Oxidation of low density lipoprotein by ferritin at lysosomal pH and its possible role in atherosclerosis

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Declaration

All of the data presented in this thesis is my own work. None of the content has been submitted for a diploma, degree or other qualifications at any other University. The use of all materials from other sources have been acknowledged.

Oluwatosin Ololade Ojo

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Publications and Posters

Full paper

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Full papers to be prepared and submitted

- Vitamins C and E do not effectively inhibit lysosomal LDL oxidation by ferritin at lysosomal pH**.**
- Effects of LDL degradation on its oxidation by ferritin at lysosomal pH
- LDL oxidation by ferritin in lysosomes of macrophages: effects on macrophage function in relation to atherosclerosis.

Abstract

Atherosclerosis leading to coronary heart disease and thrombotic stroke is the leading cause of death worldwide. Oxidised low density protein is considered important by many in this disease. Our laboratory has shown that LDL can be oxidised by iron in the lysosomes of macrophages. Others have demonstrated the presence of oxidised products similar to those formed *in vitro* by catalytically active iron in advanced atherosclerotic lesions. Ferritin is the body's main iron storing protein. The role of ferritin in lysosomal LDL oxidation and explored the possible consequences for atherosclerosis were investigated. Ferritin oxidised LDL effectively at lysosomal pH (pH 4.5), much faster than at pH 7.4, as shown by increased formation of oxidised lipids (HPLC and tri-iodide assay) and conjugated dienes (automated spectrophotometry). Ferritin spontaneously released iron at lysosomal pH and iron chelators supressed the oxidation of LDL by ferritin.

The degradation of apolipoproteinB-100 and cholesteryl esters of LDL speeded up the oxidation of LDL by ferritin. The lysosomotropic antioxidant cysteamine was shown to be a more appropriate antioxidant to effectively inhibit LDL oxidation by ferritin compared to ascorbate, α-tocopherol and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) and N, N′- diphenyl 1,4-phenylenediamine (DPPD).

The role of ferritin in lysosomal LDL oxidation within macrophages and macrophage function were accessed. Incubation with ferritin and LDL led to increased intracellular lipid, ceroid and lipid peroxidation in lysosomes. Ferritin and LDL treatment increased glycolysis and THP-1 macrophage-like cells became metabolically activated, as shown by a Seahorse analyser. Ferritin-oxidised LDL induced reactive oxygen species formation and apoptosis in macrophages, suggesting LDL oxidation by ferritin in lysosomes might be atherogenic.

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List of Abbreviations

AAS	Atomic absorption spectrophotometer
Acetyl LDL	Acetylated LDL
ADH	Autosomal dominant hypercholesterolaemia
BHT	Butylated hydroxytoluene
BP	Bathophenthroline
CAD	Coronary artery disease
CHD	Coronary heart disease
CLOOH	Cholesteryl linoleate hydroperoxide
CVD	Cardiovascular Disease(s)
DHE	Dihydroethidium
DMT 1	Divalent metal transporter 1
DTPA	Diethylenetriaminepentaacetic acid
DPPD	N, N'- diphenyl – p-phenylenediamine
EC	Endothelial cells
ECM	Extracellular matrix
FCS	Fetal calf serum
FH	Familial hypercholesterolaemia
HD	Huntington Disease
HDL	High density lipoprotein
HDS	High density Solution
HIF-1α	Hypoxia-inducible factor
HMDM	Human monocyte derived macrophages
HNE	Hydroxynonenal
IDL	Intermediate density lipoprotein

iNOS	Inducible nitric oxide synthase
PL	Phospholipid
PMA	Phorbol 12-myristate-12-acetate
IFN γ	Interferon γ
IHD	Ischaemic heart disease
LDL	Low density lipoprotein
LIP	Labile iron pool
LOX1	Lectin-type oxidised LDL receptor 1
MCSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MMP	Matrix metalloproteinase
MI	Myocardial infarction
MPO	Myeloperoxidase
oxLDL	oxidised low density lipoprotein
TG	Triglycerides
TGF-β	Tumour growth factor β
TNF-α	Tumour necrosis factor α
PCSK9	Proprotein convertase subtilisin-kexin 9
PDGF	Platelet derived growth factor
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid(s)
SDS	Sodium dodecyl sulphate
SMase-LDL	Sphingomyelinase-aggregated LDL
SMC	Smooth muscle cells

SRA	Scavenger receptor A
SRB1	Scavenger receptor class B member 1
SREBP	Sterol regulatory binding protein
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl
WHHL	Watanabe-Heritable Hyperlipidaemic
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein

