Small dense LDL in Healthy Adult and Diabetic Populations

A dissertation submitted in partial fulfilment of the requirement for the award of

M.Sc. in Biomedical Science

To

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Abbreviations

BMI	Body mass index
CHD	Coronary heart disease
CRP	C reactive protein
CVD	Cardiovascular disease
HDL	High density lipoprotein
LDL	Low density lipoprotein
ox-LDL	Oxidised low density lipoprotein
sdLDL	small dense low density lipoprotein
vLDL	Very low density lipoprotein

Access to Contents

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Section A:

Literature Review

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Literature Review

MSc Biomedical Science

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Contents of Literature Review

1. Introduction

Cardiovascular diseases (CVD) are disorders of the heart and blood vessels and represent the number one cause of death globally. The estimated economic burden of CVD on society in Europe is estimated to be €169 billion annually (Leal et al., 2006). CVD include coronary heart disease (CHD), cerebrovascular disease and congenital heart disease. Atherosclerosis is a chronic inflammatory disease and involves the build up of fatty deposits on the arterial wall. It is the main physiological precursor of CHD.

The association between lipids and CHD has been well documented. Two of the main forms of lipids are cholesterol and triglycerides. Plasma lipids are insoluble in water and are transported through the blood bound to lipoproteins. Lipoproteins are classified according to their size. Two of the smaller lipoproteins are high density lipoprotein (HDL) and low density lipoprotein (LDL), while the larger lipoproteins are chylomicrons, very low density lipoproteins (VLDL) and intermediate density lipoproteins (IDL). A fasting lipid profile is currently one of the most extensively used tools for predicting CHD (Mora et al. 2008). However, studies have shown that over half of all myocardial infarctions occur in individuals whose cholesterol levels are normal to moderately raised (Rifai et al. 2001). It has also been suggested that a nonfasting triglyceride may better predict CHD risk, as this may be associated with delayed clearance of chylomicron particles (Mora et al. 2008). HDL and LDL comprise of distinct subclasses which differ in many respects. Small dense LDL (sdLDL), a component of LDL, is emerging as an independent risk factor for the development of atherosclerosis. A common form of dyslipidemia termed "lipid triad" has been introduced to describe three lipid abnormalities: increased triglyceride levels, decreased HDL cholesterol and the presence of sdLDL particles (Rizzo et al. 2005). This lipid triad has been designated the "atherogenic lipoprotein phenotype" as it commonly occurs in individuals with cardiovascular disease.

The metabolic syndrome is a collection of risk factors including obesity, high blood pressure, increased cholesterol and insulin resistance. The American Heart Association and National Heart, Lung and Blood Institute have proposed that the risk factors associated with the metabolic syndrome directly promote atherosclerosis development (Rizzo et al. 2007). There is evidence to suggest that sdLDL is a

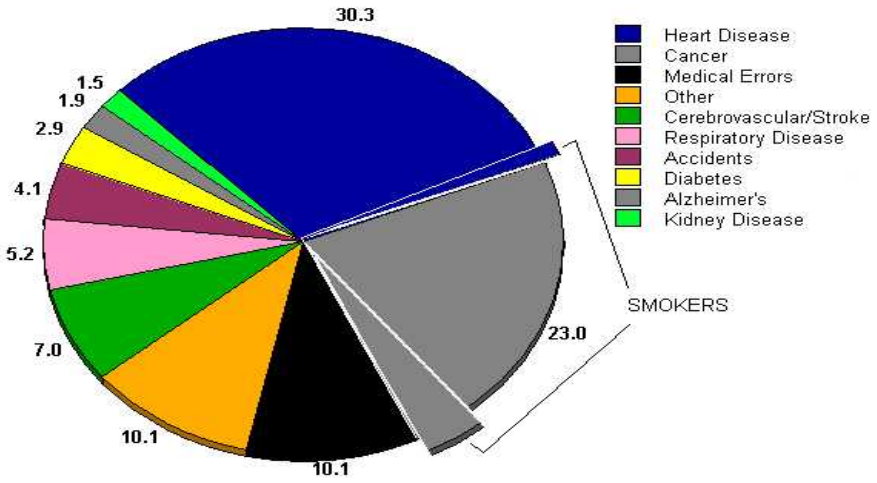
member of these metabolic risk factors and an increased fraction of sdLDL particles is characteristic of diabetes dyslipidemia (Berneis et al. 2005).

In 2008 Koba et al. found that a high concentration of sdLDL is a potent risk factor of CHD and is superior to LDL measurement. Consequently the inclusion of sdLDL analysis in routine CHD screening could serve to enhance risk prediction values. To date no study has identified a normal range for sdLDL among an apparently healthy population and diabetic population.

2. Coronary Heart Disease

CHD, a narrowing of the coronary arteries, is one of the leading causes of morbidity and mortality in both the developing and developed worlds with over 7 million deaths per year (see figure 1). 80% of these deaths occur in developing countries (Boutayeb & Boutayeb 2005). By 2020 it is predicted that CHD will be the main cause of death worldwide (Scott 2002). In younger life men have a higher incidence of CHD but as women get older their risk rises. Consequently, in later life men and women's risk of CHD is almost equal (www.nlm.nih.gov/medlineplus/print/ency/article/007115.htm). Studies involving animal models suggest that CHD develops as a result of the deposition of lipids in the vessel wall in response to inflammation due to injury or infection (Chiltion 2004).

Figure 1: Showing leading causes of death worldwide



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2.1 Atherosclerosis

Atherosclerosis, the build up of fatty deposits on the arterial wall is the main physiological precursor of CHD. These fatty deposits, atherosclerotic lesions, build up in medium and large sized arteries gradually resulting in a reduction or total block of blood flow leading to ischemia of the brain (cerebral arteries), extremities (peripheral arteries) and the heart (cardiac arteries). Atherosclerotic lesions can be found at any stage of an individual's lifetime.

CHD is a multifactorial disease with genetic and environmental factors playing important roles (Roheim et al., 1995). The initial event in the development of atherosclerosis appears to be repeated injury to the arterial wall through various mechanisms, leading to endothelial dysfunction. The mechanisms involved include infection, free radicals, toxins and physical stresses which can occur with high blood pressure and/or high blood lipid levels (Stoll et al., 2006).

The response-to-injury hypothesis is the most widely accepted theory for the development of atherosclerosis. Under normal circumstances circulating leucocytes adhere poorly to vascular endothelium. However, upon repeated injury the homeostasis of the endothelium is disrupted resulting in increased adhesiveness of leucocytes, particularly T cells and macrophages to the inner surface of the arterial wall. This is facilitated by the up-regulation of leucocyte adhesion molecules e.g. L-selectin and endothelial adhesion molecules e.g. intracellular adhesion molecule-1 (ICAM-1) and E-selectin. The disrupted endothelium shows increased permeability to lipoproteins. This increased permeability is mediated by prostacyclin, angiotensin II,endothelin, platelet derived growth factor (PDGF) and nitric oxide (NO) (Reiner Z & Tedeschi-Reiner E, 2001). The disrupted endothelium displays procoagulant properties and has the ability to form cytokines and growth factors.

Inside the arterial wall lipids, particularly LDL, are oxidised to oxidised LDL (ox-LDL). ox-LDL acts as a chemoattractant for monocytes and can inhibit macrophage mobility. ox-LDL also facilitates the accumulation of cholesterol esters (Chiton 2004). Macrophages are one of the main effector cells in atherosclerosis. They upregulate scavenger receptors and engulf large amounts of ox-LDL via these receptors thereby transforming into foam cells (Stoll 2006). These foam cells release growth factors which promote migration of smooth muscle cells. They also continue to accumulate lipids. It is the gradual accumulation of smooth muscle cells, foam

cells and T cells which form the fatty streak (see figure 2). Platelet adhesion and aggregation also occurs at this stage. The fatty streak is the hallmark of early-stage atherosclerotic lesions (Fan 2003). As the fatty streak develops it is covered by a fibrotic cap which contains extracellular matrix and smooth muscle cells (see figure 3). The lipid core of advanced atherosclerotic lesions are formed when foam cells undergo secondary necrosis. The fibrotic cap gives the plaque mechanical stability and separates the lipid rich thrombogenic core from the lumen and circulating blood ((Reiner Z & Tedeschi-Reiner E, 2001). When these plaques are exposed by erosion or rupture they trigger acute thrombotic events which are the main causes of stroke and myocardial infraction (Gleissner et al. 2007).

