DHA-RICH FISH OIL AND REGULAR MODERATE EXERCISE: A COMBINED INTERVENTION TO IMPROVE CARDIOVASCULAR, METABOLIC AND INFLAMMATORY BIOMARKERS IN OBESITY

Alison M Hill

B App Sc. (Hons) Human Movement

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ABSTRACT

The current obesity epidemic has intensified research on lifestyle interventions aimed at combating obesity and associated cardiovascular (CV) and metabolic risk. This clustering of risk factors with obesity is known as the "Metabolic Syndrome" (MS). There is now a large body of evidence detailing the ability of omega-3 fatty acids (n-3 FA) and regular moderate exercise to independently ameliorate several CV risk factors; however the combination of these interventions may be a more effective strategy in reducing CV risk than either treatment alone. This thesis describes the independent and combined effects of supplementation with docosahexaenoic acid (DHA) rich fish oil, and regular moderate exercise, on CV, metabolic and inflammatory biomarkers.

Sedentary, overweight volunteers (BMI > 25kg/m^2) with mild hypertension (140/90 – 160/100mmHg), elevated plasma triglycerides (TAG) (>1.6mmol/L) or elevated total cholesterol (TC) (>5.5mmol/L) were recruited in three cohorts for a 12-week intervention trial. Subjects were randomised to one of the following interventions: fish oil, fish oil and exercise, sunflower oil (placebo), sunflower oil and exercise. Subjects consumed 6 g/day of DHA-rich fish oil (26% DHA, 6% EPA; ~1.9g n-3 FA) or sunflower oil. The exercise groups walked 3 days/wk for 45 min, at 75% age-predicted maximal heart rate (HR). Outcome measures were assessed and compared across each intervention group at Weeks 0, 6 and 12, with the exception of body composition, heart rate variability (HRV) and immune functions, which were assessed at Weeks 0 and 12 only. Apart from the consumption of allocated capsules, all subjects were instructed to maintain their normal diet during the study. If not asked to exercise as part of the intervention subjects were also instructed to maintain their normal level of physical activity.

Supplementation with DHA rich fish oil resulted in substantial increases in total long chain n-3 FA and DHA levels in erythrocyte membranes, accompanied by reduction of TAG, increase of high-density lipoprotein (HDL) cholesterol and reduction of superoxide production by stimulated neutrophils. Both the increase in HDL and the decrease in superoxide production were correlated with the change in erythrocyte DHA. Endothelium dependent arterial vasodilation (assessed by flow-mediated dilatation, FMD), HRV and HR response to exercise were also improved in subjects supplemented with the DHA-rich fish oil. Regular moderate intensity exercise, either alone or in addition to the DHA-rich fish oil supplementation, had no effect on these parameters, although it improved the compliance of small resistance arteries. Interestingly, however, both DHA-rich fish oil and regular exercise reduced body fat and these effects were additive when the interventions were combined. The change in fat mass was accompanied by an increase in fat oxidation during exercise, as measured by the respiratory exchange ratio. For the population as a whole, reductions in total and abdominal fat mass were associated with reductions in blood pressure.

In summary, this study is the first to evaluate the metabolic and CV benefits that can be achieved by combining n-3 FA supplementation from fish oil and regular aerobic exercise in overweight/obese adults. While this combination did not produce any synergistic effects, several independent benefits were attained. The high compliance rate (>85%) within this study indicates that this intervention is well tolerated and may therefore be sustainable in the longer term. Future research should evaluate the mechanisms underlying the n-3 FA - mediated improvements in body composition.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to a copy of my thesis being made available in the University Library.

Alison M Hill

31st March 2007

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GLOSSARY OF ABBREVIATIONS

AA	Arachidonic Acid
ACSM	American College of Sports Medicine
AHA	American Heart Association
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
Apo B	Apolipoprotein B
ATP III	Adult Treatment Panel III
β-receptor	Beta-Adrenoreceptor
BIA	Bioelectrical Impedance
BMI	Body Mass Index
BP	Blood Pressure
° C	Degrees Celsius
Ca^{++}	Calcium
CDC	Centers for Disease Control and Prevention
CHD	Coronary Heart Disease
Con A	Concanavalin A
COX	Cyclooxygenase
CPT-1	Carnitine Palmitoyl Transferase-1
CRP	C-Reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DART	Diet and Reinfarction Trial
DBP	Diastolic Blood Pressure

DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
DXA	Dual Energy X-ray Absorptiometry
ECG	Electrocardiogram
EDTA	Ethyldiaminetetraacetic Acid
ELISA	Enzyme Linked Immunoabsorbent Assay
eNOS	Endothelial NO Synthase
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FAT/CD 36	Fatty Acid Translocase
FMD	Flow-Mediated Dilatation
fMLP	f-met-leu-phe
FXR	Farnesol X Receptor
FO	Fish Oil
FOX	Fish Oil + Exercise
g	Gram
GTN	Glyceryl Trinitrate
GTN-D	Glyceryl - Trinitrate Mediated Dilatation
HbA_{1c}	Glycosylated Hemoglobin
HBSS	Hanks' Balanced Salt Solution
HDL	High Density Lipoprotein
HFP	High Frequency Power
HNF-4α	Hepatic Nuclear Factor-4α
HOMA	Homeostasis Model Assessment
HPEPE	Hydroperoxyeicosapentaenoic Acid
HPETE	Hydroperoxyeicosatetraenoic Acid

HR	Heart Rate
HRV	Heart Rate Variability
IAAT	Intra Abdominal Adipose Tissue
ICAM-1	Intracellular Adhesion Molecule-1
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IFNγ	Interferon Gamma
IGT	impaired glucose tolerance
IL-1β	Interleukin 1 Beta
IL-1ra	Interleukin 1 receptor agonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
ISAK	International Society for the Advancement of Kinanthropometry
kg	Kilogram
kJ	Kilojoule
K^+	Potassium
LA	Linoleic acid
LNA	Alpha-linolenic acid
LDL	Low Density Lipoprotein
LAC	Large Artery Compliance
LFP	Low Frequency Power
LOX	Lipoxygenase
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LT	Leukotriene

LTA_4	Leukotriene A_4
LTA_5	Leukotriene A_5
LTB_4	Leukotriene B_4
LTB_5	Leukotriene B_5
LTC_4	Leukotriene C_4
LTC_5	Leukotriene C_5
LTD_4	Leukotriene D_4
LTD_5	Leukotriene D_5
LTn	Lymphotoxin
LXR	Liver X receptor
mg	Milligram
mL	Millilitre
mm	Millimetre
mRNA	Messenger Ribonucleic Acid
MAP	Mean Arterial Pressure
MI	Myocardial Infarction
MIP	Macrophage Inflammatory Protein
MNL	Mononuclear
MS	Metabolic Syndrome
n-3 FA	Omega-3 FA
n-6 FA	Omega-6-FA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NATA	National Association of Testing Authorities of Australia
NBT	Nitroblue Tetrazolium
NCEP	National Cholesterol Education Program
NF-κB	Nuclear Factor-Kappa-Beta
NHMRC	National Health and Medical Research Council

NNS95	1995 Australian National Nutrition Survey
NO	Nitric Oxide
OD	Optical Density
OGTT	Oral Glucose Tolerance Test
PA	Physical Activity
PAI-1	Plasminogen Activator Inhibitor-1
PBMC	Peripheral Blood Mononuclear Cells
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGH_2	Prostaglandin H_2
PGH_3	Prostaglandin H_3
PGJ_2	Prostaglandin J_2
PGJ_3	Prostaglandin J_3
PHA	Phytohaemagglutinin
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PP	Pulse Pressure
PPAR	Peroxisome Proliferator-Activated Receptor
PPL	Post-prandial Lipemia
QUICKI	Quantitative Insulin-Sensitivity Check Index
RCT	Randomised Controlled Trial
RER	Respiratory Exchange Ratio
ROS	Reactive Oxygen Species
s	second
SAC	Small Artery Compliance
SBP	Systolic Blood Pressure

SEM	Standard Error of Mean
SREBP-1c	Sterol Regulatory Element Binding Protein
SO	Sunflower Oil
SOX	Sunflower Oil + Exercise
SVR	Systemic Vascular Resistance
TAG	Triglyceride
TC	Total Cholesterol
TNFα	Tumor Necrosis Factor Alpha
TVI	Total Vascular Impedance
TX	Thromboxane
TXA_2	Thromboxane A_2
TXA_3	Thromboxane A_3
µL	Microlitre
VCAM-1	Vascular Cell Adhesion Molecule-1
VLCD	Very Low Calorie Diet
VLDL	Very Low Density Lipoprotein
VO_2	Oxygen Uptake
VCO_2	Carbon Dioxide Output
WC	Waist Circumference
WHO	World Health Organisation
WHR	Waist to Hip Ratio
WLFO	Weight Loss with Fish Oil
WLPO	Weight Loss with Placebo Oil

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PUBLICATIONS ARISING FROM THIS THESIS

Papers – Published

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Buckley JD, **Hill AM**, Coates AM, Howe PRC. Simpler diet and exercise strategies for managing obesity in "Physical activity and obesity". Editors Hills AP, Bryne NM and King NA. Smith-Gordon and Co, London (Published August 2006).

Papers - In Press

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Ninio DM, **AM Hill**, JH Smith, K Murphy, J Buckley, DA Saint, PRC Howe. Fish oil enhances heart rate variability in overweight adults. Cardiac Soc ANZ, Brisbane. Heart, Lung Circ 2004;13:S82

Davison K, **Hill A**, Thorp A, Worthley C, Murphy K, Howe P, Buckley J. Digital volume pulse (DVP) analysis of vasodilator function during exercise. *The Australian Society for Medical Research (SA Division) Annual Scientific Meeting*, 4th June 2004; abstr. O31.

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Hill AM, Buckley JD, Murphy KJ, Saint DA, Morris AM & Howe PRC Combined effects of omega-3 supplementation and regular exercise on body composition and cardiovascular risk factors. *Nutrition Society of Australia, 29th Annual Scientific Meeting*, December 2005, Melbourne, Australia.

Hill AM, Buckley JD, Murphy KJ, Saint DA & Howe PRC. Omega - 3 supplementation enhances body fat loss during exercise. *2nd International Symposium on Triglycerides and HDL: Role in Cardiovascular Disease and the Metabolic Syndrome*, July 2005, New York City, USA.

Hill A, Buckley J, Murphy K, Saint D, & Howe P. Cardiovascular & Metabolic benefits following omega-3 fatty acid supplementation and exercise. *Australasian Section of the American Oil Chemists Society, Bi-Annual Meeting*, December 2004, Adelaide, Australia.

Hill A, Murphy K, Saint D, Buckley J, Howe P. Combined benefits of omega-3 and exercise for cardiovascular health. Australian Atherosclerosis Society Annual Scientific Meeting, September 2004, Barossa Valley, Australia.

Hill AM, Murphy KJ, Saint DA, Buckley JD, Howe P. Combined effects of omega-3 (ω3) and moderate exercise on body fat and cardiovascular (CV) risk factors. *The Australian Society for Medical Research (SA Division) Annual Scientific Meeting*, 4th June 2004; abstr. O30.

Poster Presentations

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1 INTRODUCTION

1.1 OVERVIEW

Omega-3 fatty acids (n-3 FA) are naturally occurring polyunsaturated fatty acids found in fish, fish oil and green vegetables. These FA, along with omega-6 (n-6) FA, found primarily in meat and dairy products are considered essential for the human body and must be consumed in the diet.

Linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (LNA; 18:3n-3) are the precursors for the n-6 and n-3 FA families, respectively and through a series of elongation and desaturation steps produce arachidonic acid (AA, 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3), respectively. Additional metabolism of EPA produces the n-3 FA docosahexaenoic acid (DHA, 22:6n-3). Omega-3 FA are important for human health in that they promote infant cognitive development, and in later years they maintain physical, mental, and reproductive fitness (Simopoulos, 1991).

Omega-3 FA have been used extensively for the prevention and treatment of cardiovascular disease (CVD) and inflammatory disorders, such as arthritis and psoriasis. More recently, it has been suggested that n-3 FA may have metabolic benefits, although these are yet to be confirmed.

The rapid rise in the prevalence of obesity in recent times indicates that it is now the most common risk factor for CVD. However, although obesity is a strong, independent predictor of CVD and diabetes, it is the clustering of obesity with other CVD risk factors that is of most concern since this clustering increases CVD risk by more than the sum of the individual risk factors alone. This clustering has been termed the “Metabolic Syndrome”

(MS), and it encompasses a number of physiological and metabolic abnormalities, such as dyslipidemia, hypertension, hyperglycaemia, and obesity, that relate to insulin resistance and CVD (Grundy, 2006).

Recommended strategies for the prevention and treatment of MS include diet and lifestyle change (Lichtenstein *et al.*, 2006). Dietary recommendations have typically focused on reducing energy intake, particularly intake of saturated and *trans* fats. Similarly, lifestyle recommendations are aimed at increasing physical activity, however compliance with caloric restriction and regular physical activity is often poor. This has stimulated interest in developing specific dietary modifications and patterns of physical activity that are achievable for the majority of obese people, while at the same time delivering cardiovascular (CV) and metabolic benefits.

Given the broad range of physiological benefits that are known to derive from consuming n-3 FA, increasing their intake in the diet might provide a simple therapeutic intervention for combating CV risk. Similarly, regular physical activity can ameliorate these same CV risk factors, and additionally, may play a role in counteracting obesity, even when performed at relatively modest levels. However, the potential for a modest level of regular physical activity and an increased intake of n-3 FA to act in combination to enhance these known CV, and possible metabolic benefits, is yet to be explored. Furthermore, these interventions may impact on several inflammatory processes that contribute to the atherosclerotic process.

The aim of this thesis was to examine the independent and combined effects of n-3 FA supplementation and a modest level of regular physical activity and on CV, metabolic and inflammatory risk factors in an overweight / obese population.

1.2 OMEGA-3 FATTY ACIDS (n-3 FA)

1.2.1 Description, sources & metabolism of n-3 FA

Dietary fat comprises three types of fat, which are classified by the number of double bonds or degree of saturation: saturated fat, monounsaturated fat and polyunsaturated fat. Polyunsaturated fats can be divided into two additional classes based on the position of the double bond in relation to the omega terminal: n-6 and n-3. LA and LNA are the precursors for the n-6 and n-3 FA families, respectively (**Figure 1.1**). These FA cannot be synthesized by animals and are therefore considered essential and must be obtained through the diet. Although plants (including nuts and seeds) provide good sources of LA and LNA, their low fat content means they contribute little to the overall dietary intake of these FA. In comparison, plant oils and margarines provide a more substantial contribution although these contain significantly more LA than LNA (Holub, 2002). LA and LNA undergo a series of desaturation and elongation steps that yield AA and EPA, respectively (**Figure 1.2**). Additional metabolism of EPA produces the n-3 FA DHA. AA is also obtained from the diet in animal products such as meat and dairy, while the greatest source of EPA and DHA is from fish.

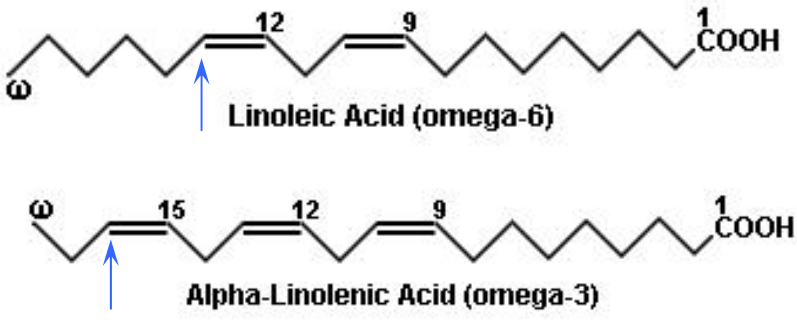

Figure 1.1 Structure of linoleic (LA) and alpha-linolenic acid (LNA). [1]

[1] LA is an n-6 FA with two double bonds, the first occurring at the sixth carbon from the methyl end. LNA is an n-3 FA with three double bonds, the first occurring at the third carbon from the methyl end.

NOTE: This figure is included on page 4 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 Metabolic pathway of the conversion of linoleic acid (18:2n-6, LA) and linolenic acid (18:3n-3, LNA). [1]

[1] From Holub & Holub, 2004.

⇡ Indicates retroconversion.

1.2.2 Biochemical effects of n-3 FA

Omega-3 FA are thought to exert their biological effects through alterations in the lipid membrane environment (i.e. an increase in membrane fluidity) or by acting directly as substrates for metabolism (Demaison & Moreau, 2002). Until recently the beneficial effects of n-3 FA were predominantly attributed to changes in eicosanoid synthesis. However there is evidence that in addition to eicosanoid formation, n-3 FA, by altering membrane fluidity, can egulate gene expression (Clarke, 2000) and modulate ion channels (Simopoulos, 1999).

1.2.2.1 Eicosanoid synthesis

Eicosanoids are a group of physiologically active signaling molecules that exert several biological effects. There are four families of eicosanoids, the prostaglandins (PG), prostacyclins, thromboxanes (TX) and leukotrienes (LT), and several series within each family. Eicosanoids derived from the n-6 FA, AA, are pro-inflammatory and pro-aggregatory, and promote vasoconstriction and leukocyte chemotaxis and adherence, whereas n-3 FA, more specifically EPA, derived eicosanoids are less inflammatory and less pro-aggregatory (Leaf & Weber, 1988).

AA is released from the cell membrane by phospholipase A_2 where it is oxygenated by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. The metabolism of AA by COX leads to the production of the two series PG and TX (PGE_2 and TXA_2). Alternatively, metabolism by the LOX pathway produces four-series LT (LTB_4) and hydroperoxy derivatives. EPA disrupts this synthetic pathway by preventing the mobilisation of AA from cell membranes by phospholipase A_2 (Obata *et al.*, 1999) and directly competing with AA for metabolism by COX and LOX enzymes, resulting in the production of the three series PG and TX (PGE_3 and TXA_3) and the five-series LT (LTB_5) (James *et al.*, 2000). EPA also leads to an increase in total prostacyclin by increasing PGI_3 without a reduction in PGI_2. Both PGI_3 and PGI_2 are potent vasodilators and inhibitors of platelet aggregation (Simopoulos, 2002). This metabolic pathway is presented in **Figure 1.3**. While DHA is not a direct substrate for eicosanoid metabolism there is evidence that DHA may exert anti-inflammatory effects by i) inhibiting gene expression of enzymes involved in eicosanoid synthesis (VanRollins, 1995), ii) inhibiting expression of inflammatory mediators (De Caterina *et al.*, 2000; Calder, 2005) or iii) conversion into highly anti-inflammatory compounds (i.e. resolvins) (Hong *et al.*, 2003).

Figure 1.3 Metabolic pathway of eicosanoid synthesis from arachidonic acid (20:4n-6) and icosapentaenoic acid (20:5n-3). [1]

[1] Adapted from Simopoulous, 2002.

Abbreviations: PG, prostaglandin; TX, thromboxane COX, cyclooxygenase; LOX, lipoxygenase; HPETE, ydroperoxyeicosatetraenoic acid; HPEPE, ydroxyeicosapentaenoic acid, LT, leukotriene.

1.2.2.2 Modulation of ion channels

Omega-3 FA may reduce the risk for sudden cardiac death (Burr *et al.,* 1989; Siscovick *et al.*, 1995; Albert *et al.*, 1998; de Lorgeril *et al.,* 1999; 1999) by decreasing the occurrence of fatal sustained ventricular arrhythmia. This relationship has been clearly demonstrated in animal models (Nair *et al.*, 1997), and is related to an increase in membrane and free levels of n-3 FA (Siscovick *et al.,* 2003). Higher n-3 FA levels alter cardiac ion channel function, thereby modifying the cardiac action potential and reducing vulnerability to ventricular arrhythmia (Siscovick *et al.*, 2003). Xiao and colleagues (Xiao *et al.*, 2005) have demonstrated that n-3 FA can inhibit the fast, voltage-dependent sodium currents and the L-type calcium currents, leading to a voltage-dependent shift of the steady state inactivation potential, and prevention of calcium overload in heart cells (Leaf *et al.,* 2003).

1.2.2.3 Regulation of gene expression

There is now substantial evidence that n-3 FA can modulate inflammation and energy (lipid and glucose) metabolism, either by directly influencing gene transcription or through changes in cell signalling mediated via changes in the cell-lipid membrane (Clarke *et al.,* 1997). *In vitro* experiments have demonstrated that DHA can inhibit the expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (De Caterina *et al.,* 1994). E-selectin slows and tethers rolling leukocytes to the endothelium, while ICAM-1 and VCAM-1 mediate their attachment (Springer, 1990). Omega-3 FA may also influence inflammatory gene expression by altering cell signalling upstream of nuclear factor kappa β (NF-κβ). NF-κβ is a transcription factor that regulates the production and expression of several cell adhesion molecules and inflammatory cytokines (De Caterina *et al.*, 2006). A key component of the NF-κβ activation pathway is protein kinase C (PKC). Inhibition of PKC will lead to a reduction in the activity of NF-κβ. Both EPA and DHA can inhibit PKC activation (May *et al.*, 1993), which may therefore influence the production and expression of inflammatory cytokines and adhesion molecules.

The mechanism by which n-3 FA regulate fuel partitioning is likely through their action as ligands for several nuclear receptors. These receptors include liver X receptor (LXR), hepatic nuclear factor-4α (HNF-4α), farnesol X receptor (FXR), peroxisome proliferator-activated receptors (PPARs – α, β, γ1 and γ2) and sterol regulatory element binding protein (SREBP)-1c. Triglyceride (TAG) synthesis is predominantly influenced by the activity of SREBP-1c, which stimulates the synthesis of lipogenic enzymes including fatty acid synthase and acetyl-CoA carboxylase (Horton *et al.*, 2002). This transcription factor is directly controlled by LXR, and indirectly by FXR. The activation of FXR and inhibition of LXR by n-3 FA leads to a reduction in SREBP-1c gene transcription and results in a decrease in hepatic TAG synthesis (Davidson, 2006). Additionally, activation of PPARα can suppress the LXR-SREBP-1c pathway (Yoshikawa *et al.*, 2003). Furthermore, n-3 FA may inhibit SREBP-1c directly by reducing the concentration of precursor and mature SREBP-1c protein and SREBP-1c mRNA (Xu *et al.*, 1999).

By acting as ligands for PPARs, n-3 FA can also induce the transcription of genes encoding proteins for thermogenesis and fatty acid oxidation, such as lipoprotein lipase, acetyl-CoA carboxylase-2, fatty acid translocase, carnitine palmitoyl transferase-1 (CPT-1), and mitochondrial uncoupling protein 3 [as reviewed by Clarke (Clarke, 2000)]. PPARs are also involved in high-density lipoprotein (HDL) metabolism. Activation of PPARα may increase HDL by up-regulating the synthesis of apolipoprotein A-I and A-II (Vu-Dac *et al.*, 1995; Vu-Dac *et al.*, 1998). The regulation of these nuclear receptors by n-3 FA can effectively mediate a shift in fuel metabolism, away from storage and toward oxidation. This increase in fatty acid oxidation and thermogenesis may also have additional benefits in improving insulin sensitivity and enhancing glucose uptake (Clarke, 2000).

1.2.3 Health benefits of n-3 FA

1.2.3.1 Healthy development and prevention of chronic disease

Both AA and DHA play a major role in growth and functional development of the brain and retina (Innis, 2003) and deficiencies in DHA and AA during infant development can lead to abnormalities in visual acuity and learning difficulties (Horrocks & Yeo, 1999). In later life, the importance of n-3 and n-6 FA in the diet is related to the bioactivity of AA, EPA and DHA, and their role in the prevention of chronic disease. Increased intake of n-3 FA has shown beneficial effects in several inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel disease and psoriasis, which are associated with changes in eicosanoid metabolism (Simopoulos, 2002). A reduction in the production of pro-inflammatory and pro-aggregatory eicosanoids may also explain some of the associated CV benefits of n-3 FA (Grimm *et al.,* 2002).

1.2.3.2 Counteracting CVD – Evidence from Epidemiological studies & Randomised Controlled Trials (RCTs)

Bang & Dyerberg first highlighted the possible cardio-protective effects of n-3 FA in 1971 (Bang *et al.*, 1971; Bang & Dyerberg, 1972). Since then, several studies have demonstrated that n-3 FA intake from fish is associated with reduced mortality from coronary heart disease (CHD) (Kromhout *et al.*, 1985; Dolecek & Granditis, 1991; Rodriguez *et al.*, 1996; Daviglus *et al.*, 1997; Hu *et al.*, 2002). Zhang et al. (Zhang *et al.*, 1999) investigated the relationship between fish consumption and all cause mortality in 36 countries, and similarly reported an inverse relationship between fish consumption and mortality from all-causes, ischemic heart disease and stroke. However not all studies have demonstrated a beneficial association between fish consumption and mortality from CHD (Ascherio *et al.*, 1995; Morris *et al.*, 1995; Albert *et al.*, 1998). It has been suggested that this may be due to methodological differences (including differences in experimental design, study end points, intervention and reference populations, and quantification of fish intake), the possible adverse effects of high

levels of methyl mercury, and small control or reference population sample sizes (Kris-Etherton *et al.*, 2003).

The Diet and Reinfarction Trial (DART) and the GISSI-Prevenzione Study are perhaps the most referenced RCTs investigating the role of n-3 FA in secondary prevention of CHD. The DART trial (Burr *et al.*, 1989) demonstrated a 29% reduction in two year all-cause mortality in recovered myocardial infarction (MI) patients advised to increase their intake of fatty fish to two serves per week. After two years their intake of EPA had increased fourfold and averaged 2.4 g/week, compared to 0.6 g/week in the control group. The GISSI-Prevenzione Study (GISSI-Prevenzione Investigators, 1999) was designed to investigate the independent and combined effects of fish oil and vitamin E on morbidity and mortality after MI. Patients supplemented with 1 g/day of fish oil (0.85-0.88 mg EPA+DHA) had a 20% reduction in risk of overall mortality and a 30% reduction in risk of mortality from CVD. Supplementation with vitamin E showed no effect on risk reduction, nor did it provide any additional benefits above that observed with fish oil supplementation alone. Bucher et al (Bucher *et al.*, 2002) reviewed the outcomes of several RCTs and reported a risk ratio of 0.7 for sudden death and fatal MI, and 0.8 for non-fatal MI in patients supplemented with n-3 FA compared with placebo or control. Hooper et al. (Hooper *et al.*, 2004) recently challenged these results in a *Cochrane Review*, however the Hooper review has been criticised based on the inclusion of the Dart II trial and the omission of biomarker-based studies. Psota and colleagues (Psota *et al.*, 2006), in their excellent review of dietary n-3 FA intake and CV risk, conclude that there is substantial evidence for a beneficial effect of dietary n-3 FA on CVD, and suggest that if the methodologically flawed DART-II trial had been excluded from the Cochrane meta-analysis it would provide evidence for continued support of recommendations to increase dietary fish consumption for the treatment and prevention of CVD (Lichtenstein *et al.*, 2006).

1.2.4 Current & recommended intakes of n-3 FA

Dietary patterns have changed dramatically in industrialized societies compared with the Hunter-gatherer, with substantial increases in dietary fat, largely due to increased intake of saturated, trans and n-6 FA, with a concurrent reduction in n-3 FA intake (**Figure 1.4**) (Simopoulos, 1995). Optimal dietary intake of n-6 FA and n-3 FA should be ~2:1, however it is estimated that the typical Western diet has an n-6: n-3 FA ratio of 15:1 to 16.7:1 (as reviewed by Simopoulos 2006 (Simopoulos, 2006)). This imbalance favors the formation of AA and the production of pro-inflammatory and pro–aggregatory eicosanoids. The World Health Organisation (WHO) has recommended an n-6: n-3 FA ratio of between 5:1 and 10:1 (FAO/WHO, 1993). However, there is some disagreement as to whether the n-6: n-3 FA ratio provides an adequate representation of the overall health of the diet, and consequently recommended intake levels may be more appropriate (Kris-Etherton *et al.*, 2004).

NOTE: This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4 Hypothetical schematic diagram of the change in total dietary fat intake and the contribution of different fatty acids.[1]

[1] From Simopoulous 1995

Based on estimates from the 1995 Australian National Nutrition Survey (NNS95) the mean intake of total long chain n-3 FA (EPA + docosapentaenoic acid (DPA) + DHA) in Australia is 246 mg/day (Howe *et al.*, 2006). However, the median intakes are substantially lower (160 mg for men, 90 mg for women) (Howe, unpublished research). These median values have been adopted by the National Health and Medical Research Council (NHMRC) as adequate intakes (AI) for Australian adults. The NHMRC also recommends target intakes for Australian adults as 610 mg/day for men and 430 mg/day for women (National Health and Medical Research Council, 2006) for the prevention of chronic disease. These values are based on the 90th percentile of current population intake, indicating that only 10% of Australians meet the intake of n-3 FA recommended for the prevention of CVD (Howe, unpublished research). Recommendations for North America, published by the American Heart Association (AHA) suggest an n-3 FA intake of 1g/day for patients with CHD, and 2-4 g/day for individuals requiring TAG lowering. For CVD risk reduction, the AHA recommends 500-mg/d n-3 FA or consumption of oily fish twice per week (Kris-Etherton *et al.*, 2003).

Changes in dietary intake of EPA and DHA are reflected in the FA profile of several tissues. EPA and DHA are readily incorporated into the membranes of erythrocytes and are present for the life of the cell (Brown *et al.*, 1991b; Brown *et al.*, 1991a). Erythrocytes are therefore a useful marker for assessing FA status and there is good evidence that erythrocyte FA composition reflects FA levels in other tissues, particularly cardiac tissue (Harris *et al.*, 2004; Ninio *et al.*, 2005). This relationship has provided the basis for the development of the Omega-3 Index (Harris & von Schacky, 2004), which correlates erythrocyte n-3 FA (EPA + DHA) level with risk for CV mortality. The Omega-3 Index (Harris & von Schacky, 2004) suggests that the greatest protection against CV mortality is with erythrocyte n-3 FA (EPA + DHA) levels >8% of total fatty acids. Supplementation with n-3 FA from foods or fish oil can result in a near doubling of DHA in erythrocyte membranes (Murphy *et al.*, 2007). Comparable changes have been observed in platelets, mononuclear (MNL) cells (Mantzioris

et al., 2000; Di Stasi *et al.*, 2004) and neutrophils (Kew *et al.*, 2004), although there is some degree of variability in the time course for incorporation between different cell types (Di Stasi *et al.*, 2004).

1.3 PHYSICAL ACTIVITY (PA)

1.3.1 Description of PA

Physical activity refers to any form of movement that results in an increased energy output from the resting condition, and therefore includes leisure and non-leisure activities. Moderate-intensity exercise correlates with 60-79% of maximal heart rate (HR), whereas vigorous exercise is equivalent to 80-89% of maximal HR (Wilmore & Costill, 1999).

1.3.2 Health benefits of PA

1.3.2.1 Counteracting CVD – Evidence from Epidemiological studies & Randomised Controlled Trials (RCTs)

Morris (Morris & Heady, 1953; Morris *et al.*, 1953) and Paffenberger (Paffenbarger & Hale, 1975; Paffenbarger *et al.*, 1978) demonstrated early evidence for an association between physical activity and reduced mortality. Since then, several long-term epidemiological studies have been conducted to determine CV mortality in sedentary compared with active individuals (Powell *et al.*, 1987; Berlin & Colditz, 1990). These studies generally confirm a reduced risk of overall and/or CVD mortality in physically active individuals compared to sedentary people. Data from the Framingham study provides additional support for the prevention of CVD and an increase in life expectancy in physically active individuals (Franco *et al.*, 2005). Physical or cardiorespiratory fitness is also associated with a reduced incidence of death (Blair *et al.*, 1995; Lee *et al.*, 1999; Laukkanen *et al.*, 2001), and both of these demonstrate a strong inverse relationship for risk of fatal and non-fatal health outcomes (Blair *et al.*, 2001; Kohl, 2001). Furthermore, physical fitness predicts mortality independently of overweight or obesity (Wei *et al.*, 1999). An increase in energy

expenditure of 1000 kcal / week (equivalent to 1 hr of moderate-intensity walking on 5 days) is associated with a 20-30% reduction in risk of all-cause mortality (Lee & Skerrett, 2001).

1.3.3 Current and recommended levels of PA

Several health and government authorities from Australia (Department of Health and Aged Care; *National Physical Activity Guidelines for Australia*) and the United States [Centers for Disease Control and Prevention (CDC) and the American College of Sports Medicine (ACSM)] recommend at least 30 minutes of moderate-intensity physical activity on most, preferably all days of the week to achieve health benefits (Pate *et al.*, 1995; Department of Health and Aged Care, 1999; Thompson *et al.*, 2003). These requirements may be accumulated throughout the day in 10-15 minute blocks, or in a single 30-minute session. In addition, these guidelines acknowledge that participation in some vigorous activity can attain additional health and fitness benefits over and above that attained from regular moderate-intensity activity (Department of Health and Aged Care, 1999). For weight control or weight reduction, it is estimated that 60-90 min/day of moderate-intensity activity is required (Jakicic & Otto, 2006). However this required frequency and duration of exercise has obvious implications for exercise compliance, particularly with respect to work and family commitments. In 2000 in the United States, it was determined that only 32% of adults engaged in regular leisure-time physical activity (Barnes & Schoenborn, 2003) and in 2003, over 54% of US adults failed to meet the minimum recommended level of physical activity (Centers for Disease Control and Prevention (CDC), 2005). In Australia, several surveys have been conducted to determine the prevalence and progression of physical inactivity. The *2000 National Physical Activity Survey* determined that the proportion of Australians who did not undertake sufficient activity for health benefits had increased by 5% since 1997 to 54%, 15% of whom were classified as sedentary (Australian Institute of Health & Welfare (AIHW), 2004). Data from the *2001 National Health Survey* reported that 30.6% of men and 32.3% of women were physically inactive, as defined by participation in less than 15 minutes/week of

leisure-time exercise (Australian Institute of Health & Welfare (AIHW), 2005). More recently, the *2005-2006 Multi-Purpose Household Survey* was conducted to evaluate regular participation (defined as two or more times per week) in sports and physical activity (Australian Bureau of Statistics (ABS), 2007). The survey found 29% of the population were regular participants in sports and physical recreation activities, with walking the most popular activity.

1.4 EFFECTS OF n-3 FA & PA ON CV, METABOLIC & INFLAMMATORY BIOMARKERS

There are an overwhelming number of studies investigating the effects of n-3 FA and PA on CV risk factors including hypertension, dyslipidemia, glucose tolerance, insulin sensitivity, endothelial function, inflammation and immune function. Consequently, it is not the intention that this review be an exhaustive analysis of these individual studies, but will rather identify and discuss the general body of knowledge concerning the effects of n-3 FA and PA on risk factors for CVD.

1.4.1 CV biomarkers

1.4.1.1 Blood Lipids

1.4.1.1.1 Overview

Currently, Australian recommendations (National Heart Foundation & Cardiac Society of Australia and New Zealand) are to lower fasting TAG below 1.5 mmol/L (Tonkin *et al.*, 2005) as there is evidence that elevated TAG is an independent risk factor for CVD (Austin *et al.*, 1998; Cullen, 2000). An increase in available TAG can contribute to CVD by altering lipoprotein density (Rizzo & Berneis, 2006). Small dense lipoproteins are associated with an increased risk for coronary artery disease (Austin *et al.*, 1988; Lamarche *et al.*, 1997; St-Pierre *et al.*, 2005). Although not conclusive, TAG may contribute to other pathological

processes associated with CV disease such as increased inflammation, impaired fibrinolysis, increased coagulability and endothelial dysfunction (Bersot *et al.*, 2006). Furthermore, elevated TAG often present with low HDL and elevated small, dense LDL, coined "the lipid triad" or atherogenic dyslipidemia (Grundy, 1998), and this phenotype is associated with increased risk for CVD (Austin *et al.*, 1988). However, irrespective of the presence of hypertriglyceridaemia, elevated serum LDL and reduced HDL are independently associated with an increased risk for coronary events (Abbott *et al.*, 1988; Neaton & Wentworth, 1992; Stamler *et al.*, 2000).

The most recognised mechanism by which HDL prevents atherosclerosis is through reverse cholesterol transport. This process involves the extraction of cholesterol from atherosclerotic plaques, foam cells and macrophages and its subsequent transportation by HDL to the liver where it is converted to bile acids and excreted (Toth, 2005). HDL may also impact on other antiatherothrombotic mechanisms to reduce LDL oxidation, inhibit platelet aggregation and expression of vascular adhesion molecules, and stimulate nitric oxide (NO) and prostacyclin production (Toth, 2005). National guidelines in Australia recommend that HDL concentrations be > 1.0 mmol/L (Tonkin *et al.*, 2005).

There is substantial evidence that reducing LDL cholesterol can reduce the risk for developing CV disease (Grundy *et al.*, 2004). Currently, the National Heart Foundation recommends an LDL concentration of < 2.5 mmol/L (Tonkin *et al.*, 2005) but is considering lowering the recommended target to 2.0 mmol/L. LDL, particularly small dense LDL which is more readily oxidised, plays a key role in the development of atherosclerosis (St-Pierre *et al.*, 2005; Rizzo & Berneis, 2006). The process of atherosclerosis begins with injury to the endothelium and movement of LDL from plasma into the artery wall where it accumulates Trapped LDL are then oxidised, triggering recruitment of monocytes from the blood which differentiate into macrophages and engulf the modified LDL particle, forming a cholesterol-filled foam cell (Barter, 2005). Macrophages perpetuate the formation of foam cells by releasing cytokines and growth factors that stimulate the expression of adhesion molecules,

and enhance smooth muscle cell growth and proliferation, eventually leading to plaque formation (Damjanov, 2000).

1.4.1.1.2 Effects of n-3 FA on blood lipids

Several reviews have documented the effects of n-3 FA on blood lipids ((Harris, 1989; Schmidt *et al.*, 1993; Harris, 1996; Harris, 1997). With respect to this study, the review by Harris in 1997 (Harris, 1997) is most pertinent. This paper reports the effects of placebo-controlled trials administering <7 g/day of n-3 FA on normolipidaemic (TAG < 2.0 mmol/L) and hypertriglyceridaemic (TAG ≥ 2.0 mmol/L) individuals. These results are summarised in **Table 1.1**.

Table 1.1 Effect of n-3 FA supplementation (<7 g/day) on blood lipids (% change from baseline). [1]

Design	Type	TC	TAG	LDL	HDL
Crossover	**Hypertriglyceridaemic**	↓ 1.0	↓ 33.8	↑ 10.8	↑ 1.2
	Normolipidaemic	↑ 1.4	↓ 25.2	↑ 4.5	↑ 2.9
Parallel	**Hypertriglyceridaemic**	↑ 2.5	↓ 25.2	↑ 5.2	↓ 0.1
	Normolipidaemic	↑ 2.5	↓ 25.4	↑ 4.5	↑ 2.8

[1] As reviewed by Harris 1997.

It is well established that supplementation with n-3 FA rich fish oil (1.1g/day up to 7 g/day n-3 FA) consistently lowers TAG in both normo-and hypertriglyceridaemic individuals (Harris, 1997). The TAG lowering effect is more pronounced in subjects with elevated TAG levels (Schmidt *et al.*, 1993), and the reduction is dose-dependent (Schmidt *et al.*, 1990; Harris, 1996; Robinson & Stone, 2006). Relatively low doses (1.5-2.8 g/day n-3 FA) have

also shown to be effective (Agren *et al.*, 1996; Davidson *et al.*, 1997). The reduction in TAG results from an increase in TAG oxidation, together with a reduction in the production of very LDL (VLDL) (Kinsella *et al.*, 1990b) and inhibition of hepatic TAG synthesis and secretion (Davidson, 2006).

Some studies have reported a decrease in total cholesterol (TC) following intervention with high doses of n-3 FA, most likely due to a concomitant reduction in saturated fat intake (Harris, 1989). In general, TC remains relatively stable following supplementation but with changes in the balance of LDL and HDL, as fish oil tends to increase LDL and LDL particle size (Table 1.1) in patients with hypertriglyceridaemia (Contacos *et al.*, 1993; Suzukawa *et al.*, 1995; Mori *et al.*, 2000a; Woodman *et al.*, 2003b). Larger LDL particles are less atherogenic than smaller, denser sub-fractions (Austin *et al.*, 1988; Lamarche *et al.*, 1997; St-Pierre *et al.*, 2005). An increase in LDL particle size has also been demonstrated in statin-treated hyperlipidaemics (Nordoy *et al.*, 2001), and indicates that n-3 FA may be a useful adjunct to Statin treatments.

The effects of n-3 FA on HDL are not entirely clear. A 1997 review by Harris (Harris, 1997) reported no effect of n-3 FA on HDL cholesterol, as did a *Cochrane Review* in 2004 (Hooper *et al.*, 2004). However this has been challenged by a more recent meta-analysis showing a net increase in HDL of 0.08-0.13 mmol/L in most studies (Robinson & Stone, 2006). Although doses ranged from 0.045 to 5.4 g/day EPA + DHA, a dose-response effect was not apparent. In both normo- and hypertriglyceridaemic individuals, reductions in TAG have been associated with an increase in HDL (Miller *et al.*). Given this association it is surprising that the effects of n-3 FA on HDL are inconsistent. Nevertheless, although total HDL may be unchanged, there is some evidence that n-3 FA may alter HDL sub-fractions (Mori & Woodman, 2006), particularly by increasing HDL-2 (Lungershausen *et al.*, 1994). The potential increase in HDL may result from a decrease in lipid transfer protein activity (Abbey *et al.*, 1990), and an increase in the activity of lipoprotein lipase (LPL) via activation of PPAR-α, β and γ (Davidson, 2006).

1.4.1.1.3 Effects of PA on blood lipids

Several meta-analyses have reviewed the effects of regular aerobic exercise training on lipids and lipoproteins (Halbert *et al.*, 1999; Durstine *et al.*, 2001; Leon & Sanchez, 2001; Carroll & Dudfield, 2004; Kelley *et al.*, 2005). Carroll & Dudfield (Carroll & Dudfield, 2004) reported that regular aerobic exercise was effective in reducing TAG (12%) and increasing HDL (4.1%) in overweight/obese, sedentary adults with symptoms of dyslipidemia. Similar changes in HDL (4.6%) were reported by Leon & Sanchez (Leon & Sanchez, 2001), however these authors indicated that changes in other lipid parameters were far less consistent and of smaller magnitude than those reported above. Durstine et al. (Durstine *et al.*, 2001) reported reductions in TAG between 4-22% and increases in HDL from 4-37%, with no effects on LDL or TC. They suggest that changes in these lipids may be dependent on training volume, with a minimum expenditure of 1200 kcal/week required to elicit these improvements. Furthermore, subjects with normal to high levels of HDL may benefit most from training interventions (Durstine *et al.*, 2001). In a recent meta-analysis, Kelley et al. (Kelley *et al.*, 2005) concluded that regular aerobic exercise was effective in reducing TAG (11%) in previously sedentary, overweight/obese adults. Although variable, these meta-analyses indicate that regular aerobic exercise may induce favourable changes in TAG and HDL, but not LDL or TC. However, there is evidence to suggest that exercise may be ineffective in raising HDL in subjects with isolated low HDL levels (Couillard *et al.*, 2001). Regular moderate exercise may also change LDL particle size without any change in total LDL concentration (Kraus *et al.*, 2002).

The exercise-induced reductions in TAG are likely mediated by changes in the activity of LPL. LPL is located on the walls of capillaries in most tissues, but is found in particularly high quantities in adipose, heart and skeletal muscle. LPL determines the rate of TAG catabolism (Eckel, 1989), with its activity increasing the release of fatty acids for uptake by tissues. During exercise the activity of LPL in skeletal muscle increases (Nikkila *et al.*, 1978) and TAG move from the circulation and into the exercising muscle. The plasma TAG

lowering effect of exercise may be prolonged for up to 3 days and consequently any additional exercise within this time will cause a further reduction in TAG levels (Mougis, 2006). An increase in LPL, in addition to a decrease in hepatic lipase (Stubbe *et al.*, 1983), results in a net reduction in HDL catabolism (Mendoza *et al.*, 1991).

1.4.1.2 Blood Pressure (BP)

1.4.1.2.1 Overview

It is estimated that 30% of Australians have elevated BP (National Heart Foundation Australia, 2004a), defined as systolic BP > 140 mm Hg and diastolic > 90 mm Hg. Resting BP, at levels >110 mm Hg systolic, demonstrates a consistent, graded relationship with several CVD outcomes including mortality, MI, stroke, heart failure, and renal dysfunction (Lewington *et al.*, 2002). Consequently individuals should attempt to reduce their BP to normal levels (systolic < 120 mm Hg, diastolic < 80 mm Hg) (Lichtenstein *et al.*, 2006). Lifestyle modification (diet and exercise) is recommended as first-line intervention for hypertension, although more aggressive treatment with drug therapy is recommended when absolute risk of CVD is high (National Heart Foundation Australia, 2004b). The effectiveness of anti-hypertensive medication in reducing risk for CVD has been demonstrated repeatedly (Williams, 2005), and epidemiological studies have shown a reduction in the prevalence of elevated BP, which is likely due to increased use of anti-hypertensive medication (Gregg *et al.*, 2005). Changes to diet and exercise, can lower BP (Dickinson *et al.*, 2006; Lichtenstein *et al.*, 2006), but also have an additional benefit of reducing body weight. Hypertension is strongly associated with obesity; systolic and diastolic BP increases linearly with body mass index (BMI) (Aneja *et al.*, 2004). Epidemiological studies have shown that the prevalence of hypertension is two-fold greater in obese compared to normal weight individuals (Gregg *et al.*, 2005). Hypertension is also associated with endothelial dysfunction, which has been implicated in the development of atherosclerosis (Landmesser *et al.*, 2004).

