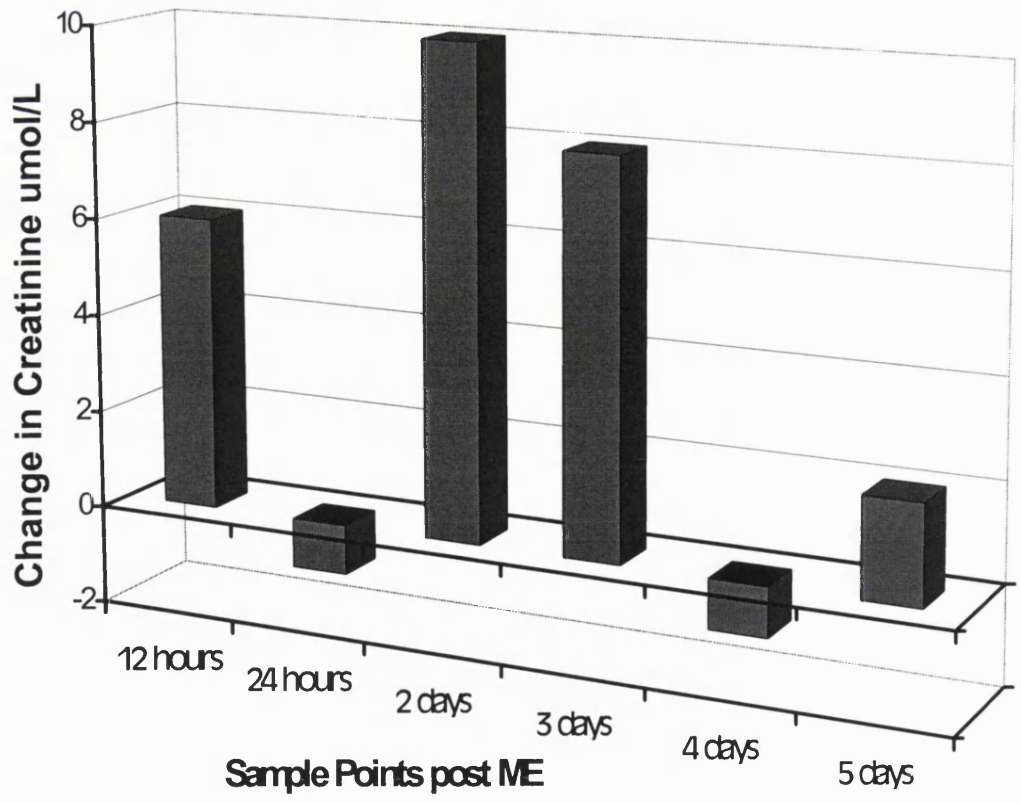
Figure 4.3c Change in acute phase response proteins and Lp(a) over five days after the military exercise

Table 4.3.11 Creatinine μmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	89 (8.8)	95 (8.4)	6	0.0001	***
G2	9	87 (6.4)	86 (8.3)	-1	0.5801	ns
G3	12	88 (11.8)	98 (13.4)	10	0.0055	**
G4	15	87 (7.5)	95 (10.1)	8	0.0045	**
G5	39	92 (9.9)	91 (9.7)	-1	0.5748	ns
G6	41	87 (10.2)	89 (9.7)	2	0.3457	ns

Table 4.3.11 shows the mean change in serum creatinine levels for all the recruit groups after the ME, while figure 4.3d shows the change (delta) in creatinine for each recruit group.

Figure 4.3d combined Creatinine data



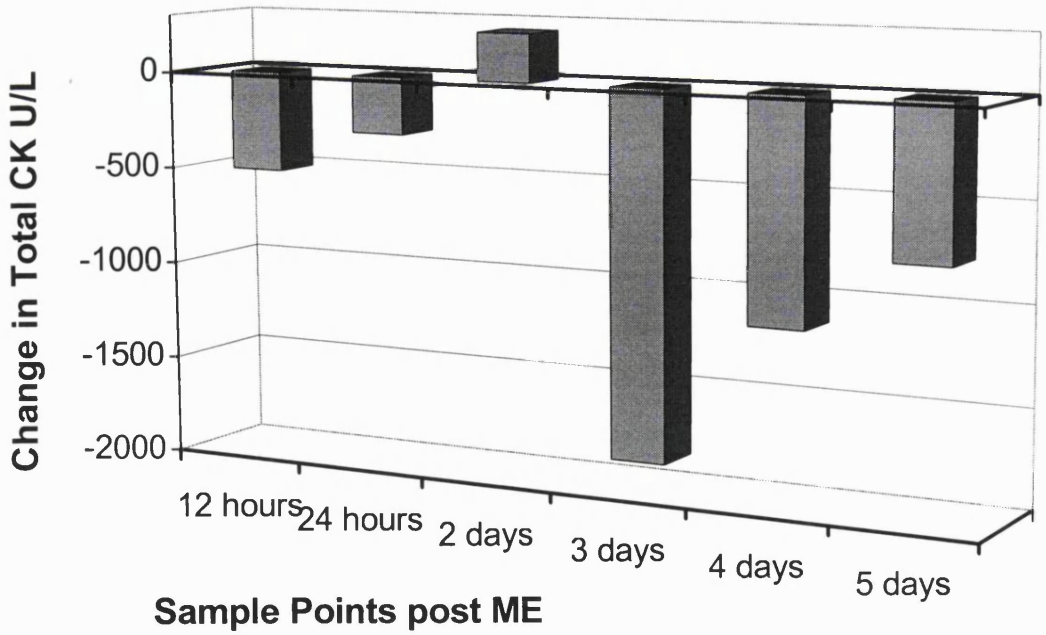
Creatinine levels were increased in many of the recruits after the ME. The increases were significant 12 hours, 2,3, and 5 days after the exercise. The increase in creatinine levels suggests that the ME was vigorous enough to produce muscle permeability changes in these young men.

Table 4.3.12 Total creatine kinase results U/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	1009 (1093)	495 (322)	-514	0.0013	**
G2	9	829 (926)	537 (269)	-292	0.4297	ns
G3	31	386 (170)	633 (537)	247	0.0081	**
G4	15	2390 (4530)	539 (612)	-1851	0.1476	ns
G5	38	1524 (1271)	398 (332)	-1126	<0.0001	***
G6	41	1468 (628)	698 (1063)	-770	0.0158	*

Table 4.3.12 shows the mean change in to total creatine kinase levels after the ME, while figure 4.3e below shows the change in total CK after the ME.

Figure 4.3e Combined total CK data



Total creatine kinase levels were significantly reduced in all the recruits sampled after the ME, apart from group 3 who displayed a significant increase. These large reductions are due to the very high levels of total CK seen in many of the recruits before the training programme or ME. A pattern of increased levels of total CK following strenuous exercise might be expected after the ME. This unexpected observation could in part be due to the recruits being inoculated shortly before the pre exercise samples were taken. The raised CK might also be because of local muscle damage due to the intra muscular injection or due to high levels of exertion by the recruits prior to their arrival at Bassingbourn.

This would also help explain the slightly raised levels of CRP seen in the pre exercise samples. Creatinine levels were increased in many of the recruits after the ME. These increases were significant 12 hours, 2, 3, and 5 days after the exercise. The increase in creatinine levels suggests that the ME was vigorous enough to produce muscle permeability changes in these young men.

Table 4.3.13 Urate mmol/L

	n	Pre (±S.D.)	Post (±S.D.)	Δ	p	Sig.
G1	41	0.47 (0.105)	0.41 (0.069)	-0.06	0.0009	***
G2	10	0.39 (0.072)	0.35 (0.058)	-0.04	0.0029	**
G3	12	0.46 (0.056)	0.45 (0.059)	-0.01	0.8648	ns
G4	15	0.42 (0.079)	0.33 (0.071)	-0.09	0.0066	**
G5	38	0.48 (0.079)	0.36 (0.071)	-0.12	<0.0001	***
G6	42	0.45 (0.076)	0.39 (0.059)	-0.06	0.0001	***

Figure 4.3e Combined Urate data

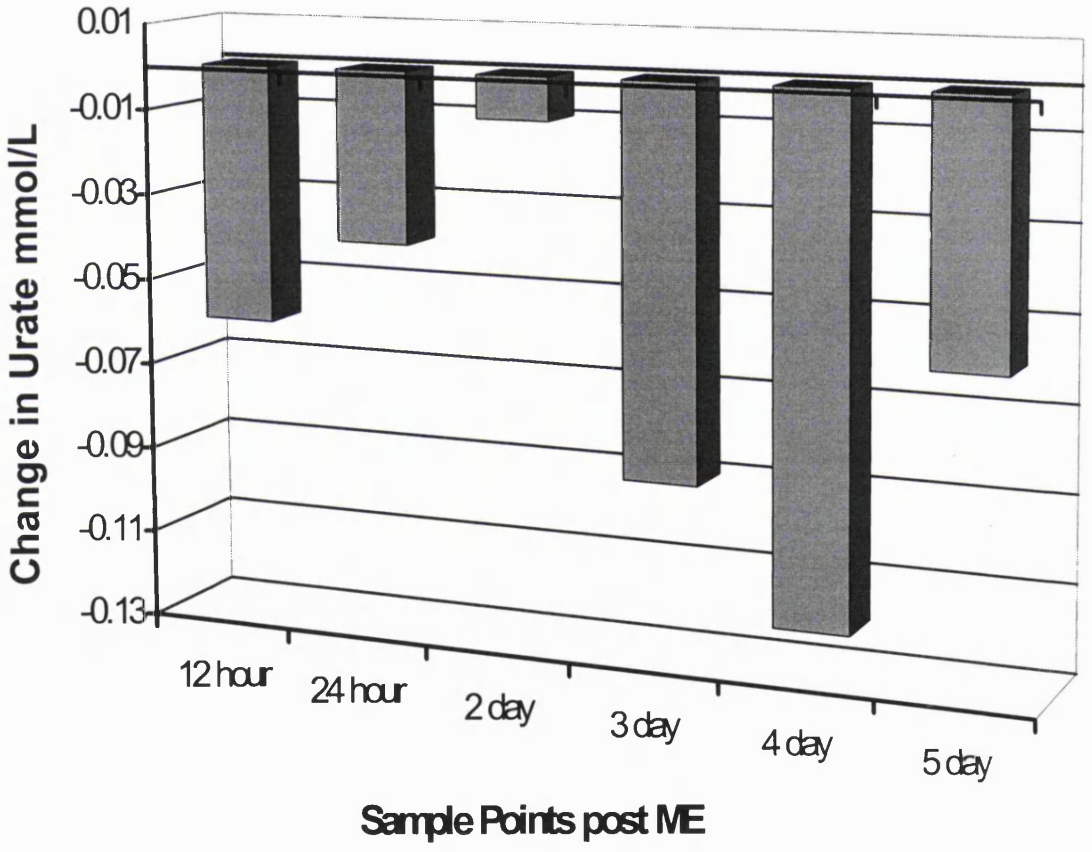
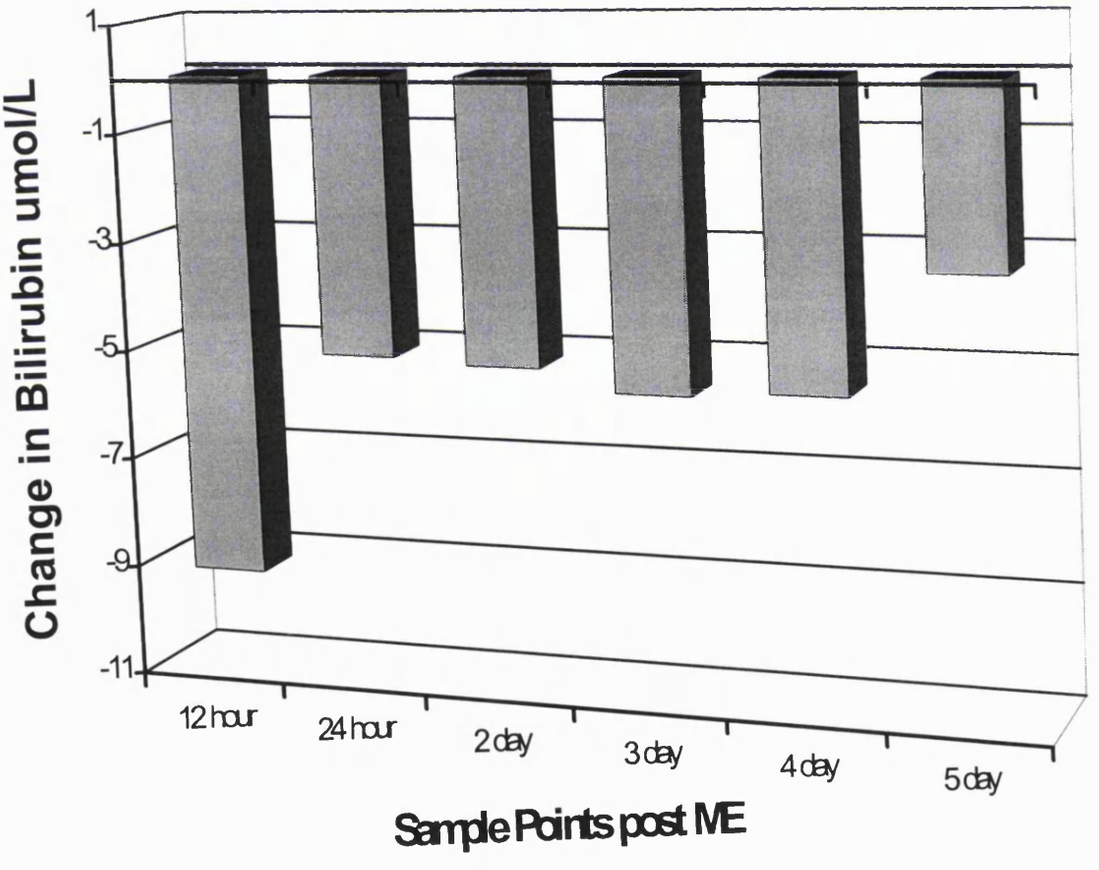