Figure 2

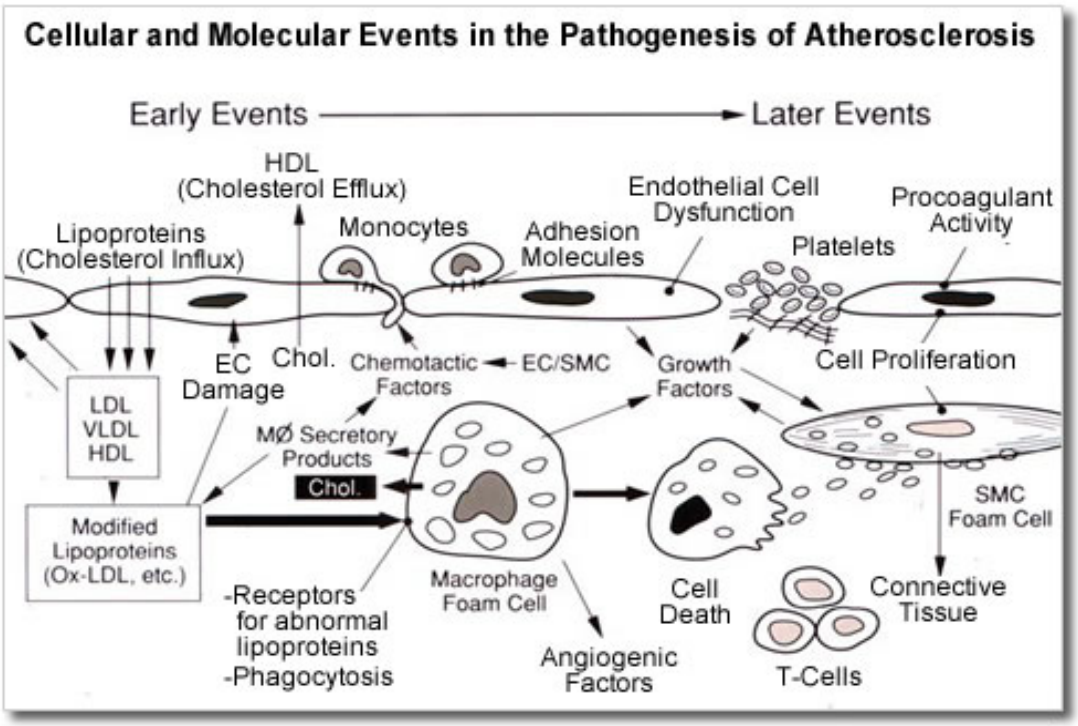

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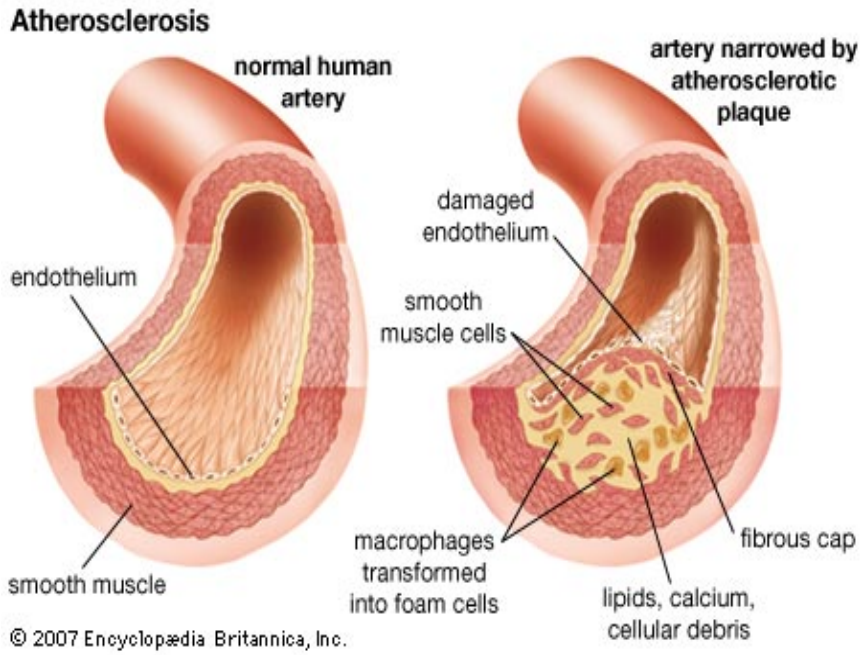
Figure 3



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2.2 Risk Factors for Atherosclerosis and the Development of CHD

Several risk factors for the development of atherosclerosis have been identified. The risk factors for CHD can be divided into two categories:

1. Risk factors – Those that have been proven to increase a persons chance of developing CHD
2. Risk markers – The association with CHD has been shown but the cause and effect association are yet to be proven (table 1) (Yusuf et al., 2001)

Table 1: Proven and presumed risk factors for CHD

Risk Factors:	Risk Markers:
Elevated cholesterol levels	Elevated levels of homocysteine
Elevated LDL cholesterol levels	Elevated prothrombic factors e.g. fibrinogen
Smoking	Elevated lipoprotein (a)
Physical inactivity	Physiological factors e.g. stress
Obesity	Family history
Diet	Age
Hypertension	
Diabetes	

Adapted from Yusef et al., 2001 & Humphries et al., 2007

The risk factors such as hyperlipidaemia, hypertension and smoking are also known as classic risk factors. These risk factors alone do not fully explain the risk of development of CHD. Consequently, other novel factors must be involved (Troughton et al., 2007).

Family History: Familial hypercholesterolemia (FH) is a genetic disorder which is characterised by abnormally elevated levels of cholesterol due to the accumulation of LDL in the plasma and cholesterol deposition in tendons. Patients are at an increased risk of atherosclerosis (Marais 2004). In FH there are single gene changes present which lead to accelerated atherosclerosis. However atherosclerosis in patients without familial hypercholesterolemia is more likely to be influenced by multiple genes (Seo & Goldschmidt-Clermont 2008). Several recent studies have concentrated on identifying genetic markers to predict the risk of future CHD in individuals without FH however, to date no study has found any marker which could have widespread clinical usefulness (Humphries et al., 2007 & Talmud et al., 2008).

C reactive protein (CRP): Recent studies have also been concentrating on the association of certain inflammation markers, particularly C-reactive protein (CRP). CRP is an acute phase reactant which is synthesised primarily by the hepatocytes of the liver. One of the many functions of CRP is as an opsonin at sites of tissue injury. CRP has frequently been detected in atherosclerotic plaques (Ecomonou et al., 2005). It binds foreign particles such as bacteria causing the activation of the complement system. There is evidence of chronic low grade systemic inflammation during the development of CHD and CRP is a marker of low grade systemic inflammation (Danesh et al., 2000). Studies have shown that CRP can be used as an independent predictor of adverse cardiovascular disease (Packard & Libby 2008).

Diet & Lifestyle: Smoking is also long recognised as a risk factor for the development of CHD. One explanation is that smokers are exposed to a range of harmful substances e.g. carbon monoxide and free radicals. These can impact on the process of atherogenesis and thrombosis (Cullen et al., 1998). Smoking is also thought to elicit an inflammatory response in cells involved in atherosclerosis and is thought to trigger the endothelial expression of adhesion molecules e.g. vascular adhesion molecule-1 (VACM-1) (Packard & Libby 2008). A diet high in saturated fat has also been recognised as a risk factor for the development of CHD.

Homocysteine: Homocysteine is an amino acid which is derived from the metabolism of methionine. It has been suggested that hyperhomocysteinaemia alone does not increase an individuals risk of developing atherosclerosis, however it does appear to increase the risk when an individuals with one or more pre-existing CHD risk factor (Troughton et al., 2007).

3. Established risk factors for coronary heart disease

Lipids are either endogenous i.e. synthesised by the body or exogenous i.e. derived from food. They are typically carried in the bloodstream as soluble protein complexes called lipoproteins. Lipoproteins are classified according to their density. There are 2 main classes of lipoprotein which contain mostly cholesterol: HDL and LDL, and 3 main classes of lipoprotein which are triglyceride rich: chylomicrons, very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL). Approximately 70% of plasma cholesterol is incorporated into LDL and 20% in HDL (Mayne 1994).

3.1 Cholesterol

Cholesterol is essential for membrane integrity, the synthesis of bile, vitamin D and other steroid hormones e.g. aldosterone and cortisol. Cholesterol synthesis is both endogenous and exogenous. Synthesis occurs mainly in the liver, however all nucleated cells have the ability to synthesise cholesterol if needed through the synthesis of enzymes that produce cholesterol. Up to 60% of dietary cholesterol is absorbed in the gut however this varies between individuals (Marais 2004).