Chapter 1- General introduction

1.1 Importance of Subject Area

Cardiovascular diseases (CVD) which arise from disorders of the heart, arteries and other blood vessels remain one of the leading causes of death worldwide, despite scientific advances. In the year 2012, the global burden of CVD had increased to 17.9 million from a previous value of 14.4 million in 1990 (WHO, 2015). Atherosclerosis, characterised by the pathological transformation of the arteries is the underlying cause of the majority of the deaths arising from CVD (Stone, 2012). It is a complex chronic disease in which there is an accumulation of fibrofatty deposits made up of lipids and fibrous element mainly in the inner lining (intima) of arteries (Lusis, 2000, Wang and Bennett, 2012). Atherosclerosis is a multifactorial disease; studies conducted over the past five decades have revealed different genetic and environmental factors associated with the disease (Goldbourt and Neufeld, 1988, Assmann *et al*., 1999, Gerhard and Duell, 1999). Until recently, CVD has been overshadowed by infectious diseases such as HIV/AIDS. This is evident by its omission in the United Nations Millennium Development Goals in 2000.

CVD in the UK has declined since the early 1970s but despite the reduction, it remains relatively high when compared to other western European countries. CVD still remains the biggest killer in the UK with about 180,000 deaths in 2010 (Townsend *et al.*, 2012). It has been predicted that by the year 2030, non-communicable diseases will be responsible for three-quarters of death occurring worldwide (Fuster and Kelly, 2010). In low and middle-income countries, CVD alone will be responsible for more deaths compared to other diseases (Beaglehole and Bonita, 2008). Due to the high mortality rate and incidence of heart diseases, the EU economy is estimated to spend almost 196 billion Euros a year on CVD (Nichols *et al*., 2012). Over the last decades, there has been a significant rise in cardiovascular diseases mainly

in developing countries. These changes have been attributed to lifestyle changes and nutritional transition (Herrington *et al*., 2016). The clinical consequence of atherosclerosis, heart attack and thrombotic strokes continues to be a huge burden. In spite of the progress made in decreasing mortality rate in the UK, the National Health Service in England incurred a cost of about 6.8 billion pounds from spending on CVD between the year 2012 and 2013 (Bhatnagar *et al*., 2015). CVD continues to be a burden on the country, in terms of cost and health.

Despite the lifestyle modification initiatives and development of effective drugs that can effectively reduce some of the risk associated with the disease such as high plasma cholesterol and blood pressure (Olsen *et al*., 2016, Cesena *et al.*, 2017), atherosclerosis and its clinical consequence, still remains a global burden (Herrington *et al*., 2016). The global burden of atherosclerotic cardiovascular disease in people living with HIV have been recently been suggested to be double that of the healthy population, as they are twice at risk of developing cardiovascular disease (Shah Anoop *et al.*, 2018). Hence the scenario of atherosclerotic cardiovascular disease is of global concern. The key issues to be addressed include systemic inflammation, clotting factors, hyperlipidaemia and the oxidation of low density lipoprotein (LDL) (Sharma *et al.*, 2013). LDL is believed to be a major contributor to the pathogenesis of atherosclerosis (Lewington *et al*., 2007).

1.2 Overview of Atherosclerosis

1.2.1 Histology of human artery

The large artery is made up of three distinct layers surrounding the arterial lumen. Each of these layers is made up of different cells and extracellular matrix (ECM). The innermost layer adjacent to the lumen is called the intima and is bound by a single layer of endothelial cells (ECs) on the side of the lumen and bordered on the peripheral side by the internal

elastic lamina, a sheet of elastic fibres. The intima is made up of proteoglycans and collagen as the main connective tissue matrix (Lusis, 2000). ECs are the main cellular component of this layer, although smooth muscle cells (SMCs) are sometimes found in this layer. The EC regulate a number of events including vascular tone (Sandoo *et al*., 2010) and leukocyte movement (Muller, 2014), among others. After the internal elastic lamina is the second layer, the media (the middle layer) which consist of layers of SMCs and elastic lamellae. The number of layers present is dependent on the size of the artery. The ECM, which binds the SMCs to each other, contains mainly collagen and elastic fibres with a lesser amount of proteoglycans. The outermost layer, the adventitia contains a loose matrix of SMCs, fibroblast, and collagen (Lusis, 2000, Stocker and Keaney, 2004).

1.2.2 Pathogenesis of Atherosclerosis

1.2.2.1 Features of atherosclerotic lesion

Atherosclerosis is characterised by hardening of the arteries which arises from the accumulation of fibrofatty deposits (made up of lipids and fibrous element) in the inner lining and the middle lining of the arteries (Lusis, 2000). These abnormalities in the arteries (atherosclerotic lesions) are classified based on their histological features and composition. The lesion is classified into six types and designated with roman figures according to their stage and sequential progression.