Table 4.3.14 Bilirubin μmol/L

	n	Pre (± S.D.)	Post (± S.D.)	Δ	p	Sig.
G1	37	14.2 (6.1)	4.7 (3.4)	-9.5	<0.0001	***
G2	8	12.3 (13.0)	4.6 (5.4)	-7.7	0.0437	*
G3	11	13.9 (4.0)	6.3 (2.6)	-7.6	0.0001	***
G4	14	10.0 (5.4)	3.7 (1.4)	-6.3	0.0013	**
G5	38	10.7 (5.3)	4.9 (4.0)	-5.8	<0.0001	***
G6	41	8.7 (4.5)	5.4 (3.4)	-3.3	<0.0001	***

Figure 4.3f Combined Bilirubin data



The data displayed in tables 4.3.13 and 4.3.14 above are shown in figures 4.3e and 4.3f. This data shows that after the ME serum levels of bilirubin were significantly reduced in all the recruits. The fall in bilirubin is accompanied by similar reductions in urate concentrations. These reductions are also significant in many of the recruits tested except in recruit group 3 (2 day) where the fall in urate is not statistically significant.

Bilirubin and urate are known to act as antioxidants, and it is possible that the large reductions in both these metabolites are as a result of free radical damage due to the strenuous nature of the ME.

4.4.1 Pre exercise results

The large number of young men taking part in the study allowed a number of markers of premature CHD risk to be assessed in the pre training data. The relationship between serum Lp(a) concentration and the other markers was assessed in these young men. The table below shows the pre exercise lipid, Lp(a), fibrinogen and smoking habits of the army recruits. All data is expressed as mean and standard deviation, lipoprotein (a) and triglyceride results are expressed as median and range.

Table 4.4.1 Pre exercise data

	n	Mean / Median	S.D. / Range
Total Cholesterol	318	3.86	0.771
LDL-C	316	2.09	0.734
HDL-C	316	1.33	0.260
Triglycerides	318	0.84	0.23 – 4.03
Lipoprotein (a)	198	0.12	0.00 – 1.05
Fibrinogen	243	261	49.8
Smokers / Non-smokers	178 / 140		

The possible influence of the cigarette smoking on serum lipids, fibrinogen and Lp(a) was investigated by comparing the pre exercise data of the smoking and non-smoking recruits.

Table 4.4.2 Pre exercise smoking / non-smoking results

	Smokers (S.D.)	Non-smokers (S.D.)
n	178	140
Total Cholesterol	3.91 (0.81)	3.78 (0.69)
LDL-C	2.16 (0.77)	1.99 (0.63)
HDL-C	1.31 (0.26)	1.36 (0.26)
Triglycerides	1.01 (0.35-4.03)	0.92 (0.23-3.1)
Lipoprotein (a)	0.14 (0.0-0.94)	0.09 (0.0-1.05)
Fibrinogen	266 (50)	256 (49)

Table 4.4.2 compares the lipid, Lp(a) and fibrinogen concentrations of the smoking and non-smoking recruits. These results show that the serum / plasma concentrations of these analytes were very similar in both groups. Although the smokers have lower HDL and higher levels of TC, LDL and fibrinogen none of these differences was statistically significant. The smoking habits of these young men have not influenced the concentration of fibrinogen, or any of the other analytes measured. The lack of difference between smokers and non-smokers may be because they were all young (mean age 18.8 years) and also that many of these young men were not heavy smokers. These circumstances might therefore suggest that none of these young men had been exposed to the detrimental effects of long term cigarette smoke.

The pre exercise data was used to correlate Lp(a) levels with serum lipids and fibrinogen. This correlation was carried out using a Spearman test for paired non-parametrically distributed data. Table 4.4.3 shows the results of the Spearman correlation between Lipoprotein (a) and the other analytes.

Table 4.4.3 Pre exercise Spearman correlation results

All Lp (a) Data	Pairs	Spearman r	p Value	Sig
Total Cholesterol	198	0.1143	0.1088	ns
LDL Cholesterol	197	0.1931	**0.0065**	**
HDL Cholesterol	197	-0.2084	**0.0033**	**
Triglycerides	198	0.0888	0.2132	ns
Fibrinogen	155	0.05274	0.5145	ns

The results show a small but significant positive correlation between LDL and Lp(a) and a significant negative correlation between HDL and Lp(a). The raised levels of Lp(a) are associated with higher LDL and lower HDL concentrations. In these young men the cholesterol levels would not be regarded as risk factors but the increased Lp(a) concentrations could be considered a possible risk. There was no correlation between Lp(a) and TC, Tg and fibrinogen.

In order to study the relationship between raised Lp(a) concentration and the other variables more closely the Lp(a) data was subdivided into four groups of approximately equal size. The Spearman correlation was then repeated for the recruits with the highest Lp(a) values. Table 4.4.4 shows the mean, S.D., median and range for the analytes in the highest Lp(a) quartile.

Table 4.4.4 pre exercise highest Lp(a) group results

	n	Mean / Median	S.D. / Range
Lipoprotein (a)	50	0.44	0.27 – 1.05
Total Cholesterol	51	3.88	0.731
LDL-C	49	2.17	0.671
HDL-C	50	1.30	0.319
Triglycerides	50	0.86	0.36 – 2.26
Fibrinogen	40	265	53.3

The data in table 4.4.5 shows the Spearman correlation between the highest lipoprotein (a) group and the other analytes.

Table 4.4.5 Highest Lp(a) group Spearman correlation

	Pairs	Spearman r	p Value	Sig
Total Cholesterol	51	0.3613	**0.0092**	**
LDL Cholesterol	49	0.4496	**0.0011**	**
HDL Cholesterol	50	-0.3210	**0.0230**	*
Triglycerides	51	0.1947	0.1710	ns
Fibrinogen	41	0.3212	**0.0406**	*

The results for all the pre data (Table 4.4.3) show that there is a significant correlation between serum Lp(a) levels and LDL and HDL. This significant association is increased for those recruits with high levels of Lp(a). Table 4.4.5 shows a significant relationship between high Lp(a) concentration and fibrinogen is present although there is only a small increase in the median fibrinogen concentration when compared with the overall pre exercise data. The negative correlation between HDL and Lp(a) is maintained at high Lp(a) concentrations.

It is important to note that in the highest Lp(a) group the mean / median levels of cholesterol, Tg and fibrinogen are no different to those for the recruit group as a whole. This indicates that although raised Lp(a) levels correlate significantly with total, HDL, and LDL, and fibrinogen they are not associated with increased levels of these analytes in these young men.

4.5.1 Data Correction

Many studies into the effects of exercise have employed correction factors to the data in order to account for changes in fluid volume due to dehydration and changes body weight between the start and end of the study (Foger et al 1994, Kantor et al 1987, and Davis et al 1992). The body weight of the recruits was not recorded at the end of the study therefore no correction for this change could be made. Dehydration following intense exercise leads to haemo-concentration and could produce falsely high levels of serum proteins.

The possible effects of haemo-concentration following this type of exercise are most likely to be seen in the samples taken 12 and 24 hours after the exercise, where the recruits are still recovering from the exhaustive effects ME. Any dehydration occurring in these recruits would be offset by the fact that they had access to both water and food rations throughout the exercise, thus helping to offset any dehydration. The supervisors of the training programme are well aware of the dangers of dehydration and a great deal of care is taken to ensure the recruits do not become dehydrated during the ME. The recruits sampled in the days following the ME had more time to recover from any dehydration brought on by the ME and as such the impact of dehydration would not have a large influence on the results.

It is also important to note that if haemo-concentration had occurred its effects would be to increase the concentration of all the serum proteins measured. Increased levels of serum albumin in particular would indicate dehydration, but this was not seen.

Many of the recruits displayed both significant increases and decreases in serum proteins at any time point suggesting that when the samples were taken from the recruits they were not dehydrated.

There were a number of limitations and constraints on this study due to the large number of recruits involved, and the military surrounding where the trial was conducted. The pressure on the recruits and their instructors to complete all the parts of the training on occasion made obtaining serum and citrated blood samples from all the recruits a low priority. The limitations of the military setting also had benefits in that the environment was controlled and consistent for all these young men. During the ten weeks the lifestyle of these young recruits will have changed dramatically. A number of these lifestyle factors were under the control of the military including sleeping patterns, work, exercise, diet and recreation, and these factors were similar for all the recruits.

CHAPTER 5

GARLIC STUDY RESULTS

5.1.1 Statistical analysis

This results section shows the pre and post supplementation data for the placebo and garlic groups. This section also contains data obtained from participants who provided pre and post treatment samples but did not take either placebo or garlic tables. All the results are expressed as mean, standard deviation (S.D.), and n. Data with non-parametric distribution are expressed as median, range, and n. A students paired t test was used to compare all the parametrically distributed pre and post data. Non-parametric distributed data, Tg, lipoprotein (a), vitamin E, and antibodies to oxidised LDL were all analysed using the Wilcoxon signed rank test.

When comparing the relationship or correlation between the pre garlic data a Spearman test for non-parametric data was used. This test was also used to compare the two methods used to calculate the lag time of oxidised low density lipoprotein. All the statistical analysis was carried out using the GraphPad Prism™ Software (GraphPad Software Inc. California USA). The level of statistical significance achieved by each two tailed paired t test or Spearman correlation are expressed as follows:

$p>0.500$ is not significant = ns

$p<0.0500$ is significant = *

$p<0.0050$ is significant = **

$p<0.0001$ is highly significant = ***

Table 5.1.2 shows the baseline data of the participants in the trial. This includes the group to which subjects were assigned, the number of trial subjects, the gender, age distribution, and smoking habits. The level of compliance of the participants is also shown.

Table 5.1.2 Baseline results

	Garlic	Placebo	Non-Compliant
n	20	11	16
Male : Female	13:7	9:2	10:6
Age (S.D.)	56.0 (7.0)	51.9 (6.2)	53.3 (7.7)
Smoking / Non-smoking	3 / 17	4 / 7	2 / 14
Compliance	≥75%	≥75%	0%

The compliance of the study participants was determined by tablet counting. At the end of the study all of the subjects returned the unused garlic or placebo tables to their general practitioner and from this the level of compliance was calculated. The number of subjects in garlic and placebo groups was reduced in the final analysis by applying a compliance level of 75%. Many of the studies into the effects of garlic supplementation have not accounted for the degree of compliance of the participants (Neil et al 1996).

The tablet counting gave rise to a third group of subjects who provided pre and post blood samples, but who did not take any of the tablets supplied. These groups of sixteen individuals had a compliance level of 0% and were referred to as the non-compliant group.

Table 5.1.3 shows the oxidation curve parameters of both groups before and after garlic supplementation. These curve parameters include optical density at time zero (Zero OD), slope of initial phase (Slope), intercept between initial and propagation slopes (Intercept), and maximum optical density of reaction (Maximum OD).

Table 5.1.3 Oxidation curve results

	Garlic Group n=34		**Placebo Group n=16**	
	Pre	**Post**	**Pre**	**Post**
Zero OD	0.63	0.58	0.67	0.54
Slope	0.018	0.019	0.018	0.019
Intercept	0.086	-0.126	0.749	-0.780
Maximum OD	1.88	1.87	2.00	1.91

These values show how the pre and post garlic oxidation curves are the same for both groups. The mean initial and maximum OD values for both groups are similar, indicating the LDL preparation and reaction between the copper ions and the LDL is consistent. Differences in the slope and intercepts of each individual oxidation curve are to be expected as these form the basis of the main parameter being measured, lag time. The main determinant of the conjugated diene curve is the time taken for the propagation phase to commence. This lag time between the initial phase and the start of the propagation phase was calculated as described in figure 5.1.3a. The method used to calculate LDL lag time from the oxidation curve data is shown below in figure 5.1.3a.