Cholesterol levels usually increase as people get older. Men typically have higher cholesterol levels than women however, women's cholesterol levels increase with use of the oral contraceptive and after the menopause.

In 1948 the National Heart Institute began the first longitudinal study to determine the risk factors for CHD. One of the main risk factors concluded from this study was elevated serum cholesterol levels (Parodi 2009). Cholesterol levels are still widely used as a primary screening tool for determining CHD risk.

Several studies have demonstrated that there is a strong linear relationship between cholesterol and CHD risk. They have shown that lowering cholesterol levels decreases the risk of CHD. Hypercholesterolemia is associated with endothelium dysfunction. There is an excess of endothelial superoxide generation in hypercholesterolemia which can increase LDL oxidation (Grover-Páez &' Zavalza-Gómez 2009).

Elevated cholesterol levels are considered an important risk factor for the development of CHD however, doubts have been raised as to the effectiveness of cholesterol screening for determining CHD risk as many individuals who develop CHD have normal or only moderately raised cholesterol levels. In 1996 Weijenberg et al. performed a 5 year follow-up study of 820 men aged between 64-84 years. They found that cholesterol was an independent predictor of mortality from CHD although, the relationship between cholesterol and the incidence of CHD was not strong.

3.2 Triglycerides

Triglycerides (TG) are lipid fractions used for energy storage. Tirosh et al., 2007 assessed the effect of changes in triglyceride levels over a period of time and CHD risk. Samples were taken from apparently healthy men aged 26 – 45 years 5 years apart. They concluded that this measurement schedule is more beneficial for determining CHD risk rather that a single measurement. They found that there was an increase of CHD risk in people who have a consistently raised serum TG level compared to people whose initially elevated TG level was decreased upon the second measurement 5 years later.

Elevated TG levels have been associated with sdLDL and insulin resistance. All of these factors can result in increased atherosclerosis and consequently to CHD.

However, the role of triglyceride measurement as part of the lipid profile for determining CHD risk remains unclear. Sarwar et al., 2006 concluded that although an elevated triglyceride level was associated with CHD risk, when it was corrected against established risk factors the association was weakened. Similar results were found by Isles & Paterson in 2000.

3.3 High Density Lipoprotein Cholesterol

HDL particles are heterogeneous with respect to their physiochemical properties, intravascular metabolism and biological activity. HDL can be separated into 2 main subclasses: HDL_2 and HDL_3. Studies carried out on survivors of myocardial infraction and in patients with atherosclerosis found that these patients had a reduced level of

both HDL_2 and HDL_3, but there was a proportionally greater reduction of HDL_2. Other reports have found that a reduction in HDL_3 is the strongest predictor of CHD (Ronheim & Asztalos 1995).

HDL displays many antiatherogenic effects:

- HDL is involved in reverse cholesterol transport which removes cholesterol from areas of lipid accumulation. This protects against the development of atherosclerosis (Rosenson 2006).
- Kontush et al., 2003 found that it offers protection against both metal dependant and independent oxidation for LDL subclasses, including sdLDL.
- HDL_3 exhibits anti-inflammatory properties.
- The expression of adhesion molecules and the migration of monocytes in the endothelium in response to ox-LDL is inhibited by HDL. (Rosenson 2006).

Consequently, increased serum levels of HDL is believed to have protective effects against the development of CHD, while lower levels are associated with an increased risk for CHD development (Ballantyne et al., 2001).

3.4 Low Density Lipoprotein Cholesterol

LDL is involved in the transport of lipids to peripheral tissues. Lipoproteins are globular in shape and are composed of lipids and proteins. The lipoprotein particle core consists of triacylglycerol and cholesteryl ester. The plasma membrane is composed mainly of free cholesterol, apolipoproteins and phospholipids (Biggerstaff KD. & Wooten JS. 2004) (See figure 5). The transportation of lipids, primarily cholesterol from the liver to the periphery is one of the main functions of LDL. High affinity LDL receptors in the liver play a key role in the removal of LDL from circulation (roheim & Asztalos 1995).

LDL cholesterol comprises distinct subclasses that differ in respect to density, size, metabolic behaviour, atherogenicity and surface lipid composition (Rizzo & Berneis 2007). Four major subspecies of LDL have now been identified: large LDL-I, medium LDL-II, small LDL-III and very small LDL-IV (Rizzo & Berneis 2007). There are 2 distinct phenotypes of LDL particles: pattern A which has a higher proportion of larger more buoyant LDL particles and pattern B which has a higher proportion of sdLDL (Hirano et al., 2003). Approximately 30% of adult men and 5-

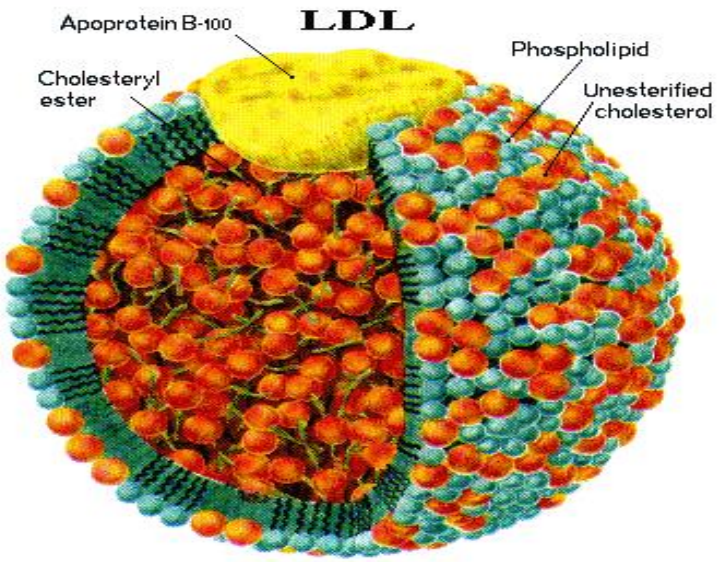
10% of young men and women <20years display pattern B phenotype. The prevalence of this phenotype in post-menopausal women is 15-25% (Rizzo & Berneis 2006). Stan et al., 2005 carried out sdLDL measurements in a population based sample of children and adolescents between 9 and 16 years of age. They found that the prevalence of pattern B was similar between sexes and across ages.

Increased total LDL has been established as a major risk factor for the development of atherosclerosis as studies have suggested that it plays an important role in the recruitment of monocytes to the vessel wall as well as the progression of macrophages to foam cells (Gleissner et al. 2007).

It has been demonstrated that 46% of first cardiovascular events occurred in people with LDL levels within the normal range (Ridker et al., 2002). However, it is the oxidised form of LDL that displays particularly more atherogenic and is more cytotoxic to the endothelium. Oxidation can occur due to co incubation with other cell types e.g. smooth muscle cells, monocytes, macrophages expressing 5-, 12-, and 15-lipoxygenase. Atherosclerotic lesions in humans have been shown to contain ox-LDL (Gleissner et al., 2007).

The main therapeutic target for management of CHD is LDL cholesterol. This is typically achieved through statin therapy. However, emerging evidence has suggested that statin drugs also have anti-inflammatory properties which have a more important role in the management of CHD than previously thought.

Figure 5.



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4 Small, dense low-density lipoprotein

4.1 sdLDL

Although LDL levels are considered to be an important risk factor for the development of CHD, many patients who develop CHD have LDL levels within the normal range (Packard & Libby 2008). One explanation for this observation is the existence of LDL subclasses.

Both environmental and genetic factors influence the expression of pattern B phenotype. Heritability ranges from 35-45% based on an autosomal dominant inheritance. Environmental factors, abdominal adiposity and oral contraceptive use are also associated with an increase in sdLDL levels. In people who are genetically predisposed to pattern B phenotype a high carbohydrate and low fat diet can induce this phenotype (Rizzo & Berneis 2006).

4.2 sdLDL and Atherogenesis

There are several factors which suggest that sdLDLs are highly atherogenic. These include:

- They display higher penetration of the arterial wall
- They have a lower binding affinity for the LDL receptor
- They have a prolonged half life compared to the larger more buoyant LDL
- They have a reduced resistance to oxidative stress compared to the larger more buoyant LDL (Hirano et al., 2003, Koba et al., 2006)
- The surface lipid layer of sdLDL has a reduced content of free cholesterol and an increased content of polyunsaturated fatty acids. This may also contribute to enhanced oxidative susceptibility (Rizzo & Berneis 2006)
- Studies have shown a 2- to 3-fold increase in risk of CHD among individuals with pattern B phenotype (Koba et al., 2002)

sdLDL has also been shown to be associated with both coronary and non-coronary forms of atherosclerosis and is a risk factor for peripheral arterial disease (Rizzo & Berneis 2007).

