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BSc (HONS) SCIENCE IN HEALTH, EXERCISE AND SPORT.  

THE MOLECULAR MECHANISMS INVOLVED IN LIPID METABOLISM DURING LOW INTENSITY EXERCISE.  

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School of Health Sciences  
Biomedical Sciences.  

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Abstract.

An important molecular mechanism by which physical activity reduces cardiovascular risk is through regulation of plasma lipids. This study aimed to investigate whether low intensity exercise modulates monocytic lipid metabolism and the activity of the nuclear transcription factor Peroxisome Proliferators-Activated Receptor-γ (PPARγ). Furthermore, this study aimed to elucidate the molecular signalling mechanisms induced through PPARγ activation.

Thirty-four sedentary adults, mean age 45.6 ± 11.1 years, participated in an eight week low intensity exercise programme consisting of walking 10,000 steps, three times a week. Compared to controls, there was a significant decrease in total cholesterol (pre-exercise: 5.73 ± 1.39 mmol/L; post-exercise: 5.32 ± 1.28 mmol/L) and a significant increase in high density lipoprotein (pre-exercise: 1.46 ± 0.47 mmol/L; post-exercise: 1.56 ± 0.50 mmol/L) after the exercise programme. There was also a significant increase in serum oxidised LDL (oxLDL) concentrations pre to post exercise (0 weeks: 554 ± 107ng/ml; 4weeks: 698 ± 134ng/ml; 8weeks: 588 ± 145ng/ml).

A significant increase in leukocyte mRNA expression for PPARγ (4 weeks: 1.8 ± 0.9 fold; 8 weeks: 4.3 ± 1.9 fold) was observed, which was reinforced by increased PPARγ DNA-binding activity post exercise (pre-exercise: 0.22 ± 0.09 OD units; post-exercise: 1.13 ± 0.29 OD units. A significant increase in gene expression was observed for the oxLDL scavenger receptor CD36 (4 weeks: 3.8 ± 0.6 fold; 8 weeks: 2.7 ± 0.5 fold). LXRα (8 weeks: 3.5 ± 0.8 fold) and two LXRα regulated genes involved in RCT, namely ABCA1 and ABCG1 were significantly upregulated after eight weeks of exercise (8 weeks: ABCA1: 3.46 ± 0.56 fold; ABCG1: 3.06 ± 0.47 fold).

The culmination of in-vitro evidence lead to the postulation of a two pathway molecular mechanism associated with oxLDL stimulation of CD36, via PPARγ in monocytic cells. A ‘short-term’ pathway (<24 hours), upregulates PPARγ (2 fold) via a transient ERK1/2 and COX-2 dependent mechanism. Whereas a ‘long-term’ pathway (>24 hours), involves the direct upregulation of PPARγ via ligands within oxLDL, which is ERK1/2 independent but still COX-2 dependent. Activation of PPARγ enables direct DNA-binding with CD36, facilitating the oxLDL to enter the cell, via CD36, exacerbating the effect and promoting the cellular clearance of oxLDL. However, between 24 and 72 hours the ‘short-term’ pathway is required to upregulate PPARγ via COX-2 and hence induces upregulation of CD36. It is possible that over several weeks of low intensity exercise the more rapid molecular pathway can be supplemented by PPARγ ligands present within oxLDL, and hence directly stimulate PPARγ gene transcription.

In conclusion this study proposes a novel molecular mechanism for low intensity exercise induced modulation of plasma lipids via cellular clearance of cholesterol that involves activation of the nuclear transcription factors PPARγ and LXRα.
Declaration.

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Effect of pre incubation with the PPARγ antagonist GW9662 (1μM) on oxLDL induced CD36 surface expression. dTHP-1 cells were pre-incubated with GW9662 (1μM) before stimulation with 1μg/mL oxLDL for 24, 48 and 72 hours. CD36 surface expression was determined by flow cytometry. Results are expressed as % change in CD36 surface expression relative to dTHP-1 cells. Data is reported as mean ± SD of three independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only, # P<0.05 two sample t-test compared to cells pre-incubated with GW9662 then treated with 1μg/mL oxLDL).

**Figure 4.12:**

Effect of oxLDL on LXRα gene expression. dTHP-1 cells were incubated with 1μg/mL oxLDL for 24, 48 and 72hours. LXRα gene expression was quantified by real time-PCR and is reported as a ratio to GAPDH. The dotted line represents mean basal LXRα mRNA expression in dTHP-1 cells. Results are expressed relative to dTHP-1 cells only and are mean ± SD of two independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only).

**Figure 4.13:**

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**Figure 4.14:**

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## Abbreviations

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<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Acetyl-Co Enzyme A</td>
</tr>
<tr>
<td>AGER</td>
<td>Advanced Glycation End-Product Receptor</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One Way Analysis of Variance</td>
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<tr>
<td>BHF</td>
<td>British Heart Foundation</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CD36</td>
<td>Cluster of Differentiation Antigen 36</td>
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<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
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<td>COX</td>
<td>Cycloxygenase</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>dTHP-1</td>
<td>Differentiated THP-1 Cells</td>
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<td>EC</td>
<td>Endothelial Cell</td>
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<tr>
<td>EDTA</td>
<td>Ethylaminediaminetetraacetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ERK</td>
<td>Extracellular Regulated Kinase</td>
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<td>FACE</td>
<td>Fast Activated Cell Based ELISA</td>
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<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
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<td>HODE</td>
<td>Hydroxyoctadecadienoic Acid</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>LDL</td>
<td>Low Density Lipoprotein</td>
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<td>Lipoprotein Lipase</td>
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<td>LXR</td>
<td>Liver X Receptor</td>
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<td>LXRE</td>
<td>LXR-Response Element</td>
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<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
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<td>MM6</td>
<td>Mono-Max-6</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor-κ Beta</td>
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<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>NSAIDs</td>
<td>Nonsteroidal Anti-Inflammatory Drugs</td>
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<tr>
<td>oxLDL</td>
<td>Oxidised Low Density Lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-Kinase</td>
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<tr>
<td>15d-PGJ₂</td>
<td>15-Deoxy-Δ12,12-Prostaglandin J₂</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
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<tr>
<td>PPRE</td>
<td>PPAR-Response Element</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PUFAs</td>
<td>Polyunsaturated Fatty Acids</td>
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<tr>
<td>RCT</td>
<td>Reverse Cholesterol Transport</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
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<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
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<tr>
<td>sE</td>
<td>Soluble Endothelial</td>
</tr>
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<td>sP</td>
<td>Soluble Platelet</td>
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<tr>
<td>SR-A</td>
<td>Scavenger Receptor A</td>
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<td>SR-B</td>
<td>Scavenger Receptor B</td>
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<tr>
<td>T2D</td>
<td>Type-2 Diabetes</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA Buffer</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human Acute Monocytic Leukemia Cell Line</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis factor</td>
</tr>
<tr>
<td>TZDs</td>
<td>Thiazolidinediones</td>
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<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<tr>
<td>VO_{2max}</td>
<td>Maximal Oxygen Consumption</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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<tr>
<td>VLDL-TG</td>
<td>Very Low Density Lipoprotein Triacylglycerol</td>
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Chapter 1 – Introduction
1.1. The sedentary lifestyle and its costs.

It has become increasingly clear that the majority of the chronic diseases faced today in western societies are fundamentally associated with the prevalence of sedentary lifestyles (Murphy et al., 2006). Despite the well publicised benefits of regular exercise, the majority of westernised individuals fail to undertake enough physical activity to confer health protective benefits. For instance, The Department of Health, (2004) reported that only 37% of men and 24% of women in the UK met the current physical activity guidelines, as suggested by the UK government. Physical inactivity is defined as ‘lack of physical activity beyond that associated with daily living and functioning (Warden and Fuchs, 2008). Not only do sedentary lifestyles increase the risk of developing chronic diseases but they are associated with a significant economic burden (Zhu, 2008). The cost of physical inactivity in England and Wales including the direct costs of treatment for the major lifestyle related diseases and the indirect costs caused through sickness and absences from work have been estimated at £8.2 billion per year (Department of Health, 2004). These costs do not include the contribution of inactivity to obesity which itself has been estimated at £2.5 billion annually (Department of Health, 2004).

The UK government has set a target in England and Wales for 70% of the population to be undertaking 30 minutes of physical activity on at least 5 days a week by 2020 (Strategy Unit, 2002). A daily total of 10,000 steps has been extensively promoted by governments and health charities worldwide, for several decades. For adults 10,000 steps equates to a distance of approximately 8 kilometres and consumes between 300 and 400 calories of energy (Choi et al., 2007). The benefits of this form of exercise have been demonstrated in hypertensive individuals, where a decline in blood
pressure after achieving 10,000 steps per day was reported (Iwane et al., 2000). In relation to a diabetic population, walking 10,000 steps per day led to significant weight loss and improved insulin sensitivity (Yamanouchi et al., 1995).

### 1.2. Health and psychological benefits of physical activity and exercise prescription.

The World Health Report, (2002) estimates that around 3% of disease burden in developed countries is caused by physical inactivity and that over 20% of coronary heart disease (CHD), 16-17% of colon cancer, 15% of diabetes, 12-13% of strokes and 11% of breast cancer in developed countries is due to physical inactivity.

Extensive evidence demonstrates the benefits of regular physical activity in the prevention, management and even the treatment of some of the most prevalent and clinically significant diseases. These include diabetes (Lehmann et al., 2001), cardiovascular diseases (CVD) (Roberts and Bernard, 2005), obesity (Warburton et al., 2006) and cancer (Hu et al., 2004). For the UK alone epidemiological data suggests that approximately 40% of deaths linked to CHD are associated with inadequate physical activity. Overall, people who are physically inactive have nearly twice the risk of developing CHD when compared to active people. Hence encouraging sedentary people to take regular physical activity such as walking could significantly reduce the number of deaths from CHD.

Regular physical activity also has the potential to improve the status of several cardiovascular risk factors, including body composition, blood pressure, plasma
lipids, cardiorespiratory fitness, quality of life and perceived well being (Kirk et al., 2004). In healthy individuals, exercise has been shown to reduce hyperglycaemia, insulin resistance, hypertension and dyslipidaemia, and in conjunction, these led to a protective effect against cardiovascular disease (Thomas et al., 2008). Individuals with type 2 diabetes are encouraged to increase physical activity levels, due to its potential to increase insulin sensitivity (Thomas et al., 2008). Studies in non-diabetic individuals demonstrate that exercise reduces hyperglycaemia and body fat and protects against developing cardiovascular disease, highlighting the therapeutic appeal of exercise, due to its low cost and non pharmacological nature (Thomas et al., 2008). Chan et al., (2004) reported significant reductions in key anthropometric parameters, namely weight, BMI and waist girth, as a result of regular walking in individuals with sedentary jobs.

While the physiological evidence has extolled the health benefits of physical activity, psychological studies have revealed a much more complex picture. A positive association between exercise and self-esteem has been extensively established (McAuley et al., 1997). Exercise has been used as part of a treatment programme for clinical depression (LaFontaine et al., 1992), with physical activity having a positive influence on depression.

1.3. Physical activity reduces morbidity and mortality from cardiovascular disease.

The inverse relationship between physical activity and mortality from cardiovascular disease (CVD) within the general population is well established. Despite this, individuals are still failing to engage in sufficient physical activity to evoke
cardioprotective benefits. Previous epidemiological literature has shown that high levels of physical activity are associated with significantly reduced rates of mortality through CVD (Kujala et al., 1998 and Anderson et al., 2000). Tanasescu et al., (2003) demonstrated that walking in men with type-2 diabetes was inversely associated with total mortality. Therefore the use of these epidemiological studies as a tool is vital for promoting engagement in physical activity.

1.4. Physical activity, lipid metabolism and inflammation.

Previous literature has reported contrasting findings regarding the effects of physical activity on lipid metabolism, mainly due to the variations of mode and intensity of exercise employed. Therefore reproducibility of results using the same mode and intensity of exercise as well the same participant status remains limited. In terms of walking as a mode of exercise, previous studies including Woolf-May et al., (1998), Murtagh et al., (2005), Murphy et al., (2006) and Tully et al., (2007) has all shown that this mode of exercise was insufficient to evoke cardioprotective benefits on lipid profiles. However, Murphy et al., (2002) and Halverstadt et al., (2007) have both reported significant reductions in total cholesterol after adherence to a chronic walking programme. The effect of low intensity physical activity on inflammation has received minimal attention and therefore warrants further investigations. However, more intensive exercise (>60% VO$_{2\text{max}}$) has been shown to increase key mediators of inflammation, including IL-6 (Ullum et al., 1994 and Keller et al., 2003).
1.5. Prevention and treatment of cardiovascular disease (CVD).

1.5.1. Pharmacological interventions.

Medication prescribed for the treatment, management or prevention of CVD was estimated to cost the NHS £2.88 billion in 2006 (BHF, 2006). Statins have been widely used in the treatment of atherosclerosis due to their lipid regulating effects and concurrent anti-inflammatory properties, thus reducing cardiovascular complications. Statins regulate plasma lipids by blocking the conversion of HMG-CoA to mevalonic acid which attenuates the biosynthesis of cholesterol, resulting in as much as a 20-40% reduction in plasma total cholesterol and low density lipoprotein (LDL) cholesterol (Bonetti et al., 2003).

Angiotensin-converting enzyme (ACE) inhibitors inhibit the development of atherosclerosis by interrupting the expression of adhesion molecules and cytokines within atherosclerotic plaques (Paoletti et al., 2004). Both isoforms of cyclooxygenase, namely COX-1 and COX-2, play important roles in inflammation and are abundantly expressed by cells within atherosclerotic lesions. Aspirin, which inhibits COX isoenzymes, has been shown to have anti-platelet and anti-inflammatory properties and has been shown to reduce the risk of cardiovascular episodes (Ridker et al., 1997). However, there are controversies regarding the safety and effectiveness of COX inhibitors in reducing inflammation and hence atherosclerosis.

The fibrate class of drugs are ligands for the fatty acid receptor Peroxisome Proliferator-Activated Receptor-α (PPARα) and were introduced for the treatment of
hyperlipidaemia (Vosper et al., 2002). Fibrates once bound to PPARs can alter lipoprotein metabolism and inflammation in atherosclerosis. PPARα activation by fibrates leads to decreased hypertriglyceridemia by increasing lipoprotein lipase (LPL) expression (Fruchart et al., 1999).

1.5.2. Dietary interventions.

In terms of lifestyle interventions that can reduce inflammation associated with the development of CVD, Chrysohoou et al., (2004) reported that adherence to a Mediterranean diet for 12 months significantly reduced key inflammatory mediators, including CRP. The Mediterranean diet used included non-refined cereals and products, fruit, vegetables, olive oil, low fat dairy products, fish, poultry, potatoes, olives, pulses, nuts, eggs, red meat and wine.

1.6. Cardiovascular disease.

Cardiovascular disease (CVD) is the name given to a group of disorders of the heart and blood vessels, including hypertension, coronary heart disease, cerebrovascular disease and peripheral vascular disease (Hardman and Stensel, 2003). The two most common types of CVD are ischaemic heart disease (IHD) or CHD and stroke. CVD is the leading cause of mortality in the UK, accounting for approximately 194,000 deaths in 2006 (BHF, 2007), a figure that equates to approximately 1 in 3 deaths. In terms of premature mortality, CVD is one of the leading causes, with 30% of male and 22% of female premature deaths being accredited to CVD (BHF, 2006). As well as its huge morbidity and mortality rates, CVD has considerable economic
consequences. For instance, Luengo-Fernandez et al., (2006) reported that in 2006 the economic burden to the NHS was in the region of £15.7 billion. This equates to almost £250 per person in the UK, with hospitalisation of individuals with conditions associated with CVD accounting for 72% of the £15.7 billion bill.

1.7. Risk factors for cardiovascular disease.

Cardiovascular disease (CVD) is a complex, multifactorial disease consisting of both genetic and environmental factors all leading to the predisposition of cardiovascular complications. A number of risk factors have been identified, including hyperlipidaemia, hypertension, smoking, diabetes mellitus (DM), obesity, age and genetic predisposition.

Hyperlipidaemia represents a substantial risk factor for cardiovascular disease and is characterised by alterations in the profile of plasma lipoproteins, including elevated total cholesterol, triglyceride and low density lipoprotein (LDL) levels as well as low high density lipoprotein (HDL) levels (Collins, 2006). Specifically, there is a strong positive correlation between raised plasma LDL levels, decreased HDL levels and the development of atherosclerosis (Nagy et al., 1998).

Hypertension is a well known risk factor for cardiovascular disease, however the pathological and molecular mechanisms by which elevated blood pressure leads to CVD remains uncertain. There is some experimental evidence, however, to suggest that hypertension may promote endothelial expression of cytokines and stimulate inflammation (Chae et al., 2001). Endothelial dysfunction in the arterial vessel walls
is associated with hypertension, thus these changes may alter vascular functions, accelerating the progression of atherosclerosis (Schiffrin, 2002).

Cigarette smoking contributes significantly to cardiovascular morbidity and mortality, impacting on all phases of atherosclerosis, from endothelial dysfunction to clinical atherosclerotic events (Ambrose and Barua, 2004). Smoking increases inflammation, thrombosis, and oxidative stress.

Diabetes mellitus (DM) substantially magnifies the risk of developing cardiovascular disease (Glass, 2001). Although type 2 diabetes (T2D) is associated with increased atherosclerosis, the pathogenesis within a diabetic population is multifactorial. Hyperglycaemia and changes in lipid profile, increase oxidative stress, glycoxidation, endothelial dysfunction, inflammation, and accelerate the proatherogenic state (Blaschke et al., 2006).

Obesity which is characterised by increased adipocyte number and mass, results from a chronic imbalance between calories consumed and expended, leading to increased storage of excess energy in the form of adipocyte intracellular triglyceride stores (de Ferranti and Mozaffarian, 2008). It is understood that cellular mechanisms associated with obesity induce inflammatory mediators, including tumour necrosis factor-α (TNFα) and C-reactive protein (CRP), which predispose individuals to develop atherosclerosis. Additionally, the spiralling global epidemic of obesity is contributing to the development of metabolic abnormalities, e.g. the metabolic syndrome (as defined by obesity, insulin resistance and low HDL) (Alberti et al., 2005).
Increasing age is an important risk factor for CVD (Booth et al., 2006). A fundamental mechanism that predisposes elderly individuals to CVD is a reduction in blood vessel elasticity, which often leads to hypertension and hence CVD. Vascular ageing is mainly characterised by endothelial dysfunction, an alteration of endothelium dependent signalling processes or vascular remodelling (van der Loo et al., 2000). A number of underlying mechanisms have been postulated for the effects of ageing on CVD, including increased production of reactive oxygen species (ROS), inactivation of nitric oxide (NO), and subsequent formation of peroxynitrite (van der Loo et al., 2009).

There is increasing evidence of a strong genetic component to CVD, with relatives of cardiovascular disease patients having an approximate six fold increased risk of premature cardiovascular complications (Puddu et al., 2005). As cardiovascular disease is a multifactorial disease, it is regarded as being polygenic in nature (Stephens and Humphries, 2003). Polymorphisms in a number of key risk factor genes have now been identified, including apolipoprotein E (ApoE), cholesteryl ester transfer protein (CETP), hepatic lipase, LDL receptor and lipoprotein lipase (LPL) (Tosi et al., 2007). Specifically, a mutation in the lipoprotein lipase gene results in elevated levels of triglycerides and decreased levels of HDL, ultimately leading the hyperlipidaemia (Mailly et al., 1995 and Pimstone et al., 1995).

In order to combat the problems of atherosclerosis, risk factors for CVD need to be addressed with interventions, including diet, physical activity and smoking cessation. Initiating and maintaining these lifestyle changes has been shown to be complex and hence the clinical administration of lipid lowering and anti-inflammatory
pharmacological agents has been widely adopted, which have been effective at reducing cardiovascular events even with their attendant costs and potential side effects (Libby, 2006).

1.8. Plasma lipids and lipoproteins as cardiovascular risk factors.

1.8.1. Cholesterol.

Cholesterol (C$_{27}$H$_{45}$OH) is a white waxy sterol lipid that occurs in all animal cells and is composed of three regions, a hydrocarbon tail, a ring structure region with four hydrocarbon rings and a hydroxyl group (Figure 1.1). It has an important structural role in cell membranes, is the precursors for steroid hormones in the adrenal gland and is the precursor for bile acids in the liver (Tabas, 2002). Cholesterol is insoluble in water and hence is transported in the blood as a lipoprotein complex.

Cholesterol is the precursor molecule for the synthesis of steroids, including oestrogen and progesterone in women and testosterone in men. Cholesterol is also the precursor for bile acids, where the liver modifies cholesterol to produce bile, which is then stored in the gall bladder. The bile is then released into the small intestine to aid in the digestion of fat (Byrne, 1991). Intracellular cholesterol homeostasis is exquisitely regulated and depends on the balance between cholesterol synthesis, influx, degradation, cholesterol ester formation and translocation of cholesterol to the plasma membrane for efflux (Tang et al., 2004).
Figure 1.1: The structure of cholesterol. Cholesterol comprises a tetracyclic ring system with a double bond in one of the rings and a free hydroxyl group. Reproduced from Berg et al., (2006).

The synthesis of cholesterol is complex requiring 26 separate reactions beginning with acetyl-CoA. Synthesis occurs primarily in the liver and involves reductive condensation, cyclisation, hydroxylation, shifts of H and CH$_3$ groups, conversion of three CH$_3$ groups to CO$_2$ and the introduction and migration of double bonds (Champe et al., 2004). Excess cholesterol is mainly excreted from the body via secretion as bile salts (Marinetti, 1990).

Cholesterol and other blood lipids are fat soluble and therefore cannot be transported freely in the aqueous medium of the blood. Hence, they are packaged into lipoproteins, which are spherical molecular complexes that transport and regulate blood lipids (Durstine et al., 2002). Nearly all of the cholesterol in the blood is transported bound to LDL and HDL (Figure 1.2). Lipoproteins are structurally a thin outer shell surrounding a central area of lipid molecules. The shell contains proteins,
phospholipids and free cholesterol, which are responsible for making the particle soluble in the blood.

Figure 1.2: Summary of cholesterol transport. Modified from Crestani et al., (2004). LDL = Low density lipoprotein; HDL = High density lipoprotein.

1.8.2. Low density lipoproteins.

Low density lipoproteins (LDL) are the principal carriers of serum cholesterol, normally transporting approximately 60 to 80% of the total cholesterol, mainly in the form of cholesteryl esters (Dudek, 2006). The British Heart Foundation, (2007) state that individuals should aim for a plasma LDL concentration of below 2mmol/L and a total cholesterol level of below 4mmol/L. The LDL molecules are larger, less dense and less stable than HDL. LDL contains apolipoprotein B100 (ApoB100) molecules
(Figure 1.3), which act as ligands for LDL receptors, hence promoting cellular cholesterol uptake (Blasiole et al., 2008). LDL also consists of a hydrophobic core consisting of polyunsaturated fatty acid (PUFAs) and esterified cholesterol molecules. This core is surrounded by a structural shell of phospholipids and unesterified cholesterol. Approximately half the fatty acids in LDL are PUFAs, including linoleic acid. These PUFAs are protected against free radical attack and oxidation by antioxidants, primarily α-tocopherol (Mertens and Holvoet, 2001).

LDL is formed from very low density lipoprotein (VLDL) via intermediate density lipoprotein (IDL) and can pass through the junctions between capillary endothelial cells and attach to LDL receptors on cell membranes. This is followed by internalisation and lysosomal degradation with the release of free cholesterol (Marshall, 2000). LDL receptors are saturable and are down regulated by an increase in intracellular cholesterol (Marshall, 2000). In early work Carew et al., (1976) provided substantial evidence that cholesterol transported by LDL may infiltrate the arterial intima and contribute to the atherosclerotic process. Macrophages can take up modified forms of LDL via scavenger receptors, including CD36 and SR-A1 (Mauldin et al., 2006) a process that occurs at normal LDL concentrations but is enhanced when LDL concentrations are increased. When macrophages become overloaded with cholesterol they are converted to lipid laden ‘foam cells’, the classic component of atherosclerotic plaques (Chinetti et al., 2001). The uptake of oxidised LDL by macrophages in the arterial wall is recognised as an important event in the pathogenesis of atherosclerosis.
Figure 1.3: The structure of a low density lipoprotein (LDL) particle. A = ApoB100 (22%), B = Triglycerides (6%), C = Cholesterol esters (42%) and D = Phospholipids (22%) and free cholesterol (8%). Modified from Ioannou, (2001).

1.8.3. High density lipoproteins.

High density lipoprotein (HDL) is the smallest and densest class of lipoprotein containing virtually half protein (Figure 1.4). Most HDL particles are synthesised by the liver and small intestine, but they can be generated within the bloodstream from VLDL and chylomicrons (Brewer, 2004). HDL has often been referred to as ‘good’ cholesterol, since high levels of HDL reduce an individual’s tendency to develop atherosclerosis (Rader, 2006). HDL has a protective role in blood vessels as it removes excess cholesterol from the arterial wall (Stemerman, 2000). The protective effect of HDL against the development of atherosclerosis, results from its anti-inflammatory and anti-oxidant properties (Paoletti et al., 2004). Mineo et al., (2006) suggested that HDL promotes the production of prostacyclin, a prostaglandin synthesised in the endothelium from arachidonate, which inhibits platelet adhesion to the inner walls of arteries.
HDL represents several lipoprotein species (HDL$_{1}$, HDL$_{2}$, HDL$_{3}$ and HDL$_{4}$) that differ in their protein and lipid content, shape, structure and density. HDL$_{-2}$ is thought to be more protective against atherosclerosis and is increased by exercise and weight loss (Hardman, 1999). Garrido-Polonio et al., (2004) reported that HDL accepts free non-esterified cholesterol from peripheral tissues, including arterial walls. The free cholesterol that is taken up by HDL is quickly altered to a cholesterol ester, which prevents it from re-entering the arterial wall. It is generally recommended that plasma HDL concentrations are above 1mmol/L (BHF, 2007).
1.8.4. Triglycerides.

Triglycerides consist of a glycerol moiety, each hydroxyl group of which is esterified to a fatty acid. Triglycerides constitute the major storage form of fat in adipocytes (McArdle et al., 2007). In nature these compounds are synthesised by enzyme systems, which determine that a centre of asymmetry is created about carbon-2 of the glycerol backbone (Figure 1.5).

The sequence of reactions involved in triglyceride synthesis, is referred to as esterification, initially involving a fatty acid substrate being attached to coenzyme A to form fatty acyl-CoA (McArdle et al., 2007). This is then transferred to glycerol in the form glycerol 3-phosphate. In successive reactions, two additional fatty acyl-CoAs become linked to the single glycerol backbone as the triglyceride molecule is formed.

![Figure 1.5: The structure of a triglyceride molecule. Reproduced from Child, (2009).](image-url)
Hydrolysis or more specifically termed lipolysis describes triglyceride catabolism to yield glycerol and the energy rich fatty acid molecules (McArdle et al., 2007). Lipolysis involves the addition of water in three distinct hydrolysis reactions, each catalysed by hormone sensitive lipase. The mobilisation of fatty acids via lipolysis predominates under conditions of low to moderate intensity exercise, low calorie dieting, fasting, cold stress or prolonged exercise that depletes the body’s glycogen reserves. The fatty acids released during lipolysis can re-esterify to triglycerides following their conversion to a fatty acyl-CoA or exit from the adipocyte, enter the blood and combine with the blood protein albumin for transport to tissues throughout the body. The glycerol released during lipolysis can not be reused by adipocytes, instead it exits the cell and circulates in the blood. Hence plasma glycerol concentrations provide an invalid index of the degree of lipolysis (McArdle et al., 2007).

Hydrolysis of dietary triglycerides takes place in the small intestine, catalysed by pancreatic lipase. Lipoprotein lipase, an enzyme located on the walls of capillaries catalyses the hydrolysis of the triglycerides carried by the bloods lipoproteins. The fatty acids released by lipoprotein lipase can be taken up by adjacent adipose tissue and muscle cells for resynthesis to triglycerides for energy storage (McArdle et al., 2007). It is generally recommended that plasma triglyceride levels are below 1.7mmol/L (BHF, 2007).
1.9. The role of lipids in atherosclerosis.

Atherosclerosis is a multifactorial chronic inflammatory disease, characterised by disturbed interplay between circulating and vascular cells and the accumulation of lipid rich plaques in the intima of the arteries of the body (Ferreira et al., 2007). In atherosclerosis the normal homeostatic functions of the endothelium are altered, initiating an inflammatory response (Paoletti et al., 2004). Atherosclerotic lesions often referred to as “plaques”, are the result of an complex accumulation of cholesterol, cholesterol esters, phospholipids, live and dead cells, calcium and other components including, collagen, elastin and proteoglycans (Figure 1.6). The development of an atherosclerotic plaque depends on the interplay of the cellular components of the immune system such as monocytes, cytokines and cell adhesion molecules.

Ross, (1999) proposes a ‘response to injury’ theory detailing the initiation and progression of atherogenesis that highlights the essential roles of monocytes/macrophages in the development of atherogenesis. Injury in this instance is perceived as activation of endothelial cells, potentially due to simultaneous and systemic inflammatory conditions (e.g. elevated levels of LDL in the vessel wall), chronic infection, free radical production and/or irregular mechanical forces caused by disturbed blood flow. As a result of this endothelial activation or disturbance in normal endothelium homeostatic function, areas of the endothelium are predisposed to lesion formation, through the transmigration of monocytes (Ross, 1999). Monocytes then differentiate into macrophages, a cardioprotective event intended to remove accumulated inflammatory metabolites, including oxidised LDL (oxLDL). This process of differentiation is associated with the upregulation of scavenger
receptors, that normally function to recognise and internalise pathogens and apoptotic cells (Li and Glass, 2004).

**Figure 1.6: Cross section of a human artery.** a) 1 = Lumen, 2 = Endothelium, 3 = Internal elastic lamina, 4 = Tunica media and 5 = Tunica externa. b) Cross section of a coronary artery with an atherosclerotic plaque that is severely restricting blood flow. 6 = Lipid plaque, 7 = Lumen and 8 = Fibrous cap. Reproduced from www.chemo.net, (2009).

However, specific scavenger receptors recognise certain modified forms of lipids and ingestion of excessive amounts of oxLDL by macrophages initiates a process generating lipid laden ‘foam cells’ that are incapable of exiting the vessel wall (Ferreira *et al.*, 2007). Monocytes increase the expression of scavenger receptors such as scavenger receptor A (SR-A) and CD36, which in turn internalise modified lipoproteins (Lucas and Greaves, 2001). There has been considerable interest in identifying the scavenger receptors implicated in the development of atherosclerosis,
with molecular cloning identifying several receptors that are expressed by monocytes/macrophages. These include SR-A, CD36, SR-B1, CD68, LOX-1 and Galectin-3.

In terms of the relative contribution to atherosclerosis, CD36 and SR-A are thought to be the most predominant in terms of uncontrollable cholesterol influx. Studies with both knockout CD36 and SR-A1 mice have shown significantly reduced internalisation of cholesterol. T-lymphocytes also enter the intima and secrete specific cytokines and stimulate the expression of certain adhesion molecules that subsequently amplify the inflammatory process, via recruitment of additional circulating leukocytes (Paoletti et al., 2004). The death of lipid laden foam cells leads to the formation of a necrotic cholesterol rich core and rupture of this advanced lesion can lead to a thrombus and ultimately acute myocardial infarction (Li and Glass, 2004).

1.10. The role of macrophages in atherosclerosis.

Macrophages are bone marrow derived phagocytic cells that are critical for tissue homeostasis and are found in virtually all tissues (Lucas and Greaves, 2001). Tissue macrophages are derived from circulating blood monocytes that migrate into the tissues in response to inflammatory signals. The macrophage accumulates cholesteryl esters and triglycerides in cytoplasmic droplets, leading to development of the characteristic foam cell morphology (van Reyk and Jessup, 1999). These early vascular lesions are described as fatty streaks. The macrophage is an early and persistent feature of atherosclerosis and is therefore likely to play a key role in the
development and progression of the disease. This role may involve, scavenging of lipoproteins and extracellular debris, the production of inflammatory mediators such as cytokines and extracellular matrix degrading enzymes and the generation and degradation of modified forms of LDL. Another crucial role of the macrophage in the development of the atherosclerotic plaque is the increased expression of the scavenger receptor CD36 in response to changes in plasma oxLDL.

1.11. Inflammation and atherosclerosis.

A broad definition of inflammation is the complex biological response of tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by an organism to remove the injurious stimuli as well as initiating the healing process for the tissue. Although inflammation is the body’s natural response to stimuli such as infection, there is increasing focus on the role of inflammation in the pathogenesis of atherosclerosis (Petersen and Pedersen, 2005). It has been proposed that the aetiology of atherosclerosis is associated with low grade chronic inflammation (Petersen and Pedersen, 2005). In atherosclerosis the biochemical markers associated with low grade chronic inflammation are increased levels of CRP and certain inflammatory cytokines such as interleukin-6 (IL-6) and TNFα (Ross, 1999). IL-6 and CRP have related roles in the inflammatory response, as IL-6 induces CRP production in the liver by activating janus kinases (Heikkila et al., 2007).

Under normal conditions endothelial cells (EC) resist adhesion by leukocytes, however exposure to key CVD risk factors, such as hyperlipidaemia, smoking,
obesity, insulin resistance or physical inactivity, initiates a highly coordinated and well regulated process increasing the expression of adhesion molecules by EC’s, which promotes the attachment of leukocytes to the arterial wall (Libby, 2006). One candidate for the increased interaction between leukocytes and the endothelium is vascular cell adhesion molecule-1 (VCAM-1), which binds monocytes and T lymphocytes. The increased expression of VCAM-1 is thought to be due to accumulation of modified lipoproteins, inducing proinflammatory pathways involving the nuclear transcription factor nuclear factor-κB (NF-κB) and the inflammatory cytokines interleukin-1β (IL-1β) and TNFα. Selectins, which mediate leukocyte rolling during early stage inflammation are expressed on leukocytes, platelets and endothelial cells (Granger et al., 2004). Lesions advance as a result of endothelial transmigration of monocytes, which subsequently differentiate into macrophages, a process designed to remove inflammatory components from the bloodstream.

Once adhered to the arterial endothelium, monocytes penetrate the endothelial lining and enter the intima of the vessel wall (Figure 1.7). Over expression of monocyte chemoattractant protein-1 (MCP-1) and intracellular cell adhesion molecule 1 (ICAM-1) also aids in the recruitment of monocytes to the arterial wall. Once inside the intima, monocytes mature into macrophages, exhibit increased expression of scavenger receptors promoting the uptake of lipoproteins. Cholesterol esters accumulate in the cytoplasm and the macrophage becomes a lipid rich foam cell, ultimately leading to the formation of a fatty streak. At the same time macrophages multiply and release numerous growth factors and cytokines, thus amplifying and sustaining proinflammatory signals (Libby, 2006).
Figure 1.7: The recruitment of mononuclear cells to the atherosclerotic plaque and some of the functions of these cells in the mature atheroma (Libby, 2002). VCAM-1 = Vascular Cell Adhesion Molecule-1, CCR2 = Chemokine (C-C motif) Receptor 2, MCP-1 = Monocyte Chemotactic Protein-1, M-CSF = Mononuclear Phagocyte Colony-Stimulating Factor, MMPs = Matrix Metalloproteinases and ROS = Reactive Oxygen Species.

The progression of fatty streaks to an atherosclerotic plaque can often take decades to evolve, during which symptoms and complications may escape clinical diagnosis. During this progression a number of proinflammatory cytokines have been identified to be actively involved in this transition, including IL-1α, IL-6, IL-18, M-CSF and receptors such as CD40L (Libby, 2006). The development of atherosclerotic plaques would not be such a major health issue if it was not for plaque ruptures and thrombosis. The physical disruption of atherosclerotic plaques through fractures in the fibrous cap results in thrombus formation. Inflammation interferes with the
integrity of this fibrous cap in two ways, by blocking the creation of new collagen fibres and by stimulating the damage of existing collagen.