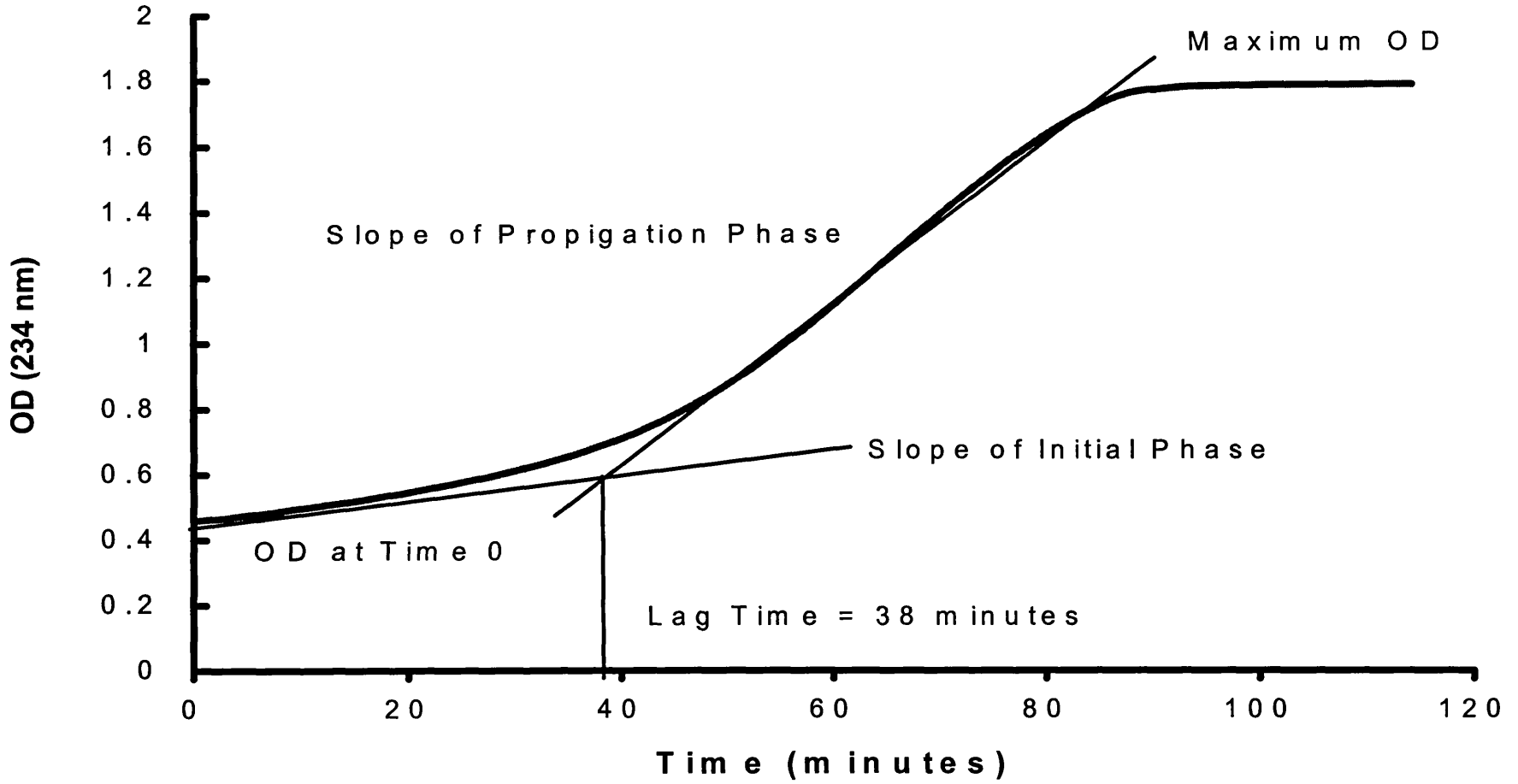


Figure 5.1.3a isolated LDL oxidation curve. This figure shows how lag time is calculated using the method of Esterbauer et al 1989, modified by McDowell et ai 1995.

Two methods of calculating lag time were used in this study. The first method plotted the OD data against time and lines of intercept between the initial and propagation were drawn by hand using pencil a ruler. The second method calculated the point of intercept between the initial and propagation phases using a computer programme set up by Mr. P Butowski (Anthra Pharmaceuticals Inc. UK). The data from both calculation methods are shown in table 5.1.4.

Table 5.1.4 Drawn and calculated lag time results

	Garlic Group n=34		**Placebo Group n=16**	
	Pre (S.D.)	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
Calculated Lag Time (min)	29.9 (8.2)	36.9 (11.9)	30.4 (11.9)	44.2 (15.5)
Drawn Lag Time (min)	39.9 (8.0)	46.4 (12.5)	42.8 (15.2)	54.4 (13.4)

The hand drawn method produced lag time data that was consistently longer than the calculated data. The spectrophotometer measured the optical density in four cuvettes simultaneously, producing data points every three minutes for each cuvette. Therefore a limited number of data points was produced for each reaction cuvette. The limited number of data points meant that the computer programme had difficulty in drawing a straight line along the slope of the initial phase. This led to the programme calculating a short initial phase slope and therefore shorter lag time values overall.

In order to compare the lag time calculation methods the pre study data from both groups were combined and the Spearman correlation for paired data was carried out.

Spearman r = 0.9708, p<0.0001*, n = 50.**

The results show a 97% correlation between the two methods of lag time calculation based on the pre lag time data. The lag time results used and referred to in the rest of this thesis are the drawn lag time data. The lag time data from both groups are shown in Table 5.4, along with the other indicators of oxidative status measured during the trial.

Table 5.1.5 Markers of lipid peroxdaion results

	Garlic Group n=20		**Placebo Group n=11**	
Mean/Median	**Pre (S.D.)**	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
Lag Time	39.9 (8.0)	46.4 (12.5)	42.8 (15.2)	54.4 (13.4)
Minutes	**p = 0.0134***		**p = 0.0002*****	
Vitamin E μmol/L	26.8	25.6	25.2	23.7
Range	(17.0-40.7)	(14.5-40.5)	(19.5-41.2)	(18.2-29.7)
	p = 0.2685 ns		p = 0.8953 ns	
oLAb mU/ml	293	299	221	328
Range	83-1392	125-1420	103-1126	119-1168
	p = 0.9331 ns		p = 0.5571 ns	

The results in table 5.1.5 show that a significant increase in lag time occurred in both garlic and placebo groups, with the increase in the placebo group reaching a higher degree of significance that that of the garlic group.

Although vitamin E is the major antioxidant of LDL there was a small decrease in the plasma concentration of this antioxidant in both groups by the end of the study. The levels of antibodies to oxidised LDL were unchanged in the garlic group while the placebo group displayed an increase in the concentration of these antibodies but the increase was not statistically significant.

5.1.6 Vitamin E extraction results

In order to test the effectiveness of the extraction procedure all of the plasma samples to be assayed had the δ-tocopherol internal standard added to them. The mean and S.D. of the peak area and retention time for both α, and δ-tocopherol are shown below.

Table 5.1.6 Vitamin E extraction data

Mean (S.D.)	Peak Area	Retention Time (minutes)
α-Tocopherol	2.52 (2.98)	3.25 (0.499)
δ-Tocopherol	2.83 (0.30)	5.79 (0.543)

The measured concentration of internal standard was then compared with set value, and the coefficient of variation of the internal standard was also calculated. Internal standard (δ-tocopherol) set value = 40.8μmol/L = 100%

Mean measured δ-tocopherol value = 38.4μmol/L (± 3.85μmol/L)

Set Value = 40.8μmol/L / 100 = 0.408μmol/L = 1%

Measured Value = 38.4μmol/L / 0.408 = 94.1

Therefore 94% of the δ-tocopherol present in the samples was successfully extracted. The coefficient of variation for the measured δ-tocopherol value was calculated to be 10%.

5.1.7 Analysis of Isolated LDL

Once the LDL had been prepared the total protein concentration was determined and an aliquot was stored for analysis. The total protein concentration of the isolated LDL (Iso.-LDL) was used as a basis for preparing the LDL for the oxidation reaction. Total cholesterol, apolipoprotein B and lipoprotein (a) concentrations were determined in the frozen aliquots of isolated and desalted LDL. The results of this analysis are shown in table 5.1.7.This analysis was set up to test the consistency of the LDL preparation and to examine the possible confounding effects of Lp(a) on the copper ion induced LDL oxidation.

Table 5.1.7 Isolated LDL results

	Garlic Group n=20		**Placebo Group n=11**	
	Pre (S.D.)	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
Total Protein	0.19 (55)	0.19 (43)	0.18 (33)	0.19 (38)
g/L	p = 0.8256 ns		p = 0.1394 ns	
Iso-LDL Apo B	0.33 (0.08)	0.34 (0.10)	0.34 (0.06)	0.34 (0.07)
g/L	p = 0.5861 ns		p = 0.9217 ns	
Iso-LDL Chol.	0.98 (0.36)	0.89 (0.24)	1.23 (0.36)	1.29 (0.61)
mmol/L	p = 0.2468 ns		p = 0.5420 ns	
Iso-LDL Lp(a)	0.05	0.06	0.06	0.06
Range	0.04-0.07	0.03-0.08	0.04-0.14	002-0.13
g/L	p = 0.1393 ns		p = 0.8311 ns	

Table 5.1.7 shows how the total protein yield from the LDL isolation was consistent for both groups before and after the garlic supplementation. The immunoturbidometric assay used to determine the apolipoprotein B levels gave consistently higher results for the apo B protein than the coomassie based total protein assay. Low density cholesterol concentrations were not significantly changed after the garlic supplementation. The control group did have higher levels of LDL cholesterol when calculated according to the Friedewald formula compared to the measured value.

The concentration of Lp(a) was not significantly different between groups, nor was it effected by the garlic supplementation. The Lp(a) concentration in the isolated LDL was not different between the groups before or after treatment. Lp(a) did not significantly alter the oxidation characteristics of either group. This suggests that Lp(a) does not confound the results of LDL oxidation.

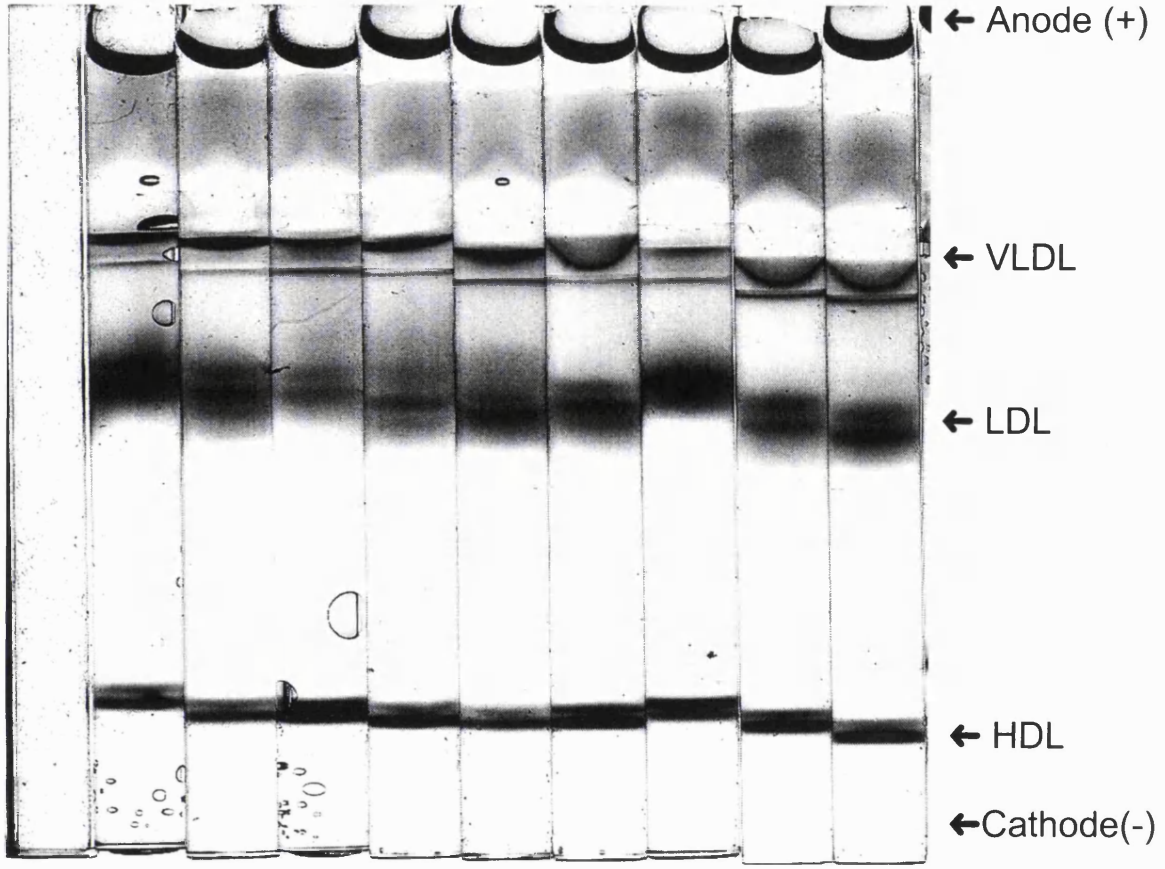
5.1.8 LDL subfractions analysis

The analysis of the distribution of LDL subfractions was carried out following the manufacturer's instructions (Quantimetrix Corp USA). This method resolved the LDL subfractions into two bands clearly visible to the eye. When the rods were analysed using imaging densitometery these LDL bands could be resolved into five LDL subfractions.