1.4.1.2.2 Effects of n-3 FA on blood pressure

Numerous meta-analyses of randomised controlled trials have reported a BP lowering effect of n-3 FA (Appel *et al.*, 1993; Morris *et al.*, 1993; Geleijnse *et al.*, 2002; Dickinson *et al.*, 2006). Morris et al. (Morris *et al.*, 1993) examined 31 placebo-controlled studies and reported a mean reduction in systolic and diastolic BP (SBP, DBP) of -3.0/-1.5 mmHg. Appel et al. (Appel *et al.*, 1993) reported mean changes of -1.0/-0.5 mmHg in normotensive individuals and -5.5/-3.5 mmHg in untreated hypertensives, and in a meta-analysis of double-blind trials, Geleijnse et al. (Geleijnse *et al.*, 2002) concluded that moderate doses of fish oil (mean dose: 3.7 g/day) were moderately effective in lowering BP (-2.1/-1.6 mmHg). More recently, Dickinson et al. (Dickinson *et al.*, 2006) evaluated the effectiveness of several lifestyle interventions including fish oil, in lowering BP. These RCTs enrolled mild hypertensive subjects (baseline BP $\geq$ 140/90) for a period of at least eight weeks in duration. Fish oil supplements were predominantly EPA rich (60%), providing between 0.1 - 17 g/day n-3 FA and resulted in a mean reduction of –2.3 and –2.2 mmHg for SBP and DBP, respectively.

Omega-3 FA are more effective in lowering BP in hypertensive subjects than normotensive individuals (Appel *et al.*, 1993; Morris *et al.*, 1993; Geleijnse *et al.*, 2002) Some (Morris *et al.*, 1993), but not all reviews (Appel *et al.*, 1993) have demonstrated a dose-response relationship between n-3 FA and BP reduction and it has also been suggested that EPA and DHA may have different BP lowering properties. Morris et al. (Morris *et al.*, 1993) reported that per gram of fatty acid, DHA was slightly more effective than EPA in lowering BP (-1.5/-0.77 mmHg versus –0.93/-0.53 mmHg). However, the results of studies investigating the effects of purified EPA and DHA on BP have been inconsistent (Grimsgaard *et al.*, 1998; Mori *et al.*, 1999a; Woodman *et al.*, 2002). Omega-3 FA exert their hypotensive effects by altering vascular reactivity (Chin & Dart, 1995) and improving autonomic function. Evidence for the latter is provided by reports of increased heart rate variability (HRV)

(Christensen, 2003) and lowered resting HR (Dallongeville *et al.*, 2003; Mozaffarian *et al.*, 2005) following increased intake of dietary n-3 FA rich fish or fish oil.

1.4.1.2.3 Effects of PA on blood pressure

In general, aerobic exercise appears to be more effective in reducing BP in hypertensive subjects (Arroll & Beaglehole, 1992; Fagard, 2001; Kelley *et al.*, 2001a; Whelton *et al.*, 2002; Fagard, 2006), although small effects have also been shown in normotensive populations (Kelley & Tran, 1995; Fagard, 2001; Kelley *et al.*, 2001a; Whelton *et al.*, 2002; Fagard, 2006) (**Table 1.2**). These reductions are related to improvements in endothelial function and baroreflex control of sympathetic nerve activity (Narkiewicz & Somers, 1997).

1.4.1.3 Arterial Function

1.4.1.3.1 Overview

The maintenance of vascular homeostasis is dependent on several key functions performed by the endothelium. These include non-selective ion permeability, the regulation of vascular tone and structure, thrombosis and thrombolysis, prevention of leukocyte and platelet adhesion and secretion of vasoconstrictor (e.g. Endothelin-1 (E-1)) and vasodilatory (e.g. NO) mediators (Celermajer, 1997; Landmesser *et al.*, 2004). Injury to the endothelium can result in adhesion of leukocytes, vasoconstriction, lipid deposition, smooth muscle cell migration and proliferation, and a decrease in NO bioactivity (Cohn, 2001). These and other extrinsic factors (e.g. hypertension, hyperlipidaemia, hormone and glucose regulation) can lead to structural remodelling of the artery, and arterial stiffness (Zieman *et al.*, 2005). Measures of arterial stiffness and vascular reactivity are typically used to assess arterial function. Vascular reactivity can be assessed via several techniques. Common methods include so-called invasive techniques that assess blood-flow in response to drug infusion and the non-invasive methods of flow-mediated dilatation (FMD) and pulse wave velocity.

Table 1.2 Meta-analyses reporting the effects of intervention with regular aerobic exercise on blood pressure. [1]

Review	Subject Type	Study No.	Participant No.	Study Design	Reduction in Blood Pressure (SBP / DBP) mmHg		
					Overall	*Normotensive*	*Hypertensive*
Arroll & Beaglehole, 1992.	Adults: H	13	389	CT		7.0 / 5.8	10.3 / 7.5
Kelley & McClellan, 1994.	Adults: H	9	245	RCT			7.0 / 6.0
Kelley & Tran, 1995.	Adults: N	35	1,076	RCT		4.5 / 3.8	
				CT		2.8 / 5.0	
				NC		4.7 / 1.7	
Halbert *et al*., 1997.	Adults: N & H	29	1533	RCT	4.7/ 3.1		
Fagard, 1999.	Adults: N & H	44	2,674	RCT	3.4 / 2.4		
Kelley, 1999.	Women: N & H	10	732	RCT	2.0 / 1.0		
Hagberg *et al.*, 2000.	Adults: H	62	1284	RCT			11.0 / 8.0
Fagard, 2001.	Adults: N & H	44	2,674	RCT		2.6 / 1.8	7.4 / 5.8
Kelley *et al.*, 2001a.	Adults: N & H	47	2543	RCT		2.0 / 1.0	6.0 / 5.0
Kelley *et al.*, 2001b.	Adults: N & H	16	650	RCT or CT	3.0 / 2.0		
Whelton *et al*., 2002.	Adults: N & H	53	2419	RCT	3.8 / 2.6	4.0 / 2.3	4.9 / 3.7
Fagard, 2005.	Adults: N & H	44	2674	RCT	3.4 / 2.4		
Dickinson *et al.*, 2006.	Adults: H	20	1270	RCT	4.6 / 2.4		
Fagard, 2006.	Adults: N & H	72	3936	RCT	3.0 / 2.4	1.9 / 1.6	6.9 / 4.9

[1] Abbreviations: N = normotensive, H = hypertensive, RCT = randomised controlled trial, CT = controlled trial, NC = no control,

1.4.1.3.2 Effects of n-3 FA on arterial function

Invasive studies have shown that supplementation with fish oil improves coronary (Fleischhauer *et al.*, 1993) and forearm (McVeigh *et al.*, 1993; Tagawa *et al.*, 1999; Mori *et al.*, 2000b; Okumura *et al.*, 2002; Morgan *et al.*, 2006) vasodilator response following infusion of acetylcholine. Additionally, Khan et al. (Khan *et al.*, 2003) reported an improvement in vascular response to acetylcholine administered via skin perfusion, following supplementation with n-3 FA. Other studies have demonstrated that fish oil supplementation significantly increases endothelial-dependent dilatation assessed by FMD in both children (Engler *et al.*, 2004) and adults (Goodfellow *et al.*, 2000). These studies suggest that n-3 FA improve endothelial function via NO-mediated mechanisms. In addition to being a potent vasodilator, NO plays an integral role in maintaining vascular health, and inhibits leukocyte adhesion, smooth muscle cell proliferation and platelet activity (Moncada & Higgs, 2006). There is also evidence that n-3 FA may act via NO-independent pathways. DHA, but not EPA has been shown to increase forearm blood flow response to the endothelial-independent vasodilator, sodium nitroprusside (Tagawa *et al.*, 1999; Mori *et al.*, 2000b). An earlier study by Nestel et al. (Nestel *et al.*, 2002) reported that both EPA and DHA increased systemic arterial compliance (or elasticity) and a recent preliminary study suggests that EPA may attenuate the age-related increase in arterial stiffness (Tomiyama *et al.*, 2005).

The ability of n-3 FA to modify eicosanoid synthesis leads to an increase in the production of vasodilatory eicosanoids (James *et al.*, 2000). Supplementation of the diet with EPA leads to a reduction in platelet aggregation and a reduced production of potent vasoconstrictors, while increasing the production of prostacyclin. Omega-3 FA also facilitate improvements in endothelial function by increasing endothelial production of NO (Harris *et al.*, 1997) EPA induces the translocation of endothelial NO synthase (eNOS) (Omura *et al.*, 2001), which regulates the production of NO from L-arginine (Palmer *et al.*, 1988). Production of NO is also influenced by eicosanoid synthesis as prostacyclin facilitates its

release by endothelial cells (Shimokawa *et al.*, 1988). Omega-3 FA may also mediate dilatation via a NO and prostacyclin independent pathway, through endothelial derived hyperpolarizing factors (Conde *et al.*, 2006). Additional mechanisms by which n-3 FA facilitate improvements in endothelial function include changes to selective ion channels, e.g. Ca^{++} and/or K^{+} (Asano *et al.*, 1998) and membrane fluidity (Mori, 2006). Omega-3 FA can alter the expression of inflammatory cytokines [e.g. tumor necrosis factor alpha (TNFα) and interleukin (IL)-1β] and vascular adhesion molecules, decrease superoxide anion generation and inhibit smooth muscle cell growth and proliferation (Abeywardena & Head, 2001). Furthermore, n-3 FA may induce haemostatic changes by decreasing platelet aggregation (Knapp, 1997).

1.4.1.3.3 Effects of PA on arterial function

Exercise improves FMD in patients with coronary (Walsh *et al.*, 2003; Edwards *et al.*, 2004) and peripheral (Brendle *et al.*, 2001) artery disease, type I diabetes (Fuchsjager-Mayrl *et al.*, 2002), mild hypertension (Moriguchi *et al.*, 2005) and metabolic syndrome (Lavrencic *et al.*, 2000), as well as in healthy individuals (Clarkson *et al.*, 1999). Improvements in endothelial-dependent dilatation following exercise training are thought to be due to an increase in the release of NO (Higashi *et al.*, 1999). Impaired NO release is associated with reduced exercise capacity (Maxwell *et al.*, 1998). NO is released by the endothelium in response to shear stress (Rubanyi *et al.*, 1986), which escalates during exercise as vessel blood flow increases (Laughlin, 1987). It is suggested that repeat exposure to shear stress may promote adaptations that improve endothelial sensitivity and thereby enhance NO release (Maiorana *et al.*, 2003). Furthermore, regular exercise training may improve endothelial function by up-regulating eNOS protein expression (Hambrecht *et al.*, 2003). Exercise induced shear stress releases additional vasodilators, such as prostacyclin and endothelial derived hyperpolarizing factor (Niebauer & Cooke, 1996). Exercise training also decreases plasma E-1, a potent vasoconstrictor released by endothelial cells (Maeda *et al.*,

2003). Regular aerobic exercise can improve coronary artery compliance (Cameron & Dart, 1994; Tanaka *et al.*, 1998; Tanaka *et al.*, 2000; Parnell *et al.*, 2002; Hayashi *et al.*, 2005), but the effect on small, peripheral vessels is less clear with some studies reporting an improvement (Mason *et al.*, 2006) while others do not (Hayashi *et al.*, 2005).

1.4.1.4 Other CV biomarkers

1.4.1.4.1 Overview

Anomalies in procoagulant factors (i.e., increases in fibrinogen and factor VII), anti-fibrinolytic factors [i.e., increases in plasminogen activator inhibitor-1 (PAI-1)] and platelet aberrations can contribute to the pathogenesis of CVD and venous thrombotic events (Warkentin, 1995; Gensini *et al.*, 1998). Other independent risk factors for CVD, all-cause and cardiac mortality include an elevated resting HR and reduced HRV (Dyer *et al.*, 1980; Kleiger *et al.*, 1987; Dekker *et al.*, 2000; Jouven *et al.*, 2001; Janszky *et al.*, 2004).

1.4.1.4.2 Effects of n-3 FA on other CV biomarkers

Although there is evidence for a reduction in platelet aggregation, there is limited evidence for a beneficial effect of n-3 FA on other markers of haemostasis, such as fibrinogen, Factor VIII, and von Willebrand factor (as reviewed by Kris-Etherton et al. (Kris-Etherton *et al.*, 2003)). In comparison, there is a clear relationship between increased intake of n-3 FA and improvements in resting HR and HRV. Previous studies have demonstrated that both regular fish consumption (Dallongeville *et al.*, 2003) and supplementation with fish oil can lower resting HR (Mozaffarian *et al.*, 2005). An increase in n-3 FA intake can increase HRV in the elderly (Holguin *et al.*, 2005), those with chronic renal failure (Christensen *et al.*, 1998), MI survivors (Christensen *et al.*, 1996) and healthy men (Christensen *et al.*, 1999). Increased HRV protects against fatal arrhythmia. The anti-arrhythmic effect of n-3 FA may be related to the increased incorporation of n-3 FA into cardiac membranes (Harris *et al.*, 2004), which increases membrane fluidity and thereby modifies ion currents and cell excitability (Demaison & Moreau, 2002).

1.4.1.4.3 Effects of PA on other CV biomarkers

Exercise training has been shown to lower resting HR (Wilmore *et al.*, 2001; Carter *et al.*, 2003), increase HRV (Carter *et al.*, 2003; Sandercock *et al.*, 2005), reduce HR response during exercise (Wilmore *et al.*, 2001; Carter *et al.*, 2003), and improve HR recovery following exercise (MacMillan *et al.*, 2006; Streuber *et al.*, 2006). The mechanisms behind these adaptations are not entirely clear, but may be related to an improvement in autonomic tone and CV adaptations that could eventually lead to a reduction in cardiac afterload and an increase in cardiac preload (Wilmore & Costill, 1999).

1.4.2 Metabolic biomarkers

1.4.2.1 Total & abdominal obesity

1.4.2.1.1 Overview

The prevalence of obesity has risen rapidly in recent years (Flegal, 1999; Rennie & Jebb, 2005; Thorburn, 2005) and has been described by the WHO as a global epidemic (World Health Organization, 2000). Obesity is defined as a condition of excess body weight, specifically adipose tissue, of sufficient magnitude to induce adverse health consequences (Spiegelman & Flier, 2001).

Measures of overweight and obese are simply determined by BMI, which is calculated using the following equation: [weight (kg)/height (m^2)]. The WHO defines overweight as a BMI $\geq$ 25 kg/m^2 and obesity as a BMI $\geq$ 30 kg/m^2 (World Health Organization, 2000). These specific cut-offs allow for the classification of individuals who are at increased risk of morbidity and mortality from CVD and allows for comparison within and across populations. It does not however distinguish between weight as lean tissue or fat, or recognise differences in fat distribution.

Techniques such as underwater weighing, doubly labeled water, dual energy x-ray absorptiometry (DXA) and bioelectrical impedance (BIA), have been used to assess body composition and give a more informative assessment than does BMI. However these methods are limited by cost and accessibility, and are therefore not appropriate for large-scale population trials. Nevertheless, they have fostered the development of reference values for body composition, more specifically percent body fat, relative to age and gender. In normal young adults, healthy body fat levels are considered to be 12-20% of total body weight in males and 20-30% of total body weight in females, with obesity classified as levels > 25% in males and > 33% in females (Bray, 1998).

National health surveys conducted in the United States (Marcason, 2007) estimates that nearly 61% of the population are overweight, 24% of which are obese, and 3.0% extremely obese. Similar observations have been made in Australia (overweight 39%, obese 20.8%) (Cameron *et al.*, 2003) and Britain (Women; overweight 34.7%, obese 23.8%, Men; overweight 45.5%, obese 23.6%) (Department of Health, 2004; Rennie & Jebb, 2005). In comparison Hodge et al. (Hodge *et al.*, 1994) reported obesity prevalence rates of nearly 75 % in urban Samoan women. For Asian countries such as China and Malaysia, the prevalence is substantially lower, but has increased rapidly in recent years. In Malaysia, 20.7 % of the population is overweight and 5.8 % obese (Ismail *et al.*, 2002), while in China these figures are 14.7 % and 2.6 %, respectively (Wu, 2006).

Obesity increases the risk for developing hypertension, dyslipidemia and glucose intolerance, and is associated with an increased risk of type II diabetes, CHD, respiratory disease and some cancers (Pi-Sunyer, 1999). Additional risk factors for CVD associated with obesity include microalbuminuria (de Jong *et al.*, 2002), elevated levels of uric acid (Bonora *et al.*, 1996) and inflammatory markers such as fibrinogen and C-reactive protein (CRP) (Kahn *et al.*, 2006). Obese individuals are also more likely to develop sleep apnea (Vgontzas *et al.*, 1994), have impaired fertility (Pasquali, 2006), and musculoskeletal disorders (Wearing *et al.*, 2006).

The secretion of inflammatory cytokines and bioactive mediators, such as CRP, TNFα, IL-6, PAI-1, angiotensinogen, adiponectin, leptin, resistin, lipoprotein lipase, retinol binding protein and cholesterol ester transfer protein (Trayhurn & Wood, 2004) by adipose tissue may provide an additional link between obesity (or excess adipose tissue) and development of CVD (Van Gaal *et al.*, 2006). The secretion of these inflammatory mediators impacts negatively on multiple functions including immune function, insulin sensitivity, appetite and energy balance, BP, lipid metabolism, angiogenesis and haemostasis (Ronti *et al.*, 2006). Furthermore, it is now recognised that atherosclerosis is an inflammatory condition, mediated by several adipokines including TNF-α, IL-6, CRP, PAI-1 and leptin (Ronti *et al.*, 2006).

While overall obesity, predominantly defined by BMI, is associated with numerous risk factors for CVD, the relative distribution of body fat may be far more important. There is now substantial evidence to support the associations between both total abdominal and intra abdominal adipose tissue (IAAT), and metabolic abnormalities such as insulin resistance, hypertension and hypertriglyceridaemia (Wong *et al.*, 2003; Karter *et al.*, 2005; Warren *et al.*, 2006). Furthermore total and IAAT may convey greater risk for chronic disease and mortality than overall body weight or body fat alone (Warren *et al.*, 2006), due to its increased lipolytic activity and direct drainage of free FA into the portal vein (Wajchenberg, 2000), whereas subcutaneous fat drains into the systemic circulation.

Given the associated CV risk, several investigators have sought to determine longitudinal trends in abdominal adiposity. The majority of these studies have used waist to hip ratio (WHR) or waist circumference (WC) as indices of abdominal or central obesity. For Caucasians, a WHR >1.0 in men and >0.85 in women indicates abdominal adiposity (WHO:, 1997). A waist circumference of > 102 cm in men and > 88 cm in women is associated with an increased risk for CVD (WHO:, 1997). Abdominal adiposity, measured by WC, is strongly associated with obesity-related health risks (Rexrode *et al.*, 1998; World Health Organization,

2000; Zhu *et al.*, 2002), and may better predict CV risk factors than does BMI (Zhu *et al.*, 2002).

Longitudinal studies have reported significant increases in WHR in Swedish women (Lissner *et al.*, 1998) and Finnish men and women (Lahti-Koski *et al.*, 2000). Abdominal obesity, measured by WC, rose by 3.3% and 3.6% in a small German community of men and women, which increased central obesity prevalence rates to 20% and 20.5%, respectively. Park et al. (Park *et al.*, 2007) observed an increase in the prevalence of central obesity (determined by WC) in Korean men and women between 1998 and 2001, and in China, a cohort study reported that 43.7% of subjects with normal WC in 1992 became high WC in 2002 (Wu *et al.*, 2005). Lorenzo et al. (Lorenzo *et al.*, 2005) showed an increased prevalence of elevated WC in Mexican men (7.5%) and women (3.9%) from 1990 to 1999. Perhaps the most alarming data from this report was that in 1999, the prevalence of women with an elevated WC was 86%. Surveys conducted in Australia between 1989 and 2000 have also reported an increase in abdominal adiposity (WC) from 14 - 21% in men and 16 - 28% in women (Dixon & Waters, 2003). Not surprisingly, the greatest increases in abdominal obesity have been observed in the United States. Data from the National Health Examination Surveys revealed a significant increase in WC in both men (12.7% - 38.3%) and women (19.4% - 59.9%) between 1960 and 2000 (Okosun *et al.*, 2004): In 2004, it was estimated that the age-adjusted prevalence of abdominal obesity (WC) was 42.4% in men and 61.3% in women (Li *et al.*, 2007).

1.4.2.1.2 Effects of n-3 FA on total and abdominal obesity

As discussed earlier, n-3 FA may mediate changes in energy metabolism by acting as ligands for nuclear receptors involved in the regulation of genes from key metabolic pathways, such as lipid metabolism (Desvergne & Wahli, 1999). The modulation of PPAR target genes, including CPT-1 (Flachs *et al.*, 2005), fatty acyl-CoA oxidase and mitochondrial uncoupling protein 3 (Baillie *et al.*, 1999), indicates that n-3 FA may enhance fat loss by

stimulating a less efficient fatty acid oxidation pathway. Animal trials have shown that fat mass deposition is reduced following feeding with n-3 FA (Hill *et al.*, 1993; Baillie *et al.*, 1999; Ruzickova *et al.*, 2004). However in human studies the evidence is less clear, as some (Couet *et al.*, 1997; Groh-Wargo *et al.*, 2005), but not all studies indicate that n-3 FA can reduce body fat with (Fontani *et al.*, 2005) or without concomitant dietary restriction (Krebs *et al.*, 2006). Furthermore, only two studies (Groh-Wargo *et al.*, 2005; Krebs *et al.*, 2006) were suitably designed and well controlled. Couet et al. (Couet *et al.*, 1997) may have had an order effect, while Fontani et al. (Fontani *et al.*, 2005) did not include an n-3 FA control group and did not allow for a washout period in their crossover design. They also assessed changes in body composition with measures of skin fold thickness, the sensitivity of which is reduced with increasing adiposity.

1.4.2.1.3 Effects of PA on total and abdominal obesity

The addition of exercise to dietary restriction induces greater long-term reductions in body weight than does diet alone (Miller *et al.*, 1997), although in comparison, physical activity alone produces relatively small changes in body weight (Garrow & Summerbell, 1995; Miller *et al.*, 1997; Wing, 1999). Bouchard et al (Bouchard *et al.*, 1990) provided evidence that exercise alone can reduce body weight. Healthy, male adults were monitored over two weeks to establish habitual dietary intake before beginning a 100-day study that required them to exercise on a cycle ergometer twice a day at 55% of VO_2 max, six days a week, with constant dietary intake. At the end of the intervention period subjects had lost 8 kg, the majority of which was a loss of fat, estimated from skin folds. While these results are impressive, they are likely to be heavily influenced by the environment within which the study was conducted, i.e. a controlled research facility, and the study population (healthy young males). It is unlikely that the same magnitude of change would be achieved in a real-life setting, with overweight/obese subjects. Indeed studies examining the effect of aerobic exercise with constant energy intake on body composition in overweight adults have shown

positive, although varied results (**Table 1.3**). In comparison to Bouchard et al. (Bouchard *et al.*, 1990), studies conducted over a similar time frame have generally shown smaller reductions in body weight and/or fat mass (Table 1.3). As reviewed by Wing et al (Wing, 1999), exercise (when compared to no treatment controls) can produce modest, 1-2 kg reductions in body weight, without change to background diet.

The mechanism behind exercise-induced changes in fat mass is complex, and essentially involves the mobilisation and subsequent breakdown of TAG from adipose tissue. During exercise, several hormones, including epinephrine and glucagon, are released, leading to the activation of hormone sensitive lipase, which breaks down TAG into glycerol and FA. Glycerol can be metabolised into pyruvate or glucose. Free FA diffuse into the blood where they bind to serum albumin, a soluble protein molecule that enables transportation of FA to the cytosol of tissues otherwise inaccessible (heart, skeletal muscle). Within the cytosol, FA are involved in a series of enzymatic reactions that enable their transportation into the mitochondria where they are oxidised. Transportation of FA into the mitochondria is the rate-limiting step for oxidation. Exercise can impact on FA mobilisation, transportation and oxidation in several ways.

There is evidence suggesting that beta-adrenoreceptor (β-receptor) function in obese subjects is impaired (Schiffelers *et al.*, 2001). Endurance training can increase the sensitivity of these receptors, particularly in abdominal adipose tissue (De Glisezinski *et al.*, 1998; Moro *et al.*, 2005). An improvement in β-receptor sensitivity to circulating catecholamines may increase lipolytic activity and may explain demonstrated reductions in abdominal obesity following exercise training (Ross *et al.*, 2000a; Ross *et al.*, 2004). Insulin action in obese and healthy individuals is improved with exercise training (Hawley, 2004), and the reduction in circulating blood insulin promotes lipolysis (Large & Arner, 1998). Tunstall et al. (Tunstall *et al.*, 2002) reported that exercise training can facilitate an increase in FA transport across muscle and mitochondrial membranes, by up-regulating the gene expression and protein content of key enzymes, such as fatty acid translocase (FAT/CD 36) and CPT-1. Furthermore,

several significant CV and muscular adaptations contribute to the increase in fat oxidation. For example, exercise training improves blood flow and thereby increases oxygen delivery to muscles, increases mitochondrial size and number, and decreases lactate production for a given workload (Wilmore & Costill, 1999). These adaptations have a net effect of increasing FA transportation and oxidation.

1.4.2.2 Glucose uptake & insulin sensitivity

1.4.2.2.1 Overview

Plasma glucose is linearly associated with increased risk for developing diabetes and CVD (Coutinho *et al.*, 1999). Even in the non-diabetic range, blood glucose level is a significant risk factor for CVD (Levitan *et al.*, 2004). The WHO defines diabetes as a fasting plasma glucose level ≥ 7.0 mmol/L, and this represents an increased risk for microvascular and CV complications and increased premature mortality (World Health Organisation, 2006). Between 1999–2000, the total prevalence of abnormal glucose tolerance in the Australian population was 23.7 %, with diabetes, impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) contributing 7.4, 5.8 and 10.6 %, respectively (Dunstan *et al.*, 2002). These figures are not dissimilar to estimates from other countries (Unwin *et al.*, 2002), but represent almost a doubling in the prevalence of diabetes since 1981 (Glatthaar *et al.*, 1985). Hyperglycaemia contributes to the pathogenesis of CVD via several mechanisms including enhanced formation of advanced glycation end products, increased production of reactive oxygen species (ROS), and activation of PKC (Brownlee, 2005), all of which can lead to endothelial injury and dysfunction.

Table 1.3 Summary of select studies reporting the effect of exercise (without dietary restriction) on total body composition in overweight/obese subjects.[1]

	Duration	Non-exercise Control	Subjects			Exercise				Change (kg)	
			N (Total:Ex)	*Age**	*BMI**	*Mode*	*Frequency*	*Duration*	*Intensity*	*Weight*	*Fat Mass*
Donnelly *et al.*, 2003.	16 months	Y	74 : 16 M	22 ± 4	29.7 ± 2.9	W	5 d/wk	45 min	75% HR reserve ↓	5.2±4.7	4.9±4.4
			: 25 F	24 ± 5	28.7 ± 3.2					0	0
Irwin *et al.*, 2003.	12 months	Y	131 : 81 F	61	30.5 ± 31	MI (W)	5 d/wk	45 min	60-75% HR max ↓	1.3	1.4
Miyatake *et al.*, 2002.	12 months	N	31 : 31 M	46.2 ± 6.8	28.6 ± 2.2	W	7 d/wk	increase daily steps by 1000	↓	~3.7	~2.8
Ross *et al.*, 2000a.	3 months	Y	52 : 16 M	45 ± 7.5	32.3 ± 1.9	W/J	7 d/wk	time to expend 700kcal.	80% HR max ↓	7.5	6.1
Ross *et al.*, 2004.	14 weeks	Y	54 : 17 F	43.2 ± 5.1	32.8 ± 3.8	W/J	7 d/wk	time to expend 500kcal.	80% HR max ↓	6.1±1.2	6.7±1.9
Savage *et al.*, 2003.	4 months	N	15 : 15 M/F	62.5 ± 9.7	31 ± 3.1	W	5-7 d/wk	60-90 min	50-60% peak VO2 ↓	4.6	3.6
Melanson *et al.*, 2004.	24 weeks	N	41 : 19 M/F	42.4 ± 4.4	30.6 ± 0.5	W	5 d/wk	time to expend 500kcal.	MI ↓	~0.4	~1.7
Cox *et al.*, 2004.	16 weeks	Y	51 : 10 M	43 ± 4.2	30.5 ± 3.9	C	3 d/wk	30 min	75% HR reserve ↓	1.55	0.74
Nieman *et al.*, 2002.	12 weeks	Y	91 : 21 F	45.6 ± 1.1	32.3 ± 1.1	W	5 d/wk	45 min	60-80% HR max ↓	~1.0	~1.3
Gordon *et al.*, 1997.	12 weeks	N	48 : 14 M/F	50 ± 8	~34	MI (W)	3-5 d/wk	30-45 min	60-85% HR max ↓	1.0±1.8	0.8
Schwartz, 1987.	3 months	N	26 : 14 M	31.6	not reported	W/J	5 d/wk	40 min	70-85% HR reserve ↓	2.8	3.5
Williams *et al.*, 1990.	12 months	Y	130 : 46 M	30-59	~29	W/J	5 d/wk	40-50 min	not reported ↓	4.0±3.9	4.6±3.5
Tully *et al.*, 2005.	12 weeks	Y	26 : 17 M/F	55.5 ± 4	28.1 ± 4	W	5 d/wk	30 min	brisk ↑	0.46±2	not measured
Keller & Trevino, 2001.	24 weeks	Y	36 : 12 F	18-45	36.2 ± 5.4	W	3 d/wk	30 min	50% HR reserve ↓	1.36	not measured
			: 12 F	18-45	33.2 ± 8.3	W	5 d/wk	30 min	50% HR reserve ↑	1.36	not measured
Hill et al. 2007 (in press).	12 weeks	Y	65 : 30 M/F	48.9 ± 8	33.7 ± 5	W	3 d/wk	45 min	75% HR max ↓	1.02±2.7	0.9±1.8

[1] PubMed keyword search: aerobic exercise or physical activity or walking and weight loss and overweight Limits: Adult: 19-44 years, Middle Aged: 45-64 years, English, Randomized Controlled Trial, Humans. Abbreviations: C= cycling, W = walking, MI = moderate intensity, J = jogging, HR = heart rate, VO_2 = oxygen consumption.

* Indicates values are for exercising subjects.

† 111 to 185% of ideal body weight. Average body fat = 30.7 %.

1.4.2.2.2 Effects of n-3 FA on glucose uptake & insulin sensitivity

There is considerable evidence from animal studies to support the beneficial role of n-3 FA in glucose uptake and insulin sensitivity. In rats, elevated plasma and skeletal muscle TAG are associated with peripheral insulin resistance and impaired insulin action (Thorburn *et al.*, 1989; Storlien *et al.*, 1991). Storlien et al. (Storlien *et al.*, 1987) showed that fish oil prevented insulin resistance in rats fed a high fat diet. The mechanism by which n-3 FA prevent insulin resistance may be related to reductions in plasma and muscle TAG levels. Additional mechanisms by which n-3 FA improve glucose uptake and insulin sensitivity in rodents include changes in membrane fluidity and stability, insulin signalling, and regulation of gene transcription (Ye *et al.*, 2001; Lombardo & Chicco, 2006). However it is not entirely clear whether these benefits and their related mechanisms translate to humans.

Early data in type II diabetics, albeit from uncontrolled trials, suggested that n-3 FA may adversely effect glycaemic control and insulin release (Kasim *et al.*, 1988; Borkman *et al.*, 1989; Friday *et al.*, 1989). This potential negative effect of n-3 FA has since been thoroughly reviewed and dismissed (Friedberg *et al.*, 1998; Montori *et al.*, 2000; Farmer *et al.*, 2001; Nettleton & Katz, 2005); modest doses (1-2 g/day) of n-3 FA do not adversely effect glycaemic control or insulin release in diabetic patients. However some authorities still recommend caution when administering fish oil to diabetics as high doses (> 10 g/day) may raise plasma glucose (Nettleton & Katz, 2005). Although n-3 FA intake is inversely correlated with the development of glucose intolerance (Feskens *et al.*, 1991), there appears to be little effect of n-3 FA on glycaemic control or insulin sensitivity in healthy adults (Giacco *et al.*, 2006; Vandongen *et al.*, 1993).

1.4.2.2.3 Effects of PA on glucose uptake & insulin sensitivity

Insulin is secreted in response to elevated blood glucose levels and facilitates the transportation of glucose into adipose and skeletal muscle through the activation of glucose transporters such as GLUT 4 (Mougis, 2006). In type II diabetics, insulin stimulated GLUT4

translocation is impaired (Zierath *et al.*, 1996). O'Gorman et al. (O'Gorman *et al.*, 2006) reported an increase in whole body insulin-mediated glucose disposal in type II diabetics following exercise training, which was associated with an increase in GLUT4 protein content. Exercise-induced changes in intramuscular lipids, particularly diacylglycerol (DAG) may also play a role in regulating insulin sensitivity (Bruce *et al.*, 2006). DAG activates PKC, which inhibits the activity of key enzymes located on insulin receptors, thus inhibiting insulin action (Griffin *et al.*, 1999). Improving fatty acid metabolism in muscle (as done with exercise) would reduce TAG concentration and prevent DAG formation. Sriwijitkamol et al. (Sriwijitkamol *et al.*, 2006) recently demonstrated that in individuals with type II diabetes exercise training was associated with a reduction in the activation of the inhibitor NF-κβ pathway, which has been implicated in the pathogenesis of insulin resistance.

In a recent systematic review, Shaw et al. (Shaw *et al.*, 2006) concluded that aerobic exercise training was effective in reducing plasma glucose in overweight/obese individuals by 0.2 mmol/L compared to no treatment. Bouele et al. (Boule *et al.*, 2001) investigated the effects of regular exercise on glycosylated haemoglobin (HbA_{1c}), an indicator of glycaemic control, in type II diabetics. Despite variable reports in the studies reviewed, they concluded that exercise was effective in reducing HbA_{1c} in this population. This modest effect of aerobic exercise on reducing HbA_{1c} has also been reported by Snowling and Hopkins (Snowling & Hopkins, 2006). Additionally, exercise training may improve beta-cell function in type II diabetics (Dela *et al.*, 2004). In healthy but overweight individuals, Weiss et al. (Weiss *et al.*, 2006) reported an improvement in glucose tolerance and insulin sensitivity following exercise-induced weight loss (Mean: 5.8±2.5 sessions/wk, 62±18 minutes per session, @ 71±9% HR max). Katzel et al (Katzel *et al.*, 1995) compared the effect of diet-induced weight loss and regular aerobic exercise without weight loss in sedentary, obese men. Nine months of exercise training failed to produce any changes in fasting glucose or insulin concentrations or glucose response to an oral glucose tolerance test (OGTT). These effects were in contrast to those seen in the diet-induced weight loss group. Changes in body weight may therefore be

the primary driver behind improvements in insulin sensitivity and glucose tolerance. Indeed, excess adiposity, particularly abdominal adiposity is associated with metabolic impairments including insulin resistance, which improves when adipose tissue is reduced (Ross *et al.*, 2000a). However, several studies have shown that regular exercise can improve insulin sensitivity without changes in body weight in healthy obese adults (Lamarche *et al.*, 1992; Cox *et al.*, 1999; Boule *et al.*, 2005; Bruce *et al.*, 2006). Furthermore, Hasbum et al. (Hasbum *et al.*, 2006) have shown that regular moderate exercise can reduce fasting plasma glucose and insulin and increase insulin sensitivity in healthy non-obese subjects.

1.4.3 Inflammatory biomarkers

1.4.3.1 Eicosanoids, cytokines & neutrophil functions

1.4.3.1.1 Overview

As previously discussed, eicosanoids have different inflammatory effects depending on whether they are synthesised from AA or EPA. In addition to their effects on platelet aggregation and vascular reactivity (dilatation/constriction), eicosanoids can influence cytokine production. Cytokines are involved in numerous inflammatory processes, including the regulation of immune cell functions (chemotaxis, adherence and respiratory burst) and vascular activation (**Table 1.4)** (Borish & Steinke, 2003).

1.4.3.1.2 Effects of n-3 FA on eicosanoids, cytokines & neutrophil functions

The ratio of AA to EPA determines which series of eicosanoids are produced in the greatest abundance. As previously described in 1.2.2.1, a high concentration of AA will result in increased synthesis of the pro-inflammatory PGE_2, LTB_4 and pro-aggregatory TXA_2, whereas a high concentration of EPA will result in increased synthesis of the less inflammatory PGE_3, LTB_5 and TXA_3 (James *et al.*, 2000). Additionally, eicosanoids influence the production of several inflammatory cytokines (Kinsella *et al.*, 1990a; Tilley *et al.*, 2001). PGE_2 inhibits production of interferon gamma (IFN-γ), TNFα, IL-2, IL-1, and IL-6, while

LTB_4 has opposing effects and increases production of IFN-γ, TNFα, IL-2, IL-1, and IL-6 (Calder & Grimble, 2002). In this respect, AA has some anti-inflammatory activity, as it is the precursor for PGE_2.

Table 1.4 Summary of select inflammatory cytokines and cytokine receptor functions. [1]

Cytokine	Pro / Anti-inflammatory	Function
TNFα	Pro	• Kills tumour cells; • Induces vascular adhesion molecules; • Mediates several neutrophil functions (chemotaxis, adherence and respiratory burst)
IFNγ	Pro	• Modest antiviral activity, • B cell activation; stimulates antigen presentation; • Stimulates monocyte cytokine production and functions (chemotaxis, adherence and respiratory burst); • Stimulates killing by neutrophils and NK cells
IL-1	Pro	• Up-regulates genes encoding for cytokines, including its own, IL-6, IL-2 and vascular adhesion molecules; • Induces enzymes required for synthesis of inflammatory mediators (leukotrienes, prostaglandins and NO); • Stimulates T-cells and B-cell proliferation
IL-2	Pro	• T cell growth factor; • Activator of cytotoxic T cells, NK cells, B cells and macrophages
IL-6	Pro / Anti	• Mediates T-cell and B lymphocyte activation and differentiation; • Inhibits IL-1 and TNF synthesis; increases IL-1ra synthesis
IL-4	Anti	• Enhances antigen-presenting capacity of B cells; • Decreases production of IL-1, IL-6 and TNFα and increase IL-1ra synthesis
IL-10	Anti	• Inhibits production of several cytokines including TNFα, IFNγ, IL-6, IL-1α and IL-1β
IL-1ra	Anti	• Binds to IL-1 receptors; no biological activity - therefore down-regulates IL-1 activity

[1]As described by Borish & Steinke.

Supplementation with n-3 FA has been shown to reduce IL-1, IL-6 and TNFα production from stimulated whole blood (Espersen *et al.*, 1992; Cooper *et al.*, 1993) and MNL cells (Endres *et al.*, 1989; Meydani *et al.*, 1991; Gallai *et al.*, 1995). However other studies have shown no effect (Blok *et al.*, 1997; Yaqoob *et al.*, 2000; Thies *et al.*, 2001a; Kew *et al.*, 2004). Similar controversies exist for measures of neutrophil function (chemotaxis,

adherence and respiratory burst), as some (Lee *et al.*, 1985; Schmidt *et al.*, 1991; Sperling *et al.*, 1993; Varming *et al.*, 1995; Luostarinen & Saldeen, 1996; Andrioli *et al.*, 1999) but not all studies (Guarini *et al.*, 1998; Healy *et al.*, 2000; Miles *et al.*, 2004) have shown a positive effect of n-3 FA on these parameters. As mentioned previously, n-3 FA may modulate the expression of other inflammatory markers, including E-selectin, ICAM-1 and VCAM-1 (De Caterina *et al.*, 1994), which has implications for endothelial function.

1.4.3.1.3 Effects of PA on eicosanoids, cytokines & neutrophil functions

Epidemiological studies have confirmed that regular physical activity is associated with a reduced presence of several systemic inflammatory markers, primarily IL-6, CRP and fibrinogen (Abramson & Vaccarino, 2002; Colbert *et al.*, 2004; Panagiotakos *et al.*, 2005a; Mora *et al.*, 2006) and some (Adamopoulos *et al.*, 2001; Larsen *et al.*, 2001; Goldhammer *et al.*, 2005) but not all studies (Nicklas *et al.*, 2004; Marcell *et al.*, 2005; Niebauer *et al.*, 2005) have shown a reduction in markers of systemic inflammation following intervention with physical activity.

The inflammatory response to acute strenuous exercise has been well documented and is described in **Figure 1.5**. The most notable change is in IL-6, which is released by MNL cells and skeletal muscle (Steensberg *et al.*, 2002), and increases dramatically during exercise (Febbraio & Pedersen, 2002). While IL-6 is sometimes classified as a pro-inflammatory cytokine it has several anti-inflammatory actions as it inhibits the production of TNFα and IL-1 (Tilg *et al.*, 1997) and stimulates production of IL-1 receptor agonist (ra) and IL-10 (Steensberg *et al.*, 2003). The net response to acute exercise is therefore anti-inflammatory (Tilg *et al.*, 1997). Consequently, it is possible that the overall anti-inflammatory effect of regular physical activity may be mediated by these acute bouts of exercise (Petersen & Pedersen, 2005).

NOTE: This figure is included on page 40 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.5 Plasma cytokine response to strenuous exercise [1].

[1] From Kasapis & Thompson (2005).

A comprehensive review of acute exercise effects on neutrophil functions, with particular focus on degranulation and respiratory burst activity has been published by Peake (Peake, 2002). This review cites evidence that regular physical activity or training can attenuate the respiratory burst response to acute exercise. The mechanism by which exercise alters superoxide production is linked to the priming and activation of neutrophils and includes changes to a number of intra- and extracellular parameters (Pyne, 1994). Briefly, these include changes to cell surface receptor expression, signal transduction, promotion of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex assembly and activation, and the presence of hormones, cytokines and other stimulants (e.g. lipopolysaccharide, LPS) (Pyne, 1994; Peake, 2002).

1.4.4 Clustered risk factors i.e. the Metabolic Syndrome (MS)

1.4.4.1 Overview

The MS refers to a clustering of physiological and metabolic abnormalities that relate to insulin resistance and CVD. Several organisations have sought to provide guidelines to allow profiling of patients with MS, with the first of these being released in 1988 by the WHO (Alberti & Zimmet, 1998). Since then, various organisations, including the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP), 2001), and more recently the International Diabetes Federation (IDF) (Federation, 2005) have published their own definitions, each with slightly different criteria (**Table 1.5)**.

Despite the inconsistency in defining MS, there is strong evidence that both the risk of developing CVD and all-cause and CV mortality (Isomaa *et al.*, 2001; Lakka *et al.*, 2002; Malik *et al.*, 2004) is greater in individuals with MS (diagnosed by WHO or NCEP) than without. This is likely due to the independent association of abdominal obesity, elevated TAG, reduced HDL, raised BP and raised fasting blood glucose with an increased risk of developing CVD (Lippel *et al.*, 1981; Fuller *et al.*, 1983; Hubert *et al.*, 1983; Larsson *et al.*, 1984; Antikainen *et al.*, 1998; Austin *et al.*, 1998; Rexrode *et al.*, 1998; Coutinho *et al.*, 1999; Franceschini, 2001; Lewington *et al.*, 2002; Wilson *et al.*, 2002). Furthermore, the sum of individual risk factors attributes less risk for CVD than does diagnosis with MS (Grundy, 2006).

Using the NCEP guidelines, the prevalence of MS in the United States has been estimated to be 33.7 %, rising to 39.0 % using the IDF criteria (Ford, 2005). Similar discrepancies exist for estimates in Australia: 15 % for ATP III and 22.8% using IDF criteria (Adams *et al.*, 2005). In Asian populations the prevalence of MS is estimated at 15 -20 %, but varies with ethnicity (Park *et al.*, 2007).