4.3 sdLDL as a predictor of future cardiovascular events

Numerous studies have been carried out to determine if sdLDL could be used as a reliable and independent marker to predict an individuals risk for future cardiovascular events. Some researchers believe that measurement of cholesterol subfractions may be superior in determining CHD risk than the standard lipid profile currently in use (Krauss 2005). Koba et al., 2002 found a strong association between sdLDL and various types of CHD. They demonstrated that the sdLDL level was related to the extent and severity of coronary lesions and was independent of both traditional and non-traditional risk factors. Koba et al., 2006 also found that sdLDL is an independent risk factor. They demonstrated that increases in sdLDL cholesterol correlated with progressively more severe coronary sclerosis, while total LDL did not. They also concluded that it was the amount of sdLDL rather than particle size alone that is more strongly linked to CHD severity.

A further study reported that a greater proportion of sdLDL is an independent risk predictor of CHD and an elevated concentration of large LDL is associated with a low risk of CHD (Koba et al. 2008). Similarly Gentile et al., 2008 analyzed the relationship between sdLDL and early atherosclerosis in a group of women aged 30-69 years and found that sdLDL is a marker of early atherosclerosis, independent of other covariants. Pauciullo et al., 2009 also found that elevated sdLDL levels are related to CHD risk, independent of other risk factors.

The Quebec cardiovascular study was a 5 year follow up study of 2,103 men who were initially free of heart disease. Over the course of the study 114 men had heart attacks. For analysis they separated the men into 3 groups depending on their LDL particle size. They found that the men with the lowest LDL particle size (≤25.64nm/0.000025mm) had a 3.6 fold increase in risk of having a heart attack when compared to the group with the largest LDL diameter (>26.05nm). St-Pierre et al., 2004 conducted a 13 year follow up from the Quebec study. They measured both sdLDL and large LDL. They found no increased risk in patients with elevated large LDL particles while there was an increased risk in patients with accumulated levels of sdLDL.

Norata et al., 2009 studied different lipoprotein subclasses in a healthy population. They evaluated whether different lipoprotein subclasses affected the expression of chemokines, adhesion molecules and endothelial cells differently in participants with comparable total LDL cholesterol levels. They found similar expression of some inflammatory genes in all sdLDL subclasses, irrespective of whether the participant was pattern A or pattern B phenotype.

5. Diabetes

Diabetes mellitus (DM) is a metabolic disorder which results from a defect in insulin action, secretion or both. There are two types of DM, type 1 and type 2. Type 1 is associated with cessation of insulin secretion while type 2 DM occurs due to insulin resistance. Normally glucose levels rise after eating and insulin is released from the pancreas. This release of insulin results in increased glucose disposal and decreased hepatic glucose output. However, in type 2 diabetes insulin resistance results in increased hepatic glucose output and decreased glucose utilisation. The prevalence of diabetes is increasing. According to the World Health Organisation the prevalence of diabetes in 2000 was 171 million people worldwide, but this is expected to increase to 366 million by the year 2030 (http://www.who.int/diabetes/facts/world_figures/en/).

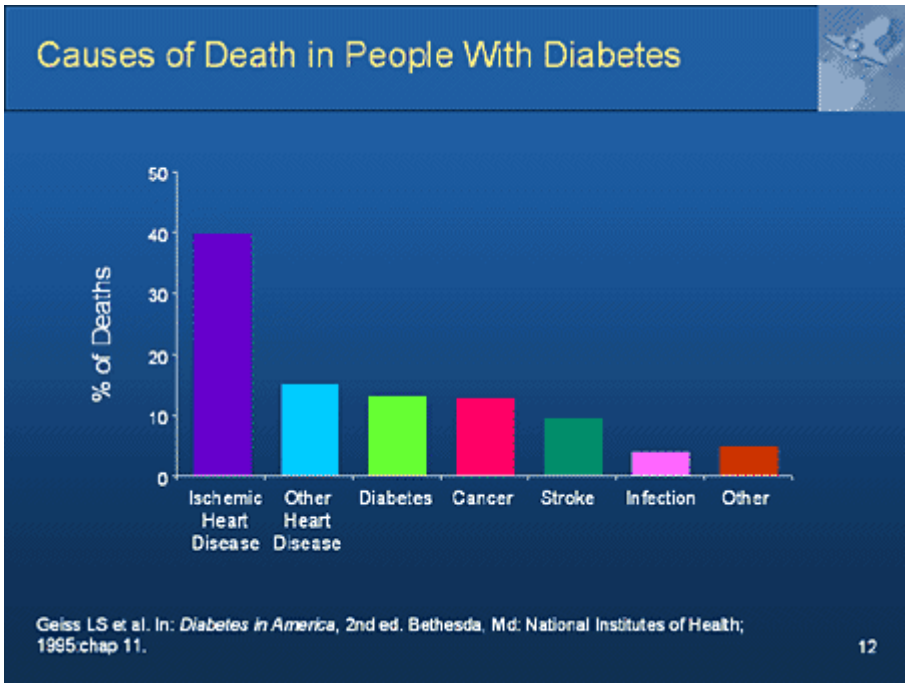
5.1 Diabetes and Coronary Heart Disease

In the adult diabetic population the prevalence of CHD can be as high as 55%, with DM being an independent risk factor for CVD in both men and women. Patients with both type 1 and type 2 diabetes, as well as people in the prediabetic stage and with the metabolic syndrome have an increased risk for CVD (Berry et al., 2007). CHD is the leading cause of death among patients with diabetes (see figure 5). People with

diabetes have an increased mortality and morbidity from CHD. Typically CHD occurs at a younger age in diabetics and men and women are equally affected (Tan 1999).

A study carried out on newly diagnosed type 2 diabetics found that a high fasting plasma glucose was a significant predictor of cardiovascular mortality, independently of other risk factors (Tan 1999).

Figure 5.



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5.2 Diabetes and small dense-LDL

Diabetic dyslipidemia is characterised by increased triglycerides, low HDL, and an increased fraction of sdLDL. It has been shown that an increase in peak size of LDL is associated with a decreased risk for the development of type 2 DM, while people with pattern B phenotype and an increased risk for the development of the disease, irrespective of age, BMI and glucose tolerance (Rizzo & Berneis 2007). Alabakovska et al., 2008 compared the LDL subclass profile in diabetic children and compared the

results with a healthy control group. They found that there was an increased frequency of pattern B phenotype among the diabetic children. They concluded that this is associated with an increased risk of atherosclerosis in diabetic children.

CHD risk factors only account for some of the excessive risk of CVD among diabetic patients. Consequently, it is thought that there is a relationship between hyperglycaemia associated with diabetes and CVD (Berry et al., 2007). Ogita et al., 2008 carried out a study to demonstrate the effect of insulin on sdLDL. Participants were given 75g oral glucose tolerance test (OGTT) and oral fat tolerance test (OFTT). They found that sdLDL levels were significantly reduced after the OGTT but remained unchanged after the OFTT. They concluded that insulin is a key modulator of sdLDL and can be explained by the ability of insulin to increase LDL receptor activity. As patients with DM have either insulin resistance or insufficient insulin production their sdLDL levels would be affected, particularly after eating. Similarly Stan et al., 2005 found an increased prevalence of sdLDL among children with insulin resistance when compared to children with normal insulin activity.

6. Aims

As already stated sdLDL is emerging as a reliable predictor of future coronary events in apparently healthy men and women. This study involves measuring sdLDL levels in an apparently healthy adult population ranging in age from 20-60. sdLDL levels will also be measured from a diabetic population within the same age range. Approximately 30 – 40 samples from each age range will be collected. As well as measuring for sdLDL the blood samples collected will also be analysed for total cholesterol, triglycerides, HDL and LDL for comparison purposes. Questionnaires will be completed by each participant to allow for the collection of specific data such as age, sex, weight, height, smoking status, lipid lowering medication, family history of CHD and history of hypertension. This data will then be used for comparison reasons as part of the study.

The aims of this study are:

- To identify normal ranges of sdLDL within the following age groups: 20-29years, 30-39 years, 40-49 years, and 50-60 years.
- To identify factors which may influence sdLDL levels i.e. BMI, smoking, age, sex
- To identify any correlation between sdLDL and cholesterol, triglycerides, HDL and LDL
- To identify any differences between sdLDL levels in an apparently healthy population and a diabetic population

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Section B:

Scientific Paper

Small dense LDL cholesterol in healthy adult and diabetic populations

Abstract

Background: Atherosclerosis, a chronic inflammatory disease which involves the build up of fatty deposits on the arterial wall is the main physiological precursor of coronary heart disease (CHD). The association between lipids and CHD has been well documented. Small dense low density lipoprotein (sdLDL) is emerging as a reliable and independent risk marker of (CHD). Diabetes has long since been recognised as a risk factor for CHD. This study aims to establish normal ranges of sdLDL in a healthy adult population and diabetic populations.