Figure 5.1.8a

Non-gradient Acrylamide Rod Electrophoresis

This figure shows the Lipoprint™ non-gradient acrylamide gel rods stained with Sudan Black B. The image is obtained by scanning the rods using a Bio-Rad GS-700 imaging densitometer.



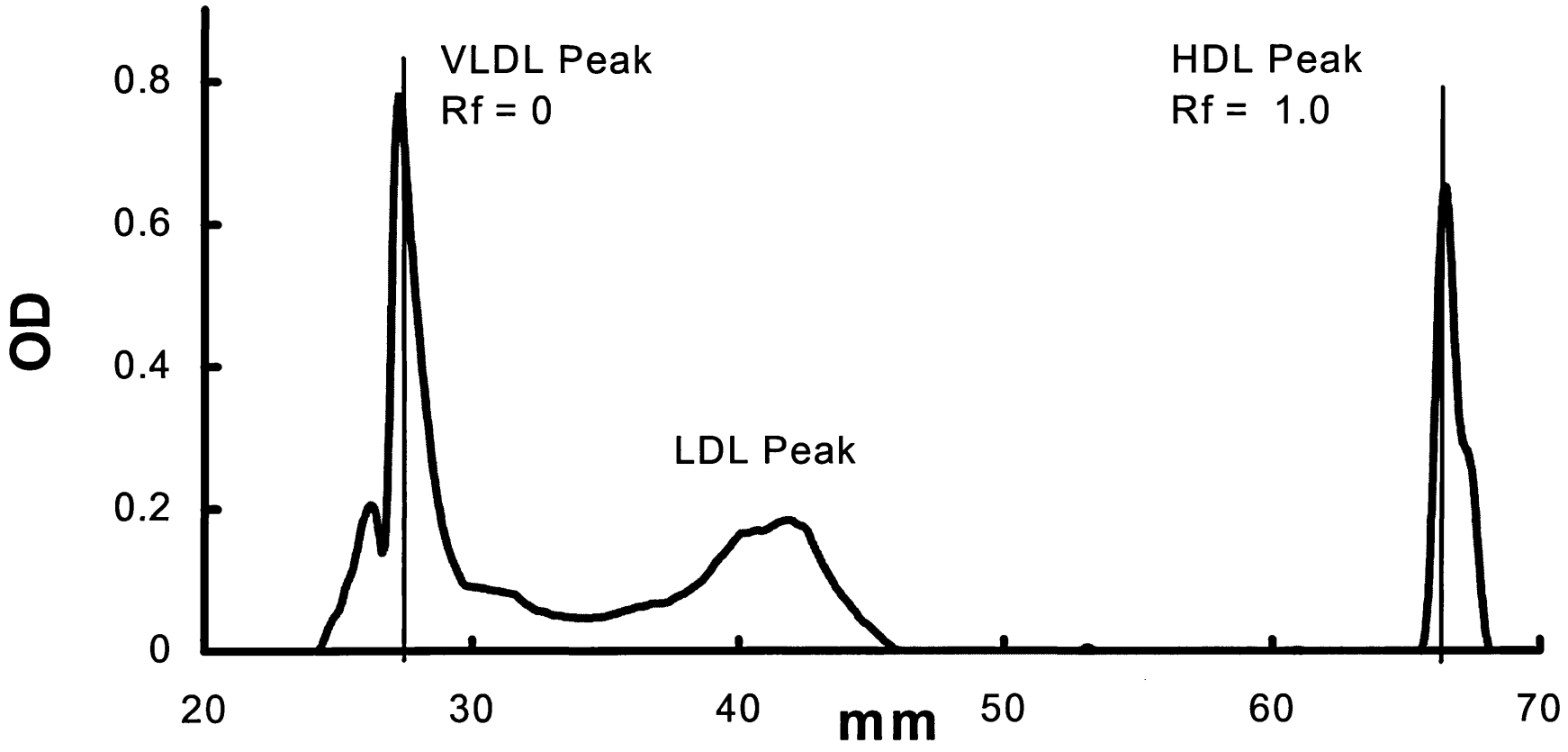


Figure 5.1.8b Acrylamide rod lipoprotein profile. This figure shows the VLDL, LDL and HDL peaks separated within the rod and analysed by imaging densitometery.

Figure 5.1.8b shows a single rod lipoprotein profile. The non-gradient acrlyamide rod resolved the VLDL, LDL, and HDL peaks. Using the manufacturer's template the VLDL peak is assigned an Rf value of zero while the HDL peak is assigned an Rf value of one. This allows the subfractions within the LDL peak to be assessed. VLDL and HDL peaks were easily identified within each rod and the presence of intermediate density lipoproteins could also be detected. The position of VLDL and LDL peaks within each rod was consistent from rod to rod.

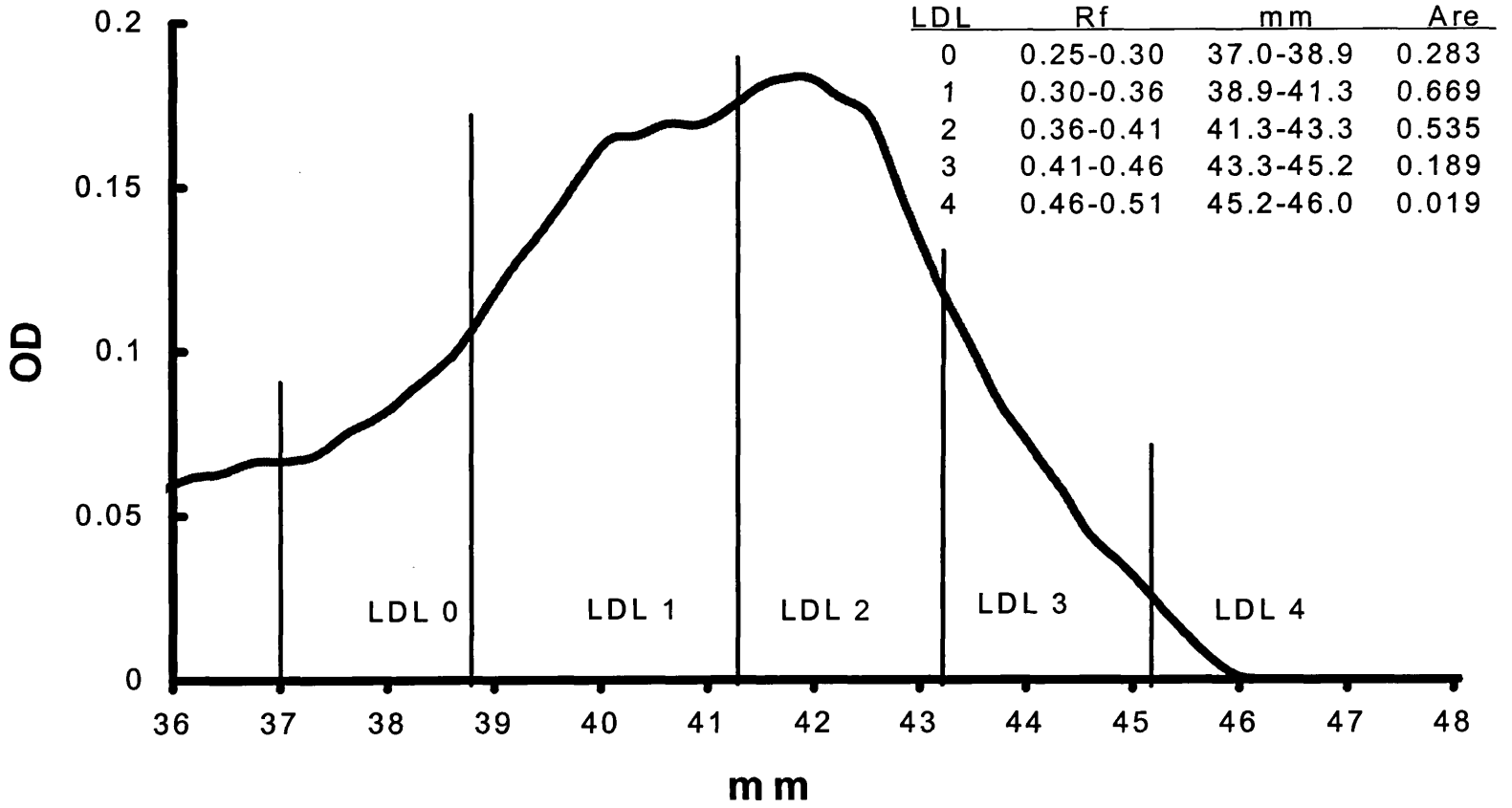


Figure 5.1.8c Acrylamide rod LDL peak profile. This figure shows how using the manufacturers template the LDL peak can be subdivided into a number of LDL fractions. Rf is calculated based on the position of LDL relative to VLDL and HDL.

Figure 5.1.8c shows a typical LDL profile with LDL 1 and 2 making up the majority of the area, LDL 3 and 4 are also present but in much smaller amounts. Lipoproteins of intermediate density are resolved in the LDL 0 region, this fraction is denoted midband A by the manufacturer. The LDL subfraction data was expressed as integrated area under the curve as calculated using the BioRad molecular analyst software.

5.1.9 LDL subfraction results

The results in table 5.1.9 show that there was no change in subfraction profiles after treatment when analysed using the manufacturer's template. Apart from a significant increase in the area of the LDL 1 subfraction in the placebo group no other significant changes were seen. Overall there was an increase in the area of many of the subfractions in the group receiving garlic. There was a similar pattern of non-significant increases in the areas of many of the placebo group subfractions. There were no significant changes in the area of the dense LDL subfractions. Very few of the participants had defined subfractions in the dense region of the rod. A paired t test analysis was not performed on the LDL 4 and 5 dense subfractions because of the small number of subjects.

Table 5.1.9 LDL subfraction results

	Garlic Group n=20		Placebo Group n=11	
Mean	**Pre (S.D.)**	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
LDL 0	0.47 (0.17)	0.52 (0.18)	0.59 (0.15)	0.66 (0.13)
	p = 0.2513 ns		p = 0.2016 ns	
LDL 1	0.77 (0.21)	0.88 (0.23)	0.85 (0.13)	1.00 (0.29)
	p = 0.0636 ns		**p = 0.0186***	
LDL 2	0.53 (0.18)	0.59 (0.21)	0.42 (0.23)	0.47 (0.20)
	p = 0.0812 ns		p = 0.4131 ns	
LDL 3	0.26 (0.19)	0.29 (0.19)	0.14 (0.07)	0.11 (0.07)
	n=18 p = 0.8406 ns		n=8 p = 0.6875 ns	
LDL 4	0.10 (0.11)	0.12 (0.07)	0.06	0.08
	n=14		n= 1	
LDL 5	0.09 (0.03)	0.06 (0.02)		
	n=3			

5.1.10 Combined LDL subfraction results

The areas from each of the LDL subfractions were combined and expressed using the method of Musliner and Krauss 1988. This involved combining the data from each individual rod and expressing the combined data in terms of either light or dense LDL

Light LDL = LDL 1 + LDL 2 ($d > 1.038$ Kg/L)

Dense LDL = LDL 3 + LDL 4 + LDL 5 ($d < 1.038$ Kg/L)

Table 5.1.10 below shows the results of the combined LDL subfraction analysis, LDL 0 (midband A) was not included in the combined area data.