Table 1.5 Metabolic Syndrome definitions [1]

WHO	ATP III	IDF
Diabetes, IFG, IGT, or insulin resistance (assessed by clamp studies) **& at least two of the following criteria:**	**Any three or more of the following criteria:**	**Central obesity [1] Plus any two of the following four factors:**
• ***Waist-to-hip ratio:*** M >0.90, W >0.85	• ***Waist-circumference (cm):*** M >102 , W >88	
• ***Serum TAG (mmol/L)*** >1.7 **or** • ***HDL cholesterol (mmol/L):*** M <0.9, W <1.0	• ***Serum TAG (mmol/L)*** >1.7	• ***TAG level (mmol/L)*** >1.7 [3]
• ***Blood pressure (mmHg)*** >140/90 mmHg	• ***Blood pressure (mmHg)*** >130/85	• ***Blood pressure (mmHg)*** SBP >130 or DBP >85 [2]
	• ***HDL cholesterol (mmol/L):*** M <1.0 , W <1.3	• ***HDL cholesterol (mmol/L):*** M <1.03, W <1.29 [3]
• ***Urinary albumin excretion:*** rate >20 g/min or albumin/creatinine ratio >30 mg/g	• ***Serum glucose (mmol/L):*** >6.1 mmol/L (>5.6 mmol/l may be applicable)	• ***Fasting plasma glucose*** (*mmol/L)* > 5.6 [3]

[1] Central obesity defined as waist circumference >94 cm for Europid men and 80 cm for Europid women, with ethnicity specific values for other groups

[3] or specific drug treatment for this abnormality

[3] or previously diagnosed type II diabetes

1.4.4.2 Treatment strategies for MS

The primary goal for treatment of the MS is to prevent the development of diabetes and CVD, and therefore treatment should address all underlying mechanisms of the MS (Chew *et al.*, 2006).

1.4.4.2.1 Pharmacological intervention

Approved pharmacological treatment for the MS are restricted to the use of drugs that target the individual risk factors, i.e. antihypertensive, lipid-lowering, weight-loss, anti-platelet and hypoglycaemic agents (Grundy, 2006). However several drugs aimed at targeting the MS as a whole are currently under development. These include weight reduction drugs (such as rimonabant), PPAR–alpha agonists (e.g. fibrates which lower TAG) and PPAR-gamma agonists (e.g. thiazolidinediones which improve insulin sensitivity) (Grundy, 2006).

1.4.4.2.2 Lifestyle intervention

Lifestyle change is recommended as first line intervention for MS and guidelines include dietary modification, weight reduction, increased physical activity, and smoking cessation (Grundy *et al.*, 2005; Stone & Saxon, 2005). Epidemiological data suggests that physical activity can play an important role in counteracting MS, as the prevalence of MS and type II diabetes is significantly lower in individuals who are more physically active (Dunstan *et al.*, 2005; Ford *et al.*, 2005). Regular physical activity may also attenuate or reverse the CVD process (Hambrecht *et al.*, 1993; Niebauer *et al.*, 1997) and when compared to usual care, exercise-based cardiac rehabilitation is associated with reduced all-cause and cardiac mortality (Taylor *et al.*, 2004).

The effectiveness of lifestyle interventions (diet and physical activity) in preventing the development of diabetes and reducing the incidence of MS have been demonstrated in several studies (Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002; Orchard *et al.*, 2005). Diet and lifestyle recommendations specific for CVD risk reduction have been recently revised and published by the AHA (Lichtenstein *et al.*, 2006). These dietary recommendations are

detailed in **Table 1.6.** Of particular relevance to this thesis is the recommendation to consume oily fish at least twice per week, and participation in regular physical activity. Similarly, the Heart Foundation of Australia recommends regular physical activity and the consumption of fish, twice a week, as part of a healthy lifestyle to reduce the risk of developing CVD. The potential for physical activity and n-3 FA (as contained in fish or fish oil) to prevent the development of MS is likely enhanced by their ability to impact on multiple CV, metabolic and inflammatory markers. However, few studies (Warner *et al.*, 1989; Brilla & Landerholm, 1990) have evaluated the effectiveness of these two interventions in combination.

Table 1.6 American Heart Association (AHA) diet and lifestyle recommendations for CVD risk reduction. [1]

AHA Dietary Guidelines
Balance calorie intake and physical activity to achieve or maintain a healthy body weight.
Consume a diet rich in vegetables and fruits.
Choose whole-grain, high-fibre foods.
Consume fish, especially oily fish, at least twice a week
Limit your intake of saturated fat to <7% of energy, *trans* fat to <1% of energy, and cholesterol to <300 mg per day by • choosing lean meats and vegetable alternatives; • selecting fat-free (skim), 1%-fat, and low-fat dairy products; and • minimising intake of partially hydrogenated fats.
Minimize your intake of beverages and foods with added sugars.
Choose and prepare foods with little or no salt
If you consume alcohol, do so in moderation
When you eat food that is prepared outside of the home, follow the AHA Diet and Lifestyle Recommendations

[1] Recommendations updated in 2006

1.4.4.3 Combined n-3 FA & PA interventions

Only two studies have investigated the combined effects of n-3 FA and regular moderate-intensity exercise on CV risk factors, without modifying background diet (Warner *et al.*, 1989; Brilla & Landerholm, 1990). The details for these two studies are contained in **Table 1.7**. Warner and colleagues (Warner *et al.*, 1989) investigated the effect of 12 weeks of aerobic exercise training (walk/jog 3 d/week x 45-50 min at 75-80% HR max) in hyperlipidaemic persons allocated to one of four treatments: fish oil (8.5 g/day EPA, 6 g/day DHA), fish oil & exercise, corn oil or control. Percent body fat, as measured by underwater weighing was reduced only in the combined fish oil and exercise group. Similar changes in TAG and HDL were observed in the combined intervention (fish oil and exercise) and fish oil groups, but greater reductions of serum LDL and apolipoprotein B concentrations were seen following the combined intervention than with fish oil alone. Unfortunately, the design of this study did not enable comparison with an exercise only group so it was not possible to determine whether the reduction in body fat was due entirely to the exercise, or to some synergistic effect of n-3 FA and exercise. Brilla & Landerholm (Brilla & Landerholm, 1990) reported that 10 weeks of exercise training (aerobic activities 3 d / week, 60 min at 70-85% of maximal HR) and fish / fish oil supplementation had no effect on HDL, LDL, TC, TAG or percent body fat in young adults. This lack of effect cannot be attributed to study design as there were placebo and exercise controls, but may be due to the recruitment of relatively lean subjects with low levels of body fat (15-22%) and a normal range of blood lipoprotein lipids.

Table 1.7 Combined n-3 FA and exercise intervention details.

	Warner *et al.*, 1989.	Brilla & Landerholm, 1990.
Population		
Number	34	32
Age	27-63 yr (mean: 49 yr)	19-34 yr (mean: not reported)
Health status	Hyperlipidaemic	Healthy males
Intervention		
Study duration	• 12 weeks	• 10 weeks
General	• Maintain entry activity level (regular exercisers were excluded) • Decrease dietary fat intake to compensate for oil	• Sedentary entry criteria • Maintain usual diet
Group Allocation	• Control • Corn Oil : 50ml/d Mazola corn oil • Fish Oil: 50ml/d max EPA fish oil - ~14.5g/day n-3 FA • Fish oil & Exercise: fish oil plus exercise (walk/jog 3 d/week x 45-50 min at 75-80% HR max)	• Control • Fish/Fish Oil: 4g/day on 5 days/salmon steaks on 2 days - ~4g/day n-3 FA • Exercise: (aerobic 3 d/week x 60 min at 70-85% HR max) • Fish oil & Exercise
Measures		
	• Lipids all groups: TAG, HDL, LDL, TC • Lipids Fish oil & Exercise and fish oil only: Apo B • Platelet counts & bleeding times • VO_2 • Body composition	• Lipids: TAG, HDL, LDL, TC • VO_2 and HR • Body composition

1.5 AIMS OF THESIS

Intervention trials have demonstrated that n-3 FA and regular moderate-intensity exercise can independently modify several risk factors for MS, including blood lipid profiles, hypertension, endothelial function, inflammation, hyperglycaemia and obesity. These benefits undoubtedly contribute to their protection against CV and all-cause mortality. However there exists a paucity of data on the combined effects of these two interventions.

The overall aim of this doctoral thesis was to investigate the individual and combined effects of a moderate dose of n-3 FA from fish oil and regular moderate-intensity exercise on risk factors for MS. This was addressed by conducting a randomized, double blind placebo controlled intervention in an overweight / obese population with risk factors associated with MS. The outcomes of this thesis may be important in terms of informing health policy in relation to the use of n-3 FA and exercise for the management and / or prevention of MS.

STUDY DESIGN & SUBJECTS 2

2.1 STUDY DESIGN

2.1.1 Overview

This study aimed to determine the effect of n-3 FA supplementation from fish oil, exercise and the combination of these two interventions on a number of CV and metabolic risk factors and inflammatory markers associated with MS. The intervention was of parallel design with a placebo control. Treatment was administered double blind over a 12 week duration to allow sufficient time to detect changes in metabolic parameters, particularly body composition. Outcome measures were assessed and compared across each intervention group at Weeks 0, 6 and 12. Subjects attended two clinic visits at each of these time points This testing frequency was selected as several cardiovascular parameters (e.g. TAG, BP), as well as erythrocyte fatty acid profiles, can change within six weeks of supplementation with n-3 FA. **Figure 2.1** presents the general protocol used for the intervention trials. Ethics approval for this study was obtained from the University of Adelaide (Appendix A) and the University of South Australia (Appendix B) and approval to conduct the body composition scans using DXA was obtained from the Environmental Protection Agency (Appendix C). The Therapeutic Goods Administration was also notified of the trial and of the intention to use glyceryl trinitrate (GTN) (Appendix D). Written informed consent was obtained from all subjects prior to their participation.

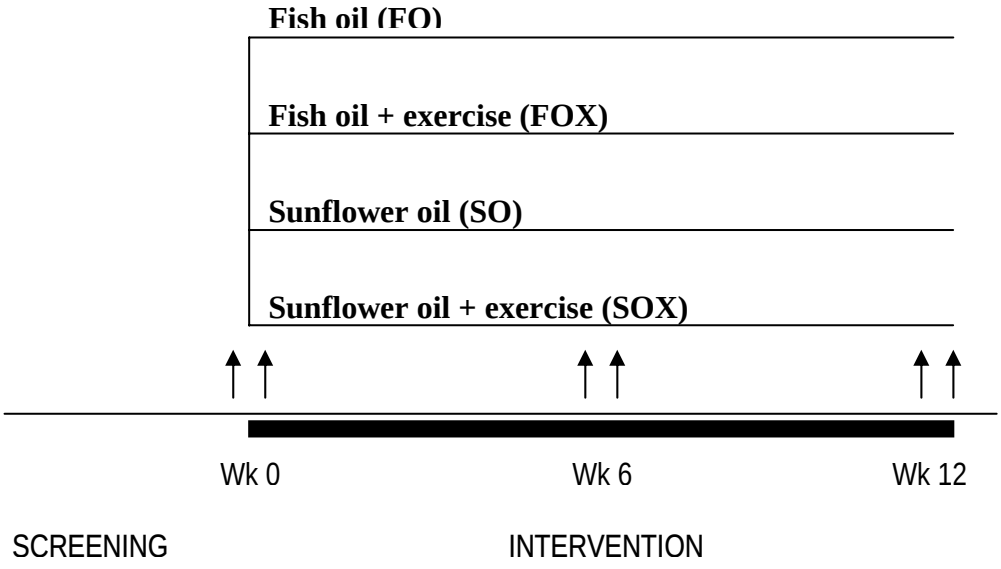

Figure 2.1 Study design and time line.

↑ Indicates clinic visit. For group allocation and description see section 2.2.3

2.1.2 Outcome measures

Primary outcome measures for this study included;

- Erythrocyte fatty acid profiles
- Blood lipids - TAG
  - HDL
  - LDL
  - TC
- Arterial Function - Arterial compliance
  - Endothelial dependent and –independent dilatation
- BP
- Body composition - Weight and BMI
  - Fat and lean mass

Secondary outcome measures for this study included;

- Blood glucose & insulin
- Exercise parameters - Respiratory exchange ratio (RER) & fat oxidation
  - HR
  - Oxygen consumption (VO_2)
- HRV
- Neutrophil functions - Cytokine production
  - Respiratory burst activity
  - Adherence
  - Bactericidal activity
  - Chemotaxis

2.1.3 Power calculation

The calculation of subject numbers was based on the well-established TAG lowering effects of fish oil. Thus from previous estimates of variance in TAG assessment, 80 subjects provided 80% power at $P<0.05$ to detect a 0.2 mmol/L (10%) change in TAG.

2.1.4 Statistical analysis

Statistical analysis was performed using *Statistica for Windows* (Version 5.1, StatSoft Inc, Tulsa, USA). Baseline comparisons for age, gender and height were made using one-way ANOVA (FO vs. FOX vs. SO vs. SOX). The effects of the treatments on the dependent measures were analysed using three-factor repeated-measures ANOVA with oil treatment (fish oil or sunflower oil) and exercise treatment (exercise or non-exercise) being the between group factors and time (Weeks 0, 6 and 12) being the within subject repeated measurement. In the case of FMD, because the magnitude of the dilatory response of the brachial artery is inversely related to the resting arterial diameter (Pyke *et al.*, 2004) and resting arterial diameter varied between Weeks 0, 6 and 12, the resting diameter at each of

Weeks 0, 6 and 12 was included in the model as a changing covariate. Where ANOVA indicated a significant main effect differences between individual means were identified post-hoc using Tukey's post-hoc test.

To optimise the analysis of differences between treatments, where appropriate, a nested ANOVA design was used to examine changes in dependent variables from baseline with the oil and exercise treatments nested in time. This analysis was used when there were multiple time points (i.e. weeks 6 and 12) which were significantly different from baseline, but not from each other.

Pearson product-moment correlation coefficients were used to identify relationships between variables. A P value of ≤ 0.05 was considered statistically significant. Results are presented as means ± SEM.

2.2 SUBJECTS

2.2.1 Entry criteria

To assess the effects of the intervention on selected CV parameters, which constituted our primary outcome measures, it was necessary to recruit males and females with a particular risk profile. The primary essential inclusion criteria were a sedentary lifestyle and overweight or obesity as defined by a BMI ≥ 25 kg/m^2. These two criteria carry an increased likelihood of having dyslipidemia and elevated BP, and each of these are associated with an increased risk of developing CVD, possibly due to their role in the MS (Grundy, 2006). In addition to being overweight/obese, subjects were required to exhibit at least one other CV risk factor. These inclusion criteria are detailed in **Table 2.1**. Apart from having a BMI ≥ 25 kg/m^2 and one other risk factor for CVD, subjects were essentially healthy without known renal or CVD, Type I or Type II diabetes. Exclusion criteria are listed in **Table 2.2**.

Table 2.1 Study inclusion criteria

Criteria	Value
Age	25-65 years
BMI	$\geq$ 25 kg/m^2
Activity level	Sedentary ($\leq$1 session/week of regular aerobic
Plus one or more of the following:	
Fasting TAG	>1.6 mmol/L
Fasting TC	>5.5 mmol/L
BP	systolic ≥140 mmHg - $\leq$ 160mm Hg diastolic ≥90 mmHg - $\leq$ 100 mmHg

Table 2.2 Study exclusion criteria

Criteria
• Consumption of two or more fatty fish (e.g. sardines, tuna, salmon) meals per week or fish oil capsules. • Antihypertensive or hypolipidaemic medication. • Type I or II diabetes • Regular participation in aerobic exercise (>1 session/week) during the six months prior to the study • Smoking • Pregnancy (subjects receive radiation exposure from DXA) • Known coronary condition (heart failure, arrhythmia, cardiac valve abnormality, stroke), peripheral vascular, renal or any other CVD • Phosphodiesterase inhibitor medication (e.g. Viagra, Levitra; potential interaction with nitric oxide and GTN administered during assessments of endothelial function) • Suspected non-compliance with exercise or dietary requirements • Allergies to fish or sunflower products.

2.2.2 Recruitment & screening

Subjects were recruited in three separate cohorts between July 2003 and September 2004. Subjects were recruited through information flyers placed on university and hospital notice boards, newspaper advertisements and articles, and radio and television interviews. Interested volunteers completed the following screening procedures to determine their suitability for inclusion:

A. Telephone Screening

The telephone interview enabled an initial assessment of subject suitability. Subjects were asked their weight and height (to estimate BMI), smoking status, current exercise and dietary habits (particularly fish consumption) and several general health questions including regular medications and supplements. Potential subjects were also given a brief description of the study and the experimental procedures involved.

B. Health and Lifestyle Questionnaire

Following the telephone interview, eligible subjects were sent a detailed information sheet (Appendix E) and a diet and lifestyle questionnaire (Appendix F). In addition to general information such as name, age, gender and contact details, the questionnaire also requested specific details about current illnesses, medication use, family history, diet and alcohol intake, exercise and smoking status.

C. Clinic Visit

Subjects deemed suitable for the study as assessed by their completed questionnaires were contacted by telephone and asked to attend a screening visit at the Nutritional Physiology Research Centre at the University of South Australia and the ATN Centre for Metabolic Fitness. The screening visit included an interview and some data collection. An example of the screening visit data sheet is shown in Appendix G.

1. An explanation of the visit and study requirements, including answering of any volunteer questions in relation to the requirements of the study.

2. Completion of volunteer consent forms (Appendix H).

3. Measurement of height and weight.

Weight was measured to the nearest 200g using an electronic scale (Tanita 'Ultimate Scale 2000', Tokyo, Japan) with subjects in minimal clothing and without shoes. Height was measured to the nearest 0.1 cm using a wall mounted telescopic stadiometer (Seca, 220, Vogel & Halke, Humberg, Germany) with subjects in stockinged or bare feet. These measures were used to calculate BMI.

4. Measure of waist and hip circumferences

Waist and hip measures were taken using a plastic fibre tape measure with subjects standing with arms relaxed by their side and balanced on both feet. The tape was held tight to the skin but without compression of tissue. Hip circumference was measured according to the guidelines of the International Society for the Advancement of Kinanthropometry (ISAK), as described by Norton & Olds (Norton & Olds, 1996), and was taken at the circumference level with the greatest posterior protuberance of the buttocks. Waist circumference was measured just above the iliac crest as recommended in the National Institute of Health Guidelines (Wang *et al.*, 2003).

5. Measure of resting BP.

Resting BP was measured using automated oscillometry (Omron Healthcare Inc., Bannockburn, USA) and determined by the mean of three measurements recorded at one-minute intervals after sitting for 10 minutes.

6. Collection of a fasting blood sample (8 ml).

Blood was collected into two 4 ml tubes, one of which contained EDTA and the other sodium fluoride/potassium oxalate tubes, which enabled respective lipid and glucose concentrations to be determined.

D. Medical and Exercise Stress Test

Once subjects had satisfied the initial entry criteria they underwent a medical examination, which included electrocardiogram (ECG) (Nihon Kohden, Tokyo, Japan) monitoring by a cardiologist during a graded exercise test. This exercise test required subjects to select a comfortable walking speed, which they maintained for 3 minutes at 0% gradient. The treadmill gradient was then increased incrementally every 3 minutes until a near age-predicted maximum HR (approx 85-90 %) was reached. HR and BP were monitored throughout the test. This confirmed their suitability for exercise training and also served as a familiarisation session for the exercise testing protocol. Subjects who demonstrated an abnormal ECG were excluded from participation.

2.2.3 Randomisation

Subjects were allocated to one of four groups, with each group being balanced for gender, BMI and TAG. The groups were then randomly assigned to one of four treatments. Two groups took 6 g/d of tuna fish oil (Hi-DHA®; Nu-Mega Ingredients Pty Ltd, Brisbane, Australia), which provided 260 mg of DHA and 60 mg of EPA in each 1g capsule (i.e. 1560 mg DHA + 360 mg EPA / day), and two groups took 6 g/d of placebo oil (sunflower oil) for 12 weeks. All capsules were identical in colour, shape and flavour (peppermint essence was added to all capsules during the manufacturing process) and were administered double blind.

Half of the subjects assigned to each oil treatment also participated in a program of regular physical activity. Subjects allocated to an oil plus exercise group (FOX and SOX) were required to run or walk for 45 minutes, 3 times per week at a HR which corresponded to 75% of their age predicted maximum (208 - (0.7 x age)) (Tanaka *et al.*, 2001). Subjects were provided with individual HR monitors (Polar F1; Polar Electro, Kempele, Finland) to facilitate their exercising at the appropriate HR.

The four groups were therefore identified as;

Sunflower oil (SO)

Fish oil (FO)

Sunflower oil and exercise (SOX)

Fish oil and exercise (FOX)

Subject enrolment and attrition patterns for the study population as a whole (cohorts have been combined) are described in **Figure 2.2**.

Outcome measures were assessed and compared across each intervention group at Weeks 0, 6 and 12, with the exception of body composition which in order to decrease exposure to ionising radiation was assessed at Weeks 0 and 12 only. Subjects attended two clinic visits at each of these time points where fasting blood samples, CV and anthropometric data were collected.

2.2.4 Compliance & retention

All subjects were instructed to maintain their normal diet during the study. If not asked to exercise as part of the intervention, subjects were also instructed to maintain their normal level of physical activity. To assist in monitoring of usual dietary intake and physical activity, subjects completed a 3-d physical activity diary (adapted from Bouchard et al. (Bouchard *et al.*, 1983)) and a weighed food record on two weekdays and one weekend day prior to attending the clinic at each of the three time points. To facilitate accurate recording of dietary intake, subjects were provided with a set of digital food scales. The use of these scales was demonstrated to all subjects during a group information session. Subjects were asked to record all food and drink (including vitamins, minerals, water etc) consumed during the 3-day period. Each food item was entered into and analysed using *Foodworks Professional Edition* (Xyris Software, Version 3.02, Highgate Hill, Australia). This program calculated total energy, macro and micronutrient intake. At each visit and at regular intervals throughout the trial, subjects were questioned about changes to diet and physical activity and other factors such as smoking, medication and supplement use.

At the clinic visits at Weeks 0 and 6, subjects were provided with a container of capsules. The number of capsules within this container was enough for a six week period and consequently compliance was assessed by counting the number of capsules remaining at each six week time point. Compliance was therefore confirmed by capsule count, erythrocyte fatty acid analysis, monitoring of regular participation in supervised exercise sessions, and completion of weighed food records and physical activity diaries.

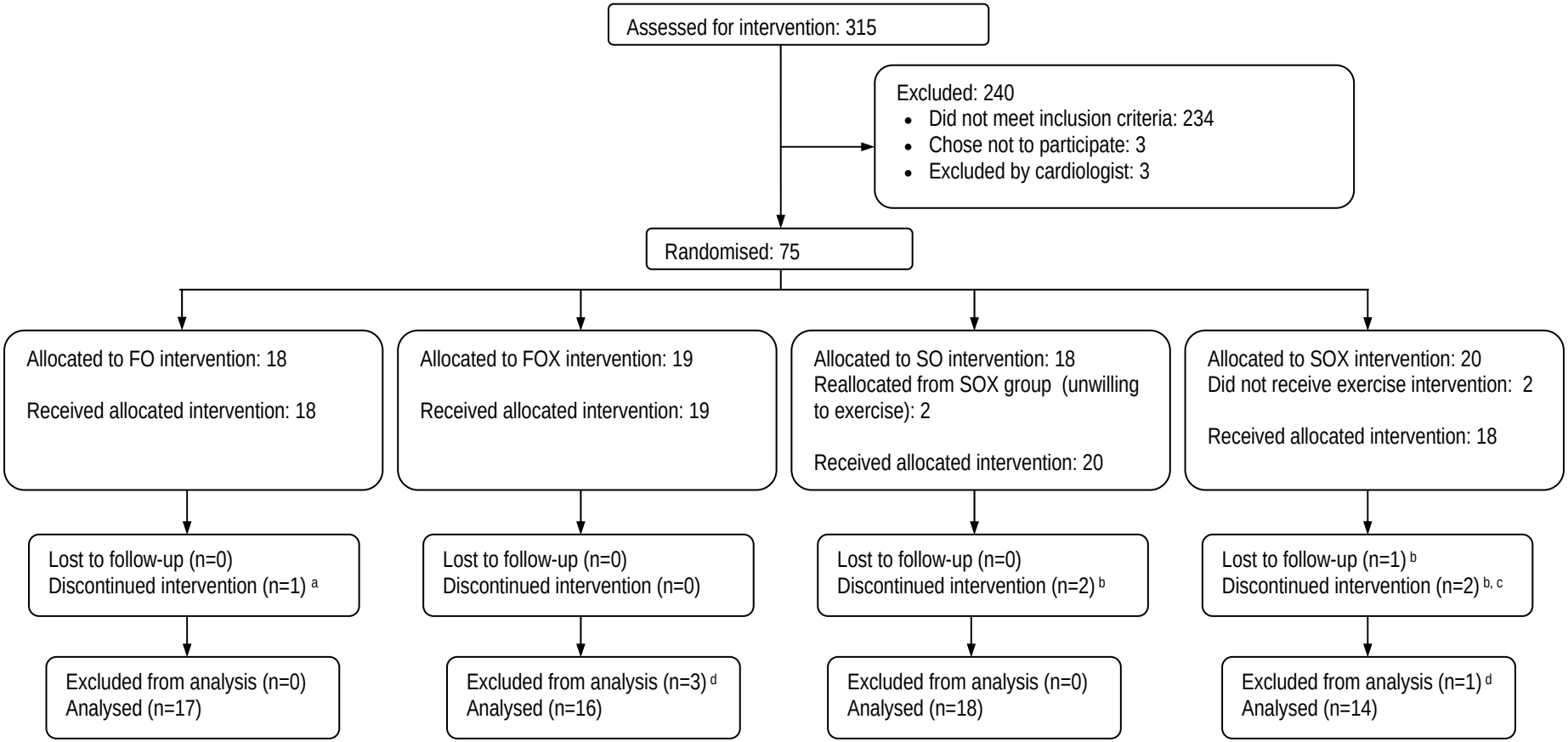

Figure 2.2 Subject recruitment and attrition patterns. [1]

[1] Abbreviations: FO = fish oil, FOX = FO and exercise, SO = sunflower oil, SOX = SO and exercise. Reasons for subject withdrawal: *a)* did not take required number of capsules, *b)* change in work/family circumstances, *c)* could not meet exercise intensity and *d)* did not comply with exercise protocol.

2.2.5 Treatment group profiles

Eligible subjects were randomised according to the protocol described in section 2.2.3 and as summarised in Figure 2.2. A total of 65 subjects completed all requirements of the study. **Table 2.3** shows the profiles of these subjects, grouped by treatment, upon entry to the study. There were no differences between groups (one-way ANOVA) for any of these characteristics. More importantly, groups were almost identical for both characteristics by which they were blocked (age, gender, BMI and TAG) and those that they were not (% body fat, BP and TC). Subjects were on average obese (BMI>30) with high levels of body fat, the latter likely influenced by the greater proportion of female subjects enrolled in the study. As previously mentioned, in addition to being overweight or obese, subjects were required to have at least one additional CV risk factor; elevated BP, TAG and/or TC. As each subject differed in the number of risk factors they exhibited, when averaged, the group means indicated only borderline risk. The recruitment of individuals who satisfied each entry criteria would have been preferable; however given the increasing prevalence of hypertensive and hyperlipidaemic medications within the general population, the recruitment of such subjects would be extremely difficult.

Table 2.3 Subject entry characteristics by treatment group.

	Treatment Group			
	FO (n=17)	**FOX (n=16)**	**SO (n=18)**	**SOX (n=14)**
Sex (M:F)	6 : 11	5 : 11	7 : 11	6 : 8
Age (years)	52 ± 2	47 ± 2	51 ± 2	51 ± 2
BMI (kg/m^2)	34 ± 1	34 ± 2	35 ± 1	33 ± 1
% Body Fat	44 ± 2	44 ± 2	44 ± 2	42 ± 2
Systolic BP (mmHg)	129 ± 3	132 ± 4	128 ± 3	132 ± 3
Diastolic BP (mmHg)	72 ± 2	78 ± 3	73 ± 2	76 ± 2
TC (mmol/L)	6.7 ± 0.3	6.2 ± 0.3	6.3 ± 0.4	6.6 ± 0.4
TAG (mmol/L)	1.7 ± 0.2	1.9 ± 0.4	1.7 ± 0.2	1.9 ± 0.2

BODY COMPOSITION 3

3.1 INTRODUCTION

The increasing prevalence of overweight and obesity brings with it significant economic cost (Visscher & Seidell, 2001) due to obesity's association with a number of CV disease risk factors, including hypertension, dyslipidemia, inflammatory markers and glucose intolerance (Poirier *et al.*, 2006). Weight loss is recommended as a primary means for correcting several of these factors. Even small changes in body weight (5-10 %) can induce significant improvements in metabolic abnormalities such as elevated BP, lipids and glucose (Goldstein, 1992; Mertens & Van Gaal, 2000).

Caloric restriction is the primary method recommended for achieving weight loss. However severe caloric restriction is difficult to maintain and if not combined with exercise, can result in substantial loss of lean tissue (Garrow & Summerbell, 1995). Similarly, some intense exercise programs may be unsuitable for obese and sedentary individuals. Consequently it is imperative that an achievable diet and exercise program to reduce obesity and CV risk be identified and implemented. Animal studies have indicated that n-3 FA may prevent fat accumulation (Hill *et al.*, 1993; Baillie *et al.*, 1999; Ruzickova *et al.*, 2004), so the combination of n-3 FA and a modest level of physical activity may therefore provide an alternate strategy for reducing body fat and CVD risk.

3.2 AIM

To assess the independent and combined effect of n-3 FA supplementation and regular moderate exercise on body composition in subjects with risk factors for MS.

3.3 METHODS

All clinical assessments were taken under fasting conditions (10-12 hr) and where possible, pre- and post-intervention measures were performed at similar times of the day.

3.3.1 Anthropometry

Measures of height and weight are as described in section 2.2.2 as part of the screening protocol. Height taken at Visit 1, Week 0 was used throughout the intervention to calculate BMI. Weight was measured at each visit and the two visits at each time point (i.e. Weeks 0, 6 and 12) were averaged.

3.3.2 Total body and abdominal composition

Body composition (fat, lean and bone tissue) was assessed in all subjects using DXA (*Lunar Prodigy*: General Electric, Madison, USA) at Week 0 and Week 12. Subjects were asked to remove all materials that could attenuate the x-ray beam, including jewellery items, glasses and underwear containing wire. Subjects were scanned wearing a hospital gown and were positioned supine in accordance with the manufacturer's instructions. Prior to each scan, the DXA scanner was calibrated according to the standard procedures recommended by the manufacturer. Abdominal adiposity was determined using the method described by Carey et al. (Carey *et al.*, 1996), which defines a region of interest using bony landmarks.

The errors for assessments of body composition by DXA were 0.87% for %body fat, 0.53 kg (1.6%) for fat-mass, 1.05 kg (2.3%) for lean mass, and 0.02 g/cm2 (1.3%) for bone mineral density, performed on consecutive days on 11 overweight/obese subjects not involved in this study.

3.3.3 Exercise testing

A graded exercise test was used to assess changes in heart rate, measured at 15-second intervals using a Polar Accurex Plus HR monitor (Sports Tester, Polar Electro, Finland), and gas exchange, monitored at 30-second intervals by indirect calorimetry (Ametek S-3A/I and CD-3A, Pittsburgh, USA). Subjects began the test by self-selecting a moderate walking speed at 0% incline on an electronic treadmill (Quinton Instruments, Model Q65, Washington, USA). After 10 minutes, the incline was increased to 5.0% for 5 minutes and then 10.0% for an additional 5 minutes. At the end of each workload, subjects were seated for 2 minutes while BP and HR were recorded. During exercise, metabolic (oxygen uptake [VO_2]), carbon dioxide output [VCO_2], respiratory exchange ratio [RER]) and cardiovascular (HR) responses were measured.

3.3.4 Diet analysis

Dietary data were collected and analysed as described in section 2.2.4. Dietary intake was assessed by weighed food record on two weekdays and one weekend day prior to attending the clinic at each of the three time points (i.e. Weeks 0, 6 and 12), with the average of the three days at each time point used for statistical analysis.

3.4 RESULTS

3.4.1 Subjects

There were no significant differences (one-way ANOVA) between groups for weight, BMI, % body fat, fat and lean mass, or energy intake (kJ) at baseline (**Table 3.1**).

Table 3.1 Baseline (Week 0) measures of body composition and energy intake (kJ).

	Treatment Group			
	FO (n=17)	**FOX (n=16)**	**SO (n=18)**	**SOX (n=14)**
Weight (kg)	97 ± 4.7	98 ± 5.5	98 ± 5	96 ± 3.8
BMI (kg/m^2)	34 ± 1	34 ± 2	35.1 ± 1.2	33 ± 1
% Body Fat	43.9 ± 1.6	43.5 ± 2.4	43.72 ± 1.93	41.5 ± 2.2
Fat Mass (kg)	40420 ± 2204	40961 ± 3449	40964 ± 2511	37822 ± 1947
Lean Mass (kg)	52343 ± 3458	53105 ± 3821	53161 ± 3375	54241 ± 3517
Energy Intake (kJ)	8895 ± 679	9739 ± 921	10549 ± 769	9937 ± 642

3.4.2 Effects of n-3 FA and/or exercise on energy intake

There were no effects of oil, exercise treatment, or time on macronutrient intake, and consequently total energy intake during the intervention period (**Table 3.2**). ANOVA revealed a trend toward a difference in energy intake between the fish oil and sunflower oil supplemented groups (**Figure 3.1,** oil x time interaction, *P*=0.09), due primarily to an overall tendency for energy intake to increase in the fish oil groups and decrease in the sunflower oil groups by Week 12 (Figure 3.1).

Table 3.2 Profile of macronutrient intake by treatment group.

		Treatment Group			
		FO (n=17)	**FOX (n=16)**	**SO (n=18)**	**SOX (n=14)**
Energy Intake (kJ)	*Week 0*	8895 ± 679	9739 ± 921	10549 ± 769	9937 ± 642
	Week 6	8674 ± 632	9189 ± 925	10329 ± 850	10703 ± 1053
	Week 12	9348 ± 804	10208 ± 1253	9334 ± 511	10297 ± 1199
Protein (g)	*Week 0*	95.4 ± 7.0	95.1 ± 7.3	103.5 ± 5.5	104.6 ± 9.4
	Week 6	93.8 ± 5.7	95.0 ± 9.1	96.7 ± 5.9	103.2 ± 8.2
	Week 12	98.2 ± 7.5	100.0 ± 11.3	90.0 ± 4.1	104.0 ± 10.7
Carbohydrate (g)	*Week 0*	209.4 ± 21.0	253.0 ± 26.5	290.2 ± 29.6	280.3 ± 19.3
	Week 6	215.2 ± 23.5	240.0 ± 28.5	284.5 ± 33.1	286.9 ± 29.0
	Week 12	214.4 ± 23.0	256.6 ± 35.1	240.3 ± 17.5	272.0 ± 31.0
Alcohol (g)	*Week 0*	9.9 ± 4.0	20.6 ± 7.4	9.8 ± 3.2	11.6 ± 4.3
	Week 6	12.4 ± 4.8	14.8 ± 4.4	10.0 ± 3.0	18.9 ± 6.1
	Week 12	13.9 ± 5.2	21.8 ± 7.5	9.1 ± 3.6	20.1 ± 7.2
Total Fat (g)	*Week 0*	9.10 ± 8.9	86.0 ± 8.8	94.8 ± 7.4	80.8 ± 7.1
	Week 6	81.8 ± 7.4	81.8 ± 9.2	95.9 ± 8.2	94.2 ± 10.9
	Week 12	97.6 ± 9.4	92.9 ± 12.9	92.7 ± 6.5	88.8 ± 13.0
Saturated Fat (g)	*Week 0*	34.2 ± 3.9	33.4 ± 4.1	37.2 ± 3.6	30.3 ± 3.0
	Week 6	31.4 ± 3.5	31.5 ± 4.4	35.8 ± 3.1	37.5 ± 5.5
	Week 12	38.2 ± 3.7	38.2 ± 6.2	34.8 ± 3.0	36.3 ± 6.5
Monounsaturated Fat (g)	*Week 0*	34.8 ± 3.7	32.5 ± 3.2	33.6 ± 2.4	30.5 ± 3.3
	Week 6	30.0 ± 2.8	29.7 ± 3.3	33.8 ± 3.6	33.5 ± 3.9
	Week 12	35.8 ± 3.8	32.3 ± 4.6	34.9 ± 2.6	31.4 ± 4.4
Polyunsaturated Fat (g)	*Week 0*	13.8 ± 1.4	12.8 ± 1.5	15.4 ± 2.1	12.7 ± 1.6
	Week 6	12.8 ± 1.4	13.2 ± 1.4	17.2 ± 2.1	14.7 ± 1.8
	Week 12	13.9 ± 1.7	14.0 ± 2.4	15.6 ± 1.1	13.6 ± 1.8

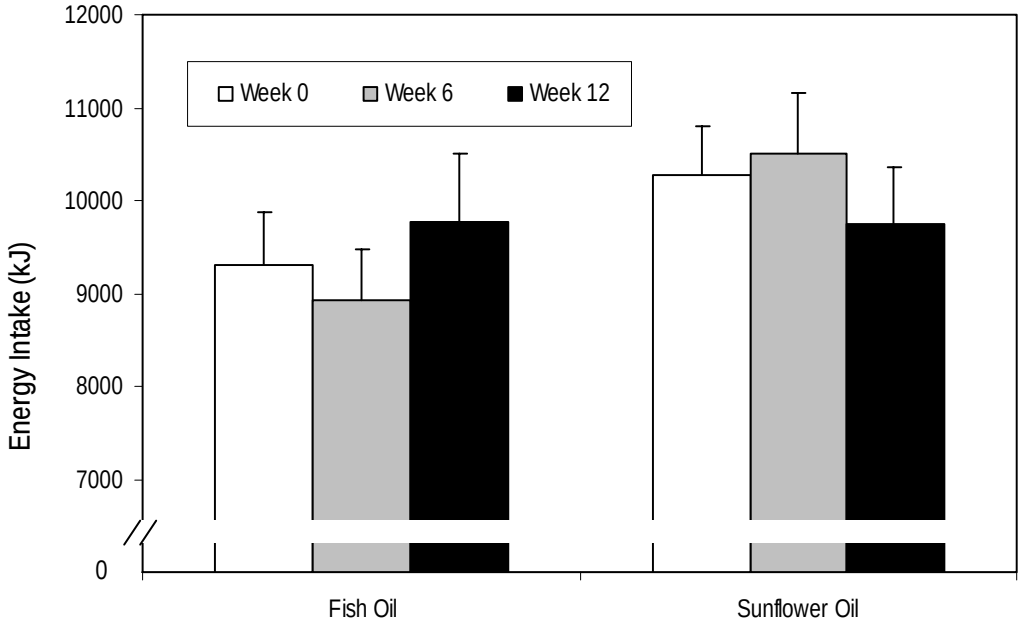

Figure 3.1 Energy intake by oil treatment at Weeks 0, 6 and 12 of intervention (oil treatment x time, *P*=0.09).

3.4.3 Effects of n-3 FA and/or exercise on body weight and BMI

Three-factor ANOVA of body weight did not reveal a significant exercise x oil x time interaction (*P*=0.30) or oil x time interaction (*P*=0.15). However, there was a significant exercise x time interaction (*P*=0.049) (**Figure 3.2**). Post-hoc analysis (Tukey test) indicated that after 12 weeks of intervention body weight had decreased in exercising subjects (*P*=0.036), but not in non-exercising subjects (*P=0.*998), resulting in body weight being significantly lower in exercising subjects compared with non-exercising subjects (Figure 3.2, *P*=0.0001). Body weight was reduced by 1.02 ± 0.49 kg in exercising subjects compared to a 0.13 ± 0.29 kg increase without exercise.

BMI data were analysed with the same method described for changes in body weight. ANOVA showed a strong trend toward an exercise x time interaction (*P*=0.058). To account for any differences in starting BMI, data were analysed using nested ANOVA, which showed that the change in BMI in exercising subjects was significantly different to non-exercising subjects (*P*=0.042).

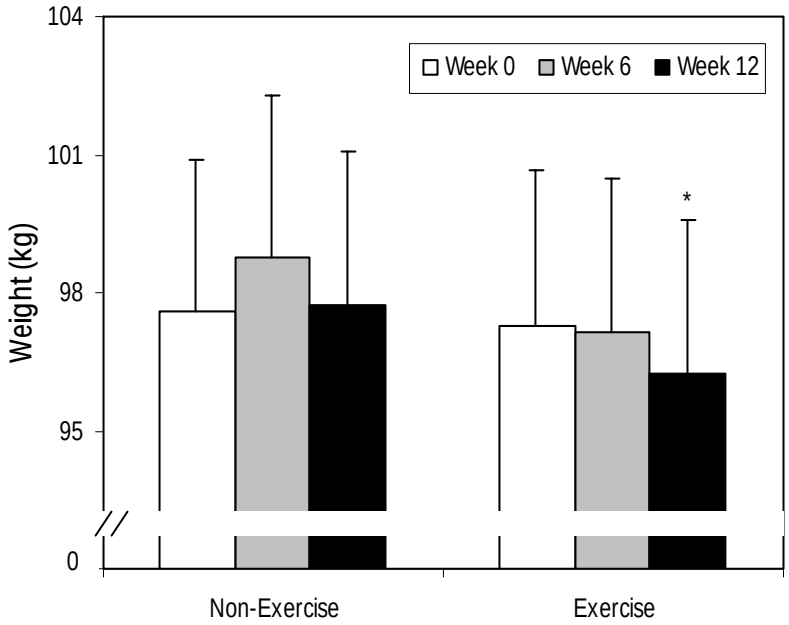

Figure 3.2 Effect of exercise training or no exercise on body weight. *Significant reduction in body weight in exercising compared to non-exercising subjects (exercise treatment x time, *P*=0.049).

3.4.4 Effects of n-3 FA and/or exercise on total body composition (fat and lean mass)

DXA was performed to allow for assessment of effects on body composition. There were no effects of oil treatment (oil x time, *P*=0.26) or exercise (exercise x time, *P*=0.38) or the combination (oil x exercise x time, *P*=0.50) on lean mass.

While ANOVA did not detect any significant 3-factor interactions for fat mass (oil x exercise x time), there were separable effects of exercise (exercise x time, *P*=0.005) and oil (oil x time, *P*=0.043) (**Figure 3.3**). The average reductions in fat mass with fish oil and exercise were 667 ± 346 g and 930 ± 332 g respectively, compared to an increase of 440 ± 320 g in non-exercising or 297 ± 328 g in sunflower oil controls. The additive effect is reflected in the FOX group which showed an average reduction of 1571 ± 390 g. Changes in fat mass were inversely correlated with changes in DHA content of erythrocytes (r = -0.347, *P* = 0.021)

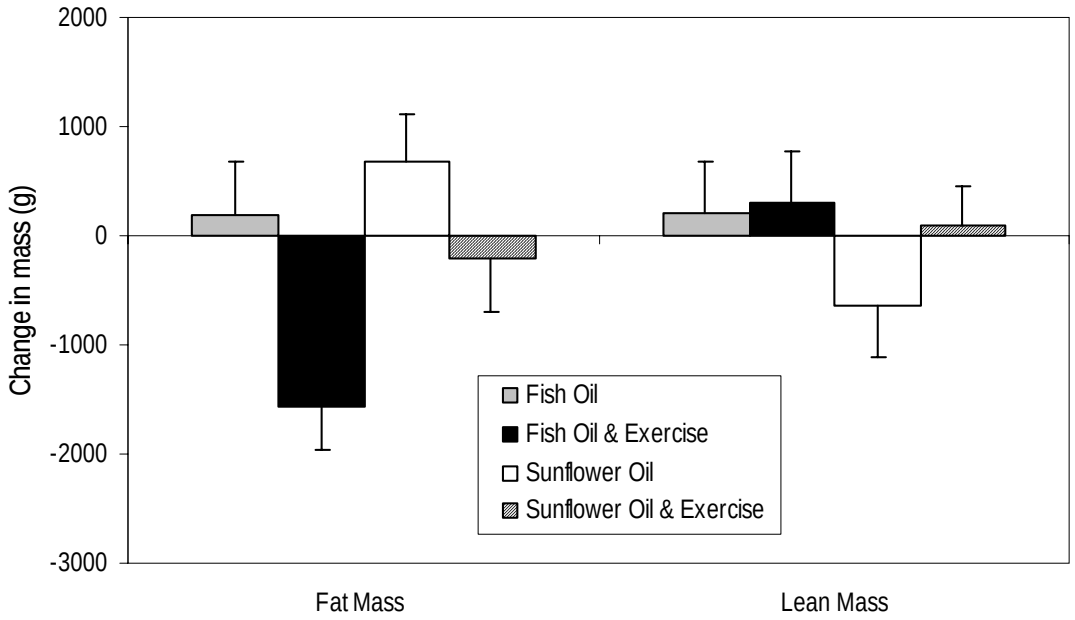

Figure 3.3 DXA assessed changes in total body fat and lean mass by treatment group. Fat mass was independently reduced by fish oil supplementation (*P*=0.043) and exercise training (*P*=0.005) by Week 12 of intervention.