The first observable change is the type I lesion which contains enough lipids to trigger the formation of scattered foam cells (lipid-laden macrophages). These changes are seen more in areas that are susceptible to the lesion and capable of adaptive thickenings, (the thickening are common in everyone at birth but are only formed in response to mechanical force) (Stary *et al*., 1995). The accumulation of the scattered foam cells leads to progression to type II lesions,

fatty streaks (which are accumulated lipid-filled macrophages) which are formed at this stage. Continuous formation of lipid-laden cells (foam cells) and macrophages death leads to the formation of Type III lesions which contain extracellular droplets of lipids in addition to the foam cells. These early stage lesions can be observed within the first decade of life (Stary, 1983, Stary *et al*., 1995). The extracellular lipids in type III form the basis for the larger lipid core that characterises the type IV lesion. The core lipid is separated from the lumen by the thin tissue layer, which advances and becomes thickened to form a fibrous connective tissue (the lesion cap). They mature into the more advanced lesion (Type VI lesion) characterised by calcification of the fibrous region and appearance of ulcerations that are often visible (Stary *et al*., 1995).

1.2.2.2 Aetiology

The aetiology of atherosclerosis is often complex. In the last fifteen decades, there have been concerted efforts by researchers to explain the series of complex events that are associated with the onset of the disease. To explain these events several hypotheses have emerged over the years. Early studies proposed passive deposition of cellular components. The early hypothesis of atherosclerosis **"incrustation hypothesis"** by Rokitansky suggest that an atherosclerotic event arises as a result of deposition of fibrin in the arterial intima (Rokitansky, 1849). Virchow in 1856 proposed the **"lipid infiltration hypothesis''** which suggests the deposition of lipids in the arterial wall is responsible for the disease. The deposited lipid is taken up by macrophages and SMCs resulting in the formation of foam cells (Virchow, 1989).

Ross and Glomset in 1973, proposed the **"response to injury hypothesis''**. They suggested that the early event in atherosclerosis is due to endothelial damage which has a negative impact on normal endothelial function (Ross and Glomset, 1973). The injury increases the adhesion

of leukocytes and platelets to the endothelium. The recruited leukocytes bring about an inflammatory response by releasing a number of cytokines and growth factors. The inflammatory area is often associated with smooth muscle cells proliferation and migration from the media giving rise to an intermediate lesion. The macrophages and T lymphocytes recruited are the principal mediators of the early inflammatory response (Jonasson *et al*., 1986, Keaney Jr, 2000). The hallmark of atherosclerosis involves the uptake of LDL by macrophages and to some extent SMC to form foam cells (lipid-laden macrophages). The accumulation of foam cells leads to the continuation of the inflammatory response and formation of a localised lesion. Cellular apoptosis or necrosis occurs as a result of the continued inflammatory process. The associated release of growth factor, cytokines and proteolytic enzymes bring about the enlargement of the lesion. Blood flow can be impaired at some point when the artery is unable to compensate for the encroachment of the lumen.

The response-to-injury hypothesis previously believed the key initiating event in atherosclerosis is as a result of the endothelium desquamation (Stocker and Keaney, 2004). Later on, there was evidence that when atherosclerotic lesions develop, they are covered by an endothelial cell layer that is intact. This led to the refinement of this hypothesis. In 1999, Ross proposed that endothelial dysfunction can initiate atherosclerosis through enhanced permeability of the endothelium to LDL (Ross, 1999). This hypothesis was challenged by the fact that more LDL entered the normal artery than the amount that accumulated there (Carew *et al.*, 1984). The rate of entry was similar but areas with a large accumulation of lipoproteins are prone to developing lesions due to their ability to retain apoB-containing lipoproteins which are atherogenic in nature (Schwenke and Carew, 1989, Schwenke and Zilversmit, 1989). These observations lead to the development of an alternative hypothesis, the **‘‘response to retention hypothesis’’** which suggests that the inciting event for atherosclerosis is the retention of

lipoprotein with the recognition that endothelial permeability may have a role in the initiation of the disease if a portion of the infiltrated material is retained. Other functional modification of the endothelial layer which has been documented often appear later. For example, in rabbits, the expression of the vascular cell adhesion molecule-1 (VCAM-1) by endothelial cells on lesions appeared after four days of severe hypercholesterolaemia and foam cell formation (Li *et al*., 1993). On the other hand, aggregation and retention of lipoproteins were observed within hours of the onset of hypercholesterolaemia. This lead to the conclusion the endothelial changes during atherosclerosis such as expression of cellular adhesion molecules (CAM) is likely to be a consequence of the lipoprotein retained initially within the arterial wall (Williams and Tabas, 1998).