Table 5.1.10 Combined LDL subfraction results

	Garlic Group n = 20		**Placebo Group n = 11**	
Mean	**Pre (S.D.)**	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
LDL 1+2	1.29 (0.29)	1.47 (0.31)	1.25 (0.35)	1.53 (0.48)
	p = 0.0129*		p = 0.1230 ns	
LDL 3+4+5	0.316 (0.29)	0.366 (0.28)	0.108 (0.11)	0.108 (0.09)
	p = 0.7855 ns		p = 0.9375 ns	

The combined subfraction data table 5.1.10 shows a significant increase in the overall area of the light subfractions in the garlic group. The light fraction of the placebo group was also increased but this was not statistically significant. There was a small increase in the dense fraction in the garlic group and no change in the area of the placebo group.

Table 5.1.11 Lipid and lipoprotein results

	Garlic Group n=20		Placebo Group n=11	
Mean/Median	**Pre (S.D.)**	**Post (S.D.)**	**Pre (S.D.)**	**Post (S.D.)**
TC	7.10 (0.64)	6.79 (0.81)	6.95 (0.61)	7.00 (0.75)
mmol/L	p = 0.0986 ns		p = 0.8774 ns	
LDL	4.97 (0.57)	4.77 (0.68)	5.11 (0.62)	5.11 (0.69)
mmol/L	p = 0.2045 ns		p = 0.9971 ns	
HDL	1.22 (0.27)	1.23 (0.30)	1.03 (0.29)	1.15 (0.31)
mmol/L	p = 0.7736 ns		**p = 0.0005*** **	
Tg	1.65	1.62	1.77	1.47
Range	(0.69-4.93)	(0.57-3.76)	(0.68-2.77)	(0.73-2.75)
mmol/L	**p = 0.0412***		p = 0.2402 ns	
Apo A1 g/L	1.18 (0.44)	1.07 (0.34)	1.19 (0.22)	1.11 (0.34)
	p = 0.2494 ns		p = 0.2720 ns	
Apo B g/L	1.64 (0.55)	1.52 (0.46)	1.51 (0.44)	1.44 (0.26)
	p = 0.3175 ns		p = 0.7167 ns	
Lp(a) g/L	0.11	0.18	0.04	0.04
Range	(0.0-0.70)	(0.0-0.70)	(0.0-0.49)	(0.0-0.45)
	p = 0.1937 ns		p = 0.8125 ns	

The lipid and lipoprotein data, excluding the Lp(a) results, shown in table 5.1.11 have been previously reported by Neil et al 1996. This earlier report used the same data and level of compliance for both groups but had greater numbers of subjects in each group (n =28). Table 5.1.11 shows the lipid and lipoprotein data for the participants of the oxidation part of the study.

Overall, the data shown here using smaller groups is similar to that previously reported. The decrease in total and LDL cholesterol seen in the garlic group was not significant, a small but significant decrease triglycerides was also seen. The increase in the HDL cholesterol concentration was the only significant change that occurred in the placebo group.

Garlic supplementation did not significantly alter the concentration of Lp(a) in serum over the study period. The results show that six months garlic supplementation does not significantly alter the serum lipid profiles in the middle aged participants of the study.

5.1.12 Non-compliant group results

As stated earlier a number of subjects in this study were excluded from the garlic and placebo groups because of non-compliance. The sixteen excluded participants took no tablets at all during the six-month trial. This non-compliant group was made up of three control subjects and thirteen garlic subjects all of whom provided pre and post study blood samples. Table 5.1.12 below shows the lag time, vitamin E, lipid and apolipoprotein data for this group. Given that no significant change in the LDL subfraction profiles of the garlic or placebo groups occurred the LDL subfraction data from the non-compliant group was not included in this table.

Table 5.1.12 Non-compliant group results (n=16)

Mean/Median	Pre (S.D.)	Post (S.D.)	p Value	Sig.
Lag Time (mins.)	41.4 (12.0)	46.8 (9.1)	**0.0120**	*
oLAb mU/L	393	416	0.1816	ns
Range	(139-2600)	(115-2342)		
Vitamin E μmol/L	18.8	19.0	0.6919	ns
Range	(12.7-24.7)	(12.2-25.5)		
TC	6.80 (0.47)	6.69 (0.61)	0.4107	ns
LDL	4.72 (0.44)	4.65 (0.55)	0.5363	ns
HDL	1.09 (0.31)	1.08 (0.24)	0.9348	ns
Tg	2.17	1.62	0.4835	ns
Range	(0.69-4.93)	(0.57-3.76)		
Apo A1 g/L	1.21 (0.34)	1.13 (0.43)	0.4152	ns
Apo B g/L	1.49 (0.43)	1.63 (0.43)	0.2858	ns
Lp(a) g/L	0.17	0.16	0.7736	ns
Range	(0.0-0.70)	(0.0-0.70)		

The non-compliant subjects displayed the same significant increased in lag time at the end of the study period seen in the garlic and placebo groups. This increase was not accompanied by any significant change in vitamin E or in the concentration of antibodies to oxidised LDL. The levels of serum lipids and apoproteins did not change significantly in the non-compliant subjects. This suggests that the basis for the increase in lag time is not associated with garlic supplementation.

5.2.1 Pre study Spearman correlation results

In order to investigate the relationship between the lag time and the other markers of oxidation the pre study data was combined and a Spearman correlation performed. This combination of data provided information on a group of fifty individuals rather than the smaller treatment groups. The relationship between lag time and the concentration of vitamin E and antibodies to oxidised LDL was investigated in this way. The small number of smokers (n=9) compared to non-smokers (n=38) in the study meant that the possible effect of smoking on the markers of oxidation in the pre study data was not investigated.

Table 5.2.1 Lag time Spearman correlation

	n	r	p value
Vitamin E μmol/L	46	0.3226	**0.0287***
oLAb mU/ml	50	-0.03141	0.8286

A significant association between lag time and vitamin E was seen in the pre study data. This correlation is not investigated in the post garlic samples as the number of subjects in each group is small and because lag time increased with treatment and vitamin E levels did not. The increase in lag time may be related to an increase in other antioxidants or to a change in the fatty acid composition of the LDL particle itself. There was no significant correlation between lag time and the concentration of antibodies to oxidised LDL.

It has been reported that differences in the levels of oLAb are seen between men and women and with age (Tatzber et al 1995). This was investigated by comparing the levels of oLAb in the men and women taking part in the study, again the pre study data was combined to increase the numbers for this comparison.

Table 5.2.2 oLAb Spearman correlation results

	n	Mean (S.D.)	Median	Range
Male	32	513 (536)	290	83 – 2600
Female	18	682 (670)	434	129 – 2600

Mann Whitney t test p value = 0.3631 ns

Age (years)	n	r	p value
oLAb mU/ml	50	-0.1867	0.1941

Table 5.2.2 shows that women have higher mean and median oLAb concentrations that men. The range of oLAb values is greater in this group than in the garlic and placebo groups alone because of the inclusion of two non-compliant subjects with particularly high antibody levels. Comparing the median values tested the statistical significance of this difference using a Mann Whitney t test. The comparison shows no statistical difference between male and female levels of antibodies. The relationship between age and oLAb levels was also investigated using a Spearman correlation for non-parametrically distributed data. The correlation below shows no relationship between age and antibody concentration in the pre study participants.

CHAPTER 6

DISCUSSION

6.1 Army study introduction

The army exercise study set out to record the effects of a ten week training programme including an intense two day military exercise on groups of young male army recruits. The large number of recruits taking part in this study allowed us to investigate the effect of both these forms of exercise on serum lipids, glucose, lipoprotein (a), and fibrinogen. The acute phase response elicited by the acute exercise and its effect on lipoprotein (a), and fibrinogen and the role of bilirubin and urate as possible markers of oxidative stress were also investigated in these young men.

6.1.1 Effects of ten week training on serum lipids and glucose

The effect of the ten-week training protocol on the recruits can best be seen in the group sampled five days after the ME (Group 6). The changes to lipid and carbohydrate metabolism can also be seen in the other recruit groups but these results are influenced by the effects of the acute response to the ME.

No direct measure of increased fitness was obtained on these recruits for example increase in VO_2 max. Improvement in VO_2 as an indicator of improved fitness is not always associated with favourable alterations in serum lipid concentrations (Nye et al, 1981 and Despres et al, 1994). The training programme was specifically designed to increase the fitness and stamina of these young men and to test their ability to carry out tasks of military nature while involved in prolonged submaximal exercise. The whole purpose of the military exercise was to assess the fitness of the recruits and without training these young men would be unable to complete such a physically demanding exercise.

The recruits provided non-fasting samples at the end of the training programme and analysis showed that total cholesterol increased significantly, LDL displayed a very small increased while HDL showed a small non-significant reduction. Tg levels increased significantly in the last recruit group tested. The increase in TC and Tg is within the VLDL/chylomicron fraction and may therefore be partly of dietary origin. Fasting VLDL triglyceride levels generally fall as a result of exercise training. The training-induced changes may be confounded by the lack of fasted blood samples, changes in plasma volume and also by possible alcohol consumption during the last days of training. The most consistent change that occurred in the recruits at the end of the training was the reduction in serum glucose concentrations. The change in TC and Tg are in terms of overall CHD risk small in these young men.

Other investigators have reported improvements in serum lipid profiles as a result of regular exercise or exercise training (Wei et al 1997, Houmard et al 1994, Wood et al 1988). These improvements include lower serum TC and LDL and Tg combined with increased HDL. The reduction in serum glucose seen at the end of the training, although not statistically significant is perhaps an indicator of how the training programme improved energy metabolism in these young men. In the review by Holloszy 1996 it is stated that during exercise endurance trained individuals spare plasma glucose and liver glycogen. Depletion of glycogen stores results in the inability to continue to exercise vigorously. Carbohydrate sparing is therefore, one of the most important mechanisms by which training increases the ability to perform prolonged strenuous exercise.

The slower rate of utilisation of glycogen is made possible by training induced adaptations to endurance exercise that result in an increase in the proportion of energy derived from fat oxidation during prolonged exercise. The increase in fitness as a result of the training programme did not improve the lipid profiles of these young men. From epidemiological comparisons, the changes in serum lipid concentration in the final recruit group do not represent an increased risk of premature CHD. The initially favourable serum lipid profile is an important factor in the effects improved fitness can have. The more deteriorated the lipid profile, the greater will be the probability of observing a significant response to training (Despres et al 1994). The leanness, youth and initially low TC and Tg levels meant that the impact of the exercise regime on serum lipid concentrations was very limited in these recruits.

6.1.2 Effects of ten week training on lipoprotein (a)

Lp(a) levels were unchanged in the recruits sampled five days after the ME when compared with pre training levels. The cardiovascular risk in young Finns study examined levels of activity and Lp(a) in 2464 young men and women. The results demonstrated that increased physical activity is associated with lower levels of serum Lp(a) (Taimela et al 1994).

A number of other studies have produced conflicting reports regarding the effects of exercise training on serum Lp(a) concentration. Ponjee et al 1995 and Holme et al 1996 both reported that Lp(a) levels were increased following exercise training. Lobo et al 1992 studied the effects of exercise and estrogen therapy on Lp(a) and reported that exercise alone had a minimal non-significant effect on Lp(a) levels.

Szymanski et al 1996 reported that Lp(a) did not correlate with fitness or any fibrinolytic variable including plasminogen activator inhibitor (PAI-1) in men with varying fitness levels after treadmill exercise. Halle et al 1996 compared Lp(a) levels in endurance trained athletes, power athletes, and sedentary control subjects and found only minor non-significant differences in Lp(a) concentration.

The type and the duration of the exercise training performed by the recruits does not compare with the studies mentioned above in that some of these reports studied older populations undergoing exercise training that was much less intense than that of the army recruits. The Lp(a) assay results reported for these recruits are also supported by participation in an external quality assurance scheme. The results presented here show that the ten weeks training undertaken by these recruits did not alter their Lp(a) concentration.

6.1.3 Effects of ten weeks training on fibrinogen

The recruit group sampled at the end of training displayed a significant increase in plasma fibrinogen concentration. This is an unexpected result as many previous studies have associated increased fitness and activity with lower plasma fibrinogen concentrations.

Epidemiological studies have shown an inverse relationship between physical activity and fibrinogen concentration, while longitudinal studies have demonstrated reduced levels of fibrinogen following exercise (Ernst 1993). Reports have stated that lower fibrinogen levels are seen in subjects who are more active, and that the most physically fit individuals, as determined by VO_2 max, have persistently lower fibrinogen concentrations when compared with those who are less fit (MacAuley et al 1996). A study investigating a combination of exercise and diet in post-menopausal women however did not lower fibrinogen concentration (Svendsen et al 1996).