3.4.5 Effects of n-3 FA and/or exercise on abdominal region composition

ANOVA confirmed that there were no 3-factor (oil x exercise x time, *P*=0.65) or 2-factor (oil x time, *P*=0.20; exercise x time, *P*=0.58) interactions for abdominal lean mass. However there was a significant exercise x time interaction (**Figure 3.4**, *P*=0.004) for abdominal fat mass, which decreased in subjects allocated to an exercise group.

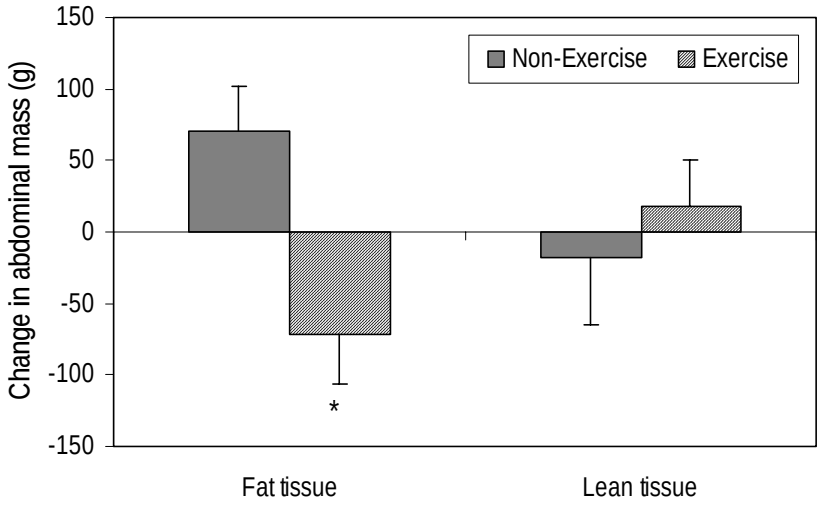

Figure 3.4 DXA assessed changes in abdominal fat and lean mass after exercise training or no exercise for twelve weeks. *Significant reduction in abdominal fat in exercising compared to non-exercising subjects (exercise treatment x time, P=0.004).

3.4.6 Effects of n-3 FA and/or exercise on RER

Four-factor ANOVA (oil x exercise x workload x time) assessed changes in RER following intervention. This analysis detected a significant 3-factor (exercise x workload x time, P<0.031) and 2-factor (exercise x time, P<0.034) interaction for the effect of exercise on RER. Given the complexity of the post-hoc analysis, data are reported for exercise x time interactions only. **Figure 3.5** shows the RER (measured by indirect calorimetry) for each workload at Week 0 and after 6 and 12 weeks of intervention for the exercise and non-exercise treatment groups. Within the exercise group, RER was significantly lower at Week 6 (P<0.021) compared with baseline (Week 0). However there were no further reductions in RER by Week 12, although this remained significantly different from Week 0 (Week 12 vs. Week 6, P<0.975; Week 12 vs. Week 0, P<0.002). Pearson's correlation showed a significant relationship between the change in fat mass and the average change in RER across all workloads (**Figure 3.6**).

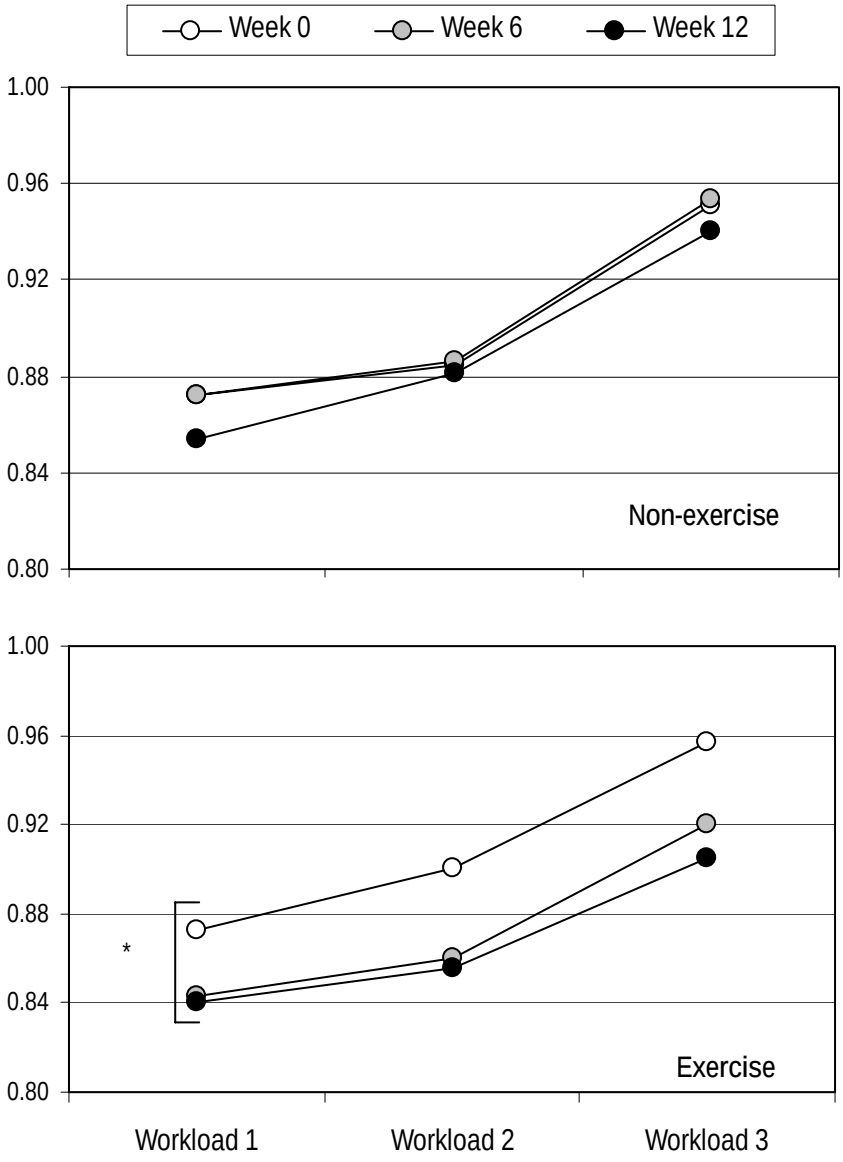

Figure 3.5 RER during 3 exercise workloads at Week 0 and after 6 and 12 weeks of exercise training or no exercise. * Indicates Week 0 significantly different to Weeks 6 and 12 (Tukey test, $P<0.021$ and $P<0.002$, respectively).

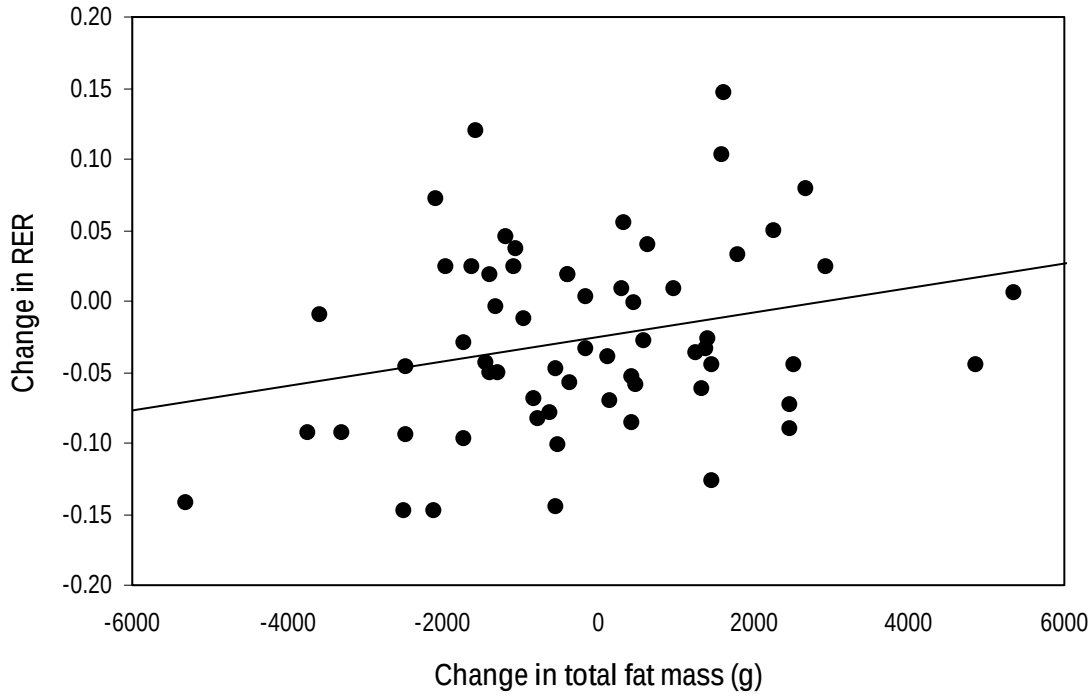

Figure 3.6 Relationship between the change in total body fat mass and average change in RER across all workloads. Week 12 of intervention showed a significant decrease in fat mass with a reduction in RER (r = 0.266, *P* = 0.038).

3.4.7 Effects of n-3 FA and/or exercise on fat oxidation during exercise

Given the positive effect of exercise on RER, we sought to determine the effect of intervention on fat oxidation. Data collected by indirect calorimetry were converted using the equation developed by Frayn et al. (Frayn, 1983). ANOVA detected a significant two-factor exercise x time interaction (**Figure 3.7**, *P*<0.034). As expected, the change in fat oxidation mirrored the changes seen for RER. Post-hoc analysis indicated that by Week 6, fat oxidation had increased significantly in the exercise group (*P*=0.016), with no further increases by Week 12, although this remained significantly elevated compared to Week 0 (*P*=0.0024).

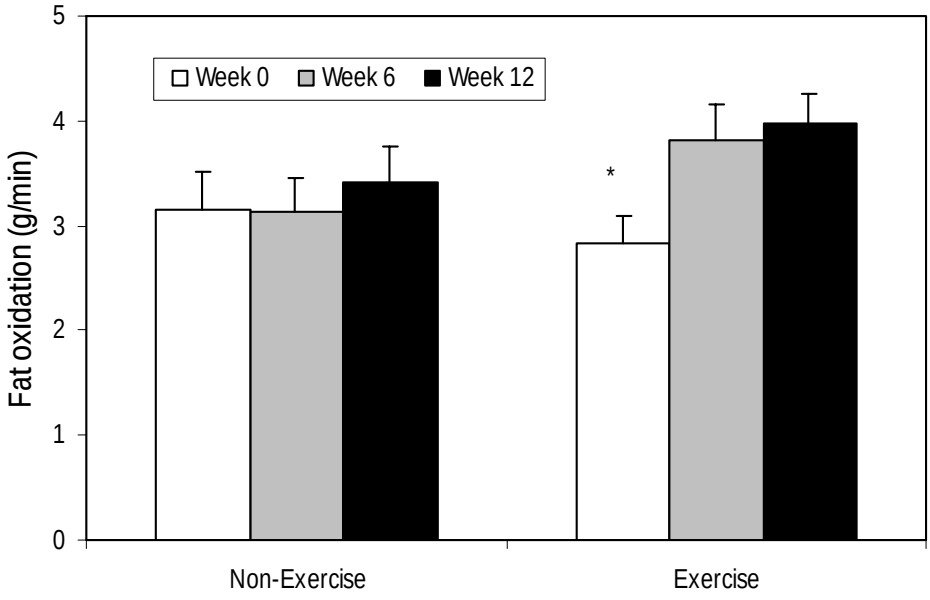

Figure 3.7 Fat oxidation during exercise at Week 0 and after 6 and 12 weeks of exercise training or no exercise. * Indicates Week 0 significantly different to Weeks 6 and 12 (Tukey test, $P<0.016$ and $P<0.003$, respectively).

3.4.8 Associations between measures of body composition and metabolic variables

Table 3.3 shows the correlation coefficients for the relationships between the change in several measures of body composition and metabolic variables for all subjects by Week 12 of intervention. Some of these relationships are presented graphically in **Figures 3.8 – 3.12**.

Table 3.3 Pearson's correlations between changes in various measures of body composition and metabolic variables by Week 12 of intervention.

	Body weight		**BMI**		**% Body Fat**		**Total body fat mass**		**% Abdominal fat**		**Abdominal fat mass**	
	R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
TAG (mmol/L)	0.193	0.137	0.186	0.152	0.231	0.073	0.235	0.069	0.181	0.163	0.026	0.844
HDL (mmol/L)	-0.005	0.972	-0.025	0.850	-0.058	0.658	-0.044	0.735	-0.145	0.266	-0.111	0.394
TC (mmol/L)	0.341	0.007*	0.365	0.004*	-0.002	0.988	0.192	0.138	-0.007	0.955	0.196	0.130
SBP (mmHg)	0.109	0.404	0.134	0.305	0.371	0.003*	0.339	0.007*	0.300	0.019*	0.343	0.007*
DBP (mmHg)	0.165	0.204	0.185	0.153	0.348	0.006*	0.371	0.003*	0.274	0.033*	0.279	0.030*
LAC (ml/mmHg x 10)	-0.063	0.632	-0.073	0.576	-0.173	0.184	-0.145	0.265	-0.138	0.290	-0.128	0.327
SAC (ml/mmHg x 100)	0.156	0.231	0.174	0.181	-0.009	0.948	0.076	0.562	-0.058	0.656	-0.037	0.775
FMD (absolute) (mm)	-0.052	0.693	-0.062	0.634	-0.120	0.358	-0.111	0.394	-0.096	0.462	-0.082	0.531
Resting HR (bpm)	0.422	0.001*	0.430	0.001*	0.239	0.063	0.347	0.006*	0.155	0.233	-0.101	0.438
Glucose (mmol/L)	0.281	0.028*	0.277	0.031*	0.114	0.383	0.221	0.086	0.178	0.169	0.082	0.531
Insulin (uU/mL)	0.265	0.039*	0.261	0.042*	0.252	0.050*	0.324	0.011*	0.280	0.029*	-0.039	0.766
HOMA	0.290	0.024*	0.285	0.026*	0.253	0.049*	0.352	0.005*	0.292	0.022*	0.044	0.734
Beta cell function (%)	0.032	0.808	0.033	0.798	0.104	0.425	0.101	0.440	0.042	0.748	-0.174	0.180
QUICKI	-0.178	0.170	-0.172	0.184	-0.038	0.770	-0.123	0.346	-0.089	0.495	0.020	0.880

* Indicates significant relationship ($P<0.05$).

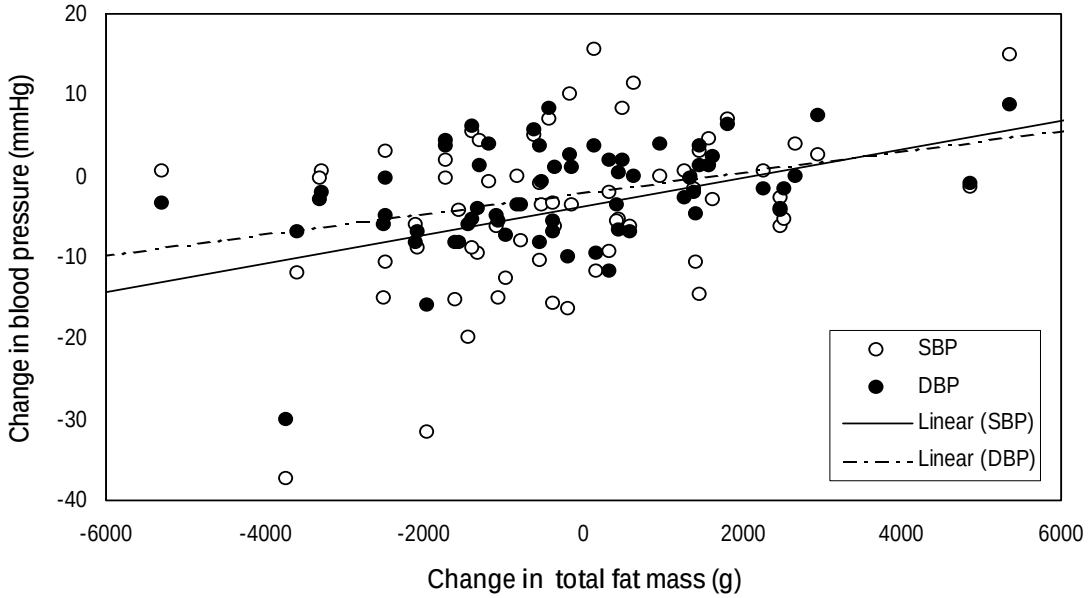

Figure 3.8 Relationship between changes in total body fat mass and SBP and DBP (SBP; r = 0.339, *P* = 0.007; DBP; r = 0.371, *P* = 0.003).

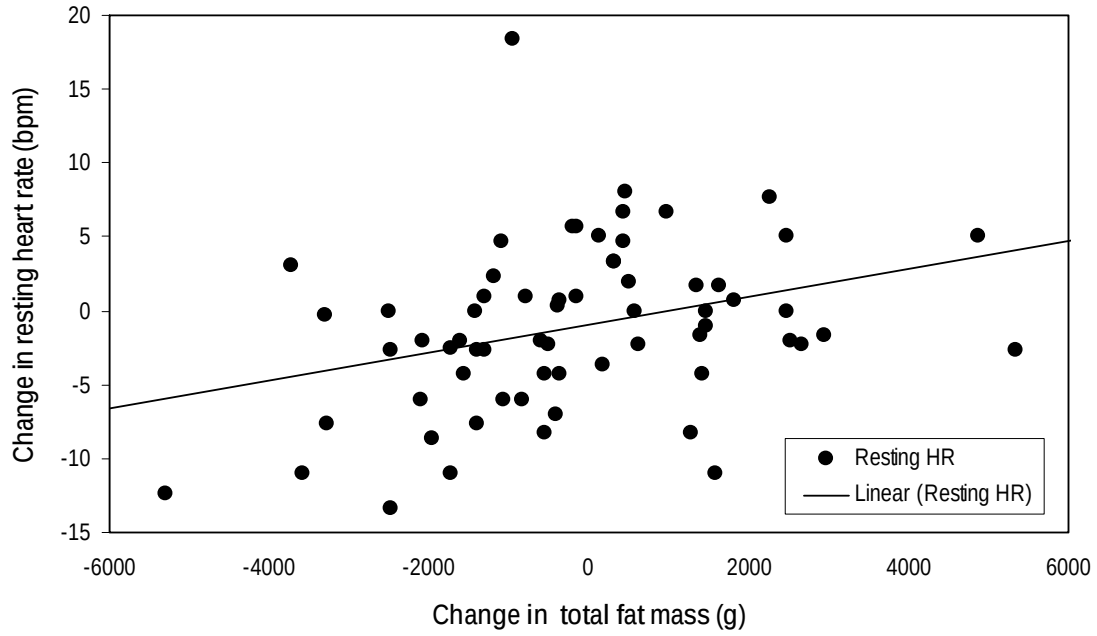

Figure 3.9 Relationship between the change in total body fat mass and resting (r = 0.347, *P* = 0.006).

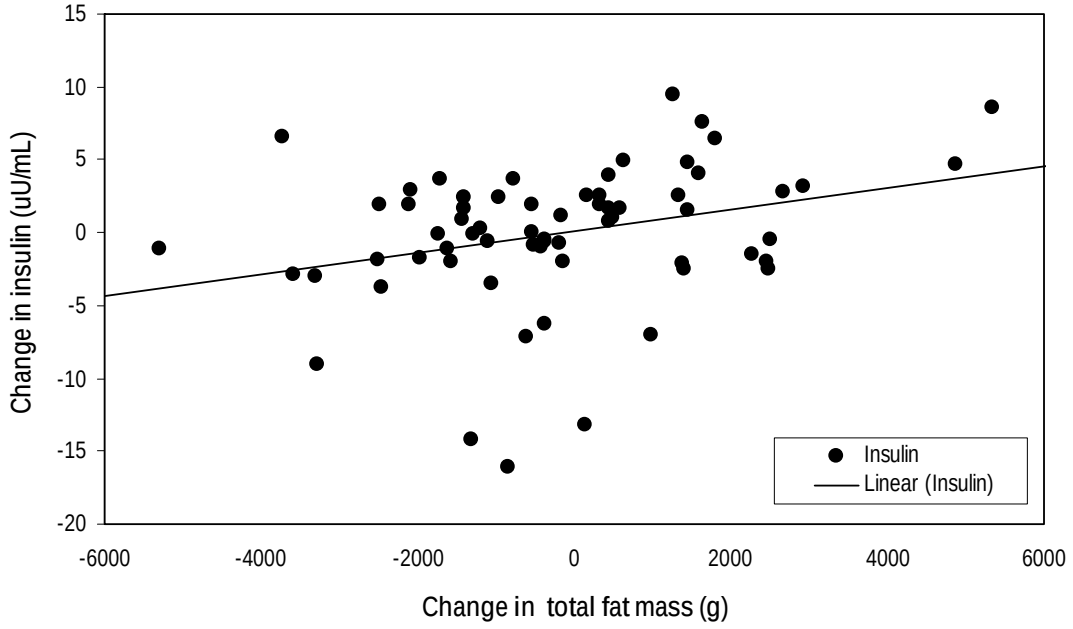

Figure 3.10 Relationship between the change in total body fat mass and plasma insulin (r = 0.324, *P* = 0.011).

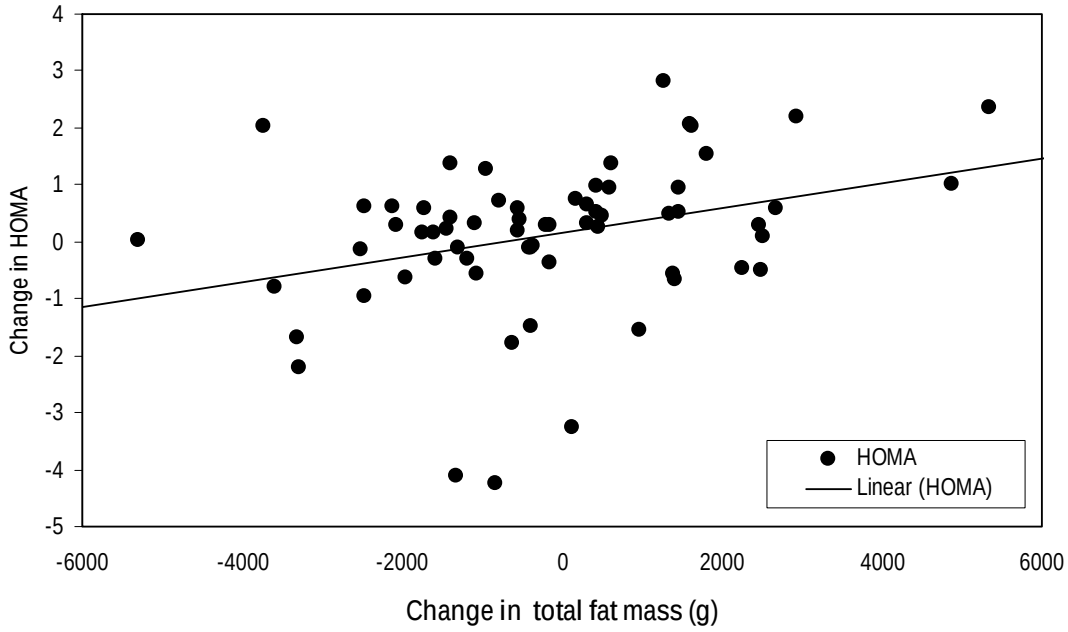

Figure 3.11 Relation between the change in total body fat mass and HOMA (r = 0.352, *P* = 0.005).

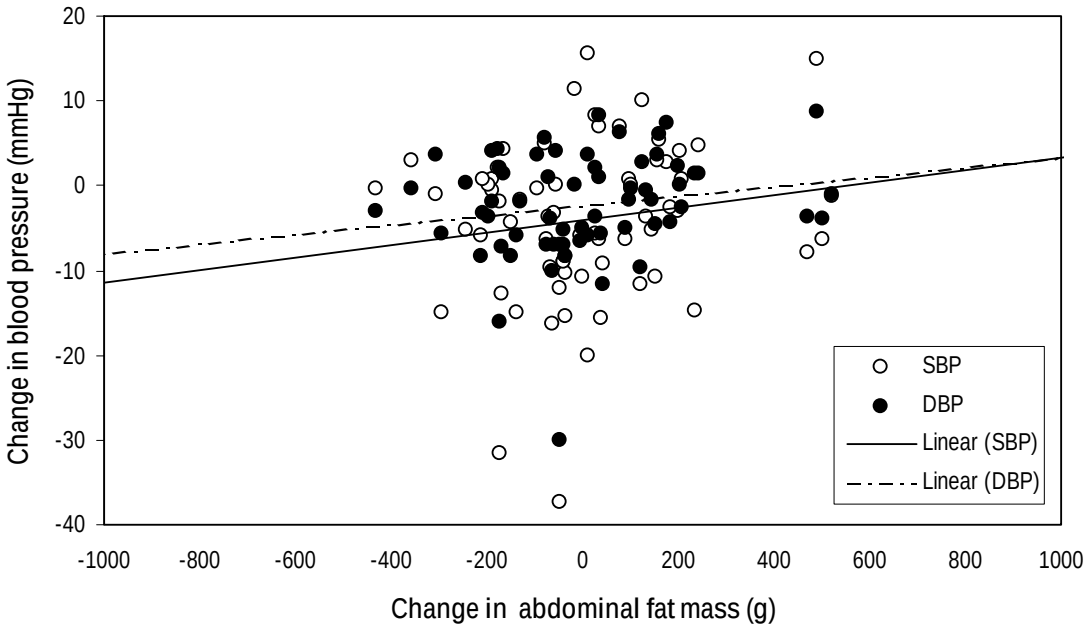

Figure 3.12 Relation between the change in abdominal fat mass and SBP and DBP (SBP; r = 0.343, *P* = 0.007; DBP; r = 0.279, *P* = 0.03).

3.5 DISCUSSION

3.5.1 Exercise induced changes in body composition

Caloric restriction has been established as the best non-pharmacological method for short-term weight loss, however its efficacy long-term is less convincing (Miller *et al.*, 1997). The addition of exercise to dietary restriction provides greater long-term changes in body weight than does diet alone (Miller *et al.*, 1997). In comparison, most studies find that physical activity alone produces relatively small changes in body weight (Garrow & Summerbell, 1995; Miller *et al.*, 1997; Wing, 1999). Ross and colleagues (Ross *et al.*, 2000b) suggest that these reports are likely to be confounded by studies that fail to adequately control and measure dietary intake or energy expenditure (Garrow & Summerbell, 1995; Miller *et al.*, 1997). While the extent of weight loss that can be achieved through exercise is still being debated, it is clear that physical activity plays a key role in preventing weight gain following an initial weight loss (Hill & Wyatt, 2005).

The assessment of the effect of exercise on body weight or body fat is often made difficult by study design; where exercise is usually combined with a dietary intervention and compared to diet alone or a non-intervention control (Rice *et al.*, 1999; Janssen *et al.*, 2002). Furthermore, subjects allocated to exercise groups are often of considerably lower weight and younger age than subjects in diet or diet plus exercise groups (Ross *et al.*, 2000b). It has been estimated that physical activity alone can induce modest, 1-2 kg reductions in body weight without concomitant changes in diet (Wing, 1999). To ensure that changes in body weight and /or composition were due to treatment, subjects in the present study were also asked to maintain their usual dietary intake. Analysis of weighed food diaries at each of the three intervention time points (Week 0, 6 and 12) showed that there were no differences in macronutrient intake and therefore total energy intake between any of the treatment groups; dietary intake did not change during the study.

In this study, exercise produced a modest change in body weight, primarily due to a loss of fat mass (0.93 kg). This reduction in body weight is smaller than that reported in other studies with overweight/obese subjects (Ross *et al.*, 2000a; Donnelly *et al.*, 2003; Savage *et al.*, 2003; Ross *et al.*, 2004), and is likely due to the magnitude of exercise undertaken and the duration of the exercise intervention. The exercise intervention in this study (3 d/week x 45 min @ 75% maximal HR) was of substantially less volume than the majority of these trials (Table 1.3 in Chapter 1). However when compared to studies with subjects of similar age, BMI and exercise protocols (Gordon *et al.*, 1997; Cox *et al.*, 2004), the effects on body weight and body fat was not dissimilar. This modest change in fat mass may be attributed at least partly to an improvement in fat oxidation during exercise, as demonstrated by a reduction in the respiratory exchange ratio during exercise and an increase in calculated fat oxidation. Exercise induces several adaptations that can contribute to improvements in fat oxidation, including an increase in β-receptor function (De Glisezinski *et al.*, 1998; Moro *et al.*, 2005), decreased circulating insulin which in turn increases lipolysis (Large & Arner, 1998) up-regulation of genes involved in FA oxidation (Tunstall *et al.*, 2002) and an increase

in blood flow (Maiorana *et al.*, 2003), thus improving delivery of oxygen and nutrients (glucose/fat) to sites of metabolism.

Although the exercise protocol in this trial produced only a modest reduction in body weight and fat mass, it may play a positive role in preventing weight gain, particularly over a longer duration. This is supported by observations from the current study. Subjects allocated to a non-exercise group showed a trend toward an increase in body weight and fat mass (+440g) during the intervention period, an effect that was counteracted in the exercise group.

Furthermore, exercise can play a significant part in reducing abdominal obesity. Total abdominal and IAAT are strongly associated with several metabolic abnormalities including insulin resistance, hypertension and hypertriglyceridaemia and may convey greater risk for chronic disease and mortality than overall body weight of fat alone (Warren *et al.*, 2006). The increased risk for metabolic complications observed with excess IAAT is likely due to its increased lipolytic activity and direct drainage of free FA into the portal vein(Wajchenberg, 2000). The mechanism behind exercise-induced changes in IAAT of overweight/obese subjects may be an increase in lipolytic activity due to improved sensitivity of β-receptors to circulating catecholamines.

Ross and colleagues have demonstrated that daily exercise, without weight loss, can significantly reduce total abdominal and visceral fat in obese men (Ross *et al.*, 2000a) and women (Ross *et al.*, 2004). More recently, Kay & Singh (Kay & Fiatarone Singh, 2006) reviewed the relationship between abdominal fat and physical activity and concluded that although limited, there is evidence that physical activity can reduce total abdominal and IAAT in overweight/obese subjects. However, they also indicated that these changes were more readily observed with the use of imaging techniques (computed tomography and magnetic resonance imaging) over anthropometry. Although the abdominal fat loss in this trial was marginal, our data support the positive effect of exercise on reducing abdominal adiposity.

3.5.2 Omega-3 FA induced changes in body composition

There are few randomised controlled trials investigating the effect of n-3 FA supplementation from fish or fish oil on body composition. Two trials have shown that n-3 FA can prevent body fat accumulation (Couet *et al.*, 1997; Groh-Wargo *et al.*, 2005). Couet et al. (Couet *et al.*, 1997) assessed the energy intake of 6 healthy (BMI 23.9±1.6), young (age 23±2 y) adults over three weeks while they consumed a control, ad libitum diet. Following a 3-month break, subjects were asked to consume the same diet over three weeks but 6 g/day of visible fat was substituted with 6g/day of fish oil (1.1 g/day EPA, 0.7 g/day DHA). Body composition was measured at the beginning and end of each 3-week intervention period. Fish oil feeding resulted in a significantly greater reduction in fat mass during the fish oil supplementation period (-0.88 ± 0.16 kg) than during the control period (-0.3 ± 0.34 kg). Basal lipid oxidation was also increased following supplementation with fish oil. However this study did not account for an order effect of intervention, and therefore changes in body composition may have been due to seasonal effects. In a well-designed study, Groh-Wargo and colleagues (Groh-Wargo *et al.*, 2005) investigated the effect of formula supplemented with DHA (0.15-0.27 g/100g of total fatty acids) and AA on growth and body composition in 57 pre-term infants. After 12 months, infants fed the DHA and AA-supplemented formulas had significantly more lean mass and less fat mass than the non-supplemented control group.

In comparison, the majority of studies investigating the effects of diets supplemented with n-3 FA have failed to observe any additional effects on body weight or fat mass above those induced by diet alone (Mori *et al.*, 1999b; Fontani *et al.*, 2005; Krebs *et al.*, 2006). Fontani et al. (Fontani *et al.*, 2005) allocated 33 healthy subjects to one of 2 diets, Zone or INRNIF (diet suggested by Italian National Research Institute for Nutrition and Foods), for 70 days. In a crossover design, subjects were randomised to receive either 4 g/day of fish oil (1.6 g/day EPA, 0.8 g/day DHA) or olive oil for the first 35 days, and then consumed the other oil for the last 35 days. There was a reduction in fat mass in both diet groups, although this

reduction was greater in the Z diet group. However, the addition of n-3 FA did not provide any additional effect on fat mass. This study did not include an n-3 FA control group and did not allow for a washout period in their crossover design. They also assessed changes in body composition with measures of skin fold thickness, the accuracy of which is reduced with increasing adiposity. Krebs et al. (Krebs *et al.*, 2006) investigated the potential synergistic effect of a 24-week intervention combining 5 g/day of fish oil (1.3 g/day EPA, 2.9 g/day DHA) with caloric restriction on weight loss in 93 overweight women. Subjects allocated to a weight loss group (weight loss with placebo oil, WLPO or with fish oil, WLFO) showed a reduction in body weight and fat mass from baseline by Week 12 of intervention, and this was maintained to Week 24. However, there were no differences between the WLPO or WLFO groups in the magnitude of weight or fat loss. This study also did not include an n-3 FA control group and did not appear to control for the effect of exercise.

In a recent study, Kunesova et al. (Kunesova *et al.*, 2006) showed that the addition of n-3 FA (2.8 g/day n-3 FA) to a very low calorie diet (VLCD + n-3 FA) resulted in greater BMI loss and reduction in hip circumference in obese women than did VLCD alone. In addition to the VLCD, patients also participated in 60 min of light-moderate physical activity daily. Interestingly, subjects in the VLCD group had a starting body weight that was 20 kg heavier than the VLCD + n-3 FA group. This study did not provide any indication of compliance or method for determining exercise intensity and given the difference in body weight it could be speculated that the greater reduction in BMI in the VLCD + n-3 FA group may have been due to differences in exercise volume. Mori et al. (Mori *et al.*, 1999b) observed no additional effects of n-3 FA from fish (3.65 g/day n-3 FA) on weight loss by caloric restriction in overweight, treated hypertensive subjects. Furthermore, there were no changes in body weight with fish intake (3.65g/day n-3 FA) alone. Had Mori et al. (Mori *et al.*, 1999b) assessed changes in body composition in addition to changes in body weight, they may have provided some earlier insight into the effect of n-3 FA on fat and lean mass,

particularly as there is some evidence that fish oil supplementation may protect against a reduction in lean tissue during weight loss (Kriketos *et al.*, 2001).

In the present study fish oil supplementation resulted in a loss of fat mass that was independent of exercise. It is reported that n-3 FA, can effectively mediate a shift in fuel metabolism, away from storage and toward oxidation, by up regulating several nuclear receptors, such as PPARs and SREBP (Davidson, 2006). Several studies have shown that n-3 FA decrease hepatic lipogenesis and TAG production (Harris & Bulchandani, 2006) and increase fatty acid oxidation in liver, muscle and adipose tissue (Ukropec *et al.*, 2003; Flachs *et al.*, 2005) by increasing expression of PPAR target genes involved in fatty acid transport and oxidation, including CPT-1 (Flachs *et al.*, 2005), fatty acyl-CoA oxidase and mitochondrial uncoupling protein 3 (Baillie *et al.*, 1999). This indicates that n-3 FA may enhance fat loss by stimulating a less efficient fatty acid oxidation pathway. However there is a paucity of studies investigating the effects of n-3 FA on fat metabolism and the associated mechanisms in humans.

3.5.3 Correlations with metabolic variables

In this study, changes in body weight and fat mass were positively associated with changes in TC, resting HR, SBP, DBP, glucose, insulin, and insulin resistance (as measured by HOMA). Modest weight loss (5-10% of initial body weight) has been shown to reduce a number of CV risk factors such as elevated BP, lipids and glucose and to increase insulin sensitivity (Goldstein, 1992; Mertens & Van Gaal, 2000; Utzschneider *et al.*, 2004). However, it is encouraging that even small changes in body weight can improve other CV risk factors.

3.5.4 Combined intervention trials & summary

There has been limited evidence that fish oil supplementation can reduce body fat in overweight/obese subjects, with little attempt to control for the influence of physical activity. Only two trials (refer to section 1.4.4.3) have investigated the effect of n-3 FA supplementation from fish and/or fish oil in combination with aerobic exercise training on body composition (Warner *et al.*, 1989; Brilla & Landerholm, 1990). The outcomes of these trials are limited by their study design. Warner and colleagues (Warner *et al.*, 1989) did not include an exercise only control group while Brilla and Landerholm (Brilla & Landerholm, 1990) enrolled a lean, healthy male cohort. This study is therefore the first to effectively evaluate the combined effect of regular aerobic exercise and n-3 FA on body composition in overweight/obese subjects. Both fish oil and exercise independently reduced body fat, which produced an additive effect on fat loss in subjects taking both fish oil and exercise (FOX group). Further research is required to identify the exact mechanisms behind these changes.

4 BLOOD PARAMETERS

4.1 INTRODUCTION

LDL and HDL are of great importance to cardiovascular health. HDL is responsible for transporting cholesterol from tissue depots to the liver where it is converted to bile acids and excreted. In contrast, LDL plays a key role in atherosclerosis as oxidised LDL initiates the atherosclerotic process by injuring the endothelial lining, and is then involved in the subsequent plaque formation. Elevated serum LDL and reduced HDL are independently associated with an increased risk of coronary events (Abbott *et al.,* 1988; Neaton & Wentworth, 1992; Stamler *et al.,* 2000). There is also evidence that elevated TAG are an independent risk factor for CVD (Austin *et al.,* 1998; Cullen, 2000), and predictor CHD mortality (Asia Pacific Cohort Studies Collaboration, 2004). This association may be related to its coupling with low HDL and high LDL. An increase in available TAG can contribute to CVD by altering lipoprotein density (Rizzo & Berneis, 2006). Small dense LDL are associated with an increased risk of coronary artery disease (Austin *et al.,* 1988; Lamarche *et al.,* 1997; St-Pierre *et al.,* 2005). Therefore the reduction in LDL density, the increase in HDL and the decrease in TAG resulting from n-3 FA would seem to be beneficial for reducing risk of CVD.

Elevated plasma glucose and insulin resistance are independent predictors of developing Type II Diabetes (Lillioja *et al.,* 1993; The Expert Committee on the Diagnosis and Classification of Diabetes, 2003). Insulin resistance is associated with an increased risk of developing glucose intolerance, dyslipidemia, essential hypertension, and procoagulant and proinflammatory states, all of which increase the risk of CVD (Reaven, 2006). Consequently

lifestyle interventions that modulate insulin and glucose, thus preventing the development of diabetes are of great interest to health professionals.

4.2 AIM

To determine the independent and combined effects of n-3 FA supplementation and regular moderate-intensity exercise on blood lipids, glucose and insulin in subjects with risk factors for MS.

4.3 METHODS

4.3.1 Blood sample collection

Blood samples were collected at each clinic visit following an overnight fast (10-12 hr). During the fast subjects were asked to avoid all food and beverages other than water. Samples were taken via venepuncture of an antecubital vein by a trained phlebotomist with subjects in a seated or supine position. Blood was collected into the appropriate Vacutainer ® tubes (Becton, Dickinson and Company, Franklin Lakes, USA) for subsequent analysis.

Blood collection tubes were spun by centrifuge at 4000 rpm for 10 min at 4 C. The plasma and serum was removed and stored at –80 °C until analysis. Erythrocytes were subsequently washed with isotonic saline (0.9%) to remove all plasma or serum and the buffy coat. Samples were re-spun and the excess saline removed. This process was repeated twice. The isolated and washed red blood cells were then frozen in 1 ml aliquots at –80 °C until analysis.

4.3.2 Erythrocyte fatty acid analysis

Frozen red blood cells were thawed, then lysed in hypotonic 0.01M Tris EDTA buffer, pH 7.4 and the membrane lipids were extracted and transmethylated according to the method of Lepage & Roy (Lepage & Roy, 1988). Fatty acid methyl esters (FAME) were analysed using a Shimadzu gas chromatograph 20A (Shimadzu Corporation, Kyoto, Japan)

fitted with a flame ionisation detector and a 50m BPX70 column (0.32 mm ID and 0.25 μm film thickness, SGE, Australia). Individual fatty acids were identified by comparison with known FAME standards (Nuchek Prep Inc, MN, USA) and expressed as a percentage of total fatty acids.

4.3.3 Analysis of lipoprotein lipids

Plasma TAG (including second day repeats) and TC concentrations were measured on an automated Cobas-Bio centrifugal analyser using reagents from Roche Diagnostica (Indianapolis, USA). HDL cholesterol was measured by an enzymatic colorimetric test on a Hitachi Autoanalyser system 911 (Hitachi, Tokyo, Japan) using Roche Diagnostica test kits (Indianapolis, USA). LDL was calculated using the Friedewald equation (Friedewald *et al.*, 1972).

4.3.4 Analysis of glucose and insulin

The concentration of plasma glucose was measured using enzymatic colorimetric kits (Roche Diagnostica, Indianapolis, USA) on an automated Cobas-Bio centrifugal analyser. Serum insulin concentration was determined by radioimmunoassay using a Human Insulin Specific Assay (Linco Research, St. Charles, USA). The glucose and insulin concentrations were used to calculate the homeostasis model assessment (HOMA), quantitative insulin-sensitivity check index (QUICKI) and beta-cell function as described by Cutfield et al. (Cutfield *et al.*, 2003).

4.4 RESULTS

4.4.1 Effects of n-3 FA and/or exercise on erythrocyte fatty acid concentrations

Fatty acid profiles of erythrocytes were measured in 44 subjects, due to technical difficulties leading to loss of data. Data are presented for Weeks 0, 6 and 12 in **Table 4.1**. There were no differences between groups prior to intervention (Week 0). To determine the effects of treatment, erythrocyte fatty acid composition data were analysed by 3-factor ANOVA (oil x exercise x time), and this analysis detected several one- and two-factor interactions (Table 4.1). Across all subjects, there was a significant reduction in oleic acid (18:1n-9) by Week 6, which was maintained to Week 12 (P<0.001). Following supplementation with fish oil there was a reduction in linoleic acid (18:2n-6) (oil x time interaction, P<0.001) and a trend toward a decrease in (20:4n-6) (P=0.067). Post-hoc analysis of the exercise x time interaction indicated that by Week 12 subjects allocated to a non-exercise group showed a reduction in AA (P<0.025).

The percentage of long chain n-3 FA (EPA+DPA+DHA) in erythrocytes increased substantially in both fish oil treated groups: from 10.7% to 13.7% (of total fatty acids) in the FO group and from 10.4% to 13.5% (of total fatty acids) in the FOX group and resulted in a significant oil x time interaction (**Figure 4.1** P < 0.001). This increase was entirely attributable to the change in DHA (22:6n-3), which increased by 85% in FO and 86% in FOX compared with –6% in SO and 9% in SOX (oil x time interaction, P<0.001). DHA and total long chain n-3 FA remained unchanged in subjects supplemented with sunflower oil (P=0.92). For all subjects, there was a marginal reduction in DPA (22:5n-3) at Week 6, which returned to baseline levels by Week 12. EPA (20:5n-3) did not change in any of the treatment groups.

Table 4.1 Erythrocyte fatty acid composition (% of total fatty acids) by treatment group.