The link between blood flow and atherogenesis in the response-to- injury hypothesis prompted the studies on the effect of shear stress on cultured endothelial cells. Many alterations have been reported *in vitro* (Williams and Tabas, 1998), such as increased endothelial cell division, production of growth factors and adhesion molecules. Findings from *in vitro* studies support the contribution of shear stress to alterations observed in the endothelium in atherogenesis. *In vivo* studies, however, demonstrated that endothelial alterations induced by shear stress are not enough by themselves for the initiation of atherosclerosis. Possibly, stress-induced endothelial changes can contribute to the pathogenic process of atherosclerosis. The most relevant changes identified at pre-lesional sites are altered proteoglycan structure and retention of lipoprotein (Keaney Jr, 2000). The retention of lipoprotein appears to be closely associated with the constituents of the extracellular matrix. The apolipoprotein B-100 (apoB-100) component of LDL is retained within the arterial wall with tight linkage to proteoglycans that promote lipoprotein retention (Williams and Tabas, 1998).

Lipoprotein oxidation has also been proposed to be central to atherogenesis. The discoveries that modified LDL was toxic to endothelial cells (Hessler *et al*., 1983) and that ox-LDL is taken up faster by macrophages (Steinbrecher *et al.*, 1984) lead to **"the oxidative modification hypothesis''**, which proposed that LDL enters the intima, becomes oxidised and can cause endothelial dysfunction, inflammation and foam cell formation (Steinberg *et al*., 1989). The hypothesis is discussed in detail in (section 1.4.1). All the hypotheses have attempted to explain the complex nature of the cause of atherosclerosis focusing on a critical initiating event. A common feature is the deposition of lipids, particularly LDL. The oxidative hypothesis, in addition, showed the importance of oxidative events in the genesis of the disease.

1.2.3.2 Lesion initiation, inflammatory response and foam cell formation.

Studies with the cultured cells have suggested the ways in which the disease process begins and progresses. The major cause of atherosclerosis is believed to be the accumulation of lipoprotein in the subendothelial intima (Tabas *et al*., 2007). The accumulation of LDL, oxLDL, and other forms of modified LDL is believed to be the primary event of initiation. The build-up of oxLDL contributes to the infiltration of macrophages and formation of foam cells in the intima. Lipoprotein with an additional apolipoprotein (a) namely lipoprotein (a) (Lp(a)) has also been confirmed to be atherogenic (Morrisett, 2000). Studies with cultured cells have also suggested the initiating role of endothelial cells in mediating inflammation. oxLDL is toxic to the cells and hence induce endothelial damage.

The toxicity of oxLDL elicits a compensatory response from the immune system. The endothelial cells (ECs) subsequently become pro-inflammatory cells, thereby increasing the endothelial adherence of leukocytes and platelets through cell adhesion molecules (CAM),

such as selectins, and the production of growth factors, such as cytokines. The signalling proteins (cytokines and chemokines) trigger the movement of lymphocytes and monocytes to the site of activation on the surface of ECs. Chemokines play a major role in movement into the subendothelial region after binding to CAM on the surface. Vascular cell adhesion molecule 1 (VCAM-1) is specific for binding monocytes and T lymphocytes found in the newly developing lesion. Chemokines such as interferon (IFN γ), macrophage colony-stimulating factor (M-CSF), and monocyte chemoattractant protein-1 (MCP-1) are commonly found in the atherosclerotic lesion (Libby, 2002, McLaren *et al*., 2011, Ilhan and Kalkanli, 2015)**.** The role of CAM and growth factors in atherosclerosis has been shown by studies mice lacking selectins (Dong, 1998, Collins, 2000) and MCP-1 (Gu, 1998, Gosling *et al*., 1999, Ohman *et al*., 2010). There was a reduction in atherosclerotic lesions of apoE deficient mice lacking both intercellular adhesion molecule (ICAM)-1 and P-selectin (Collins, 2000).

Macrophages do not take up native LDL enough to generate foam cells but oxLDL (Goldstein *et al*., 1979a). Foam cells are formed from the uptake of massive amount of oxLDL. The mechanisms of oxidation of LDL and its relation to atherosclerosis is discussed in detail in section 1.4. In the event of atherosclerosis, lymphocytes and monocytes are recruited to the arterial wall. The importance of inflammation in atherosclerosis has been confirmed clinically and experimentally. Many lines of evidence support the important role of the inflammatory cells especially monocyte derived macrophage (Lusis, 2000, Wang *et al*., 2012, Ilhan and Kalkanli, 2015) due to the fact that cholesterol-enriched macrophages are a major indication of the early atherosclerotic lesion. The link between atherosclerosis and inflammation was recently reaffirmed by a large human trial (CANTOS trial) by Ridker et al which demonstrated that anti-inflammatory therapy, canakinumab significantly reduced incidents of cardiovascular disease without any influence on the level of lipids compared to placebo (Ridker *et al*., 2017).