Methods: One hundred and sixty four apparently healthy adults aged 20-60 years and eighty one diabetic patients had blood samples taken for cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL) and sdLDL measurement. Blood samples were collected and centrifuged at 3000rpm for 10 minutes. All serum samples were stored frozen at -80ºC until analysis on the Roche Hitachi modular system.

Results: Females aged 20-60 and males aged 20-49 had a sdLDL reference range of 0.3 – 1.4 mg/dL. Males aged 50-60 had a range 0.3 – 1.8mg/dL. Significant correlations were found between sdLDL and established markers of CHD. Mean sdLDL for type 1 and type 2 diabetics were 0.62 and 0.65 respectively. Diabetic patients on lipid lowering therapy were found to have significantly lower sdLDL levels when compared to healthy individuals. No significant difference was found between healthy individuals and diabetic patients not on lipid lowering therapy

Conclusion: sdLDL levels were significantly higher in older men .A significant increase in sdLDL was also seen in individuals with a body mass index (BMI) >25. This could increase the risk of CHD in these individuals. A significant decrease in sdLDL levels among diabetic patients on lipid lowering medication was evident. This could offer protection against CHD in these patients.

Abbreviations

ANOVA	Analysis of Variance
BMI	Body Mass Index
CHD	Coronary Heart Disease
H_2O_2	Hydrogen Peroxide
HRT	Hormone Replacement Therapy
HSDA	Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline
O_2	Oxygen
sdLDL	Small Dense Low Density Lipoprotein

Introduction

Coronary heart disease (CHD) is a major cause of morbidity and mortality in both the developed and developing world. By 2020 it is predicted that CHD will be the main cause of death worldwide (1). It occurs due to the development of atherosclerotic lesions which result in the arteries becoming narrower.

There are many risk factors for the development of CHD. These include elevated cholesterol levels, elevated LDL cholesterol, hypertension, smoking, obesity and diabetes (2). Currently a fasting lipid profile is the most extensively used tool to identify persons at risk of CHD. A fasting lipid profile consists of total serum cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol.

Several studies have shown that there is a positive correlation between elevated total cholesterol levels and CHD (3, 4). However, other studies have shown that over half of all myocardial infarctions occur in individuals whose cholesterol levels are normal to moderately raised (5). Simons et al. 2001 found that total cholesterol levels were a weak predictor of CHD in individuals younger than 75years (6).

The role played by triglycerides in CHD remains unclear. It has been shown that triglycerides are associated with CHD, however, when it is adjusted against other established risk factors the relationship weakened.

HDL cholesterol is synthesised primarily in the liver and small intestine and has been shown to have a variety of antiatherogenic properties. These include the inhibition of LDL oxidation by HDL-bound PON1 and the transport of cholesterol from cells in the arterial wall to the liver (7).

LDL cholesterol is the main transporter of cholesterol. LDL cholesterol particles are heterogeneous with respect to their size and can be divided into three categories; large buoyant LDL, intermediate LDL and small dense LDL. Elevated LDL cholesterol has been implicated in the initiation and progression of atherosclerotic lesions. Oxidised LDL is now recognised to be particularly atherogenic as it has been demonstrated in atherosclerotic lesions but it is not present in the healthy arterial wall. Scavenger cells of macrophages can recognise oxidised LDL which induces subendothelial lipid accumulation and foam cell formation; both of which have been established as early hallmarks of atherosclerosis.

As a high proportion of CHD occurs in persons with normal to moderately raised cholesterol levels recent studies have concentrated on identification of other

independent markers of CHD. Small dense LDL cholesterol is emerging as a possible risk marker. sdLDL has been shown to be highly atherogenic as it displays a higher penetration of the arterial wall, has a lower binding affinity to the LDL receptor, shows reduced resistance to oxidative stress and has a prolonged half life compared to the larger more buoyant LDL (8, 9). When compared to a control group LDL size in CHD patients were shown be markedly smaller. These patients were also shown to have significantly higher sdLDL cholesterol levels than the control group (10, 9). Traditionally, LDL particle size was measured to determine sdLDL concentration. These methods required the use of salt density gradient ultracentrifugation which made it unsuitable for routine clinical analysis. The advancement of automated methods for the determination of sdLDL has allowed for its rapid and reliable estimation in serum samples and it has been suggested that quantification of sdLDL is a more sensitive marker of CHD than LDL particle size determination (11).

The American Heart Association and National Heart, Lung and Blood Institute have proposed that the metabolic syndrome represents a collection of metabolic risk factors that directly promote atherosclerosis development (12). There is evidence to suggest that sdLDL is a member of these metabolic risk factors and an increased fraction of sdLDL particles is characteristic of diabetes dyslipidemia (13). Diabetic patients have a two- to three fold increased risk for CHD with females and males being affected equally (14). To compensate for this phenomenon diabetic patients deemed at risk of CHD development are prescribed statins which have the ultimate goal of lowering LDL cholesterol while increasing HDL cholesterol.

This study is concerned with establishing a normal range for sdLDL in an apparently healthy adult population consisting of both males and females aged between 20 and 60 years. Establishment of normal ranges are of vital importance in modern medicine. As sdLDL is emerging as an important risk marker for CHD, establishment of normal ranges will enable sdLDL to be routinely used in a clinical setting. Inclusion of sdLDL as a risk marker of CHD could allow for early identification of individuals at risk of developing atherosclerosis and consequently CHD. As diabetes has long been associated with an increased risk of CHD this study will also compare sdLDL levels between apparently healthy individuals and a population of diabetic patients.

Materials and Methods

Participant Recruitment

Participants for the establishment of an sdLDL normal range were recruited through personal invitation of hospital staff in Letterkenny General Hospital. Individuals were deemed eligible to participate if they were free from any known illness, were not on any lipid lowering drugs had not been diagnosed with CHD and were between the ages of 20 and 60 years.

The medical ethics committee of Letterkenny General Hospital approved all participant recruitment and data collection procedures. All suitable candidates were given an ethically approved patient information sheet. Upon agreeing participants signed a consent form and completed a self-administered questionnaire which included the following details: sex, date of birth, smoking status, use of lipid lowering drugs, history of hypertension, family history of CHD, history of diabetes and family history of diabetes. Participant's height and weight were also recorded at the time of blood sampling.

Diabetic patients were recruited through personal invitation of patients attending two weekly diabetic clinics in Letterkenny General Hospital. All participants were given the same patient information sheet, questionnaire and consent form as before.

Blood Measurements

Fasting blood samples were drawn by the phlebotomists in Letterkenny General Hospital. Blood was collected into serum tubes with clot activator and gel for serum separation (Becton-Dickinson Vacutainer Systems, N.J., U.S.A.) for the analysis of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol and sdLDL cholesterol. After collection blood samples were allowed to stand for 30 minutes to allow for clot activation, according to manufactures guidelines. Samples were then centrifuged at 3000rpm for 10 minutes. The separated serum was aliquoted into plain plastic tubes (1.5mL) and stored frozen at -80ºC until analysis.

Total Cholesterol Methodology

Total cholesterol concentrations were measured using an enzymatic assay (Roche Diagnostics, GmbH, Germany) performed on the Hitachi Modular analyser (Roche Diagnostics, GmbH, Germany). Cholesterol esterase cleaves cholesterol esters yielding free cholesterol and fatty acids. Free cholesterol is converted by O_2 with the aid of cholesterol oxidase to cholest-4-en-3-one and H_2O_2. The H_2O_2 created forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity, which is directly proportional to the concentration of cholesterol, can be measured photometrically at 505nm.

Triglyceride Methodology

Triglyceride concentrations were measured using an enzymatic assay (Roche Diagnostics, GmbH, Germany) performed on the Hitachi Modular analyser (Roche Diagnostics, GmbH, Germany). Triglyceride levels are determined using a lipoprotein lipase which allows for the complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and H_2O_2 . H_2O_2 reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff. The colour intensity, which is directly proportional to triglyceride concentration, can be measured photometrically at 505nm.