		Treatment Group			
		FO (n=12)	***FOX (n=13)***	***SO (n=7)***	***SOX (n=12)***
Oleic	*Week 0*	10.98 ± 0.11	11.06 ± 0.29	10.18 ± 0.32	11.33 ± 0.47
Acid (18:1n-9) [1]	*Week 6*	10.34 ± 0.18	10.36 ± 0.23	10.05 ± 0.46	10.54 ± 0.25
	Week 12	10.40 ± 0.21	10.47 ± 0.27	10.19 ± 0.41	10.54 ± 0.27
Linoleic	*Week 0*	7.63 ± 0.22	6.99 ± 0.23	6.95 ± 0.23	7.11 ± 0.28
Acid (18:2n-6)[1, 3]	*Week 6*	6.97 ± 0.14	6.41 ± 0.21	7.30 ± 0.25	7.73 ± 0.23
	Week 12	6.61 ± 0.27	6.40 ± 0.22	7.36 ± 0.22	7.36 ± 0.23
Arachidonic	*Week 0*	11.92 ± 0.39	11.70 ± 0.48	12.01 ± 0.49	11.61 ± 0.59
Acid (20:4n-6)[1, 2]	*Week 6*	11.99 ± 0.46	12.09 ± 0.30	12.12 ± 0.50	12.61 ± 0.30
	Week 12	10.66 ± 0.32	11.21 ± 0.37	11.83 ± 0.87	12.22 ± 0.39
Eicosapentaenoic	*Week 0*	1.76 ± 0.06	1.62 ± 0.07	1.59 ± 0.10	1.70 ± 0.06
Acid (20:5n-3)	*Week 6*	1.71 ± 0.05	1.59 ± 0.07	1.58 ± 0.12	1.61 ± 0.04
	Week 12	1.60 ± 0.06	1.53 ± 0.05	1.55 ± 0.12	1.64 ± 0.05
Docosapentaenoic	*Week 0*	4.96 ± 0.21	4.76 ± 0.16	4.74 ± 0.16	5.18 ± 0.19
Acid (22:5n-3)[1]	*Week 6*	4.66 ± 0.13	4.56 ± 0.13	4.58 ± 0.29	4.87 ± 0.10
	Week 12	4.71 ± 0.15	4.70 ± 0.12	4.54 ± 0.30	5.00 ± 0.12
Docosahexaenoic	*Week 0*	3.99 ± 0.25	3.97 ± 0.29	3.98 ± 0.29	3.88 ± 0.24
Acid (22:6n-3)[1, 3]	*Week 6*	6.18 ± 0.32	6.40 ± 0.21	3.99 ± 0.26	4.36 ± 0.18
	Week 12	7.39 ± 0.22	7.22 ± 0.26	3.75 ± 0.33	4.28 ± 0.22
Total n-3 FA [1, 3]	*Week 0*	10.71 ± 0.23	10.35 ± 0.24	10.30 ± 0.50	10.76 ± 0.21
(EPA+DPA+DHA)	*Week 6*	12.55 ± 0.35	12.56 ± 0.30	10.15 ± 0.58	10.85 ± 0.28
	Week 12	13.71 ± 0.33	13.45 ± 0.26	9.85 ± 0.50	10.92 ± 0.28

[1] Significant main effect of time, $P<0.05$

[2] Significant exercise x time interaction, $P<0.05$.

[3] Significant oil x time interaction, $P<0.05$.

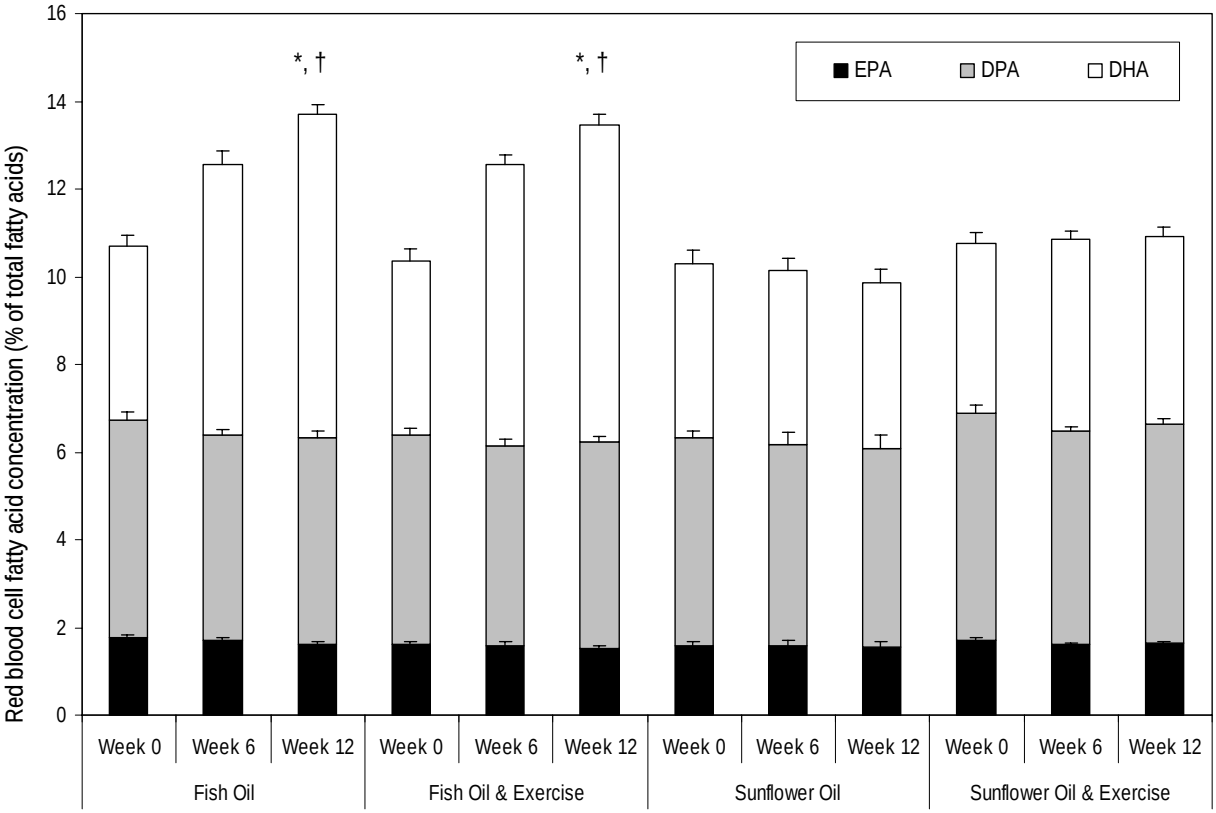

Figure 4.1 Erythrocyte fatty acid levels by treatment group. * Indicates DHA significantly different from Week 0 and sunflower oil groups at all time points, $P<0.0002$ (Tukey's post-hoc analysis). † Indicates total n-3 FA significantly different from Week 0 and sunflower oil groups at all time points, $P<0.0002$ (Tukey's post-hoc analysis).

4.4.2 Effects of n-3 FA and/or exercise on lipoprotein lipids

For all lipid parameters, one-way ANOVA did not detect any significant differences between intervention groups at Week 0 (**Table 4.2**). Changes in lipids were assessed using three-factor repeated-measures ANOVA. While ANOVA did not detect any significant 3-factor interactions (oil x exercise x time), it did show an effect of exercise (exercise x time, $P<0.014$) and oil (oil x time, $P<0.033$) on TC and HDL, respectively. Exercise was associated with a marginal increase in TC, possibly due to an increase in HDL, but post-hoc analysis failed to find any differences between means.

Fish oil significantly increased HDL (~10 %) compared with sunflower oil (~3% increase) (**Figure 4.2**, $P<0.05$). Changes in TAG were also observed, with maximum reductions achieved by Week 6, and maintained to Week 12. Thus changes in TAG were assessed by nested analysis (i.e. oil and exercise nested in time) and showed that fish oil supplementation reduced TAG (~14%) compared with sunflower oil (~5% increase) ($P = 0.03$) (**Figure 4.3**). For all subjects, the magnitude of change in TAG was associated with baseline (Week 0) TAG levels ($r = -0.4073$, $P = 0.001$) (**Figure 4.4**). An inverse correlation existed between TAG and HDL levels at baseline ($r = -0.51$, $P = 0.001$), and this association was still present at Week 12 ($r = -0.48$, $P = 0.001$), however no relationship existed between changes in HDL and changes in TAG ($r = 0.14$, $P = 0.27$). Regression analysis showed that the changes in both total long chain n-3 FA and DHA content in erythrocytes from Week 0 to Week 12 were correlated with the changes in HDL over this same period (n-3 FA; $r=0.377$, $P=0.012$, DHA; $r=0.322$, $P=0.033$) (**Figure 4.5**) but not with changes in TAG (n-3 FA; $r=-0.23$, $P=0.13$, DHA; $r=-0.20$, $P=0.19$). LDL remained unchanged following intervention.

Table 4.2 Plasma lipoprotein lipid concentrations (mmol/L) by treatment group.

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
HDL [1, 3]	*Week 0*	1.34 ± 0.09	1.37 ± 0.10	1.35 ± 0.12	1.18 ± 0.11
	Week 6	1.56 ± 0.14	1.50 ± 0.11	1.38 ± 0.15	1.22 ± 0.12
	Week 12	1.47 ± 0.13	1.53 ± 0.13	1.38 ± 0.13	1.19 ± 0.09
LDL	*Week 0*	4.31 ± 0.19	4.18 ± 0.31	4.32 ± 0.18	4.09 ± 0.25
	Week 6	4.57 ± 0.21	4.26 ± 0.37	3.95 ± 0.37	4.11 ± 0.22
	Week 12	3.87 ± 0.36	4.41 ± 0.18	3.94 ± 0.15	4.35 ± 0.27
TC [2]	*Week 0*	5.98 ± 0.21	5.94 ± 0.34	6.01 ± 0.23	5.65 ± 0.26
	Week 6	6.34 ± 0.20	5.97 ± 0.39	5.93 ± 0.33	5.70 ± 0.25
	Week 12	5.62 ± 0.36	6.27 ± 0.20	5.66 ± 0.22	5.92 ± 0.30
TAG [4]	*Week 0*	1.66 ± 0.18	1.93 ± 0.43	1.73 ± 0.20	1.88 ± 0.22
	Week 6	1.53 ± 0.19	1.52 ± 0.24	1.84 ± 0.18	1.82 ± 0.18
	Week 12	1.43 ± 0.18	1.62 ± 0.36	1.71 ± 0.16	1.88 ± 0.26

[1] Significant main effect of time, $P<0.05$

[2] Significant exercise x time interaction, $P<0.05$.

[3] Significant oil x time interaction, $P<0.05$.

[4] Significant oil treatment nested in time, $P<0.05$.

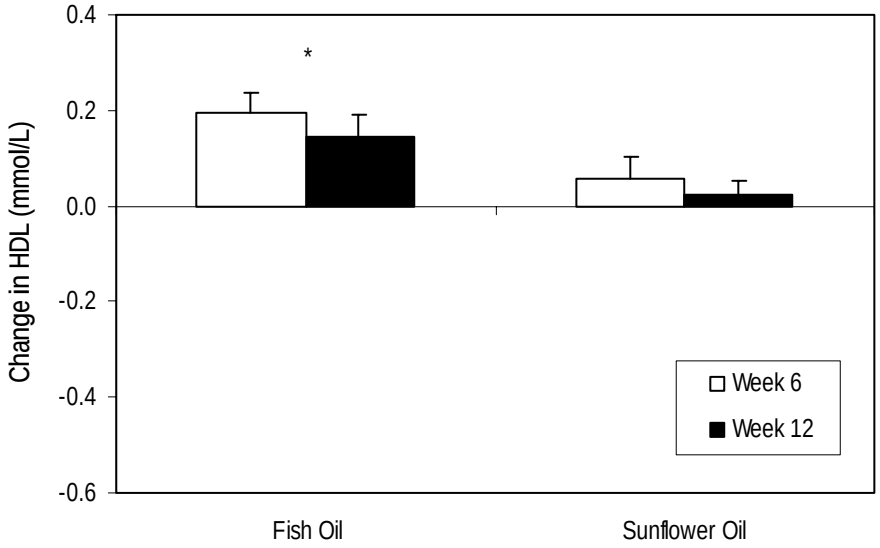

Figure 4.2 Change in HDL (mmol/L) by oil treatment. * Fish oil treatment significantly different from sunflower oil (two-factor oil x time interaction, $P<0.05$).

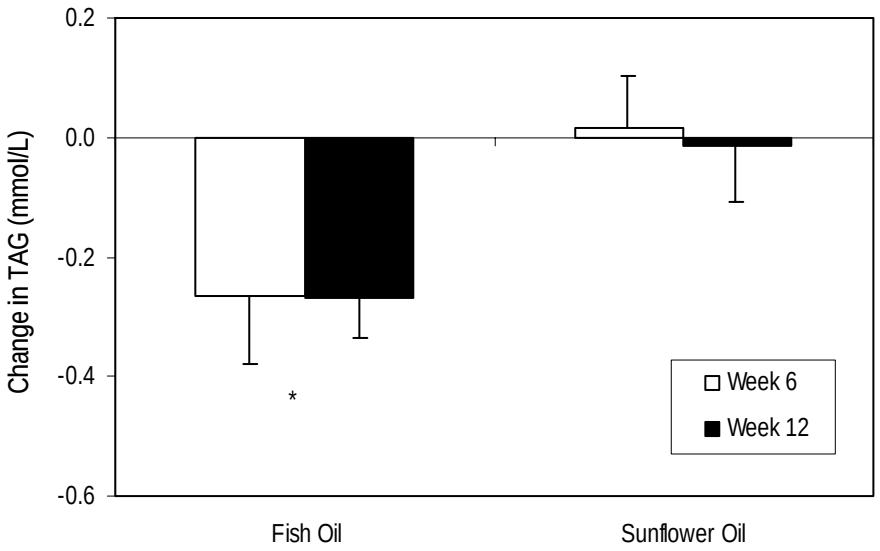

Figure 4.3 Change in TAG (mmol/L) by oil treatment. * Analysis of oil treatment nested in time showed a significant reduction in TAG with fish oil supplementation compared with sunflower oil, $P=0.03$.

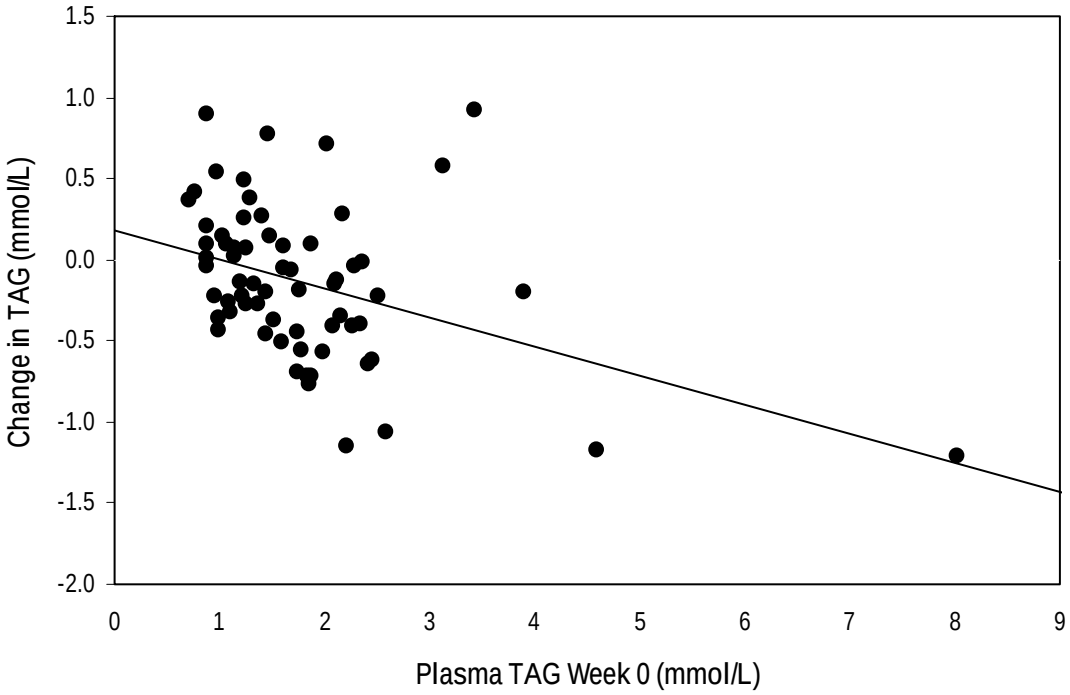

Figure 4.4 Relationship between change in TAG and baseline (Week 0) TAG levels (r = -0.4073, *P* = 0.001).

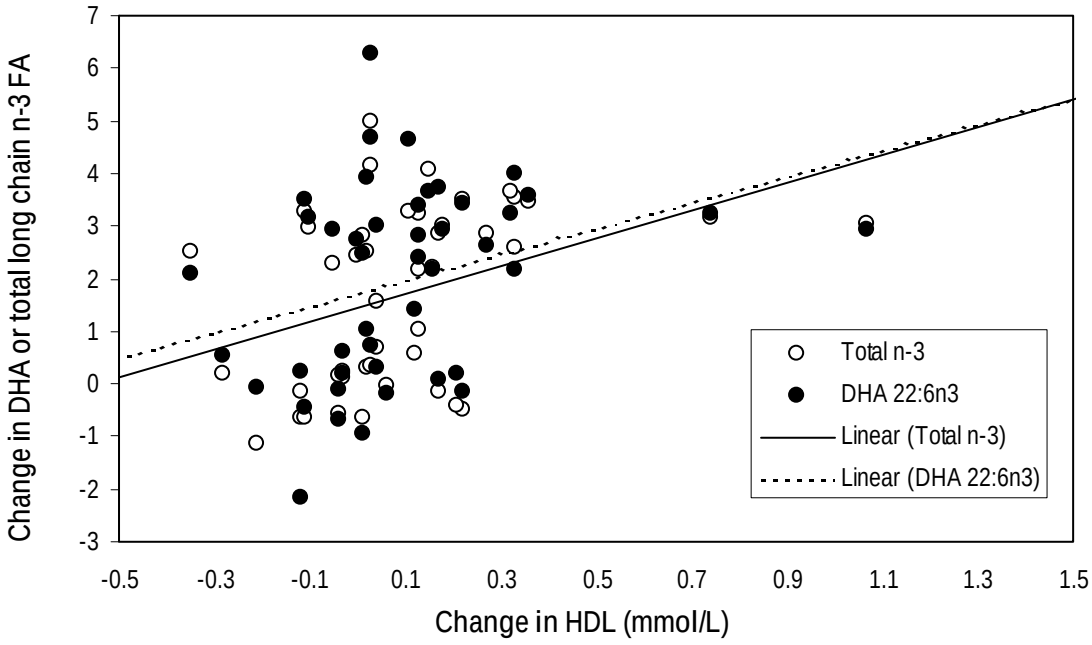

Figure 4.5 Relationship between changes in total long chain n-3 FA or DHA and HDL (total n-3 FA, r = 0.377, *P* = 0.012; DHA, r = 0.322, *P* = 0.033).

4.4.3 Effects of n-3 FA and/or exercise on glucose & insulin

There were no differences in plasma glucose or insulin markers between treatment groups prior to intervention (Week 0) (**Table 4.3**). ANOVA failed to detect any meaningful effects of treatment on glucose, insulin or measures of insulin sensitivity (Table 4.3). Across all subjects, there was a time dependent increase in plasma glucose concentration (main effect of time, $P<0.0023$), such that by Week 6 glucose concentration had increased ~0.2 mmol/L. However there were no further increases in glucose by Week 12. Significant exercises x oil interactions were detected for QUICKI ($P<0.028$) and beta-cell function ($P<0.033$), but post-hoc analysis failed to detect any differences between means for either parameter.

Table 4.3 Plasma glucose, serum insulin and measures of insulin sensitivity (HOMA, Beta cell function, QUICKI) by treatment group.

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
Glucose (mmol/L) [1]	*Week 0*	5.12 ± 0.20	5.06 ± 0.17	5.00 ± 0.11	4.79 ± 0.13
	Week 6	5.41 ± 0.22	5.16 ± 0.11	5.11 ± 0.12	5.08 ± 0.14
	Week 12	5.34 ± 0.23	5.08 ± 0.11	5.32 ± 0.10	5.00 ± 0.12
Insulin (uU/mL)	*Week 0*	16.16 ± 2.01	10.97 ± 1.16	14.39 ± 2.08	14.41 ± 2.23
	Week 6	17.98 ± 2.17	9.86 ± 0.81	15.17 ± 2.49	16.39 ± 2.21
	Week 12	16.23 ± 2.03	10.19 ± 0.69	14.46 ± 2.31	14.88 ± 1.71
HOMA	*Week 0*	3.78 ± 0.57	2.51 ± 0.32	3.24 ± 0.52	3.17 ± 0.62
	Week 6	4.42 ± 0.65	2.26 ± 0.19	3.46 ± 0.59	3.70 ± 0.55
	Week 12	4.01 ± 0.66	2.30 ± 0.17	3.44 ± 0.59	3.35 ± 0.44
Beta-cell Function (%) [2]	*Week 0*	225.33 ± 27.92	148.33 ± 18.18	203.48 ± 28.19	234.56 ± 24.81
	Week 6	208.70 ± 32.89	126.51 ± 12.80	203.47 ± 34.06	238.47 ± 37.40
	Week 12	198.44 ± 26.33	139.74 ± 13.97	167.97 ± 25.94	209.98 ± 24.56
QUICKI [2]	*Week 0*	0.32 ± 0.01	0.34 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
	Week 6	0.31 ± 0.01	0.34 ± 0.00	0.33 ± 0.01	0.32 ± 0.01
	Week 12	0.32 ± 0.01	0.34 ± 0.00	0.33 ± 0.01	0.32 ± 0.01

[1] Significant main effect of time, $P<0.05$

[2] Significant oil x exercise interaction, $P<0.05$.

4.5 DISCUSSION

4.5.1 Fatty acid composition of erythrocytes

Supplementation with DHA rich tuna oil substantially increased total long chain n-3 FA content in erythrocyte membranes. The change in total long chain n-3 FA was entirely due to a rise in DHA, which increased progressively during the 12-week intervention trial. This linear response relationship between dietary intake of DHA and incorporation into erythrocytes is well established (Brown *et al.*, 1991a), and indicates that subjects in this study were compliant with the intervention. Ninio et al. (Ninio *et al.*, 2005) demonstrated that the FA composition of erythrocytes reflects the FA levels in cardiac tissue and this may protect against stretch-related atrial fibrillation. Moreover, the Omega-3 Index (Harris & von Schacky, 2004) indicates that the greatest protection against CV mortality is with erythrocyte n-3 FA (EPA + DHA) contents >8%. In the present study, supplementation with fish oil increased erythrocyte EPA + DHA content to more than 8% by Week 12 of intervention.

4.5.2 Omega-3 FA induced changes in blood lipids

The present study confirmed the reported TAG lowering effect of fish oil. Harris (Harris, 1997) reviewed the hypotriglyceridaemic effects of n-3 FA in humans, and reported that intakes of ~4g/day decreased serum TAG by 25-30%, and that this reduction showed a dose-response relationship. The 14% reduction in TAG with ~1.9 g/day n-3 FA in this study fits well with the magnitude of TAG reduction one would expect based on data from the Harris review (Harris, 1997). We achieved a maximum TAG lowering effect by Week 6 of intervention, despite the continued incorporation of DHA into erythrocytes beyond this time point. Furthermore, we did not observe a significant relationship between changes in plasma TAG and incorporation of DHA into erythrocyte membranes, although this is consistent with some other studies (Bonaa *et al.*, 1992; Leigh-Firbank *et al.*, 2002; Geppert *et al.*, 2006).

While doses >3 g/day of n-3 FA appear to induce greater reductions in TAG (Schmidt *et al.*, 1990; Harris, 1996), lower doses have also shown to be effective (Agren *et al.*, 1996; Davidson *et al.*, 1997). Davidson et al. (Davidson *et al.*, 1997) supplemented hyperlipidaemic subjects with 1.52 or 2.50 g/day of DHA for 6 weeks and reported a 17-21% reduction in TAG, which was of similar magnitude in both groups. Agren et al. (Agren *et al.*, 1996) reported significant reduction in TAG with fish or fish oil (EPA+DHA and DHA-only) providing 2.28 g/day or less of n-3 FA in healthy males. Supplementation with low dose DHA (1.52 g/day) can also lower TAG in subjects with low HDL (Maki *et al.*, 2005). The TAG lowering effect of n-3 FA appears to be greatest in subjects with elevated TAG at baseline (Schmidt *et al.*, 1993). This trend has also been demonstrated in individuals with normal lipid profiles (Geppert *et al.*, 2006), and is confirmed in the present study.

The reduction in TAG seen with n-3 FA is due to several mechanisms including changes to FA availability, TAG synthesis and clearance. The function of n-3 FA as ligands for several nuclear receptors has been discussed in previous chapters. Briefly, following feeding with n-3 FA, the activity of several genes in liver, adipose tissue and skeletal muscle are altered such that they favour TAG oxidation over storage (Power & Newsholme, 1997; Flachs *et al.*, 2005). Supplementation with n-3 FA inhibits hepatic TAG synthesis and secretion, and decreases VLDL production (Kinsella *et al.*, 1990b).

EPA and DHA appear to have similar TAG lowering effects, but may alter HDL sub-fractions differently; DHA increases HDL_2 while EPA decreases HDL_3. In this study, we observed an increase in total HDL, possibly due to a decrease in lipid transfer protein activity (Abbey *et al.*, 1990) and an increase in the activity of LPL (Davidson, 2006). Based on the evidence of Mori et al. (Mori & Woodman, 2006) we can speculate that the changes in HDL seen in this study oil may reflect an increase in HDL_2 as subjects were supplemented with a DHA-rich fish. In contrast to other studies (as reviewed by Harris (Harris, 1997)) that have reported an increase in LDL, we did not observe any changes in total or LDL cholesterol.

4.5.3 Exercise induced changes in blood lipids

Regular moderate intensity exercise either alone or in addition to fish oil supplementation, failed to induce any change in TAG, HDL or LDL in this study. Exercise did however produce a small increase in TC, although this effect was not of sufficient magnitude to be detected by post-hoc analysis. These results are in disagreement to those reported in several meta-analyses (Halbert *et al.*, 1999; Durstine *et al.*, 2001; Leon & Sanchez, 2001; Carroll & Dudfield, 2004; Kelley *et al.*, 2005). For instance Carroll & Dudfield (Carroll & Dudfield, 2004) reported that regular aerobic exercise was effective in reducing TAG (12%) and increasing HDL (4.1%) in overweight/obese, sedentary adults with symptoms of dyslipidemia. Exercise frequency was 3-5 sessions per week, for 30-60 minutes with a median energy expenditure of 1600 kcal/week, indicating that most subjects exercised at the higher end of this range. In comparison, exercising subjects in this study would have expended approximately 1090 – 1310 kcal / week (based on equations from Warburton et al. (Warburton *et al.*, 2006)). It is therefore possible that the training stimulus used in this study was not of sufficient magnitude to induce a change in these lipid parameters. Furthermore, not all subjects in this study had dyslipidemia, a characteristic that may produce most beneficial results.

4.5.4 Combined n-3 FA & exercise effects on blood lipid profiles

To date, only five trials have investigated the effect of n-3 FA supplementation, without additional dietary modifications, in combination with an acute exercise bout (Thomas *et al.*, 2000; Smith *et al.*, 2004; Thomas *et al.*, 2004) or aerobic exercise training (Warner *et al.*, 1989; Brilla & Landerholm, 1990) on blood lipids. These acute exercise studies were limited to assessing effects on LDL and HDL subfractions (Thomas *et al.*, 2004) and post-prandial lipemia (PPL) in healthy sedentary (Thomas *et al.*, 2000) and recreationally active (Smith *et al.*, 2004) males. Thomas et al (Thomas *et al.*, 2004) reported that the combination of n-3 FA supplementation and acute exercise increased total HDL beyond exercise alone,

and this combination was also shown to be effective in reducing PPL response in active (Smith *et al.*, 2004) but not sedentary individuals (Thomas *et al.*, 2000).

Two studies have investigated the effects of aerobic exercise training in combination with n-3 FA from fish and/or fish oil on blood lipids (Warner *et al.*, 1989; Brilla & Landerholm, 1990) (see table 1.8 for details). Warner and colleagues (Warner *et al.*, 1989) reported that their combined intervention (fish oil and exercise) and fish oil groups showed similar changes in TAG and HDL, but serum LDL and apolipoprotein B concentrations were reduced more following the combined intervention than with fish oil supplementation alone. Contrary to Warner et al. (Warner *et al.*, 1989), Brilla & Landerholm (Brilla & Landerholm, 1990) reported that fish / fish oil supplementation had no effect on HDL, LDL, TC or TAG in young adults.

4.5.5 Omega-3 FA induced changes in glucose and insulin

Animal models have consistently demonstrated the beneficial effects of n-3 FA on insulin resistance (Lombardo & Chicco, 2006), and have been used to elucidate the corresponding mechanisms, which may relate to changes in insulin action and/or sensitivity. However these benefits do not appear to translate to humans.

We did not observe any changes in plasma glucose concentration following intervention with n-3 FA. Similarly, Giacco et al. (Giacco *et al.*2006) reported that fish oil supplementation (3.6 g/day n-3 FA) did not change glucose tolerance, insulin sensitivity or secretion, or beta-cell function in healthy, non-diabetic adults. Furthermore, in a similar study population to our own, 12-weeks supplementation with 1.3-2.6 g/day n-3 FA did not alter fasting plasma glucose or insulin (Vandongen *et al.*, 1993). It is therefore not surprising that we did not observe any changes in fasting plasma glucose and insulin concentration or insulin resistance (as predicted by HOMA) following n-3 FA supplementation in this study.

4.5.6 Exercise induced changes in glucose and insulin

An acute bout of exercise reduces plasma glucose and increases insulin action, effects which are sustained into the post-exercise period (Albright *et al.*, 2000). The collective effect of several bouts of acute exercise (i.e. exercise training) may therefore perpetuate these metabolic improvements and likely explain the benefits shown in several intervention trials (Lamarche *et al.*, 1992; Cox *et al.*, 1999; Boule *et al.*, 2005; Bruce *et al.*, 2006; Weiss *et al.*, 2006). However, the exercise intervention in this study did not induce any changes in fasting plasma glucose and insulin concentration or insulin resistance (as predicted by the homeostasis model assessment). Houmard et al. (Houmard *et al.*, 2004) reported that ~170 min/wk of exercise resulted in greater improvements in insulin sensitivity than 115 min/week in sedentary overweight/obese individuals, although fasting plasma glucose did not change with either exercise prescription. Subjects in the current trial were prescribed 135 min/week. It is possible that the exercise prescription was not of sufficient magnitude to elicit changes in insulin sensitivity. Furthermore, many of the studies investigating changes in insulin sensitivity and glucose tolerance have used intravenous or oral administration of glucose or the euglycemic insulin clamp technique. While these methods each have several limitations, they are superior to fasting plasma glucose and insulin in their ability to calculate insulin sensitivity (Monzillo & Hamdy, 2003). These methodological differences may contribute to the discrepancies between studies.

4.5.7 Combined n-3 FA & exercise effects on glucose and insulin

Delarue et al. (Delarue *et al.*, 2003) investigated whether fish oil supplementation (1.83 g/day n-3 FA for 20 days) could alter fuel selection during exercise. Omega-3 FA supplementation caused a reduction in both the rate of plasma glucose disappearance and hepatic glucose production during exercise, and although not significant, there were trends toward an increase in fat oxidation with a concomitant reduction in carbohydrate oxidation. An increase in lipid oxidation may eventually lead to a decrease in insulin resistance. Dunstan

et al (Dunstan *et al.*, 1997) investigated the effect of an 8-week low fat diet (30% daily energy intake) with or without a daily fish meal (3.6 g/day n-3 FA) and low or moderate exercise on glycaemic control in dyslipidaemic, diabetic subjects. While fish intake increased HbA_{1c} and self-monitored glucose, these effects were attenuated by moderate exercise. These two studies provide additional support for combined diet and exercise interventions to ameliorate CV risk.

4.5.8 Summary and conclusion

In summary, this study confirms that regular supplementation with a moderate dose of DHA rich fish oil can reduce plasma TAG and increase HDL, whereas regular moderate intensity exercise either alone or in addition to n-3 FA supplementation, had no effect on these lipid parameters. We suggest that the exercise prescription in this study was not of sufficient magnitude to elicit such changes in TAG and HDL. Omega-3 FA mediated reductions in TAG are dose dependent and the magnitude of change appears to be influenced by baseline concentrations before intervention. Furthermore, n-3 FA, exercise or the combined intervention did not alter TC or LDL. A limitation of this study was that we did not measure HDL and LDL sub-fractions, which may be altered by both n-3 FA and exercise. As demonstrated in other studies, we found no effect of DHA-rich fish oil on fasting plasma glucose or insulin, or associated predictions of insulin sensitivity. In comparison, exercise is often associated with an improvement in glucose tolerance and insulin sensitivity, although this was not supported by the current research. Again, we suggest that this is due to the modest exercise intervention prescribed, but may also reflect methodological differences in the calculation of insulin sensitivity.

5 ARTERIAL FUNCTION

5.1 INTRODUCTION

The endothelium has several key functions, which are critical for maintaining vascular homeostasis. These include non-selective ion permeability, the regulation of vascular tone and structure, thrombosis and thrombolysis, prevention of leukocyte and platelet adhesion and secretion of vasodilatory (e.g. NO) and vasoconstrictor (e.g. E-1) mediators (Celermajer, 1997; Landmesser *et al.*, 2004). Endothelial injury and dysfunction, as seen in several disease states such as hypertension and hypercholesterolemia (Landmesser *et al.*, 2004), can disrupt haemostasis and thereby contributes to the progression of atherosclerosis (Toborek & Kaiser, 1999).

Injury to the endothelium can result in adhesion of leukocytes, vasoconstriction, lipid deposition, smooth muscle cell migration and proliferation, and a decrease in NO bioactivity (Cohn, 2001). These and other extrinsic factors (e.g. hypertension, hyperlipidaemia, hormone and glucose regulation) can lead to structural remodelling of the artery, and arterial stiffness (Zieman *et al.*, 2005). Arterial stiffness is an independent predictor of CV events and a risk factor for CVD (Laurent *et al.*, 2001; van Popele *et al.*, 2001; Boutouyrie *et al.*, 2002; Kingwell *et al.*, 2002; Grey *et al.*, 2003; Oliver & Webb, 2003), and can be assessed non-invasively by pulse contour analysis. This technique assesses conductance / conduit (coronary) and resistant (peripheral) artery compliance, based on a modified Windkessel model of the human vascular system (Cohn, 2001).

The technique of FMD is an assessment of shear stress induced changes in conduit artery diameter, typically assessed in the brachial artery. The change in artery diameter is suggested to be due to endothelial release of NO. Following short-term occlusion of the forearm, reperfusion initiates an increase in blood flow through the brachial artery, which causes an increase in shear stress that stimulates the release of NO from endothelial cells (Joannides *et al.*, 1995). FMD in the brachial artery is closely related to coronary endothelial function (Anderson *et al.*, 1995) and is a predictor of CV events in at risk populations (Gokce *et al.*, 2002; Gokce *et al.*, 2003). In addition to vasodilatation, NO is an independent inhibitor of platelet activity, leukocyte adhesion and smooth muscle cell proliferation (Moncada & Higgs, 2006). Consequently, a change in NO bioactivity can contribute to the development of arterial stiffness.

Palmieri et al. (Palmieri *et al.*, 2005) recently demonstrated an association between greater FMD and higher sub-maximal and maximal aerobic performance. During exercise vasodilatation increases dramatically, thereby increasing blood flow and transportation of oxygen, which facilitates aerobic performance. An increase in blood flow will also have implications for delivery of dietary fats and other nutrients to sites of metabolism.

5.2 AIM

To determine the independent and combined effects of n-3 FA supplementation and regular moderate exercise on arterial function and BP in subjects with risk factors for MS.

5.3 METHODS

All clinical assessments were taken under fasting conditions (10-12 hr) and where possible, pre- and post-intervention measures were performed at similar times of the day.

5.3.1 FMD and Glyceryl-Trinitrate Mediated Dilatation (GTN-D)

To ensure relaxation, subjects lay supine for a minimum of 10 minutes in a dimly lit room prior to beginning any assessments. Endothelial function was assessed using FMD via ultrasound (Corretti *et al.*, 2002). For the production of reactive hyperemia, a sphygmomanometer cuff was placed around the mid-point of the forearm (i.e. distal to the scanned part of the artery) and inflated to a pressure of 200 mmHg for 5 minutes. Images of the artery were taken prior to cuff inflation, 10 seconds prior to cuff release, 10 seconds post cuff release and then every 30 sec for an additional 3 min. For the assessment of endothelium-independent vasodilatation, 300μg of glyceryl trinitrate (GTN: Anginine™, Sigma Pharmaceuticals Pty Ltd, VIC, Australia) was administered sublingually following recording of baseline images. One minute after GTN administration, the first image was taken. Images were then taken every minute for 10 min.

Artery images were acquired by a single operator using two dimensional B-mode ultrasound (LOGIQ 5, GE Medical Systems, Wisconsin, USA) with a high frequency transducer (12 MHz) placed 5-10 cm above the elbow on the right arm. Optimal imaging of the artery has been described by Raitakari & Celermajer (Raitakari & Celermajer, 2000). Images were recorded and stored as 3-second video loops, and the video frame that corresponded with end-diastole was used to assess arterial diameter. At each time point, the same landmark along the artery wall was used as reference from which measurements were made. Arterial diameter was measured using digital calipers (LOGIQ 5 1.1X software GE Medical Systems, Wisconsin, USA) and was determined to be the maximum perpendicular distance between the intima (**Figure 5.1**). FMD and GTN-D responses were calculated as the maximal artery diameter (post occlusion or GTN administration, respectively) minus baseline diameter. Previous studies within our laboratory have produced an intra-operator reliability of 5.0% for FMD and 7.1% for GTN-D.

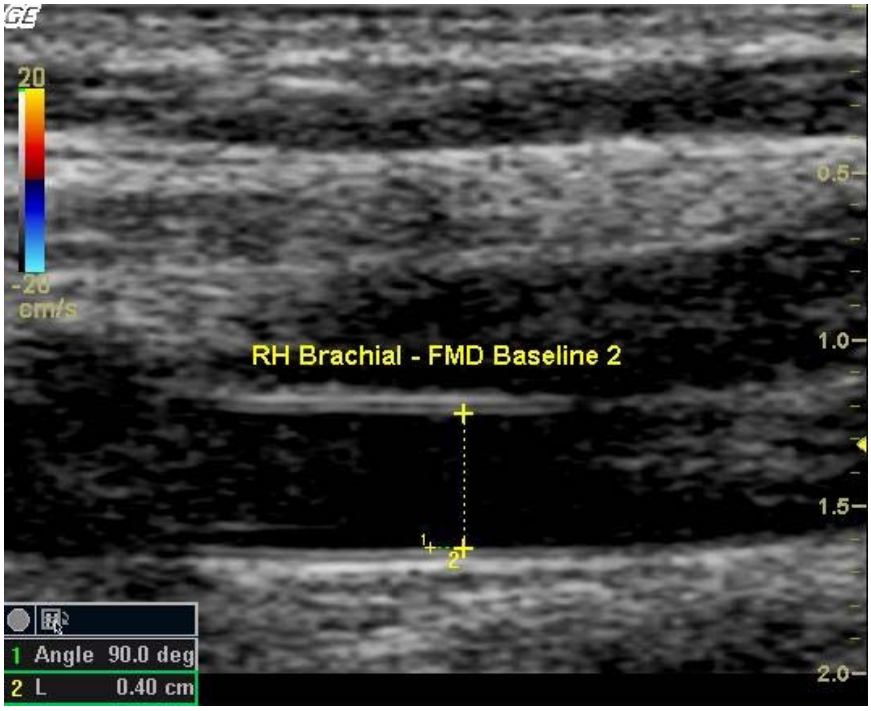

Figure 5.1 Analysis of arterial diameter using digital calipers.

Diameter was determined to be the maximum perpendicular distance between the intima.

5.3.2 Arterial Compliance and BP

BP and compliance of large and small arteries were assessed using the HDI/*Pulsewave* CR-2000 Cardiovascular Profiler (Hypertension Diagnostics Inc. (HDI), Eagan, USA) with subjects in a supine position following a minimum 10-minute rest period. This technique involves the placement of a small pressure transducer over the radial pulse and the simultaneous application of a BP cuff on the contra lateral upper arm. The waveforms obtained via the transducer are calibrated to systolic and diastolic pressure (**Figure 5.2**). A computed Windkessel model of the human vascular system (**Figure 5.3**) is applied to determine central (C_1; large) and peripheral (C_2; small) arterial compliance (Cohn, 2001), and systemic vascular resistance (SVR; calculated as mean arterial pressure / cardiac output) during the diastolic decay portion of the cardiac cycle. Total vascular impedance (TVI) is calculated as the impedance function of the modified Windkessel model, which incorporates values for C_1; C_2 and SVR (HDI Hypertension Diagnostics, 2001).

Hypertension Diagnostics Inc. (Eagan, USA) has defined arterial elasticity reference values for normotensive and hypertensive individuals (data were obtained from a prospective, controlled clinical trial that screened for hypertension). Average elasticity values for C_1 (large artery compliance, LAC) and C_2 (small artery compliance, SAC) vessels in normotensive individuals are 16.1 ± 6.1 and 7.0 ± 3.0, respectively. However, it is well established that arterial compliance decreases with increasing age. Zimlichman et al. (Zimlichman *et al.*, 2005) recently published arterial compliance values specific to age and gender. The average age of the male and female participants (n=65) in the present study was 50.2 ± 1.0 yrs. Average values are therefore estimated to be >13.6 for LAC and >5.4 for SAC.

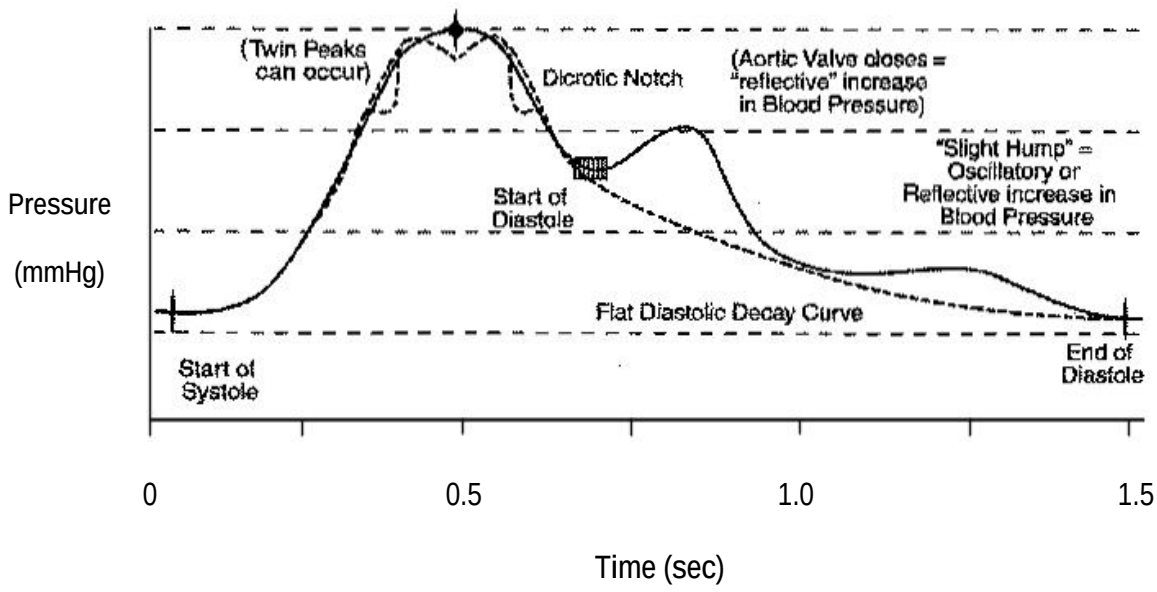

Figure 5.2 Schematic diagram of certain key features of the arterial BP waveform used to determine arterial compliance.

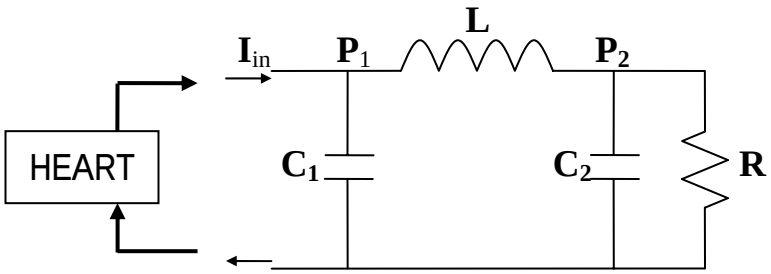

Figure 5.3 Schematic diagram of the modified Windkessel model. This model is used to determine C_1, C_2; and systemic vascular resistance.

Definitions:

P_1 – Proximal Arterial Pressure

P_2 – Distal Arterial Pressure

C_1 – Large Artery Elasticity Index (capacitive arterial compliance)

C_2 – Small Artery Elasticity Index (oscillatory or reflective arterial compliance)

L – Inertance of the Blood

R – Systemic Vascular Resistance

I_{in} – Flow into the arterial system during systole

I_{in} = 0 (or no flow) during diastole

5.4 RESULTS

Sixty-five subjects completed the assessments for BP, arterial compliance and FMD. GTN-D was assessed in 62 subjects; one subject refused GTN administration at Week 12 and images of sufficient quality were not acquired on two subjects.

5.4.1 Effects of n-3 FA and/or exercise on endothelial-dependent & -independent dilatation

FMD and GTN-D dilatation were used to measure endothelial-dependent and total vasodilatation respectively. Changes in arterial diameter induced by this technique were reported as absolute changes or calculated as percent changes from baseline diameter. There were no differences between groups at baseline for resting brachial artery diameter (P=0.142), percent (P=0.59) or absolute (P=0.41) FMD (with baseline diameter included as a covariate)

or GTN-D (percent, *P*=0.47; absolute, *P*=0.74) (**Table 5.1**). Three-factor ANOVA detected a significant oil x exercise x time interaction for baseline diameter (**Figure 5.4**, *P*=0.005). Post-hoc analysis showed multiple significant differences between groups at various time points (Weeks 0, 6 and 12). These differences were likely due to slight changes in the location of the artery image between visits. This supports the inclusion of resting brachial artery diameter as a covariate for determination of changes in FMD as the magnitude of the dilatory response to FMD is reported to be inversely related to the resting brachial artery diameter (Pyke *et al.*, 2004). To determine whether this relationship was present in the current study population, baseline diameter at each visit (Weeks 0, 6 and 12) was correlated with % change in diameter as measured by FMD. Baseline diameter was inversely related to peak FMD response (**Figure 5.5**; r = 0.273, *P* = 0.000).