However, the role of oxidised LDL as a mediator of inflammation in macrophages hence promoting atherosclerotic events (Schwarz *et al*., 2017, Lara-Guzmán *et al*., 2018) has remained of interest to researchers.

1.2.3.4 Atherosclerotic lesion macrophages

As described above, under atherosclerotic conditions, monocytes are recruited into the arterial wall. They differentiate into macrophages in a process regulated by specific cytokines with M-CSF as the most common growth factor involved (Waldo *et al.*, 2008). Macrophages are phagocytic cells found in nearly all tissues, which function in the removal of cellular debris, clearance of necrotic cells and apoptotic cells. They originate from myeloid progenitor cells that produce the neutrophils (another phagocytic cell) and dendritic cells involved in antigen presentation (Mosser and Edwards, 2008). Macrophages play a key role in all the stages of lesion development and progression. They are the major type of cells present in early lesions, contribute to the progression of the lesion through the rapid uptake of oxidised or modified LDL and the remnants of lipoproteins leading to the formation of foam cells (Lusis, 2000, Mosser and Edwards, 2008).

Modified LDL is taken up through receptors that recognize a wide range of ligand (scavenger receptors). The scavenger receptors are regulated by cytokines such as tumour necrosis factor (TNF-α) and interferon (IFN-ϒ) and region for oxidised fatty acids. The peroxisome-proliferator activated receptor (PPAR), a transcription factor containing a binding discovery of scavenger receptors confirmed the cells as the starting point for the formation of fatty streak, an indication of the onset of the disease and the centre for development of plaque (Libby, 2002, McLaren *et al*., 2011). The emergence of atherosclerotic lesion is largely dependent on monocyte-derived macrophages, with macrophages as the most abundant type of cells present

in atherosclerotic plaque, they possess the ability to contribute to the progression of atherosclerosis (Ilhan and Kalkanli, 2015). Due to the great influence they have on inflammation, cholesterol accumulation and transformation into lipid-laden foam cells, formation of necrotic core and degradation of the ECM.

1.2.3.5 Formation of fibrous plaques.

Vascular smooth muscle cells (VSMCs) contribute to the development of the fatty streaks into a more advanced form (fibrous plaques), this is usually covered with a fibrous cap. The core of the fibrous cap is made up of mainly cholesterol and its esters (Lusis, 2000), modified LDL and cells that are prone to apoptosis and necrosis due to their inability to obtain enough nutrient for their survival (McLaren *et al*., 2011). The SMCs contribute to the growth of the plaque through the formation of the fibrous cap and the production of extracellular matrix (ECM) (Wang *et al*., 2012). SMCs facilitate the migration of lymphocytes and monocytes by producing adhesion molecule (VCAM-1). Growth factors and cytokines such as platelet-derived growth factor (PDGF), MCP-1, transforming growth factor-β (TGF-β) produced by T cells and macrophages are important in the activation of leukocytes, the production of ECM, promotion of endothelial dysfunction and enhancing the proliferation of SMCs (Libby, 2002)

Recent studies have demonstrated the important role of CD40 (protein present on antigen presenting cells) expressed by T cells and macrophages in promoting advanced lesions. The CD40 interacts with its ligand to promote the production of proteases, which can degrade the matrix, adhesion molecules and inflammatory cytokine (Lusis, 2000, McLaren *et al*., 2011, Wang *et al*., 2012). Although the interaction was first seen in T and B cells, it has also been demonstrated in ECs and SMCs (Lusis, 2000). The formation of the fibrous cap is highly

dependent on the migration and proliferation of SMCs, which is in turn regulated by factors that are produced by T cells, endothelial cells and resident macrophages.

1.2.3.6 Plaque progression and vulnerability

It has been known for about four decades that plaque rupture is not only a determinant of clinical events but also a factor for the progression of the plaque. The association of angina and MI with plaque rupture in arteries with reduced blood flow was demonstrated by Davies and Thomas; the studies showed that the clinical manifestation was not only due to the narrowing of the lumen but also the change in morphology of the plaque. Pathological studies revealed that clinical events are triggered mainly by the vulnerability of the plague and its composition rather than the event of stenosis (narrowing of the blood vessel) (Davies and Thomas, 1985, Libby, 2002).

Advanced atherosclerotic plaques are classified as stable or vulnerable. As the plaque continues to build up, sudden rupture or break off can occur and the plaque becomes unstable. The plaques that are likely to become unstable and prone to present symptoms are termed vulnerable plaques. The progression of atherosclerosis later present symptoms which occur when the coronary blood flow is decreased to the point that the metabolic needs of the organs cannot be met. Restriction of blood flow to the heart or the brain may be due to blood thrombus formation (thrombosis) that can obstruct blood follow. The disturbance of blood flow initially presents as angina (pain from the heart) described as pressure, pain or squeezing arising from the heart. This can be stable for several years but the sudden rupture of the plaque brings about acute coronary clinical events of unstable angina, heart attack or myocardial infarction(MI) meaning (death of the heart muscle) (Arroyo and Lee, 1999). An acute event, such as thrombotic stroke, can occur when there is reduced blood flow to the brain.