Oxidative stress is a characteristic of the inflammatory response during atherosclerosis, during which reactive oxygen species (ROS) are generated by numerous inflammatory enzymes, including NAD(P)H oxidase and nitric oxide synthase. During inflammation, ROS promotes the adhesion of leukocytes to the endothelium by various mechanisms. These include the production of inflammatory mediators and activation of nuclear transcription factors encoding endothelial adhesion molecules or cytokines.

1.12. CD36.

CD36 is an 88kd glycoprotein that is a member of a family of receptors that also includes SR-B1/CLA-1, a HDL receptor (Nicolson and Hajjar, 2004). CD36 was originally identified as a platelet membrane glycoprotein and a receptor for thrombospondin (TSP-1) (Li et al., 1993). However, CD36 is also expressed by monocyte/macrophages, endothelial cells, adipocytes, skeletal muscle, dendritic cells and breast and retinal pigment epithelium (Febbraio et al., 2001). *In-vivo* CD36 is involved in diverse processes, including recognition of apoptotic cells, fatty acid transport, cell matrix interactions and anti-angiogenic actions (Marsche et al., 2003). In addition to TSP-1, CD36 recognises a broad variety of ligands including oxidised LDL (oxLDL), anionic phospholipids, apoptotic cells, collagen, *Plasmodium falciparum*-infected erythrocytes, long chain fatty acids and LDL modified monocyte generated reactive nitrogen species (Nicolson and Hajjar, 2004).
Much interest has focused on the components of oxLDL that are recognised by and bind to macrophage scavenger receptors. CD36 recognises lipid moities of oxLDL, in contrast to SR-A1, which recognises the oxidised apoprotein portion of the lipoprotein. However, Boullier et al., (2000) demonstrated that the binding of oxLDL to CD36 is mediated by oxidised phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. Macrophage scavenger receptors play a significant role in atherosclerotic foam cell development because of their ability to bind and internalise oxLDL. Studies have demonstrated that macrophages deficient in CD36 take up significantly less oxLDL compared to controls (Febbraio et al., 1999).

An animal study undertaken by Wei., et al (2005) demonstrated that exercise can regulate CD36 gene expression and the genes involved in reverse cholesterol transport (RCT). However, the evidence as to the effect of exercise on CD36 or oxLDL in humans is limited. For instance in a study involving 10 sedentary men who exercised for 8 weeks at 50% VO$_{2\text{max}}$, there was no significant change in oxLDL levels, although a significant decrease in total cholesterol and LDL was reported (Wang and Chow, 2004). Bonen et al., (2000) demonstrated that fatty acid uptake during exercise is subject to short term regulation by muscle contraction and involves the translocation of FAT/CD36 from intracellular stores, in a similar manner to GLUT-4 regulation.

Foam cell formation will not prevail in the presence of LDL, due to a feedback mechanism down regulating LDL receptor gene transcription in response to LDL exposure (Nicholson and Hajjar, 2004). Whereas the mechanism responsible for the
induction of CD36 after exposure to oxLDL is due to oxLDL’s ability to activate the nuclear transcription factor PPARγ (Tontonoz et al., 1998 and Nagy et al., 1998).

1.13. Oxidised low density lipoprotein.

Oxidised low density lipoproteins (oxLDLs) represent a commonly used marker of oxidative damage and has been shown to play a major role in atherosclerosis and hence CVD (Cesari et al., 2005). oxLDL is regarded as a potential risk factor for atherosclerotic inflammation, with numerous studies reporting significantly increased levels of oxLDL in individuals with cardiovascular disease (Toshima et al., 2000 and Ehara et al., 2001). oxLDL is a chemoattractant for circulating monocytes, both directly and indirectly via stimulation of the release of MCP-1 from endothelial cells (Cushing et al., 1990). oxLDL is also a chemoattractant for T cells, hence atherosclerotic plaques contain primarily monocytes and T cells. The promotion of inflammation seen with oxLDL is partly due to uptake by scavenger receptors and the subsequent activation of Nuclear Factor-κB (NF-κB) (Boullier et al., 2001). The differentiation of monocytes into macrophages is promoted by oxLDL, through the enhancement of macrophage colony-stimulating factor (MCSF) from endothelial cells. Unlike native LDL, oxLDL is immunogenic and cytotoxic, especially to endothelial cells, hence the reduction in endothelial integrity after exposure to oxLDL (Young and McEneny, 2001). However, Zhao et al., (200) demonstrated that low levels of oxLDL are cytoprotective, through mechanisms involving increased levels of the intracellular antioxidant glutathione (GSH).
There is strong evidence that the oxidation of LDL promotes the atherogenic process. oxLDL particles act as ligands for CD36 on macrophages, which can be converted to cholesterol loaded foam cells (Dhariwal and Steinbrecher, 2000). During oxidation of LDL, the oxidised phospholipid becomes covalently bonded to the apoprotein (Nicholson and Hajjar, 2004). There are three main mechanisms by which in-vivo LDL can be oxidised, namely by lipoxygenases, myeloperoxidase and metal ions (Figure 1.8). Over expression of 15-lipoxygenase in the endothelium accelerates atherosclerosis, whereas disruption of the 12/15-lipoxygenase genes attenuates atherosclerosis in ApoE knockout mice (Cyrus et al., 1999). Activated phagocytes secrete myeloperoxidase which generates reactive species.

Oxidation of LDL by metal ions, including Cu$^{2+}$, occurs in three phases; consumption of endogenous antioxidants, peroxidation of LDL and formation of reactive aldehydes (Mertens and Holvoet, 2001). However, during aerobic conditions the nitric oxide (NO), inhibits copper mediated oxidation of LDL, via conversion to nitrite. When oxidant defences become depleted, NO interacts with superoxide anion to form peroxynitrite anion, which oxidises LDL. The production of peroxynitrite results in negative feedback, hence peroxynitrite oxidises the cofactor for NO synthase and therefore decreases NO production.

The concentration of cholesterol in high density lipoproteins (HDLs) is known to correlate inversely with the risk of developing cardiovascular disease (Olchawa et
The precise mechanism by which HDL protects against cardiovascular complications remains uncertain, however HDL has been shown to participate in anti-atherogenic activities through the ability to act as acceptors of cholesterol in the first step of reverse cholesterol transport (RCT) (Packard and Rader, 2005). RCT is a multistep dynamic process comprising of the removal of excess cholesterol from peripheral tissues, including arterial wall macrophages and delivery to the liver for excretion (Figure 1.9) (Olchawa et al., 2004). A number of specific pathways of cholesterol efflux have been defined, including 1) efflux to lipid-free apolipoproteins, particularly apoA-I, mediated by ABCA1; 2) efflux to mature HDL particles mediated by ABCG1; and 3) efflux to mature HDL particles by other pathways including SR-BI as well as passive diffusion (Rader et al., 2009). Both LXRα and PPARγ agonists have been shown to synthetically promote macrophage cholesterol efflux in-vitro through upregulation of ABCA1 (Chinetti et al., 2001 and Rader et al., 2009) and hence may provide a therapeutic means of increasing RCT given the widespread use of fibrates and glitazones.

1.15. Peroxisome proliferator-activated receptors.

Peroxisome proliferator-activated receptors (PPARs) are a class of lipid activated nuclear transcription factors that are included in the steroid hormone receptor super family (Touyz and Schifrin, 2006). PPARs instigate pleiotropic effects on intra and extracellular lipid metabolism, glucose homeostasis, cell proliferation, control of inflammation and arteriosclerosis (Elangbam et al., 2001). PPARs are activated by specific ligands and are highly dependent upon their phosphorylation status, reportedly induced by protein kinase A (PKA) and C (PKC), mitogen-activated
protein kinase (MAPK) and 5’ adenosine monophosphate-activated protein kinase (AMPK) (Touyz and Schifrin, 2006). Growth factors, including platelet derived growth factor (PDGF), have been shown to phosphorylate PPARs via the MAP kinase signalling pathways and hence decrease PPAR transcriptional activity (Paoletti et al., 2004).

**Figure 1.9: Summary of the pathways involved in reverse cholesterol transport.**

ABCA1 = ATP-binding cassette transporter A1, ABCG1 = ATP-binding cassette transporter G1, FC = Free cholesterol, CE = Cholesterol ester, A-I = Apolipoprotein A1, CETP = Cholesteryl ester transfer protein, PLTP = Phospholipid transfer protein, VLDL = Very low density lipoprotein, LDL = Low density lipoprotein, LDLR = Low density lipoprotein receptor and SR-B1 = Scavenger receptor B1.
1.15.1. The gene structure of peroxisome proliferator-activated receptors.

PPARs contain both a conserved DNA binding and ligand binding domain characteristic of all nuclear transcription factors (Li and Glass, 2004). The DNA binding domain or C domain, consists of two zinc finger motifs that control sequence specific recognition of response elements in direct target genes. PPARs bind to specific DNA response elements as heterodimers with retinoid X receptor (RXRs). In terms of competition among steroid receptor family members, RXRs are not only the heterodimer partner of PPARs but also for a number of receptors including LXR, T3 and vitamin D3 receptors (Clarke et al., 1999).

The ligand binding domain (LBD) controls ligand binding with other proteins and mediates either transcriptional activation and/or transrepression (Figure 1.10). Certain steroid hormones bind to PPARs with high affinity, whereas natural ligands, including certain fatty acids and cholesterol metabolites, bind with relatively low affinity. PPAR ligands not only regulate the DNA binding activity of PPARs by binding to the ligand domain, but also function to regulate phosphorylation cascades that ultimately determine the phosphorylation status of PPARs (Clarke et al., 1999).

Figure 1.10: General gene structure of PPARs demonstrating its various domains. Reproduced from Boitier et al., (2003)
The amino-terminal A/B region encodes a ligand independent transcriptional activation function domain (AF-1) and it has been shown that its phosphorylation state contributes to the modulation of PPAR activity, by affecting the receptor/ligand affinity. For example activation of the ERK-mitogen activated protein kinase (MAPK) increases the phosphorylation state of PPARs, altering their DNA-binding activity (Cabrero et al., 2002). Mutational analysis by Werman et al., (1997) highlighted that the activation domain within PPARγ is located in the amino terminal ligand independent region of the protein transcription factor. However, Adams et al., (1997) documented that MAPK phosphorylation of PPARγ inhibits both ligand dependent and ligand independent transactivation of PPARγ. Finally the D region encodes a flexible hinge region, thought to allow independent movement of the LBD relative to the DNA binding domain.

1.15.2. Isoforms of peroxisome proliferator-activated receptors.

To date, three different isotypes have been identified, namely, PPARα, PPARβ/δ and PPARγ. PPARα is primarily expressed in brown adipose tissue, liver, kidney, duodenum, heart, skeletal muscle and endothelial cells. It is pivotal in the control of lipoprotein metabolism, fatty acid oxidation and the cellular uptake of fatty acids. PPARα is activated by natural ligands, including polyunsaturated fatty acids and by synthetic ligands, including fibrates, such as fenofibrate and fibrozil (Desvergne and Wahli., 1999).

PPARβ/δ is expressed in numerous tissues, including skeletal muscle and brown adipose tissue. Until recently, the physiological functions of PPARβ/δ remained
unclear, however PPARβ/δ has been implicated in the regulation of the fatty acid burning capacities of skeletal muscle and adipose tissue, by controlling the expression of genes involved in fatty acid uptake, β-oxidation and energy uncoupling (Luquet et al., 2005).

PPARγ is highly expressed in monocytes/macrophages, adipocytes, brown and white adipose tissue and to a lesser extent, in the large intestine, retina and some parts of the immune system. PPARγ regulates genes involved in adipocyte differentiation and lipid uptake and storage. Natural activators of PPARγ include the prostaglandin D2 derivative 15-deoxy-Δ 12,14-prostaglandin J2 (15d-PGJ₂) and forms of oxidised linoleic acids, including 9- and 13(S)-HODE (Touyz and Schifrin., 2006). Synthetic ligands for PPARγ include the anti-diabetic insulin sensitisers thiazolidinediones (TZDs), such as pioglitazone, rosiglitazone and troglitazone (Touyz and Schifrin, 2006).

1.15.3. The regulation of peroxisome proliferator-activated receptors.

Upon activation, PPARs form heterodimers with another nuclear transcription factor, retinoid X receptor (RXR) and bind to specific PPAR response elements (PPREs) in the promoter region of their target genes (Touyz and Schifrin, 2006). This process regulates gene function, which includes activation or repression of gene expression (Figure 1.11). In the unliganded state, the PPAR/RXR heterodimer is associated with a multiprotein corepressor complex that contains histone deacetylase activity. Once a PPAR ligand binds to the receptor, the corepressor complex dissociates and a
coactivator complex, containing histone acetylase activity, is recruited to the PPAR/RXR heterodimer.

Figure 1.11: Intracellular mechanisms of PPAR transactivation and transrepression, which modulates inflammation and controls carbohydrate and lipid homeostasis. Modified from Li and Glass, (2004) and Touyz and Schifrin, (2006). PPAR = Peroxisome Proliferator Activated Receptor, RXR = Retinoid X Receptor, PPRE = Peroxisome Proliferator Response Element.
PPARs have been shown to regulate gene expression through a number of transcriptional activities. Firstly PPARs bind to response elements in the presence of ligands and coactivator proteins and initiate transcription. Secondly PPARs negatively regulate gene expression in a ligand dependent manner by antagonising the activities of other signal dependent transcription factors, including NF-κB. This mechanism does not involve binding to DNA and is referred to as transrepression and is critical in the anti-inflammatory actions of nuclear transcription factors.

1.15.4. Synthetic activation of peroxisome proliferators-activated receptors.

Thiazolidinediones (TZDs) activates PPARγ, leading to the induction of glucoregulatory molecules and enhanced insulin sensitivity. Activation of PPARγ inhibits inflammatory processes, including cytokine production (Ricote et al., 1998). Specific ligands of PPARγ, including rosiglitazone has been shown to increase endothelium dependent vasodilation, suggesting that PPARγ activation enhances NO production and protects against vascular injury (Collins et al., 2001).

1.16. Peroxisome proliferator-activated receptors γ.

PPARγ agonists have been shown to enhance the expression of PPARγ in macrophages and subsequently inhibit synthesis of (SR-A), partly through the inhibition of the nuclear transcription factor NF-κB (Ricote et al., 1998). PPARγ dependent inhibition of NF-κB has also been implicated in the anti-inflammatory inhibition of key cytokines, including TNFα and IL-6 (Ricote et al., 1998). The action of PPARγ ligands within the endothelium may reduce leukocyte accumulation
in the vascular wall, ultimately leading to a potential anti-atherosclerotic action. Two isoforms of PPARγ have been identified (Li and Glass, 2004). PPARγ2 is specifically expressed in adipose tissue and differs from PPARγ1 by the presence of 30 additional amino acids that characterise its tissue specific capabilities.

It is clear that PPARγ agonists possess anti-inflammatory properties that are both dependant and independent of PPARγ activation. It has been demonstrated that the endogenous PPARγ agonist 15d-PGJ2 inhibits NF-κB activation by preventing the degradation of its inhibitory factor IκBα. (Cabrero et al., 2002). In addition 15d-PGJ2 is also able to inhibit NF-κB activation by alkylating p50/p65 dimers.

1.17. The role of PPARs in metabolism and atherosclerosis.

PPAR agonists appear to beneficially modulate plasma lipid profiles by reducing total cholesterol, LDL and increasing HDL. Both PPARα and PPARγ modulate lipoprotein profiles by increasing the expression of lipoprotein lipase (LPL) and decreasing the expression of ApoCIII, an inhibitor of LPL activity (Bocher et al., 2002). Both PPARα and PPARγ agonists has been shown to be involved in the regulation of lipid efflux, primarily through upregulation of genes involved in reverse cholesterol transport (RCT) (Rigamonti et al., 2008). However the precise molecular pathways responsible for the control of lipid homeostasis remains to be fully elucidated. PPARs, in addition to their roles in lipid homeostasis regulate glucose metabolism. Including regulation of gluconeogenesis via stimulation of pyruvate dehydrogenase kinase 4 (PDK4) (Wu et al., 2001).
PPARs have been shown to regulate the expression of genes involved in fatty acid uptake, ketone body synthesis and for enzymes implicated in the peroxisomal β-oxidation pathway, including acyl-CoA oxidase (Schoonjans et al., 1996). Fatty acid metabolism is regulated by the rate of mitochondrial fatty acid uptake and PPARα plays a significant role in upregulating key genes involved in this mechanism (Yu et al., 1998). PPARα play a major role in liver fatty acid oxidation, especially during fasting and exercise, as the enhanced release of fatty acids from adipose tissue is used as a vital energy source (Desvergne et al., 2004). The role of PPARs in triglyceride metabolism is focused around the hypotriglyceridemic effect of PPARα as a result of increased lipoprotein lipolysis and fatty acid oxidation (Gervois et al., 2000).

PPAR activation has both direct and indirect effects on the modulation of atherosclerosis. The potential of PPARs to modulate the atherosclerotic process may involve a coordinated interaction between the indirect effects on glucose and fat metabolism and the direct effects on improving insulin sensitivity and reducing inflammation (Plutzky, 2003). PPARs have been shown to regulate adipokines, which participate in the development of insulin resistance, endothelial dysfunction and atherosclerosis (Lau et al., 2005). The use of PPARγ null mice has emphasised the necessity of PPARγ for adipocyte differentiation (Desvergne et al., 2004). PPARγ have been implicated in the modulation of insulin resistance and the regulation of macrophage function (Hamm et al., 1999 and Ricote et al., 1998).
1.18. Peroxisome proliferators-activated receptor and liver X receptor pathway in reverse cholesterol transport.

Liver X receptors (LXRs) belong to a subclass of nuclear hormone receptors that are critical for the control of lipid homeostasis (Tontonoz and Mangelsdorf, 2003). LXRs form heterodimers with retinoid X receptors (RXR) and are activated by a number of oxysterols, namely 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol and 24(S), 25-epoxycholesterols (Tontonoz and Mangelsdorf., 2003). There is evidence that LXRα mRNA expression is dependant upon activation of PPARγ, hence leading to a proposed “cross-talk” model between the LXRα and PPARγ. This is focused around the activation of PPARγ which leads to a direct increase in the expression of human LXRα, via an identified PPRE in the LXRα promoter region (Laffitte et al., 2001).

Two subtypes of LXRs have been identified, LXRα and LXRβ. LXRα is expressed in the liver, spleen, kidney, adipocytes, macrophages and small intestine, whilst LXRβ is ubiquitously expressed (Yoshikawa et al., 2003). Similar to other nuclear transcription factors, LXRs possess a zinc finger DNA-binding domain and a ligand binding domain (Tontonoz and Mangelsdorf, 2003). The binding of LXRs with a ligand triggers a molecular conformational change that promotes interaction with coactivator proteins, including RXR, which facilitates the activation of specific target genes associated with lipoprotein metabolism, such as LPL and ApoE (Zhang et al., 2001 and Laffitte et al., 2001).

LXRs regulate intracellular cholesterol levels by activating the expression of the ATP-binding cassette transporters, including ABCA1, ABCG1 and ABCG4, which
modulate cholesterol efflux and initiate RCT from peripheral tissues (Li and Glass, 2004). Therefore, LXR activation promotes the removal of excess cholesterol in macrophages, hence reducing the cytotoxic effects associated with cholesterol accumulation (Repa and Mangelsdorf, 2000).

LXR agonists may exert their anti-atherogenic effects not only by promoting cholesterol efflux, but also by limiting the production of inflammatory mediators in the artery wall (Tontonoz and Mangelsdorf, 2003). LXRs have been identified as regulators of inflammation by repressing key inflammatory genes, including IL-6 and COX-2. However, it is unknown whether this regulation is via direct interaction between LXR and its corresponding LXREs or whether LXR causes transrepression of inflammatory mediator genes (Rigamonti et al., 2008).
1.18. General aims.

Exercise is regarded as having beneficial effects on plasma lipids and lipoproteins. However, the molecular mechanisms responsible for the cardioprotective effects associated with regular physical activity are not fully understood. This is particularly the case for low intensity exercise, where cardioprotective factors are likely to be of significant clinical benefit to the general population. Therefore this study aimed to:

1. Investigate the effects of low intensity exercise on plasma lipids, lipoproteins and inflammatory mediators associated with cardiovascular risk.

2. Elucidate the molecular mechanisms responsible for the anti-atherogenic effects associated with low intensity exercise.

The use of “exercise prescription” is increasingly seen as important in both the prevention and treatment of some of the most important public health problems affecting western populations. Exercise is a low cost non-pharmacological intervention in the treatment of CVD and therefore, increasing our understanding of the mechanisms by which low intensity exercise mediates its beneficial effects will have important consequences in clinical practice.
Chapter 2.

The effect of low intensity exercise on plasma lipids and lipoproteins and inflammatory markers in normal sedentary adults.
2.1. Introduction.

Physical activity is considered to have important and diverse health benefits in terms of reducing cardiovascular risk. Walking is a form of low intensity exercise that has been shown to have numerous cardioprotective benefits (Murphy and Hardman, 1998). Walking is a rhythmic, dynamic, aerobic activity of large skeletal muscles that confers the multifarious benefits, with minimal adverse effects (Morris and Hardman, 1997). There is very little evidence that a training frequency as low as two days per week elicits improvements in cardiovascular risk factors, however Murphy et al., (2006) have recently published evidence to shed some light on this rather neglected area of research. They found that 45 minutes of self-paced walking, two days per week, for 8 weeks decreased systolic blood pressure, but had no significant effects on fitness, body mass, waist/hip circumference, diastolic pressure or plasma lipoproteins in previously sedentary adults.

Studies investigating the effects of low intensity aerobic exercise on lipids and lipoproteins have produced conflicting findings. For instance, several groups have shown no significant changes in lipoprotein concentrations after 6 and 12 weeks of walking, respectively (Branth et al., 2006 and Tully et al., 2007). However, in a recent study by Tsekouras et al., (2007), a single bout of brisk walking decreased basal very low density lipoprotein triacylglycerol (VLDL-TG) concentration in males, an effect that was associated with increased plasma clearance of lipoproteins. These inconsistencies may in part be explained by the initial baseline lipid/lipoprotein level, variety of training stimulus and the lack of dietary analysis undertaken.
Compared to chronic endurance based exercise, less information exists supporting resistance training as a modulator of plasma lipids. Previous studies have been contradictory with some showing cardioprotective effects with regular resistance training, while others have found no associated benefits (Trejo-Gutierrez and Fletcher, 2007). A possible explanation for these discrepancies is variations in energy expenditure during resistance exercises in the different exercise studies.

There is increased evidence that physical activity has anti-inflammatory effects, whether this is in the form of aerobic endurance or resistance based training. These associated effects have been shown to occur in several tissues including, monocytes, skeletal muscle and adipocytes (Flynn and McFarlin, 2006). Many of the health benefits of exercise are influenced by the balance of stress mediators and growth factors, including the soluble selectins, sE and sP (Schwindt et al., 2007). However, Petridou et al., (2007a) reported that exercise failed to reduce plasma adhesion molecule concentrations in obese and non-obese adults. The proinflammatory cytokines interleukin-6 and TNFα are known to be systematic measurable markers of low-grade inflammation associated with atherosclerosis and have been shown to be correlated with cardiovascular risk (Rothenbacher et al., 2003). Adiponectin, an adipocytokines with important metabolic effects plays a protective role against the development of atherosclerosis by suppressing inflammatory processes on the vascular endothelium (Yokoyama et al., 2004). It has been suggested that adiponectin has potential anti-atherogenic and anti-inflammatory properties, in the light that adiponectin inhibited the expression of TNFα induced monocyte adhesion molecules (Ekmekci and Ekmekci, 2006).
It remains unclear whether exercise influences oxidant production and the antioxidative capacity in monocytes (Wang et al., 2006). A previous study by Urso and Clarkson, (2003) indicated that strenuous exercise generates an imbalance between ROS and antioxidant defence, possibly culminating in oxidative stress. oxLDL mediated redox status in monocytes is affected by acute exercise in an intensity dependent manner. Thus high intensity exercise may promote oxLDL induced monocyte ROS production, possibly by reducing superoxide dismutase (SOD) activity and reduced glutathione (GSH) content. However the precise mechanisms and intensity thresholds responsible for the overall oxidative environment reported during exercise remains unclear.
2.1.1. Aims.

The primary aim of this chapter is to investigate the hypothesis that low intensity exercise is capable of regulating plasma lipid and lipoprotein levels and inflammatory markers in healthy but previously sedentary adults. This hypothesis will be investigated by:

1. Determining the effect of an 8 week low intensity aerobic exercise programme on anthropometric parameters.
2. Determining the effect of an 8 week low intensity aerobic exercise programme on plasma lipid and lipoprotein concentrations.
3. Establishing the influence of an 8 week low intensity exercise programme on the secretion and expression of key leukocyte and platelet derived inflammatory cytokines, adhesion molecules and the adipokine, adiponectin.
2.2. Methodology.

2.2.1. Participants.

A cohort of 34 inactive healthy adults (18 male and 16 female), mean age 45.6 ± 11.1 years; body weight 83.56 ± 18.13 kg; body mass index [BMI] 26.78 ± 5.11 kg/m²; % body fat 33.78 ± 10.25; blood pressure (systolic 133.12 ± 17.59: diastolic 88.5 ± 11.3 mmHg) means ± SD) were recruited to participate in the study. Individual’s height and weight were measured using Seca® electronic scales and a Seca® height monitor (Birmingham, UK), respectively. Percentage body fat was measured using a body fat monitor (Tania, Hoofddorp, Netherlands) and by skinfold calipers (Baty, West Sussex, UK). When using the calipers the Durnin & Womersley protocol was applied and triplicate readings at each of the four skinfold sites were recorded (Durnin and Womersley, 1974). Measurement of blood pressure in triplicate was taken five minutes apart using an automated device (Boso Medicus Uno Automatic Blood Pressure Monitor, Jungingen, Germany) after the subjects had been seated for five minutes, with the average of the readings being used. Exclusion criteria for participation in the low intensity exercise programme was any history of cardiovascular disease, a physically active lifestyle, the taking of any medication in the previous three months, smoking and age > 60 years. A physically active lifestyle was defined as ‘any form of physical activity undertaken a minimum of 3 times per week, lasting at least 30 minutes per session, that is planned, structured and repetitive for the purpose of improving or maintaining one or more components of physical fitness’. Ethical Approval was granted by the School of Health Sciences Ethical Committee at the University of Wales Institute, Cardiff, UK, with all participants completing an informed consent form and health and dietary questionnaires.
2.2.2. Pre-health screening.

To monitor any possible cardiovascular adaptations to the exercise programme, \( \text{VO}_{2\text{max}} \) was estimated using a submaximal predictor test (Rockport Fitness Walking Test), which was conducted before and after the exercise programme. In the week prior to the submaximal predictor test, participants were familiarised with walking on the treadmill (Monark 8424E, Varberg, Sweden) at various speeds. The Rockport fitness walking test consisted of a four minute warm up, after which participants were instructed to walk one mile (1609 meters) on the treadmill as fast as possible. The time taken to complete the one mile walk was recorded along with the participant’s heart rate upon completion of the walk. In all cases heart rate was measured throughout using a Polar S410 HR monitor (Polar Electro, Finland). \( \text{VO}_{2\text{max}} \) was estimated using the following equation; \[ 132.853 - (0.0769 \times \text{Weight}) - (0.3877 \times \text{Age}) + (6.315 \times \text{Gender}) - (3.2649 \times \text{Time}) - (0.1565 \times \text{Heart rate}) \].

2.2.3. Design of the exercise programme.

Participants were assigned to either an exercise or control group, with equal numbers of males and females in the exercise and control groups (9 men and 8 female). The exercise group (n=17) underwent a low intensity exercise programme that consisted of walking 10,000 steps three times per week for eight weeks. Low intensity exercise was defined as brisk walking resulting in an increase in heart rate and perspiration, equivalent to approximately 40\% \( \text{VO}_{2\text{max}} \). Participants selected their own walking speed during each exercise session. All walking sessions were completed on a treadmill under constant supervision. Participants in the control group (n=17) continued with their sedentary lifestyle for the duration of the study.
2.2.4. Dietary analysis.

Dietary diaries were completed by all participants over a 3 day period before and immediately after commencing the exercise programme. All diet data was analysed using the NetWisp 3.0 (Tinuviel Software Systems, Cheshire, UK) computer software package to determine the relative composition of the participant’s diet before and after the exercise programme.

2.2.5. Fractionation for the collection of leukocytes.

Fasting blood was sampled at baseline and after four and eight weeks of exercise. Blood samples were collected 24 hours before the commencement of the exercise study or 48 hours after an exercise session. At each time point, blood samples were collected by venepuncture from the antecubital vein with minimal tourniquet into anticoagulated EDTA vacutainers and serum separator tubes (SST) (Becton-Dickenson, Oxford, UK). Whole blood was fractionated by centrifugation at 1000 x g for 15 minutes at room temperature (RT). A sterile plastic transfer pipette was used to aspirate off the plasma down to ~1mm from the buffy layer and stored at -80°C. The buffy layer containing leukocytes was carefully removed using a circular motion and added to 1.3mL of RNAlater® (Applied Biosystems, Warrington, UK). Samples in RNAlater® were vortexed and stored at 4°C overnight, then stored at -80°C. Serum was obtained from blood collected into SST collection tubes, allowed to clot for 30 minutes, centrifuged at 1000 x g for 10 minutes at RT and the resulting serum supernatant aliquoted and stored at -80°C.
2.2.6. **Biochemical analysis of plasma samples.**

A Kodak Ektachem DT60 II (Axis-Shield, Cambridgeshire, UK) was used to determine values of total cholesterol, triglycerides and high density lipoprotein (HDL) in the plasma samples. Low density lipoprotein (LDL) was calculated using the Friedewald equation (Friedewald et al., 1972). Samples were analysed within one month of freezing.

2.2.7. **Technique for the use of the Kodak Ektachem DT60 II.**

Once the desired lipoprotein slide is loaded onto the machine, 10µL of a subjects sample is spotted onto the slide and the corresponding lipoprotein values is given. The sample spreads evenly and diffuses into the reagent layers. The cholesterol in the sample undergoes a series of reactions in the slide to produce a coloured compound. The intensity of the colour is proportional to the amount of cholesterol in the sample.

2.2.7.1. **Analysis of total cholesterol.**

During the quantification of total cholesterol the proportion of cholesterol dissociates from its lipoprotein carriers after which the cholesterol esters are hydrolysed to free cholesterol, which then undergoes a series of reactions beginning with oxidation by cholesterol oxidase. In the final reaction a coloured dye is produced and by measuring the amount of absorbance from the dye layer after incubation, the amount of cholesterol present in the sample is therefore determined.
2.2.7.2. Analysis of triglycerides.

Analysis of triglycerides involves their hydrolysis by the enzyme lipase to form glycerol and fatty acids.

2.2.7.3. Analysis of HDL.

The analysis of HDL involves the precipitation of LDL and VLDL using dextran sulphate and magnesium chloride to isolate HDL. After centrifugation the HDL lipoproteins remains in the supernatant and the non HDL fractions form a pellet on the bottom of the tube.

2.2.7.4. The analysis of LDL.

The individual subject’s plasma LDL levels were not measured using the Kodak Ektachem DT60 II, instead they were calculated using the Friedewald equation; [LDL = Total Cholesterol – (HDL + Triglycerides/5)].

2.2.8. Quantification of serum oxLDL by ELISA.

Prior to analysis participants serum samples were diluted 1 in 10 (i.e. 50μL + 450μL) with assay buffer. All reagents, including standards, controls and buffers were brought to room temperature (RT) and prepared using the manufactures instructions (Oxford Biosystems, Oxford, UK). Prior to analysis, 100μL of standard, control or diluted sample in duplicate were added to their respective wells. The plate was subsequently sealed tightly and incubated for 2 hours at 37°C. Wells were then washed (5x) with diluted wash buffer and blot dried to remove any remaining liquid,
after which 100μL of conjugated anti-oxidised LDL (oxLDL) antibody was added to each well, except the blanks. The plate was again sealed and incubated at 37°C for 1 hour. The wells were washed (x5) with diluted wash buffer. Substrate (100μL) was then added to each well, followed by 30 minutes incubation at RT in the dark. After which 50μL of stop solution (sulphuric acid) was added to stop the reaction and the plate shaken well. Colorimetric results were quantified by spectrophotometry immediately using the absorbance 450nm with a reference of 620nm (Dynex Technologies, VA, USA).

2.2.9. ELISA quantification of serum adiponectin.

Prior to analysis participants serum samples were diluted 1 in 50 (i.e. 10μL + 490μL) with Calibrator Diluent RD6-39. All reagents and standards were brought to room temperature (RT) and prepared as directed by the manufactures instructions (R&D Systems, Abingdon, UK). After which 100μL of Assay Diluent was added to each well and then 50μL of either standard, control or sample was added to their respective wells. The plate was subsequently sealed and incubated for 2 hours at RT. Wells were then washed (x4) with wash buffer and blot dried to remove any remaining liquid and 200μL of adiponectin conjugate added to each well. The plate was sealed with an adhesive strip and incubated at RT for 2 hours. The wells were further washed (x4) with wash buffer. Next, 200μL of substrate solution was added to each well and incubated at RT in the dark for 30 minutes. After which 50μL of stop solution (sulphuric acid) added to each well to stop the reaction and the plate gently shaken. Colorimetric results were quantified spectrophotometrically at 450nm (Dynex Technologies, VA, USA).
2.2.10. ELISA quantification of serum sE and sP selectin.

Prior to analysis, participants serum samples were diluted 1 in 10 (i.e. 30μL + 270μL) with Calibrator Diluent RD6-11 for soluble endothelial selectin (sE selectin) and diluted 1 in 20 (i.e. 15μL + 285μL) with sample diluent for soluble platelet selectin (sP selectin). All reagents and standards were brought to room temperature (RT) and prepared as directed by the manufactures instructions (R&D Systems, Abingdon, UK). For sE selectin only 100μL of assay diluent was added to each well before 100μL of standard, control or diluted sample was added to the designated wells in both the sE and sP selectin plates. For sP selectin, 100μL of diluted sP selectin conjugate was added to each well. The plates were tightly sealed with an adhesive strip and incubated at RT for 1 hour for sP selectin and 2 hours for sE selectin. After which time the sP selectin plate was washed (x3) with 300μL wash buffer and blot dried to remove any remaining liquid. The sE selection was washed (x4) with 400μL wash buffer and blot dried to remove any remaining liquid. For sP selectin 100μL of substrate and for sE selectin 200μL of substrate were added to its corresponding plate, covered with a new plate strip and incubated for 15 minutes (sP selectin) or 2 hours (sE selectin) at RT. Finally for the sP selectin assay 100μL of stop solution (sulphuric acid) was added to each well to stop the reaction and colorimetric results were quantified spectrophotometry using the absorbance 450nm and correction wavelength 620nm (Dynex Technologies, VA, USA). The sE selectin plate was washed (x4) with 400μL wash buffer, after which 200μL of substrate solution added to each well and incubated for 30 minutes at RT in the dark. Finally 50μL of stop solution was added and colorimetric results quantified as per sP selectin.
2.2.11. ELISA quantification of serum IL-6.

Interleukin-6 (IL-6) was determined by standard ELISA (R&D Systems, Abingdon, UK). During the plate preparation 100μL of mouse anti-human interleukin-6 (IL-6) capture antibody diluted to 2μg/mL in PBS was added to each of the 96 wells on the plate and incubated at room temperature (RT) overnight. Capture antibody was aspirated from each well, washed (x3) with 400μL wash buffer and blot dried to remove any remaining liquid. The plate was blocked by the addition of 300μL of reagent diluent, which was incubated at RT for 1 hour. After which wells were washed (x3) with 400μL wash buffer as described above. The first stage of the assay involved adding 100μL of sample or standard in reagent diluent to their corresponding wells. The plate was sealed with a new adhesive strip and incubated for 2 hours at RT. Wells were washed as described before and 100μL of detection antibody diluted in reagent diluent was added to each well. Again the plate was sealed with a new adhesive strip and incubated for 2 hours at RT. The plate was subsequently washed as detailed before and 100μL of streptavidin-HRP was added to each well and incubated for 20 minutes at RT in the dark. The aspiration and wash steps were again repeated and 100μL of substrate solution was added and incubated again for 20 minutes at RT in the dark. Finally 50μL of stop solution (sulphuric acid) was added to each well and colorimetric results were quantified by spectrophotometry immediately using the absorbance 450nm, with correction wavelength set at 540nm (Dynex Technologies, VA, USA).
2.2.12. ELISA quantification of serum TNF-α.