A significant two-factor oil x time interaction was observed for FMD (absolute change) when resting brachial artery diameter at each of Weeks 0, 6 and 12 was used as a changing covariate (**Figure 5.6**, *P*=0.022). Post-hoc analysis showed a significant improvement in FMD with fish oil by Week 12 (*P*=0.008). Significance remained when expressed as a percentage (**Figure 5.7**; oil x time interaction, *P*=0.05), using resting brachial artery diameter as a changing covariate. There were no effects of exercise, oil or the combined treatment on GTN-D.

Table 5.1 Endothelial-dependent and-independent measures of vascular function by treatment group.

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
Baseline Diameter (cm) [2]	*Week 0*	0.385 ± 0.018	0.401 ± 0.020	0.370 ± 0.011	0.425 ± 0.017
	Week 6	0.357 ± 0.017	0.377 ± 0.020	0.349 ± 0.013	0.376 ± 0.019
	Week 12	0.368 ± 0.017	0.399 ± 0.019	0.367 ± 0.014	0.383 ± 0.020
FMD (%)	*Week 0*	3.70 ± 0.76	3.85 ± 0.71	4.82 ± 0.60	4.64 ± 0.96
	Week 6	6.22 ± 0.86	5.35 ± 0.48	6.25 ± 0.58	4.54 ± 0.72
	Week 12	6.57 ± 0.77	5.29 ± 0.56	5.27 ± 0.84	5.39 ± 0.66
FMD (cm) [1]	*Week 0*	0.013 ± 0.003	0.015 ± 0.003	0.018 ± 0.002	0.020 ± 0.004
	Week 6	0.021 ± 0.003	0.020 ± 0.002	0.021 ± 0.002	0.017 ± 0.003
	Week 12	0.024 ± 0.003	0.021 ± 0.002	0.019 ± 0.003	0.020 ± 0.002
GTN-D (%)	*Week 0*	22.631 ± 1.45	22.04 ± 1.44	25.06 ± 1.90	21.85 ± 1.69
	Week 6	24.44 ± 1.72	23.76 ± 1.95	28.40 ± 1.51	23.35 ± 2.08
	Week 12	22.59 ± 1.53	22.07 ± 1.51	23.92 ± 1.33	23.46 ± 1.44
GTN (cm)	*Week 0*	0.083 ± 0.003	0.085 ± 0.005	0.090 ± 0.007	0.084 ± 0.004
	Week 6	0.084 ± 0.005	0.084 ± 0.005	0.096 ± 0.004	0.085 ± 0.006
	Week 12	0.081 ± 0.004	0.086 ± 0.005	0.085 ± 0.004	0.087 ± 0.005

[1] Significant oil x time interaction, $P<0.05$.

[2] Significant oil x exercise x time interaction, $P<0.05$.

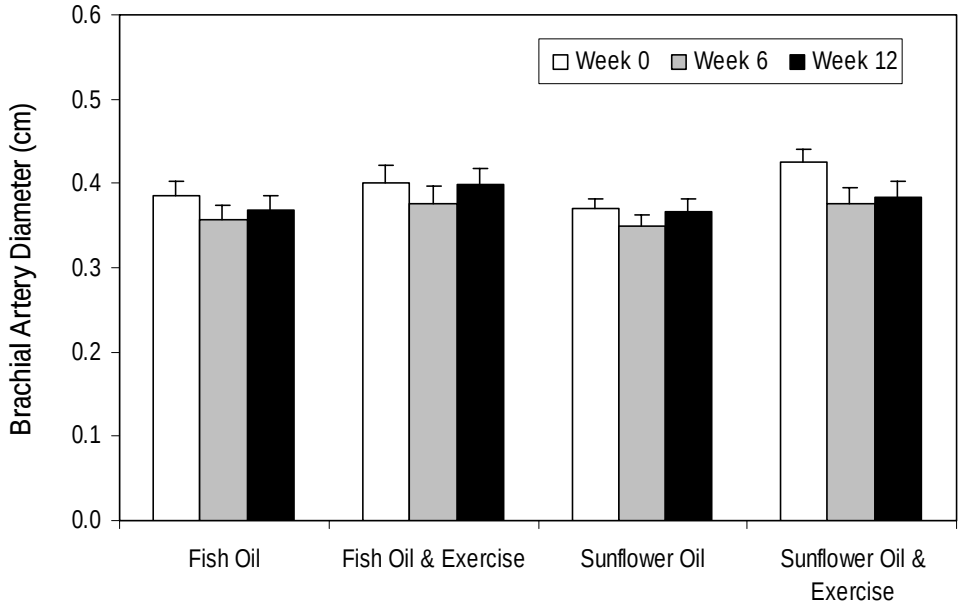

Figure 5.4 Ultrasound assessed resting brachial artery diameter measured at 0, 6 and 12 Weeks of intervention. A significant oil x exercise x time interaction was detected, $P=0.005$.

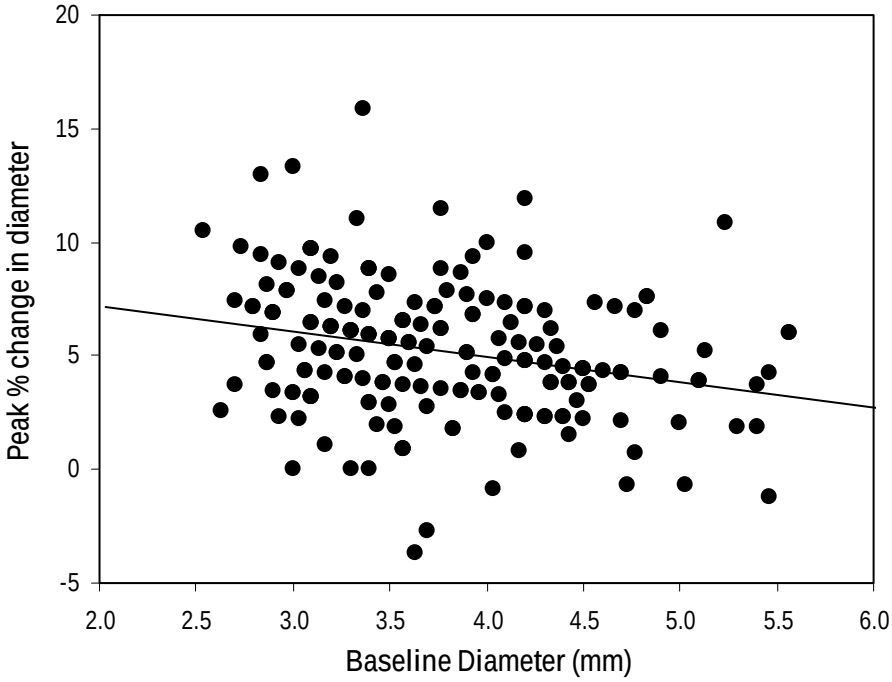

Figure 5.5 Relationship between baseline brachial artery diameter and peak FMD response (r = 0.273, $P < 0.001$).

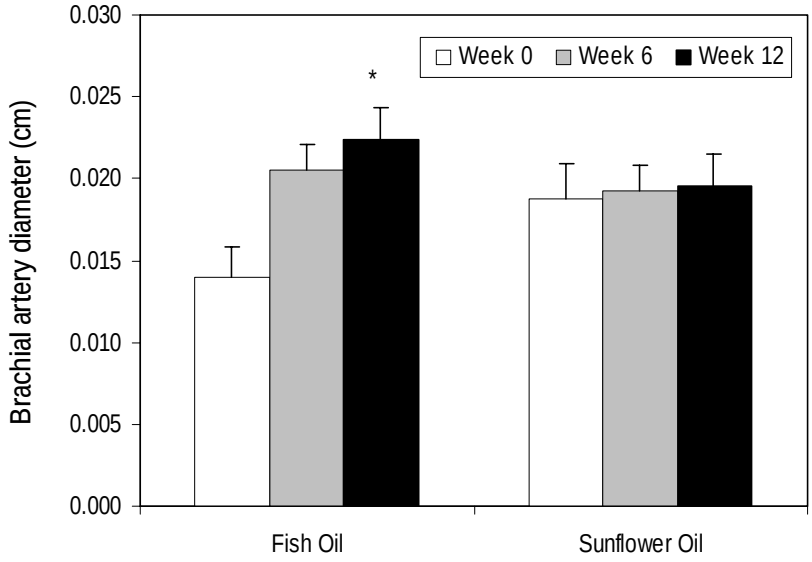

Figure 5.6 FMD mediated absolute change in brachial artery diameter (cm) following intervention with DHA rich fish oil or sunflower oil. *Diameter increased significantly by Week 12 in the fish oil supplemented group, (oil x time interaction, P=0.022).

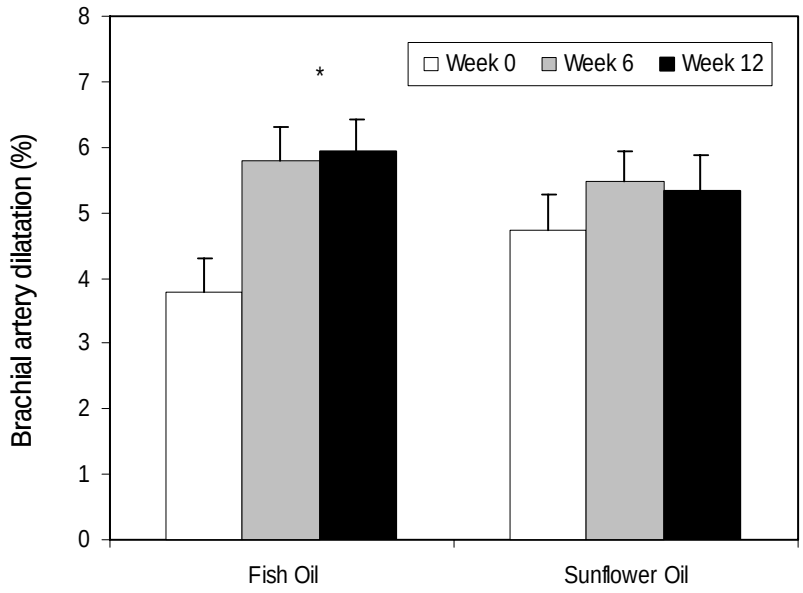

Figure 5.7 FMD mediated percent change in brachial artery diameter following intervention with DHA rich fish oil or sunflower oil. *Diameter increased significantly by Week 12 in the fish oil supplemented group (ANCOVA; oil x time interaction, *P*=0.05).

5.4.2 Effects of n-3 FA and/or exercise on measures of arterial compliance

There were no differences between groups at baseline for any measures of arterial compliance (**Table 5.2**). Three-factor ANOVA detected a significant main effect of time for systemic vascular resistance (*P*<0.006), which was increased at Week 6 before dropping to below baseline levels at Week 12. Neither LAC nor TVI were improved by oil, exercise, or the combined intervention. Regression analysis showed that changes in LAC were associated with changes in resting HR (r = -0.287, *P* = 0.023). A significant two-factor exercise x time interaction was detected for SAC (**Figure 5.8**; *P*=0.041). Post-hoc analysis showed a significant increase in SAC in subjects who undertook the exercise training program (*P*=0.05), with exercise increasing SAC by 26 ± 8% compared with 1 ± 4% in the non-exercise group.

Table 5.2 Measures of arterial compliance by treatment group.

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
LAC (ml/mmHg x 10)	*Week 0*	15.7 ± 0.8	16.3 ± 1.0	16.6 ± 1.0	16.3 ± 1.0
	Week 6	16.5 ± 1.1	16.6 ± 1.0	15.9 ± 0.9	16.8 ± 1.1
	Week 12	17.2 ± 0.8	16.8 ± 1.0	15.8 ± 0.8	16.5 ± 1.2
SAC (ml/mmHg x 100) [2]	*Week 0*	7.8 ± 0.8	7.3 ± 0.9	6.9 ± 0.8	7.7 ± 1.1
	Week 6	7.5 ± 1.0	7.7 ± 0.8	7.3 ± 1.0	8.1 ± 0.8
	Week 12	7.9 ± 0.8	8.7 ± 1.0	6.4 ± 0.7	9.0 ± 0.8
SVR (dyne•sec•cm^{-5}) [1]	*Week 0*	1309.9 ± 60.2	1333.1 ± 54.7	1280.3 ± 54.5	1335.3 ± 42.2
	Week 6	1317.6 ± 53.8	1392.2 ± 50.1	1315.4 ± 66.2	1306.8 ± 38.4
	Week 12	1238.5 ± 58.8	1311.9 ± 60.1	1267.7 ± 55.0	1297.6 ± 34.1
TVI (dyne•sec•cm^{-5})	*Week 0*	132.2 ± 6.8	128.9 ± 7.4	131.1 ± 7.8	129.6 ± 8.4
	Week 6	130.6 ± 7.6	133.0 ± 5.7	137.2 ± 9.2	127.5 ± 8.8
	Week 12	123.2 ± 5.3	130.8 ± 6.6	129.6 ± 5.7	129.8 ± 8.3

[1] Significant time effect, $P<0.05$.

[2] Significant exercise x time interaction, $P<0.05$.

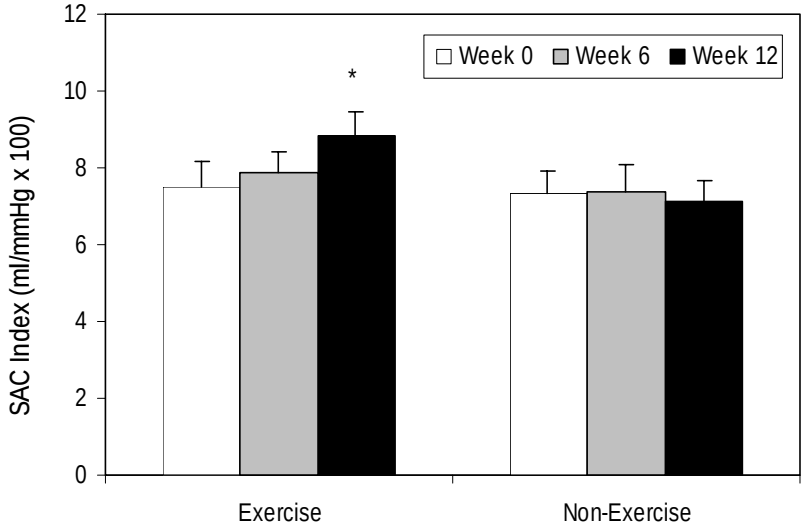

Figure 5.8 Effect of exercise training or no exercise on SAC. *SAC increased significantly following exercise training, two-factor exercise x time interaction, $P<0.05$.

5.4.3 Effects of n-3 FA and/or exercise on BP

There were no differences between treatment groups for SBP, DBP, mean arterial pressure (MAP), or pulse pressure (PP) at baseline (P>0.34). Three-factor ANOVA detected significant main effects of time for SBP (P<0.0007), DBP (P<0.0009), MAP (P<0.0001) and PP (P<0.049). While all of these parameters decreased during the intervention, there were no significant effects of treatment (oil, exercise or the combination) for SBP, DBP or MAP (P>0.20). A significant oil x exercise x time interaction was detected for PP (P=0.028), although post-hoc analysis failed to detect any meaningful differences (**Table 5.3**). **Figure 5.9** shows the mean changes in SBP and DBP for each treatment group by Week 12 of intervention.

Table 5.3 Measures of blood pressure by treatment group. [1]

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
SBP (mmHg) [1]	*Week 0*	128.8 ± 3.5	131.9 ± 4.3	128.0 ± 3.1	132.4 ± 2.7
	Week 6	128.8 ± 3.4	134.2 ± 4.6	130.1 ± 3.4	130.2 ± 3.3
	Week 12	123.7 ± 3.3	126.1 ± 3.8	126.2 ± 3.4	128.5 ± 3.3
DBP (mmHg) [1]	*Week 0*	72.5 ± 2.5	77.6 ± 2.8	72.6 ± 1.8	75.9 ± 2.3
	Week 6	73.9 ± 2.1	78.8 ± 3.1	72.5 ± 1.9	76.0 ± 2.7
	Week 12	71.2 ± 2.3	73.0 ± 2.9	71.4 ± 2.1	73.2 ± 2.4
MAP (mmHg) [1]	*Week 0*	93.1 ± 2.8	96.5 ± 3.2	91.5 ± 2.9	94.5 ± 2.5
	Week 6	93.7 ± 2.7	98.7 ± 3.7	93.2 ± 2.7	93.7 ± 2.7
	Week 12	90.1 ± 3.2	91.9 ± 3.2	89.6 ± 2.5	91.8 ± 2.6
PP (mmHg) [1,2]	*Week 0*	56.4 ± 1.9	54.2 ± 2.0	55.5 ± 1.8	56.5 ± 2.1
	Week 6	54.9 ± 2.0	55.5 ± 2.0	57.7 ± 2.2	54.2 ± 1.8
	Week 12	52.5 ± 1.7	53.1 ± 1.8	55.1 ± 1.7	55.4 ± 2.3

[1] Significant time effect, P<0.05.

[2] Significant exercise x oil x time interaction, P<0.05.

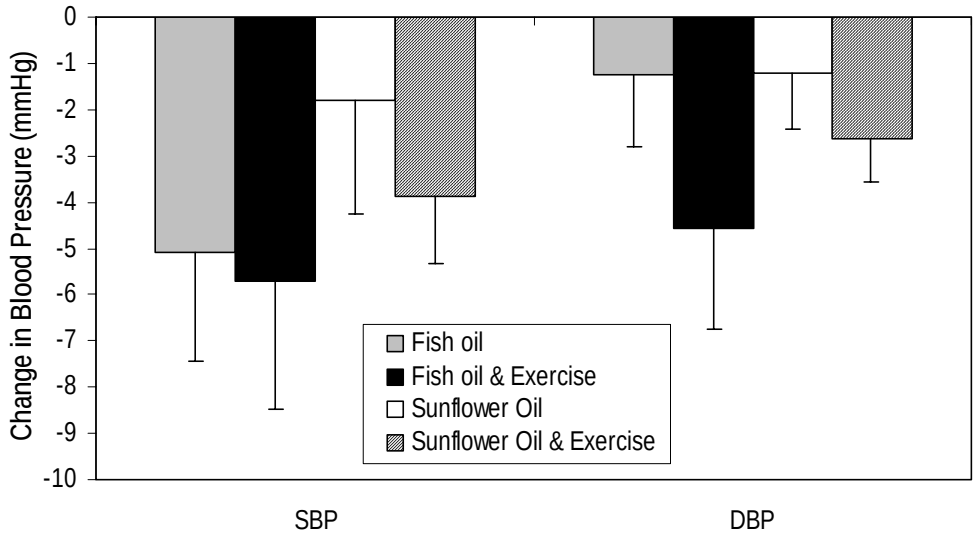

Figure 5.9 Change in SBP and DBP by treatment group.

5.5 DISCUSSION

5.5.1 Omega-3 FA induced changes in arterial function

Several studies have demonstrated that n-3 FA can independently improve arterial function (McVeigh *et al.*, 1993; Tagawa *et al.*, 1999; Goodfellow *et al.*, 2000; Mori *et al.*, 2000b; Okumura *et al.*, 2002; Khan *et al.*, 2003; Engler *et al.*, 2004), however FMD assessed changes have been inconsistent. The inconsistency between studies may be due to differences in study populations. Woodman et al. (Woodman *et al.*, 2003a) suggested that medication use and state of insulin resistance might have prevented improvements in vascular function in their diabetic subjects. Similarly, n-3 FA may be limited in their effects in healthy individuals (Yosefy *et al.*, 2003; Dyerberg *et al.*, 2004). This study provides additional evidence for a positive effect of n-3 FA on endothelial-dependent dilatation (Goodfellow *et al.*, 2000; Engler *et al.*, 2004).

The improvement in endothelial function seen in the current study is likely due to increased endothelial production of NO (Harris *et al.*, 1997) and/or vasodilatory eicosanoids (James *et al.*, 2000). The ratio of AA to EPA determines which series of eicosanoids are produced in the greatest abundance. A ratio favouring higher concentration of AA will result in increased synthesis of the pro-inflammatory PGE_2, LTB_4 and pro-aggregatory TXA_2, whereas a high concentration of EPA will result in increased synthesis of the less inflammatory PGE_3, LTB_5 and TXA_3 (James *et al.*, 2000). Both AA and EPA also determine which series of prostacylin is produced; AA produces PGI_2, EPA produces PGI_3. Both series are potent anti-aggregatory and vasodilatory agents and thereby counteract the activity of TXA_2 and LTB_4 (Kinsella *et al.*, 1990b). Supplementation with n-3 FA selectively reduces TXA_2 with minimal effect on PGI_2 production (von Schacky *et al.*, 1985), resulting in a net increase in PGI. Kelley et al. (Kelley *et al.*, 1999) reported a reduction in LTB_4 and PGE_2 from stimulated human peripheral blood mononuclear cells (PBMC) following feeding with DHA, although it is unclear whether this is a direct action of DHA or due to retroconversion of DHA to EPA. DHA also inhibits TXA_2 synthetase (Abeywardena *et al.*, 1991). Supplementation with fish oil therefore leads to a reduction in platelet aggregation and a decrease in synthesis of potent vasoconstrictors, while concomitantly increasing the production of PGI_3, all of which favours healthy endothelial function.

Omura et al. (Omura *et al.*, 2001) demonstrated that EPA directly affects endothelium-dependent vasorelaxation by inducing Ca^{2+}-independent activation and translocation of eNOS. In endothelial cells, this enzyme controls the production of NO from the amino acid L-arginine (Palmer *et al.*, 1988). EPA increases NO production by cultured human endothelial cells, and prevents glucose-mediated NO inhibition (Okuda *et al.*, 1997). Incorporation of DHA into cell membranes may induce changes in membrane fluidity, calcium flux, and the synthesis and release of NO (Mori, 2006).

Endothelial function is a strong determinant of arterial compliance, which in turn influences BP. Few studies have investigated the effect of n-3 FA on arterial compliance. Nestel et al. (Nestel *et al.*, 2002) reported a 36 % and 27% improvement in SAC in dyslipidaemic subjects following a 7-week intervention with 3g/day EPA and DHA, respectively. LAC, also measured by pulse-contour analysis, was significantly improved following a 6-week intervention with fish oil in Type 2 diabetic subjects (McVeigh *et al.*, 1994). However, n-3 FA supplementation in this study did not significantly improve arterial compliance, nor did fish oil improve systemic arterial compliance in healthy individuals (Dyerberg *et al.*, 2004). The reasons for these inconsistencies are unclear, but may be due to differences in measurement techniques.

5.5.2 Exercise induced changes in arterial function

The exercise intervention in this trial did not improve FMD. Improvements in endothelial-dependent dilatation following exercise training are mediated by an increase in the release of NO (Higashi *et al.*, 1999), and exercise training has shown to increase peak FMD response in patients with metabolic syndrome (Lavrencic *et al.*, 2000), coronary (Walsh *et al.*, 2003; Edwards *et al.*, 2004) and peripheral (Brendle *et al.*, 2001) artery disease, type 1 diabetes (Fuchsjager-Mayrl *et al.*, 2002), mild hypertension (Moriguchi *et al.*, 2005) and in healthy young men (Clarkson *et al.*, 1999). Repeat exposure to shear stress may promote adaptations that improve endothelial sensitivity, such as an increase in the release of NO (Maiorana *et al.*, 2003), prostacyclin and endothelial derived hyperpolarizing factor (Niebauer & Cooke, 1996). Regular exercise can up-regulate eNOS protein expression (Hambrecht *et al.*, 2003) and reduce plasma E-1 release from endothelial cells (Maeda *et al.*, 2003).

Regular exposure to shear stress may induce structural adaptations that reverse or prevent age-associated arterial stiffening. The coronary vessel diameter is enlarged in athletes compared with matched controls (Pelliccia *et al.*, 1990), and in healthy individuals exercise training increases conduit vessel diameter (Dinenno *et al.*, 2001). The change in lumen

diameter therefore reduces blood flow induced shear stress and normalises NO activity. This may explain why studies of medium to short duration more consistently report improvements in NO mediated dilatation than interventions of longer duration (Maiorana *et al.*, 2003). This mechanism of structural change, without an improvement in NO activity, is consistent with the results from this study; exercise improved small artery compliance but did not improve FMD. Tanaka & Safar (Tanaka & Safar, 2005) suggest that short-term exercise interventions may induce "qualitative" structural changes, specifically the breakage of glucose/collagen protein cross-links, which can reduce arterial stiffness. However, in a similar population, Lavrencic et al. (Lavrencic *et al.*, 2000) showed an improvement in FMD with exercise training, albeit at higher intensities of exercise than was employed in the present study. In comparison, Kobayashi et al. (Kobayashi *et al.*, 2003) reported that three months of stationary cycling improved FMD in the posterior tibial artery but not in the brachial artery, suggesting a localised effect of training.

Higher aerobic fitness is associated with reduced central arterial stiffness (Vaitkevicius *et al.*, 1993; Tanaka *et al.*, 1998). In a recent study, Mason et al. (Mason *et al.*, 2006) demonstrated that participation in three or more episodes of vigorous activity per week was associated with greater small artery compliance. Using a similar exercise protocol to the current study (3-4 d /wk x 45 min @ 75% HR reserve), Hayahsi et al. (Hayashi *et al.*, 2005) evaluated the effect of a 16-week intervention on central and peripheral arterial stiffness in healthy, sedentary men. While reducing carotid artery stiffness, femoral artery compliance, determined by ultrasound analysis, did not change. With respect to the current study, these results are difficult to compare as the femoral artery is a large conduit artery and is therefore incorporated within the estimate for C_1 or LAC, which was not improved by the exercise intervention in this study. Consequently, these differences are likely due to variations in measurement techniques, as well as the duration and intensity of exercise interventions.

5.5.3 Combined effects of n-3 FA & exercise on arterial function

Recently it has been shown that fish oil can increase FMD, as well as increase arterial dilatation and blood flow to skeletal muscle during exercise (Walser *et al.*, 2006). The improvement in endothelial function (FMD) may be beneficial in reducing the risk of future CV events and development of CVD. In addition, the increased blood flow during exercise has implications for FA delivery to sites of metabolism (i.e. skeletal muscle) during physical activity. It is therefore possible that some of the change in body composition with n-3 FA supplementation in this study may have been due to improved blood flow increasing the delivery of fats to skeletal muscle which, in conjunction with exercise and n-3 FA induced changes in gene expression, may have facilitated fat oxidation during exercise. This proposition is indirectly supported by our own observation of an improvement in FMD and small arterial compliance, indicating an improvement in vasodilatory capacity, in response to n-3 FA supplementation and exercise respectively. However, as described in Chapter 3, we observed no increase in fat oxidation during exercise as a result of fish oil supplementation. Any increase in fat oxidation during exercise must therefore be small i.e. below the sensitivity of the method. It is possible that n-3 FA rich fish oil may cause an increase in resting metabolic rate (Couet *et al.*, 1997), thereby increasing fat oxidation during rest. Unfortunately basal metabolic rate was not measured in the present study and we were unable to confirm the results of Couet et al. (Couet *et al.*, 1997).

5.5.4 Omega-3 FA induced changes in BP

The hypotensive benefits of n-3 FA have been evaluated in several meta-analyses (Appel *et al.*, 1993; Morris *et al.*, 1993; Geleijnse *et al.*, 2002; Dickinson *et al.*, 2006). Their effects appear to be greatest in hypertensive individuals and with moderate to high doses. Moreover, several trials have since investigated the benefits of EPA versus DHA. Morris et al. (Morris *et al.*, 1993) reported that per gram of fatty acid, DHA was slightly more effective than EPA in lowering BP (-1.5/-0.77 mmHg versus –0.93/-0.53 mmHg). Mori et al. (Mori *et*

al., 1999a) supplemented overweight, hyperlipidaemic men with 4g/day or purified EPA or DHA for 6 weeks. They concluded that DHA, but not EPA, was effective in lowering 24 hr ambulatory BP. This group later reported that DHA, but not EPA, improved forearm vascular reactivity, and suggest that this may partially explain the hypotensive effects observed with DHA (Mori *et al.*, 2000b). However supplementation with 4g/day DHA did not change BP in type 2 diabetics with treated hypertension (Woodman *et al.*, 2002). Similarly, Howe et al. (Howe *et al.*, 1999) reported no effect of 2.08 g/day of DHA on clinic or ambulatory BP in moderately hypertensive subjects. Other studies in healthy subjects have reported no change in BP following supplementation with 4 g/day (Grimsgaard *et al.*, 1998) or 0.75-1.5 g/day (Conquer & Holub, 1998) of DHA. Furthermore, Nestel et al. (Nestel *et al.*, 2002) failed to show any change in BP despite an improvement in systemic arterial compliance. These results are not dissimilar to our own as despite an improvement in dilatory function, we did not observe any significant reductions in resting BP following supplementation with DHA rich fish oil. As indicated in the aforementioned meta-analyses, the lack of effect in these and the current study may be due to low n-3 FA dose and the recruitment of normotensive subjects. Additionally, methodology used to assess BP (ambulatory versus single clinic measure), use of hypertensive medication and arterial health, with particular reference to the diabetic subjects recruited by Woodman et al. (Woodman *et al.*, 2002), may have also influenced the outcomes of the individual studies discussed above.

5.5.5 Exercise induced changes in BP

The mechanisms behind the hypotensive benefits of exercise are related to their effects on endothelial function, several of which are described above, and include structural and neurohumoral changes (Hamer, 2006). An additional factor not previously mentioned is the improvement in baroreflex control of sympathetic nerve activity (Narkiewicz & Somers, 1997). Nevertheless, despite an improvement in arterial compliance, the present study failed to show a significant effect of exercise training on lowering BP. It is suggested that the anti-

hypertensive effects of regular exercise result from the accumulated effects of single, acute exercise bouts, which lower BP for a period afterward (Narkiewicz & Somers, 1997). Indeed the ACSM prescribes 30 min of accumulative or continuous, moderate aerobic exercise (40-60% VO_{2max} reserve), supplemented with resistance training on most, preferably all, days of the week (Pescatello *et al.*, 2004). It is therefore possible that more frequent exercise sessions would have provided a training stimulus of sufficient volume to elicit a significant reduction in BP. Based on the relatively greater effects of exercise on hypertensive versus normotensive individuals, the recruitment of an exclusively hypertensive population would have likely also influenced the magnitude of change in BP.

5.5.6 Summary & conclusion

There have been few studies that have investigated the combined benefit of regular aerobic exercise and n-3 FA supplementation on endothelial function and BP. The results of this study confirmed that regular supplementation with a moderate dose n-3 FA from fish oil can improve FMD. Regular moderate intensity exercise, either alone or in addition to the fish oil supplementation, had no effect on these parameters, although it did improve the compliance of small resistance arteries. Although not significantly different from each other, supplementation with n-3 FA, exercise, and the combination, showed trends toward reductions in BP (see Figure 5.9). In addition to reducing risk for development of CV disease, these improvements in arterial function may facilitate delivery of oxygen and FA to working muscles, thereby increasing exercise capacity and fat oxidation during exercise, an effect that could explain the observed changes in body composition with exercise, but not n-3 FA.

6 INFLAMMATION & IMMUNE FUNCTION

6.1 INTRODUCTION

The body's response to injury is mediated by a cascade of inflammatory events that are generally well ordered and controlled. However, if these responses become uncontrolled, they can lead to host damage and disease. Much research has reported a positive association between n-3 FA and markers of inflammation (Calder, 2002; Lopez-Garcia *et al.*, 2004; Ferrucci *et al.*, 2006). Similarly, several epidemiological studies support a link between regular physical activity and the presence (or absence) of a number of inflammatory markers (Abramson & Vaccarino, 2002; Ford, 2002; Panagiotakos *et al.*, 2005a; Mora *et al.*, 2006). Given these relationships, research has sought to determine the specific effects of n-3 FA and exercise on the inflammatory process; including neutrophil and MNL cell functions, and the mechanisms underlying these effects.

While neutrophils are essential for host defence, they also contribute to tissue damage (Smith, 1994) and have been identified in several inflammatory conditions (Smith, 1994) and as a cause of injury during myocardial ischemia-reperfusion (Vinten-Johansen, 2004). In this study, we determined the immunodulatory effects of exercise and n-3 FA from fish oil using several *ex vivo* measures of immune function; namely neutrophil chemotaxis, adherence, bactericidal activity and the respiratory burst.

6.2 AIM

To determine the independent and combined effects of n-3 FA supplementation and regular moderate exercise on immune response in subjects with risk factors for MS.

6.3 METHODS

Part of the data collected for this trial was performed in conjunction with Carrie Worthley, an Honors student in the Discipline of Physiology at the University of Adelaide. Subjects involved in this study were also enrolled in the main trial and therefore eligibility criteria and methods for subject recruitment were identical. As for other parameters, subjects were fasted (10-12 hr) and where possible, pre- and post-intervention measures were performed at similar times of the day.

6.3.1 Blood sample collection

Subject preparation and sample collection are as described above in Chapter 4. Blood samples were collected in heparinised tubes at Weeks 0 and 12 and transported on ice to the Department of Immunopathology, Children, Youth & Women's Health Services.

6.3.2 Leukocyte preparation

Blood samples were separated into MNL cells and neutrophils, >96% purity and >99% viable, by centrifugation on Hypaque-Ficoll of density of 1.44 (Ferrante & Thong, 1982). This was prepared by mixing Ficoll 400 (Amersham Pharmacia, Uppsala, Sweden) with sodium diatrozoate (Sigma Aldrich, St Louis, USA) and Angiografin (Schering Pty ltd, Sydney, Australia). Leukocyte functions were conducted under conditions accredited by the National Association of Testing Authorities of Australia (NATA). This ensures a limited degree of variability between testing conducted at different times.

6.3.3 Neutrophil functions

Superoxide anion production was measured spectrophotometrically using the nitroblue tetrazolium (NBT) reduction test (Gill, 2004). Briefly, 100 μL of neutrophils ($5x10^6$/mL) in Hanks' balanced salt solution (HBSS) were treated with 250 μL of phorbol myristate acetate (PMA) (0.4 μg/mL), or diluent (250 μL of HBSS). 500 μL of NBT solution (Sigma-Aldrich; St Louis, USA) was added to each tube and allowed to incubate for 20 min at

37°C. The tubes were then centrifuged (600g x 5 min) and neutrophils resuspended in 200 μL of ethanol:PBS (1:1, v/v) to lyse cells. Following incubation at ambient temperature for 30 min, the 200 μL suspensions were transferred to wells in a microtitre tray. Absorbance was measured at 570 nm using a Dynatech MR 7000 plate reader (Dynatech Laboratories, Chantilly, USA).

Neutrophil chemotaxis was determined by measuring neutrophil migration under agarose using the chemotactic agent f-met-leu-phe (fMLP) (Sigma Aldrich), as previously described (Hii *et al.*, 2004). Bactericidal activity was assessed by determining viability of bacteria at 0 and 60 min, following incubation of neutrophils with complement treated *Staphylococcus aureus* (strain 6571; National Collection of Type Cultures, Oxford, England), as described previously (Ferrante *et al.*, 1993). Neutrophil adherence was measured by adherence to plasma-coated plates, according to the method of Powell *et al*. (Powell *et al.*, 1997).

6.3.4 Cytokine production from stimulated MNL

MNL were cultured in the presence or absence of the T lymphocyte mitogens, phytohaemagglutinin (PHA, 2μg/ml) (Murex Diagnostics, Dartford, England) or Concanavalin A (Con A, 40μg/ml) (Sigma) (Costabile *et al.*, 2005) for 72h at 37^{o}C in 5% CO_2-air and high humidity. Cytokine production (lymphotoxin, [LTn], interferon gamma [IFNγ], and interleukin [IL]-2) was measured in the culture supernatants by ELISA using cytokine-specific monoclonal antibodies, as described by Costabile et al. (Costabile *et al.*, 2005). Cytokine production by monocytes (TNFα, IL-1β, IL-6) was examined by stimulating MNL with bacterial lipopolysaccharide (LPS, 1μg/ml) for 24h as above) (Ferrante *et al.*, 1997). Absorbance at 450/750 nm was read using a Dynatech MR 7000 plate reader (Dynatech Laboratories, Chantilly, USA). The limit of detection for all cytokines was

approximately 0.02ng/mL. The inter-assay CVs were <8% for LTn, IL-1β, IL-2 and IL-6, and <16% for IFNγ and TNFα. The intra-assay CVs were <6% for all cytokine assays.

6.4 RESULTS

6.4.1 Subjects

Data for the sub-studies on neutrophil function and cytokine production were attained from 40 (2nd and 3rd cohorts) and 26 (2nd cohort) subjects, respectively. Subject recruitment and attrition patterns for the study are described in Chapter 2. **Table 6.1** displays baseline characteristics by treatment group for subjects involved in the study on neutrophil function. There were no differences between the four intervention groups for any of these characteristics (one-way ANOVA), and these are representative of the smaller cytokine production study population (n=26, data not shown) and the trial population overall (n=65, see Chapter 2).

Table 6.1 Neutrophil function and cytokine production sub-study subject entry characteristics by treatment group.

	Treatment Group			
	FO (n=11)	***FOX (n=10)***	***SO (n=11)***	***SOX (n=8)***
Sex (M:F)	3 : 8	2 : 8	4 : 7	2 : 6
Age (years)	52 ± 2	49 ± 2	50 ± 3	51 ± 2
BMI (kg/m^2)	35 ± 2	34 ± 2	35 ± 1	34 ± 1
% Body Fat	45 ± 1	46 ± 2	45 ± 2	45 ± 3
Systolic BP (mmHg)	128 ± 5	132 ± 5	128 ± 3	133 ± 3
Diastolic BP (mmHg)	70 ± 3	77 ± 3	72 ± 2	78 ± 3
TC (mmol/L)	6.8 ± 0.5	6.1 ± 0.5	6.3 ± 0.6	6.9 ± 0.6
TAG (mmol/L)	1.6 ± 0.3	1.5 ± 0.2	1.5 ± 0.2	1.8 ± 0.3

6.4.2 Effects of n-3 FA and/or exercise on erythrocyte fatty acid concentrations

Due to technical difficulties fatty acid profiles of erythrocyte membranes for 13 subjects were lost, providing data for this parameter in only 27 subjects at Weeks 0, 6 and 12. However, given that the variance in this measure was small, the data obtained is likely to be representative of the changes for all subjects. ANOVA identified differences between treatment groups at baseline for the concentration of DPA and total long chain (EPA+DPA+DHA) n-3 FA in erythrocytes (**Table 6.2**). To determine the effect of treatment, erythrocyte fatty acid concentration data were analysed by 3-factor ANOVA (oil x exercise x time). Where a difference was detected at baseline, changes were analysed by ANCOVA, using baseline values as a covariate. Several one- and two-factor interactions were detected by this analysis and these are shown in Table 6.2.

Briefly, the change in fatty acid concentration in this sub-group exhibited an almost identical profile to that described for the whole cohort in Chapter 4. The percentage of total n-3 FA in erythrocytes rose progressively in the FO and FOX groups (oil x time interaction, $P<0.001$) due mainly to an increase in DHA (**Figure 6.1**; oil x time interaction, $P<0.001$). There was no change in DHA or total n-3 FA in the sunflower oil groups. Post-hoc analysis detected a marginal reduction in EPA for all subjects during the intervention (main effect of time, $P<0.013$), but failed to identify any differences between means for changes in DPA.

Table 6.2 Erythrocyte fatty acid composition (% of total fatty acids) by treatment group.

		Treatment Group			
		FO (n=7)	***FOX (n=9)***	***SO (n=4)***	***SOX (n=7)***
EPA[2, 3]	*Week 0*	1.75 ± 0.07	1.57 ± 0.07	1.46 ± 0.11	1.73 ± 0.11
	Week 6	1.71 ± 0.05	1.49 ± 0.05	1.39 ± 0.11	1.62 ± 0.06
	Week 12	1.62 ± 0.07	1.49 ± 0.06	1.36 ± 0.12	1.63 ± 0.07
DPA[1, 3]	*Week 0*	4.66 ± 0.15	4.50 ± 0.11[a]	4.49 ± 0.11	5.30 ± 0.30[a]
	Week 6	4.49 ± 0.12	4.33 ± 0.13	4.11 ± 0.28	4.92 ± 0.12
	Week 12	4.48 ± 0.13	4.48 ± 0.10	4.00 ± 0.24	4.99 ± 0.16
DHA[2, 4]	*Week 0*	3.83 ± 0.19	3.97 ± 0.34	3.40 ± 0.15	3.53 ± 0.32
	Week 6	6.12 ± 0.16	6.30 ± 0.29	3.53 ± 0.33	4.19 ± 0.22
	Week 12	6.89 ± 0.17[c]	7.35 ± 0.33	3.62 ± 0.14	4.20 ± 0.17
Total n-3 FA[1, 2, 4]	*Week 0*	10.24 ± 0.25	10.04 ± 0.27	9.34 ± 0.16 [a]	10.56 ± 0.20 [a]
	Week 6	12.32 ± 0.22	12.13 ± 0.32	9.02 ± 0.52	10.73 ± 0.35
	Week 12	13.00 ± 0.22[, c]	13.33 ± 0.33[, c]	8.98 ± 0.38	10.82 ± 0.34

[1] Treatment groups with the same superscript letter were significantly different at baseline, $P<0.05$

[2] Significant main effect of time, $P<0.05$

[3] Significant oil x exercise interaction, $P<0.05$.

[4] Significant oil x time interaction, $P<0.05$.

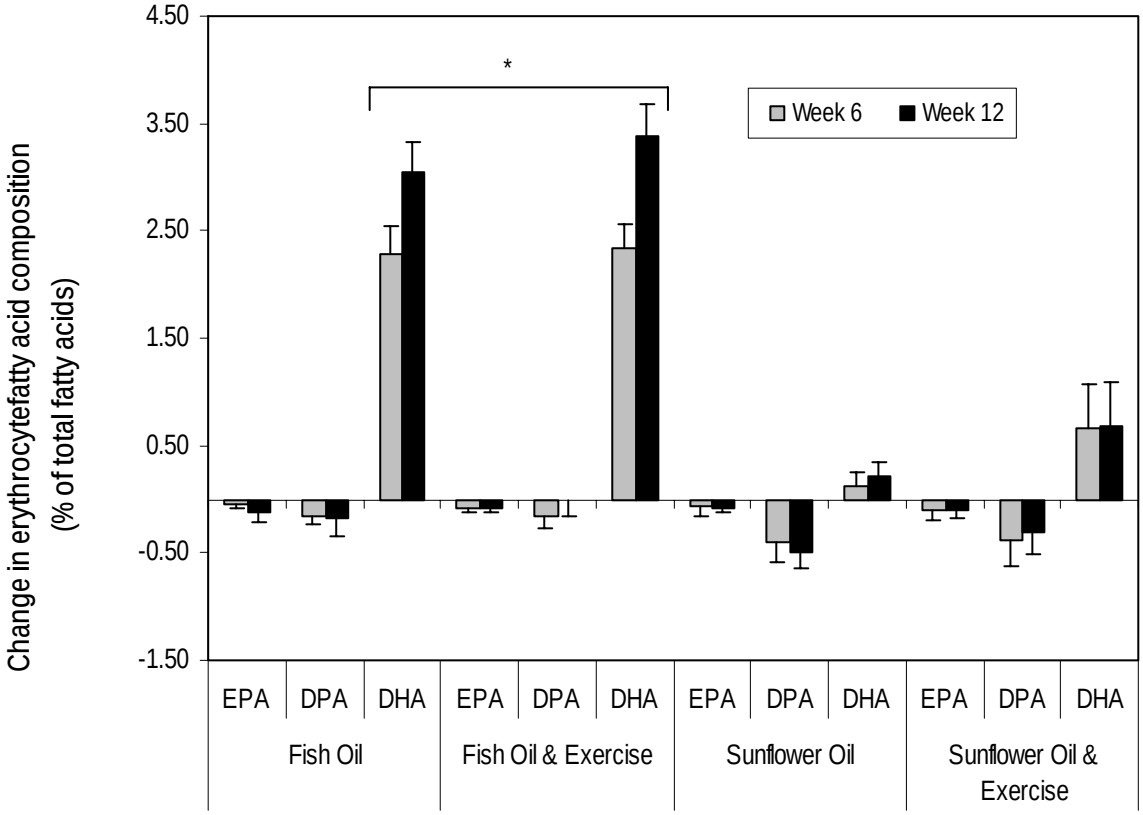

Figure 6.1 Change in erythrocyte fatty acid composition by treatment group. *Significant increase in DHA in the fish oil and fish oil and exercise groups compared with the sunflower oil or sunflower and exercise groups (oil x time interaction, $P<0.001$).