Stable plaque consists of a thick fibrous cap, a small lipid core with few inflammatory cells, as opposed to the vulnerable plaques that are characterised with a thin fibrous cap, considerably large lipid core and numerous macrophages and T cells (Arroyo and Lee, 1999, Wang and Bennett, 2012). The stability of the plaque is dependent on the thickness of the fibrous cap, whose structure is maintained by matrix synthesis and degradation. Low synthesis and increased degradation of the matrix lead to a weakening of the fibrous cap, both of these processes are likely to be influenced by inflammatory cells (Arroyo and Lee, 1999). For example, macrophages are the main cells in lesions that produce matrix metalloproteinase (MMP). The MMPs are zinc dependent enzymes that degrade the ECM. T cells produce interferon γ (IFN-γ) which inhibits the synthesis of matrix by SMCs. ECM components are mainly produced by SMCs, hence the migration and proliferation of SMCs influence the stability of the plaque (Lusis, 2000, McLaren *et al*., 2011).

The intima of advanced atherosclerotic lesions becomes mineralized as their complexity increases. Mineralization of the lipid core is regulated by enzymes involved in regulation of calcification and bone tissue formation (ossification) (Wang et al., 2012). Calcification and neovascularisation (formation of new blood cells), can also influence the stability of atherosclerotic lesion (Lusis, 2000). The clinical consequence such as heart attack and stroke that occur in atherosclerosis are not usually due to the blockage of the lumen by the build-up of the advanced plaque but a manifestation of an event arising from plaque rupture or endothelial erosion and the initiation of thrombosis. The progression of atherosclerotic lesions from onset to well-advanced form is depicted in Fig. 1.1. Maintaining the stability of advanced plaques, hence preventing rupture seems a plausible way of preventing the occurrence of acute clinical events (Lusis, 2000, Halvorsen *et al*., 2008). The many factors involved in the destabilisation of the plaque makes this challenging.

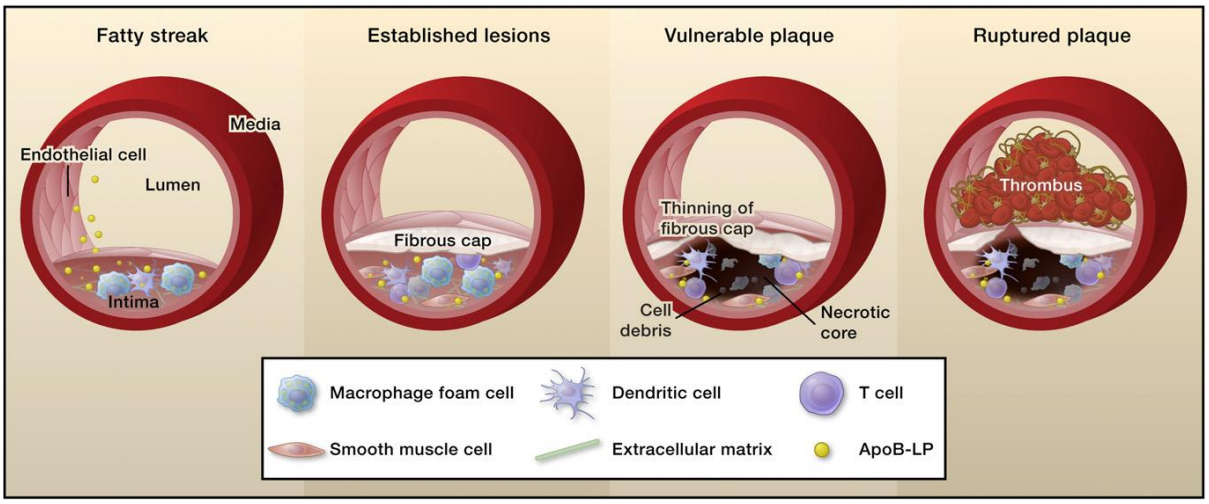


Figure 1.1: Schematic representation of the progression of atherosclerotic lesion.

The normal human coronary artery is characterised by three layers. The development of fatty streak arises from the deposition of lipids (Apo B-LP) mainly oxLDL which incites the recruitment of inflammatory cells. The accumulation of the lipids and the inflammatory cells (mainly lipid-laden macrophages) leads to a formation of a lipid core. As the lesion progresses, SMCs and T-cells infiltrate the intima leading to the formation of established lesions. The SMCs migration and proliferation leads to the formation of a fibrous cap. The vulnerable plaques (with thinning fibrous cap) results in a formation of necrotic core from the accumulated apoptotic cells. The thinning of the fibrous cap leads to the formation of unstable plaques susceptible to rupture, hence the development of a thrombus, which eventually brings about the acute coronary clinical event such as unstable angina, heart attack. Adapted from (Moore and Tabas, 2011).