Plasma fibrinogen increased by a mean of 10% in the recruits sampled at the end of the study. The report by Montgomery et al 1996 suggested that the recruits with the $G\text{-}_{435}\text{-}A$ polymorphism of the β-fibrinogen gene displayed an enhanced response to acute exercise. This study reported a reduction in fibrinogen for the same recruit group data (n=11). The results presented here a based in a larger sample number (n=27) than considered in the earlier study. The 10% increase in fibrinogen although statistically significant is well within the reference range for the assay. This increase in terms of epidemiological evidence is small and may not contribute to an increased risk of premature CHD.

6.1.4 Effects of ten week training on other serum analytes

(Total CK, creatinine, albumin, bilirubin and urate)

All of the other serum analytes measured in the final recruit group reflect the changes that have occurred as a result of training and the ME. Serum albumin and CRP levels were reduced five days after the ME, both these analytes were still exhibiting the effects of the acute response.

Total CK levels are significantly reduced five days after the ME, creatinine levels are elevated but indicate a slow return to pre ME levels. Bilirubin and urate show the most interesting changes to occur as result of the training and the ME. These changes are more as a result of the physical stress of the ME that the overall training regime. The basis for these changes is discussed in detail below (section 6.2.3).

6.2.1 Effects of acute exercise on lipids and glucose

The responses of each recruit group sampled after the ME reflects the strenuous nature of the military exercise. The training programme undertaken in the weeks leading up to the ME also affected these responses. The acute effects on serum lipids can be seen in the recruits sampled after twelve hours, while recovery from the intense exercise are seen in the samples taken up to four days post ME. Twelve hours after the ME there was a significant increase in TC, both HDL and LDL displayed small non-significant increases. Serum Tg was significantly increased in this recruit group. The consumption of food and changes in plasma volume may also contribute to the changes seen twelve hours post ME.

Twenty-four and forty-eight hours after the ME serum levels of cholesterol were reduced while Tg continued to increase non-significantly. Three to four days after the ME levels of total, LDL and HDL begin to increase while Tg levels continued to increase. Serum glucose levels were significantly reduced in the recruit groups sampled twelve and twenty-four hours after the ME. This pattern of reduced glucose concentrations was continued in all the other recruit groups tested.

Studies into the acute effects of exercise have focused on the lipid changes that occur in the minutes and hours after a bout of exercise. There are also differences in the type, intensity and duration of the exercise protocols undertaken. Investigations by Lithell et al 1984, Davis et al 1992, Foger et al 1994, all followed the effects of acute exercise on serum lipids in groups of healthy men for a period of days after the exercise.

The study by Lithell et al is of particular interest because it also reported the effects of military field manoeuvres on a group of sixteen recruits over a period of five days. All of these studies reported reductions in TC and LDL levels up to 2 days after exercise, followed by a gradual return to pre exercise levels. Tg levels were reduced in the hours and days after the bout of exercise while HDL levels, particularly the HDL_2 subfraction, were increased up to 48 hours after a bout of exercise. The increase in fat oxidation particularly metabolism of FFA from adipose and skeletal muscle is an important effect of endurance training (Hollosky 1996).

This increase in fat oxidation allows glucose and glycogen to be spared and contributes to the energy needed to complete the ME. Acute exercise also increases the activity of lipoprotein lipase (LPL) and reduces the action of hepatic triglyceride hydrolase (HTGL) (Foger et al 1994, Kantor et al 1987). An increase in the activity of muscle LPL coupled with a reduction in the activity of HTGL should lead to a reduction in triglyceride but this reduction was offset by the uncontrolled access to food and possibly alcohol in the final days of training. The samples taken in the hours after the ME may also be effected by changes in plasma volume.

The recruits were not fasted prior to sampling and presented with initially low triglyceride values. The intensity and duration of the ME may have given rise to a depletion of skeletal muscle triglyceride stores. This in turn may have contributed to the increasing triglyceride levels in all recruit groups after the ME. Serum levels of glucose were reduced in all the recruits in the hours and days following the ME. The reductions reached statistical significance after twelve hours, and one and three days.

The fall in glucose concentrations seen in all the recruits in the days following the ME are as a result of metabolic changes due to the training protocol. The response of each recruit to the ME shows how training improved the use of energy substrates during intense exercise. These lower glucose levels are a result of fitness training rather than an acute response, and have been reported in a number of studies (Houmard et al 1994, Coggan et al 1995, Philips et al 1996)

6.2.2 Effects of acute exercise on the acute phase response

The strenuous nature of the ME produced a clear acute phase response that could be seen in the recruits sampled up to three days after the exercise had finished. Serum levels of CRP increased after 12 hours and remained elevated (>5U/L) for up to three days. None of the increases in CRP were statistically significant due to the raised levels seen in the pre exercise samples.

Fibrinogen concentrations also increased significantly after 24 hours and remained elevated up to three days after the ME. Serum albumin concentrations displayed a clear negative response following the ME and were significantly reduced for up to four days. These changes in serum proteins clearly indicate an acute phase response in all the recruits brought on by the physical exertion of the military exercise.

Lp(a) concentrations displayed little change in any of the recruit groups tested during the acute phase response and none of these changes were statistically significant. Lp(a) concentrations have been reported to increase as part of the acute phase response (Craig 1992, Lude 1993, Norma 1994).

Comparisons between patients with high and low CRP concentrations showed Lp(a) levels to be significantly higher in the raised CRP group (Min 1997). In patients with unstable angina Lp(a) was raised but in the absence of an acute phase response (Oshima 1991), while the acute response brought on by myocardial infarction was shown not to alter Lp(a) (Mbewu 1993).

All of these studies examined Lp(a) changes in older subjects presenting with an acute response brought on by a number of different stimuli, and these stimuli were usually associated with acute clinical disorders. Little data has been published examining the effects of an exercise induced acute phase response and Lp(a) in a young healthy population. Studies have reported the effects of short periods of treadmill running on Lp(a) concentration. The results shown that Lp(a) did not change in response to this stimulus (Hubinger et al 1996). The results on these young army recruits presented here support the idea that Lp(a) does not act as an acute phase protein in response to acute exercise.

6.2.3 Effects of APR on other serum analytes

(Total CK, creatinine, albumin, bilirubin and urate)

There were significant increases in the serum concentration of creatinine in the hours and days following the ME. Total CK levels would also be expected to increase due to muscle permeability changes but in these recruits but the opposite is seen. The high levels of total CK seen in the pre exercise samples explain these large reductions in total CK. The reasons of the unexpected change in total CK maybe due to intramuscular injections received by the recruits just before the initial blood samples were taken.

The intramuscular injection prior to the pre exercise sample is a good example of how the military setting of the study introduced unforeseen analytical complications. A recent report studied the effect of a 100Km ultra-marathon on thirteen male runners, the samples were obtained one hour before and ten minutes after the race. This study described a significant increase in total bilirubin after the race as a result of haemolysis and changes in hepatic function (DePaz et al 1995). Bilirubin and urate levels were both significantly reduced during the acute phase response. These reductions may be as a result of the action of these metabolites as antioxidants. Oxygen free radicals are released in muscles through mitochondrial oxidative phosphorylation and from inflammatory cells during exercise induced oxidative stress. This stress in turn may trigger and stimulate a number of metabolic events that involve the antioxidant defence system. Both bilirubin and urate are important participants in this antioxidant defence mechanism along with vitamin E and C (Jacob et al 1996, Moller et al 1996). The significant reduction in the levels of these metabolites suggests that the military exercise led to oxidative stress and antioxidant response in these young men.

6.3.1 Army recruits study pre exercise results

The data obtained from the recruits before they began their training provided an opportunity to investigate the associations between Lp(a), serum lipids and fibrinogen. Much of the data into the associations between these risk factors has been obtained from older populations with clinically detectable CHD. Raised levels of Lp(a) are independently associated with increased risk of premature CHD (Seed 1996). Increased levels of Lp(a) particles have been shown to correlate with raised levels of fibrinogen in older men with CHD (Mikhailidis et al, 1996). Fibrinogen levels have been significantly correlated with raised TC and Tg in men with essential hypertension (Fogari et al 1994).

The possible effect of cigarette smoke in these recruits was investigated by comparing the pre exercise data of the smokers and non-smokers. The results show no differences in the levels of any of the analytes compared. This suggests that these young men have not yet been exposed to cigarette smoke for long enough for its harmful effects to be seen. The overall data (n=197) shows a significant positive Spearman correlation between Lp(a) concentration and LDL, and a significant negative correlation between Lp(a) and HDL is also seen. These associations have not previously been reported. Fibrinogen, triglyceride and cholesterol showed a weak associated with Lp(a). Applying a cut off limit for the highest Lp(a) values ($\geq$0.30g/L) and repeating the correlation did not significantly alter the Spearman correlation seen in the whole group.

Subdividing the Lp(a) data into four groups of approximately equal size broadened the range of Lp(a) values from 0.30–1.05g/L to 0.27–1.05g/L and increased the number of pairs in the correlation from 47 to 50. The cholesterol, triglyceride and fibrinogen levels of the high Lp(a) group were no different when compared with the group as a whole.

The Spearman analysis of this high Lp(a) group produced a significant correlation between Lp(a) and TC, HDL, LDL cholesterol and fibrinogen. The analysis suggests that the level at which Lp(a) correlates significantly with other markers of premature CHD risk can vary depending on the cut off limits applied. Given the trends seen here raised Lp(a) merits further investigation and could offer a means of identifying those individuals at possible risk of premature CHD where other conventional markers indicate no risk.

6.4.1 Garlic study introduction

The garlic study set out to determine the effects of garlic supplementation on several indicators of lipid peroxidation, including isolated LDL lag time, oLAb, LDL subfraction distribution and vitamin E concentration. The trial was double blind and used a large dose (900mg/day), of odour controlled garlic tablets (Kwai™), containing a standard amount of allicin (0.6%).

The only other study to investigate the antioxidant properties of dietary garlic was conducted by Phelps et al 1993. This double blind trial used the same Kwai garlic table preparation at a lower dose (600mg /day) in a group of ten subjects over a two week period. The results showed a significant decrease in thiobarbituric acid reactive substances (TBARS), suggesting the garlic may have antioxidant properties. A number of in vitro studies have suggested that extracts of garlic can reduce free radical generation (Torok et al 1994) and can activate nitric oxide synthase in isolated platelets to produce nitric oxide and thus reduce platelet aggregation (Indrajit 1995).

Compared to other studies investigating the antioxidant effects of garlic this trial was carried out over a much longer period (six-months). The garlic tablets were odour controlled. The placebo tablets were coated in a tiny amount of garlic to make them indistinguishable from the garlic tables. The study also monitored the level of compliance by tablet counting at the end of the trial. By applying a compliance level of 75% any possible antioxidant effect could be more reliably attributed to garlic. This reduced the number of subjects in each group to garlic (n=20), placebo (n-11).

The application of compliance created a third group of participants who had supplied pre and post study blood samples but had failed to take any of the garlic or placebo tables provided. The results from the third or non-compliant group were important when it came to explain the results from the treatment groups.

6.4.2 Garlic study antioxidant effects

The results of the study into the antioxidant effects of garlic produced some unexpected findings. Both garlic and placebo groups showed a statistically significant increase in LDL oxidation lag time over the treatment period. The non-compliant group also displayed a significant increase in lag time. The increase in lag time was not associated with an accompanying increase in the concentration of vitamin E, the main antioxidant of LDL (Hoffman 1995).

The main indicator of oxidative status in this study was isolated LDL lag time. A hand drawn and a computer based method were used to calculate lag time. Both methods showed that an increase in LDL oxidation lag time had occurred at the end of the six-month trial. These two methods produced lag time data that was highly correlated (97%), but the computed based method produced consistently shorter lag times overall. The difference in lag times was because the computer programme had difficulty in constructing a straight line along the slope of the initial phase when the number of data points was limited, for this reason the hand drawn data was preferred.

Analysis of the isolated LDL indicated the isolation procedure produced low density lipoprotein that was consistent in terms of its protein, cholesterol and Lp(a) content. None of these variables were significantly altered after the treatment period. The Lp(a) content of the LDL did not significantly alter the oxidation characteristics of the LDL. (Kleinveld et al 1996). The LDL isolation procedure involved both concentration and dilution steps which varied among individuals and made comparisons and with serum levels of these analytes impractical.

Using the nongradient acrylamide rod method LDL was subdivided into five subfractions. The data was expressed according to the manufacturers protocol and showed changes in the LDL subfraction profile in both garlic and placebo groups. The garlic group displayed non-significant increases in the area of all the subfractions while, the placebo group showed a similar pattern of increased area which was significant for the LDL 1 subfraction. The individual subfraction areas when combined and expressed using the notation adopted by Musliner et al 1988 showed a significant increase in the area of the light LDL subfraction in the garlic group. The placebo group showed a similar but non-significant increase in this region. No significant change in the area of the dense LDL subfraction was seen in either group. These change in the area of the light LDL subfraction profile may explain the increase in lag time seen in both groups.