6.4.3 Effects of n-3 FA and/or exercise on cytokine production

Con A and PHA were used to stimulate T cells in the MNL fraction to produce the cytokines LTn, IFN-γ and IL-2. LPS was used to stimulate cytokine production (TNFα, IL-1β, and IL-6) by monocytes. Cytokine production did not differ between groups at Week 0 (**Table 6.3**), and was not affected by either oil or exercise treatment after 12 weeks of intervention (3-factor ANOVA). For all subjects, ANOVA showed a main effect of time on the production of nearly all cytokines (Table 6.3). There was a time dependent increase in TNFα, IL-1, and IL-6, while the production of IL-2 and LTn (with both PHA and Con A stimulation) decreased by Week 12.

Table 6.3 Cytokine production from stimulated neutrophils by treatment group.

		Treatment Group			
		FO	***FOX***	***SO***	***SOX***
LTn (ng/mL) [1]	*Week 0*	8.52 ± 2.13	13.41 ± 6.62	7.28 ± 1.00	10.43 ± 2.26
	Week 12	5.91 ± 1.54	5.39 ± 0.81	5.91 ± 1.22	5.58 ± 0.99
Stimulant:	*Change*	-2.61 ± 1.41	-2.10 ± 1.36	-1.36 ± 1.78	-4.85 ± 2.24
Con A	*n*	7	5[2]	7	6
LTn (ng/mL) [1]	*Week 0*	30.26 ± 4.30	30.04 ± 6.53	36.71 ± 6.96	32.37 ± 7.27
	Week 12	24.24 ± 5.65	15.95 ± 2.94	23.46 ± 4.54	17.93 ± 4.83
Stimulant:	*Change*	-6.02 ± 4.61	-14.10 ± 6.15	-7.76 ± 2.84	-14.44 ± 8.01
PHA	*n*	7	6	6[2]	6
IFNγ (ng/mL)	*Week 0*	23.46 ± 5.92	26.39 ± 7.53	18.38 ± 3.78	23.96 ± 6.03
	Week 12	21.52 ± 5.80	31.52 ± 1.13	23.31 ± 6.04	26.59 ± 6.47
Stimulant:	*Change*	-1.94 ± 2.95	5.14 ± 8.10	4.93 ± 6.01	2.63 ± 6.59
Con A	*n*	7	6	7	6
IFNγ (ng/mL)	*Week 0*	17.50 ± 5.32	24.66 ± 10.25	15.64 ± 6.38	14.09 ± 3.57
	Week 12	16.56 ± 3.03	18.26 ± 5.44	14.77 ± 5.88	15.83 ± 6.19
Stimulant:	*Change*	-0.94 ± 3.60	-6.41 ± 7.73	-0.88 ± 7.18	1.74 ± 4.55
PHA	*n*	7	6	7	6
IL-2 (ng/mL) [1]	*Week 0*	0.80 ± 0.40	0.71 ± 0.28	1.88 ± 0.78	0.63 ± 0.33
	Week 12	0.45 ± 0.25	0.24 ± 0.08	0.51 ± 0.16	0.18 ± 0.05
Stimulant:	*Change*	-0.35 ± 0.36	-0.45 ± 0.23	-1.29 ± 0.80	-0.58 ± 0.35
PHA	*n*	7	5[2]	6[3]	5[2]
IL-1β (ng/mL) [1]	*Week 0*	0.37 ± 0.19	0.44 ± 0.20	0.65 ± 0.35	0.46 ± 0.20
	Week 12	1.30 ± 0.30	1.16 ± 0.35	1.00 ± 0.15	1.56 ± 0.33
Stimulant:	*Change*	0.67 ± 0.34	0.73 ± 0.28	0.36 ± 0.37	1.10 ± 0.34
LPS	*n*	6[2]	6	7	6
IL-6 (ng/mL) [1]	*Week 0*	3.00 ± 0.85	5.80 ± 2.34	4.97 ± 2.28	4.55 ± 2.12
	Week 12	8.22 ± 1.60	7.25 ± 0.65	7.54 ± 1.81	11.33 ± 1.63
Stimulant:	*Change*	5.22 ± 2.22	1.45 ± 1.83	2.57 ± 2.74	6.78 ± 2.44
LPS	*n*	7	6	7	6
TNFα (ng/mL) [1]	*Week 0*	0.42 ± 0.16	0.32 ± 0.08	0.56 ± 0.16	0.60 ± 0.23
	Week 12	2.08 ± 0.37	1.53 ± 0.52	1.82 ± 0.39	2.15 ± 0.60
Stimulant:	*Change*	1.67 ± 0.45	1.21 ± 0.50	1.26 ± 0.35	1.54 ± 0.61
LPS	*n*	7	6	7	6

[1] Significant main effect of time, $P<0.05$.

[2] One subject excluded due to insufficient blood sample.

6.4.4 Effects of n-3 FA and/or exercise on neutrophil function

There were no significant differences between groups for any measures of neutrophil function at Week 0 (**Table 6.4**).

Table 6.4 Measures of neutrophil functions by treatment group.

		Treatment Group			
		FO	***FOX***	***SO***	***SOX***
Superoxide production [2]	*Week 0*	0.14 ± 0.02	0.15 ± 0.02	0.12 ± 0.02	0.13 ± 0.01
(OD_{570nm})	*Week 12*	0.11 ± 0.02	0.10 ± 0.02[3]	0.14 ± 0.03	0.13 ± 0.02
	Change	-0.03 ± 0.02	-0.05 ± 0.02	0.02 ± 0.02	0.01 ± 0.02
	n	11	10	11	8
Bactericidal activity [3]	*Week 0*	96 ± 1	96 ± 1	96 ± 1	93 ± 1
(% killing)	*Week 12*	93 ± 1[4]	95 ± 1	94 ± 1	96 ± 1
	Change	-3 ± 1	-1 ± 1	-1 ± 2	2 ± 2
	n	10[4]	10	10[4]	7[4]
Chemotaxis	*Week 0*	2.15 ± 0.05	2.17 ± 0.07	2.16 ± 0.06	2.07 ± 0.07
(mm/90min)	*Week 12*	2.03 ± 0.08	2.23 ± 0.07	1.98 ± 0.09	2.11 ± 0.10
	Change	-0.13 ± 0.11	0.06 ± 0.11	-0.18 ± 0.11	0.04 ± 0.12
	n	11	10	11	8
Adherence [1]	*Week 0*	0.17 ± 0.04	0.23 ± 0.05	0.25 ± 0.04	0.21 ± 0.02
(OD_{570nm})	*Week 12*	0.31 ± 0.05[3]	0.32 ± 0.04	0.26 ± 0.04	0.27 ± 0.03[3]
	Change	0.15 ± 0.06	0.09 ± 0.05	0.01 ± 0.06	0.06 ± 0.02
	n	10[4]	10	10[4]	8

[1] Significant main effect of time, $P<0.05$

[2] Significant oil x time interaction, $P<0.05$

[3] Significant exercise x time interaction, $P<0.05$.

[4] Subjects excluded due to insufficient harvesting of cells or technical problem with assay.

6.4.4.1 Superoxide Production

While ANOVA did not detect any significant 3-factor interactions (oil x exercise x time), an effect of oil treatment (oil x time, $P<0.05$) on superoxide production was identified (**Figure 6.2**). Post-hoc analysis showed that by Week 12 superoxide production had decreased significantly in subjects supplemented with fish oil (-19.5 ± 8.5%, $P<0.041$) but not sunflower oil (+10.7 ± 14%, $P=0.82$), and the changes in superoxide production were negatively correlated with changes in DHA content in erythrocyte membranes (**Figure 6.3**; r = -0.385, P = 0.047).

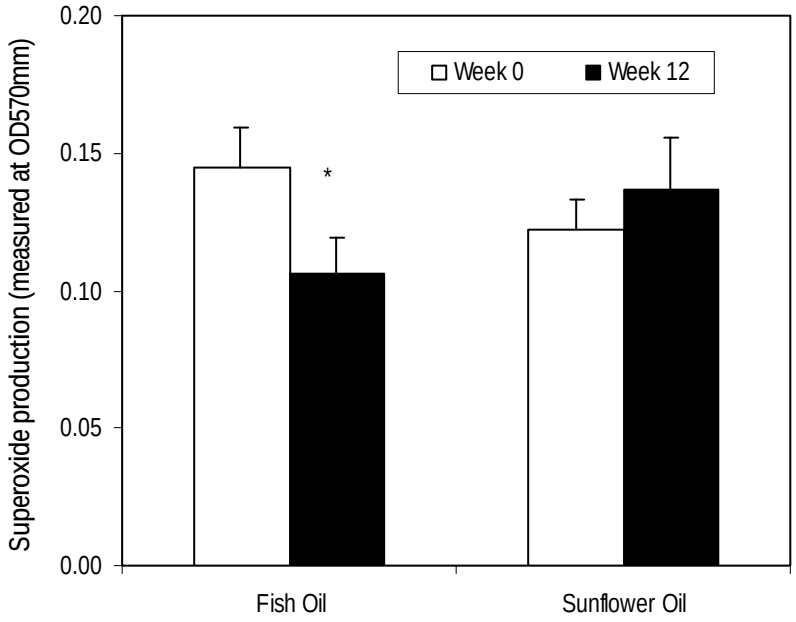

Figure 6.2 Effect of DHA-rich fish oil or sunflower oil on superoxide production.

* Significant reduction in superoxide production by Week 12 with fish oil, $P=0.041$.

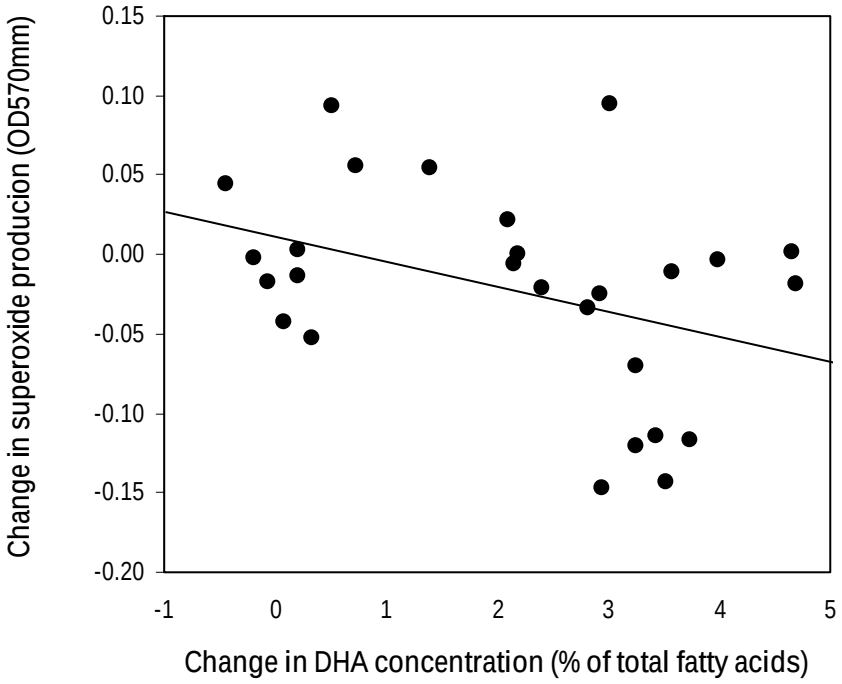

Figure 6.3 Relationship between change in superoxide production and incorporation of DHA into erythrocyte membranes (r = -0.385, *P* = 0.047).

6.4.4.2 Bactericidal Activity

There were no significant 3-factor interactions (oil x exercise x time) for bactericidal activity. There was however a significant exercise x time interaction (**Figure 6.4**, *P*<0.013), with subjects allocated to a non-exercise group showing a significant decrease in bactericidal activity by Week 12 (Tukey test, *P*<0.002). There was no change in subjects who undertook exercise (*P*=0.99).

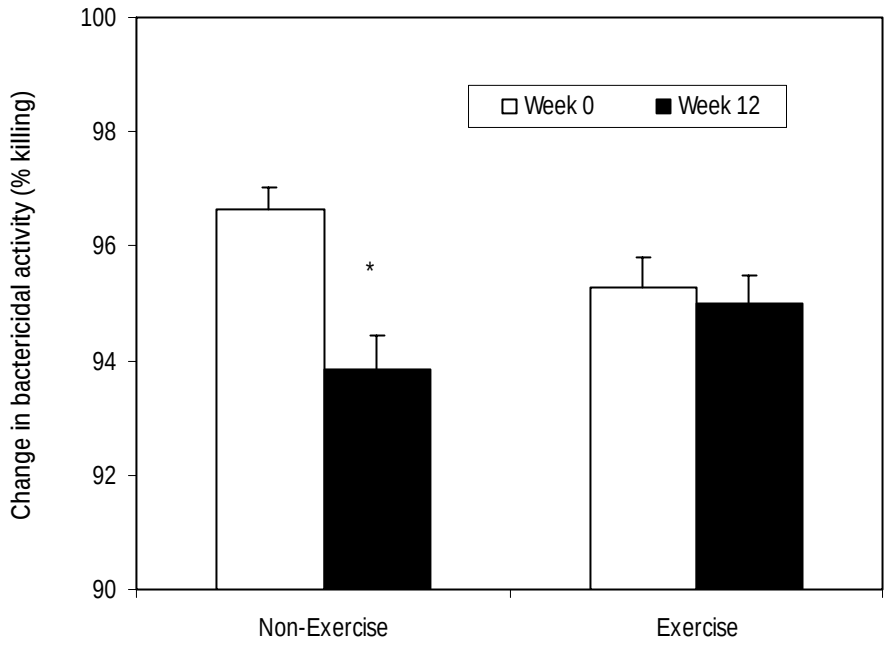

Figure 6.4 Effect of exercise training or no exercise on bactericidal activity. * Significant reduction in bactericidal activity by Week 12, in non-exercising subjects, P=0.002.

6.4.4.3 Chemotaxis and Adherence

There were no 3-factor interactions (oil x exercise x time) for chemotaxis or adherence. For all subjects, there was a main effect of time on adherence, which increased during the intervention (main time effect, P<0.008). ANOVA indicated a trend toward an effect of exercise treatment on chemotaxis (**Figure 6.5**; 2-factor exercise x time interaction, P<0.083) with exercise tending to maintain chemotaxis compared with a reduction in the non-exercise groups.

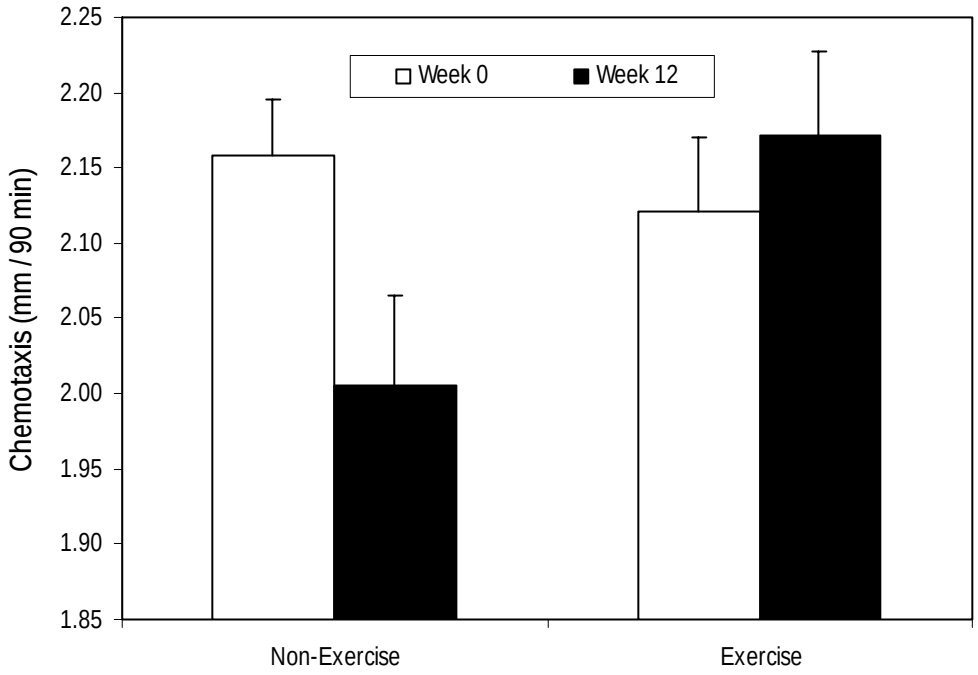

Figure 6.5 Effect of exercise training or no exercise on chemotaxis. There was a trend toward a reduction in chemotaxis in non-exercising subjects (exercise x time interaction, $P<0.083$).

6.5 DISCUSSION

6.5.1 Omega-3 FA induced changes in cytokine production

The typical Western diet contains around ten times as much n-6 FA (predominantly LA) than it does the n-3 FA, LNA. LA is converted through a series of chain elongation and desaturation steps to produce AA, which is a primary substrate for eicosanoid synthesis. The metabolism of AA by COX leads to the production of PGE_2 and TXA_2. Alternatively, metabolism by LOX produces LTB_4 and hydroperoxy derivatives. These eicosanoids can stimulate the production of several cytokines (IL-2, IFN-γ, TNFα, IL-1, and IL-6) (Calder & Grimble, 2002). Thus diets rich in LA are likely to result in a greater content of AA in cell membranes and therefore promote the synthesis of pro-inflammatory eicosanoids and cytokines.

EPA is another substrate for COX and LOX. It disrupts this inflammatory pathway by preventing the mobilisation of AA from cell membranes by phospholipase A_2 (Obata *et al.*, 1999) and directly competing with AA for metabolism by COX and LOX enzymes, resulting in the production of the less inflammatory PGE_3 and LTB_5, and the less pro-aggregatory TXA_3 (James *et al.*, 2000) (for pathway see Chapter 1, Figure 1.3). In comparison, DHA may exert anti-inflammatory effects by i) acting as a substrate for conversion into highly anti-inflammatory compounds (i.e. resolvins) (Hong *et al.*, 2003), ii) inhibiting gene expression of enzymes involved in eicosanoid synthesis (VanRollins, 1995), or iii) inhibiting expression of inflammatory mediators (De Caterina *et al.*, 2000; Calder, 2005). The ability of EPA and DHA to alter membrane fluidity (Sipka *et al.*, 1996) and cell signaling pathways (Calder, 2005) may also provide additional avenues by which they exert their anti-inflammatory effects.

This study investigated the effect of supplementation with DHA-rich tuna fish oil on T cell and monocyte responses to PHA/ConA and LPS, respectively. Supplementation failed to induce any change in the T cell production of T helper1 cytokines, LTn, IFN-γ and IL-2, or the monocyte production of IL-1β, TNFα and IL-6. These results are in contrast to some other studies which have reported effects of EPA and DHA on cytokine production (Endres *et al.*, 1989; Meydani *et al.*, 1991; Gallai *et al.*, 1995; Trebble *et al.*, 2003), but may be explained by several factors. Studies investigating the anti-inflammatory effects of n-3 FA have typically used EPA-rich fish oil. Meydani et al. (Meydani *et al.*, 1991) reported a reduction in TNFα, IL-1β and IL-6 from stimulated MNL cells following 12 weeks of supplementation with 2.4g/day of EPA rich fish oil in young and older women. Trebble et al. (Trebble *et al.*, 2003) found a U-shaped dose-response relationship between EPA intake and cytokine production, wherein maximum inhibition occurred with 1g/day of EPA-rich oil. However, a dose-response curve for DHA is yet to be established.

Our finding of the lack of effect on cytokine production might also have been due to the use of a relatively low dose of n-3 FA. Several studies have shown a reduction in cytokine production with high doses of n-3 FA (Endres *et al.*, 1989; Kremer *et al.*, 1990; Gallai *et al.*, 1995; Kelley, 2001), whereas Blok et al. (Blok *et al.*, 1997) failed to observe any differences in plasma cytokine concentration when compared to placebo in healthy monks supplemented with various doses of fish oil (0, 3, 6 or 9g/day) for one year. Alternatively, the fact that the volunteers in the present study did not have any inflammatory conditions might also explain our finding. Indeed studies showing a decrease in IL-1β, TNFα, IL-2 and IFNγ production by stimulated MNL using comparable or lower doses of n-3 FA to the present study, have used subjects with diseases/conditions characterised by elevated cytokine production (Espersen *et al.*, 1992; Cooper *et al.*, 1993) or EPA rich oils (Meydani *et al.*, 1991; Trebble *et al.*, 2003). It is possible that the supplementation in this trial may therefore have been of insufficient dose to induce any changes in cytokine production, particularly as participants in this trial were healthy and did not present with raised serum cytokines at baseline. Indeed, Thies et al (Thies *et al.*, 2001a) and Schmidt et al. (Schmidt *et al.*, 1996) reported no effect of supplementation with low doses of DHA (0.7g/day) or EPA+DHA (0.5 &1g/day) on the production of TNFα, IL-1β or IL-6 in healthy young (Schmidt *et al.*, 1996) and older adults (Thies *et al.*, 2001a). Therefore effects on cytokine production at lower doses of n-3 FA might only be evident in patients with inflammatory conditions, while high doses are required to elicit changes if cytokine levels are not elevated.

6.5.2 Exercise induced changes in cytokine production

In the current study, regular physical activity did not produce any changes in cytokine production following 12-weeks of intervention. Comparison of results with other studies is made difficult by two key differences; most studies investigating changes in cytokine production have focused on short-term or acute exercise or have looked at cytokine concentrations in plasma. In this study, cytokine production was measured from T cells and

monocytes stimulated with PHA/ConA and LPS, respectively. One relevant study investigated the effect of supervised physical activity (mean duration 2.5 h/week, various aerobic exercise modalities) on cytokine production in subjects with risk factors for CVD (Smith *et al.*, 1999). They showed a reduction in IFNγ and TNFα, and an increase in IL-4 and IL-10 from PHA stimulated blood MNL cells.

Other studies which have examined the effect of exercise on cytokine production have reported inconsistent findings. Fairey et al. (Fairey *et al.*, 2005) reported that 15 weeks of regular exercise training (stationary cycling 3d/week @ 70-75% peak VO_2, increasing to 35 min) did not effect PHA stimulated cytokine production in postmenopausal women survivors of breast cancer. Baum et al. (Baum *et al.*, 1999) trained healthy subjects for 12 weeks (running 3-5 h/wk) and observed an increase in IL-1β and IL-6 from LPS stimulated cells, with no change in IL-2 or IFNγ (stimulated by staphylococcal enterotoxin B). Following 12 weeks of training (30 min/day at 65-70% VO_2 max, 4-5 d/wk), Rhind et al. (Rhind *et al.*, 1996) observed a reduction in IL-2 from PHA stimulated PBMC in both trained and control subjects, with a significantly greater reduction in trained individuals. However, this protocol differed in that it required subjects to perform a 60 min exercise bout prior to blood sampling, which would have increased inflammatory cytokine production. In cyclists, 6 months of training (~500km/wk) attenuated IL-2 production from PHA stimulated PBMC (Baj *et al.*, 1994). In a comparative study, McFarlin et al. (McFarlin *et al.*, 2006) showed that LPS stimulated production of IL-6, IL-1 and TNFα was significantly lower in active (defined by the Paffenbarger Physical Activity Questionnaire and a modified sub-maximal treadmill test) compared to inactive subjects.

Should cytokine production from MNL cells be modified substantially following exercise training, this could be reflected in the cytokine levels in plasma. However, as mentioned above, studies investigating the effect of regular or chronic exercise training on inflammatory markers have shown mixed results (Adamopoulos *et al.*, 2001; Larsen *et al.*, 2001; Nicklas *et al.*, 2004; Goldhammer *et al.*, 2005; Marcell *et al.*, 2005; Niebauer *et al.*,

2005). Consequently, given the great disparity between studies there is much scope for future investigation.

The inflammatory response to acute strenuous exercise has been well documented and typically consists of an increase in IL-6 (Febbraio & Pedersen, 2002). While IL-6 is classified as a pro-inflammatory cytokine it has several anti-inflammatory actions. IL-6 inhibits the production of TNFα and IL-1 (Tilg *et al.*, 1997) and stimulates production of IL-ra and IL-10 (Steensberg *et al.*, 2003). The net response to acute exercise is therefore considered anti-inflammatory, and the overall anti-inflammatory effect of regular physical activity may be mediated by these acute bouts of exercise (Petersen & Pedersen, 2005). A reduction in fat mass, as can occur with regular physical activity, may also potentially reduce inflammation, as obesity, particularly abdominal obesity, is associated with increased levels of circulating TNFα, IL-6 and CRP (Fantuzzi, 2005; Panagiotakos *et al.*, 2005b). However in the current study, exercise resulted in only a small change in fat mass and it is therefore unlikely that this would have induced any significant changes in cytokine production.

6.5.3 Combined n-3 FA & exercise induced changes in cytokine production

Toft and colleagues (Toft *et al.*, 2000) recruited 20 endurance-trained men to consume 6g/day of fish oil (3.6g n-3 FA) or no supplement for 6 weeks prior to competing in the Copenhagen Marathon. Plasma levels of TNFα, IL-6, IL-ra and serum transforming growth factor-β_1 were measured before, immediately after and 1.5 and 3.0 hours post-exercise. With respect to our work, this study provided two important outcomes. Firstly, they showed that following the 6-week supplementation period cytokine levels did not differ between the fish oil and control groups and secondly that fish oil supplementation failed to attenuate the cytokine response to acute exercise. This demonstrates that high-intensity endurance training in combination with n-3 FA does not influence cytokine production.

With respect to studies investigating the effects of n-3 FA and / or exercise on cytokine production, variations in protocol may partially explain the reported differences, as

investigators have used a range of MNL stimulants. Furthermore, the test protocol in this study also required the application of a large volume of stimulus to the cells, which may have masked any potential treatment effects.

6.5.4 Omega-3 FA induced changes in neutrophil function

Previous studies have reported decreases in neutrophil adhesion (Lee *et al.*, 1985; Andrioli *et al.*, 1999), chemotaxis (Schmidt *et al.*, 1991; Sperling *et al.*, 1993) and superoxide anion production with n-3 FA supplementation (Varming *et al.*, 1995; Luostarinen & Saldeen, 1996), but several investigators reported no effect of n-3 FA on any of these measures of neutrophil function (Guarini *et al.*, 1998; Healy *et al.*, 2000; Miles *et al.*, 2004). In this study, we observed a significant reduction in superoxide production by stimulated neutrophils following 12 weeks of n-3 FA supplementation, but failed to show any effect on chemotaxis, adherence or bactericidal activity. Previous studies have shown decreases in superoxide production of 14-64% following 4-6 weeks of supplementation with EPA-rich oil providing > 4g n-3 FA/day (Fisher *et al.*, 1990; Varming *et al.*, 1995; Luostarinen & Saldeen, 1996), These studies provided substantially more n-3 FA than in the present study. Conversely, Healy et al (Healy *et al.*, 2000) reported that lower doses of n-3 FA (0.27-2.25g/day) did not influence superoxide production by neutrophils. This finding is supported by Thies et al. (Thies *et al.*, 2001a) who reported that the neutrophil respiratory burst response to *E.coli* remained unchanged following 12-weeks of supplementation with a low dose of DHA (0.7g/day) or EPA+DHA (1g/day). Although differences in the methods by which ROS are measured may explain some of the variation in the magnitude of change in superoxide production, it is more likely to be a function of the neutrophil stimulus used. Varming et al. (Varming *et al.*, 1995) and Fisher et al. (Fisher *et al.*, 1990) used particles acting on neutrophil surfaces while we and others who observed a smaller effect used the soluble stimulus PMA which acts on intracellular PKC (Luostarinen & Saldeen, 1996).

The method by which DHA induces a reduction in superoxide production is not fully understood but may be related to its incorporation into cell membranes. Following supplementation, both DHA and EPA are readily incorporated into PBMC phospholipids, and some studies have reported a simultaneous reduction in AA with EPA (Thies *et al.*, 2001b) and DHA (Kelley *et al.*, 1999). Other studies have reported increased incorporation of n-3 FA into neutrophils (Luostarinen & Saldeen, 1996; Healy *et al.*, 2000; Kew *et al.*, 2004; Gorjao *et al.*, 2006) and these compositional changes are similar to those seen in plasma phospholipids (Kew *et al.*, 2004). While we did not measure the fatty acid composition of neutrophil membranes, it is reasonable to assume that DHA incorporation into these cells would have mirrored the increase seen in erythrocyte membranes. Evidence for this relationship may be seen in the negative correlation between superoxide production and the increased incorporation of DHA in erythrocyte membranes.

Increasing the n-3 FA concentration of neutrophil membrane phospholipids, may alter the ability to activate signaling molecules such as PKC. PKC exists in the form of several isozymes, the activation of which is mediated by lipids, the most well known being the second-messenger DAG (Spitaler & Cantrell, 2004). DHA, by replacing AA in the sn-2 position of the phospholipid, can lead to the production of DHA-containing DAG, possibly compromising PKC translocation/activation. Indeed Madani et al. (Madani *et al.*, 2001) reported different in vitro activation of PKC isozymes with DHA-containing DAG versus AA-containing DAG. Furthermore, activation of the small guanosine 5'-triphosphate binding protein Rac, a component of the neutrophil oxidase, is promoted by intracellular AA (Ferrante *et al.*, 2005). Replacing AA with DHA in the phospholipid may impair this part of the oxidase activation process.

The importance of reducing ROS is linked to its' role in CV disease, more specifically atherosclerosis. ROS, including superoxide, increases oxidized LDL (Hansson & Libby, 2006) and microvessel permeability (Zhu *et al.*, 2005), and up-regulates endothelial expression of cell adhesion molecules (de Prost & Hakim, 1997). Within the endothelium, NO

bioavailability is reduced in the presence of excess ROS (Landmesser *et al.*, 2006). Given the importance of NO as a vasodilator, this can have a negative impact on vascular function. Furthermore, ROS have also been implicated as activators of NF-κβ (Brand *et al.*, 1997), a transcription factor that regulates the production and expression of several cell adhesion molecules and inflammatory cytokines (De Caterina *et al.*, 2006).

6.5.5 Exercise induced changes in neutrophil function

As for changes in cytokine production, the majority of studies investigating the effect of exercise on neutrophil functions have used short-term or acute exercise as a stimulus. Other studies have attempted to determine the effect of regular physical activity by comparing inactive and active subjects. Consequently there is a paucity of data detailing the effect of intervention with regular moderate exercise on neutrophil functions, particularly in previously sedentary individuals.

Pyne et al. (Pyne *et al.*, 1995) compared the oxidative burst activity of neutrophils from sedentary and elite swimmers at rest and showed a substantial reduction in oxidative burst activity in the athletes. Similarly, ROS production (determined by luminol-enhanced chemiluminescence) was significantly reduced in elite cyclists following a 6-month intensive training period (Smith *et al.*, 1990). In the same group of cyclists, ROS production before and after an acute exercise bout was considerably lower compared to sedentary individuals. Benoni et al. (Benoni *et al.*, 1995a) also reported significantly lower superoxide anion release in active compared to inactive subjects following an acute exercise bout. However, in a separate study, the same group reported that superoxide anion release increased during the sports season of elite basketball players (Benoni *et al.*, 1995b). To determine the effect of an acute exercise bout and exercise training on cell functions, Suzuki et al. (Suzuki *et al.*, 1996) studied the effect of 1 week of repeated daily acute exercise in untrained men. Acute exercise caused a significant increase in ROS, which was reduced with repeated daily exercise, although this trend was not significant.

While we observed no effect of regular aerobic training on superoxide production, we did observe a protective effect of exercise on bactericidal activity. As for other measures of neutrophil function, there is limited data on the effect of aerobic exercise training on this parameter. Lewicki et al. (Lewicki *et al.*, 1987) reported that bactericidal activity of trained sportsmen was significantly reduced compared to untrained individuals. Conversely, Benoni et al. (Benoni *et al.*, 1995b) observed an increase in bactericidal activity during the sports season of elite basketball players, which returned to baseline levels at the end of the season. Although significant, the change in bactericidal activity observed in this trial was marginal and unlikely to be of clinical significance.

6.5.6 Combined n-3 FA & exercise induced changes on neutrophil function

Using a rat model, Robinson & Field (Robinson & Field, 1998) investigated the effect of n-3 FA supplementation and regular moderate exercise (swimming) on immunocompetence. Both exercise and n-3 FA supplementation independently increased the proportion of T and B cells that were activated after ConA stimulation. However, this activity was suppressed when the treatments were combined. Natural killer cell cytotoxic activity was greater in rats supplemented with n-3 FA, but exercise was also shown to attenuate this activity. The authors conclude that immune responses to n-3 FA may be dependent on physical activity levels.

6.5.7 Summary and conclusion

In summary, the present study found that supplementation with DHA rich fish oil reduces superoxide production from stimulated neutrophils, while regular moderate exercise training protects bactericidal activity. Neither of these functions were enhanced by the combination of interventions. However it is unclear whether these changes are of biological significance. Other neutrophil functions (viz chemotaxis, adherence and cytokine production) were unaffected by intervention. Supplementation with ~2g/day n-3 FA therefore did not

inhibit immune function in this study. Differences between this and other studies reporting a positive effect of n-3 FA supplementation on cytokine production and neutrophil functions may be due to several factors, including fish oil dose and composition and the presence of inflammatory conditions within a study population. For exercise, most studies have investigated the effects of acute or short-term exercise and changes in systemic cytokine levels and not cytokine production from stimulated cells.

Protocol variations may also partially explain these differences as a range of MNL stimulants including LPS (Cooper *et al.*, 1993; Trebble *et al.*, 2003) and PHA (Smith *et al.*, 1999) have been used. Furthermore, the small sample size and large inter-individual subject variation may have limited our ability to detect effects of intervention (fish oil or exercise) compared to controls. Potential treatment effects may also have been masked by the application of a large volume of stimulus to the MNL cells.

7 HEART RATE

7.1 INTRODUCTION

HRV is a non-invasive measure of the beat-to-beat variation in normal heartbeat, which provides a reliable assessment of autonomic control of the heart and reflects the interplay between sympathetic and parasympathetic activity. This interpretation is based on the analysis of frequency bands within the HRV power spectrum (Malik *et al.*, 1996). These frequency bands derive from differences in parasympathetic and sympathetic control of HR. Under parasympathetic (or vagal) control, the sinus node response is short and effects can occur within 1 s (Borst & Karemaker, 1983; Koizumi *et al.*, 1985), whereas the maximal sympathetic response occurs after a few seconds (Koizumi *et al.*, 1985). In consequence, rapid (approximately 0.15 Hz) fluctuations in heart rate are almost solely mediated by cardiac vagal outflow (Akselrod *et al.*, 1981; Pomeranz *et al.*, 1985), while sympathetic excitation contributes markedly to lower-frequency regulation (Hayano *et al.*, 1991; Pagani *et al.*, 1997). Thus the term low frequency power (LFP) is associated with sympathetic tone, while high frequency power (HFP) reflects parasympathetic activity (Akselrod *et al.*, 1981).

A reasonable conceptual model for the processes controlling HRV is that sympathetic tone operates on an extended time frame whereas vagal tone contributes to beat-to-beat (i.e. high frequency) alterations in HR. The continual adjustment in vagal tone is thought to be due to the baroreceptor reflex. Therefore HRV is a simple surrogate of arterial baroreceptor reflex function. Reductions in HRV can reflect impairments at various levels in the baroreceptor reflex arch, including loss of baroreceptor sensitivity due to increased arterial stiffness at the site of the afferent baroreceptor reflex stretch receptors in the aorta and the

carotid. A decrease in baroreceptor sensitivity may also influence BP as the fine-tuning of BP is regulated by the beat-to beat feedback of cardio-vagal activity from the central baroreflex arch.

The importance of HRV has been illustrated in population studies; a reduced HRV predicts all-cause and cardiac mortality in both the general population (Tsuji *et al.*, 1996; Dekker *et al.*, 1997; Dekker *et al.*, 2000) and in persons with established coronary disease (Kleiger *et al.*, 1987; Janszky *et al.*, 2004). Similarly, resting HR, under inhibitory control by the vagus, is also a measure of parasympathetic function (Marieb, 1998), and an elevated resting HR is an independent risk factor for CVD and sudden death (Dyer *et al.*, 1980; Kannel *et al.*, 1987; Palatini *et al.*, 1999; Palatini & Julius, 1999; Jouven *et al.*, 2001).

Interventions that modify autonomic control toward parasympathetic dominance have desirable effects on CV health and can improve prognosis following coronary events (Curtis & O'Keefe, 2002). Indeed, regular aerobic exercise lowers resting HR (Wilmore *et al.*, 2001; Carter *et al.*, 2003), improves HRV (Carter *et al.*, 2003; Sandercock *et al.*, 2005) and reduces the HR response to sub-maximal exercise (Wilmore *et al.*, 2001; Carter *et al.*, 2003). Similarly, regular consumption of n-3 FA from fish or fish oil is associated with lower resting HR (Dallongeville *et al.*, 2003; Mozaffarian *et al.*, 2005) and can increase HRV in several subject populations including the elderly (Holguin *et al.*, 2005), those with chronic renal failure (Christensen *et al.*, 1998), MI survivors (Christensen *et al.*, 1996) and in healthy men (Christensen *et al.*, 1999). However the majority of these studies have used EPA-rich oils and consequently there is a paucity of data evaluating the effectiveness of DHA-rich fish oil in improving HRV.

Given their association with parasympathetic tone, measures of resting HR and HRV are therefore effective tools for assessing the success of interventions aimed at enhancing arterial baroreflex regulation of BP through improvements in vagal tone. In comparison to resting HR and HRV, a reduction in the HR response during sub-maximal exercise may reflect CV adaptations that improve cardiac efficiency.

7.2 AIM

This chapter will discuss 2 studies, the aims of which were:

- STUDY 1 - To determine the independent and combined effects of supplementation with n-3 FA (from DHA-rich fish oil) and regular moderate exercise on resting HR and HR response to exercise in subjects with risk factors for MS. and
- STUDY 2 - To determine the independent and combined effects of supplementation with n-3 FA (from DHA-rich fish oil) and regular moderate exercise on HRV in subjects with risk factors for MS.

Data collected for study 1 was from the main cohort of subjects, whereas HRV data were collected from a smaller sub-study cohort.

7.3 METHODS - STUDY 1

Eligibility criteria and methodology for subject recruitment has been previously described in Chapter 2. All clinical assessments conducted pre and post intervention were performed under fasting conditions and where possible at similar times of the day.

7.3.1 HR response to exercise

A graded exercise test was used to assess changes in heart rate, measured at 15-second intervals using a Polar Accurex Plus HR monitor (Sports Tester, Polar Electro, Finland), Subjects began the test by self-selecting a moderate walking speed at 0% incline on an electronic treadmill (Quinton Instruments, Model Q65, Washington, USA). After 10 minutes, the incline was increased to 5.0% grade for 5 minutes and then 10.0% grade for an additional 5 minutes. During a 2 min rest period at the end of each workload, subjects were seated while BP and HR were recorded. Steady state HR was determined by averaging the HR readings from the last minute of each workload.

7.3.2 Resting HR

Resting HR data were collected using the HDI/*Pulsewave* CR-2000 Cardiovascular Profiler (Hypertension Diagnostics Inc., Eagan, MN, USA), the same instrument described in Chapter 5 used for the assessment of BP and arterial compliance.

7.3.3 Statistical analysis

Details of statistical analysis are provided in Chapter 2.

7.4 RESULTS – STUDY 1

7.4.1 Effects of n-3 FA and/or exercise on resting HR

There were no differences between groups at baseline (Week 0) for resting HR (P>0.96) (**Table 7.1**). The change in resting HR by Weeks 6 and 12 are presented in **Figure 7.1**. Despite an average reduction of 3.4 bpm in the FOX group at Weeks 6 and 12, nested ANOVA failed to detect any significant interactions between treatments or over time. However, there was a strong trend toward a reduction in resting HR by fish oil (.P>0.09).

Table 7.1 Resting HR (bpm) measured at Weeks 0, 6 and 12 by treatment group.

		Treatment Group			
		FO (n=17)	***FOX (n=16)***	***SO (n=18)***	***SOX (n=14)***
Resting HR (bpm)	*Week 0*	62.3 ± 1.6	62.0 ± 2.2	61.0 ± 1.8	62.0 ± 2.3
	Week 6	61.9 ± 1.6	58.6 ± 2.4	61.4 ± 2.0	61.9 ± 2.4
	Week 12	61.1 ± 1.6	58.6 ± 2.3	61.9 ± 1.4	61.0 ± 2.3

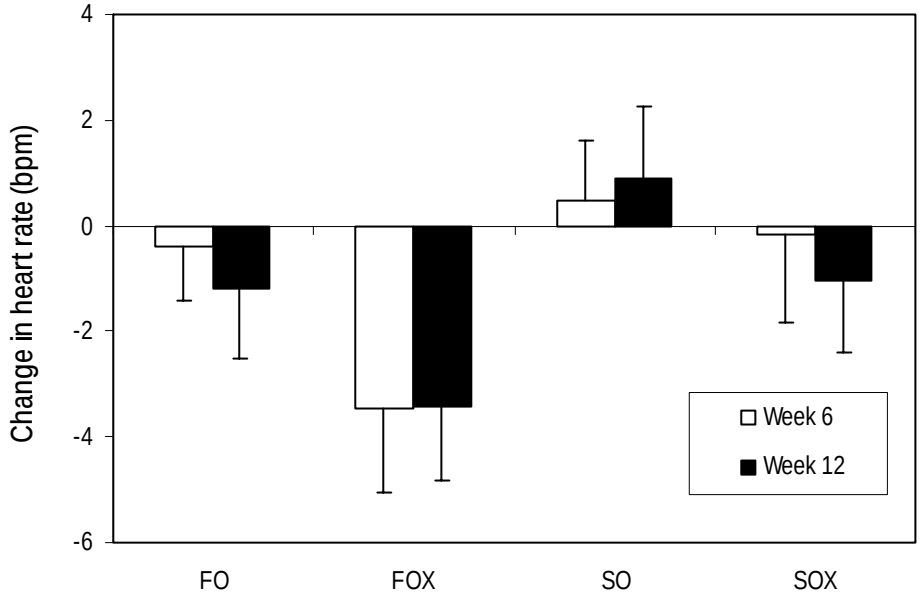

Figure 7.1 Change in resting HR by treatment group. Nested ANOVA did not detect any significant treatment interactions.

7.4.2 Effects of n-3 FA and/or exercise on HR response to exercise

Treatment groups did not differ in their HR response to exercise at baseline (P>0.80). The effects of treatment on HR response during a multi-stage (3 workloads) exercise test were assessed by 4-factor ANOVA (oil, exercise, workload and time). For the study population as a whole, time (P<0.001) and workload (P<0.001) effects were detected, with HR increasing as workload intensity increased, and the magnitude of the HR response to exercise decreasing progressively over the intervention period. **Figure 7.2** shows the HR response at each workload for the four treatment groups. For each treatment group, the change in HR from Week 0 to Week 12 at the three workloads was of similar magnitude (P>0.86). Nested analysis revealed that fish oil significantly attenuated the HR response to exercise (-9.4 bpm) compared with sunflower oil (-3.7 bpm) (Figure 7.2: P=0.0003).