Tumor necrosis factor-α (TNFα) was determined by standard ELISA (R&D Systems, Abingdon, UK). All reagents and standards were brought to room temperature (RT) and prepared as directed by the manufactures instructions. Assay Diluent (100μL) was added to each well and then 50μL of either standard, control or sample was added to their respective wells. The plate was subsequently sealed and incubated for 3 hours at RT. Wells were then washed (x3) with 400μL diluted wash buffer, blot dried to remove any remaining liquid and 200μL of TNFα conjugate was then added to each well and the plate was again sealed tightly with a new adhesive strip and incubated at RT for 2 hours. As before the wells were washed (x3) with 400μL diluted wash buffer and 50μL of substrate solution was then added to each well and incubated at RT in the dark for 1 hour. Subsequently 50μL of amplifier solution was added to each well and incubated for 30 minutes at RT in the dark. After which 50μL of stop solution (sulphuric acid) was added to each well to stop the reaction and the plate was gently shaken. Colorimetric results were quantified by spectrophotometry immediately using the absorbance 490nm, with correction wavelength set at 650nm (Dynex Technologies, VA, USA).

2.2.13. Statistical analysis.

Data is expressed as mean ± standard deviation (SD) unless otherwise specified. When analysing multiple comparisons within groups, a one-way analysis of variance (ANOVA) was applied to normally distributed data and the Kruskal-Wallis test for data deemed non-normally distributed. For non multiple comparisons between exercise and control groups, two sample t-tests were used when data was assumed to
be normally distributed, otherwise a Mann-Whitney test was used. All statistical calculations were carried out using Minitab 14® and significance levels were set at $P<0.05$. The sample size for this study was determined using data generated from a provisional study and using the change in plasma cholesterol as the primary outcome. The power calculation assumed at least 0.6 mmol/L increase in HDL cholesterol expression with a standard deviation of 0.7 mmol/L, a required power of 0.8 (80%) and using a two-sample t-test for comparisons. These figures suggested a sample size of at least 16 in each group was required to undertake this study. The actual study involved the recruitment of 17 subjects in both the control and exercise group.
2.3. Results.

2.3.1. Effect of low intensity exercise on anthropometric measures.

Previous studies have reported conflicting evidence as to the desirable duration and intensity of physical activity required to induce significant changes in anthropometric measures. There were no significant differences in any anthropometric measures after eight weeks of low intensity exercise and between the exercise and control groups at either baseline or post exercise (Table 2.1). The intensity of this exercise programme did not lead to any significant adaptations in physical fitness as estimated by VO$_{2\text{max}}$, even though a non significant increase was observed after exercise (Table 2.1). There were also no significant changes in the composition of the exercise or control subject’s diet throughout the study.

2.3.2. Effect of an eight week low intensity exercise programme on plasma lipids and lipoproteins.

To investigate whether the exercise programme employed in the present study was capable of regulating plasma lipid and lipoprotein levels, total cholesterol, high density lipoprotein (HDL), triglycerides and low density lipoprotein (LDL) were quantified at baseline, and after both four and eight weeks of exercise. As can be seen in table 2.2, mean plasma total cholesterol was significantly reduced (7.2%; $P=0.041$) in subjects who undertook the eight week exercise programme (baseline 5.73 ± 1.39mmol/L; week 8: 5.32 ± 1.28mmol/L). A significant increase (6.8%; $P=0.042$) was observed in HDL after exercise (baseline: 1.46 ± 0.47mmol/L; week 8: 1.56 ± 0.50mmol/L), while non significant reductions in both triglycerides
(13.6%) and LDL (11.4%) were observed in the subjects who undertook the exercise programme (triglycerides baseline: 3.83 ± 2.74mmol/L; week 8: 3.31 ± 2.25mmol/L; LDL baseline: 3.50 ± 1.34mmol/L; week 8: 3.10 ± 1.12mmol/L; Table 2.2). In contrast, no significant changes in any of these lipids or lipoproteins were observed in the inactive control participants. No significant changes in plasma lipid and lipoprotein levels were observed after four weeks of the exercise programme in either the exercise or control group.

Table 2.1: Anthropometric measurements at baseline and after 8 weeks of participation in the low intensity exercise study.

<table>
<thead>
<tr>
<th></th>
<th>Exercise (n=17)</th>
<th>Controls (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week Number</strong></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>44.94 (10.01)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>169.32 (2.46)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>83.56 (18.13)</td>
<td>83.28 (18.12)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.78 (5.11)</td>
<td>26.68 (5.15)</td>
</tr>
<tr>
<td><strong>% Body Fat</strong></td>
<td>33.78 (10.25)</td>
<td>33.3 (9.94)</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>134.14 (18.74)</td>
<td>133 (16.9)</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>89.43 (11.89)</td>
<td>91.44 (11.9)</td>
</tr>
<tr>
<td><strong>VO₂max (ml/kg⁻¹/min⁻¹)</strong></td>
<td>35.49 (6.94)</td>
<td>38.23 (4.33)</td>
</tr>
</tbody>
</table>

Data is presented as means and (S.D).
Table 2.2: Plasma lipid and lipoprotein levels in the exercise and control groups at baseline, week 4 and week 8.

Data is presented as means and (S.D).

* Denotes significant difference between baseline and week eight ($P<0.05$ two sample t-test).

* Triglyceride results reported as median and inter-quartile range, due to non-normal distribution of data.

In terms of correlations involving exercise induced modulations in lipids and lipoproteins, there were significant positive correlations between changes in total cholesterol vs LDL (Table 2.3) and oxLDL (Table 2.3) after exercise. Interestingly there was a significant correlation between exercise induced changes in HDL vs oxLDL (Table 2.3).
### Table 2.3: Correlation analysis between exercise induced modulations in plasma lipids.

<table>
<thead>
<tr>
<th>Correlations between changes in lipoproteins</th>
<th>R value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol vs LDL*</td>
<td>0.986</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Total cholesterol vs oxLDL*</td>
<td>0.772</td>
<td>0.025</td>
</tr>
<tr>
<td>HDL vs oxLDL*</td>
<td>-0.710</td>
<td>0.048</td>
</tr>
<tr>
<td>HDL vs Triglycerides*</td>
<td>-0.734</td>
<td>0.038</td>
</tr>
</tbody>
</table>

* Denotes significant difference between variables \((P<0.05)\).

### 2.3.3. The effect of low intensity exercise on serum adiponectin concentrations.

Insulin resistance and obesity are both known to be associated with reductions in serum adiponectin concentrations. Adiponectin is believed to play a protective role against the development of atherosclerosis by suppressing inflammatory processes on the vascular endothelium. This is emphasised by the evidence that adiponectin concentrations are lower in individuals with cardiovascular disease. Therefore it was decided to investigate whether low intensity exercise affects circulating levels of this cardiovascular risk marker. There were no significant changes in serum adiponectin concentrations after adherence to the eight week low intensity exercise programme in either the exercise or control groups (Figure 2.1). There were also no significant differences in serum adiponectin levels between the exercise and control groups at all time points.
Figure 2.1: The effect of low intensity exercise on serum adiponectin concentrations. Serum adiponectin levels were quantified by ELISA at baseline and 4 and 8 weeks for the control and exercise group. Results are expressed as mean ± SD.

2.3.4. The effect of low intensity exercise on serum sE and sP selectin concentrations.

Both soluble endothelial (sE) and soluble platelet (sP) selectins are adhesion molecules mediating the first step in leukocyte migration into the subendothelial space (Dong et al., 1998). There were no significant exercise induced modulations in both sE and sP selectins after adherence to an eight week low intensity exercise programme in both the exercise and control groups (Figures 2.2a and 2.2b). Further analysis also revealed no significant differences in sE and sP concentrations between the exercise and control groups at all time points.
Figure 2.2: The effect of low intensity exercise on serum sE and sP selectin concentrations. Both serum sE (a) and sP (b) selectin levels were quantified by ELISA at the times indicated in samples taken from both the exercise and control groups. Results are expressed as mean ± SD.
2.3.5. The effect of low intensity exercise on serum IL-6 concentrations.

The proinflammatory cytokine interleukin 6 (IL-6), is involved in the modulation of immune cell function and migration, initiating and amplifying the acute phase and stress responses (Moldoveanu et al., 2000). The local production of this molecule coordinates the function of innate and adaptive immune cells, including the interactions with vascular endothelial cells, differential expression of cell surface effector molecules, growth, and differentiation. Therefore, to investigate whether the adherence to an eight week low intensity exercise programme increases the potential for proinflammatory mediators to initiate proatherogenic events, serum IL-6 was quantified. Figure 2.3 demonstrates that there were no significant changes in serum IL-6 concentrations after either four or eight weeks of low intensity exercise. In a similar manner there were no significant changes in the control group and no significant difference between the exercise and control group at all time points.
Figure 2.3: Effects of low intensity exercise on serum IL-6 concentrations. IL-6 serum levels were quantified by ELISA at the times indicated in samples from both the exercise and control groups. Results are expressed as mean ± SD.

2.3.6. The effect of low intensity exercise on serum TNFα concentrations.

Tumour necrosis factor-α (TNFα), another proinflammatory cytokine was quantified at the protein level after adherence to the eight week low intensity exercise programme. The result for TNFα supports the previous result for IL-6, in that low intensity exercise had no significant effect on TNFα serum levels after both four and eight weeks (Figures 2.4). There was also no significant change in serum levels of TNFα in the control group during the eight weeks. At baseline, week four and eight there was no significant differences between the exercise and control groups for serum levels of TNFα.
Serum TNFα levels were quantified by ELISA in serum samples from the exercise and control groups. Results are expressed as mean ± SD.

**2.3.7. Effect of low intensity exercise on serum oxLDL concentrations.**

Exercise at certain durations and intensity is considered to instigate an oxidative environment, therefore it was decided to further investigate whether exercise affected serum oxLDL levels. After four weeks of exercise, there was a 31% increase in serum oxLDL levels which reverted to near its baseline level in the exercise group after eight weeks (baseline: 554 ± 107ng/ml; week 4: 698 ± 134ng/ml; week 8: 588 ± 145ng/ml; Figure 2.5). This result suggests that during the early stages of a low intensity aerobic exercise programme a significant increase in LDL oxidation occurs. There were no significant changes in serum oxLDL levels in the control group at any
of the time points investigated and no significant difference between the exercise and control group at all time points measured.

**Figure 2.5: The effect of low intensity exercise on serum oxLDL levels.** Serum oxLDL levels were quantified by ELISA at the times indicated in samples taken from individuals in both the exercise and control groups. The change in oxLDL observed in the exercise group was deemed significant (* $P=0.0001$, Kruskall-Wallis). Results are expressed as median and the inter-quartile range.
2.4. Discussion.

This study investigated the effect of an eight week low intensity exercise programme on plasma lipids and lipoproteins and inflammatory markers in disease free sedentary adults. During the exercise programme no significant change in % body fat pre to post exercise was observed in either the exercise and control group (Table 2.1). The lack of body mass changes in the present study is also consistent with previously reported walking studies using subjects with normal weight (Santiago et al., 1995 and Murtagh et al., 2005). However in another study (Wang and Chow, 2004), a significant decrease in % body fat after eight weeks of training at 50% of maximal oxygen consumption ($\text{VO}_{2}\text{max}$) was observed. The differences in the effects of low intensity exercise on % body fat may be a reflection on the population characteristics, as Wang and Chow, (2004), used only men with a mean age of 21 years. It however, remains questionable whether substantial modulations in body fat after adherence to a low intensity exercise programme should be expected, especially over such a short time frame.

No significant changes were recorded in either diastolic or systolic blood pressure after completion of the eight week low intensity exercise programme (Table 2.1). This is in agreement with other walking based interventions, including Murphy and Hardman, (1998), Chan et al., (2004) and Murtagh et al., (2005). The individuals in the present study were normotensive at baseline. Therefore, it was unlikely that any significant changes in blood pressure in response to exercise would be achieved since changes in blood pressure reported as a result of engaging in exercise are greater in hypertensive individuals rather than normotensive ones (Murtagh et al., 2005). However, Wang and Chow, (2004) and Tully et al., (2007) have reported that 8 and
12 weeks of physical activity, respectively, significantly reduced both diastolic and systolic blood pressure in healthy inactive adults. As observed with weight, any change in blood pressure associated with exercise may be attributed to the type and intensity of exercise and also to subject characteristics.

Even though there was an increase in predicted VO$_{2\max}$ pre to post exercise, this was deemed non significant. The increase in predicted VO$_{2\max}$ in both the exercise and control groups could be explained by the fact that the individuals may have been more accustomed to completing the walking based VO$_{2\max}$ predictor test on the treadmill during their second test. Duncan and Pozehl, (2003) and Murphy et al., (2006) reported similar results to those reported here, in that there were no significant adaptations in aerobic fitness during an eight week walking programme. However, Couillard et al., (2001) and Murphy et al., (2002) both reported significant increases in predicted VO$_{2\max}$ after 20 and 6 weeks of exercise, respectively. As with the current study, VO$_{2\max}$ predictor tests were used and the validity of these tests is somewhat debatable. This is due to the fact that these types of VO$_{2\max}$ predictor tests use a number of assumptions to estimate individuals VO$_{2\max}$.

Adherence to the eight week low intensity exercise programme resulted in a significant reduction in plasma total cholesterol concentrations (Table 2.2). This is in agreement with Halverstadt et al., (2007) who reported a significant decrease in total cholesterol after 24 weeks of exercise in healthy sedentary adults. Also Murphy et al., (2002) published data highlighting the atheroprotective effects of a 6 week walking programme, including significant reductions in total cholesterol. However, one of the first walking based studies to report on the effects of physical activity on
blood lipids was conducted by Santiago et al., (1995), who described how 40 weeks of brisk walking failed to significantly modulate plasma total cholesterol concentrations. Studies by Woolf-May et al., (1998) and Woolf-May et al., (1999) also reported no changes in total cholesterol compared to controls after 18 weeks of walking. Interestingly, Wang and Chow, (2004) described an increase in total cholesterol after an acute bout of strenuous exercise as well as a significant decrease in total cholesterol after eight weeks of exercise training. Vuorimaa et al., (2005) however reported significant decreases in total cholesterol immediately after acute exercise. In addition Blumenthal et al., (1991) described no significant changes in total cholesterol after 12 weeks of either strength or aerobic training in middle aged sedentary females. This suggests that exercise intensity is an important factor that influences plasma levels of total cholesterol.

A non significant reduction in plasma triglyceride concentrations was observed in individuals completing the eight week low intensity exercise programme (Table 2.2). Even though this exercise induced modulation was statistically non significant, it nevertheless corresponds with the overall atheroprotective effects of exercise on plasma lipids and lipoproteins reported here. Duncan and Pozehl, (2003) and Murphy et al., (2006) also report no significant changes in plasma triglyceride levels after walking 3 to 4 times a week for 6 months and 8 weeks respectively. However, Murphy et al., (2002) reported that a 6 week walking programme significantly reduced plasma triglyceride levels. A study on men with initially high levels of triglycerides demonstrated exercise training significantly reduced plasma triglyceride levels (Couillard et al., 2001), suggesting that baseline levels of triglycerides maybe a good indicator to whether exercise training will significantly modulate plasma
concentrations of triglycerides. This study demonstrated significant correlations between all baseline lipid parameters and their exercise induced modulations and is further evidence that baseline lipid levels are indicators of the effect of exercise on plasma lipid levels.

Table 2.2 demonstrates that the eight week low intensity exercise programme reduced plasma low density lipoprotein (LDL) levels pre to post exercise, however this exercise induced modulation was deemed non significant. The present study’s result is reinforced by Tully et al., (2007) who also documented no significant modulations in LDL after a 12 week walking based exercise programme. Woolf-May et al., (1998), Murtagh et al., (2005) and Murphy et al., (2006) have also published data that supports this study’s findings, in that walking did not significantly modulate plasma LDL levels. However, the exercise based study by Halverstadt et al., (2007) reported a significant decrease in LDL, but after 24 weeks of aerobic exercise in previously sedentary adults.

After completion of the eight week low intensity exercise programme plasma levels of high density lipoprotein (HDL) in previously sedentary but healthy adults were significantly increased. This potential anti-atherogenic modulation has also been reported in a previous walking based study by Murphy et al., (2002). However, in several other walking based studies there were no reported significant exercise induced modulations in HDL (Murphy et al., 2006) and Tully et al., 2007). Table 2.2 highlights that there were no significant changes in the lipoprotein profiles of all individuals after 4 weeks of the present low intensity exercise programme. Suggesting that a minimum of eight weeks of low intensity exercise is required for
any atheroprotective modulations in lipids to occur. There were also no significant changes in plasma lipid concentrations in the control participants, who maintained their inactive lifestyles for the duration of the study.

The relationship between exercise and high density lipoproteins (HDL) is that individuals with higher levels of aerobic fitness generally tend to have higher concentrations of HDL compared to their sedentary counterparts (Couillard et al., 2001; Olchawa et al., 2004 and Trejo-Gutierrez and Fletcher, 2007). The current study reinforced the trend reported in the literature that the highest HDL responses with exercise are found in those individuals with the lowest HDL concentrations at baseline ($R = -0.706, P = 0.05$). Couillard et al., (2001) reported that regular endurance exercise training may be particularly helpful in men with low HDL and elevated triglyceride levels. As previously inactive healthy men were asked to adhere to an exercise training regime lasting 20 weeks, with a relative exercise intensity starting at 55% VO$_{2\text{max}}$ and finishing at 75% VO$_{2\text{max}}$. Individuals with both low HDL and high triglyceride concentrations showed a significant improvement in lipid profile.

Table 2.3 highlights a significant positive correlation between exercise induced mediations in total cholesterol and LDL. LDL remains the most important clinical lipid target for the clinician in the prevention of cardiovascular disease and the non-pharmacological benefits of exercise on reducing LDL and hence total cholesterol is of considerable importance (Trejo-Gutierrez and Fletcher, 2007). The exercise induced changes in total cholesterol were significantly correlated with oxLDL, reinforcing the understanding that as total cholesterol decreases with exercise,
oxLDL is also reduced. Interestingly, changes in the lipoprotein HDL as a result of participation in low intensity exercise were negatively correlated with oxLDL levels, hence as oxLDL is reduced, HDL increases. These significant correlations between changes in cholesterol, plasma lipoproteins and oxLDL during exercise are limited in terms of causality, since correlations only indicate associations between variables. However, these correlations are further evidence that engagement in physical activity promotes the metabolic clearance of LDL from within the plasma through interactions between oxLDL and HDL, hence RCT.

Adiponectin, a member of the adipocytokine family of proteins has been previously shown to be reduced with obesity and diabetes (Hulver et al., 2002). Low adiponectin levels have been suggested to be a potential risk marker for cardiovascular disease (Rothenbacher et al., 2005). Even though the individuals in this study were considered non-diabetic, it was still deemed worthwhile that the effects of chronic low intensity exercise on serum adiponectin levels were quantified. This was especially warranted as minimal evidence exists regarding the effects of engaging in low intensity exercise on in-vivo adiponectin levels. There is rather conflicting evidence in terms of the effect of exercise intensity, duration and mode on adiponectin concentrations, especially as there has been differences in exercise induced modulations in adiponectin concentrations between healthy and pre-diabetic/diabetic individuals.

Figure 2.1 illustrates that eight weeks of low intensity exercise had no significant effect on serum adiponectin levels in the exercise group and also there was no change within the control group. This finding is reinforced by Hulver et al., (2002)
and Boudou et al., (2003) who reported that 6 months and 8 weeks of exercise, respectively, did not significantly modulate serum adiponectin levels. Hulver et al., (2002) did reinforce the understanding that insulin and adiponectin levels are inversely correlated, both before and after exercise. However, Kriketos et al., (2004) reported that 10 weeks of low to medium intensity exercise caused a significant increase in plasma adiponectin levels. A major dissimilarity between the two studies is that the individuals recruited in the study by Kriketos et al., (2004) were borderline obese (BMI 30.7 ± 0.7kg/m²). Therefore baseline body weight may significantly influence how exercise modulates adiponectin levels in specific populations. To add even more complexity to the story, Jurimae et al., (2005) reported that after 20 minutes of high intensity rowing, trained athletes plasma adiponectin levels were significantly decreased. However, 30 minutes post exercise adiponectin levels had significantly increased compared to baseline values. Therefore, it appears that exercise duration and intensity determines the response of adiponectin to exercise.

In the current exercise study both four and eight weeks of low intensity exercise did not cause any significant alterations in either serum soluble endothelial (sE) or soluble platelet (sP) selectin concentrations (Figure 2.2). Suggesting that adhesion molecules released from endothelial cells and platelets, were unaffected by the low intensity exercise programme. Wang et al., (2005) also reported no significant exercise induced modulations in either sE or sP selectins in healthy sedentary males. In terms of acute exercise, Schwindt et al., (2007) reported that short duration exercise in young males increased both sE and sP concentrations. However, Smith et al., (2000) demonstrated that resistance exercise significantly reduced serum sP selectin, for up to 6 days after exercise. A study by van Eeden et al., (1999) reported
similar results to Smith et al., (2000) as decreases in selectins were observed after a maximal aerobic test in healthy trained adults.

Interleukin-6 (IL-6) is a pleiotropic, abundantly expressed cytokine involved in cell to cell signalling within the immune system (Keller et al., 2003). Until recently the major focus surrounding IL-6 function has been on the immune response, however lately there has been considerable attention regarding the changes in IL-6 both at the gene and protein level, in response to exercise. Serum IL-6 levels were not significantly modulated after adherence to the current eight week low intensity exercise programme, in sedentary healthy adults (Figure 2.3). Few studies have been conducted on the effects of chronic low intensity exercise on plasma/serum IL-6 concentrations. In terms of the acute effects of exercise on plasma IL-6 levels, Ullum et al., (1994) and Keller et al., (2003) have both demonstrated that exercise significantly increased plasma IL-6 levels. Ullum et al., (1994) reported that one hour of cycling at 75% VO2max in moderately trained men caused a significant increase in plasma IL-6 concentrations. Keller et al., (2003) also implemented an acute high intensity exercise programme consisting of 3 hours of cycling at 65% VO2max in sedentary but healthy males.

In a similar manner to the results for the other inflammatory cytokine quantified, namely IL-6, serum TNFα levels were not significantly modulated during the eight week low intensity exercise programme (Figure 2.4). It has been reported that completion of a marathon causes an approximate 3 fold increase in plasma TNFα levels (Starkie et al., 2001a). However, 3 hours of cycling at 75% VO2max significantly reduced plasma TNFα in healthy male individuals (Starkie et al.,
This adds to the complexity regarding the effects of various exercise durations and intensities on inflammatory markers. Taken together, both the serum IL-6 and TNFα results from this study demonstrate that low intensity exercise lasting eight weeks does not significantly alter IL-6 or TNFα expression at the protein level. Therefore, this form of chronic exercise could be perceived as having no adverse proinflammatory effects.

Exercise with its associated hypoxic conditions and increased metabolic activity has been reported to increase oxidative stress (Shern-Brewer et al., 1998; Ross, 1999 and Wei et al., 2005). However, the evidence surrounding the association between exercise duration, intensity and oxidative stress remains controversial. Therefore it was decided to investigate whether the low intensity exercise programme affected the oxidised form of LDL (oxLDL), used as a long-term marker of oxidative stress, in individual’s serum samples. Figure 2.5 demonstrates that the eight week low intensity exercise programme resulted in a significant increase in serum oxLDL levels. These results suggest that the oxidative conditions during exercise maybe responsible for increasing oxLDL. A previous study by Wang et al., (2005) suggests that only medium to high intensity exercise is capable of significantly modulating plasma oxLDL levels. However, a number of differences exist between the current study and the one undertaken by Wang et al., (2005). For instance, the anthropometric characteristics were different between the two exercised based studies, as Wang et al., (2005) recruited only men (age 24.2 ± 0.6 years; body weight 68.1 ± 2.1 kg) and the duration of the exercise was a single 40 minute bout only. The study by Afzalpour et al., (2008) demonstrated that 8 weeks of exercise between 60 and 85% maximal heart rate did not significantly alter serum oxLDL concentrations.
in untrained individuals. This reinforces the understanding that the effects of exercise at differing intensities on the oxidation of LDL remains complex and warrants further investigations.

The uncertainty regarding the effects of exercise intensity and duration on oxidative stress is demonstrated by the evidence that acute high intensity exercise has produced conflicting results to that of chronic low intensity exercise (Goto et al., 2003 and Rush et al., 2003). As part of metabolic processes under normal conditions, cells produce free radicals and reactive oxygen species (ROS). The body’s antioxidant system neutralises these free radicals by the use of specific enzymes including glutathione peroxidase, vitamin A and C and flavonoids (Urso and Clarkson, 2003). Participation in exercise causes an imbalance between ROS and antioxidants leading to oxidative stress (Finaud et al., 2006). However, recent evidence by Wang et al., (2006) suggests that low and moderate exercise attenuates the suppression of the monocyte’s ability to act in an anti-oxidative capacity.

Shern-Brewer et al., (1998) and Liu et al., (1999) reported that healthy subjects are resistant to LDL oxidation in response to aerobic training over several months, whereas an overall shorter exercise duration (i.e. completion of a marathon) elevated plasma LDL oxidation. Shern-Brewer et al., (1998) attributed an increased susceptibility of LDL to undergo oxidation in exercised individuals compared to their sedentary counterparts, to increased amounts of lipid peroxides (Figure 1.8). The enhanced rate of LDL oxidation seen in exercised individuals is suggestive of an ongoing oxidative clearance of LDL in the plasma during exercise. This may account for the lipid lowering effects seen with regular exercise.
In summary participation by previously sedentary healthy adults to an eight week low intensity exercise programme resulted in a significant decrease in total cholesterol and a significant increase in HDL, characteristic of improved plasma lipid profiles. These atheroprotective modulations in plasma lipids were independent of changes in anthropometric measures, including BMI, % body fat and aerobic fitness. The increase in the oxidised form of LDL (oxLDL) also observed here, may provide a trigger for the molecular mechanisms associated with the improved lipid profiles associated with low intensity exercise. Finally, despite previously reported potentially inflammatory nature of engaging in certain forms of exercise, no significant modulations in key inflammatory mediators investigated, namely the cytokines IL-6, TNFα and the enzyme COX-2 were seen.
Chapter 3.

The effect of an eight week low intensity exercise programme on PPARα/γ and LXRα activation.
3.1. Introduction.

Adherence of monocytes to the endothelial wall, and their migration into the subendothelial space in response to chemoattractants, is crucial in the development of atherosclerosis, since it can result in the accumulation of cholesterol that is characteristic of atherosclerotic macrophage foam cells (Ross, 1999). Concurrent with the migration of circulating monocytes into the subendothelial space (Figure 1.7), a programme of differentiation into macrophages takes place that involves the actions of a number of nuclear transcription factors. These include the peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs). Activation of PPARs and LXRs leads to the upregulation of PPAR response element (PPRE) and LXR response element (LXRE) bearing target genes (Li et al., 2002 and Branth et al., 2006). For example, the PPAR isoform PPARγ can be activated by lipids derived from oxLDL, including 9-Hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) (Nagy et al., 1998 and Tontonoz et al., 1998). The accumulation oxLDL within monocytes/macrophages can lead to increased binding of PPARγ to a PPRE within the promoter of the gene encoding CD36. The lipid scavenger receptor CD36 possesses the ability to recognise and internalise modified forms of LDL including oxLDL, upregulation of which leads to increased uptake of oxLDL, via a positive feedback pathway (Nagy et al., 1998 and Chawla et al., 2001b).

Oxidation renders LDL unrecognisable to the LDL receptor and encourages its uptake by scavenger receptors on macrophages in an unregulated manner (Kavanagh et al., 2003). Macrophage scavenger receptors such as CD36, therefore play a significant role in atherosclerotic foam cell development because of their ability to
bind and internalise oxLDL (Collot-Teixirera et al., 2007). CD36 is expressed in many cell types relevant to the progression of atherosclerosis, including monocytes/macrophages, microvascular endothelial cells, adipocytes, skeletal muscle cells, dendritic cells and epithelial cells, and its expression is known to be increased in cells within atherosclerotic lesions (Kunjathoor et al., 2002 and Collot-Teixirera et al., 2007). However, there is evidence for anti-atherosclerotic benefits associated with the upregulation of macrophage CD36 mRNA expression, as stimulation of macrophages with CD36 ligands has been shown to reduce plasma cholesterol levels (Marleau et al., 2005).

While cholesterol is an essential component of cellular membranes, in excess it is toxic to cells. Therefore, the reverse cholesterol transport (RCT) pathway is critical for maintaining cholesterol homeostasis, via transport of excess cholesterol for peripheral tissue to the liver for excretion (Mahley et al., 2006). ATP-binding cassette (ABC) transporters are a superfamily of proteins critical to RCT that use ATP as a source of energy to transport substrates between different cellular compartments including the monocyte/macrophage (Dean et al., 2001). ABC transporters are defined by the presence of nucleotide binding domains containing two conserved peptide motifs known as Walker A and Walker B that are present in many proteins that utilise ATP (Oram and Lawn, 2001). The human ABCA1 gene is composed of 50 exons, which encode 2261 amino acid residues (Santamarina-Fojo et al., 2000). The ABCA1 protein is a full size ABC transporter containing two transmembrane domains and two intracellular nucleotide binding domains (Cavelier et al., 2006). The ATP-binding cassette transporter A1 (ABCA1) plays a key role in reverse cholesterol transport (RCT), during which oxLDL taken in by macrophages
is exported from the potential atherosclerotic site as a component of lipid poor apolipoproteins, a rate limiting step in both HDL formation and cholesterol efflux (Wong et al., 2008).

To maintain lipid homeostasis, ABCA1 is tightly regulated both transcriptionally and posttranslationally (Cavelier et al., 2006). Deficiencies of ABCA1 causes Tangier disease and familial HDL deficiency, both of which are characterised by low levels of HDL. Tangier patients suffer from the excess accumulation of cholesteryl esters in various tissues such as the tonsils, liver, spleen and intestinal mucosa (Chinetti et al., 2001). Like Tangier disease patients, ABCA1 knockout mice exhibit HDL deficiencies and reduced cellular cholesterol efflux activity (McNeish et al., 2000). Agonists of LXR augment ABCA1 expression through a transcriptional cascade which is ultimately dependent upon LXR activation (Isoda et al., 2007).

ATP-binding cassette transporter G1 (ABCG1) is highly expressed in monocytes (Cavelier et al., 2006) and was recently identified as being a mediator of macrophage cholesterol efflux and the formation of mature high density lipoprotein (HDL) but not lipid depleted apolipoproteins (Vaughan and Oram, 2005). The human ABCG1 gene is composed of 23 exons and has multiple transcripts (Langmann et al., 2000). Experiments using siRNA for ABCG1 demonstrated a reduction in cholesterol efflux, however ABCG1 overexpression causes an increase in HDL mediated cholesterol efflux and a reduction in cellular cholesterol (Mauldin et al., 2006). Both ABCA1 and ABCG1 genes are LXRE bearing target genes activated by the nuclear receptor LXRα (Chinetti et al., 2001) and both ABCA1 and ABCG1 have been
proposed to function synergistically to promote cellular cholesterol efflux (Wong et al., 2008).

Studies on the effects of exercise on RCT are limited and have been focused around quantifying HDL as a direct measure of RCT. This is reinforced in the studies by Leaf, (2003) and Olchawa et al., (2004) who have focused on the effects of exercise on RCT within the liver and have used concentrations of HDL as in-direct measures of RCT. Therefore, the precise mechanisms responsible for the increase in RCT associated with a physically active lifestyle remain unclear. Furthermore, metabolic regulation of ABCA1 expression in leukocytes may contribute to the atheroprotective effect of physical exercise, as ABCA1 expression in these cells plays a key role in determining susceptibility to atherosclerosis (Hoang et al., 2008).
3.1.1. Aims.

The primary aim of this chapter is to investigate the hypothesis that low intensity exercise regulates lipid metabolism through activation of the lipid regulating transcription factor PPAR. Furthermore, the secondary aim of this chapter is determine if PPAR activation is associated with activation of the nuclear transcription factor LXRα and reverse cholesterol transport (RCT). These aims will be investigated through:

1. Determining the effect of an 8 week low intensity exercise programme on the mRNA expression of the scavenger receptors CD36 and SR-A1.
2. Determining PPARα and PPARγ mRNA expression and activity in response to adherence to an 8 week low intensity exercise programme.
3. Investigating whether 8 weeks of low intensity exercise stimulates the expression of a number of inflammatory mediators, including AGER and COX-2.
4. Investigating the effects of a low intensity exercise programme on LXRα activation and reverse cholesterol transport (RCT), through quantification of ABCA1, ABCG1 and ApoE gene expression.
3.2. Methodology.

For participants, pre-health screening, design of exercise programme and dietary analysis, refer to sections 2.2.1, 2.2.2, 2.2.3 and 2.2.4.

3.2.1. Determination of white blood cell counts in participants samples.

Total leukocyte counts were measured on whole blood using an automated Beckman Coulter counter (Coulter, Buckinghamshire, UK).

3.2.2. Isolation of RNA from participant’s leukocytes.

The extraction of total RNA was carried out using an Ambion® RiboPure™-Blood Kit (Applied Biosystems, Warrington, UK) according to the manufacturer’s instructions. Briefly, samples were thawed, vortexed and centrifuged at 13200 x g for 1 minute. The supernatant was removed and lysis and sodium acetate solutions were added and the mixture vortexed and acid-phenol:chloroform was added with further vortexing. Samples were left to stand for 5 minutes at room temperature (RT) and the tubes were centrifuged at 13’200 rpm for 1 minute and the upper aqueous phase containing the RNA was transferred to a fresh RNase free collection tube. To this 500–600μL of 100% ethanol was added to each tube, a filter cartridge was inserted into a new RNase free collection tube and 700μL of the sample was carefully applied in the middle of the cartridge. The tubes were centrifuged for ~20 seconds and the flow through was discarded. This procedure was repeated, after which 700μL of wash solution 1 was applied to the cartridge assembly and centrifuged for ~10
seconds. The flow through was again discarded and the filter cartridges were washed twice with wash solution 2/3 for ~10 seconds each. After these washes the filter cartridges were centrifuged at 13200 rpm for 1 minute to remove the last traces of wash solution. The filter cartridges were transferred to fresh RNAse collection tubes and pre-heated elution solution was applied to the centre of filters. Tubes were left to stand at RT for 30 seconds and then centrifuged for 35 seconds at 13’200 rpm. The filter cartridges were removed and the RNA stored at -80°C.

RNA was quantified and checked for purity using the ratio of its absorbance at 260:280nm using an Amersham Biosciences Gene Quant Pro Spectrophotometer (Buckinghamshire, UK) (only samples of ratio > 1.8 being deemed suitable for use), and by visualisation on 1% denaturing agarose electrophoresis gels. Genomic DNA contamination was controlled for by using extracted nucleic acid directly as templates in control PCR reactions.

3.2.3. Conversion of RNA to cDNA.

RNA samples were converted to cDNA using an Applied Biosystems® High-Capacity cDNA Archive Kit (Warrington, UK) and stored at –20°C. Briefly, 10μL of diluted RNA (all participants samples were diluted to 30ng/μL), was added to 10μL of 2X RT Master Mix. Samples were mixed by simple pipette mixing and briefly centrifuged to eliminate any air bubbles. Tubes were placed in an Applied Biosystems 96-Well GeneAmp® PCR System 9700 (Warrington, UK) and the following thermal cycling was applied. Step 1: 10 minutes at 25°C; step 2: 120 minutes at 37°C; step 3: 5 seconds at 85°C and step 4: 4°C for ∞.
3.2.4. Storage of primers.