HDL Methodology

HDL cholesterol concentrations were measured by homogenous enzymatic colorimetric test (Roche Diagnostics, GmbH, Germany) performed on the Hitachi Modular analyser (Roche Diagnostics, GmbH, Germany). In the presence of O_2, cholesterol is oxidised by cholesterol oxidase to Δ4-cholestenone and H_2O_2. H_2O_2, in the presence of peroxidase reacts with 4-amino-antipyrine and HSDA to form a purple-blue dyestuff. The colour intensity is directly proportional to HDL cholesterol concentration and is measured photometrically at 505nm.

LDL Methodology

LDL cholesterol concentrations were measured by homogenous enzymatic colorimetric test (Roche Diagnostics, GmbH, Germany) performed on the Hitachi Modular analyser (Roche Diagnostics, GmbH, Germany). In the presence of O_2 cholesterol is oxidised by cholesterol oxidase to Δ4-cholestenone and H_2O_2. H_2O_2 in

the presence of peroxidase reacts with 4-amino-antipyrine and HSDA to form a purple-blue dyestuff. The colour intensity is directly proportional to LDL cholesterol concentration and is measured photometrically at 505nm.

sdLDL Methodology

sdLDL cholesterol was measured using Randox Laboratories s LDL-EX"SEIKEN" kit. It is based on a two step enzymatic colorimetric assay. In the first step non-sdLDL lipoproteins are decomposed by a surfactant and sphingomelinase (SPC). The cholesterol released from these non-sdLDL lipoproteins is then degraded to water and oxygen by the action of enzymes. In the second step, another surfactant releases cholesterol only from sdLDL particles and cholesterol released form sdLDL is then subjected to enzymatic reactions. H_2O_2 produced from the reaction with cholesterol esterase and cholesterol oxidase then develop a purple-red colour with the coupler in the presence of peroxidase (POD). The colour intensity, which is directly proportional to sdLDL cholesterol concentration, can be measured photometrically at 505nm.

Statistical Methodology

The sample size was calculated using a power analysis. All statistical analysis on data from the study was conducted using Minitab Version 15. Independent t-tests were carried out to determine differences in sdLDL levels within body mass index groups (<25 and >25), males and females and family history of CHD groups. This test was also used in the same instances for cholesterol, triglycerides, HDL and LDL. A one-way analysis of variance (ANOVA) was used to determine differences in each analyte between the four age groups. Correlation, regression analysis and a fitted line plot were also carried out on Minitab to determine correlation between sdLDL and the other analytes. Reference ranges were calculated using Anderson-Darling A^2 equation.

Results

A total of 164 apparently healthy fasting subjects were sampled between the ages of 20 and 60 years. Of these 88 (54%) were female and 76 (46%) were male. 12% of the sampled population had cholesterol levels >6.2mmol/L. According to Roche Diagnostics (Roche Diagnostics, GmbH, Germany) cholesterol results above 6.2mmol/L are deemed to be high, consequently these elevated values were removed from all statistical analysis. Therefore, the total population number for healthy subjects was 144 including 75 (52%) females and 69 (48%) males. Baseline characteristics of the participants are summarised in Table 1.

The diabetic population consisted of a total of 81 participants. Of these 31 (38%) were female and 50 (62%) were male, 34 (42%) were type 1 and 47 (58%) were type 2). Baseline characteristics of the participants are summarised in Table 2.

sdLDL results

sdLDL results were found to be normally distributed for both males and females (see figure 1a and 1b). A one-way ANOVA was used to compare sdLDL levels among the four age groups (20-29, 30-39, 40-49 and 50-60 years). No significant difference was found between female age ranges ($P > 0.050$). Males aged 50-60 years were found to have significantly higher sdLDL cholesterol when compared to all other age groups of males and females ($P<0.01$) (see figure 2). Females aged 20-60 years had a mean value of 0.68 mg/dL with a 95% confidence interval (CI) of 0.62 - 0.73mg/dL. Males aged 20-49 years were found to have a mean value of 0.76 with a 95% CI of 0.75 – 0.90 while males aged ≥50 years had a mean value of 1.00. Due to the limited numbers in this sample population it was not possible to obtain reliable 95% CI.

With regard to BMI the population was split into two groups: BMI <25 and BMI >25. Typically BMI groups are split into four groups i.e. BMI <18.5, BMI 18.5–24.9, BMI 25-29.9 and BMI >30, however as there was so few individuals falling into the lowest and highest groups it was decided to divide the population into two groups for statistical analysis purposes. A significant difference in sdLDL results was found between the two groups ($P<0.01$), participants with a lower BMI having a lower sdLDL level.

Using regression analysis significant correlations were found between sdLDL and other established markers of CHD i.e. cholesterol ($R = 0.741$, $P<0.001$), triglycerides ($R = 0.640$, $P<0.001$), HDL ($R = 0.311$, $P<0.001$) and LDL ($R = 0.722$, $P<0.001$).

A t-test comparing smokers with non-smokers did not reveal any significant difference in sdLDL levels; however, only 22 (15%) individuals were smokers. A summery of all t-test and ANOVA results can be found in Table 3.

There was no significant difference in sdLDL levels between Type 1 and Type 2 diabetics. However, diabetic patients who were on lipid lowering treatment had a significantly lower sdLDL level compared to patients who were not on treatment ($P<0.05$). When diabetic patients were separated into two BMI groups no significant difference in sdLDL levels was observed.

Cholesterol

Cholesterol results were found to be normally distributed with the mean cholesterol concentration at 5.00mmol/L in females and 5.16 mmol/L in males. In females a significant difference was found between all age groups ($P<0.01$), except between group 1 (20-29 years) and group 2 (30-39 years). Cholesterol levels in females were found to be higher with increasing age. No significant differences in male cholesterol levels among the different age groups were found. Cholesterol levels showed no significant differences between the two BMI groups (i.e. BMI <25 and BMI >25) for both males and females.

There was no significant difference in cholesterol levels between type 1 and type 2 diabetics. Diabetic patients on lipid lowering treatment had a significantly lower cholesterol level compared to patients not on treatment ($P<0.01$). There was no significant difference in cholesterol levels between the two BMI groups for diabetic patients.

Triglycerides

Triglycerides were found to be normally distributed with mean concentration of 1.04 for females and 1.38 for males. Statistical analysis highlighted a significant difference in triglyceride levels among males and females ($P<0.001$), with males having a higher triglyceride level than females. No significant difference in triglyceride levels among the four age groups was observed for either sex. There was no significant difference in triglyceride levels between both male BMI groups while females with a higher BMI had a significantly higher triglyceride level ($P<0.001$).

No significant difference in triglyceride levels between type 1 and type 2 diabetics was observed. There was also no significant difference between patients on lipid

lowering treatment and those not on treatment. Diabetic patients with a BMI >25 had a significantly higher triglyceride level ($P<0.05$).

HDL

HDL levels were normally distributed in the study population. There was a significant difference between the two sexes ($P<0.01$) with females having a higher concentration (mean value of 1.69 for females and 1.39 for males). There was s significant difference in HDL levels between the two BMI groups for both females and males ($P<0.001$ and $P<0.05$ respectively).

Type 1 diabetic patients had a significantly higher HDL level compared to type 2 diabetics ($P<0.001$). Diabetic patients on lipid lowering treatment had significantly lower HDL levels compared to those not on any lipid lowering treatment ($P<0.05$). Diabetic patients with a BMI <25 had a significantly higher HDL level ($P<0.005$).

LDL

LDL cholesterol levels were normally distributed in the study population. A significant difference was seen between males and females ($P<0.01$). Males had a mean concentration of 3.18 while females had a mean concentration of 2.91. A one-way ANOVA highlighted a significant difference in LDL concentration among the youngest age group and in females 40 years and older. A significant difference in LDL levels were observed between the two BMI groups ($P<0.05$).

Diabetic patients on lipid lowering therapy had a significantly lower LDL level compared to patients not on treatment ($P<0.001$). No significant difference in LDL levels was observed between type 1 and type 2 diabetic patients.

Discussion

This present study was concerned with sdLDL levels in apparently healthy adult and diabetic populations in North West Donegal and is the first of its kind in this region. Some of these findings collaborate with other studies carried out on sdLDL.

This study has shown that sdLDL levels in healthy females aged 20-50 years and males aged 20-49 years should be in the range of 0.3 – 1.40 mg/dL, while males aged 50 - 60 years should have sdLDL levels within the range 0.3 – 1.80 mg/dL.

Previous studies have found higher levels of sdLDL in males compared to premenopausal females, with sdLDL levels in females typically increasing after onset of menopause (15). In this study only males 50 years and older displayed a significant increase in sdLDL. This is in accordance with trends seen in onset of CHD which typically occurs in older age groups (16, 17). No significant difference was found between pre- and post-menopausal females. However, the development and progression of CHD in females can be delayed for up to ten years in females compared to males (15). Foder & Tzerovska 2004 acknowledged that although the risk of CHD in females increases after the menopause, their risk is not equal to that of males until both groups are in their 80s (18).