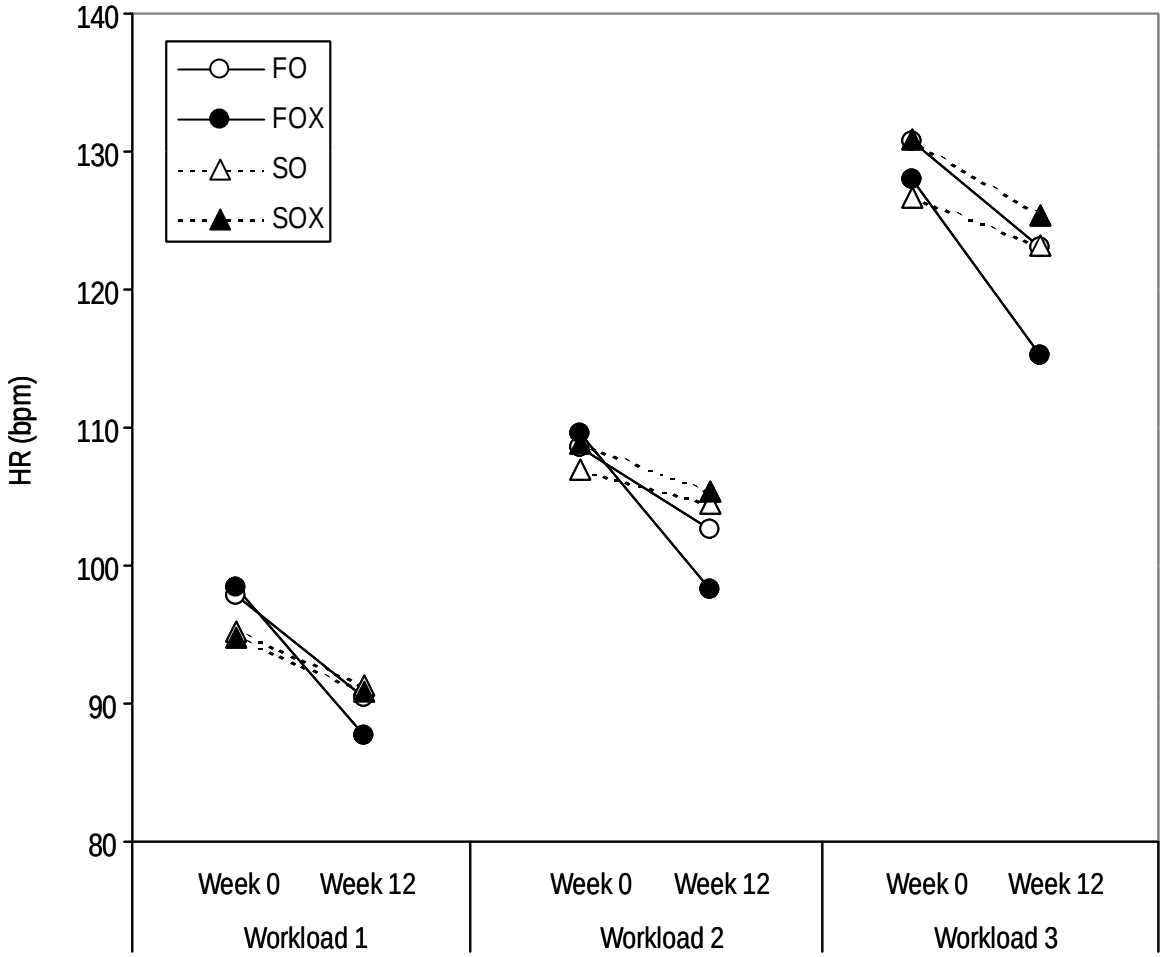

Figure 7.2 Change in HR response to sub-maximal exercise by treatment group. Fish oil significantly attenuated the HR response to exercise (P<0.0003).

7.5 METHODS - STUDY 2

The study of HRV was performed in conjunction with Dr Daniel Ninio, a cardiologist and PhD candidate in the Discipline of Physiology at the University of Adelaide, as part of his candidature. Subjects involved in this study were also enrolled in the main trial and therefore eligibility criteria, methodology for subject recruitment and testing conditions were identical to those described earlier.

7.5.1 HRV

7.5.1.1 Data Collection

ECG leads were placed above the first intercostal space to the right of the sternum and at the eighth intercostal space on the right anterior axillary line. An earthing lead was placed on the abdomen of sufficient distance from the negative and positive leads to prevent electromagnetic distortion. Subjects were required to rest in a supine position for a period of 10 min before ECG recording began. ECG signals were amplified using a PowerLab Bioamplifier (ADInstruments, Bella Vista, Australia) and data recorded at 1 KHz sample rate, with filters set at 20 Hz low pass and 0.3 Hz high pass. A threshold-crossing detector on the differentiated ECG signal measured time of R wave occurrence [as recommended by Friesen et al. (Friesen *et al.*, 1990)]. R-R intervals and the derived power spectrum were calculated by the software package Chart v5 for Windows (ADInstruments, Bella Vista, Australia). ECG data were recorded for a 20 min period. All data were collected according to the Task Force guidelines for HRV (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).

7.5.1.2 Data Analysis – Frequency Domain analysis

LFP corresponds to cycles between 0.04-0.15 Hz and is predominantly representative of sympathetic activity, while HFP is from 0.15-0.40 Hz. and is almost entirely determined by parasympathetic activity (Akselrod *et al.*, 1981). These frequency domains (LFP and HFP) can also be presented as a ratio (LFP/HFP). Frequency domains were transformed to normalised units (nu) by the HRV recording software. The formula used to calculate normalised units was:

$$\text{Power [Frequency (nu)]} = \frac{\text{Power (Frequency) value}}{(\text{Total Power} - \text{Very Low Frequency Power} - \text{Very High Frequency Power}) * 100}$$

7.5.1.3 Data Analysis Statistical Analysis

Details of statistical analysis are provided in Chapter 2.

7.6 RESULTS – STUDY 2

7.6.1 Subjects

Data for this sub-study were attained from 46 subjects. Baseline characteristics for these subjects are listed in **Table 7.2**. This cohort of subjects exhibited similar characteristics to subjects in the entire study population (see Chapter 2). There were no differences between the four intervention groups for any of these characteristics in the HRV sub-study cohort (Table 7.2).

Table 7.2 Heart rate variability sub-study subject entry characteristics by treatment group.

	Treatment Group			
	FO (n=13)	***FOX (n=10)***	***SO (n=14)***	***SOX (n=9)***
Sex (M:F)	4 : 9	4 : 6	6 : 8	3 : 6
Age (years)	52 ± 2	48 ± 1	51 ± 3	50 ± 3
BMI (kg/m^2)	35 ± 2	33 ± 2	34 ± 1	33 ± 1
% Body Fat	45 ± 2	42 ± 3	43 ± 2	44 ± 3
Systolic BP (mmHg)	126 ± 4	130 ± 5	126 ± 3	131 ± 4
Diastolic BP (mmHg)	71 ± 3	77 ± 3	72 ± 2	75 ± 3
TC (mmol/L)	6.9 ± 0.4	6.1 ± 0.5	6.0 ± 0.4	7.1 ± 0.5
TAG (mmol/L)	1.5 ± 0.1	2.1 ± 0.7	1.7 ± 0.3	2.1 ± 0.3

7.6.2 Effects of n-3 FA and/or exercise on HRV

There were no differences between groups at baseline (Week 0) for any measures of HRV (P>0.21). ANOVA indicated several 2-factor interactions (**Table 7.3**). Fish oil supplementation increased HFP (oil x time interaction, P<0.011) (**Figure 7.3**), while exercise x time interaction (P<0.012) was detected for LFP (**Figure 7.4**). Post-hoc analysis showed a significant increase in HFP in subjects supplemented with fish oil (P = 0.022) such that by week 12, HFP in this group was significantly greater than in subjects supplemented with sunflower oil (P = 0.002). The decrease (i.e. reduction) in LFP occurred in the non-exercise group. Post-hoc analysis indicated that in non-exercising subjects LFP decreased, but despite this decrease, actual LFP values were not different from those for exercising subjects at Week 12. It is hypothesised that this reduction was simply associated with elevated sympathetic excitability at baseline, which by Week 12 had dropped to similar levels to the other treatment groups. Although it did not reach statistical significance, there was a strong trend toward an improvement in the HFP/LFP ratio (P<0.062).

Table 7.3 Measures of heart rate variability by treatment group.

		Treatment Group			
		FO (n=13)	***FOX (n=10)***	***SO (n=14)***	***SOX (n=9)***
HFP (nu) [1]	*Week 0*	37.07 ± 6.01	38.32 ± 5.39	37.62 ± 3.39	37.41 ± 4.12
	Week 12	45.18 ± 6.36	43.32 ± 5.60	34.69 ± 4.50	36.70 ± 1.89
LFP (nu) [2]	*Week 0*	59.03 ± 5.99	52.14 ± 4.89	60.17 ± 3.64	54.64 ± 3.59
	Week 12	48.07 ± 6.45	54.01 ± 5.17	54.46 ± 5.75	57.25 ± 3.86
Ratio (HFP/LFP)	*Week 0*	3.16 ± 1.00	1.93 ± 0.47	1.99 ± 0.27	1.26 ± 0.20
	Week 12	1.99 ± 0.63	2.01 ± 0.49	2.68 ± 0.96	1.68 ± 0.27

[1] Significant oil x time interaction, $P<0.05$.

[2] Significant exercise x time interaction, $P<0.05$.

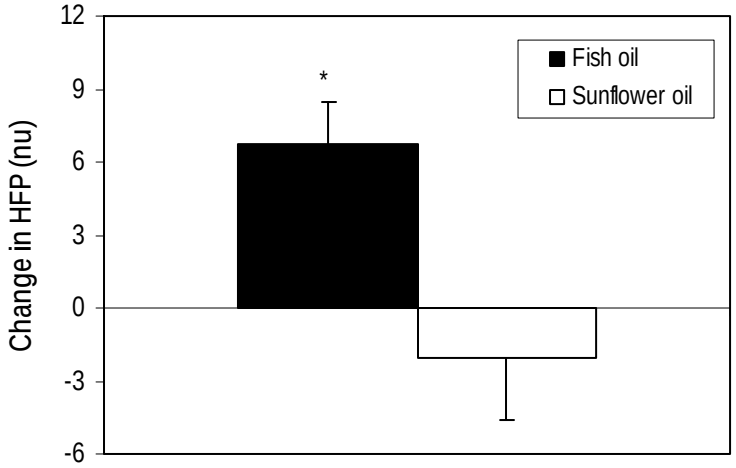

Figure 7.3 Change in HFP by oil treatment. *Fish oil significantly increased HFP compared to sunflower oil ($P<0.05$).

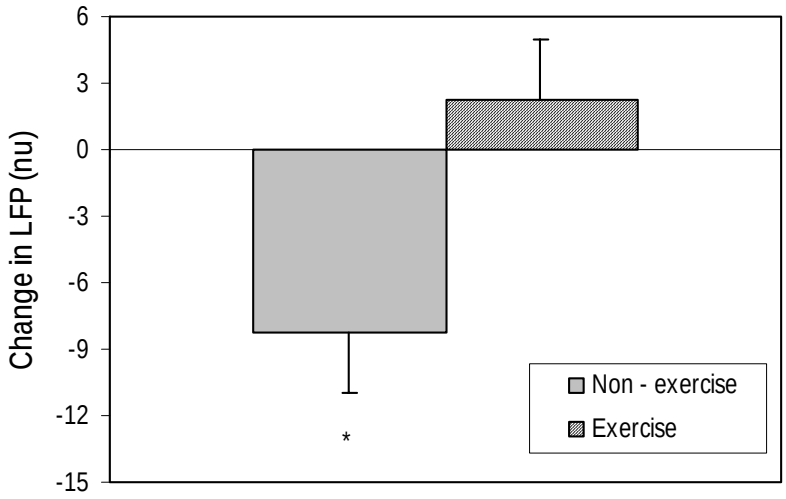

Figure 7.4 Change in LFP by exercise treatment. *Significantly greater reduction in LFP in non-exercising compared to exercising subjects (P<0.05).

7.7 DISCUSSION

7.7.1 Effect of n-3 FA and exercise on resting HR, HR response to exercise & HRV

Several epidemiological studies have demonstrated an inverse association between regular fish consumption and death from coronary heart disease, especially nonsudden death from MI (Kromhout *et al.*, 1985; Dolecek & Granditis, 1991; Daviglus *et al.*, 1997; Hu *et al.*, 2002). Additional evidence for the cardio-protective benefits of n-3 FA has come from randomised controlled trials, notably the Diet and Reinfarction Trial (DART) (Burr *et al.*, 1989) and GISSI-Prevenzione Study (GISSI-Prevenzione Investigators, 1999). These trials reported significant reductions in all-cause mortality (29 and 20%, respectively) following increased intake of dietary fish (Burr *et al.*, 1989) and fish oil supplementation (GISSI-Prevenzione Investigators, 1999). The mechanisms behind these benefits are not entirely clear but may at least in part, reflect antiarrhythmic activity and changes in autonomic function.

Previous studies have demonstrated that both regular fish consumption (Dallongeville *et al.*, 2003) and supplementation with fish oil can lower resting HR (Mozaffarian *et al.*, 2005). In a large population based trial, using non-parametric analysis, Mozaffarian et al. (Mozaffarian *et al.*, 2006) evaluated the association between HR and usual dietary intake of n-3 FA (EPA + DHA). They observed a steep decline in HR with doses up to 0.3 g/day, followed by a gradual reduction with doses increasing to 1.5 g/day. Comparing those who did not consume fish to the highest fish intake (~1.07 ± 0.44 g/day n-3 FA) was associated with a 3.2 bpm difference in resting HR. However, despite an average reduction in resting HR of 3.4 bpm in the FOX group, we did not observe any significant effects of fish oil in this study. In their meta-analysis, Mozaffarian et al (Mozaffarian *et al.*, 2005) identified several factors influencing the magnitude of HR reduction. Fish oil had a greater effect in individuals with a baseline HR ≥ 69 bpm (-2.5 vs. HR < 69; -0.4 bpm), and in interventions of ≥ 12 weeks duration. These factors, in conjunction with the substantially smaller sample size may explain the lack of effect in this study.

Supplementation with n-3 FA from DHA-rich fish oil significantly attenuated the HR response to sub-maximal exercise, an effect that was independent of exercise training. Using the same duration and dose of fish oil as the present study, Buckley et al. (Buckley *et al.*, 2005) reported that fish oil significantly reduced the HR response to maximal exercise in athletes. This effect has also been observed in exercising horses (O'Connor *et al.*, 2004) and in patients with stable coronary artery disease (Mehta *et al.*, 1988), although with substantially greater doses of n-3 FA (5.4 g/day). In contrast, two short-term studies found no effect of fish oil in reducing peak HR during exercise (Vacek *et al.*, 1989; O'Keefe *et al.*, 2006). O'Keefe et al. (O'Keefe *et al.*, 2006) reported that fish oil can accelerate the return to a normal HR after exercise. HR recovery after exercise is an independent predictor of mortality (Cole *et al.*, 2000). It is possible that the attenuated HR response during exercise in this study is due to an increase in cardiac efficiency, reflecting changes in cardiac pre- and post-load and stroke

volume. These factors may be influenced by n-3 FA mediated changes in blood vessel resistance and blood viscosity and the incorporation of n-3 FA into cardiac tissue.

There has shown to be a distinct relationship between HRV and levels of n-3 FA (measured in cholesteryl esters, granulocytes and platelets) in both healthy individuals (Christensen *et al.*, 1999; Brouwer *et al.*, 2002) and those at risk of CV events (Christensen *et al.*, 1997; Christensen *et al.*, 1998; Christensen *et al.*, 2001). Supplementation with n-3 FA has also been shown to increase HRV in several subject populations including the elderly (Holguin *et al.*, 2005), those with chronic renal failure (Christensen *et al.*, 1998), MI survivors (Christensen *et al.*, 1996) and in healthy men, but not healthy women (Christensen *et al.*, 1999). Results from the present study are in agreement with the studies described above; DHA-rich fish oil supplementation increased HFP and is therefore indicative of an improvement in parasympathetic tone. This is of particular relevance as overweight individuals generally have enhanced sympathetic activity and reduced baroreflex sensitivity compared with non-obese populations (Karason *et al.*, 1999). To our knowledge, this is the first study to investigate the effect of a DHA-rich fish oil supplement on HRV in healthy overweight/obese subjects without a history of CVD.

Animal studies show DHA is preferentially incorporated into myocardial membranes (McLennan, 2001) and is more effective than EPA in preventing ischemia-induced arrhythmias (McLennan *et al.*, 1996). Several studies have reported a decrease in HR with DHA, but not EPA (Grimsgaard *et al.*, 1998; Mori *et al.*, 1999a; Woodman *et al.*, 2002), and this reduction has been related to an increase in serum phospholipid DHA (Grimsgaard *et al.*, 1998), which increases membrane fluidity. Omega-3 FA induced changes in membrane fluidity may elicit anti-arrhythmic effects by modifying ion currents, and associated changes in cell excitability (Demaison & Moreau, 2002). Erythrocyte EPA + DHA content is highly correlated with cardiac n-3 FA content (Harris *et al.*, 2004) and although independent correlations for DHA were not reported, fish oil supplementation resulted in similar changes in the content of DHA in erythrocytes and cardiac tissue (Harris *et al.*, 2004). In rabbits,

feeding DHA-rich fish oil substantially increased the incorporation of n-3 FA in RBC and cardiac tissue (atrium and ventricle) (Ninio *et al.*, 2005). The n-3 FA content (%) of these tissues was almost identical at the end of the 12-week trial, and entirely due to the increase in DHA. Furthermore, the incorporation of n-3 FA in the atrium was associated with protection against stretch-related atrial fibrillation.

Exercise training has been shown to lower resting HR (Wilmore *et al.*, 2001; Carter *et al.*, 2003), increase HRV (Carter *et al.*, 2003; Sandercock *et al.*, 2005), reduce HR response during exercise (Wilmore *et al.*, 2001; Carter *et al.*, 2003), and improve HR recovery following exercise (MacMillan *et al.*, 2006; Streuber *et al.*, 2006). The mechanisms behind these adaptations are not entirely clear, but regular exercise appears to increase parasympathetic activity while decreasing sympathetic activity, the underlying effect being an improvement in autonomic tone. Exercise also induces a number of CV adaptations including cardiac hypertrophy (of which an increase in left ventricle size is most prominent), increased stroke volume and cardiac output (particularly during exercise), and changes in blood flow, volume and pressure, which results from increased capillarisation, greater opening of existing capillaries and more effective blood redistribution (Wilmore & Costill, 1999). These adaptations can eventually lead to a reduction in cardiac afterload and an increase in cardiac preload.

7.7.2 Summary & conclusion

The majority of studies investigating the effects of intervention with n-3 FA or exercise have focused on subjects at risk for future cardiac events, such as those with diabetes, renal disease or a history of MI. To our knowledge, this is the first trial to investigate the effects of supplementation with DHA-rich fish oil in overweight/obese subjects without a history of CVD. These results confirm the benefits of n-3 FA for improving parasympathetic tone, as evident by the increase in HFP and a decreased HR response during exercise. The

latter may also reflect an increase in cardiac efficiency, influenced by factors such as resistance to blood flow and blood viscosity, both of which are modified by n-3 FA. We did not however, show an improvement in any of these parameters following regular exercise training. This lack of effect is likely due to the relatively light training volume.

8 GENERAL DISCUSSION

8.1 KEY OUTCOMES FOR THIS STUDY

In addition to confirming, and providing further insight into some of the well-known CV and metabolic effects of n-3 FA and regular aerobic exercise, the work described in this thesis also identified a novel effect of n-3 FA on improving body composition, which has not been previously described and may be beneficial to human health.

8.1.1 Benefits of n-3 FA supplementation in an overweight population with increased metabolic risk

Following supplementation with DHA-rich fish oil, the proportion of long chain n-3 FA, particularly DHA, in erythrocytes increased substantially. This increase was associated with an increase in HDL cholesterol and a significant reduction in TAG which, as may be expected, was greater in individuals with high pre-treatment TAG levels (Chapter 4). These changes are likely to translate to a reduced CV risk (Mozaffarian & Rimm, 2006).

Apart from some improvements in lipid parameters, supplementation with DHA-rich fish oil also improved HRV (Chapter 7) and endothelial function (FMD) (Chapter 5). Endothelial dysfunction can lead to a decrease in compliance of the carotid artery and aorta, resulting in a decrease in baroreceptor sensitivity, reflected by low HRV. This increase in coronary stiffness can lead to left ventricular hypertrophy (Boutouyrie *et al.*, 1995). Although the improvement in endothelial function in this study was not accompanied by any change in BP, it is plausible that long-term treatment may result in a reduction in BP by all of the above mechanisms i.e. by preventing arterial stiffness and coronary and arterial hypertrophy.

Additionally, HR at a given work load during sub-maximal exercise was reduced following DHA-rich fish oil supplementation, suggesting that the n-3 FA improved cardiorespiratory fitness (Chapter 7).

While many studies have reported the immune and anti-inflammatory benefits of fish oil supplementation, few have focused specifically on the effects of DHA-rich fish oil on neutrophil function. The work described in this thesis also showed that DHA-rich fish oil can reduce superoxide production by stimulated neutrophils, where the magnitude of reduction is proportional to the change in DHA or long chain n-3 FA content of erythrocytes (Chapter 6).

Perhaps one of the most important findings of this thesis was that, without any changes in diet, DHA-rich fish oil supplementation independently reduced body fat (Chapter 3). Subjects who were allocated to the sunflower oil (placebo) treatments gained body fat as a result of continuing to consume their usual diet that had made them overweight / obese in the first place, while subjects who consumed the fish oil supplements, in addition to consuming a background diet that was not different from the subjects consuming the sunflower oil, lost body fat. This finding is important because it indicates that DHA-rich fish oil supplementation may provide a simple, and sustainable, method for reducing body fat in overweight / obese populations. Longer-term studies should be conducted to confirm the efficacy, and determine the sustainability, of n-3 FA supplementation using fish oil as a strategy for reducing obesity.

8.1.2 Potential benefits of regular aerobic exercise in an overweight population with increased metabolic risk

Regular exercise is a cornerstone in the management of overweight and obesity and its benefits for CV and metabolic health outcomes are widely recognised. However, it appears that the levels of exercise required to reduce body fat may be unrealistic for many individuals. The aerobic exercise program employed in this thesis was modest, only requiring subjects to exercise three times per week for 45 min at moderate intensity. The high level of compliance suggests that this level of activity is sustainable, at least for 12 weeks, for the majority of

overweight / obese people who are generally unaccustomed to undertaking regular exercise. Despite this level of physical activity, subjects who undertook the exercise program achieved modest reductions in total body fat and abdominal fat mass (Chapter 3). These reductions most likely resulted from the well-described increase in fat oxidation during exercise, which occurred as subjects improved their fitness through exercise training. Therefore, this exercise program provided a metabolic benefit in that it increased the ability to oxidise fat during exercise.

In terms of improving CV function, the exercise program independently improved the compliance of small arteries (Chapter 5). Although no effect of exercise on BP was seen in the current study, this improvement in small artery compliance may ultimately contribute to BP reduction, particularly in subjects with elevated BP, if exercise training is sustained for a longer duration.

While n-3 FA reduced neutrophil superoxide activity, which might potentially reduce tissue damage, regular aerobic exercise assisted in maintaining neutrophil bactericidal activity (Chapter 6). These independent effects of n-3 FA and exercise suggest that n-3 FA may reduce the potential for excess superoxide to cause tissue damage, while exercise ensures that, despite this reduction, neutrophils maintain their capability to destroy bacteria, and therefore continue to protect against infection.

8.1.3 Potential benefits of combined regular aerobic exercise and n-3 FA in an overweight population of increased metabolic risk

Both n-3 FA and regular aerobic exercise have multiple beneficial effects on several CV, metabolic and inflammatory biomarkers. Although there was no evidence of synergism, there appeared to be an additive effect of n-3 FA and exercise on reductions in body fat, providing further support for combining diet and physical activity interventions for the management of CVD risk.

8.2 PUBLIC HEALTH IMPLICATIONS

Current physical activity guidelines for Australia and North America recommend participation in 30 min (continuous or cumulative) of moderate-intensity activity on most, preferably all days of the week to achieve health benefits, with longer durations (60-90 min) advised for body weight loss or maintenance. While these recommended levels of physical activity will generally result in improvements in body composition and reduce CV and metabolic risk, they generally exceed what one might expect an overweight/obese population who are not accustomed to regular exercise to undertake. Indeed, current data suggests that only approximately 50% of people comply with recommended levels of physical activity. The physical activity intervention employed in this thesis differed from these guidelines in both duration and frequency of activity; and attempted to prescribe a level of physical activity which could be sustained, at least for 12 weeks, by a sample of subjects who were not accustomed to undertaking regular exercise. Indeed, we were successful in achieving this, since > 85% of subjects complied with the exercise (and supplementation) requirements of the study.

The major CV benefits attained from supplementation with DHA-rich fish oil in this trial were a reduction in TAG and an improvement in endothelial function. Supplementation provided ~1.9g/day n-3 FA, which is 10-fold greater than the NHMRC defined adequate intakes (160 mg for men, 90 mg for women) for Australian adults (National Health and Medical Research Council, 2006). Furthermore, this is 4-fold greater than the NHMRC recommended target intakes (610 mg/day for men and 430 mg/day for women) for the prevention of chronic disease. Although higher than the recommended target intake for Australians, this study indicates that there is potential for a) similar health benefits to be attained from lower doses of n-3 FA or b) greater benefits over a longer time frame. However, these recommended target intakes are based on percentiles of current population intake, and

their relevance to health outcomes is uncertain. Consequently, a dose-response study is warranted to evaluate the effectiveness of lower doses of DHA.

In addition to fish oil capsules, n-3 FA are easily obtained from fish, particularly oily fish such as salmon (Mozaffarian & Rimm, 2006). Although concentrations vary between fish sources, 100-200g/day of salmon would provide an equivalent n-3 FA dose to that administered in this study. However while the health benefits of regular fish consumption are seemingly well established, there is some concern as to the potential risks of fish consumption, particularly relating to mercury exposure. As yet, health effects from low levels of mercury exposure (as seen with fish consumption) are yet to be established, but the evidence suggests that the benefits of fish consumption greatly exceed the potential risks (Mozaffarian & Rimm, 2006).

While the exercise protocol in this study induced modest fat loss, it did not impact on other CV risk factors (e.g. blood lipids and endothelial function) known to be improved by regular physical activity. However when undertaken in conjunction with n-3 FA supplementation, not only did we observe an improvement in these risk factors, but we saw an additional benefit on body fat, suggesting that this combination can convey multiple health benefits.

8.3 STUDY LIMITATIONS

A limitation of this study was the inclusion of subjects with varied CV risk profiles, i.e. not every subject was hypertensive or hypertriglyceridaemic, which therefore limited the extent to which n-3 FA and exercise could modify these risk factors. Additionally a longer study duration would have further distinguished this from other n-3 FA intervention trials, the majority of which run from 6-12 weeks, and would have enabled us to determine the longer-term effect of n-3 FA supplementation. Furthermore, although we observed improvements in several CV, metabolic and inflammatory biomarkers, it is unclear whether these are clinically relevant, particularly the changes in superoxide production and bactericidal activity.

8.4 FUTURE DIRECTIONS

The results of this study suggest that both exercise and n-3 FA from fish oil can favourably modify several CV risk factors. Of particular interest, and requiring more thorough investigation is the finding that supplementation with DHA-rich fish oil can reduce body fat. Supplementation with n-3 FA is a simple intervention which, based on data from the present study, can be adhered to by most overweight / obese people and might therefore provide a potential strategy for longer-term obesity management. In animal studies, changes in fat mass have been attributed to an increase in peroxisomal and mitochondrial fatty acid oxidation (Baillie *et al.*, 1999). However the exact mechanisms of action and their relevance to humans are yet to be elucidated. In order to fully evaluate the public health implications of this preliminary finding, a controlled dose-response study over a longer time frame is warranted.

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APPENDICES

NOTE: Appendices A, B, C and D are included in the print copy of the thesis held in the University of Adelaide Library.

APPENDIX E

Ms Alison Hill
University of Adelaide
Frome Rd, Adelaide
Ph (08) 8303 3223
Email:
alison.hill@adelaide.edu.au

UNIVERSITY OF SOUTH AUSTRALIA
Division of Health Sciences

VOLUNTEER INFORMATION SHEET

Effect of omega 3 fatty acids and exercise on cardiovascular risk factors

Purpose of Study

The majority of middle-aged to elderly Australians are overweight, with high blood pressure (BP) and elevated blood fat and blood glucose concentrations. These characteristics represent an increased risk of cardiovascular disease. The performance of regular exercise and taking fish oil supplements which incorporate omega-3 polyunsaturated fatty acids have each been shown to improve cardiovascular risk, but no studies have examined whether combining both of these treatments is more effective than either treatment alone.

The aim of this project is to investigate the combined effect of omega-3 polyunsaturated fatty acid (omega-3) supplementation and moderate regular exercise on the improvement of cardiovascular risk factors. In particular we will investigate effects on blood pressure, blood vessel function, abdominal fat deposition, and blood fats in people with risk factors for cardiovascular disease.

What it Involves

The study will be conducted at the Nutritional Physiology Research Facility located at the University of South Australia (City East Campus). If you would like to participate in the study, you will be asked to complete a health questionnaire and have your blood pressure and body measurements taken. You will also be asked to provide a small fasting blood sample (40mL, equivalent to 2 tbspn) which will be taken by an experienced blood taker similar to a routine blood test. This will be used to measure your blood triglyceride and total cholesterol level and to measure blood glucose and blood insulin, to ensure you meet the particular inclusion criteria. At this time you will also have explained to you the study protocol, including how to complete a Physical Activity and Food Frequency Diary.

If you participate in the study, you will be randomly assigned to one of four groups. One group will maintain their normal lifestyle and take fish oil supplements, another group will maintain their normal lifestyle and take sunflower oil supplements, a third group will undertake a walking/jogging exercise program for 45 min 3 times per week and take sunflower oil supplements, and the final group will undertake a walking/jogging exercise program for 45 min 3 times per week and take fish oil supplements.

The study will run over a 12-week period and you will be required to attend the clinic for testing at week 0, 6 and 12 over two days of the intervention period (total of 6 visits in 12 weeks). At each vist, you are expected to have fasted overnight (min. 10-12 hours without eating, water is ok) and breakfast will be supplied following testing. We will measure your resting heart rate and blood pressure, weigh you and measure your height. A venous blood sample (40mL, equivalent to 2 tbspn) will also be taken so that we can measure your blood triglyceride and cholesterol levels, blood glucose and insulin concentrations and red blood cell membrane lipid composition, and various inflammatory markers,

including neutrophil functional responses. As a precaution, blood thinning agents, such as aspirin and warfarin, should not be taken 3 days prior to giving your blood sample.

On the first visit you will have your blood vessel function assessed by flow mediated dilatation (ultrasound) and arterial compliance. You will be required to lie still on your back for 10-15 minutes before the test will begin. Arterial compliance (stretch and elasticity) will be measured by having your right wrist placed in a comfortable support, a sensor will then be positioned on the skin where there is a visible pulse and a blood pressure cuff is also placed on the left upper arm to allow for repeated blood pressure measurements. Three measurements will then be recorded with each test lasting approximately 5 minutes each. Once these tests are complete, the sensor will be removed and the pressure cuff placed around the lower right arm, which will be inflated and held for 5 minutes. After 5 minutes, the cuff will be released and a probe (attached to an ultrasound machine) will be placed over the large artery in the arm (brachial artery) and the change in diameter of the artery in response to the release of the cuff will be measured. After a 10-minute recovery period, you will be given a nitroglycerin tablet (300 μg) that will be placed under the tongue for 3 minutes. Nitroglycerin dilates blood vessels and therefore increases blood flow through the vessels. After 3 minutes, if not completely dissolved, the tablet will be removed and your arterial dilation in response to this drug will be assessed by ultrasound. This session will last approximately one hour.

On your second visit, you will again have your weight measured and a blood sample taken. Body composition will then be assessed using a method called Dual Energy Xray Absorptiometry (DEXA), which will involve you lying on a special bed while a scanner moves along and scans your body. This procedure will take about 15 min.

Using a different technique, digital volume pulse oximetry (DVP), you will again have your endothelial function assessed at rest and after exercise. DVP is a simple, non-invasive test that involves placing a small, painless clip containing an infrared light on the index finger of the right hand. You will have three baseline measures recorded while seated (after a 10 minute rest period), which will take approximately 5 minutes. You will then partake in a walking exercise on a treadmill. The initial part of the test will last for 10 minutes and will be at a speed equivalent to a brisk walk. If you have been allocated to one of the exercise groups you will participate in the second stage of exercise testing. This test will involve walking at a comfortable pace on a treadmill as it gets steeper and steeper until you cannot go any further. You will be asked to wear a heart rate monitor, which sits conformably mid chest, while walking on the treadmill as a safety precaution to ensure you don't over exert yourself. All exercise tests will be conducted under medical supervision, and as an additional precaution you will have your heart monitored by electro-cardiogram (ECG).

During the walking test you will have a mouthpiece similar to that used by scuba divers in your mouth so that we can measure the gases that you are breathing in and out. If you are involved in the second stage of exercise testing, we will also prick one of your fingers periodically to get small samples of blood so that your blood lactate concentration can be measured. The information that we gain during this test will be used to set your walking/jogging program. This test will last approximately 30 minutes.

Possible Risks

All procedures will be carried out by qualified personnel. However, in order for you to make an informed decision, the risks associated with procedures are set out below: You may seek further clarification from the medical practitioner who will be present during exercise tests.

Nitroglycerin: is a non-prescription drug, which can be purchased over the counter at any pharmacy. It is predominately used to prevent or relieve chest pain and works by relaxing blood vessels to the heart, so the blood flow and oxygen supply to the heart is increased. The glyceryl trinitrate can cause a headache in 60% of people and in a minority it may cause, dizziness, nausea or vomiting. In less than 1% of people it may cause a skin rash. To prevent the likelihood of any of these side effects occurring, nitroglycerin will be administered to you on an empty stomach and there will be a qualified doctor available at all times throughout testing. It is also advisable you do not drive a car, drink alcohol or operate heavy machinery a couple of hours after taking the nitroglycerin

Blood sampling: will be taken by venepuncture and by fingerprick. The risks associated with these procedures are:

- infection - although all of the needles will be sterile and all reasonable precautions will be taken, in any situation involving penetration of the skin there is a slight risk of infection.
- blood clotting - insertion of a needle into a blood vessel involves a risk of a blood clot forming which can travel through the circulation and block a smaller blood vessel somewhere else. However, the danger of this occurring is considered to be remote.
- bruising - it is possible that you may experience slight bruising around the area where the needle was inserted. This is nothing to worry about as any such bruising should clear up within a few days.

Blood thinning agents, such as aspirin, warfarin and ginko, should not be taken three days prior to sampling.

Dual Energy Xray Absorptiometry (DEXA): will be used to assess body fat levels. DEXA involves the administration of a very low dose of radiation, which is not much more than would be encountered in everyday life. The exact entrance dose is 0.08 µSv for the two scans combined. This dosage is exceptionally low, less than would normally be received as daily background radiation. In Sydney, daily background radiation is approximately 6 µSv/day.

Exercise: If you are allocated to one of the two groups that will undertake the treadmill exercise testing, because you have risk factors for cardiovascular disease you are at an increased risk of suffering a cardiovascular incident during this type of high intensity exercise. As a precaution, you will be asked to attend a pre-trial, supervised exercise screening visit, where a cardiologist will assess your response to exercise and ultimately whether it is safe for you to participate in the trial. This visit will also serve as a familiarisation session, to ensure that you know what to expect when you attend your visits during the trial.

Blood Pressure: High blood pressure increases the risk of cardiovascular disease. The most common forms of treatment are through diet or lifestyle (including exercise) modification and drug therapy. If at any stage you are concerned about your blood pressure in relation to your participation in the study, you should consult your doctor who will advise you on appropriate management. If your blood pressure is 140/90 mmHg or greater and you have risk factors for cardiovascular disease, you should speak to your doctor before participating. As a precaution, if your blood pressure measurement exceeds 160/100 mmHg at any stage during the study, you will be withdrawn and referred to your doctor.

All information collected as part of the study will remain confidential and no information that could lead to identification of any individual will be released. Participants in the study may withdraw at any stage. This is a volunteer based study, and therefore no payment for participation will be made. However, on completion of the study you will be provided with a copy of your individual results.

Prior to your participation in the study, we will be contacting your General Practitioner to confirm your suitability in the study. However, contact will only be established once your approval for this procedure has been given.

Further Information

If you would like to participate in the study, or require more information to help you arrive at a decision, please contact: Ms Alison Hill *B App Sci., Hons*. Discipline of Physiology, University of Adelaide or Dr Karen Murphy *B App Sci., Hons., PhD.* Division of Health Sciences, University of South Australia on 8303 3223

E-mail: alison.hill@adelaide.edu.au karen.murphy@adelaide.edu.au

If you, or any member of your family, would like to discuss any ethical concerns about the study with a person not directly involved in the conduct of the study, please refer to the attached "Contacts for information on project and independent complaints procedure" form.

APPENDIX F

THE UNIVERSITY OF ADELAIDE AUSTRALIA

UNIVERSITY OF SOUTH AUSTRALIA
Division of Health Sciences

Diet and Lifestyle Questionnaire

Title: Effect of omega-3 fatty acid supplementation in combination with exercise on cardiovascular risk in the metabolic syndrome

In this questionnaire general questions are asked about your health, diet and some background information about yourself. All information will be kept strictly confidential. If you have any concerns about the questions in this form or have difficulty in answering any questions please do not hesitate to contact
Ms. Alison Hill (8303 3223)

1. Title: Dr Mrs Mr Miss Ms

2. Full name: _____

3. Date: _____

4. Address: _____

_____

5. Telephone Nos: **Home:** _____

Work: _____

Mobile: _____

6. Date of birth:_____day_____month_____year

7. Age:_____

PLEASE COMPLETE IF KNOWN:

Weight:_____kg **Height:**_____cm BMI**:**_____

Blood Pressure: _____ **Pulse:**_____

Total Cholesterol Level (if known) :_____ **Date last taken:**_____

Triglyceride Level (if known) :_____**Date last taken:**_____

PLEASE COMPLETE THE FOLLOWING DETAILS :

8. Doctor's Information

DOCTOR's NAME_____

Name of Practice _____

Address _____

_____ Post Code _____

Telephone_____

Would you be happy for us to notify your doctor of your involvement in this study and if necessary contact them directly (please tick) **Yes** ☐ **No** ☐

9. Have you had, or do you have :	**Yes**	**No**	**Not sure**
Arrhythmia	☐	☐	☐
High blood pressure	☐	☐	☐
Angina	☐	☐	☐
Heart attack	☐	☐	☐
Stroke	☐	☐	☐
High cholesterol (>6mmol/L)	☐	☐	☐
High triglycerides (>2mmol/L)	☐	☐	☐
Diabetes	☐	☐	☐
Peripheral vascular disease	☐	☐	☐
Cardiac valve abnormality	☐	☐	☐
Hyperglycaemia	☐	☐	☐

Please give details (and medication, if any):_____

_____

Are you undergoing treatment for any of the above conditions? **Yes** ☐ **No** ☐

If yes please give details:_____

_____

10. Do you have a family history of :	**Yes**	**No**	**Not Sure**
Obesity	❑	❑	❑
Heart disease	❑	❑	❑
Stroke	❑	❑	❑
High blood pressure	❑	❑	❑
Diabetes	❑	❑	❑
Hyperlipidemia	❑	❑	❑

11. Do you have any communicable diseases? (such as HIV and Hepatitis A, B or C)

Yes ❑ No ❑

12. Have you ever been a smoker ?

Yes ❑ No ❑

If yes, how long ago did you give up :_____

OR

If you **currently still smoke** how many cigarettes approx. would you smoke in a day:_____

How many years have you been a smoker? _____

13. Do you take any medication regularly ?

- Eg: do you regularly use aspirin, lipid and or blood pressure lowering drugs, drugs for diabetes or phosphodiesterase inhibitor medication (e.g. Viagra, Levitra) etc?

Yes ❑ No ❑

If yes, please give details: _____

_____

14. Do you regularly take vitamin, mineral or other dietary supplements?

Eg. Cod liver oil or fish oil capsules, Vitamin C etc.

Yes ❑ No ❑

If yes, please give details of dose and frequency of consumption:_____

_____

15. Would you consume more than two standard glasses of any alcoholic beverage :

Daily ☐ A few days a week ☐

Once a week ☐ Occasionally ☐

Rarely or never ☐ If yes, type of beverage:_____

16. Do you exercise :

Yes ☐ No ☐

17. What sort of exercise do you do each week? (please list responses)

How often do you do this type of exercise?

How long does each session last?

How would you describe your intensity or effort during exercise (light, moderate, hard)?.

18. Do you eat red meat regularly ?

Daily ☐ A few days a week ☐

Once a week ☐ Occasionally ☐

Rarely or never ☐ If yes, type of meat and quantity:_____

_____

19. Do you eat fish regularly ?

Daily ☐ A few days a week ☐

Once a week ☐ Occasionally ☐

Rarely or never ☐ If yes, type of fish and quantity (e.g 100g):_____

_____

*If **Female** please answer **Questions 20-23**.* *If **male** please go to **Question 24**.*

20. Would you consider yourself to be postmenopausal?

Yes ☐ No ☐

21. Are you, or do you suspect that you may be pregnant?

Yes ☐ No ☐

22. Are you currently on hormone replacement therapy (HRT)? (eg. Oral oestrogen- Premarin; oestrogen patch)

Yes ☐ No ☐

23. Have you ever taken HRT?

Yes ☐ No ☐

If so, please indicate when you last used HRT (please tick)?

- ☐ less than a month ago
- ☐ 3-6 months ago
- ☐ 6-8 months ago
- ☐ 8-12 months ago
- ☐ more than 12 months ago

24. Would you be able to attend two 1.5 hour long testing sessions a day apart?

Yes ☐ No ☐

25. What two days per week would suit you best?

Please list:_____

26. What time would best suit you for testing?

7.30-8.45 am ☐ **1**1.15-12:30pm ☐

8.45-10.00 am ☐

10.00-11.15pm ☐

***After hours testing may also be available on request**

Thank you for your co-operation. We will be selecting a study group of approximately 80 people, 40 females and 40 males. Please do not be offended if you are not chosen, selection into the study is based on a set list of criteria that you may have not met for whatever reason. We will be notifying everyone in due course, whether they have been selected or not. Those selected will then be asked to attend The University of South Australia City Easy Campus (Bonython Jubilee Building) on 6 occasions (over a 12 week period) to take part in the study.

APPENDIX G

THE UNIVERSITY OF ADELAIDE AUSTRALIA

UNIVERSITY OF SOUTH AUSTRALIA

Nutritional Physiology Research Group

OMEGA - EXERCISE STUDY 2004 SCREENING INTERVIEW

SCREENING ID ScOE_____ APPOINTMENT DATE & TIME _____

FIRST NAME _____ SURNAME _____

Height _____cm Weight _____kg BMI _____

Waist circumference_____cm Hip circumference_____cm WHR _____

DOB ____/___/____

STUDY CRITERIA:

Inclusion Criteria:

- ☐ Between 25 – 65 yrs of age
- ☐ BMI > 25
- ☐ Waist circumference males >102 cm/females < 88 cm
- ☐ BP > 140/90, < 160/110
- ☐ Triglyceride > 2.3mmol/L
- ☐ Total Cholesterol > 5.5mmol/L

Exclusion Criteria

- ☐ High level of physical activity – moderate exercise for 30 min duration >3x/wk
- ☐ History of peripheral vascular disease, stroke, cardiovascular or renal disease
- ☐ Known presence of Type 1 or 2 diabetes
- ☐ Height: <101cm or >255cm (limitation of Cardiovascular Profiler instrument)
- ☐ Eat fatty fish meal > 2 times/week
- ☐ Take anti-hypertensive and / or lipid lowering medication
- ☐ Are pregnant

BLOOD PRESSURE (3 measurements recorded, 1 min apart, seated, not talking):

Cuff size: M / L

	SYSTOLIC BP	DIASTOLIC BP	PULSE
1			
2			
3			
4			
AVERAGE			

BLOOD TEST:

Time:_____ **Fasting: Y / N** **Arm R / L**

Alcohol in last 24hrs: Y / N **Type/amount _____**

Vein Code **Comments: _____**

Collect: 1 x 4mL EDTA Tube (purple top) ☐
1 x 4mL Glucose Tube (grey top) ☐

VISITS:

What two days per week would suit you best to attend two 1.5 hour long testing sessions

Monday ☐ **Tuesday** ☐ **Wednesday** ☐ **Thursday** ☐ **day** ☐

What time would best suit you for a 1.5 hr testing session.
***Please note these are fasting visits.**

7.30-8.45 am ☐ 10.00-11.15pm ☐

8.45-10.00 am ☐ **1**1.15-12:30pm ☐

Investigator:_____ **Screening Code:**

Investigator use only

Total Cholesterol_____mmol/L Triglycerides _____mmol/L

Glucose _____mmol/L

Eligible for study ☐ **Yes** ☐ **No**

Ineligibility reason _____

APPENDIX H

Ms Alison Hill
University of Adelaide
Frome Rd, Adelaide
Ph (08) 8303 3223
Email:
alison.hill@adelaide.edu.au

UNIVERSITY OF SOUTH AUSTRALIA
Division of Health Sciences

Nutritional Physiology Research Group

University of Adelaide and University of South Australia

PROJECT CONSENT FORM

PROJECT TITLE: Effect of omega 3 fatty acids and exercise on cardiovascular risk factors

INVESTIGATORS:
Professor Peter Howe
Ms Alison Hill
Dr Karen Murphy
Dr. Jon Buckley
Dr David Saint
Dr Gary Scroop
Dr Daniel Ninio

1. I have read the Information Sheet, and the nature and the purpose of the research project and the risks inherent in my participation have been explained to me. I understand and agree to take part.

2. I understand that I may not directly benefit from taking part in the study.

3. I understand that while information gained during the study may be published, I will not be identified and my personal results will remain confidential.

4. I understand that I can withdraw from the study at any stage and that this will not affect my rights or the responsibilities of the researchers in any respect.

5. I have had the opportunity to discuss taking part in this study with a family member or friend.

6. I understand that I will not receive any payment for my assistance in this research project.

7. I confirm that I am over 18 years of age.

Name of Subject .....

Signed .....

Date .....

I have explained the study to the subject and consider that he/she understands what is involved.

Signed .....

Date .....