Desalted, lyophilised primers were reconstituted in TE buffer to give a stock concentration of 100μM. After a brief mix 50μL of stock solution was added to 450μL of ddH₂O to give a working concentration of 10μM and 50μL aliquots were taken and stored along with the stock concentration at -20°C. Once an aliquot had been thawed, it was either used or discarded, in order to minimise the degradation of the primers due to freeze thawing.

3.2.5. Reverse transcriptase polymerase chain reaction (RT-PCR).

The reverse transcription reaction mixture consisted of 5μL 10 X PCR buffer, 1.5μL 50 mM MgCl₂, 1μL 10 mM dNTP mix, 1μL of both forward and reverse primers, 0.4μL of Taq DNA polymerase, 2μL of cDNA and finally 38.1μL of RNase free water. Semiquantitative PCR was performed using the following selected primer pairs: Beta Actin (β-actin): 5’-TCCTGTGGCATCCACGAA-3’; 5’-GAAGCATTTGCGGTGGAC-3’ and CD36: 5’-GGAAGTGATGATGAACAGCAGC-3’; 5’-GAGACTGTGTTGTCTCCTCAGCG-3’ to give PCR products of 315bp and 117bp. Thermocycling was performed as follows, using an Applied Biosystems Gene Amp PCR System 9700 (Warrington, UK): primary melting: 95°C for 5 minutes followed by n cycles of 95°C for 60 seconds (secondary melting), 56°C for 60 seconds (annealing) and 72°C for 60 seconds (extension), with a final elongation step of 95°C for 7 minutes. To optimise the number of cycles needed for amplification, RT-PCR was performed with a single cDNA sample, using a number of amplification cycles ranging from 26 to 38 cycles.
With the intensity of the PCR band being representative of the quantity of PCR product.

### 3.2.6. Agarose gel electrophoresis for RT-PCR products.

A 1% w/v agarose gel was made by adding 0.3g of agarose to 30mL of 1 X TBE running buffer. The mixture was heated in a microwave to allow the agarose to dissolve and was then subsequently cooled to 50-60°C. The mixture was poured into the electrophoresis tank containing an 8 well comb. The comb was removed after 30 minutes and the electrophoresis tank was filled with 1 X TBE running buffer until the surface of the gel was just covered. To each 10mL of PCR product, 2μL of high performance RNA gel loading dye was added and then loaded into its allocated well and electrophorises undertaken at 80V for 90 minutes. Gels were stained with 0.05mg/mL ethidium bromide and photographed under UV light using a Gel Doc 2000 gel documentation system (Bio-Rad, Hertfordshire, UK). Densitometry analysis was performed on the PCR products using an AutoChemi System UVP Bioimaging System (UVP, Cambridge, UK).

### 3.2.7. Real time-PCR primer optimisation.

Optimisation of forward and reverse primer concentrations for all genes quantified was determined using a primer titre table (Table 3.1). By independently varying the forward and reverse primer concentrations, the combination of primer concentrations that provide optimal assay performance is identifiable. The primer concentrations selected should be the minimum forward and reverse primer concentrations that yield
the maximum ΔRn values and lowest Cₜ values. ΔRn is defined as the magnitude of the signal generated by the given set of PCR conditions and is determined using the following formula: (Rn+) – (Rn−). Cₜ is the fractional cycle number at which the fluorescence passes a fixed threshold.

Table 3.1: Titration of real time-PCR primers to determine the minimum concentrations of forward and reverse primers that yielded the lowest Cₜ values and highest ΔRn.

<table>
<thead>
<tr>
<th>Forward Primer (nM)</th>
<th>Reverse Primer (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300</td>
</tr>
<tr>
<td>300</td>
<td>300/300</td>
</tr>
<tr>
<td>400</td>
<td>400/300</td>
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<tr>
<td>500</td>
<td>500/300</td>
</tr>
<tr>
<td>600</td>
<td>600/300</td>
</tr>
<tr>
<td>700</td>
<td>700/300</td>
</tr>
</tbody>
</table>

3.2.8. Real time-PCR.

Real time-PCR was performed for the following genes CD36, SR-A1, CD14, PPARγ, LPL, PPARα, Acyl-CoA oxidase, IL-6, TNFα, AGER, COX-2, LXRα, ABCA1, ABCG1 and ApoE, using GAPDH as the endogenous reference. Gene expression was analysed on a Applied Biosystems 7500 Real-time PCR system (Warrington, UK) using Platinum® SYBR® Green qPCR SuperMix-UDG
(Invitrogen, Paisley, UK). For each reaction 12.5μL of Platinum® SYBR® Green qPCR SuperMix-UDG was added to 0.05μL of ROX reference dye, n μL of both the forward and reverse primers, n μL of RNase free water and 5μL of cDNA sample. (n depends upon optimisation of individual primers for each gene quantified). This was briefly centrifuged to remove any air bubbles and the following thermal cycling was performed. Activation step: 95°C for 20 seconds (hold); denaturing step: 95°C for 3 seconds; annealing/extension step: 60°C for 30 seconds, both the denaturing and annealing steps were repeated 40 times. The extension temperature was optimised for each primer set and ranged from 56 to 64°C. A dissociation step was included to check for the amplification of one single PCR product. All primers for the real time-PCR were designed using the Applied Biosystems Primer Express ® software V 2.0 (Warrington, UK). The following guidelines were considered and adhered to during the design of primer sequences. Both primer sequences should have similar if not identical % GC content and should have comparable melting temperatures (Tm), within the range of 55 - 60°C. In order to minimise the risk of primer dimer formations, sequences of identical nucleotides, especially G’s were avoided, as well as ensuring the last five nucleotides at the 3’ end of the primer contained no more than two G/C bases. Finally all potential primer sequences were screened using a Basic Local Alignment Search Tool (BLAST) search on PubMed to ensure that they were specific only to the region of the gene in question. Table 3.2 highlights the primer sequences that were used. Estimates of cDNA abundance were made using the portion of the curve for which the plot of the log input amount versus the C_T (cycle threshold) differences resulted in a slope of approximately 0, indicating that the amplicon efficiencies were approximately equal. Relative quantification of target
genes in human leukocytes was calculated using the $2^{-\Delta\Delta CT}$ formula, in which $\Delta C_T$ equals the difference between $C_T$ values for both target gene and GAPDH.

**Table 3.2: Primer sequences used in real-time-PCR analysis.**

<table>
<thead>
<tr>
<th>Gene (Accession No)</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36 (NM_001001548.1)</td>
<td>GGAAGTGATGACAGACAGAC</td>
<td>GAGACCTGTGTGTCCTCAGCG</td>
<td>117</td>
</tr>
<tr>
<td>SR-A1 (NM_138716.2)</td>
<td>ATTCGCTTTACCTCCTCTCTG</td>
<td>TCAATCTTCTCTGCCGTGAG</td>
<td>149</td>
</tr>
<tr>
<td>CD14 (NM_001040021.1)</td>
<td>GGTTCGGAAGACTTATCGACCA</td>
<td>TCTCAGTGTCAGACAGACTGAC</td>
<td>111</td>
</tr>
<tr>
<td>PPARγ (NM_138711.3)</td>
<td>CGTGAGCGAGTTTGGAA</td>
<td>CTCCTCCATTAGGAAGAGATCCCG</td>
<td>166</td>
</tr>
<tr>
<td>LPL (NM_000237.2)</td>
<td>GAGATTTCTCTGTAAGGACAC</td>
<td>CTGCCAAATGAGAATACACTTC</td>
<td>276</td>
</tr>
<tr>
<td>PPARα (NM_005036.4)</td>
<td>GACAAGGCCAGAGTTTGGAA</td>
<td>TCGACCTGTGATACACACAGCG</td>
<td>101</td>
</tr>
<tr>
<td>Acyl-CoA oxidase</td>
<td>GCACACACTTTAAGGAAGAGAGAT</td>
<td>GCCACCATTTAATGGAGGAT</td>
<td>130</td>
</tr>
<tr>
<td>IL-6 (NM_000600.2)</td>
<td>GCCTCGGAGAGTGGCTTC</td>
<td>AGTGGGCTTGTCCTGTC</td>
<td>233</td>
</tr>
<tr>
<td>TNFα (NM_00594.2)</td>
<td>CGAGGGTCCAGCCCTTTAGG</td>
<td>GTGGTTGAGAGGCACAT</td>
<td>215</td>
</tr>
<tr>
<td>AGER (NM_001136.3)</td>
<td>ATGGAAACTGAAAGAGACGCG</td>
<td>AAAATCCCTACATCTGGAGATCC</td>
<td>140</td>
</tr>
<tr>
<td>COX-2 (NM_000963.1)</td>
<td>TGAGGGTCCACCTCCCAACACA</td>
<td>GAGAAGGCTTTCCAGCCTTT</td>
<td>187</td>
</tr>
<tr>
<td>LXRa (NM_005693.1)</td>
<td>CGACACTCAGCTGCAAGCG</td>
<td>TGAGGGTCCAGCTGTC</td>
<td>141</td>
</tr>
<tr>
<td>ABCA1 (NM_005502.2)</td>
<td>GCAGCTGAGGAGATGCTGAA</td>
<td>AGTTTCTGGAAGGCTGTGTTCA</td>
<td>205</td>
</tr>
<tr>
<td>ABCG1 (NM_207627.1)</td>
<td>AGCAGAGTCTGCAAGCTCTCTT</td>
<td>CCGAGGTCAGTCACAGACTT</td>
<td>160</td>
</tr>
<tr>
<td>ApoE (NM_000041.2)</td>
<td>GTGGGCTTTAGGGTACCGCG</td>
<td>CCGGTCAGTGTGCT</td>
<td>162</td>
</tr>
<tr>
<td>GAPDH (NM_002046.3)</td>
<td>CATTGAACCTCAACTACATG</td>
<td>TCTCCATGGGTGAGAC</td>
<td>209</td>
</tr>
</tbody>
</table>

**3.2.9. Isolation of human peripheral mononuclear cells.**

For isolation of peripheral blood mononuclear cells, 10mL of blood was diluted 1:1 in RPMI, layered over 10mL of Histopaque-1077 Ficoll-Hypaque (Sigma, Dorset, UK) and centrifuged at 400 x g for 20 minutes. The mononuclear cell suspension was
carefully removed from the Ficoll-Hypaque interface, washed (x4), by centrifuge at 500 x g for 10 minutes, in 0.4mL of Active Motif phosphate inhibitor solution (Rixensart, Belgium) and 7.6mL Phosphate-Buffered Saline (PBS).

3.2.10. PPARγ DNA-binding activity assay.

PPARγ DNA-binding activity was determined using a Trans AM™ PPARγ assay kit according to the manufacturer’s instructions (Active Motif, Rixensart, Belgium). The 96 well plate provided with the PPARγ kit contained an immobilised oligonucleotide sequence for the peroxisome proliferator response element (PPRE) (5’-AACTAGGTCAAAGGTCA-3’). Any activated PPARγ present in the nuclear extract bind specifically to its corresponding nucleotides and is subsequently detected by addition of a primary antibody that recognises a specific accessible epitope on the PPARγ protein upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a colorimetric measurement quantified by spectrophotometry. In each individual well, 40μL of complete binding buffer (containing 1% herring sperm DNA) was added. For each 8μg of nuclear extract sample, 20μL of complete lysis buffer (containing 0.1% 1M DTT and 1% protease cocktail inhibitor) was added. The 20μL of diluted samples were added to their corresponding well, after which the plate was then sealed and incubated for 1 hour at room temperature (RT) with mild agitation using a rocking platform. After incubation the samples were washed (x3) with 200μL washing buffer and after each wash the plate was blot dried to remove excess buffer. Each well was then incubated with 100μL of diluted PPARγ primary antibody (1:1000 dilution in 1 X antibody dilution buffer). The plate was then sealed and incubated for a further 1 hour at RT.
The plate was washed with 200µL of washing buffer and blot dried, after which 100µL of diluted HRP-conjugated secondary antibody (1:1000) was added to each well. The plate was again sealed and incubated for 1 hour at RT. The wells were then washed (x4) with 200µL washing buffer and blot dried. The developing solution was warmed to RT and 100µL was added to each individual well and incubated for between five and ten minutes at RT in the dark until the development of the blue colour became apparent. The reaction was stopped by addition of 100µL of stop solution (sulphuric acid). Colorimetric results were quantified by spectrophotometry within five minutes, using the absorbance 450nm with a reference of 620nm (Dynex Technologies, VA, USA).

3.2.11. Bioinformatics analysis.

Sequence analyses and alignments were performed using DNASTAR™ software (Lasergene, version 7; DNASTAR Inc, Madison, WI).


Data is expressed as mean ± standard deviations (SD). When analysing multiple comparisons within groups, a one-way analysis of variance (ANOVA) was applied to normally distributed data and the Kruskal-Wallis test for data which was non normally distributed. For non multiple comparisons between exercise and control groups, two sample t-tests were used. Significance levels were set at $P<0.05$. All statistical calculations were carried out using Minitab 14®.
3.3. Results.

3.3.1. Determination of RNA integrity after extraction from participant’s blood.

Upon completion of RNA extraction from all participants’ blood samples, several were picked at random and assessed for RNA quality using a 1% denaturing agarose gel, stained with ethidium bromide. Figure 3.1 illustrates that the RNA was clearly present in both samples as identified after analysis under UV light. Both the 18s and 28s strands of ribosomal RNA are readily identifiable in figure 3.1.

![Figure 3.1: Initial assessment of RNA quality in participant’s blood.](image)

The purity of all RNA samples was quantified by using the ratio of absorbance at 260:280nm, with all having a ratio greater than 1.7. Figure 3.2 illustrates that two random participant’s RNA samples demonstrated no observed degradation by RNases. A single peak at 260nm is highly indicative that the RNA sample is free
from RNase contamination, multiple absorbance peaks would suggest RNase contamination.

![Absorbance Peaks Graph](image)

**Figure 3.2: Determination of RNA contamination in participant’s samples.** Two random participants RNA samples were quantified using a Nanodrop Spectrophotometer and absorbance was determined at various wavelengths, ranging from 220 to 350 nm. Absorbance was then plotted against wavelength to reveal a single RNA peak at 260nm.

3.3.2. Effect of low intensity exercise on cell differential count.

There was no significant change in white blood cell count after the adherence to the eight week low intensity exercise programme. Table 3.3 demonstrates that there was
no change in total white cell count or in the percentage of each leukocyte subtype after completion of the exercise programme.

Table 3.3: Effects of low intensity exercise on white blood cell count.

<table>
<thead>
<tr>
<th>Exercise Group (n=8)</th>
<th>Week Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WBC (x10⁹L)</td>
<td>7.5 (2.1)</td>
</tr>
<tr>
<td>NE (%)</td>
<td>51.6 (7.6)</td>
</tr>
<tr>
<td>LY (%)</td>
<td>34.0 (6.3)</td>
</tr>
<tr>
<td>MO (%)</td>
<td>10.1 (2.1)</td>
</tr>
<tr>
<td>EO (%)</td>
<td>3.4 (1.7)</td>
</tr>
<tr>
<td>BA (%)</td>
<td>0.9 (0.3)</td>
</tr>
</tbody>
</table>

Data is presented as means and (S.D).

3.3.3. Determination of levels of CD36 gene expression using PCR.

The following two methods were used to quantify CD36 gene expression in individual’s leukocytes:

1. Conventional RT-PCR with densitometry
2. Real time-PCR, using the $2^{\Delta\Delta CT}$ formula to determine relative gene expression.
3.3.3.1. Optimisation of CD36 RT-PCR cycle number.

Using conventional reverse transcriptase-PCR (RT-PCR) it is imperative to optimise which cycle number gives a detectable yield within the exponential phase of amplification. Optimisation of CD36 RT-PCR cycle number revealed that 30 cycles was the optimum number required to determine CD36 gene expression (Figure 3.3).

**Figure 3.3: Optimisation of CD36 RT-PCR cycle number.** CD36 mRNA was determined in a random sample using RT-PCR cycle number ranging from 26 to 36. PCR products were then run on a 1% denaturing agarose gel, stained with ethidium bromide and visualised under UV.
3.3.3.2. Preliminary investigation into the effect of low intensity exercise on CD36 mRNA expression, using RT-PCR.

The low intensity exercise programme investigated in this study resulted in a significant increase in oxLDL within the exercise group as compared to non-exercise individuals. In response to elevated plasma oxLDL levels, monocytes are known to upregulate their expression of the scavenger receptor CD36. Therefore, it was decided to investigate whether the increase in oxLDL was associated with changes in CD36 gene expression. Figures 3.4a and 3.4b illustrate typical gels for CD36 and β-actin gene expression, respectively, at baseline, and at weeks 4 and 8. CD36 gene expression was determined in two random participant’s leukocyte samples using RT-PCR, consisting of 30 thermal cycles and then run on a 1% denaturing agarose gel and visualised by UV densitometry after staining with ethidium bromide (Figure 3.4a). β-actin gene expression was again determined using RT-PCR, consisting of 34 thermal cycles and visualised using the same procedure as applied for CD36 mRNA (Figure 3.4b).
Figure 3.4: Effects of exercise on CD36 and β-actin mRNA expression. Gene expression of CD36 (a) and β-actin (b) was determined in participant’s leukocytes using RT-PCR and visualised on a 1% denaturing agarose gel and visualised under UV light. Lane 1 = 100bp ladder; lanes 2-4 (Participant x, weeks 8, 4 and 0), lanes 5-7 (Participant y, weeks 8, 4 and 0).

Figure 3.5 illustrates the percent change in CD36 gene expression relative to β-actin as determined by RT-PCR, run on a 1% denaturing agarose gel and analysed by densitometry (Figures 3.4a and 3.4b). Low intensity exercise increased CD36 mRNA expression significantly after both four (58%) and eight weeks (32%), compared to baseline.
Figure 3.5: Effect of an eight week low intensity exercise programme on CD36 mRNA expression. Both CD36 and β-actin mRNA expression in participant’s leukocytes was determined using RT-PCR and quantified using densitometry. CD36 gene expression at all time points is relative to β-actin gene expression, with the exercise induced modulations in CD36 gene expression being relative to baseline (dotted line). Results are expressed as mean ± SD (* P<0.05 ANOVA, compared to baseline), n = 5.

3.3.3. Optimisation of CD36 real time-PCR primer concentrations.

Optimisation of real time-PCR CD36 primer concentrations revealed that the optimum forward and reverse CD36 primer concentrations that yielded the maximum ∆Rn values and lowest C_T values were both 600nM (Figure 3.6).
**Figure 3.6: Optimisation of CD36 real time-PCR primer concentrations.** Real time-PCR was conducted on a single cDNA sample using various combinations of forward and reverse primers, ranging from 300nM to 600nM. The red circle highlights the optimum concentration of forward (600nM) and reverse (600nM) primers required per real time-PCR reaction. Results are expressed as means with determinants quantified in triplicate. Error bars are emitted for clarity.

3.3.3.4. Real time-PCR relative efficiency of CD36 and GAPDH.

For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target amplification (in this case CD36) and the efficiency of the reference amplification (i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how $\Delta C_T$ varies with a template dilution. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus $\Delta C_T$ in theory has a slope of approximately zero. However the absolute value of the slope of log input amount vs.
ΔC_T should be between -0.1 and 0.1. Once this is confirmed, ΔΔC_T can be used for the relative quantitation of a target gene without running standard curves on the same plate. The real-time-PCR relative efficiency between GAPDH and all target genes was quantified prior to analysis on individual’s samples. Figure 3.7 demonstrates that the slope of the line representing ΔC_T of CD36 and GAPDH and log10 cDNA is 0.0403. This corresponds to a significant efficiency between the target gene CD36 and the endogenous reference GAPDH. Therefore, all the real-time-PCR experiments using CD36 and GAPDH primers are efficient in terms of using compatible primer pairs.

Figure 3.7: Real time-PCR relative efficiency of CD36 and GAPDH. CD36 and GAPDH mRNA expression were quantified using real-time-PCR on obtained cDNA from one participant. Using a template, a dilution series of cDNA, ranging from 15 to 0.23ng was made. ΔC_T was then plotted against its corresponding log10 cDNA value. Results are expressed as mean ± SD of three individual experiments.
3.3.3.5. **CD36 real time-PCR relative efficiency.**

The second parameter that must be considered in order for the ΔΔC_T calculation to be valid is that the efficiency of a given real time-PCR amplification must be as close to 100% as possible. Real time-PCR efficiency was determined for all genes quantified in this study. Figure 3.8 demonstrates that during the real time-PCR of CD36 gene expression, 93% PCR efficiency was achieved.

\[
\text{Efficiency (E)} = 10^\left(-1 / \frac{-3.5034}{26.899}\right) - 1
\]

\[
= 10^\left(0.285437004\right) - 1
\]

\[
= 0.929465439 \text{ or } 92.9\%
\]

**Figure 3.8: CD36 real time-PCR relative efficiency.** CD36 mRNA expression was quantified using real time-PCR on a single cDNA sample, using a template dilution series of cDNA, ranging from 15 to 0.234 ng. CD36 C_T was then plotted against its corresponding log10 RNA value. Results are expressed as mean ± SD of three individual experiments.
3.3.3.6. The effect of low intensity exercise on CD36 mRNA expression.

The preliminary investigation using conventional RT-PCR revealed that low intensity exercise significantly upregulates CD36 mRNA expression in five random participant’s samples, after both four and eight weeks. Therefore it was decided to further quantify CD36 mRNA expression in all the participants’ samples, including the exercise and control groups using real time-PCR. Real time-PCR is a more automated and sensitive method compared to that of conventional PCR. As real time-PCR quantifies amplification of PCR products at the start of the exponential phase as opposed to RT-PCR which uses a point within the exponential phase. As can be seen in figure 3.9, low intensity exercise increased CD36 mRNA expression significantly in the exercise group after both 4 (3.9 fold) and 8 weeks (2.7 fold), compared to baseline. There were no significant modulations in CD36 mRNA expression in the control group. The increase in CD36 mRNA expression was also significant compared to the corresponding CD36 mRNA expression levels in the control group, at both 4 and 8 weeks.
Figure 3.9: The effect of low intensity exercise on CD36 mRNA expression using real time-PCR. Gene expression of CD36 was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* $P=0.006$ ANOVA, compared to baseline (dotted line); # $P<0.05$ two sample t-test, compared to controls), n = 17.


In order to establish whether the adherence to the eight week low intensity exercise programme uniformly upregulates scavenger receptors, with similar characteristics to CD36, SR-A1 was quantified. SR-A1, which has been reported to play a major role in the development of atherosclerosis, is also known to be regulated by the nuclear transcription factor PPARγ. Unlike the lipid scavenger receptor CD36 which is upregulated after low intensity exercise, there was no significant change in SR-A1 mRNA after either four or eight weeks of low intensity exercise, as compared to
baseline (dotted line) (Figure 3.10). No significant change in SR-A1 expression was observed within the control group and no significant difference between the exercise and control group was observed at any of the time points investigated.

Figure 3.10: The effect of low intensity exercise on SR-A1 mRNA expression. Gene expression of SR-A1 was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD, n = 17.

3.3.5. The effect of low intensity exercise on the lipid sensor CD14.

The lipid sensor and PPARγ regulated gene, CD14, was quantified in all individuals samples using real time-PCR to confirm the degree of PPARγ upregulation during low intensity exercise. There was a significant upregulation of CD14 mRNA
expression after only eight weeks of the low intensity exercise programme (Figure 3.11). There were no significant changes in CD14 gene expression in the control group throughout the study. CD14 mRNA expression was also not significantly different between groups at baseline, week 4 or 8.

![Figure 3.11: The effect of an eight week low intensity exercise programme on CD14 mRNA expression.](image)

Figure 3.11: The effect of an eight week low intensity exercise programme on CD14 mRNA expression. Gene expression of CD14 was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* $P=0.047$ ANOVA, Tukey’s pairwise analysis, compared to baseline (dotted line)), n = 17.

3.3.6. The effect of low intensity exercise on leukocyte PPARγ mRNA expression and DNA-binding activity.

After demonstrating that the low intensity exercise programme was capable of significantly increasing the oxidative form of LDL (oxLDL), which contains a
number of known PPARγ ligands, we further investigated the effect of low intensity exercise on PPARγ gene expression using real-time PCR on all cohort samples. As can be seen in figure 3.12, exercise significantly increased PPARγ gene expression after four (1.8 fold) and eight weeks (4.3 fold). No significant change in the mRNA expression of PPARγ was seen in the control group. PPARγ gene expression in the exercise group was significantly different as compared to the control group after both 4 and 8 weeks.

![Figure 3.12](image)

**Figure 3.12: The effect of low intensity exercise on leukocyte PPARγ mRNA expression.** Gene expression of PPARγ was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* P=0.0001 ANOVA, compared to baseline (dotted line); # P<0.05 two sample t-test, compared to controls), n = 17.
As the large increase in mRNA for this transcription factor is strongly indicative of increased synthesis of PPARγ protein and therefore upregulation of PPARγ target genes, (e.g. CD36), it was decided to determine whether the observed increase in mRNA for PPARγ was associated with an increase in PPARγ DNA-binding activity. Figure 3.13 demonstrates that after four weeks of a subsequent low intensity exercise programme, there was a significant increase (5 fold) in PPARγ’s ability to bind to an immobilised PPRE sequence (baseline: 0.22±0.09 OD units; week 4: 1.13±0.29 OD units). A result strongly suggestive of increased PPARγ activity.

![Figure 3.13: The effect of low intensity exercise on PPARγ DNA-binding activity.](image)

Figure 3.13: The effect of low intensity exercise on PPARγ DNA-binding activity. PPARγ DNA-binding activity was determined in nuclear extracts at baseline and after four weeks of low intensity exercise in 5 sedentary but healthy adults. Results are expressed as mean ± SD (* P<0.05 two sample t-test, compared to baseline).
3.3.7. The effects of an 8 week low intensity exercise programme on the mRNA expression of the PPARγ regulated gene, namely lipoprotein lipase.

Even though the exercise induced decrease in plasma triglyceride levels was deemed non significant, it was still decided to look at the effects of exercise on lipoprotein lipase (LPL) mRNA expression. This was to establish whether LPL, an enzyme that plays a key role in lipid metabolism, including responsibility for the hydrolysis of circulating VLDL-TG (Kiens et al., 2004), correlates with the decrease in plasma triglycerides. Figure 3.14 demonstrates that adherence to the eight week low intensity exercise programme by sedentary healthy adults, did not result in any significant changes in LPL mRNA, compared to baseline (dotted line) in either the exercise or control group. There was also no significant difference in LPL gene expression between the exercise and control groups.
Figure 3.14: The effect of low intensity exercise on lipoprotein lipase gene expression. Gene expression of LPL was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD, n = 17.

3.3.8. The effect of an 8 week low intensity exercise programme on PPARα mRNA expression.

In order to determine whether low intensity exercise can induce modulations in another nuclear transcription factor involved in lipid metabolism and homeostasis, namely PPARα, real-time PCR of the PPARα gene was conducted. Figure 3.15 demonstrates that low intensity exercise stimulated a significant (1.7 fold) increase in PPARα gene expression only after four weeks. After eight weeks of the exercise programme PPARα gene expression was not significantly different to baseline. There were no significant changes in PPARα gene expression in the control group.
throughout the study. No significant differences between exercise and control groups PPARα mRNA expression was observed at all time points quantified.

Figure 3.15: The effect of an 8 week low intensity exercise programme on PPARα mRNA expression. Gene expression of PPARα was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* P=0.012 ANOVA, Tukey’s pairwise analysis, compared to baseline (dotted line)), n = 17.

3.3.9. The effect of low intensity exercise on the PPARα regulated gene Acyl-CoA oxidase.

The low intensity exercise programme’s ability to significantly upregulate PPARα mRNA, was further investigated by also determining the levels of an important lipid regulating and PPARα regulated gene, namely acyl-CoA oxidase. However, acyl-
CoA oxidase was not significantly altered in either the exercise or control group after either four or eight weeks of the current study (Figure 3.16). There was also no significant difference in acyl-CoA oxidase gene expression between the exercise and control group.

![Graph showing Acyl-CoA oxidase gene expression](image)

**Figure 3.16:** The effect of an 8 week low intensity exercise programme on acyl-CoA oxidase mRNA expression. Gene expression of acyl-CoA oxidase was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD, n = 17.

3.3.10. The effect of an eight week low intensity exercise programme on the mRNA expression of the NF-κB regulated genes IL-6 and TNFα.

Table 3.4 demonstrates that after either four or eight weeks of low intensity exercise the gene expression of both IL-6 and TNFα was not significantly different compared
to baseline. Both of these inflammatory cytokines are known to be regulated by NF-κB and therefore this low intensity exercise programme could be perceived as being anti-inflammatory. There was also no significant change in IL-6 and TNFα gene expressions in the control group and no significant differences in gene expressions between the exercise and controls groups at all time points.

3.3.11. The effect of low intensity exercise on advanced glycated end-product receptor mRNA expression.

This study has already reported increases in the oxidative form of LDL (oxLDL), due possibly to the oxidative nature of exercise, possibly through an imbalance between ROS and antioxidants. It was therefore decided to investigate whether adherence to the eight week low intensity exercise programme causes increases in a number of inflammatory mediators. Advanced glycated end-product receptor (AGER), which is widely regarded as playing a major role in the development of atherosclerosis, was chosen to quantify due to its interactions with the proinflammatory nuclear transcription factor NF-κB. There were no significant changes in AGER mRNA expression in either the exercise or control groups after four and eight weeks of the current study (Table 3.4). There was also no significant difference in AGER gene expression between the exercise and control groups at all time points quantified.

Another inflammatory mediator quantified on the exercise and control samples was cyclooxygenase-2 (COX-2). This proinflammatory enzyme involved in the synthesis of the biological mediators prostanoids, has recently been shown to play a potential role in the upregulation of PPARγ. This is despite its known cross talking capabilities with the nuclear transcription factor NF-κB. There was no significant change in COX-2 mRNA expression after either four or eight weeks, in the exercise and control group (Table 3.4). There was also no significant difference in COX-2 gene expression between the exercise and control groups at baseline, week four or week eight.

Table 3.4: Effects of low intensity exercise on the mRNA expression of IL-6, TNFα, AGER and COX-2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exercise (n=17)</th>
<th>Control (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.01 (0.2)</td>
<td>-0.01 (0.26)</td>
</tr>
<tr>
<td>TNFα</td>
<td>-0.14 (0.19)</td>
<td>0.11 (0.19)</td>
</tr>
<tr>
<td>AGER</td>
<td>0.23 (0.20)</td>
<td>0.25 (0.14)</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.02 (0.21)</td>
<td>0.12 (0.18)</td>
</tr>
</tbody>
</table>

Data is expressed as fold change in gene expression compared to baseline and is presented as mean ± (SD).
3.3.13. The effect of low intensity exercise on leukocyte LXRα mRNA expression.

So far the combination of the findings in this chapter are suggestive of the potential proatherogenic modulations associated with low intensity exercise. In that low intensity exercise significantly increases plasma oxLDL concentrations, which is associated with upregulation of the lipid scavenger receptor, CD36 and the lipid sensor receptor CD14, possibly mediated via the nuclear transcription factor PPARγ. This is indicative of increased susceptibility of monocytes/macrophages to become lipid laden foam cells, due to the continual influx of modified lipids, including oxLDL, via CD36. It was therefore decided to investigate whether the decrease in LDL and increase in HDL reported in chapter 2, was due to a cellular mechanism facilitating the influx of proatherogenic cholesterol from the bloodstream into monocyte/macrophages and then the subsequent efflux of anti-atherogenic cholesterol back into the bloodstream.

The nuclear transcription factor Liver X receptor-α (LXRα), is a plausible candidate to facilitate this potential reverse cholesterol transport (RCT) during the current low intensity exercise programme. LXRα is also known to regulate a number of cholesterol efflux genes involved in RCT and was therefore subsequently quantified in all cohort samples. A significant increase (3.5 fold) in the gene expression of LXRα was observed after eight weeks of low intensity exercise only (Figure 3.17). No significant increase in LXRα gene expression was seen in the control group. There was a significant difference in LXRα mRNA expression between the exercise and control group at week 8 only.
Figure 3.17: The effect of low intensity exercise on leukocyte LXRα mRNA expression. Gene expression of LXRα was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* P=0.0001 ANOVA, Tukey’s pairwise analysis, compared to baseline (dotted line); # P<0.05, two sample t-test, compared to controls), n = 17.

3.3.14. The effect of low intensity exercise on leukocyte ABCA1 and ABCG1 mRNA expression.

Having already shown significant upregulation of the membrane bound scavenger receptor CD36 and the nuclear transcription factor involved in the facilitation of RCT, namely LXRα, it was decided to investigate the effects of exercise on the mRNA expression of the lipid efflux ATP binding cassette genes, namely ABCA1
and ABCG1. Both efflux genes are known to be direct targets in terms of transactivation of LXRα. Figures 3.18a and 3.18b demonstrate that mRNA expression for both transporters is significantly increased after eight weeks of low intensity exercise (ABCA1: 3.5 fold; ABCG1: 3 fold). There were no significant changes in mRNA expression of ABCA1 or ABCG1 in the control group. ABCA1 and ABCG1 mRNA expression was only significantly different between the exercise and control group after eight weeks of the study.
Figure 3.18: The effect of low intensity exercise on leukocyte ABCA1 and ABCG1 mRNA expression. Gene expression of ABCA1 (a) and ABCG1 (b) was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* P=0.0001 ANOVA, Tukey pairwise analysis, compared to baseline (dotted line); # P<0.05 two sample t-test, compared to controls), n = 17.
3.3.15. The effect of low intensity exercise on leukocyte apolipoprotein E mRNA expression.

Having already shown significant upregulation at week eight, of both LXRα and the membrane bound cholesterol efflux proteins, namely ABCA1 and ABCG1, suggestive of increased RCT, the effects of low intensity exercise on the mRNA expression of apolipoprotein E (ApoE) were quantified. ApoE is known to play a pivotal role in RCT. Figure 3.19 demonstrates that mRNA expression for ApoE is significantly increased (1.7 fold) also at week eight, in subjects that undertook the low intensity exercise programme. No change in ApoE mRNA expression was observed in the control group. ApoE mRNA expression was only significantly different between the exercise and control group after eight weeks of the study.

3.3.16. Sequence analysis of putative PPARγ and LXRα target genes.

The promoters of all genes investigated in this study were screened for the presence of PPARγ response elements, and sequences showing strong (>90%) homology to a previously reported PPRE (TGACCTnnTGACCT) by Smith, (2002), were identified in the promoter regions of the human CD36, LXRα, ABCA1 and ABCG1 genes (Accession Nos: NC_000007.12 and NC_000011.8 respectively). These preliminary analyses support previous reports that both CD36 and LXRα are encoded by PPRE bearing genes whose expression is regulated in a PPARγ dependent manner in mammalian cells (Chawla et al., 2001a and Chawla et al., 2001b). Interestingly, a sequence of moderately high (>80%) homology to the above PPRE was identified in the promoter region of the PPARγ gene itself (Accession No: NC_000003.10),
suggesting that as reported, activation of PPARγ protein by PPARγ ligands may induce increased transcription of the PPARγ gene (Fajas et al., 1997). The promoters of all genes were also screened for the presence of LXR response elements, and sequences showing strong (>90%) homology to a previously reported LXRE’s (AGGTCAnnnnAGGTCA and TGACCTCAAGTGATCC) by Li et al., (2002), were identified in the promoter regions of the human ABCA1, ABCG1 and LXRα genes (Accession Nos: NC_000009.10, NC_000021.7 and NC_000011.8, respectively).

Figure 3.19: The effect of low intensity exercise on leukocyte apolipoprotein E mRNA expression. Gene expression of ApoE was determined in leukocytes using real time-PCR. Results are expressed as mean ± SD (* P=0.006 ANOVA, Tukey’s pairwise analysis, compared to baseline (dotted line); † P<0.05 2 sample t-test, compared to controls), n = 17.
3.3.17. Exercise induced correlations between plasma lipoproteins, lipid scavenger receptors and genes involved in reverse cholesterol transport.