1.2.4 Cell death in atherosclerosis

Apoptotic cell death has been well demonstrated in atherosclerotic plaque. The consequence of apoptosis is however dependent on the cells involved, where the cells are located within the plaque and stage of progression on the atherosclerotic lesions. Apoptosis of macrophages might possibly be beneficial in early lesions, but not in advanced lesions if the apoptotic bodies cannot be phagocytosed and cause inflammation (Tabas, 2005). Smooth muscle cells and macrophages have been shown to go through apoptosis in plaques (Boyle, 1999, Akishima *et al*., 2005).

Over the years several studies have emphasised the importance of apoptosis in contributing to plaque stability and the progression of atherosclerosis (Kockx and Herman, 2000, Tabas, 2005, Martinet et al., 2011). Factors such as hypoxia, oxidative stress, cholesterol overload and IFN-γ have been implicated in instigating apoptosis in atherosclerosis (Mallat and Tedgui, 2000, Van Vre *et al.*, 2012). The increased turnover of EC in atherosclerotic plaque is linked to enhanced apoptosis. Loss of SMC can contribute to the weakening of fibrous cap and the disappearance of SMCs in plaque has been attributed to apoptosis. Apoptotic macrophages are mainly present in cellular regions which are rich in macrophages which synthesise DNA (Kockx and Herman, 2000). Macrophages represent a greater number of cell deaths occurring in atherosclerotic lesions compared to leukocytes, SMCs and ECs, making up over forty percent of cell deaths (Kolodgie *et al*., 2000).

1.2.5 Epidemiology and risk factors

Atherosclerosis affects people with certain risk factors more than others. Over the years, many risk factors have been identified as a predictive factor for the development of atherosclerotic cardiovascular disease (Homma, 2004). Epidemiological studies over five decades have focused on the associated risk factors, largely grouped as genetic and environmental factors. Genetic predisposition to the disease can commonly be associated with the family history of CVD. The importance of genetic factors has been emphasised over the years. Asides the impact of genetic variations involved in lipid metabolism, other genes that

predispose to other risks factors of atherosclerosis are equally considered of importance (Indolfi, 2002). The heritability of coronary disease and the importance of genetic factors have been suggested by studies in twins (Marenberg *et al*., 1994, Zdravkovic *et al*., 2002).

Some of the factors that are strongly correlated to atherosclerosis are non-modifiable. Non-modifiable factors such as age and genetic factors e.g. familial hypercholesterolaemia cannot be changed. Modifiable factors such as diabetes, smoking, hyperlipidaemia and hypertension can change depending on certain lifestyle changes. It is compelling to note that most risk factors for atherosclerosis are risk factors for heart failure. The involvement of some of the risk factors as predictive factors for the development of CVD is described below:

1.2.5.1 Smoking

The risk of developing CVD from smoking is dependent on the level of smoking in terms of the number of cigarettes smoked per day and the age at which the person began smoking. The link between smoking and heart diseases dates as far back as studies which clearly linked smoking to the incidence of heart attack (Doll and Hill, 1956, Hammond and Horn, 1958). Evidence from in vivo and *in vitro* studies demonstrated the ability of cigarette smoke to induce LDL oxidation (Yokode *et al.*, 1996, Yamaguchi *et al*., 2001). Cessation of smoking leads to a reduction of risk of a heart attack in smokers, in two years the risk status of ex-smokers was near that of nonsmokers(Gaziano, 1996). Mons et al showed increased association of cardiovascular mortality in current smokers compared to former smokers and never smokers with smoking cessation having a beneficial effect in reducing the risk of cardiovascular mortality (Mons *et al*., 2015). Smoking is often classified as an important risk factor for CVD

1.2.5.2 Dyslipidaemia and Hyperlipidaemia

The term hyperlipidaemia or hyperlipoproteinaemia is described as the presence of abnormal elevated concentration of lipids or lipoprotein in blood and is the most common form of dyslipidaemia (abnormal level of lipids in the blood). The abundance of lipoprotein

in plasma is of importance in atherosclerosis, as high level of atherogenic lipoprotein serves as a precursor in most form of the disease. Individuals with an elevated level of LDL due to genetic inheritance or lifestyle have been confirmed to be a high risk of myocardial infarction(Goldstein and Brown, 1986).