There was no significant change in the levels of in the garlic group, while the increase in antibody levels seen in the placebo group was not statistically significant. Reports that the concentrations of oLAb are increased by vitamin E supplementation (Meraji et al 1996) are not seen in any of the groups studied.

6.4.3 Garlic study non-compliant group results

The trial participants who provided pre and post samples but did not comply with the garlic or placebo treatment produced some interesting results. This group showed a significant increase in LDL lag time similar to the garlic and placebo groups. The serum concentration of vitamin E and oLAb were also the same in the non-compliant group as in the other two groups. Serum lipid, apoprotein and LDL subfraction results mirrored the changes of both garlic and placebo groups. Apart from the increase in lag time none of the other changes were statistically significant.

6.4.4 Garlic study lipid and Lp(a) results

The changes in lipids and apoproteins presented here are based on the groups defined as garlic, placebo and non-compliant for the oxidation part of the study. These results are similar to those previously reported by Neil et al 1996, who analysed the same subject data using a larger sample of selected participants with the same level of compliance. The change in total cholesterol for the garlic group is not as large as that reported by Neil et al 1996, and the mean reduction of 0.31mmol/L was not statistically significant. The study was initially designed to have the statistical power to detect a change in total cholesterol of 0.6mmol/L, so any change in cholesterol below this cannot be reliably attributed to garlic.

The serum concentration of lipoprotein (a) was higher in the garlic group and increased non-significantly after treatment while levels in the placebo group did not change.

6.4.5 Garlic study pre treatment results

The data provided by the participants at the start of the study gave an opportunity to correlate lag time with the other markers of oxidative status in a group of fifty subjects. The results show a significant relationship between lag time and vitamin E concentration in these pre study samples. This significant relationship was not seen for after-treatment samples as lag time increased and vitamin E levels did not. There was weak a non-significant negative correlation between lag time and antibodies to oxidised LDL.

Reports that gender and age (Tatzber 1995, Temmel 1996) affect oLAB were also investigated. The age range of the subjects (40–65 years) did not correlate with the concentration of oLAb. Female participants had higher median antibody concentrations the male participants. When compared statistically this difference was found to be non-significant. The small number of smokers participating in the study did not allow for the investigation of this risk factor and its possible effects on the other markers of oxidation measured.

6.5.1 Army recruits study conclusions

The army exercise study shows that fitness training undertaken by these young men did not increase HDL or lower Tg levels as has been previously reported. Serum glucose was significantly lower in all the recruits as a direct result of increased fitness. Training did not affect Lp(a) levels and fibrinogen concentrations showed a small increase. Bilirubin and urate levels were both reduced at the end of the study period. These reductions are not as great as those seen in the earlier recruit groups suggesting that recovery from the oxidative stress of the ME is still taking place.

The results from the recruits tested the end of training were affected by the intensity and duration of the military exercise performed five days earlier. All the recruits presented with favourable lipid profiles and low BMI, reducing the impact of the training programme on these variables. Fitness training caused a small increase in fibrinogen but did not effect Lp(a) levels. Therefore due to the youth and physical condition of the recruits the impact of increased fitness on indicators of CHD risk was not as great as has been reported for older and obese populations.

In the period after the ME a clear acute phase response was seen in all the recruits lasting up to three days. Significant increases in fibrinogen and creatine were seen along with large increases in CRP levels, while albumin concentrations were reduced. Lp(a) levels showed little change during the APR. The significant reductions in urate and bilirubin levels seen in all the recruits after the ME may be as a result of oxidative stress produced by the strenuous nature of the ME.

Serum lipid levels in the recruits sampled after 12 hours were similar to those previously reported. After this time point the pattern of lipid results suggests that the recruits were undergoing metabolic recovery after the ME. The physical condition of the recruits and the postprandial nature of the blood samples affect the changes in serum lipids.

The recruits taking part in the ME were exposed to a high level of physical exertion and stress. These levels are not comparable with the levels of physical exertion used in other studies into the effects of acute exercise on serum lipids. Thus Lp(a) did not act as an APR following an acute physical challenge in these young men but on the basis of other reports it may act as an APR when the stimulus is disease driven.

Analysis of the pre-exercise data has shown that serum Lp(a) is positively correlated with LDL and negatively correlated with HDL for all the recruits. Recruits with the highest serum concentrations of Lp(a) show a significant correlation with a number of premature CHD lipid markers. Lp(a) in these recruits was also associated with raised plasma fibrinogen levels. Lp(a) may be of use in the identification of individuals at increased risk of CHD where other markers do not clearly indicate risk.

6.5.2 Garlic study conclusions

The results of this study do not support the hypothesis that dietary garlic supplementation enhances the susceptibility of isolated LDL to copper ion induced oxidation. The increased LDL oxidation lag time seen in the placebo, garlic, and non-compliant groups suggests that the changes in lag times were not related to the dietary garlic supplementation. Serum concentrations of vitamin E did not change over the study period and therefore were not responsible for the increased lag time. The change in lag time was accompanied by a non-significant increase in the area of both light and dense LDL subfractions. Concentrations of oLAb did not change over the study period. There were small non-significant reductions in serum TC levels, but no change in levels of Lp(a)

The study participants had to visit their GP on several occasions before the trial and repeat cholesterol measurements were made and dietary advice was also given. The dietary advice could influence eating behaviour during the trial and participation in the study may give rise to a 'placebo effect' (Gøtzsche 1994). The 'placebo effect' could help to explain the changes that occur in lag time, given that the non-compliant subjects received the dietary advice and took part in the trial by supplying pre and post treatment blood samples which also displayed this change.

All of the pre samples for the study were obtained during the winter months, (December to January), while all the post samples were obtained during the summer, (May to July). Studies into the seasonal variation of vitamin E have suggested that levels of this antioxidant are subject to annual and 4-monthly rhythms.

The concentration of vitamin E was found to be lower from January to May than from August to November. The changes in vitamin E have been associated with a number of other factors including changes in serum lipid concentration, ambient air temperature, seasonal weather changes, and seasonal changes in dietary habits (Maes et al 1996). Vitamin E levels in this study do not display the reported seasonal variation. Many of the participants had lower levels of vitamin E after treatment indicating that the increase in lag time is not due to an increase in vitamin E.

The diet of the participants may change or improve during the summer months. This could lead to increased levels of monounsaturated fatty acids within the LDL particle, particularly in LDL 1 and 2, thus rendering the isolated LDL less susceptible to oxidation. Monounsaturated fatty acids have been shown to be less susceptible to oxidative modification (Reaven 1993). Changes in the concentration of other antioxidants such as vitamin C (ascorbate), urate and bilirubin may also contribute to the increase in lag time.

The findings of this study show that six months of dietary garlic supplementation did not contribute to the increase in LDL lag time. Increased dietary consumption of antioxidants other than vitamin E and seasonal factors may explain the changes seen at the end of the trial period.

Bibliography

Ariga T. Oshila S. Tamoda T. Platelet aggregation inhibitor in garlic.
Lancet 1981;i:150-151.

Austin MA, Hokanson JE, Brunzell JD. Measurement and Clinical Significance of Low Density Lipoprotein Subclasses. in N Rifai, G R Warnick (ed) Laboratory Measurement of Lipids, Lipoproteins and Apolipoproteins. Washington, DC, AACC Press, 1994,223-234.

Block E. The Chemistry of Garlic and Onions. Sci Am. 1985;252:114-119.

Campos H, Genest JJ, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, Wilson PWF, Schaefer EJ. Low density lipoprotein particle size and coronary heart disease. Arterioscler Thromb 1992;12:187-195.

Chait A. Methods for assessing lipid and lipoprotein oxidation.
Curr Opin Lipidol 1992;3:389-394.

Chandrashekhar Y, Anand IS, Exercise as a coronary protective factor.
Am Heart J. 1991;122:1723-1739.

Coggan AR, Raguso CA, Williams BD, Sidossis LS, Gastaldelli A. Glucose kinetics during high intensity exercise in endurance trained humans.
J Appl Physiol. 1995;78:1203-1207

Coggan AR. Plasma glucose metabolism during exercise: effect of endurance training in humans. Med Sci Sports Exer 1997:620-627.

Craig WY. Ledue TB. Lipoprotein (a) and the acute phase response. Clin Chim Acta. 1992;210:321-322.

Davis PG, Bartoli WP, Durstine JL. Effect of acute exercise intensity on plasma lipids and apolipoproteins in trained runners. J Appl Physiol 1992;72:914-919.

DePaz JA, Villa JG, Lopez P, Gonzalez-Gallego J. Effects of long distance running on serum bilirubin. Med Sci Sport Exer. 1995;27:1590-1594.

Despres JP, Lamarche B. Low-intensity endurance exercise training, plasma lipoproteins and the risk of coronary heart disease. J Intern Med 1994;23:7-22.

Dufaux B, Order U, Muller R, Hollmann W, Delayed effects of prolonged exercise on serum lipoproteins. Metabolism. 1986;35:105-109.

Durstine JL, Ferguson MA, Szymanski LM, Davis PG, Alderson NL, Trost SG, Pate RR. Effect of a single session of exercise on lipoprotein (a). Med Sci Sports Exer. 1996;28:1277-1281.

Ernst E. Regular exercise reduces fibrinogen levels: a review of longitudinal studies. Brit J Sport Med. 1993:27;175176.

Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in virto oxidation of human low density lipoprotein.
Free Rad Res Comm 1989;6:67-75.

Esterbauer H, Wag G, Puhl H. Lipid peroxidation and its role in atherosclerosis.
Brit Med Bull 1993;49:566-576.

Fogari R, Zoppi A, Marasi G, Vanasia A, Villa G, Associations between plasma fibrinogen levels and cardiovascular risk factors in hypertensive men.
J Cardiovasc Risk. 1994;1:341-345.

Foger B, Patsch JR. Exercise and postprandial lipaemia.
J Cardiovasc Risk. 1995;2:316-322.

Föger B, Wohlfarter T, Ritsch A, Lechletitner M, Miller CH, Dienstl A, Patsch JR. Kinetics of Lipids, Apolipoproteins, and Cholesterol Ester Transfer Protein in plasma after a bicycle marathon. Metabolism 1994;43:633-639.

Friedewald WT, Levy RI, Fredrickson DS, Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.

Gøtzsche PC. Is there a logic in the placebo? Lancet 1994;344:925-26.

Hajjar KA, Nachman RL. The role of Lipoprotein (a) in atherosclerosis and thrombosis. Ann Rev Med. 1996;47:423-442.

Halle M, Berg A, von Stein T, Baumstark MW, Konig D, Keul J. Lipoprotein (a) in endurance athletes, power athletes, and sedentary controls.
Med Sci Sport Exer 1996;28:962-9965.

Handley DA, Hughes TE. Pharmacological approaches and strategies for therapeutic modulation of fibrinogen. Thromb Res 1997;87:1-36.

Hardman AE. Exercise and the prevention of atherosclerotic, metabolic hypertensive diseases: A Review. J Sports Sci 1996;14:201-218.

Heinrich J, Sandkamp M, Kokott R, Schulte H, Assamnn G. Relationship of Lipoprotein (a) to variables of coagulation and fibrinolysis in a healthy population. Clin Chem.1991;37:1950-1954.

Hennekens CH, Rosner B, Jesse MJ, Ddolette ME, Speizer FE. A retrospective study of physical activity and coronary deaths. Int J Epidemol. 1977;62:707.

Herbert PN, Bernier DM, Cullinane EM, Edelstine L, Kantor MA, Thompson PD. High density lipoprotein metabolism in runners and sedentary men.
JAMA. 1984;252:1034-1037.

Hoffman RM, Garewal HS. Antioxidants and the prevention of Coronary Heart Disease. Arch Intern Med 1995;155:241-246.

Holloszy JO, Kohrt WM. Regulation of carbohydrate and fat metabolism during training and exercise. Ann Rev Nutr 1996;16:121-138.

Holme I. Urdal P. Anderssen S. Hjermann I. Exercise-induced increase in lipoprotein (a). Atherosclerosis 1996;122:97-104.

Houmard JA. Bruno NJ. Bruner RK. McCammon RG. Israel RG. Barakat HA. Effects of exercise training on the chemical composition of plasma LDL. Arterioscler Thromb 1994;14:325-330.

Hubinger L, Mackinnon LT. Barber L. Mccosker J. Howard A. Lepre F. Acute effects of treadmill running on lipoprotein (a) levels in males and females.
Med Sci Sport Exer. 1997;29:436-441.