There was no significant correlation between oxLDL concentrations and PPARγ mRNA expression at baseline in either the exercise or control group. However, there was a significant correlation between the change in plasma oxLDL concentrations and the change in PPARγ mRNA expression between baseline and week 8 (Table 3.5). There was no significant correlation between plasma oxLDL concentrations and CD36 mRNA expression at baseline, after 4 and 8 weeks of the low-intensity exercise programme. There was also no significant correlation between change in plasma oxLDL concentrations and change in CD36 mRNA expression in the exercise group (Table 3.5). The exercise induced change in HDL was significantly correlated with the change in LXRα mRNA expression pre to post exercise (Table 3.5). The change in both ATP-binding cassette genes, ABCA1 (Table 3.5) and ABCG1 (Table 3.4) were significantly correlated with LXRα gene expression, baseline to week 8.
Table 3.5: Exercise induced correlations between plasma lipoproteins, lipid scavenger receptors and genes involved in reverse cholesterol transport.

<table>
<thead>
<tr>
<th>Exercise induced correlations (Week 0 – 8 changes)</th>
<th>R value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxLDL vs PPARγ*</td>
<td>0.603</td>
<td>0.013</td>
</tr>
<tr>
<td>oxLDL vs CD36</td>
<td>0.599</td>
<td>0.117</td>
</tr>
<tr>
<td>LXRα vs ABCA1*</td>
<td>0.874</td>
<td>0.005</td>
</tr>
<tr>
<td>LXRα vs ABCG1*</td>
<td>0.669</td>
<td>0.049</td>
</tr>
<tr>
<td>LXRα vs HDL*</td>
<td>0.725</td>
<td>0.042</td>
</tr>
<tr>
<td>ABCA1 vs ABCG1*</td>
<td>0.803</td>
<td>0.016</td>
</tr>
</tbody>
</table>

* Denotes significant difference between variables ($P<0.05$).
3.4. Discussion.

The evidence described in chapter 2 regarding the lipid effects of an eight week low intensity exercise programme on serum oxLDL levels, lead to the investigation of low intensity exercise on the expression of cell surface lipid scavengers. Also, in the light that oxLDL possess known PPARγ ligands, the expression and activity of PPARγ was quantified. As shown in figure 3.4a, adherence to the eight week low intensity exercise programme increased leukocyte CD36 mRNA expression. There was a 58% and 32% increase in CD36 mRNA expression after four and eight weeks of low intensity exercise, respectively (Figure 3.5). This was quantified using PCR and was subsequently reinforced on all 37 participants samples using real time-PCR. Real time-PCR results in figure 3.9 demonstrate an approximate 4 and 3 fold increase in CD36 mRNA expression after four and eight weeks of adherence to the low intensity exercise programme, respectively.

This is the first ever human in-vivo study to report these specific low intensity exercise induced effects. Wei et al., (2005) have previously reported that mice exercised for 2 weeks increased the expression of CD36 (4 fold). This four fold induction of CD36 gene expression was reported in the liver and at first could be considered proatherogenic, conversely it could also be considered beneficial in terms of oxLDL clearance from the bloodstream to the liver. One of the very few human exercised based studies that have focused on CD36 is by Holloway et al., (2006), who reported increased CD36 protein expression in human skeletal muscle after 2 hours of cycling at 60% VO₂peak. This exercise induced modulation was deemed important for increasing whole body fatty acid oxidation during prolonged exercise.
The study by Yanai et al., (2007) documented a reduction in fatty acid utilisation and aerobic capacity in CD36 deficient individuals, suggesting that CD36 mediated fatty acid oxidation is an important determinant for aerobic exercise capacity. The study by Yanai et al., (2007) however failed to mention the training status of these young female individuals, as this may have influenced the results obtained during this one off exercise bout.

SR-A1, another member of the protein scavenger receptor family was quantified by real time-PCR after adherence to the eight week low intensity exercise programme. Figure 3.10 demonstrates that exercise had no significant effect on SR-A1 mRNA after either four or eight weeks of low intensity exercise. These results are similar to that reported by Wei et al., (2005), who stated that mice who exercised for 30 minutes a day for two weeks, did not exhibit any significant modulations in SR-A1 gene expression. This is suggestive of a decreased capacity to generate foam cells via the uptake of modified lipoproteins by SR-A1. The fact that out of the two scavenger receptors quantified, only CD36 was upregulated, indicates that specific ligands released during low intensity exercise upregulate specific members of the scavenger receptor family.

Figure 3.11 illustrates that after eight weeks of low intensity exercise there was a significant increase in the lipid sensor CD14. However, four weeks of the low intensity exercise programme failed to modulate CD14 mRNA expression. This membrane bound lipid sensor is also a PPARγ regulated gene (Tontonoz et al., 1998 and Shashkin et al., 2005), reinforcing the understanding that low intensity exercise increases PPARγ transcriptional activity. Interestingly, Shashkin et al., (2005)
reported that minimally oxLDL significantly interacts with CD14 and could therefore be described as a potential ligand involved in the upregulation of this lipid sensor. This study reported increased levels of oxLDL during low intensity exercise and considering the evidence from Shashkin et al., (2005) the increase in CD14 gene expression reported here could be due to increased PPARγ activity.

Figures 3.12 and 3.13 demonstrate that within four weeks, the current exercise programme is capable of inducing significant increases in the mRNA expression and DNA-binding activity of the lipid sensing transcription factor PPARγ. Real time-PCR results reported in figure 3.12 illustrate an approximate 2 and 4 fold increase in PPARγ mRNA after four and eight weeks respectively. This result is reinforced by figure 3.13, which shows a large and significant increase in PPARγ DNA-binding activity after four weeks of a subsequent low intensity exercise programme. There are a limited number of in-vivo exercise based studies focusing on the nuclear transcription factor PPARγ. However Petridou et al., (2007b) reported that eight weeks of voluntary wheel running by rats, had significant effects on the concentration of proteins playing key roles in in lipogenesis and lipolysis in rat liver, visceral fat, and subcutaneous fat. There were no differences in the liver, muscle, or adipose tissue PPARγ gene expressions between exercised and non exercised rodents. However, the DNA-binding activity of PPARγ was significantly higher in both fat deposits of the exercised rats. These findings led Petridou et al., (2007b) to the suggestion that exercise training may regulate the expression of target genes of PPARγ through PPARγ activation rather than induction, rendering the regulation of transcription more economical and flexible.
Figure 3.14 illustrates that even though there was a non significant decrease in plasma triglyceride levels (Table 2.2), lipoprotein lipase (LPL) mRNA expression was not significantly modulated by the low intensity exercise programme, after either four or eight weeks. However, Kiens et al., (2004), state that exercise lasting 90 minutes at an intensity of 60% VO$_{2\text{max}}$ results in an increase in skeletal muscle expression of LPL mRNA. This discrepancy between the two studies is possibly due to the nature of the exercise prescribed in each study. Kiens et al., (2004) used a much more demanding exercise protocol and also used individuals with a considerably higher aerobic capacity than those used in the current exercise study (55.1 vs 35.5ml/kg$^{-1}$/min$^{-1}$). This is possibly why one would expect skeletal muscle LPL mRNA to increase in response to aerobically demanding exercise, in order to utilise circulating triglycerides for energy production. Another possible factor in the discrepancy between the two studies is that Kiens et al., (2004) investigated LPL mRNA in skeletal muscle and it has been reported LPL mRNA expression in response to exercise differs significantly between tissue types (Seip et al., 1995). Therefore, LPL mRNA expression in leukocytes or adipocytes may not be significantly modulated by exercise compared to skeletal muscle expression. Suggesting a more aerobic performance based modulation in LPL mRNA expression in skeletal muscle, which may not have considerable clinical potential.

The hydrolysis of triglycerides and the subsequent generation of material for HDL formation is potentially seen as anti-atherogenic. This is reinforced by the finding by Shimada et al., (1996) who demonstrated that over expression of LPL in atherogenic animals protects against atherosclerosis. On the other hand however, evidence suggests that macrophage expression of LPL may be proatherogenic, due to its role
in binding modified lipoproteins (Tontonoz and Mangelsdorg, 2003). It has been reported that LXRα plays a role in the regulation of LPL, however this is tissue specific (Zhang et al., 2001), suggesting that both PPARγ and LXRα are very selective in which molecules they activate after stimulation during exercise.

Another member of the PPAR transcription factor family, PPARα was also quantified after adherence to the low intensity exercise programme. Figure 3.15 reveals that PPARα mRNA expression was significantly upregulated after four weeks of low intensity exercise. However, after eight weeks, PPARα gene expression was not significantly different from baseline expression. Horowitz et al., (2000) reported similar results, as 12 weeks of endurance training in sedentary females resulted in a significant 2 fold increase in PPARα protein expression in skeletal muscle. This was associated with an increase in a number of key enzymes involved in fatty acid oxidation. Horowitz et al., (2000) postulated that the increase in muscle PPARα content and the increased fatty acid delivery to muscle during exercise may both be important factors in enhancing muscle fatty acid oxidative capacity.

Acyl-CoA oxidase, which catalyses the first and rate determining step of the peroxisomal β-oxidation of fatty acids (Nakajima et al., 2002), was analysed for gene expression, before, during and after the eight week low intensity exercise programme. Results shown in figure 3.16, illustrate that acyl-CoA oxidase mRNA expression was not significantly altered from baseline during the exercise programme. There was also no significant difference in mRNA levels for acyl-CoA oxidase between the exercise and control groups at all time points. This is reinforced by the findings by Wei et al., (2005), who also reported no change in acyl-CoA
oxidase gene expression after exercise. Similarities are limited in that the study by Wei et al., (2005) lasted only two weeks, was conducted on mice and acyl-CoA oxidase mRNA expression was quantified in the liver, a different tissue type than the current studies. Even though PPARα gene expression was upregulated after four weeks of the exercise programme, Acyl-CoA oxidase, a PPARα regulated gene was however not. This suggests that while PPARα gene expression is upregulated after low intensity exercise, this upregulation does not necessarily translate to increased activity of the transcription factor. This is evidenced by the non significant modulations in the gene expression of the PPARα regulated gene Acyl-CoA oxidase after adherence to the exercise programme. However, the quantification of other PPARα regulated genes is warranted to fully clarify the role PPARα plays in the exercised induced modulations in plasma lipids. The results from the current study and from Wei et al., (2005) suggest that PPARα might be of little significance in the exercise induced beneficial effects in the monocyte previously mentioned. However, non hepatic and leukocyte tissues might be influenced differently as PPARα distribution varies substantially among various tissues.

There were no significant changes in IL-6 mRNA expression during the eight week low intensity exercise programme (Table 3.4). This reinforces the findings by Moldoveanu et al., (2000) who reported that IL-6 mRNA expression was not significantly affected by 3 hours of cycling at 65% VO2max. However Starkie et al., (2001b) reported that IL-6 mRNA was significantly increased after 60 minutes of cycling at individuals lactate threshold. After completion of the eight week low intensity exercise programme there were no significant modulations in tumour necrosis factor-α (TNFα) mRNA expression (Table 3.4). This emphasises the
findings by Moldoveanu et al., (2000) who also reported exercise did not induced modulations in TNFα mRNA expression after 3 hours of cycling at 65% VO$_{2\text{max}}$. The study by Kimura et al., (2001) also reported no modulations in TNFα mRNA after 30 minutes of cycling at 45% VO$_{2\text{max}}$, a similar intensity to that used in this current study. However there was a significant upregulation in TNFα mRNA after 30 minutes of cycling at 70% VO$_{2\text{max}}$. Taken together the IL-6 and TNFα results from table 3.4 suggest that eight weeks of low intensity exercise fails to induce the gene expression of key inflammatory cytokines. Given that PPARα and PPARγ were upregulated, one would expect minimal activation of NF-κB due to the competitive nature of the two transcription factors and the reported repressive activities of PPARγ on NF-κB (Chen et al., 2003, Chen et al., 2005 and Sasaki et al., 2005).

Alternative forms of monocytes present within peripheral blood have been recently reported (Bouhlel et al., 2007 and Charo, 2007). The two subtypes; M1, which are know to be proinflammatory and are suggested to have an important role in the development of macrophage foam cells characteristic of atherosclerosis and M2, which are regarded as anti-inflammatory. The M1 macrophage is characterised as having high levels of IL-6 and TNFα expression whereas M2 express the mRNA of these inflammatory cytokines at lower levels. Furthermore, Bouhlel et al., (2007) demonstrates that increased PPARγ activity within monocyic cells increases M2 anti-inflammatory macrophages at the expense of the M1 proinflammatory form. This study did not investigate these specific cells types further, however the ability of this form of low intensity exercise to regulate PPARγ activity and mRNA expression does support the view of another important potentially anti-inflammatory mechanism associated with low intensity exercise, mediated through PPARγ.
Having already reported that adherence to the eight week low intensity exercise programme resulted in an increase in oxLDL, which could be interrupted as increased inflammatory potential, two other proinflammatory mediators were quantified. There is a growing body of evidence postulating that advanced glycated endproducts (AGEs) and their receptors advanced glycated endproduct receptors (AGERs) are implicated in the pathogenesis of atherosclerosis (Nakamura et al., 2007). Engagement of AGER with AGEs is also shown to elicit the generation of oxidative stress and subsequently evoke inflammatory responses in endothelial cells (Nakamura et al., 2007). Table 3.4 illustrates that during the eight week low intensity exercise programme implemented in this study, there were no significant modulations in AGER mRNA expression at any of the time points investigated. There was also no significant change in AGER mRNA expression in the control group and between groups during the eight weeks. The lack of exercise induced changes in AGER mRNA is further evidence of a non inflammatory environment associated with a low intensity exercise programme.

The present study demonstrates that eight weeks of low intensity exercise does not significantly alter COX-2 mRNA expression in leukocytes (Table 3.4). There are a limited number of studies looking at the response of COX-2 during exercise, however a study by Qunidry et al., (2006) reported similar results to the current studies. Exercise had no effect on COX-2 protein expression in rat cardiac tissue after three consecutive days of moderate intensity exercise. Weinheimer et al., (2007) found that 4 and 24 hours after a session of resistance training, COX-2 mRNA was significantly upregulated compared to baseline values. However, COX-2 protein expression was not effected by the resistance training bout.
There are two main conclusions to be drawn from the AGER and COX-2 results, firstly both suggest that the low intensity exercise implemented is non inflammatory. Suggestive of minimal upregulation of the proinflammatory nuclear transcription factor NF-κB, which has competitive characteristics with PPARγ. Secondly, COX-2 is regarded as being crucial in the production of prostaglandins, of which several are known ligands of PPARγ, including 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂). Therefore, the lack of COX-2 upregulation suggests that prostaglandins may not be the main ligand activating PPARγ during chronic low intensity exercise. Also as PPARγ has been shown to inhibit COX-2 expression and activity through a negative feedback mechanism (Inoue et al., 2000 and Subbaramaiah et al., 2001), one would expect minimal upregulation in COX-2 seeing as PPARγ gene expression was unregulated at all time points quantified.

The observations by Chawla et al., (2001b) and Laffitte et al., (2001) that expression of LXRα is increased in response to PPARγ ligands, suggests that low intensity exercise may also activate LXRα via PPARγ and hence provide the link between proatherogenic cholesterol influx and anti-atherogenic cholesterol efflux. It is well regarded that activation of PPARγ leads to direct upregulation of LXRα via a PPAR binding site (PPRE), therefore exercise may not only activate CD36 via PPARγ but may also initiate reverse cholesterol transport (RCT) via the same transcription factor. There was a significant upregulation in LXRα gene expression after eight weeks of low intensity exercise (Figure 3.17). Unlike PPARγ mRNA, there was no upregulation in LXRα after four weeks of the low intensity exercise programme. This is the first study to report the finding that eight weeks of low intensity exercise upregulates the nuclear transcription factor LXRα and provides novel evidence that
suggests exercise facilitates a specific cellular mechanism responsible for reductions in plasma cholesterol.

To substantiate that low intensity exercise increases LXRα activity in light of the observation that LXRα gene expression is upregulated after exercise, two LXRα regulated genes were quantified. These LXRα regulated genes included two members of the ATP-binding cassette transporters, including ABCA1 and ABCG1. Figures 3.18a and 3.18b demonstrate that both ABCA1 and ABCG1 mRNA expressions were upregulated after eight weeks of low intensity exercise. In a similar pattern to LXRα upregulation, mRNA for both ATP binding cassette transporters was only increased after week eight, with no significant modulation observed after four weeks. Hoang et al., (2008) reported corresponding results to the present studies, in that participants with the highest physical activity habits had higher levels of ABCA1 gene expression in human leukocytes. This significant upregulation in ABCA1 gene expression in the high physical activity group was compared to individuals with low levels of physical activity. Physical activity in this case was determined using the International Physical Activity Questionnaire (IPAQ). Interestingly ABCA1 mRNA in skeletal muscle was not correlated with physical activity, suggesting distinct regulatory mechanism between the two tissue types (Hoang et al., 2008).

Another study that is in agreement with the findings reported in the current study is by Ghanbari-Niaki et al., (2007), who reported that treadmill training lasting 6 weeks in rats, significantly increased liver ABCA1 mRNA expression. The exercise induced regulation of ABCA1 expression in leukocytes may contribute to the
atheroprotective effect of physical activity, as ABCA1 expression in this cell type plays a key role in influencing atherosclerosis susceptibility. Considering RCT in general and its potential link with exercise, the study by Olchawa et al., (2004) reported that cholesterol efflux, as measured by the capacity of plasma to promote cholesterol efflux from macrophages, was 16% higher in triathletes compared to normally active controls.

PPARγ can directly and indirectly upregulate ABCA1 and ABCG1 expression by two different mechanisms. Ligands for PPARγ can indirectly upregulate ATP-binding cassette transporters by increasing the expression of LXRα, also PPARγ ligands can directly activate the ATP-binding cassette transporters (Venkateswaran et al., (2000) and Chawla et al., (2001b). There is considerable discrepancy in the evidence available regarding the effects of oxLDL on ATP-binding cassette transporter expression in various human tissues. Although the majority of the work on this particular area has been conducted in-vitro, this in-vivo study’s finding adds significant worth to this debate. The current findings and those by Chawla et al., (2001b) and Taketa et al., (2008), both in leukocytes, suggest that oxLDL upregulates mRNA expression of the ATP-binding cassette transporters, this may be either through a PPARγ dependent mechanism, which involves ligand activation of PPARγ, which in turn upregulates LXRα, leading to subsequent activation the ATP-binding cassette transporters. Alternatively, a PPARγ independent pathways, involving activation of LXRα through potent ligands from oxLDL, which ultimately leads to upregulation of certain ATP-binding cassette transporters may also be involved. However, Zhu et al., (2005) have claimed that the complete opposite occurs in endothelial cells. As oxLDL downregulates ABCA1 in human vascular
endothelial cells via ligand-dependent inhibition of LXRα. This oxLDL induced downregulation in ABCA1 in endothelial cells, may predispose cells within the vessel wall to accumulate lipids, due to the inhibition of RCT.

ApoE plays a pivotal role in modulating cellular cholesterol metabolism through facilitation of cholesterol efflux in macrophages. Therefore, ApoE mRNA expression was quantified in all cohort samples and figure 3.19 demonstrates that in a similar pattern to LXRα, ABCA1 and ABCG1 mRNA results, ApoE was significantly upregulated after eight weeks of low intensity exercise. There are limited number of studies focusing on the effects of exercise on ApoE expression, with the majority of studies focusing on different ApoE genotypes and their links with atherosclerosis. One example of this is a study by Shimada et al., (2007), who reported that exercised ApoE deficient mice had significantly smaller atherosclerotic lesions compared to those not exercised. In a similar manner to ABCA1 and ABCG1, ApoE is upregulated in the monocytic cell line, dTHP-1 cells after exposure to oxLDL, with this transactivation reportedly due to ligand-binding activation of LXRα (Laffitte et al., (2001).

Thus, as PPARγ can be activated by oxidized fatty acid species present in oxLDL, the following proposed model (Figure 5.1) would suggest that oxLDL induces the activity of PPARγ in monocytes leading to increased expression of its regulated genes (Febbraio et al., 2000 and Li and Glass, 2004). CD36 is one of these PPARγ regulated genes (Chawla et al., 2001a, Chawla et al., 2001b and Moore et al., 2001) and its upregulation would result in increased cellular uptake of oxLDL. Moreover, the presence of a PPRE sequence in the promoter of the PPARγ gene may provide an
autoregulatory loop mechanism for increased expression of PPARγ itself, such a mechanism has previously been reported for other transcription factor encoding genes, including LXRα (Li et al., 2002). Importantly, ligand activation of PPARγ also leads to primary induction of LXRα, whose activation subsequently (i.e. after 4-8 weeks exercise) triggers upregulation of ABCA1, ABCG1 and ApoE, and therefore increased reverse cholesterol transport (Chawla et al., 2001b).
Chapter 4.

The effect of oxLDL on PPARγ activation in monocytes.
4.1. Introduction.

Oxidised lipid signalling in macrophages is central to the pathogenesis of atherosclerosis and has been shown to stimulate multiple signalling pathways including activation of phosphatidylinositol 3-kinase (PI3-K)/Akt and p42/p42 mitogen-activated protein kinase (MAPK) (Chien et al., 2003). The MAPKs comprise a large family of kinases that include ERK1, ERK2, JNKs and p38 MAPKs (Liu and Liu, 2004). Phosphatidylinositol 3-kinase, a heterodimer of an adapter subunit (p85) and catalytic subunit (p110), is activated by many growth factors, including insulin (Takeda et al., 2001). One of the downstream effector molecules of PI3-K is Akt (formally known as PKB), a serine/threonine kinase, which is responsible for the signalling cascades involved in cell proliferation and cell survival (Takeda et al., 2001). It has been postulated by Miura et al., (2000) and Nishida et al., (2000) that activation of the ERK kinase pathway is regulated through PI3-K, the net result of which is the activation of specific nuclear receptor signalling pathways.

Mounting evidence suggests that nuclear receptor signalling pathways mediate many of the effects of oxidised lipids on cellular gene expression (Lehmann et al., 1997 and Tang et al., 2004). Recently PPARγ ligands have been shown to activate a number of members of the MAPK family (Han et al., 2000 and Takeda et al., 2001). As this activation was observed after several minutes rather than hours a ‘non-genomic’ or PPARγ independent effect has been proposed, due to the fact that activation occurred too rapidly for de novo synthesis to arise. Singh et al., (2005) have demonstrated that incubation of monocytic cells with the PPARγ ligand
rosiglitazone, increased the phosphorylation of Akt within 1 hour, reinforcing the idea of ‘non-genomic’ actions of PPARγ.

Cyclooxygenase (COX), which is responsible for catalysing the synthesis of prostaglandins from arachidonic acid, has two isoforms, namely COX-1 and COX-2. COX-1 is regarded as being constitutively expressed in cells, whereas COX-2 is mostly absent but is induced upon stimulation by inflammatory mediators, suggestive of an important role within inflammation (Inoue et al., 2000). However, evidence indicates that the expression of COX-2 is differently expressed in different cell types. Therefore, the molecular mechanisms that initiate the regulation of COX-2 remain to be fully elucidated.

COX-2 has been shown to possess both PPAR and NF-κB response elements within its promoter region (Meade et al., 1999). However, the transcriptional roles of these response elements remains controversial and rather tissue specific. For example evidence presented by Meade et al., (1999) suggests that PPARs enhance COX-2 expression in epithelial cells mediated via a PPAR/PPRE mechanism. However, Staels et al., (1998) have shown that inhibition of COX-2 via PPARs occurs transcriptionally as a result of PPAR repression of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) signalling. Interestingly, exposure to oxLDL has been shown to activate PPAR, while inhibiting NF-κB activity, thus providing evidence of the transrepressive activity of PPAR on NF-κB (Han et al., 2000).

NF-κB is a protein complex that acts as a nuclear transcription factor. NF-κB is found in almost all cell types and is involved in cellular responses to stimuli such as
stress, cytokines and free radicals, hence NF-κB plays a key role in regulating the immune response to infection (Gilmore, 2006). One of the most important upstream signalling mechanisms of the proinflammatory nuclear transcription factor NF-κB is activation of mitogen-activated protein kinase (MAPK). In relation to PPARγ, MAP kinases not only inactivates PPARγ by phosphorylation of its regulatory domain but also phosphorylates and regulates the activities of various transcriptional coactivators, e.g PPAR binding protein (PBP), cAMP-response element-binding protein-binding protein (CBP)/p300 and steroid receptor coactivator-1 (SRC-1) (Misra et al., 2002).
4.1.1. Aims.

The primary aim of this chapter is to investigate the hypothesis that the increase in oxLDL observed during low intensity exercise can trigger activation of PPARγ, LXRα and RCT through a signalling cascade involving the enzymes ERK1/2 and COX-2. This hypothesis will be determined in a human monocytic cell line, namely THP-1 cells through the following:

1. Investigating the effects of oxLDL on gene and surface expression of the PPARγ regulated gene CD36 in monocytic cells.
2. Determining if the effects on CD36 gene and protein expression are PPARγ dependent or independent.
3. Investigating the effects of oxLDL on LXRα gene expression in monocytic cells.
4. Elucidating the molecular signalling mechanisms by which oxLDL modulates CD36 mRNA and protein expression.
4.2. Materials and Methodology.

4.2.1. Materials.

A PPARγ agonist and antagonist Rosiglitazone and GW9662, respectively were obtained from Glaxo-Welcome (Middlesex, UK). Both were prepared as 10mM stock concentrations in dimethyl sulfoxide (DMSO) and stored at -80°C. Phorbol 12-myristate 13-acetate (PMA) was dissolved in DMSO at a stock concentration of 1mg/mL. The stock was protected from the sunlight and stored at -20°C. The use of PMA in all in-vitro experimental work was at a working concentration of 100ng/mL, diluted in RPMI 1640. PD98059 (ERK1/2 inhibitor) and NS-398 (COX-2 inhibitor) were reconstituted with DMSO, giving a stock concentration of 50mM. Both PD98059 and NS-398 were used in all experimental procedures at 50μM. All other chemicals were obtained from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

4.2.2. Cell lines.

The Mono-Mac-6 (MM6) cell line was obtained from The German Collection of Microorganism and Cell Cultures (DSMZ; Braunschweig, Germany). This particular cell line has been established from the peripheral blood of a 64 year old man with relapsed acute monocytic leukaemia. The human monocytic cell line THP-1 was obtained from The Health Protection Agency Culture Collections (Salisbury, UK). THP-1 is a promonocytic monocyte cell line derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia. THP-1 cells grow in suspension but can be differentiated into macrophage like cells by the addition of Phorbol 12-
myristate 13-acetate (PMA) at concentrations ranging from 10 - 400ng/mL (Resto-Ruiz et al., 2002, Theus et al., 2004 and Park et al., 2007).

4.2.3. Preparation of tissue culture media.

Both MM6 and THP-1 cell lines were allowed to grow and propagate in RPMI 1640 medium without L-glutamine (Invitrogen; Paisley, UK). Media was supplemented with 10% heat-inactivated Foetal Calf Serum (FCS) (Biosera, East Sussex, UK), and with 1% 200nM v/v L-glutamine, 1% v/v non-essential amino acids and 1% v/v 1mM sodium pyruvate (all supplements were purchased from Gibco BRL, Paisley, UK). Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂ for optimal growth and proliferation.

4.2.4. Cell sub-culturing and harvesting.

MM6 and THP-1 cells were seeded at a density of 0.4 X 10⁶ cells/mL and passaged when their growth was at 80% confluence (i.e. 0.8-1.0 X 10⁶ cells/mL), usually after a growth period of 48 hours (due to the 48 hour doubling time of both cell lines (Ziegler-Heitbrock et al., 1988)). All experiments were performed with cells at passage of less than 25.

4.2.5. Freezing of cell lines.

MM6 and THP-1 cells were harvested and resuspended at approximately 5 X 10⁷ cells/mL in 4% DMSO (v/v) in FBS. The cells were transferred to a sterile cryotube
(Nunc, Gibco, UK). Cells were initially frozen in their vials wrapped in insulating tissue paper at -80°C for 24 hours before transfer to liquid nitrogen for prolonged storage.

4.2.6. Thawing of cells.

Cryogenic vials containing the required cell line were removed from liquid nitrogen storage and thawed rapidly in a pre-warmed water bath at 37°C. Thawed cells were washed in 10mL of pre-warmed unsupplemented RPMI 1640 media to remove the DMSO. A small aliquot of cells were used for assessing cell viability and cell number, after which cells were centrifuged at 200 x g for 5 minutes, followed by further washes and centrifugation. Cells were resuspended in supplemented RPMI 1640 medium at the density of 4 X 10^5 cells/mL and maintained as previously described in chapter 4.2.3.

4.2.7. Determination of cell viability and proliferation.

In order to determine if the various treatments and stimuli affected cell viability, proliferation and cell viability assays were undertaken throughout, using either trypan blue exclusion or CellTiter 96®AQueous cell proliferation assay.

4.2.7.1. Trypan blue exclusion method.

Viability of cells was assessed by the trypan blue exclusion method. This exclusion test has been used to determine the number of viable cells present in a cell suspension (Chan et al., 2007). The test is based on the principle that live cells
possess intact cell membranes that exclude the trypan blue dye, whereas apoptotic cells with damaged membranes do not and hence appear blue under light microscopy. Cells were incubated with 0.4% w/v trypan blue for 10 minutes. The cell sample was then observed under the microscope and the percentage of unstained cells was representative of the percentage of viable cells within the cell culture. Cell viability was conducted prior to sub-culturing and before any experimentation and experiments were only performed when viability was greater than 95%.

4.2.7.2. CellTiter 96® AQUEOUS One Solution Cell Proliferation Assay.

Cell proliferation was determined by the CellTiter 96® AQueous one solution cell proliferation assay (Promega, Southampton, UK). This particular assay is a colorimetric based assay for determining the number of viable cells in proliferation. The assay involves the bio-reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, to a coloured formazan product, facilitated by dehydrogenase enzymes found in metabolically active cells. The assay involves adding 20μL of CellTiter 96® AQueous one solution reagent into each well of a 96 well plate containing different quantities of cells, in 100μL of culture medium. The plate was incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO₂. Spectrophotometric analysis of cell proliferation was determined at 490nm using a 96 well plate reader (Dynex Technologies, VA, USA).
4.2.8. Differentiation of THP-1 cells using PMA.

THP-1 cells were seeded to 0.5 X 10⁶/mL in fresh culture media, and 100ng/mL Phorbol-12-myristate-13-acetate (PMA) was added to the THP-1 cells. THP-1 cells (6mLs) were then aliquoted into each well of a 6 well plate and left to differentiate for 48 hours at 37°C in a humidified atmosphere with 5% CO₂. After incubation with PMA, culture media was removed and cells were washed (x2) with PBS (heated to 37°C). Subsequently 1mL of fresh culture media was added to each well and this was left for 24 hours before addition of any stimulant.

4.2.9. Oxidation of LDL.

Low density lipoprotein (LDL) (Autogen Bioclear, Wiltshire, UK), concentration 5mg/mL, was diluted in PBS to give a protein concentration of 0.1075 mg/mL. Thereafter 32μL of 12mM stock CuSO₄ was added to 568μL deionised water, to give a final concentration of 0.64 mM CuSO₄. The concentration of CuSO₄ needed to oxidise LDL is calculated by the following equation; concentration of CuSO₄ = (protein concentration of LDL x 1.6mM) / 0.25. The volume of CuSO₄ needed to oxidise 0.1075 mg/mL LDL is calculated using the following equation; volume of CuSO₄ = (volume of LDL x 22.5μL) / 600μL. Therefore 150μL of 0.64 mM CuSO₄ was added to the 4mL of diluted LDL in a falcon tube and was incubated in a water bath at 37°C for 17 hours, during which the top of the falcon tube LDL was not completely closed, in order to allow oxygen to enter. The oxidation of the lipoprotein was stopped by the addition of 150μL 1mM EDTA, giving a final concentration of 0.1 mg/mL oxidised LDL (oxLDL).
Validation of oxLDL was performed by comparing the relative absorbances of LDL and oxLDL using a spectrophotometer (PerkinElmer, Buckinghamshire, UK). Confirmation of LDL oxidation was also determined by comparing the effects of LDL and oxLDL on CD36 surface protein expression on THP-1 cells using a Cytomics FC500MPL flow cytometer ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 530$ nm; Beckman Coulter, Buckinghamshire, UK).

4.2.10. CD36 antibody optimization.

The primary mouse monoclonal CD36 antibody was obtained from Santa Cruz Biotechnology, Inc (California, USA) and was used at the following concentration, 10$\mu$g/mL of cells, after optimisation. The secondary antibody was a Fluorescein anti-mouse IgG (H+L) antibody, obtained from Vector Laboratories (Peterborough, UK) and after optimisation within the manufacturer’s concentration range of 5-20$\mu$g/mL, was added at the concentration 15$\mu$g/mL. Table 4.1 was deployed to determine the optimal combination of primary and secondary antibodies needed per flow cytometric reaction.
Table 4.1: Titration of both CD36 primary and FITC secondary to determine the optimum combination of both antibodies.

<table>
<thead>
<tr>
<th>Primary Antibody (μg/mL)</th>
<th>Secondary Antibody (μg/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2/5</td>
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<tr>
<td>5</td>
<td>5/5</td>
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<tr>
<td>10</td>
<td>10/5</td>
</tr>
<tr>
<td>20</td>
<td>20/5</td>
</tr>
</tbody>
</table>

4.2.11. Flow cytometric analysis of CD36 protein surface expression in MM6 and undifferentiated THP-1 cells.

MM6 and THP-1 cells (both 6mLs), seeded at 1 X 10⁶/mL was added to each well of a 6 well plate. Cells were then either incubated with rosiglitazone (1μM) or oxLDL (1μg/mL) for various time points ranging from 0 hours to 72 hours. Rosiglitazone (1μM) was used as a positive control, due to its known characteristic of being a PPARγ ligand. Following all incubations, cells were centrifuged, the supernatant removed and the cell pellets resuspended in 600μL ice-cold FACS buffer (PBS + 0.5% BSA). For each time point and treatment, 6 aliquots of 100μL of cells were transferred into grouping tubes (i.e. 1 X 10⁶/mL cells in each tube). Duplicate tubes were labelled as follows; 1) cells only, 2) cells and 2°Ab, 3) cells, 1°Ab and 2°Ab and 5μL of CD36 primary antibody (1°Ab) was added on ice and incubated for 30 minutes in the dark. After which 500μL of ice-cold FACS buffer was added and centrifuged. The supernatant was removed and cells resuspended in 100μL of ice
cold FACS buffer and 1µL of FITC secondary antibody (2°Ab) was added to its corresponding tubes and incubated on ice, in the dark for 30 minutes. After which a further 500µL of ice-cold FACS buffer was added and all tubes were centrifuged, the supernatant removed and cells resuspended and fixed in 500µL 4% paraformaldehyde prior to flow cytometric analysis to prevent antibody internalisation. Tubes were analysed for CD36 membrane surface expression using a Cytomics FC500MPL flow cytometer (\(\lambda_{ex} = 488\) nm; \(\lambda_{em} = 530\) nm; Beckman Coulter, Buckinghamshire, UK).

4.2.12. Flow cytometric analysis of CD36 surface expression in differentiated THP-1 cells.

After differentiation with 100ng/mL PMA, differentiated THP-1 (dTHP-1) cells were incubated with different stimuli for various time points ranging from 0 hours to 72 hours. Again the PPAR\(\gamma\) agonist rosiglitazone (1µM) and PPAR\(\gamma\) antagonist GW9662 (50µM) were used as positive and negative control, respectively. Due to their adherent nature, cells had to be scraped off from the bottom of the wells. This involved removing the culture media and the addition of 2mL of FACS buffer. Wells were then scraped to remove any adherent cells and the buffer removed and added to a falcon centrifuge tube. Cells were then centrifuged and the cell pellets resuspended in ice cold FACS buffer. After incubation with primary and secondary CD36 antibodies, as previously described in chapter 4.2.11, cells were fixed with 4% paraformaldehyde and analysed for CD36 protein membrane surface expression using a Cytomics FC500MPL flow cytometer (\(\lambda_{ex} = 488\) nm; \(\lambda_{em} = 530\) nm; Beckman Coulter, Buckinghamshire, UK).
4.2.13. RNA extraction from differentiated THP-1 cells.