The results in this study show a strong positive correlation between sdLDL and BMI in healthy individuals. This is in agreement with the findings of previous studies (19). An increased BMI is known to be associated with increased prevalence of dyslipidemia, including increased sdLDL. Uncontrolled fatty acid lipolysis from visceral adipose tissue leading to increased transport of fatty acids to the liver, where they act as substrate for VLDL synthesis, is the most likely cause of obesity related dyslipidemia (20). BMI has long been established as a risk factor for the metabolic syndrome. Increased adipose tissue is also associated with an increased expression of proinflammatory cytokines e.g. TNF-α. Proinflammatory molecules have been shown to contribute to defects in insulin signaling and consequently promote insulin resistance (20). No significant correlation between BMI and sdLDL in diabetic patients was found in this study. However, 80% of diabetic individuals with a BMI >25 were on lipid lowering treatment which was shown to be inversely correlated with sdLDL levels.

Smoking is thought to cause disruption of the endothelium, an early stage process of atherosclerosis development. Nitric oxide (NO) is an important vasodilating substance which offers protection to the endothelium against oxidation, vascular

smooth muscle cell proliferation and inflammation. Stimulators of NO production have been shown to be abnormal in persons who smoke (21). Analysis of the data in this study showed no significant difference in sdLDL between smokers and non-smokers. This could be due to the fact that only 15% of the population studied were smokers.

Family history of CHD has long been established as a major risk factor for the development of CHD. However, in this study no significant differences were found between individuals who had a family history of CHD when compared to individuals with no family history over all the parameters measured. This may be due to the fact that only 27% of individuals taking part in this study had a family history of CHD.

The results in this study show through simple regression analysis that sdLDL correlates with other established risk markers i.e. cholesterol, triglyceride and LDL. This is also in agreement with other peer review articles (19).

No significant difference in sdLDL levels among type 1 and type 2 diabetic patients was observed. It has previously been reported that type 2 diabetic patients typically have higher levels of sdLDL (17). One reason that no difference was seen in this study is that 82% of type 2 diabetic patients are on lipid lowering medication. Reinforcing this, a significant decrease in sdLDL levels was observed in diabetic patients on lipid lowering treatment compared to those not on any treatment. Statistical analysis showed no significant difference in sdLDL levels in patients not on lipid lowering therapy compared to the healthy population (see table 3).

One limitation of this study is that the sample population did not include individuals older than 60 years. Many studies have found that sdLDL levels typically increase with increasing age in females. Inclusion of individuals >60 years old could have shown results similar to previous studies. Lack of availability to information on hormone replacement therapy (HRT) use in postmenopausal females is another limitation. HRT has been widely credited with reducing incidence of CHD by increasing HDL levels and reducing LDL and triglyceride levels (22, 23, 24). The use of HRT could be another reason no significant difference was seen in females 50 years and older in this study.

Sample size was another limiting factor. Due to time constraints it was not possible to increase the study numbers. Reference ranges were calculated using Anderson-Darling A^2 reference interval equation. Typically if a study shows complete normality without skewness reference ranges are calculated as mean ± 2SD. Analysis

of the data in this study showed a slight skewness consequently it was decided to use the Anderson-Darling A^2 equation. A larger sample size might have shown complete fit to normality.

Conclusion

Many CHD events occur in individuals who have cholesterol and LDL cholesterol levels below recommended threshold levels. This phenomenon highlights the necessity to improve risk assessment procedures (25). Currently measurement of total LDL cholesterol levels are part of the profile used to assess CHD development risk. As sdLDL has emerged as the more atherogenic portion of LDL cholesterol its inclusion in routine screening methods may be of significant importance. This study highlights the fact that sdLDL correlates with other established risk markers of CHD. Other studies have found that not only does sdLDL correlate with the other markers but that it is indeed the more superior marker of carotid atherosclerosis. It has also been demonstrated that a high sdLDL level is closely related to the severity of CHD, independently of established risk factors. The same does not apply for total LDL (26).

The findings in this population based study are largely in agreement with previous studies carried out on sdLDL. However this is the first study used to establish sdLDL normal ranges in an apparently healthy adult population. Establishment of sdLDL normal ranges is the first step required before sdLDL could be considered for inclusion as a routine biochemistry test in laboratories. These findings will form the basis for more extensive work on sdLDL to be carried out in this region. As a result of this study the Cardiologist in Letterkenny General Hospital is going to include sdLDL in routine CHD risk assessment. An extensive study of sdLDL in a diabetic population is also underway in Letterkenny Hospital as it is thought measurement of sdLDL will aid in better treatment procedures for diabetic patients.

Table1. Baseline characteristics of all volunteers

	Healthy Volunteers		Diabetic Patients	
	Males	Females	Type 1	Type 2
Median Age			4.6±14.1	59.8±5.8
BMI kg/m²			25.0±3.8	31.0±5.9
Smoking status yes/no	10/53	11/68	10/25	6/39
History of hypertension yes/no	6/60	7/72	10/25	25/20
Family history of CHD	13/53	26/53	8/27	21/24
Lipid lowering therapy	0/66	0/79	16/19	37/8

Data is mean ± 1standard deviation (SD), except where otherwise stated

Table 2. Mean data for all volunteers

		Healthy Individuals				Diabetics	
		Group 1	Group 2	Group 3	Group 4	Type1	Type 2
Chol mmol/L						4.5±1.2	4.1±1.0
	M	4.8±0.7	5.2±0.7	5.3±1.0	5.7±1.0		
	F	4.7 ±0.9	4.9±0.8	5.4±1.5	5.7±0.6		
Trig mmol/L						1.4±0.8	1.7±0.9
	M	1.2 ±0.8	1.2±0.5	1.4±0.7	1.7±0.8		
	F	1.2±0.5	1.0±0.4	1.0±0.5	1.2±0.5		
HDL mmol/L						1.6±0.5	1.2±0.4
	M	1.4±0.4	1.5±0.4	1.3±0.4	1.3±0.3		
	F	1.7±0.3	1.7±0.4	1.7±0.5	1.7±0.5		
LDL mmol/L						2.3±0.9	2.1±0.8
	M	2.8±0.7	3.3±0.6	3.4±0.9	3.7±0.9		
	F	2.6±0.8	2.8±0.7	3.3±0.9	3.5±0.7		
sdLDL mmol/L						0.6±0.3	0.7±0.4
	M	0.7±0.3	0.8±0.2	0.9±0.5	1.1±0.4		
	F	0.8±0.4	0.7±0.2	0.7±0.4	0.8±0.3		

Data is mean ±1 standard deviation (SD)

Abbreviations: Group 1 = Age 20-29 yrs, Group 2 = Age 30-39 yrs, Group 3 = Age 40-49yrs, Group 4 = Age 50-60yrs, Chol = Cholesterol, Trig = Triglycerides, HDL = High density Liporotein, LDL = Low density lipoprotein, sdLDL = small dense low density lipoprotein.

Table 3: Summery of statistical findings

Subgroups	sdLDL	Cholesterol	Triglyceride	HDL	LDL
Healthy Individuals					
Male Vs Female	P<0.001	NS	P<0.001	P<0.001	P<0.01
Age 20-29yrs Vs 50-60yrs					
-Males	P<0.01	NS	NS	NS	NS
-Females	NS	P<0.01	NS	NS	P<0.01
BMI <25 Vs >25	P<0.01	NS	P<0.001	P<0.001	P<0.05
Smokers Vs					
Non-smokers	NS	NS	NS	NS	NS
History of CHD Vs					
No history	NS	NS	NS	NS	NS
sdLDL correlation		P<0.001	P<0.001	P<0.05	P<0.001
Diabetics					
Males Vs Females	NS	P<0.01	NS	P<0.001	NS
Type 1 Vs Type 2	NS	NS	NS	P<0.001	NS
BMI <25 Vs >25	NS	NS	P<0.05	P<0.001	NS
LL drugs					
- Use Vs No Use	P<0.05	P<0.01	NS	P<0.05	P<0.001
Healthy Individuals Vs Diabetics					
Not on LL drugs	NS	NS	P<0.05	NS	NS
Healthy Individuals Vs Diabetics					
On LL therapy	P<0.001	P<0.001	P<0.05	P<0.001	P<0.01