Indrajit D, Khan NS, Sooranna SR. Potent activation of nitric oxide synthase by garlic: a basis for its therapeutic applications.
Curr Med Res Opin. 1995:13:257263.

Jacob RA, Burri BJ. Oxidative damage and defence.
Am J Clin Nutr 1996;63:985S-990S.

Jialal I, Devaraj S. Low-Density lipoprotein oxidation, antioxidants, and atherosclerosis: a clinical biochemistry perspective.
Clin Chem 1996;42:498-506.

Kantor MA, Cullinane EM, Sady SP, Herbert PN, Thompson PD. Exercise acutely increases high density lipoprotein cholesterol and lipoprotein lipase activity in trained and untrained men. Metabolism 1987;36:188-192.

Klezovitch O, Scanu AM. Hererogeneity of lipoprotein (a): growing complexities. Curr Opin Lipidol 1995; 6:223-228.

Kleinveld HA, Hak-Lemmers HLM, Stalenhoef AFH, Demacker PNM. Improved measurement of low-density lipoprotein susceptibility to copper induced oxidation: Application of a short procedure for isolating low density lipoprotein. Clin Chem 1992;38:2066-2072.

Kleinveld HA, Duif PFCCM, Pekelharing HLM, van Rijn HJM. Oxidation of lipoprotein (a) and low density lipoprotein containing density gradient ultracentrifugation fractions. Biochim Biophys Acta 1996;1303:15-21.

Ledue TB. Neveux LM. Palomaki GE. Ritchie RF. Craig WY. The relationship between serum levels of lipoprotein (a) and the proteins associated with the acute phase response. Clin Chim Acta. 1993;223:73-82.

Lithell H, Schéle R, Vessby B, Jacobs I. Lipoproteins, lipoprotein lipase, and glycogen after prolonged physical activity. J Appl Physiol. 1984;57:698-702.

Lobo RA, Notelovitz M, Bernstine L, Khan FY, Ross RK, Paul WL. Lp(a) Lipoprotein: Relationship to cardiovascular disease risk factors, exercise, and estrogen. Am J Obstet Gyn. 1996;1182-1189.

MacAuley D, McCrum EE, Stott G, Evans AE, McRoberts B, Boreham CAG, Sweeney K, Trinick TR. Physical activity, physical fitness, blood pressure, and fibrinogen in the Northern Ireland health and activity survey.
J Epidemiol Comm Health 1996;50:258-263

Maes M, Weeckx S, Wauters A, Neels H, Scharpe S, Verkerk R, Demedts P, Desnyder R. Biological variability in serum vitamin E concentrations: relation to serum lipids. Clin Chem 1996;42:1824-1831.

Maher VMG, Brown BG. Lipoprotein (a) and coronary heart disease.
Curr Opin Lipidol 1995; 6:229-235.

Mbewu AD. Durrington PN. Bulleid S. Mackness MI. The immediate effect of streptokinase on serum lipoprotein (a) concentration and the effect of myocardial infarction on serum lipoprotein (a), apolipoprotein A1 and B, lipids and C-reactive protein. Atherosclerosis 1993;103:65-71.

McDowell IF, McEnemy J, Trimble ER. A rapid method for measurement of the susceptibility to oxidation of low density lipoprotein.
Ann Clin Biochem 1995;32:167-174.

Meraji S, Ziozenlova O, Resch U, Khoschsorur A, Tatzber F, Esterbaurer H. Effect of supplementation with β-carotene and α-tocopherol in Iranian subjects. Society for Free Radical Research – European Region Austrian Biochemical Society. Workshop: Oxidative Stress 5th & 6th July 1996.

Mikhailidis DP, Spyropoulous KA, Ganotakis ES, Jagroop IA, Vallance DT, Byrne DJ, Winder AF. Fibrinogen and Lipoprotein (a): associations in a population attending a cardiovascular risk clinic and effect of treatment with Ciprofibrate. Fibrinolysis. 1996;10:Suppl 1, pp 17.

Min WK. Lee JO. Huh JW. Relationship between lipoprotein (a) concentrations in patients with acute-phase response and risk analysis for coronary heart disease. Clin Chem.1997;43:1891-1895.

Moller P, Wallin H, Knudsen LE. Oxidative stress associated with exercise, psychological stress and life-style factors.
Chemi Biol Interactions 1996;102:17-36.

Montgomery HE. Clarkson P. Nwose OM. Mikhailidis DP. Jagroop IA. Dollery C. The acute rise in plasma fibrinogen concentration with exercise is influenced by the G-$_{453}$-A polymorphism of the β-fibrinogen gene.
Arterioscler Thromb. 1996;16:386-391.

Muniz N. Measurement of plasma lipoproteins by electrophoresis on polyacrylamide. Clin Chem 1977;23:1826-1833.

Musliner TA, Krauss RM. Lipoprotein subspecies and risk of coronary disease. Clin Chem 1988;34:8(B);B78-B83.

Neil HAW. Silagy CA. Lancaster T. Hodgeman J. Vos K. Moore JW. Jones L. Cahill J. Fowler GH. Garlic powder in the treatment of moderate hyperlipidaemia: a controlled trial and meta-analysis.
J Royal Coll Phys 1996;30:4; 329-334.

Noma A, Abe A, Maeda S, Seishima M, Makino K, Yano Y, Shimokawa K. Lp(a): an acute-phase reactant? Chem Phys Lipids 1994; 67/68:411-417.

Nye ER, Carlson K, Kirsten P, Rossner S. Changes in high density lipoprotein subfractions and other lipoproteins induced by exercise.
Clin Chim Acta 1981;113:51-57.

Oberman A. Exercise and the primary prevention of cardiovascular disease.
Am J Cardiol. 1985;55:10D-20D.

Orekhov AN, Grunwald J. Effects of garlic on atherosclerosis.
Nutrition 1997;13:656-663.

Oshima S. Uchida K. Yasu T. Uno K. Nonogi H. Haze K. Transient increase of plasma Lipoprotein (a) in patients with unstable angina pectoris. Does Lipoprotein (a) alter fibrinolysis? Arterioscler Thromb 1991;11:1772-1777.

Paximadas SA. Pagoni SN, Pitsavos CE, Skoumas JN, Hallaq Y, Giannakides S, Mermegas B, Toutouzas PK. Lp(a) levels during acute infections.
Art Thromb Vasc Biol. 1997;11:1772-1777.

Phelps S. Harris WS. Garlic supplementation and lipoprotein oxidation susceptability. Lipids 1993; 28:475-477.

Philips SM, Green HJ, Tarnopolsky MA, Heigenhauser GJF, Hill RE, Grant SM. Effects of training duration on substrate turnover and oxidation during exercise.
J Appl Physiol. 1996;81:2182-2191.

Ponjee GAE, Janssen EME, vanWersch JWJ. Long-term physical exercise and lipoprotein (a) levels in a previously sedentary male and female population.
Ann Clin Biochem 1995;34:181-185.

Rajman I, Kendall MJ, Cramb R, Holder RN, Salih M, Gamage MD. Investigation of low density lipoprotein subfractions as a coronary risk factor in normotriglyceridaemic men. Atherosclerosis 1996;125:231-242.

Reaven P, Parathasarathy S, Grasse BJ, Miller E, Steinberg D, Witztum JL. Effects of oleate-rich diets an the susceptibility of low density lipoproteins to oxidative modification in mildly hypercholesterolemic subjects.
Clin Invest 1993;91:668-676.

Reckless JP. Management of lipid disorders and the prevention of CHD – can we afford it?, in Betteridge DJ (ed): Lipids: Current Perspectives, vol 3. London, Martin Duntiz Ltd 1996, pp 273-296.

Redinbaugh MG, Campbell WH. Adaptation of the dye-binding protein assay to microtiter plates. Anal Biochem 1985;147:144-147.

Rice-Evans CA, Diplock AT, Symons MCR. Techniques in Free Radical Research., in RH Burdon, PH van Knippenberg.(eds): Laboratory Techniques in Biochemistry and Molecular Biology. Amsterdam, Elsevier, 1991,185-194.

Rumsey SC, Galeano NF, Arad Y, Deckelbaum RJ. Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. J Lipid Res 1992;33:15511561.

Sady SP, Thompson PD, Cullinane EM, Kantor MA, Domagala E, Herbert PN. Prolonged exercise augments plasma triglyceride clearance.
JAMA 1986;256:2552-2555.

Sanchez-Quesada JL, Ortega H, Payes-Romero, Serrat-Serrat J, Gonzalez-Sastre F, Lasuncion MA, Ordonez-Llanos J. LDL from aerobically-trained. subjects shows higher resistance to oxidative modification than LDL from sedentary subjects Atherosclerosis.1997;132:207-213.

Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian survival study (4S). Lancet 1994;334:1383-1389.

Seed M. Lipoprotein (a) – its role in cardiovascular disease. in, Betteridge JD (ed): Lipids: Current Perspectives vol 1 London,
Martin Duntiz Ltd,1996, pp69-88.

Shepherd J. Fibrates and statins in the treatment of hyperlipidaemia: an appraisal of their efficacy and safety. Eur Heart J 1995;16:5-13.

Silagy C. Neil HAW. Garlic as a lipid lowering agent - a Meta analysis.
J Royal Coll Phys 1994; 28:39-45.

Sliverman LM, Christenson RH. Amino acids and proteins, in Burtis CA, Ashwood ER. (ed): Tietz Textbook of Clinical Chemistry, 2nd edition. London, WB Saunders Company1994,pp 671-674.

Strinberg Parthasarathy S. Carnew TE. Khoo JC.Witztum JL. Modifications of Low-Density Lipoprotein that increase its Atherogenicity.
New Engl J of Med 1989; 320:915-924.

Svendsen OL, Hassager C, Christiansen C, Nielsen JD, Winther K. Plasminogen Activator Inhibitor-1, Tissue-Type Plasminogen Activator, and Fibrinogen. Effect of dieting with of without exercise in overweight postmenopausal women. Arterioscler Thromb. 1996;16:381-385.

Szymanski LM, Durstine JL, Davis PG, Dowda M, Pate RR. Factors affecting fibrinolytic potential : Cardiovascular fitness, body composition, and Lipoprotein (a). Metabolism 1996:45:1427-1433.

Taimela S, Viikari JSA, Porkka KVK, Dahlen GH. Lipoprotein (a) levels in children and young adults: the influence of physical activity. The Cardiovascular Risk in Young Finns Study. Acta Paediatrica. 1994;83:1258-1263.

Tatzber F, Esterbauer H. Autoantibodies to Oxidised Low Density Lipoprotein. in G Bellorno, G Finardi, E Maggi and CR Evans (eds): Free Radicals, Lipoprotein Oxidation and Atherosclerosis London, Richelieu Press, 1995 pp 245-262.

Temmel C, Tatzber F, Wonisch W, Lapin A. Age dependant decrease of antibodies to oxidised LDL (oLAb) in healthy people. Society for Free Radical Research – European Region Austrian Biochemical Society. Workshop: Oxidative Stress 5th & 6th July 1996.

Thompson PD, Cullinane E, Henderson LO, Herbert PN. Acute effects of prolonged exercise on serum lipids. Metabolism. 1980;29:662-665.

Torok B, Belagyi J, Rietz B, Jacob R. Effectiveness of Garlic on the Radical Activity in Radical Generating Systems. Drug Res. 1994;44:608-610.

Wallberg-Jonsson S, Uddhammar A, Dahlen G, Rantapaa-Dahlqvist S. Lipoprotein (a) in relation to acute phase reaction in patients with rheumatoid arthritis and polmyalgia rheumatica. Scand J Clin Lab Invest 1995;55:309-315.

Wei M, Macera CA, Hornung CA, Blair SN. Changes in lipids associated with change in regular exercise in free-living men.
J Clin Epidemiol. 1997;50:1137-1142.

Williams PT, Krauss RM, Vranizan KM, Wood DS. Changes in lipoprotein subfraction during diet-induced and exercise-induced weight loss in moderately overweight men. Circulation 1990;81:1293-1304.

Whicher JT. Acute Phase Proteins, Physiology and Clinical Uses.
Clin Biochem Rev 1990;11:4-9.

Wood PD, Haskell W, Klein H, Lewis S, Stern MP, Farquhar JW. The distribution of plasma lipoproteins in middle-aged male runners.
Metabolism. 1976;25:1249-1257.

Wood PD, Stefanick ML, Dreon DM, Frey-Hewitt B, Garay SC, Williams PT, Superko HR, Fortmann SP, Alberts JJ, Vranizan KM, Ellsworth NM, Terry RB, Haskell WL. Changes in plasma lipids and lipoproteins in overweight men during weight loss through dieting as compared with exercise.
N Engl J of Med 1988;319:1173-1179.