After incubation with the desired stimulus, culture media was removed using a Biochem VacuuCenter BVC 21 NT/BVC 21 NT Vario (Wertheim, Germany), 1mL of TRIzol® (Applied Biosystems, Warrington, UK) was added to each well and left for 5 minutes. Afterwards, the TRIzol® cell mixture was transferred to a 2mL RNase free centrifuge tube, briefly vortexed and stored at -80°C. After thawing, tubes were vortexed and incubated for 5 minutes at room temperature (RT), 200μL of chloroform was added and shaken vigorously for 15 seconds, after which samples were incubated for 3 minutes at RT. Samples were then centrifuged at 12’000 x g for 15 minutes (pre-cooled to 4°C). The colourless upper aqueous phase was then transferred to a fresh 2mL RNase free centrifuge tube and 500μL of isopropanol was added. Samples were again incubated at RT, this time for 10 minutes. Subsequently, samples were centrifuged at 12’000 x g for 15 minutes, at 4°C. As much as possible of the supernatant was removed and then the RNA pellet was washed by the addition of 1mL 75% ethanol. Samples were briefly vortexed and centrifuged at 7’500 x g for 5 minutes at 4°C. The ethanol was carefully removed, so as not to disturb the RNA pellet, leaving as little as possible. The RNA pellet was then dried in a fume hood for approximately 30 minutes, after which the RNA pellet was re-dissolved in 30-50μL RNase free water and stored at -80°C. Throughout this process only RNase free pipette tips, microfuge tubes and water were used, to prevent RNA contamination.

RNA was quantified and checked for purity using the ratio of its absorbance at 260:280nm using a Nanodrop Spectrophotometer ND-1000. This involved 1μL of sample being placed on the lower measurement pedestal and as the UV light passes through the sample quantity and purity of the RNA sample is determined.
4.2.14. Conversion of RNA to cDNA.

RNA samples were converted to cDNA as described in section 3.2.3.

4.2.15. Real-time PCR.

CD36, LXRα, ABCA1, COX-2 and GAPDH mRNA expression was analysed on an Applied Biosystems 7500 Real-time PCR system (Warrington, UK) using Fast SYBR® Green Master Mix (Applied Biosystems, Warrington, UK). For each reaction 5μL of Fast SYBR® Green Master Mix was added to nμL of both the forward and reverse primers, nμL of RNase free water and 1μL of cDNA sample. n depends upon optimisation of individual primers for each gene analysed. The thermal cycling protocol used was identical to the one used in section 3.2.7. Table 4.2 highlights the primer sequences that were used.

Table 4.2: Primer sequences used in real time-PCR analysis.

<table>
<thead>
<tr>
<th>Gene (Accession No)</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD36 (NM_001001548.1)</td>
<td>GGAAGTGATGATGAACAGCAGC</td>
<td>GAGACTGTGTGTCTTCAGCG</td>
<td>117</td>
</tr>
<tr>
<td>LXRα (NM_005693.1)</td>
<td>CCGCACTACATCTGCCACAGT</td>
<td>TGAGGGCGATCTTGCTCTTCT</td>
<td>141</td>
</tr>
<tr>
<td>ABCA1 (NM_005502.2)</td>
<td>GCACTGAGGAAGATGCTGAAA</td>
<td>AGTTTCTGGAAGGGCTGTTC</td>
<td>205</td>
</tr>
<tr>
<td>COX-2 (NM_000963.1)</td>
<td>TTAAAACCCACTCTAAAAACA</td>
<td>GAGAAGGTCTCCAGCTTTT</td>
<td>187</td>
</tr>
<tr>
<td>GAPDH (NM_002046.3)</td>
<td>CATTGACCTCACTACATG</td>
<td>TCTCCATGTGGTGAGAC</td>
<td>209</td>
</tr>
</tbody>
</table>
4.2.16. FACE ELISA quantification of total and phosphorylated ERK1/2.

FACE (Fast Activated Cell Based ELISA) kit (ActiveMotif, Rixensart, Belgium) was used to determine ERK1/2 phosphorylation relative to the total ERK1/2 protein in differentiated THP-1 (dTHP-1) cells, as per the manufacturer’s instructions. All reagents were brought to room temperature and prepared using the manufactures instructions prior to analysis. Briefly, THP-1 cells at a cell density of 0.5 X 10⁶ cells/mL were differentiated in a 96 well plate by the addition at 100ng/mL PMA as described in section 4.2.8. Cells were then incubated for the required amount of time with the desired stimulus in fresh media. After which cells were rapidly fixed by replacing the growth media with 100μL of 4% formaldehyde and the plate was incubated for 20 minutes at room temperature (RT). The formaldehyde was then removed and the plate washed (x3) with 200μL wash buffer (0.1% Triton X-100 in PBS), for 5 minutes with gentle shaking. After removal of the wash buffer, 100μL of quenching buffer (1% H₂O₂ and 0.1% sodium azide in wash buffer) was added and incubated for 20 minutes at RT. Removal of the quenching buffer was followed by washes (x2) with wash buffer as detailed above. All wash buffer was removed and 100μL antibody blocking buffer was added an incubated for 1 hour at RT. Following removal of the antibody blocking buffer, cells were washed (x2) with 200μL wash buffer and after removal of the wash buffer cells were incubated with 40μL of primary antibody. One plate at each time point was used for the quantification of phosphor-ERK1/2, while the other plate was used to determine total-ERK1/2 with another primary antibody. The plate was sealed with a plate strip and incubated at 4°C overnight. Any unbound primary antibody was removed by washing the cells (x3) with 200μL wash buffer for 5 minutes each. The wash buffer was replaced by addition of 100μL of diluted secondary HRP-conjugated antibody and incubated for
1 hour at RT. Unbound secondary antibody was removed with 200μL wash buffer (x3) and with 200μL PBS (x2). The remaining PBS was removed and replaced with 100μL of developing solution in each well and the plates incubated for 20 minutes at RT in the dark. This was followed by the addition of 100μL stop solution (0.5M sulphuric acid) to each well. The absorbance of each well was determined at 450nm with a reference wavelength of 655nm (Dynex Technologies, VA, USA).

The relative number of cells in each well was determined using crystal violet staining. After reading the absorbance, as described above, cells were washed (x2) with 200μL wash buffer (x2) with PBS. Excess liquid was removed and the plate air dried for 5 minutes at RT. Cells were then incubated with 100μL crystal violet solution for 30 minutes at RT. Excess stain was then removed by washing the cells with 200μL PBS (x3) for 5 minutes each, after which 100μL 1% SDS was then added to each well and incubated on a shaker at RT for 1 hour. The absorbance was read by spectrophotometry (Dynex Technologies, VA, USA) at 595nm. The values of phosphor-ERK1/2 and total-ERK1/2 were normalised for cell number and the data presented as a ratio of phosphor-ERK1/2 to total-ERK1/2.

4.2.17. Statistical analysis.

All data is expressed as mean ± standard deviations (SD) unless otherwise specified. One-way analysis of variance (ANOVA) was used within group comparisons with Tukey’s Pairwise analysis used for determining true differences and the student’s t-test was used to compare the differences between the means of the two samples.
Statistical analysis was performed using Minitab® version 14 (Minitab Inc, PA, USA) software package. Differences were deemed to be significant when $P<0.05$. 
4.3. Results.

4.3.1. Optimisation of both CD36 primary and FITC secondary antibodies.

For optimal results, the amount of primary antibody used was determined for each cell type prior to flow cytometric analysis as incorrect concentrations of antibodies dramatically affect the fluorescence of flow cytometric analysis. Figure 4.1a demonstrates that the optimum concentration of primary CD36 antibody required per reaction was 10μg/mL. After which the secondary FITC antibody was optimised with 10μg/mL primary CD36 antibody, within the manufacturers recommended range of 5-20μg/mL. The optimal concentration of secondary FITC antibody was found to be 15μg/mL (Figure 4.1b).
THP-1 cells were incubated with various concentrations of primary CD36 antibody (2 to 20µg/mL) and b) various concentrations of FITC secondary antibody (5 to 20µg/mL). CD36 expression was quantified using flow cytometry. The dotted line represents mean basal CD36 expression in untreated THP-1. Results are expressed as % change in CD36 relative to untreated THP-1 cells and are mean ± SD of three independent experiments.
4.3.2. Optimisation of LDL oxidation using CuSO$_4$.

The concentration of CuSO$_4$ and length of time needed to ensure complete oxidation of LDL was optimised using concentrations of CuSO$_4$ ranging from 0.1mM to 2mM and length of incubation ranging from 16 to 24 hours. The concentration found to maximise oxidation of LDL was 0.64mM and this was incubated with the native LDL for 17 hours at 37°C. Figure 4.2 illustrates the shift in peak absorbance between native LDL and oxidised LDL (oxLDL). Native LDL had an absorbance peak at approximately 250nm whereas the absorbance peak was shifted to 234nm after incubation with 0.64mM CuSO$_4$.

![Figure 4.2: Optimisation of LDL oxidation using CuSO$_4$ treatment.](image_url)

**Figure 4.2: Optimisation of LDL oxidation using CuSO$_4$ treatment.** LDL was oxidised with 0.64mM CuSO$_4$ for 17 hours, after which absorbance for both native LDL (1μg/mL) and oxLDL (1μg/mL) was determined. The presence of oxLDL is confirmed by the shift in the maximal peak absorbance from 250nm to 234nm.
A second experiment was used to check for successful oxidation of LDL. This involved incubating THP-1 cells with native LDL and oxLDL and measuring CD36 surface expression. Figure 4.3 demonstrates that only oxLDL, which is an important ligand for CD36 increased CD36 surface expression after 24 and 48 hours respectively. This data is strongly suggestive of CuSO$_4$ mediated LDL oxidation. Native LDL did not increase CD36 surface protein expression at either time point (Figure 4.3).

**Figure 4.3: Effect of native LDL and oxLDL on CD36 surface protein expression.** Undifferentiated THP-1 cells were simultaneously incubated with both 1µg/mL LDL and oxLDL for 24 and 48 hours and CD36 expression was determined using flow cytometry. The dotted line represents mean basal CD36 expression in THP-1 cells. Results are expressed as % change in CD36 surface expression relative to basal untreated THP-1 cells and are mean ± SD of three independent experiments (* $P<0.05$ ANOVA, compared to cells only).
4.3.3. Effect of oxLDL on CD36 surface expression in monocytic cells (MM6 cells).

MM6 cells were used as a model of human monocytic cells. The PPARγ ligand, oxLDL, was selected to test the hypothesis that incubation of MM6 cells with this oxidised lipid is capable of increasing CD36 surface expression. Figure 4.4 demonstrates that incubation of MM6 cells with either oxLDL (1μg/mL) or another PPARγ ligand, rosiglitazone (1μM) failed to increase CD36 surface expression at all time points. Due to the failure of either PPARγ ligands to upregulate CD36, another monocytic type cell line, namely THP-1 cells, was used.
Figure 4.4: Effects of oxLDL on CD36 surface expression in MM6 cells. MM6 cells were incubated with 1μg/mL oxLDL or 1μM rosiglitazone for 24, 48 and 72 hours. CD36 surface protein expression was measured by flow cytometry. The dotted line represents mean basal CD36 expression in MM6 cells. Results are expressed as % change in CD36 surface expression relative to untreated MM6 cells and are mean ± SD of two independent experiments.

4.3.4. Differentiation of THP-1 cells, using PMA.

To ensure that Phorbol 12-myristate 13-acetate (PMA) had successfully stimulated differentiation of THP-1 cells, digital camera pictures were taken before and after 48 hours of incubation with 100ng/mL PMA. PMA is an important activator of protein kinase C and is therefore routinely used as a differentiator of human leukaemia cell lines (Silverstein, 1996). Figure 4.5a is of undifferentiated THP-1 cells whereas
Figure 4.5b is THP-1 cells differentiated with PMA for 48 hours, as evidenced by their transformed adherent nature.

**Figure 4.5: Effect of 100ng/mL PMA on THP-1 cells.** THP-1 cells were incubated without (a) and with (b) 100ng/mL PMA for 48 hours and visualised for differentiation characteristics by light microscopy (x400), these characteristics include extension of the cell membrane and increased adherence. Scale bar = 100µm.

During the differentiation process, THP-1 cells increase their expression of PPARγ (Chinetti *et al.*, 1998). As monocytes mature into tissue macrophages a number of signalling pathways are triggered, which ultimately leads to upregulation of PPARγ mRNA. This increased expression is supported by figure 4.6, which demonstrates that THP-1 cells differentiated by PMA expressed approximately 30% more CD36 surface expression compared to undifferentiated THP-1 cells.
Figure 4.6: Effect of differentiation of THP-1 cells with 100ng/mL PMA on CD36 surface expression. THP-1 cells were differentiated for 48 hours with 100ng/mL PMA and alongside undifferentiated THP-1 cells, CD36 surface expression was measured by flow cytometry. Results are expressed as % change in CD36 surface expression relative to untreated THP-1 cells and are mean ± SD of two independent experiments (* P<0.05, two sample t-test, compared to undifferentiated THP-1 cells).

4.3.5. Effect of varying concentrations of oxLDL on cell proliferation.

Cell proliferation was measured using a CellTiter 96® AQUEOUS One Solution Cell Proliferation assay. After incubating dTHP-1 cells with the four different concentrations of oxLDL, there was no significant change in viability/proliferation at all time points compared to dTHP-1 cells only (Figure 4.7). A general decline in
viability was seen at each time point with increased concentrations of oxLDL, however this did not reach significance.

Figure 4.7: Effect of varying concentrations of oxLDL on differentiated THP-1 cell proliferation. dTHP-1 cells were treated with either 1 (red line), 10 (turquoise line), 20 (green line) or 40μg/mL (brown line) oxLDL for 8, 24, 48 and 72 hours and cell viability/proliferation was determined. Results are expressed as mean ± SD of three separate experiments.
4.3.6. Effect of varying concentrations of oxLDL on CD36 surface expression in differentiated THP-1 cells.

To verify that oxLDL is capable of upregulating CD36, varying concentrations of oxLDL ranging from 1μg/mL to 40μg/mL were incubated with differentiated THP-1 (dTHP-1) cells over a three day period. There was a significant increase in CD36 surface protein expression, relative to dTHP-1 cells only, after incubation with 1μg/mL oxLDL at 24, 48 and 72 hours. The maximum amount of CD36 surface protein expression was observed after 24 hours (Figure 4.8a).

Incubation with 10μg/mL oxLDL resulted in a significant increase in CD36 surface expression at 48 and 72 hours (Figure 4.8b). Unlike 1μg/mL, the peak in CD36 expression with 10μg/mL was after 72 hours. Both the concentrations of oxLDL, 20μg/mL and 40μg/mL respectively, showed similar results to 10μg/mL, in that there were significant increases in CD36 surface expression after 48 and 72 hours (Figures 4.8c and 4.8d, respectively). However there was no significant enhancement in CD36 surface expression at 24 hours with 10, 20 and 40μg/mL oxLDL.
Figure 4.8: Effects of varying concentrations of oxLDL on CD36 surface expression in differentiated THP-1 cells. dTHP-1 cells were incubated with a) 1μg/mL, b) 10μg/mL, c) 20μg/mL and d) 40μg/mL oxLDL for 24, 48 and 72 hours. CD36 surface expression was measured by flow cytometry. The dotted line represents mean basal CD36 expression in dTHP-1. Results are expressed as % change in CD36 surface expression relative to untreated dTHP-1 cells and are mean ± SD of three independent experiments (* P<0.05 ANOVA, Tukey’s pairwise analysis, compared to cells only).
4.3.7. The effect of the specific PPARγ ligand rosiglitazone and the PPARγ antagonist GW9662 on CD36 surface expression in differentiated THP-1 cells.

The effect of another PPARγ ligand, rosiglitazone (1μM), on CD36 surface expression was used to highlight the PPARγ dependent nature of CD36 upregulation and increased surface protein expression. After incubation with rosiglitazone (1μM) there was a significant increase in CD36 surface expression compared to untreated dTHP-1 cells, at all time points measured (Figure 4.9a). To investigate the possible effects that the PPARγ antagonist GW9662 may have on CD36 cell surface expression a preliminary experiment revealed that incubation with 1μM GW9662 had no effect on CD36 surface expression at all time points (Figure 4.9b). Thus strengthening the understanding that CD36 upregulation is PPARγ dependent.
Figure 4.9: Effect of rosiglitazone and GW9662 on CD36 surface expression in differentiated THP-1 cells. dTHP-1 cells were incubated with rosiglitazone (1μM) (a) and GW9662 (1μM) (b) for 24, 48 and 72 hours and CD36 surface expression was determined by flow cytometry. The dotted line represents mean basal CD36 expression in dTHP-1. Results are expressed as % change in CD36 surface expression relative to untreated dTHP-1 cells and are mean ± SD of three independent experiments (* \( P<0.05 \) ANOVA, compared to cells only).
4.3.8. Effect of a rosiglitazone and GW9662 on cell viability.

Cell viability was measured using a CellTiter 96® AQUEOUS One Solution Cell Proliferation assay. After incubating dTHP-1 cells with either the PPARγ ligand rosiglitazone (1μM) or the PPARγ antagonist GW9662 (1μM), there were no significant changes in cell viability/proliferation at all of the time points compared to dTHP-1 cells only (Figure 4.10).

Figure 4.10: Effect rosiglitazone (1μM) and GW9662 (1μM) on differentiated THP-1 cell viability. dTHP-1 cells were treated with either rosiglitazone (1μM) (red line) or GW9662 (1μM) (green line) for 8, 24, 48 and 72 hours and cell viability/proliferation was determined using a CellTiter 96® AQUEOUS One Solution Cell Proliferation assay. Results are expressed as mean ± SD of three separate experiments.
4.3.9. The effect of the PPARγ antagonist GW9662 on oxLDL mediated CD36 mRNA and surface expression.

Having previously shown that oxLDL induces CD36 surface expression, an important PPARγ regulated gene, it was decided to investigate whether the oxLDL induced expression of CD36 is inhibited by a known PPARγ antagonist, Differentiated THP-1 (dTHP-1) cells were treated with 1μM GW9662 for 1 hour before stimulation with or without 1μg/mL oxLDL for 24, 48 and 72 hours. There was a significant increase in CD36 mRNA expression compared to dTHP-1 cells only at all time points after stimulation with 1μg/mL oxLDL (Figure 4.11). There was also a significant inhibition in this oxLDL induced upregulation in CD36 mRNA after a 1 hour pre-incubation with the PPARγ antagonist GW9662 (1μM).
Figure 4.11: Effect of pre-incubation with the PPARγ antagonist GW9662 (1μM) on oxLDL induced CD36 mRNA expression. dTHP-1 cells were pre-incubated for 1 hour with GW9662 (1μM) before stimulation with 1μg/mL oxLDL for 24, 48 and 72 hours. CD36 mRNA was quantified by real time-PCR and is reported as a ratio to GAPDH. The dotted line represents mean basal CD36 mRNA expression in dTHP-1, with results expressed relative to untreated dTHP-1 cells. Data is reported as mean ± SD of three independent experiments (* $P<0.05$ ANOVA, compared to dTHP-1 cells only, # $P<0.05$ two sample t-test compared to cells pre-incubated with GW9662 then treated with 1μg/mL oxLDL).

Figure 4.12 illustrates that unlike the CD36 mRNA result, the PPARγ antagonist GW9662 did not significantly inhibit the oxLDL induced increase in CD36 surface expression after 24 hours. There was however, significant inhibition by the PPARγ antagonist on CD36 surface expression after 48 and 72 hours.
**Figure 4.12:** Effect of pre-incubation with the PPARγ antagonist GW9662 (1μM) on oxLDL induced CD36 surface expression. dTHP-1 cells were pre-incubated for 1 hour with GW9662 (1μM) before stimulation with 1μg/mL oxLDL for 24, 48 and 72 hours. CD36 surface expression was determined by flow cytometry. Results are expressed as % change in CD36 surface expression relative to dTHP-1 cells. Data is reported as mean ± SD of three independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only, # P<0.05 two sample t-test compared to cells pre-incubated with GW9662 then treated with 1μg/mL oxLDL).

4.3.10. The effects of oxLDL on LXRα mRNA expression.

From the published evidence (Chinetti *et al.*, 2001 and Chawla *et al.*, 2001b) and from a bioinformatics search, it has been suggested that PPARγ may have a PPRE
within the LXRα promoter region, suggesting that PPARγ regulates LXRα gene expression. In order to provide some evidence on this issue, 1μg/mL oxLDL was incubated with dTHP-1 cells for 24, 48 and 72 hours, after which RNA was extracted, converted to cDNA and LXRα mRNA was quantified. There was a significant increase in LXRα gene expression compared to dTHP-1 cells only at all time points measured (Figure 4.13).

**Figure 4.13: Effect of oxLDL on LXRα gene expression.** dTHP-1 cells were incubated with 1μg/mL oxLDL for 24, 48 and 72 hours. LXRα gene expression was quantified by real time-PCR and is reported as a ratio to GAPDH. The dotted line represents mean basal LXRα mRNA expression in dTHP-1 cells. Results are expressed relative to dTHP-1 cells only and are mean ± SD of two independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only).
Another PPARγ ligand was used to confirm the PPARγ dependent nature of LXRα upregulation. Figure 4.14 confirms that stimulation of dTHP-1 cells with the PPARγ ligand rosiglitazone, causes upregulation of the transcription factor LXRα. dTHP-1 cells were also treated with 1µM GW9662 alone for 24, 48 and 72 hours and LXRα gene expression was quantified. Figure 4.14 demonstrates that 1µM GW9662 had no significant effect on LXRα mRNA expression and therefore does not effect LXRα gene expression in a PPARγ independent manner.
Figure 4.14: Effect of the PPARγ agonist rosiglitazone and the PPARγ antagonist GW9662 on LXRα gene expression. THP-1 cells were differentiated for 48 hours with 100ng/mL PMA and then incubated with either rosiglitazone (1μM) or GW9662 (1μM) for 24, 48 and 72 hours. LXRα gene expression was quantified by real time-PCR and is reported as a ratio to GAPDH. The dotted line represents mean basal LXRα mRNA expression in dTHP-1 cells. Results are expressed relative to dTHP-1 cells only and are mean ± SD of two independent experiments. (* P<0.05 ANOVA, compared to dTHP-1 cells only).

4.3.11. The effect of oxLDL on the mRNA expression of the LXRα regulated gene ABCA1.

In order to confirm that 1μg/mL oxLDL not only upregulates LXRα but increases the activity of the nuclear transcription factor, dTHP-1 cells were incubated with 1μg/mL oxLDL for 24, 48 and 72 hours and ABCA1 mRNA expression was
quantified. Figure 4.15 reinforces the findings that incubation of dTHP-1 with oxLDL (1μg/mL) significantly upregulates ABCA1 mRNA expression at all time points quantified, indicative of increased transcriptional activity of LXRα (Figure 4.13).

**Figure 4.15: Effect of oxLDL on ABCA1 mRNA expression.** dTHP-1 cells were incubated with 1μg/mL oxLDL for 24, 48 and 72 hours. ABCA1 gene expression was quantified by real time-PCR and is reported as a ratio to GAPDH. The dotted line represents mean basal ABCA1 mRNA expression in dTHP-1 cells. Results are expressed relative to dTHP-1 cells only and are mean ± SD of two independent experiments. (* P<0.05 ANOVA, compared to cells only).
4.3.12. The effect of the PPARγ antagonist GW9662 on oxLDL upregulation of LXRα gene expression.

Having previously demonstrated that both PPARγ ligands, namely oxLDL and rosiglitzone increase LXRα expression it was decided to further elucidate the potential PPARγ dependent mechanism involved in LXRα upregulation. Therefore, dTHP-1 cells were pre-treated with the PPARγ antagonist GW9662 (1μM) for 1 hour and subsequently stimulated with 1μg/mL oxLDL. Incubation of dTHP-1 cells with 1μM GW9662 1 hour prior to stimulation with 1μg/mL oxLDL caused a significant decrease in LXRα mRNA compared to cells treated with oxLDL only at 48 hours and 72 hours only (Figure 4.16). There was no significant difference in LXRα mRNA after 24 hours between cells treated with 1μg/mL oxLDL only and cells that were pre-treated with 1μM GW9662 before stimulation with oxLDL (1μg/mL). Suggesting an initial PPARγ independent mechanism involved in LXRα activation.
Figure 4.16: Effect of pre-incubation with the PPARγ antagonist GW9662 on oxLDL induced LXRα mRNA expression. dTHP-1 cells were pre-incubated with GW9662 (1μM) for 1 hour before stimulation with 1μg/mL oxLDL for 24, 48 and 72 hours. LXRα mRNA was quantified by real time-PCR, and is reported as a ratio to GAPDH. The dotted line represents mean basal LXRα mRNA expression in dTHP-1 cells. Results are expressed relative to dTHP-1 cells only and are mean ± SD of three independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only, # P<0.05 two sample t-test, compared to dTHP-1 cells pre-stimulated with GW9662 then treated with 1μg/mL oxLDL).
4.3.13. The effects of oxLDL on COX-2 and CD36 gene expression during short and long term incubation.

In light of the recent publication by Taketa et al., (2008), the effects of 1 μg/mL oxLDL on COX-2 and CD36 gene expression were investigated. In their study evidence was presented that the short term (<6 hours) upregulation of PPARγ involves activation of COX-2. Therefore dTHP-1 cells were treated with 1 and 40 μg/mL oxLDL for 2, 6, 10, 24, 48 and 72 hours and COX-2 and CD36 gene expression was quantified. Figure 4.17a demonstrates that incubation of dTHP-1 cells with 1 μg/mL oxLDL causes a rapid increase in COX-2 mRNA, peaking after 10 hours. However by 24 hours COX-2 gene expression is not significantly different from untreated cells. At 48 and 72 hours COX-2 gene expression is reduced compared to dTHP-1 cells only (dotted line). In terms of CD36 gene expression, there is upregulation at 2, 6 and 10 hours, which coincides with an upregulation in COX-2. However in contrast to COX-2, CD36 gene expression remains upregulated after 24, 48 and 72 hours of stimulation with 1 μg/mL oxLDL. Incubation of cells with 40 μg/mL oxLDL resulted in similar patterns in COX-2 and CD36 gene expression as seen with 1 μg/mL oxLDL (Figure 4.17b). There was again a peak in COX-2 gene expression after 10 hours which reverts back to baseline after 24 hours and CD36 mRNA expression was increased at all time points.
Figure 4.17: Effects of 1 and 40μg/mL oxLDL on COX-2 and CD36 gene expression. dTHP-1 cells were stimulation with either 1μg/mL (a) or 40μg/mL (b) oxLDL for 2, 6, 10, 24, 48 and 72 hours. COX-2 and CD36 mRNA was quantified by real time-PCR, and is reported as a ratio to GAPDH. Results are expressed relative to dTHP-1 cells only (dotted line) and are mean ± SD of two independent experiments.
4.3.14. The effects of a specific COX-2 inhibitor on oxLDL induced upregulation of CD36.

The results from section 4.3.12 suggest that the initial upregulation in COX-2 mRNA, peaking after 10 hours may be responsible for the continued upregulation of CD36 gene expression between 24 and 72 hours. Therefore a specific COX-2 inhibitor, NS-398 was pre-incubated with dTHP-1 cells for 2 hours prior to stimulation with oxLDL. Figure 4.18 demonstrates that the upregulation in CD36 gene expression seen after stimulation with 1μg/mL oxLDL is inhibited at all time points after a 2 hour pre-incubation with the COX-2 inhibitor NS-398. Suggesting an important initial role for COX-2 in the long-term upregulation of CD36 mRNA expression.
Figure 4.18: Effect of the COX-2 inhibitor NS-398 on oxLDL induced activation of CD36. dTHP-1 cells were incubated with 1μg/mL oxLDL with or without a 2 hour pre-treatment with the COX-2 inhibitor NS-398 (50μM) for 2, 6, 10, 24, 48 or 72 hours. CD36 and COX-2 mRNA expressions were quantified by real time-PCR, and are reported as a ratio to GAPDH. Results are expressed relative to dTHP-1 cells only (dotted line) and are mean ± SD of two independent experiments.

4.3.15. The effect of oxLDL on ERK1/2 phosphorylation.

Figure 4.19a demonstrates that stimulation of dTHP-1 cells with 1μg/mL oxLDL causes a significant increase in the ratio of phosphorylated to total ERK1/2, relative to dTHP-1 cells only. This increase in ERK1/2 activity was observed at all time
points and this increased ratio of phosphorylated to total ERK1/2 was inhibited by the pre-incubation with the ERK1/2 inhibitor PD98059 (50μM). PD98059 is a well established inhibitor of ERK1/2 and has previously been shown to reduce ERK1/2 activity in THP-1 macrophages after stimulation with oxLDL (Yin et al., 2006). The results shown in figure 4.19b suggest that incubation of dTHP-1 cells with 40µg/mL increased the ratio of phosphorylated to total ERK1/2 compared to untreated dTHP-1 cells at all time points. This again was reduced when cells were pre-stimulated with the ERK1/2 inhibitor PD98059. As expected treatment of cells with the PD98059 alone resulted in no change in ERK1/2 activation. Figure 4.20 is an example of quantification of cell numbers using a violet blue stain. Lysed cells take up the violet dye and using light microscopy this was visualised.
Figure 4.19: Effects of 1 and 40μg/mL oxLDL on ERK1/2 phosphorylation relative to total ERK1/2. dTHP-1 cells were incubated with 1μg/mL (a) and 40μg/mL (b) oxLDL with or without a 1 hour pre-treatment with the ERK1/2 inhibitor PD98059 (50μM). ERK1/2 activity was measured by ELISA. Results are expressed relative to untreated cells and are mean ± SD of two independent experiments.
Figure 4.20: Crystal violet stain to determine cell numbers. Post analysis of phosphorylated or total ERK1/2, dTHP-1 cells were stained with crystal violet stain and incubated for 30 minutes and cell numbers were measured by reading the absorbance at 595nm. Scale bar = 15µm.

4.3.16. The effects of the ERK1/2 inhibitor PD98059 on oxLDL induced activation of CD36.

To determine if the oxLDL induced upregulation in CD36 mRNA was also ERK1/2 dependent, dTHP-1 cells were incubated with 1µg/mL oxLDL for 8, 24, 48 and 72 alone or pre-treated with 50µM PD98059 for 1 hour and then subsequently stimulated with 1µg/mL oxLDL. Figure 4.21 demonstrates that cells incubated with 1µg/mL oxLDL alone had significantly increased levels of CD36 mRNA at all time points. However the cells that were firstly pre-treated with 50µM PD98059 and then incubated with 1µg/mL had significantly reduced CD36 mRNA levels at 8 hours, compared to oxLDL alone. However at 24, 48 and 72 hours the pre-incubation of cells with PD98059 had no inhibitory effect on oxLDL stimulated CD36 mRNA
upregulation. Figure 4.21 also demonstrates that dTHP-1 cells incubated with 50μM PD98059 (negative control) alone had no effect on CD36 gene expression.

**Figure 4.21: Effect of the ERK1/2 inhibitor PD98059 on oxLDL induced activation of CD36.** dTHP-1 cells were incubated with 1μg/mL oxLDL with or without a 1 hour pre-treatment with the ERK1/2 inhibitor PD98059 (50μM) for 8, 24, 48 or 72 hours. CD36 mRNA was quantified by real time-PCR, and is reported as a ratio to GAPDH. Results are expressed relative to dTHP-1 cells only and are mean ± SD of two independent experiments (* P<0.05 ANOVA, compared to dTHP-1 cells only, # P<0.05 two sample t-test, compared to dTHP-1 cells pre-stimulated with PD98059 then treated with 1μg/mL oxLDL).
4.4. Discussion.

This chapter has investigated the effects of oxLDL on PPARγ and LXRα activation in monocyte/macrophages and aimed to elucidate the short and long term molecular mechanisms responsible for the oxLDL induced upregulation of PPARγ and LXRα dependent genes. Accumulation of modified low density lipoproteins (LDL), for example oxidised LDL (oxLDL) and the recruitment of monocytes into the arterial subendothelial spaces are early events in atherogenesis. Macrophages, which are derived from monocytes, influx oxLDL through specific scavenger receptor pathways, such as CD36 and hence become foam cells. The development of foam cells is a key factor in the advancement of atherosclerosis. There is evidence that oxLDL induces PPARγ activation (Nagy et al., 1998) and that certain metabolites of oxLDL, such as 9-HODE and 13-HODE are highly involved in oxLDL induced PPARγ activation. However, in light of the recent publications by Yano et al., (2007) and Taketa et al., (2008) an alternative pathway involved in PPARγ activation has been proposed. Yano et al., (2007) reported that the anti-atherogenic lipid lowering drugs namely, statins, induced a COX-2 dependent increase in 15-deoxy-Δ-12,14-prostaglandin J₂ (15d-PGJ₂) levels via both phosphorylation of p38 MAPK and ERK1/2. 15d-PGJ₂ which is another known natural ligand of PPARγ will therefore increase activation of PPARγ. Taketa et al., (2008) further elucidates this potential signalling pathway, in contrast they stimulated macrophages with oxLDL. Taketa et al., (2008) demonstrated that 40μg/mL oxLDL induced COX-2 expression via activation of ERK1/2 but not p38 MAPK and that oxLDL activation of PPARγ was inhibited by specific inhibitors of COX-2 and ERK1/2. This suggests that oxLDL induced activation of PPARγ is mediated by ERK1/2-dependent COX-2 expression.
in macrophages. The timescales involved in both studies was specific, focusing on oxLDL induced activation of PPARγ via COX-2 in the short term, i.e. up to 24 hours.

Therefore, this chapter attempted to further elucidate the molecular mechanisms involved in PPARγ activation, as measured by expression of CD36, that are mediated by treatment of macrophages with different concentrations of oxLDL. Specifically 1 and 40μg/mL oxLDL, with time points ranging from 2 to 72 hours being investigated. LXRα gene expression was also quantified, as another indicator of PPARγ activity. It is important to emphasise that the use of 1μg/mL oxLDL coincides with the physiological concentrations observed in the in-vivo based exercise intervention (i.e. 600-800 ng/mL), as reported in chapter 2.3.7 and therefore allows comparisons to be made, whereas 40μg/mL was chosen as a supra-physiological level.

Figure 4.6 provides important justification for the use of differentiated (dTHP-1) cells over undifferentiated cells, due to a significant increase in PPARγ activity. This is demonstrated by an increased expression of the PPARγ regulated gene, CD36, compared to undifferentiated cells. After stimulation of THP-1 cells with 100ng/mL PMA for 48 hours there was an approximate 30% increase in CD36 surface expression as determined by flow cytometry. Comparable results were reported by Han and Sidell, (2002) and Nakagawa et al., (1998) suggesting that differentiation of monocyte like THP-1 cells to cells that posses significant characteristics of macrophages is accompanied by an increase in CD36 surface expression, hence increased PPARγ activity.
Cell viability was determined with various concentrations of oxLDL, including 1, 10, 20 and 40μg/mL in order to discard any possible side effects the oxLDL may have on cell proliferation and hence apoptosis. It was observed that all concentrations of oxLDL used to stimulate cells did not significantly alter the viability of cells compared to their untreated counterparts (Figure 4.7). There appeared to be a time dependent decline in cell viability with all concentrations of oxLDL, however this did not reach significance.