NS = No significant difference, LL = Lipid lowering

Figure 1 a: Normality distribution of sdLDL levels in females

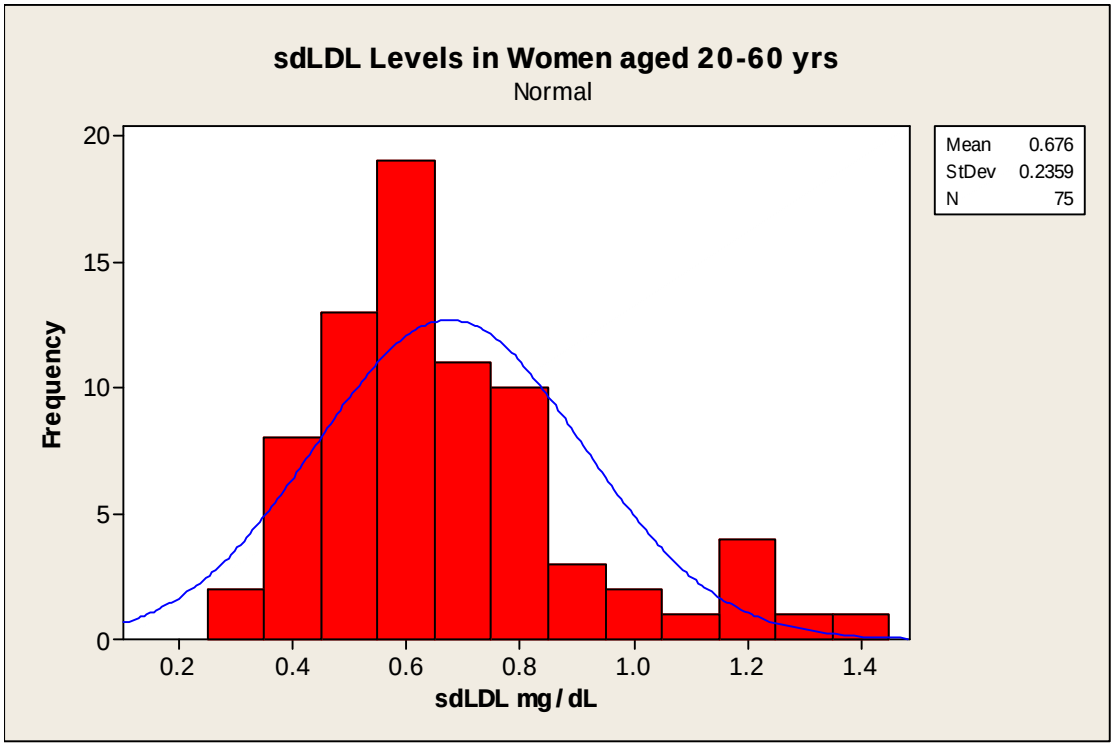


Figure 1 b: Normality distribution of sdLDL in males

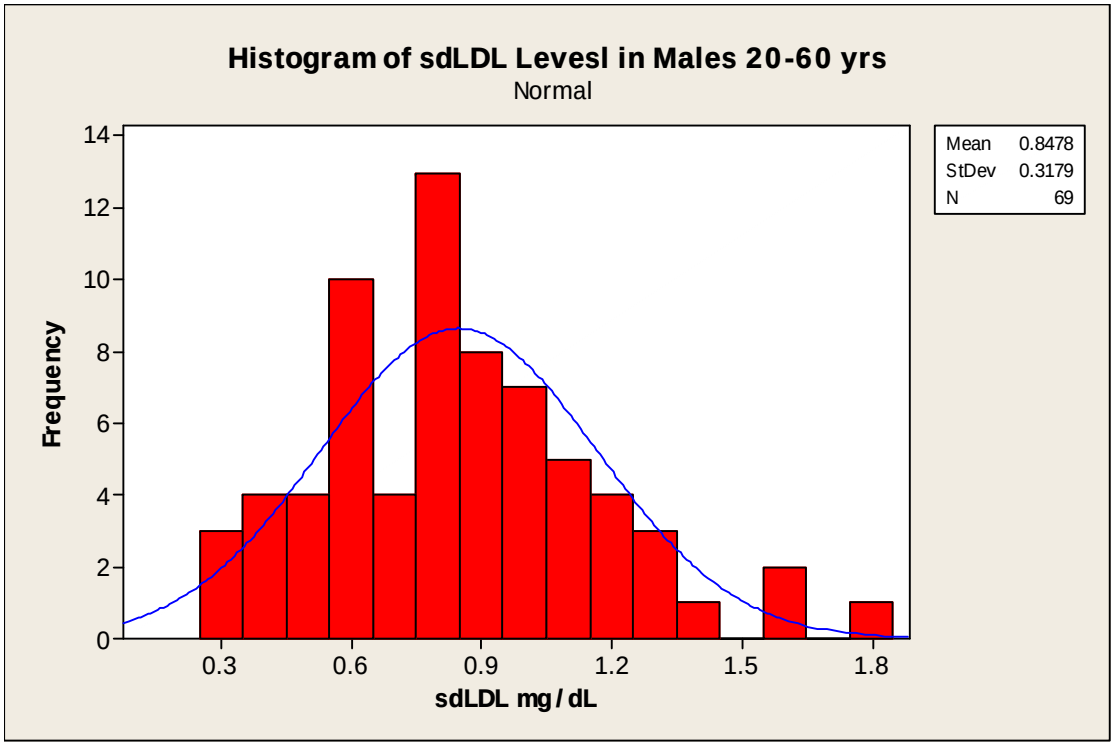
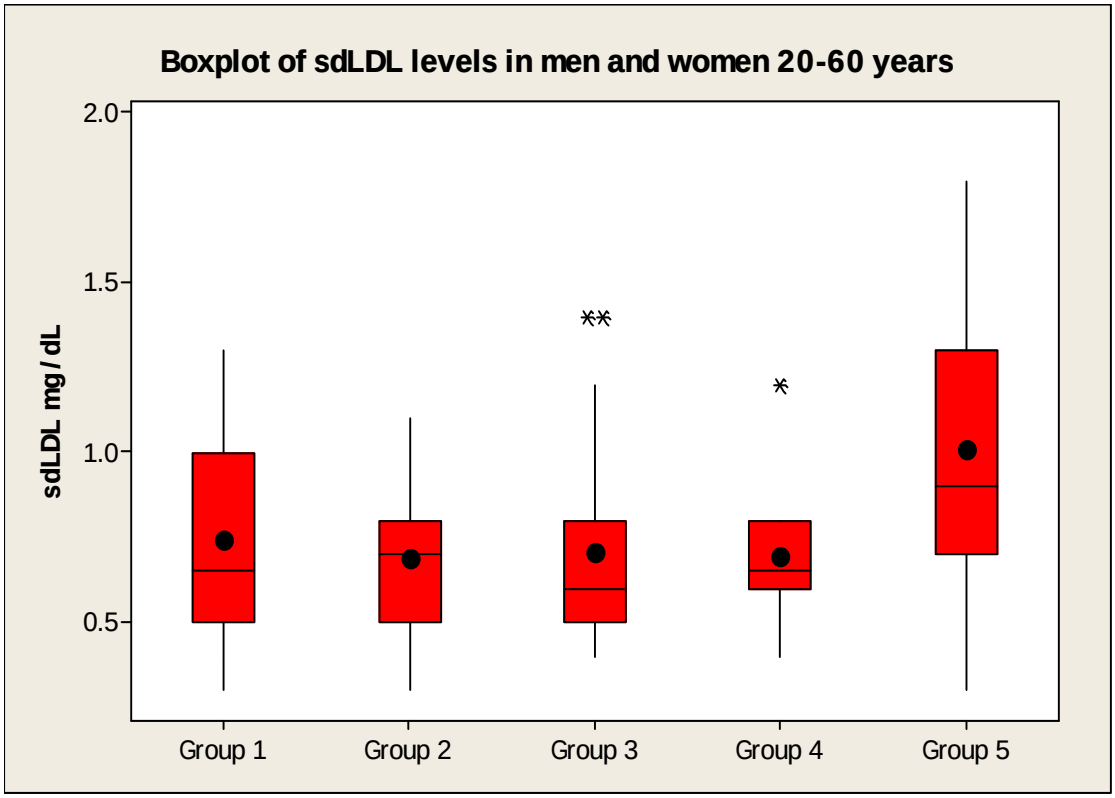


Figure 2: Boxplot showing sdLDL levels in males and females 20-60 years



Group 1: Males and females aged 20-29 years

Group 2: Males and females aged 30-39 years

Group 3: Males and females aged 40-49 years

Group 4: Females aged 50-60 years

Group 5: Males aged 50-60 years

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