As shown in Figures 4.8a to d, oxLDL at varying concentrations increased CD36 surface expression in dTHP-1 cells. Figure 4.8a demonstrates that incubation of cells with 1μg/mL oxLDL increased CD36 surface expression relative to dTHP-1 cells only at all time points. There are a minimal number of studies that have looked specifically at 1μg/mL oxLDL on either CD36 expression or ultimately PPARγ activity, however Taketa et al., (2008) reported no significant increase in PPARγ activity as measured by PPARγ-ligand binding, after incubation for 24 hours with 1μg/mL oxLDL. The current study also demonstrates that 1, 10, 20 and 40μg/mL oxLDL increased CD36 surface expression compared to dTHP-1 cells only after 48 and 72 hours. This is again reinforced by Taketa et al., (2008) who demonstrated a significant increase in PPARγ-ligand binding with 10 and 20μg/mL after 24 hours. Han et al., (1997) also reported increases in CD36 mRNA and surface expression with 25μg/mL oxLDL after 6 and 12 hours respectively. However, unlike Feng et al., (2000) and Han et al., (1997) who reported increases in CD36 mRNA after 24 hours, there was no significant increase in CD36 surface expression with 10, 20 and 40μg/mL oxLDL after 24 hours in the current study.
In order to provide more substantial evidence of the PPARγ dependent nature of CD36 upregulation, dTHP-1 cells were incubated with rosiglitazone (1μM), a well known synthetic ligand of PPARγ (Cuzzocrea et al., 2003). Figure 4.9a demonstrates that incubation of monocytic cells with 1μM rosiglitazone significantly increased CD36 surface expression by more than 70% at all time points measured. This is in agreement with many previous studies, including Li et al., (2000) who reported increased CD36 mRNA after stimulation of LDL deficient mice with rosiglitazone. Tontonoz and Nagy, (1999) also demonstrated increased CD36 expression in macrophages after treatment with the PPARγ ligand rosiglitazone. Another member of the TZD family of anti-diabetic drugs, troglitazone, which like rosiglitazone is a ligand for PPARγ, has also been shown to induce significant changes in CD36 mRNA and protein expression (Tontonoz et al., 1998 and Chen et al., 2001). The effect of the PPARγ antagonist GW9662 on CD36 surface expression was quantified and results demonstrate that incubation of dTHP-1 cells with 1μM GW9662 had no effect on CD36 expression, as measured by flow cytometry (Figure 4.9b). This is reconfirmed by Han and Sidell, (2002) who reported similar findings in THP-1 cells.

In a similar manner to oxLDL, dTHP-1 cell viability was measured after incubation of cells with rosiglitazone (1μM) and GW9662 (1μM) for 24, 48 and 72 hours (Figure 4.10). Incubation with both the PPARγ ligand and antagonist did not significantly affect cell viability and hence further experiments were allowed to proceed.

Figure 4.11 reports the effects of the specific PPARγ antagonist GW9662 on oxLDL induced expression of CD36 mRNA in dTHP-1 cells. Incubation of cells with
1μg/mL oxLDL causes a significant increase in CD36 gene expression compared to dTHP-1 cells only. This oxLDL mediated effect is evident at 24, 48 and 72 hours. This particular result confirms the previous result in section 4.3.4, which showed that incubation with 1μg/mL oxLDL induced CD36 surface expression. The PPARγ antagonist GW9662 was used to pre-incubate the cells for 1 hour before the addition of 1μg/mL oxLDL, to establish if inhibition of PPARγ reduces oxLDL upregulation of CD36. Figure 4.11 illustrates that inhibition of PPARγ by GW9662 (1μM) causes a significant reduction in CD36 gene expression compared to cells treated with oxLDL only, at all time points measured. Taken together, these results suggest that at 24, 48 and 72 hours the oxLDL induced upregulation in CD36 gene expression is mediated in a PPARγ dependent mechanism. This coincides with the publications by Nagy et al., (1998), Tontonoz et al., (1998) and Pineda Torra et al., (1999) showing that PPARγ is a critical regulator of CD36 expression. The fact that the human CD36 gene contains a PPARγ response element (PPRE) is suggestive of a direct interaction which regulates CD36 gene expression. A proposed mechanistic pathway for the oxLDL induced upregulation of CD36 gene expression starts with the internalisation of oxLDL via cell surface CD36 receptors. Once inside the cell specific ligands for PPARγ are released from the oxLDL molecules, such as 9- and 13- HODE’s and once PPARγ ligand binding has occurred in the nucleus and brought about a conformational change in PPARγ, the transcription factor becomes active. Subsequently, PPARγ can then bind to its PPRE’s upstream of its target genes and induce increased synthesis of CD36 and hence increased CD36 surface protein. Hence, initiating a positive feedback mechanism enhancing oxLDL uptake.
To further confirm the PPARγ dependent nature of oxLDL induced CD36 expression in dTHP-1 cells, CD36 surface expression was measured by flow cytometry after pre-treatment with 1μM GW9662 and subsequent incubation with 1μg/mL oxLDL. The results in figure 4.12 complement those from figure 4.11, with the exception of the 24 hour incubation. At 48 and 72 hours the PPARγ antagonist GW9662 significantly inhibits the oxLDL mediated increase in CD36 surface expression. However, at 24 hours there is no significant difference between cells treated with oxLDL and those pre-treated with GW9662 (1μM) and then subsequently incubated with oxLDL (1μg/mL). Suggesting that the increase in CD36 surface expression at 24 hours may be PPARγ independent. Regulation of CD36 expression can be mediated both pre and post transcriptionally, therefore an alternative mechanism of increasing CD36 surface expression besides transactivation is via the regulation of movement of CD36 from intracellular stores to the plasma membrane. The increase in CD36 surface expression at 24 hours in the presence of GW9662 and oxLDL is therefore unlikely to be the result of de-novo synthesis, because CD36 mRNA was inhibited by GW9662, but may be due to increased trafficking of CD36 intracellular stores from within the endoplasmic reticulum (ER). Huh et al., (1996) and Luiken et al., (2002) have previously documented that monocytes/macrophages possess significant intracellular pools of CD36.

In the current study the PPARγ ligand oxLDL, was used to stimulate LXRα gene expression in dTHP-1 cells. Figure 4.13 demonstrates that incubation of dTHP-1 cells with 1μg/mL oxLDL induced upregulation in LXRα gene expression after 24, 48 and 72 hours. LXRα has previously been proposed to be a direct target of PPARγ Chinetti et al., (2001) and Chawla et al., (2001b) via direct transcriptional regulation.
Chinneti et al., (2001) reported that numerous PPARγ ligands, including rosiglitazone and 15d-PGJ2 induced LXRα mRNA expression after exposure for 24 hours in monocytes derived macrophages. Chawla et al., (2001b) confirmed similar results, specifying that the induction of LXRα by rosiglitazone was highly specific, as there was no change in LXRβ mRNA after identical rosiglitazone stimulation. The result in Figure 4.13 reinforces the understandings of Nicholson and Hajjar, (2004) who stated that oxysterols derived from oxLDL are ligand activators of LXRα. Tang et al., (2004) also reported oxLDL induced LXRα expression in THP-1 macrophages, however this was at a much higher concentration than added in the present study, i.e. 50μg/mL. In another cell type, 3T3-L1 adipocyte type cells, Zhao et al., (2008) reported increases in LXRα and ABCA1 mRNA, 40% and 50% respectively, after stimulation for 24 hours with 25μg/mL oxLDL. They also reported that adipocytes pre-treated with oxLDL for 24 hours and then stimulated with the potent LXR agonist 22(R)-hydroxycholesterol possessed increased levels of both LXRα and ABCA1 mRNA compared to their oxLDL untreated counterparts. Dhaliwal and Steinbrecher, (2000) and Zhao et al., (2008) are both in agreement that higher concentrations of oxLDL (i.e. 50μg/mL or higher) fail to enhance the LXR-ABCA1-apoA-I pathway and hence do not stimulate cholesterol efflux in macrophages and adipocytes, respectively.

Figure 4.15 demonstrates that incubation of dTHP-1 cells with 1μg/mL oxLDL upregulates ABCA1 mRNA expression, confirming that the oxLDL increases LXRα transcriptional activity. In previous work ABCA1 mRNA and protein expression was also increased after THP-1 cells were exposed to 50mg/L oxLDL for 12 hours and increased further at 24 and 48 hours (Tang et al., 2004).
In order to confirm an association between the upregulation of LXRα in dTHP-1 cells observed with oxLDL treatment and PPARγ, the specific PPARγ ligand, rosiglitazone was incubated with dTHP-1 cells and LXRα gene expression determined. Rosiglitazone (1μM) upregulated LXRα mRNA expression by more than 2 fold at all time points used (Figure 4.14), a similar level to that observed with cells treated with oxLDL (Figure 4.11). This confirms the findings of Hirakata et al., (2004) who reported a significant upregulation of LXRα with the synthetic PPARγ ligands rosiglitazone and pioglitazone, suggesting that there is a potential PPARγ dependent pathway involved in LXRα mRNA upregulation. Figure 4.14 also demonstrates that stimulation of dTHP-1 cells with the PPARγ antagonist GW9662, has no significant effect on LXRα expression, which would suggest good specificity and minimal viability effects associated with this synthetic inhibitor.

The main question that arises from the oxLDL induced upregulation of LXRα is whether PPARγ is directly involved and/or whether a PPARγ independent pathway involving LXRα ligands from oxLDL is responsible for LXRα upregulation. Results shown in figure 4.16 demonstrate that after 24 hours cells that had been pre-incubated with the PPARγ antagonist GW9662 and subsequently stimulated with 1μg/mL oxLDL had significant upregulation in LXRα gene expression. This data is suggestive of a PPARγ independent pathway involved in oxLDL upregulation of LXRα, possibly via increased LXRα ligand binding with metabolites of oxLDL, including certain oxysterols. Laffitte et al., (2001), Whitney et al., (2001) and Li et al., (2002) have all previously reported that LXRα contains numerous LXREs and that specific LXRα ligands are responsible the autoregulation of LXRα gene expression. Therefore metabolites within oxLDL may activate LXRα and also
increase LXRα transcriptional activity. However after 48 and 72 hours, LXRα mRNA expression in cells pre-treated with 1μM GW9662 and then subsequently stimulated with 1μg/mL oxLDL was not significantly different to untreated cells only. Supporting the findings by Chawla et al. (2001b), that PPARγ ligands in oxLDL, upregulate LXRα in a PPARγ dependent mechanism. Chawla et al. (2001b), are of the opinion that the ability to regulate macrophage LXRα expression appears to be specific to PPARγ, because treatment of PPARγ knockout mice with PPARγ ligands did not significantly alter LXRα gene expression. This data suggests that the LXRα gene is a direct target for the PPARγ/RXR heterodimer. The reasoning for the switch in PPARγ mechanistic pathways (i.e. PPARγ dependent and independent) effecting LXRα gene expression remains unclear and requires further elucidation. However, this switch could be due to a deficiency in oxLDL derived oxysterols present in the cell after 24 hours and therefore LXRα gene expression reverts back to baseline (Lehmann et al., 1997 and Wong et al., 2006). Evidence to support this view is presented in figure 4.16, showing that LXRα gene expression in cells pre-treated with 1μM GW9662 then 1μg/mL oxLDL decreases progressively in a time dependent manner, suggestive of reductions in availability of oxysterols from oxLDL.

It has been recently reported that oxLDL and statin induced activation of PPARγ involves COX-2 upregulation (Yano et al., 2007 and Taketa et al., 2008). However, the precise mechanisms involved in oxLDL induced activation of COX-2 and hence PPARγ, remains poorly understood and requires further elucidation. During the present study, dTHP-1 cells were incubated with oxLDL, 1 and 40μg/mL, respectively and COX-2 and CD36 mRNA expressions were quantified over a
number of time points ranging from 2 to 72 hours. As seen in figure 4.17a and 4.17b there was a significant increase in COX-2 mRNA with 1 and 40μg/mL oxLDL at 2, 6 and 10 hours, peaking after 10 hours. This is in agreement with the studies by Norata et al., (2004) and Taketa et al., (2008) who both reported upregulation in COX-2 mRNA after 6 (in human endothelial cells) and 8 hours (in murine macrophages), respectively. However, neither study further investigated oxLDL induced COX-2 mRNA expression in the long term (i.e. longer than 8 hours). Both figures 4.17a and 4.17b show that COX-2 mRNA was not transcriptionally affected by 1 or 40 μg/mL oxLDL after 24, 48 and 72 hours. COX-2 is a member of a family of rapidly inducible response genes and therefore substantial fluctuations in COX-2 mRNA expression over time are to be expected (Inoue et al., 2000). However, using the evidence from Yano et al., (2007) and Taketa et al., (2008) that PPARγ activation is dependent upon COX-2 upregulation, one would expect CD36 mRNA to follow a similar pattern to that of COX-2. This is the case over shorter incubation periods, as after incubation with both 1 and 40 μg/mL oxLDL, CD36 mRNA is upregulated after 2, 6 and 10 hours (Figures 4.17a and 4.17b). A different picture is apparent at 24, 48 and 72 hours, in that unlike COX-2 mRNA which is decreased to baseline level or below, CD36 remains significantly upregulated. Suggesting that between 2 and 10 hours oxLDL upregulates CD36 in a COX-2 dependent manner, however after 10 and lasting as long as 72 hours, oxLDL upregulates CD36 using a COX-2 independent pathway. However, results from Eligini et al., (1999) demonstrated that COX-2 mRNA expression was only suppressed in monocyte/macrophages after stimulation for 24 hours with oxLDL (25 - 50μg/mL).
In order to determine whether the initial oxLDL induced upregulation of COX-2 (< 24 hours) influences CD36 mRNA expression after 24 hours, i.e. a maintained effect, the specific COX-2 inhibitor, NS-398 (50μM) was incubated with cells before incubation with oxLDL (1μg/mL). Figure 4.18 demonstrates that inhibition of COX-2, via the pharmacological inhibitor NS-398, fails to upregulate CD36 at all time points. Taketa et al., (2008) show a similar result after 24 hours only, as NS-398 and COX-2 siRNA significantly reduced PPARγ ligand-binding activity compared to cells treated with oxLDL only. The results from figure 4.18 suggest that the initial acute upregulation in COX-2 mRNA expression seen with 1μg/mL oxLDL is essential for the continued or maintained upregulation of CD36 gene expression, i.e. COX-2 dependent (Figure 4.22).

In order to investigate the potential of ERK1/2 phosphorylation in facilitating the oxLDL induced activation of PPARγ via COX-2, and hence upregulation of CD36, the effects of oxLDL on ERK1/2 phosphorylation over a period of 72 hours were firstly investigated. As shown in figures 4.19a and 4.19b both 1 and 40μg/mL significantly increased the ratio of phosphorylated to total ERK1/2 relative to untreated cells, at 8, 24, 48 and 72 hours. Inhibition of ERK1/2 phosphorylation by addition of the ERK1/2 inhibitor PD98059, demonstrated that the half life of PD98059 does not significantly impact on its ability to inhibit ERK1/2 phosphorylation over a time period of 72 hours. Senokuchi et al., (2004) has previously described the importance of an oxLDL-ERK1/2 pathway involved in macrophage proliferation. This is later reinforced in the literature by Namgaladze et al., (2008) who report that active ERK signalling is critical for suppression of apoptosis by oxLDL in THP-1 cells, thus providing a mechanistic explanation for the
anti-apoptotic actions of oxidised lipoproteins. Taketa et al., (2008) have previously demonstrated that in murine macrophages short term incubations of 40μg/mL oxLDL induces phosphorylation of ERK1/2 and p38 MAPK.

Prolonged exposure (> 24 hours) of dTHP-1 cells to both 1 and 40μg/mL oxLDL results in the continued upregulation of CD36 that is COX-2 dependent, but may also be ERK1/2 dependent. Hence, it was decided to investigate whether CD36 upregulation after stimulation with oxLDL is dependent upon ERK1/2 phosphorylation over a period of time ranging from 2 to 72 hours. As shown in figure 4.21, CD36 gene expression was significantly increased after 8, 24, 48 and 72 hours of stimulation with 1μg/mL oxLDL in dTHP-1 cells. When cells were pre-treated for 1 hour with the specific ERK1/2 inhibitor PD98059 (50μM) and then subsequently incubated with 1μg/mL oxLDL, there was a significant reduction in CD36 gene expression compared to oxLDL treated cells only after 8 hours. This supports the findings by Taketa et al., (2008) who reported that oxLDL-induced activation of PPARγ was inhibited by incubation with the ERK1/2 inhibitor PD98059 after 6 hours, in murine macrophages. Suggesting that the oxLDL-induced activation of PPARγ is mediated by an ERK1/2 and COX-2 dependant pathway. However, unlike Taketa et al., (2008) the present study extended the time period of the experiment and after incubation of dTHP-1 cells with firstly PD98059 (50μM) and then oxLDL (1μg/mL) there was no significant difference in CD36 gene expression after 24, 48 and 72 hours between cells treated with oxLDL alone and those pre-treated with PD98059. Indicating that from 24 hours onwards the upregulation in CD36 mRNA mediated by 1μg/mL oxLDL is an ERK1/2 independent but still a COX-2 dependent mechanism. Thus, using PD98059 inhibits
ERK1/2 activity (upstream of COX-2) and hence potential COX-2 mRNA synthesis, but cellular basal levels of COX-2 maybe responsible for the increase in CD36 expression seen in the presence of the ERK1/2 inhibitor, i.e. a partial effect. Alternatively, oxLDL after 24 hours upregulates COX-2 via an ERK1/2 independent pathway, possibly via another kinase signalling pathway. However it remains to be elucidated whether this CD36 upregulation is via an alternative COX-2 pathway, to which further molecular mechanistic work is warranted.

In summary the oxLDL induced activation of PPARγ, as measured by CD36 mRNA appears to be controlled via 2 separate mechanistic pathways over time (Figure 4.22). Firstly, between 2 and 24 hours the increase in CD36 mRNA expression is accompanied by increased ERK1/2 activity, increased COX-2 mRNA and subsequently increased PPARγ activity, i.e. ERK1/2 and COX-2 dependent. However after 24 hours, oxLDL upregulates CD36 gene expression in an ERK1/2 independent, but COX-2 dependent manner as evidenced by the prolonged upregulation in CD36 gene expression in the presence of PD98059. This suggests that oxLDL increases CD36 via an alternative pathways, possibly via the activation of PPARγ by fatty acid metabolites released from oxLDL, upon cellular internalisation.

In terms of clinical significance, the ERK1/2 and COX-2 dependent pathways involved in CD36 upregulation appears from the evidence to be short term and rather temporary, which clinically may have minimal significance. However the more long term effects of oxLDL metabolites, i.e. greater 24 hours, on CD36 upregulation has considerably more clinical potential in terms of enhancing cholesterol removal from
the bloodstream via reverse cholesterol transport. The present study contains considerable evidence of this, which agrees with and substantially adds weight to Yano et al., (2007) and Taketa et al., (2008).

**Figure 4.22**: Proposed molecular model of the importance of COX-2 in the oxLDL induced upregulation of CD36. The black arrows indicate short term signalling pathways and the blue arrows represent long term signalling. PD98059 = ERK1/2 inhibitor and NS-398 = COX-2 inhibitor.
Chapter 5 - General discussion.
5.1. *In-vivo.*

This study investigated the effects of an eight week low intensity exercise programme on plasma lipids, lipoproteins and inflammatory mediators associated with cardiovascular disease. It also aimed to elucidate the molecular mechanisms associated with lipid metabolism in previously sedentary adults undergoing low intensity exercise.

Due to exercise having diverse physiological and biochemical effects, the mechanisms underlying these effects have been difficult to elucidate. For instance, the literature regarding the effects of exercise on lipids and lipoproteins lacks consistency (Woolf-May *et al.*, 1999 and Murphy *et al.*, 2002). In particular, the effects of chronic low intensity exercise on the molecular mechanisms responsible for its anti-atherogenic effects, have received minimal attention. However, this study generates substantial evidence in relation to the effects of low intensity exercise on plasma lipids and lipoproteins. In this study, total cholesterol, low density lipoprotein (LDL) and triglycerides were reduced after both four and eight weeks, which was coupled with a significant increase in high density lipoprotein (HDL).

Exercise is widely regarded as having important and diverse benefits in terms of reducing cardiovascular risk. However, the hypoxic conditions and increased metabolic activity associated with exercise can increase the level of oxidative stress in the body over a short period of time (Ross, 1999). This oxidative state is associated with increased production of cellular hydroperoxides, reactive oxygen species (ROS) and free radicals, and thus an increase in the susceptibility of LDL to undergo oxidation. Evidence exists that the oxidation of LDL promotes the
atherogenic process (Kunjathoor et al., 2002), since oxidized LDL (oxLDL) has been identified as a potent chemoattractant triggering monocyte migration into the subendothelial space (Wang et al., 2006). The low intensity exercise programme used here, significantly increased oxLDL, suggestive of increased oxidative stress.

The results presented in this in-vivo based study provide novel mechanistic evidence regarding the atheroprotective benefits on plasma lipid profiles that could arise through participation in low intensity exercise. It is apparent from previous research that modulations in lipids and lipoproteins are dependent upon several factors including, exercise intensity, baseline lipoprotein levels and changes in body weight (Wang and Chow, 2004). However, the cardioprotective lipid and lipoprotein results presented in chapter 2, demonstrate that these adaptations arise independently of modulations in body weight. In addition, as the individuals within the cohort had lipids within the expected normal range, this suggests that this exercise programme improves lipid profiles independently of baseline lipid levels.

The majority of the evidence regarding the inflammatory nature of exercise has focused around the acute more intense bouts of exercise rather than chronic lower intensity exercise (Starkie et al., 2001a and Keller et al., 2003). Nevertheless, it was still deemed imperative to quantify key markers of inflammation, in order to ensure that the lipid profile benefits seen with low intensity exercise were not offset by increased levels of proinflammatory mediators. The non significant changes in inflammatory cytokines, namely IL-6 and TNFα and the endothelial and platelet adhesion molecules (sE selectin and sP selectin) reported are strongly supportive of the non inflammatory nature of low intensity exercise.
The significant increase in oxLDL after engagement in low intensity exercise is suggestive of enhanced lipid clearance from within the plasma and has the potential to generate important ligands for the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ). Nagy et al., (1998) and Tontonoz et al., (1998) have shown that PPARγ can be activated by lipid metabolities within oxLDL, including 9 and 13-hydroxy-octadecadienoic acids. Thus, taken together, the increase in oxLDL, its scavenger receptor, CD36 and in PPARγ, during low intensity exercise, could be interpreted as being proatherogenic, in terms of facilitating the unregulated influx of modified lipids. Activation of the lipid scavenger receptor CD36 is detrimental in terms of increasing atherosclerotic complications (Wei et al., 2005 and Collot-Teixerira et al., 2007), predominantly through the promotion of foam cell formation. Foam cell induction is mediated by the continual cellular uptake of modified lipids and lipoproteins, via CD36 (Chawla et al., 2001b). However, in contrast the upregulation of CD36 can be interpreted as being anti-atherosclerotic, through the facilitated clearance from within the bloodstream, of lipids and lipoproteins, specifically oxLDL (Marleau et al., 2005).

This study generated new information regarding the effects of low intensity exercise in regulating plasma lipids, lipoproteins and lipid transport. Exercise not only increases the expression of the potentially proatherogenic scavenger receptor CD36, via increased activation of the transcription factor PPARγ, but appears to also facilitate the upregulation of a number of membrane bound receptors indicative of reverse cholesterol transport (RCT), namely ABCA1 and ABCG1 and the nuclear transcription factor LXRα. RCT is a multifactorial process essential for maintaining cholesterol homeostasis and facilitates the transport of excess cholesterol from
peripheral tissues to the liver for excretion (Cavelier et al., 2006). Furthermore, the role of exercise in influencing RCT has received little attention and therefore the precise mechanisms responsible for the upregulation in key genes involved in RCT during exercise are not fully understood. This study proposes a novel molecular mechanism for exercise induced modulation in plasma lipids via cellular clearance of cholesterol involving activation of the transcription factors PPARγ and LXRα.

The proposed molecular mechanism illustrated in figure 5.1 incorporates the in-vivo evidence from both week four and week eight of the exercise programme. The red arrows are indicative of the exercise induced modulations seen after four weeks of low intensity exercise, which could be perceived as being potentially detrimental through increased cellular cholesterol influx and subsequent foam cell formation. Whereas the blue arrows represent the longer term effects seen with engaging in eight weeks of low intensity exercise. Coupled with the influx of cholesterol seen after four weeks, this demonstrates a potential pathway for the efflux of cellular cholesterol through activation of PPARγ and LXRα that is facilitated through upregulation of the ABC-transporters, specifically ABCA1 and ABCG1. This further culminates in a reduction in plasma LDL levels and increased HDL levels.

It appears that low intensity exercise is specific in terms of activation of genes, in particular surface scavenger receptors. Evidenced by the fact that out of the two scavenger receptors quantified, namely CD36 and SR-A1, only CD36 was transcriptionally activated. Additionally, PPARγ was shown to be upregulated and activated, but only specific PPARγ regulated genes were subsequently upregulated.
(CD36 not LPL), again supporting the specificity of the molecular pathways involved during adherence to low intensity exercise.

It remains to be elucidated whether low intensity exercise activates LXRα and hence RCT via a PPARγ dependent or independent mechanism. PPARγ has a known PPRE within the promoter of the LXRα gene and has been shown to directly activate the transcription factor (Chawla et al., 2001b). Whereas oxLDL possess key LXRα ligands, namely specific oxysterols and it has been demonstrated that these ligands can independently of PPARγ activate LXRα (Lehmann et al., 1997 and Nicholson and Hajjer, 2004). Nevertheless, this study provides new evidence that eight weeks of low intensity increases the activity of two nuclear transcription factors, namely PPARγ and LXRα, thus proposing a molecular cellular mechanism responsible for increased cholesterol clearance.

The exercise programme used in this study was well regulated and validated. Unlike a large number of previous exercise based studies (Yamanouchi et al., 1995, Iwane et al., 2000 and Tully et al., 2007) who have used pedometers and/or step diaries to quantify walking distances, this study employed a rigorous treadmill based exercise intervention. A major limitation with pedometer based studies and walking based exercise interventions, are the validity issues associated with their use in quantifying walking. The use of pedometers to quantify physical activity depends largely on the integrity of the individuals undertaking the exercise training. As with all unsupervised exercise training, there is an association with an increased dropout rate and uncertainty in whether the exercise regime was adhered to. A recent study by Bjørgaas et al., (2008) further highlights the issues surrounding the control of
walking using pedometers. Unlike this study, Bjørgaas et al., (2008) experienced a substantial drop out rate and found that the regular use of pedometers did not augment the beneficial outcomes in a physical activity intervention study in type 2 diabetics.

Figure 5.1: Proposed model of oxLDL mediated action in monocytes during exercise. 9-HODE = 9-Hydroxy-10,12-octadecadienoic acid, 13- HODE =13-hydroxy-9,11-octadecadienoic acid, PPARγ = Peroxisome Proliferator-Activated Receptor, LXRα = Liver X Receptor, ABCA1 = ATP-binding cassette transporter A1, ABCG1 = ATP-binding cassette transporter G1, oxLDL = Oxidized Low-Density Lipoprotein and HDL = High-Density Lipoprotein.
5.2. *In-vitro*.

The *in-vitro* work conducted within chapter 4 aimed to elucidate the precise mechanisms responsible for oxLDL induced upregulation of PPARγ and LXRα regulated genes, as observed with low intensity exercise (Chapter 3).

oxLDL at both concentrations investigated upregulated CD36 in monocytic cells in a PPARγ dependent manner at all time points quantified. Incubation with oxLDL (1μg/mL) resulted in a significant increase in LXRα gene expression and increased expression of an LXRα dependent gene ABCA1. Subsequently this direct relationship between oxLDL and LXRα activation was confirmed to be PPARγ dependent after 48 and 72 hours, through the use of a specific PPARγ antagonist. However, in contrast the results prior to 24 hours suggest a PPARγ independent mechanism responsible for LXRα upregulation. It remains to be fully established whether this is through a direct interaction between LXRα and oxysterol ligands and therefore requires further investigation. However, there is evidence to suggest that ligand binding of LXRα with specific oxysterols, may lead to increased DNA binding between the LXRα-ligand complex and the LXRα promoter region, via an LXRE present within the LXRα gene (Laffitte *et al.*, (2001) and Li *et al.*, (2002). Furthermore, the effects of the PPARγ activator oxLDL on CD36 and LXRα expression were also shown not to be a “jackpot effect”, but PPARγ dependent through the incubation with another rosiglitazone, a PPARγ ligand.

It has been proposed that short term (< 24 hours) induction of PPARγ involves a COX-2 dependent pathway, facilitating the production of key PPARγ ligands,
namely 15-deoxy-Δ 12,14-prostaglandin J2 (15d-PGJ2) (Taketa et al., 2008). COX-2, a well regarded rapid response gene (Subbaramaiah et al., 2001) was transiently upregulated in monocytic cells after incubation oxLDL. However, in keeping with its description this upregulation in gene expression reverted back to baseline levels within 24 hours, and remained unaffected throughout. CD36, on the other hand was upregulated at all time points quantified, reinforcing the earlier CD36 results, suggesting two distinct pathways responsible for increased PPARγ activity and CD36 upregulation. The first of these two pathways culminates in increased PPARγ activity via an ERK1/2 and COX-2 dependent mechanism. On the other hand, after 24 hours there is prolonged upregulation in CD36, indicative of PPARγ activity that may not involve an ERK1/2 and COX-2 dependent mechanism. This continued CD36 mRNA upregulation may be due to specific ligand binding of PPARγ, possibly via oxLDL metabolites known to be PPARγ ligands that include the fatty acids oxidation products, such as 9 and 13-HODEs.

It could be questioned whether the initial COX-2 dependent upregulation in PPARγ is sufficient to sustain continued upregulation in CD36. However, the inhibition of COX-2 suppresses oxLDL induced upregulation in CD36 gene expression. If the two distinct pathways responsible for PPARγ activation were entirely independent, then one would expect CD36 gene expression to be upregulated even in the presence of the COX-2 inhibitor. Therefore it would appear that the initial activation of COX-2 is essential for prolonged upregulation of CD36 gene expression.

The culmination of in-vitro evidence presented here is indicative of a molecular mechanism associated with oxLDL uptake in monocytic cells (Figure 4.22). The
diagram illustrates that oxLDL upregulates CD36 expression, through the transcription factor PPARγ, via two mechanisms. The ‘short term’ molecular pathway, (represented by the black arrows), proposes that oxLDL induces the phosphorylation of ERK1/2, which in turn activates COX-2. The precise mechanism responsible for the upregulation of COX-2 mRNA remains unclear, however, the nuclear transcription factor NF-κB is a plausible candidate, given that COX-2 can be transcriptionally regulated by NF-κB, via a NF-κB response element within the COX-2 promoter (Inoue et al., 2000). COX-2 subsequently induces the formation via arachidonic acid of 15d-PGJ$_2$, an important ligand of PPARγ.

The second and designated ‘long term’ mechanism (represented in blue) involves the direct upregulation of PPARγ via ligands within oxLDL. Ligand binding of PPARγ enhances its own gene expression through a PPRE and subsequently initiates a negative feedback pathway downregulating COX-2, via a transrepressor action (Inoue et al., 2000 and Subbaramaiah et al., 2001). Activation of PPARγ enables direct DNA-binding with CD36, facilitating the oxLDL to enter the cell, via CD36, exacerbating the effect and promoting the cellular clearance of oxLDL. Thus there is a potential means of PPARγ upregulation even in the presence of COX-2 and ERK1/2 inhibitors, i.e. through direct ligand binding. However, between 24 and 72 hours the short term pathway is required to upregulate PPARγ via COX-2 and hence instigate the upregulation of CD36. It may be the case that over several weeks of low intensity exercise the rapid molecular pathway will be supplemented by ligands within oxLDL, which are taken up by CD36 and hence directly stimulate PPARγ gene transcription.
5.3. Limitations and further work.

The low intensity exercise programme used in this study is highly dependant upon the tissue type used to quantify genes and nuclear transcription factors involved in cellular cholesterol metabolism, i.e. influx and efflux. Nevertheless, monocytic leukocytes are regarded as being influential in the initiation and development of atherosclerosis (Ross, 1999), hence monocytes can be interrupted as being an active choice of tissue in relation to the progression of atherosclerosis and therefore cardiovascular risk. The importance of the endothelial surface, skeletal muscle, adipose tissue and liver in the atherosclerotic process should however not be underestimated. However, in many cases the determination of lipid metabolism in these tissues requires tissue biopsies, which was not feasible in this study.

The benefits of the low intensity exercise programme in the sedentary individuals investigated in this study were achieved within an eight week period. Clearly, it will be important to investigate if the benefits reported here are maintained over a prolonged period of time and whether the exercise programme in conjunction with other lifestyle changes could augment the cardioprotective effects reported here. It would also be important to investigate further, how this mode and intensity of exercise can be promoted and implemented in the workplace.

Quantification of surface expression of the various receptors including CD36 and the total protein levels of genes such as ABCA1 and PPARγ would have enhanced the data generated by this study. However, in the case of many of the genes reported here the increase in mRNA was several fold, which is highly suggestive of increased protein synthesis and expression. Plasma markers of oxidative stress e.g.
thiobarbituric acid reactive substances (TBARS) and malonyldialdehyde (MDA) and the genes associated with mitochondrial oxidative stress would have informed of the overall oxidative status and the degree of oxidative stress associated with low intensity exercise in these previously inactive individuals. Finally, PPARγ activation is considered preventive in developing type-2 diabetes (T2D) in a pre-diabetic population and has been targeted pharmacologically in the treatment of T2D. Therefore, future studies investigating the physiological and biochemical effects of low intensity exercise in pre-diabetics and in T2D would be extremely advantageous, both clinically and economically.
5.4. Conclusion.

In summary, this study has demonstrated that adherence to an eight-week low intensity exercise programme significantly improves lipid profiles, coinciding with an increase in plasma oxLDL, which has the potential to serve as a trigger for stimulating lipid influx via PPARγ and thus CD36. Simultaneously increased activity in the nuclear transcription factor LXRα provides a potential coupled cellular mechanism for enhanced cellular cholesterol efflux via the ATP-binding cassette proteins ABCA1 and ABCG1, hence exacerbated reverse cholesterol transport and culminating in atheroprotective modulations in lipid profiles.

The in-vitro evidence presented postulates a potential cellular molecular mechanism for the increased activation of PPARγ and LXRα via the oxLDL trigger observed with adherence to low intensity exercise. This mechanism is time specific, hence up to 24 hours of oxLDL stimulation, PPARγ activation is dependent upon ERK1/2 and COX-2 activity, however with prolonged oxLDL exposure (> 24 hours) PPARγ activity occurs via an ERK1/2 independent but COX-2 dependent mechanism.

Finally, low intensity exercise of the intensity, duration and mode employed here is readily achievable by the majority of the general population. Walking involves minimal costs and can be easily incorporated into daily lifestyle, without a great deal of endeavour and could even be integrated into the working day. The culmination of evidence presented in this thesis further highlights the clinical and therapeutic benefits of low intensity exercise, that are non-pharmacological and of low cost to the individual and to that of the health service.
Chapter 6 – References.
6.1. References.


bicycle exercise on plasma level of interleukin-1 alpha (IL-1 alpha), tumor necrosis factor alpha (TNF alpha), and interferon gamma (IFN gamma). *Anal Sci* **17**, 593-7.


• Starkie, R. L., Arkinstall, M. J., Koukoulas, I., Hawley, J. A. & Febbraio, M. A. (2001b). Carbohydrate ingestion attenuates the increase in plasma interleukin-6,


and duplications in the LDL-receptor gene and summary of all mutations found in patients attending the Hammersmith Hospital Lipid Clinic. *Atherosclerosis* **194**, 102-11.


6.2. Publications.


6.3. Oral Presentations.

- **Butcher, L.**, Thomas, A., Backx, K., Roberts, A., Webb, R., and Morris, K. 28/05/08 to 31/05/08. Walking, PPAR’s and their regulated genes. 55\textsuperscript{TH} Annual ACSM meeting 2008. Indianapolis, Indiana USA.

- **Butcher, L.**, Thomas, A, Backx, K and Morris, K. 05/05/06. The effects of light intensity exercise program on lipids and lipoprotein concentrations in sedentary healthy adults. Speaking of Science 2006. The Graduate Centre, Cardiff University.

- **Butcher, L.** 24/06/05. The effects of a light intensity exercise program on lipid and lipoprotein concentrations in sedentary, healthy adult subjects. The 2\textsuperscript{nd} All Wales Conference in Sport, Exercise and Medicine 2005. Cardiff University, UK.

6.4. Poster Presentations.


- **Butcher, L.** Thomas, A, Backx, K and Morris, K. 02/05/07. Walking, lipid metabolism and inflammation. Speaking of Science 2007. The Graduate Centre, Cardiff University.

- **Butcher, L.** Thomas, A, Backx, K and Morris, K. 06/07/06. Low intensity exercise significantly modulates serum lipids and PPAR-gamma gene expression in healthy sedentary adults. The 3\textsuperscript{rd} All Wales Symposium on Exercise Science, Sports Medicine Research and Sports Psychology 2006. Cardiff School of Sport, Cyncoed Campus, UWIC.
7. Appendices.


Appendix B: 1. Example of Informed Consent Form.
2. Example of General Health Questionnaire.
3. Example of Dietary Questionnaire.
4. Example of Blood Questionnaire.
5. Example of Step Diary.


University of Wales Institute, Cardiff
SCHOOL OF APPLIED SCIENCES

Memo
Ref: Approval Letter/Ethics

To: Butcher, Lee, PhD
Cc: Morris, Keith, (Supervisor)
From: SCHOOL ETHICS PANEL
Date: Friday, 27 January 2006
Subject: APPLICATION FOR ETHICAL APPROVAL

Project Title
The role of exercise in regulating inflammation through activation of Peroxisome Proliferator-Activated Receptors α and γ

Your project proposal, as shown above, was amongst those considered at the most recent meeting of the School Ethics Panel, and the outcome is as follows:

Your proposal was APPROVED subject to the conditions listed below – please read carefully.

Conditions of approval

That any changes in connection to the proposal as approved are referred to the Panel.

That any untoward incident which occurs in connection with this proposal should be reported back to the Panel without delay.

Your application is approved in principal, but you need to amend your Consent form to make it easier for participants to understand. Please resubmit your amended Consent Form.

[Signature]

Dr K Jones
Chair of School Ethics Panel

PLEASE RETAIN THIS LETTER FOR REFERENCE
Informed Consent Form.

Mr Lee Butcher, a research student, currently studying at University of Wales Institute, Cardiff (UWIC) has kindly requested my participation in his research project based at the institute named above. The research is centred on the role of exercise in regulating inflammation through activation of Peroxisome Proliferator-Activated Receptors.

Between thirty and forty individual participants will partake in the investigation, each carrying out the same exercise programme and testing measures.

My participation will include:

- Carrying out a low intensity exercise programme for eight weeks. The type of exercise to be undertaken is brisk walking on the treadmill in the science laboratory at UWIC. Each exercise session should last approximately 60 – 90 minutes.
- Having blood samples (via the venapuncture method) taken 24 hours before the commencement of the exercise regime, after four weeks and 24 hours after the exercise programme finishes. The subject’s blood samples will be taken by a qualified phlebotomist inside the sports laboratory at UWIC.
- Having BMI (Body Mass Index), % body fat and blood pressure measured prior to the three blood samples being obtained.
- Fasting before the collection of the blood samples.

I understand and acknowledge the potential risks and discomfort that may occur as a result of the study if I agree to partake. Shortness of breath will be the most common discomfort experienced by most of the subjects during the completion of the exercise programme. There may also be possible discomfort during the collection of the three blood samples. I recognise that my specific participation in the research project may help to highlight any benefits that exercise has on key cardiovascular risk markers and inflammation.

I have been advised that the participation is voluntary and understand that I may withdraw from the research project at any specific time, with no penalty being enforced upon me. I have been advised that I require appropriate running shoes and clothing. I appreciate that during the study and the possible publication of the results that I will be guaranteed complete anonymity. Instead of using individuals names Lee Butcher will use corresponding numbers and codes that will only be available to him and the individual in question.
I have been advised of all the possible side effects or discomforts that I may face and that the kind of activity doesn’t involve more than a minimal risk to me the participant. I understand that any questions I have relating to the research project on my personal participation, before or after my consent will be answered by Dr Keith Morris or Lee Butcher.

Supervisor: Dr Keith Morris  Research Student: Lee Butcher
Tel: 02920416826  Tel: 02920417145
E-mail: kmorris@uwic.ac.uk  Email: L.R.Butcher2@uwic.ac.uk

I have read and understood the above information. The purpose and demands of the research project have been satisfactorily explained to me. In signing this consent form, I am not waiving any legal claims or rights. An individual copy of this consent form will be given to me.

Name of subject (please print)________________________________________________________

Subjects signature____________________________________Date________________________

I declare that I have explained to the above individual what the purpose and the methods of the study involve and any potential risks and discomfort associated with this study. I have answered any questions or referred the subject to someone who can and I have witnessed the above signature. I have provided a copy of this form to the above individual.

Researchers signature_____________________________Date________________________
General health questionnaire.

Name: .................................................................
Age: ........................................................................
Sex: M □ F □
Marital status: ........................................................

Are you a smoker? Yes/No
If yes, how many do you smoke a day?
1 – 10 □
11 – 20 □
21 – 30 □
30+ □

Do you suffer from asthma? Yes/No

Do you suffer from or have suffered from epilepsy? Yes/No

How many times have you visited the doctor within the last 12 months?
0 □ 1 – 2 □ 3 – 4 □ 5 – 6 □ 7+ □

How would you assess your present state of health?
Good □ Reasonably good □ Average □ Rather poor □ Poor □

Have you suffered from any of the following in the last 2 years?
Cancer Yes/No
Diabetes Yes/No
Stroke Yes/No
Heart attack Yes/No
Mental illness Yes/No
High blood pressure Yes/No
A muscular-skeletal problem Yes/No
If you have answered yes to any of the above questions please give details below:
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Are you currently on any form of medication? E.g. Lipid altering drugs
Yes/No
If yes please give details below:
........................................................................................................
........................................................................................................
Do you have a disability?  Yes/No
If yes please give details below:

____________________________________________________________________
____________________________________________
____________________________________________

Do you exercise on a regular basic?  Yes/No
If yes, please give details below:

____________________________________________________________________
____________________________________________
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I understand that any personal information that I disclose will be in the strictest of confidentiality. The only person able to access this information during and indeed after the research study will be Lee Butcher.

I have read this form and I understand that if there is anything which is not clear I can ask for further explanations at any time. I have answered questions about my previous and current health status to the best of my knowledge and will disclose any undue feelings or concerns about my health during the course of the study to the researcher or exercise advisor.

Printed name __________________________ Signature ______________________ Date ______

Signature researcher________________________________________ Date ______
**General dietary questionnaire.**

These questions are based on your AVERAGE daily or weekly food intake, please complete as accurately as possible.

1. Do you eat breakfast?  Yes/No

2. How much water do you drink everyday?  
   - 0 – 0.5 Litres  □
   - 0.6 – 1 Litres  □
   - 1.1 – 1.5 Litres  □
   - 1.6 – 2 Litres  □
   - 2.1 – 2.5 Litres  □
   - 2.6 + Litres  □

3. Do you have any food allergies?  Yes/No  
   *If yes, please specify,*
   
   __________________________________________________
   __________________________________________________

4. How many portions of fruit and vegetables do you eat everyday?  
   - 0  □
   - 1  □
   - 2  □
   - 3  □
   - 4  □
   - 5+  □

5. How many cups of tea and coffee do you drink each day?  
   *Please complete,*  
   **TEA_____ cups  COFFEE_____ cups**

6. How many portions of fish do you eat per week?  
   - 0  □
   - 1  □
   - 2  □
   - 3  □
   - 4  □
   - 5+  □

7. How many portions of meat do you eat per week?  
   - 0  □
   - 1  □
   - 2  □
   - 3  □
   - 4  □
   - 5+  □

8. What type of milk do you use?  
   - Don’t use milk  □
   - Skimmed  □
   - Semi-skimmed  □
   - Whole milk  □
   - Soya  □
   - Other  □ **please state________**
9. How much alcohol do you consume per wk?  
0–2 (Pints, shots or equivalent) □  
3–5 (Pints, shots or equivalent) □  
6–8 (Pints, shots or equivalent) □  
9–10 (Pints, shots or equivalent) □  
11+ (Pints, shots or equivalent) □

10. Do you take any vitamin and/or mineral supplements?  Yes/No
   If yes, which type/s and how often:
   _______________________________________________________________________
   _______________________________________________________________________

I understand that any personal information that I disclose will be in the strictest of confidentiality. The only person able to access this information during and indeed after the research study will be Lee Butcher.

I have read this form and I understand that if there is anything that is not clear I can ask for further explanations at any time. I have answered questions about my previous and current health status to the best of my knowledge and will disclose any undue feelings or concerns about my health during the course of the study to the researcher or exercise advisor.

Printed name ___________________Signature___________________ Date__________

Signature researcher_____________________________________________ Date__________
THIS QUESTIONNAIRE IS CONFIDENTIAL

QUESTIONNAIRE FOR POTENTIAL PARTICIPANTS IN PROJECTS INVOLVING BLOOD DONATION

Information given will be treated with strictest confidentiality
All questions to be answered by placing a tick in the appropriate box

1. Are you receiving any medicines? YES  NO
2. Have you had any recent illnesses or are you attending hospital outpatients? YES  NO
3. Have you had your ears pierced, been tattooed or received acupuncture in the last six months? YES  NO
4. Have you ever been advised by a doctor not to give blood? YES  NO
5. Are you or have you ever suffered from any of the following?
   Anaemia or other blood disorders YES  NO  NOT KNOWN
   Brucellosis YES  NO  NOT KNOWN
   Diabetes YES  NO  NOT KNOWN
   Epilepsy (fits) YES  NO  NOT KNOWN
   Glandular fever (in last 2 years) YES  NO  NOT KNOWN
   Hepatitis (jaundice) or been in contact with a case in the last six months YES  NO  NOT KNOWN
   Tropical disease, especially malaria YES  NO  NOT KNOWN
   Venereal disease YES  NO  NOT KNOWN
6. Is your lifestyle likely to place you at an increased risk of HIV infection (AIDS)? YES  NO
7. Please advise the phlebotomist if you have travelled outside Europe within the last six months and/or received travel vaccinations

DECLARATION

I have had explained to me, and fully understand, the reasons for donating blood.
I have not answered ‘yes’ to any of the questions listed and to the best of my knowledge am fully eligible to donate blood and do so of my own free will.

Signature of potential donor: ____________________________ Dated: ____________________________
Signature of Phlebotomist: ____________________________ Dated: ____________________________
Step Diary.

NAME:
TARGET – 10,000 STEPS PER DAY FOR 3 DAYS EVERY WEEK

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cholesterol/TG, and FFA. Hybridization of cDNAs derived from these samples allowed identification of genes that were up or down-regulated in high fat diet. C57-High fat diet sample compared with the B6 sample represented that changes of total 2434 gene expression (more than 2 folds). 1524 genes represented overexpression more than 2 fold. In contrast, 1719 genes, such as genes involved in immune response (Stat3, C1qα) represented downexpression. We also analyzed the expression profiles of genes related to ER-stress (EDEM, CHOP), lipogenesis (FAS, ACC), lipoprotein metabolism (apoB), transcription factors regulating glucose and lipid metabolism (Xbp1, PPAR) showing characteristic changes of metabolism between DIO-prone and resistant mice.

Conclusions: Lipid metabolism, inflammation, and ER-stress play roles in developing fatty liver under high fat diet, and the mechanism of DIO-resistance could be accessed with our microarray data.

RESISTANCE TO OBESEITY DEVELOPMENT IN THE LOUC RAT: A QUESTION OF IMPROVED CENTRAL LEPTIN SENSITIVITY?
C. Veyrat-Durebe1, X. Montet1, F. Rohner-Jeanrenaud1
1University of Geneva, Internal Medicine, Geneva, Switzerland
2University of Geneva, Radiology, Geneva, Switzerland

Geneic predispositions have been reported to modulate the susceptibility of individuals to develop obesity. The inbred Lou/C rat, originating from the Wistar strain, has been described as a model of resistance to obesity even if selecting a high proportion of fat (≥70%) when submitted to a self-selection diet. These two strains therefore represent valuable tools in the study of obesity-resistant and obesity-prone animal models, respectively. The aim of this work was to characterize and compare lipid metabolism, as well as leptin sensitivity, in mature Lou/C and Wistar rats under standard or high-fat (HF) diet.

Under standard diet, Lou/C rats had a lighter body weight and a lower food intake than Wistar rats, but the daily food efficiency was identical between the two strains. Under HF diet, the food efficiency was greatly increased in Wistar rats. Analysis of body composition confirmed lower fat deposits in Lou/C compared to Wistar rats, and the development of obesity in Wistar rats only under HF diet. In inguinal adipose tissue, expression of uncoupling protein 1 was detected in both standard and HF diet-fed Lou/C rats: Sensitivity to leptin, explored by acute peripheral and central injections, was significantly more marked in Lou/C rats.

In conclusion, the resistance to the development of obesity observed in Lou/C rats with age and in response to a high fat diet seems to involve optimal regulations of both food intake and metabolic homeostasis through preferential channeling of lipid nutrients toward utilization rather than storage, linked to improved central leptin sensitivity.

REGULATION OF SERUM AMYLOID A3 (SAA3) IN MOUSE COLONIC EPITHELIUM AND ADIPOSE TISSUE BY THE INTESTINAL MICROBIOTA
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Serum amyloid A (SAA) proteins are proposed mediators of inflammation in obesity exhibiting increased levels in the serum of insulin resistant mice. Using germ-free (GF), conventionally raised (CONV-R) and genetically modified mice, we found that SAA3 expression in adipose and colonic tissues was significantly higher in CONV-R and wild type mice than those that were either GF or lacking Myd88, a gene encoding an adaptor protein required for most Toll-like receptor (TLR) signaling and sensitivity to bacterial lipopolysaccharides (LPS). Electrophoretic mobility shift assays revealed reduced colonic activation of nuclear factor-kappa B (NF-kappaB) in GF mice than in CONV-R mice. Immunohistochemical detection of SAA3 in colonic epithelial cells and intracellular macrophages prompted investigation of SAA3 in macrophages (RAW 264.7) and colonic epithelial (CMT-93) cell lines. SAA3 was significantly induced by LPS in both macrophages and colonic epithelial cells, but to a greater extent in CMT-93 epithelial cells. SAA3 was also significantly induced by both recombinant TNF-alpha and IL-1beta in CMT-93 cells. Both cell lines exhibited markedly increased expression of TNF-alpha in response to LPS treatments. Our results suggest that SAA3 is augmented in mouse adipose and colonic tissue by intestinal microbes through the TLR/NF-kappaB signaling axis and that colonic surface epithelial expression of SAA3 may be part of an NF-kappaB-dependent response to gut-derived bacterial LPS. Thus, intestinal activation of SAA isoforms and other cytokines in response to microbial stimuli may have unappreciated effects related to chronic inflammation and the metabolic syndrome.

PHYSICAL ACTIVITY IMPROVES METABOLIC HEALTH BUT NOT BODY COMPOSITION: A LONGITUDINAL STUDY IN PRE-PUBERTAL CHILDREN
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Aims: To determine the extent to which physical activity is associated with changes in body composition and metabolic health in pre-pubertal children.

Methods: Non-intervention longitudinal study of 212 children (1995/96 birth cohort) recruited at school entry from 53 primary schools in Plymouth, UK. Physical activity (7-day Actigraph accelerometer), body composition (BMI, sum of five skinfolds, waist circumference) and metabolic risk (insulin resistance by HOMA-IR, triglycerides, cholesterol/HDL ratio and blood pressure - separately and combined as a composite metabolic risk z-score) were all measured on four consecutive annual occasions (5, 6, 7 and 8y).

Results: There were no associations between total physical activity, averaged over the four annual measures, and changes in body composition over the same period from 5 to 8y (BMI r=−0.04 p=0.57, skinfolds r=−0.03 p=0.70, waist circumference r=−0.06 p=0.40). However, there was a small-to-moderate inverse association between physical activity and change in metabolic risk (combined: composite z-score r=-0.19 p<0.01, separately: insulin resistance r=-0.08 p=0.25, triglycerides r=-0.16 p=0.03, cholesterol/HDL ratio r=-0.12 p=0.09, blood pressure r=-0.15 p=0.04) independent changes in body mass/fat. Mixed effects modeling showed that the improvement in composite metabolic z-score among the more active, compared to the less active children, progressed year-by-year in a linear fashion (<0.08 z-score/year, p=0.001).

Conclusions: In young children, physical activity appears to improve metabolic health even in the absence of any improvement in body mass/fat. BMI whilst popular, may not be the best outcome measure with which to judge the success, or otherwise, of any physical activity intervention/programme.

LOW-INTENSITY EXERCISE REGULATES PPARG ACTIVITY: A MOLECULAR RATIONAL FOR DIABETES PREVENTION?
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Aims: Numerous intervention trials have demonstrated that exercise can be beneficial in type 2 diabetes. However, the precise biochemical and

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molecular events that bring about these exercise-mediated effects are not fully understood. This study investigated if low-intensity exercise could mediate its effects through activation of PPARγ.

Methods: Thirty sedentary adults, mean age 45.6±11.1 years were recruited into an exercise programme (10,000 steps 3 times per week for 8 weeks).

Fasting blood was collected at 0, 4 and 8 weeks and plasma lipids, lipoproteins and gene expression (RT-PCR) was determined.

Results: The low-intensity exercise programme increased PPARγ expression (4 weeks: 1.8±0.9 fold; 8 weeks: 4.3±1.9 fold) and up-regulated PPARγ dependent genes, CD36 (4 weeks: 3.8±0.6 fold; 8 weeks: 2.7±0.5 fold), and LXRα (8 weeks: 3.5±0.8 fold). The LXR Response Element (LXRE)-bearing target genes, ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) were also elevated (ABCA1: 3.4±0.5 fold; ABCG1: 3.0±0.7 fold). ABCA1 and ABCG1 mediate reverse cholesterol transport (RCT), ensuring lipid clearance via HDL-cholesterol. In concordance with this, serum HDL-cholesterol increased (pre-exercise: 1.4±0.47 mmol/L vs post-exercise: 1.5±0.50 mmol/L) and serum oxLDL concentration increased (0 weeks: 554±107ng/ml vs 4 weeks: 698±134ng/ml, P<0.05).

Conclusion: Low-intensity exercise activated PPARγ and genes involved in RCT and transiently increased serum oxLDL (a natural activator of PPARγ). Recent studies have demonstrated that treatment with synthetic PPARγ ligands can prevent the development of diabetes. This study suggests that the ability of low-intensity exercise to activate PPARγ may provide a molecular rationale for the prevention of diabetes.

MEASURED PHYSICAL ACTIVITY BEHAVIOUR AND THE METABOLIC SYNDROME

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Aim: To assess the relationship between inactivity and risk for metabolic syndrome.

Methods: 107 healthy postal workers were recruited to wear the activPAL activity monitor for one week. Participants were non-smokers. Family history of cardiovascular disease was obtained. Socioeconomic status (SES) were obtained using the Scottish Index of Multiple Deprivation. Anthropometric and blood pressure measurements were undertaken according to WHO protocol. Fasting blood lipids and glucose were measured. Cardiorespiratory fitness was assessed using the 3-minute step test (Golding et al 1999). Participants were grouped according to achievement of current physical activity recommendations (Hasdell et al 2007), and according to the presence of metabolic syndrome as defined by ATP III. Comparative data analyses were undertaken using SPSS version 15.

Results: One achieve of physical activity recommendation compared to 11 non-achievers (χ2 = 0.001) had metabolic syndrome (RR = 1.2 (95%CI 1.1 to 1.4). Participants with metabolic syndrome were significantly less active than those without metabolic syndrome: time spent in sedentary posture (p = 0.036), time spent walking (p = 0.003), step count (p = 0.003), stepping rate (p = 0.02) and daily energy expenditure (p = 0.003). There was no significant difference between the groups in time spent standing (p = 0.195) and cardiorespiratory fitness (p = 0.34). Prevalence of positive family of cardiovascular disease was similar (χ2 = 0.754). Those with metabolic syndrome were of higher SES (p = 0.014). The number of metabolic syndrome components were directly related to the physical activity parameters (p for trend <0.05).

Conclusions: Inactivity may accurately predict the risk for metabolic syndrome.

DIET-INDUCED POST-NATAL PROGRAMMING IN RATS REGULATES SYSTEMIC AND ADIPOSE TISSUE ANGIOGENESIS SYSTEM IN ADULTHOOD

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Early life nutritional environment plays an important role in the appearance of cardiovascular diseases in adulthood, but little is known about the role of adipose tissue (AT). AT synthesizes all components of the angiogenesis system (RAS), including the insulin receptor (PI3K). We studied, using an experimental model of postnatal programming of insulin resistance obtained by postnatal feeding with continuous high fat feeding, the status of systemic and AT RAS in adult rats.

Plasma angiogenesis concentrations did not show significant variations between groups whereas plasma insulin resistance was increased by post-natal programming, but not by high fat feeding, and was found to be positively correlated to plasma insulin-to-glucose ratio. In mesenteric AT (MAT), angiogenesis and angiogenesis converting enzyme mRNAs were decreased in programmed animals fed high fat diet while angiogenesis receptor 1 (PI3K) mRNA levels were not affected. A strong (PI3K) immunoreactivity was observed in stromal cells whereas adipocytes were not labelled. (PI3K) expressing cells density was comparable between epididymal (EAT) and MAT whatever the treatment considered. In both EAT and MAT, (PI3K) expression was not changed by post-natal programming or high-fat diet alone and significantly increased in programmed rats fed high-fat diet.

In conclusion our experiments demonstrate that (PI3K) is expressed in rat AT, its levels being increased in postnatally programmed rats fed high-fat diet.
Low-Intensity Exercise Exerts Beneficial Effects on Plasma Lipids via PPARγ

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ABSTRACT

BUTCHER, L. R., A. THOMAS, K. BACKX, A. ROBERTS, R. WEBB, and K. MORRIS. Low-Intensity Exercise Exerts Beneficial Effects on Plasma Lipids via PPARγ. Med. Sci. Sports Exerc., Vol. 40, No. 7, pp. 1263–1270, 2008. Introduction: An important mechanism by which physical activity reduces the risk of cardiovascular disease is through regulating plasma lipids. We investigated whether low-intensity exercise modulates lipid metabolism and the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor α (LXRα) responsible for controlling reverse cholesterol transport (RCT). Methods: Thirty-four sedentary adults, mean age 45.6 ± 11.1 yr, participated in an 8-wk low-intensity exercise program consisting of walking 10,000 steps, three times a week. Subjects were randomly allocated to either an exercise group or a sedentary control group, and serum lipid or lipoprotein concentrations were determined. Results: Compared with controls, there was a significant decrease in total cholesterol (preexercise, 5.73 ± 1.39 mmolL–1; postexercise, 5.32 ± 1.28 mmolL–1) and a significant increase in HDL (preexercise, 1.46 ± 0.47 mmolL–1; postexercise, 1.56 ± 0.50 mmolL–1) after the exercise program. There was a significant increase in serum oxidized LDL (oxLDL) concentrations in the exercise group before and after exercise (0 wk, 554 ± 107 ngmL–1; 4 wk, 698 ± 134 ngmL–1; 8 wk, 588 ± 145 ngmL–1). A significant increase in leukocyte mRNA expression for PPARγ (4 wk, 1.8 ± 0.9-fold; 8 wk, 4.3 ± 1.9-fold) was observed, which was reinforced by increased PPARγ DNA-binding activity postexercise (preexercise, 0.22 ± 0.09 OD units; postexercise, 1.13 ± 0.29 OD units). A significant increase in gene expression was observed for the oxLDL scavenger receptor CD36 (4 wk, 3.8 ± 0.6-fold; 8 wk, 2.7 ± 0.5-fold) and LXRα (8 wk, 3.5 ± 0.8-fold). Two LXRα-regulated genes involved in RCT, namely, ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1, respectively), were significantly up-regulated postexercise (8 wk: ABCA1, 3.46 ± 0.56-fold; ABCG1, 3.06 ± 0.47-fold). Conclusion: We propose that the net effect of these changes may be to increase oxLDL uptake, to stimulate RCT, and thus to promote clearance of proatherogenic lipids from the vasculature, ultimately contributing to the cardiovascular benefits of low-intensity aerobic exercise. Key Words: WALKING, TRANSCRIPTION FACTORS, REVERSE CHOLESTEROL TRANSPORT, AEROBIC EXERCISE

Exerc...
of which leads to increased uptake of oxLDL (4,24,30). Oxidation renders LDL unrecognizable to the LDL receptor and encourages its uptake by scavenger receptors on macrophages in an unregulated manner (13); macrophage scavenger receptors such as CD36 are therefore thought to play a significant role in atherosclerotic foam cell development due to their ability to bind and internalize oxLDL (7). CD36 is expressed in many cell types relevant to the progression of atherosclerosis, including monocytes or macrophages, microvascular endothelial cells, adipocytes, skeletal muscle cells, dendritic cells, and epithelial cells, and its expression is known to be increased in cells within atherosclerotic lesions (7,15). However, there is evidence for antatherosclerotic benefits of up-regulation of macrophage CD36 mRNA expression because stimulation of macrophages with CD36 ligands has been shown to reduce plasma cholesterol levels (18).

The ATP-binding cassette transporter A1 (ABCA1) is a member of the ATP-binding cassette transporter protein superfamily that plays a key role in reverse cholesterol transport (RCT), in which oxLDL taken in by macrophages is exported from the potential atherosclerotic site as a component of lipid-poor apolipoproteins (36). Similarly, ATP-binding cassette transporter G1 (ABCG1) was recently identified as being a mediator of macrophage cholesterol efflux, in this case to mature HDL but not to lipid-depleted apolipoproteins (32). Thus, ABCA1 and ABCG1 have been proposed to function synergistically to promote cellular cholesterol efflux (36). Both ABCA1 and ABCG1 genes are LXRE-bearing target genes activated by the nuclear receptor LXRα (6).

Walking is a form of aerobic low-intensity exercise that has been shown to have numerous health benefits (21). Previously sedentary individuals have demonstrated a significant reduction in systolic blood pressure and percentage body fat after undertaking two 45-min walks per week for 8 wk (23). Another study provided evidence that walking either 20–40 or 10–15 min·d⁻¹ for 18 wk significantly reduced mean LDL concentrations (37). In another recent study, a single bout of brisk walking decreased basal VLDL triglyceride concentration in males, an effect that was associated with increased plasma clearance of the lipoprotein (38). However, some studies investigating the effects of aerobic exercise on lipids have produced conflicting findings. For instance, several groups have shown that no significant changes in lipoprotein concentrations occur after 6 and 12 wk of walking, respectively (3,31). These inconsistencies may in part be explained by the initial baseline lipid level, the variety of training stimulus, and the lack of dietary analysis.

Because exercise has the potential to generate oxidized lipids (33) and because oxLDL and its metabolites are known to be substrates for CD36 and ligands for PPARγ (1,20), we hypothesized that low-intensity exercise has the potential to stimulate RCT and thus promote clearance of proatherogenic lipids, such as cholesterol from the vascu-
completed by participants over a 3-d period before and immediately after commencing the exercise program. All diet data were analyzed using the NetWisps 3.0 (Timucul Software Systems, Cheshire, UK) computer software to determine the relative composition of the participant’s diet before and after the exercise program.

**Blood fractionation for the collection of leukocytes.** Fasting blood was sampled at baseline, after 4 and 8 wk of exercise. Blood samples were collected 24 h before the commencement of the exercise study or 24 h after an exercise session. At each time-point, blood samples were collected by venepuncture from the antecubital vein with minimal tourniquet in EDTA vacutainer and serum separator tubes (Becton-Dickenson, Oxford, UK). Whole blood was fractionated by centrifuging at 1000g for 15 min at room temperature. A sterile plastic transfer pipette was used to aspirate off the plasma down to ~1 mm from the buffy layer and stored at ~80°C. The buffy layer containing leukocytes was carefully removed using a circular motion and was added to 1.2 mL of RNAlater (Applied Biosystems, Warrington, UK). Samples in RNAlater were mixed thoroughly using a vortex and stored at 4°C overnight then stored at ~80°C. Serum was obtained from blood collected into SST collection tubes (Becton-Dickenson), centrifuged at 1000g for 10 min at room temperature, and the resulting serum supernatant was aliquoted and stored at ~80°C.

**Analysis of serum samples.** A Kodak Ektachem DT60 II (Axiss-Shield, Cambridgeshire, UK) was used to determine values of triglycerides, total cholesterol, and HDL cholesterol in the serum samples. LDL cholesterol was calculated using the Friedewald equation. Samples were analyzed within 1 month of freezing. oxLDL was analyzed using a standard ELISA (Oxford Biosystems, Oxford, UK).

**Isolation of RNA.** The extraction of total RNA was carried out using Ambion RiboPure-Blood Kit (Applied Biosystems) according to the manufacturer’s instructions.

**Real-time PCR.** RNA samples were converted to cDNA using an Applied Biosystems high-capacity cDNA archive kit and stored at ~20°C. CD36, GAPDH, PPARγ, LRXα, ABCA1, and ABCG1 mRNA expressions were analyzed on an Applied Biosystems 7500 real-time PCR system using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Paisley, UK). All primers for the RT-PCR were designed using the Applied Biosystems Primer Express software V 2.0, and the following sequences were obtained: CD36: 5'-GGAGATGTGATGATGACAGACGCACG-3', 5'-GAGACTGTGTGTCTCACGCG-3'; GAPDH: 5'-CATTGACCTCACAACTAGT-3', 5'-TTCCTCATGTGTTGGT-3'; PPARγ: 5'-CTGGGCGCGCAGTGTGAA-3', 5'-CTCCTTACATCGGAGAAGATCC-3'; LRXα: 5'-CGACTACATCTGTGCCAAGT-3', 5'-TGAGGCGCGGATCTGTGTCCT-3'; ABCA1: 5'-GACCTGAGGGAGATGC-3', 5'-AGTTCCTGGAAAGTCTTCTTCA-3'; ABCG1: 5'-AGGCAAGTGGTCTGACCTT-3', 5'-CGGAGGTGCTCAAGACCTC-3' to give PCR products of 117, 209, 166, 141, 205, and 160 bp, respectively. Estimates of cDNA abundance were made using the portion of the curve for which the slope of the log input amount versus the C₅ (cycle threshold) differences resulted in a slope of approximately 0.0, indicating that the amplion efficiencies were approximately equal. Relative quantification of target genes in human leukocytes was calculated using the 2⁻ΔΔC₅ formula, in which ΔΔC₅ equals the difference between C₅ values for both target gene and GAPDH.

**PPARγ DNA-binding activity assay.** For isolation of peripheral blood mononuclear cells, 10 mL of blood was diluted 1:1 in RPMI, layered over 10 mL of Histopaque-1077 Ficoll–Hypaque and centrifuged at 400g for 20 min. The mononuclear cell suspension was carefully removed from the Ficoll–Hypaque interface, washed four times (300g; 10 min) in 0.4 mL of active motif phosphate inhibitor solution and 7.6 mL phosphate-buffered saline. PPARγ DNA-binding activity was determined using a Trans AMTM PPARγ assay kit according to the manufacturer’s instructions (Active Motif, Rixensart, Belgium).

**Bioinformatics analysis.** Sequence analyses and alignments were performed using DNASTAR™ software (Lasergene, version 7; DNASTAR Inc., Madison, WI).

**Statistical methods.** Data are expressed as mean ± SD. When analyzing multiple comparisons within groups, a one-way ANOVA was applied to normally distributed data and the Kruskal-Wallis test for data which was non-normally distributed. For nonmultiple comparisons between exercise and control groups, two-sample t-tests were used. Significance levels were set at P < 0.05. The sample size for this study was determined using data generated from a provisional study and using the change in PPARγ gene expression as the primary outcome. The power calculation assumed at least an 80% increase in PPARγ mRNA expression with an SD of 50%, a required power of 0.9 (90%), and using a two-sample t-test for comparisons. These figures suggested that a sample size of at least 11 in each group was

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**TABLE 1. Anthropometric characteristics of exercise and control groups at baseline and after 8 wk of exercise.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exercise Pre (n = 17)</th>
<th>Change after 8 Weeks</th>
<th>Controls Pre (n = 17)</th>
<th>Change after 8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>44.94 (10.01)</td>
<td>46.12 (12.2)</td>
<td>45.95 (10.05)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.32 (7.49)</td>
<td>165.32 (7.49)</td>
<td>165.32 (7.49)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.56 (18.13)</td>
<td>83.56 (18.13)</td>
<td>83.56 (18.13)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>BMI</td>
<td>26.78 (11.11)</td>
<td>26.78 (11.11)</td>
<td>26.78 (11.11)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>% body fat</td>
<td>33.78 (10.25)</td>
<td>33.78 (10.25)</td>
<td>33.78 (10.25)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>134.14 (18.13)</td>
<td>134.14 (18.13)</td>
<td>134.14 (18.13)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>89.43 (11.10)</td>
<td>89.43 (11.10)</td>
<td>89.43 (11.10)</td>
<td>0.88 (18.35)</td>
</tr>
<tr>
<td>VO₂peak (ml/kg/min)</td>
<td>35.49 (6.91)</td>
<td>35.49 (6.91)</td>
<td>35.49 (6.91)</td>
<td>0.88 (18.35)</td>
</tr>
</tbody>
</table>

Means (SD) are shown.
low-intensity physical activity with strict energy balance control. 


Walking, PPARs And Their Regulated Genes: 763:
May 29 2:45 PM - 3:00 PM
[D-14 Free Communication/Slide - Fat Metabolism 2:
May 29, 2008 1:00 PM - 3:00 PM: ROOM: 116]

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(No relationships reported)

An important mechanism by which physical activity reduces the risk of cardiovascular
disease is through regulating plasma lipids. The ligand-dependant nuclear transcription
factors Peroxisome Proliferator Activated Receptors α and γ (PPARα and γ) are important
lipid sensors and regulators of inflammation with certain PPAR ligands having
atheroprotective properties. In this study we investigated whether low-intensity exercise
modulates the gene expression of PPARγ and α and also two of their regulated genes
involved in lipid metabolism.

PURPOSE: To determine whether walking modulates gene expression of PPARγ and
PPARα and of the PPARγ regulated gene CD14 and the PPARα regulated gene, Acyl-CoA
oxidase.

METHODS: 34 sedentary adults, mean age 45.6±11.1 years, participated in an 8-week low-
intensity exercise programme consisting of walking 10,000 steps, 3 times/week. Subjects
were randomly allocated to either an exercise group or sedentary control group. After blood
collection, mRNA extracted from leukocytes and gene expressions estimated by Real-Time
PCR.

RESULTS: No changes in anthropometric data in the exercise or control participants were
observed after the exercise programme. There was also no change in the dietary intake in
either group pre-to post exercise. A significant increase in leukocyte mRNA expression for
PPARγ (4 weeks: 1.8±0.9 fold; 8 weeks: 4.3±1.9 fold) was observed. The PPARγ regulated
gene CD14, a lipid sensor, was significantly up-regulated post exercise (4 weeks: 1.8±0.2
fold; 8 weeks: 2.1±0.4 fold). A significant increase in gene expression was also observed for
PPARα (4 weeks: 1.7±0.4). However, the mRNA of the PPARα regulated gene Acyl-CoA
Oxidase, involved in fatty acid metabolism was not significantly altered after adherence to
the exercise program.

CONCLUSION: An 8-week low-intensity exercise programme significantly increases gene
expression of both PPARγ and PPARα. Increase in gene expression of these transcription
factors has been shown to be associated with their activity. However, the increase in gene
expression of these transcription factors is associated only with an increase in gene
expression of a PPARγ regulated gene. This study suggests that the beneficial effects of
low-intensity exercise are mediated mainly through PPARγ in leukocytes.