The relationship between LDL-cholesterol and atherosclerosis is well established, lowering blood cholesterol leads to decrease incident of cardiovascular deaths in men with hypercholesterolemia (Shepherd *et al.*, 1995). Human studies on the lipid lowering drugs statins demonstrate a reduction in the incidence of CVD due to decreased LDL-cholesterol (Baigent *et al*., 2010, Mihaylova *et al*., 2012). A meta-analysis of large longitudinal cohort study showed the link between lipids and the risk of ischaemic heart disease (IHD) with LDL cholesterol having a direct association to IHD whereas HDL cholesterol was inversely related (Lewington *et al.*, 2007, Di Angelantonio *et al*., 2009). LDL is clearly causal, but it is not clear if HDL is causally protective.

1.2.5.3 Diabetes

Diabetic patients are three to five times more at risk of coronary atherosclerosis in spite of controlling other confounding factors (Mazzone *et al.*, 2008). The oxidation and glycation of LDL in diabetic patients can lead to the formation of modified LDL that is taken up by macrophages and generates foam cells which are associated with the onset of atherosclerosis (Knott *et al.*, 2003). Other risks such as abnormal lipids and hypertension are commonly found in diabetic patients (Bierman, 1992). There is an increased lipid peroxides level and increased serum cholesterol in diabetics patients (Suryawanshi *et al.*, 2006), which might predispose them to more cardiovascular disease. Both type 1 and type 2 diabetes promotes atherosclerosis with the level of blood glucose as the causal factor (Chait and Bornfeldt,

2009). Hence, diabetes is a major contributor to atherosclerosis with most deaths of diabetic patients arising from atherosclerosis.

1.2.5.4 Hypertension

Hypertension is defined as diastolic and systolic blood pressure above 90mmHg and 140mmHg, respectively. It is a risk factor for CHD, stroke and other CVD (WHO, 1996). Reduction of blood pressure (systolic blood pressure (SBP) below 140mmHg and diastolic blood pressure (DBP) below 90mmHg) has been seen to reduce the onset of CVD (Turnbul, 2003). In 2002 a longitudinal cohort study demonstrated a direct association between DPB and SBP and both IHD and stroke (Lewington *et al*., 2002). Hypertension might lead to thickening of the arterial wall or lesion fissuring. The benefit of treating hypertension was seen in both mild and severe cases at all ages within five years of treatment, the incidence of CHD and stroke was reduced to 14% and 40% respectively. The hypertension optimal treatment study demonstrated that aggressive antihypertensive therapy reduced the occurrence of a cardiovascular event in high-risk patients (Hansson *et al.*, 1998).

1.2.5.5 Age

The risk of CVD has been proven to increase with age, atherosclerosis is often regarded as a very good example of age-related disease. Atherosclerosis progresses with age, becomes more severe and universally observed more in older humans (Stout, 1987). The link is best observed when comparing the average risk of developing CVD at a certain age compared to increased age perhaps monitored over a period over ten years. Comparing the average risk of men at the age of 30-34years in the US to men at the age of 60-64years showed a seven fold increase (Wilson *et al.*, 1998). Examination of the aorta of elderly with magnetic resonance imaging (MRI) demonstrated that age might promote atherosclerosis (Chen *et al*., 2013). Age is an important non-modifiable factor, as atherosclerosis is often regarded

as part of the process of aging. Other factors such as diabetes and hypertension also increase with age.

1.2.5.6 Gender

Data from observational studies showed that males are at higher risk when compared to females of the same age. Both human and animal studies have documented the gender difference in the predisposition to CVD. The Framingham study by Kannel *et al*. showed that the rate at which males below the age of 60years developed CVD doubled that of women (Kannel *et al*., 1976). Hayashi *et al* showed that the formation of atherosclerotic lesions in male rabbits was greater than that of female rabbits fed the same high cholesterol diet (Hayashi *et al.*, 1995). The incidence is seen to accelerate in postmenopausal women, supported by the work of Kannel *et al.* which showed that the event of CVD was almost twice that of premenopausal women (Kannel *et al.*, 1976). It is controversial if sex hormones play a role in the development of CHD in women, as hormone replacement therapy did not protect against CVD in postmenopausal women (Manson *et al.*, 2003), although there is ample of evidence that oestrogen prevents atherosclerosis in animal models (Williams *et al.*, 1995). It is also evident that the deficiency of testosterone predisposes to atherosclerotic events (Oskui *et al*., 2013). Over the last decade there is greater emphasis laid on the crucial role, gender might have on the occurrence of CVD (Spence and Pilote, 2015).

1.2.5.7 Obesity

Obesity is a term used to describe excess body weight. Humans are classified as obese based on their body mass index (BMI), A range between 18.5-24.9 kg/m^2 is considered healthy or normal while values above 30 kg/m^2 are considered obese (Kopelman, 2000). Obesity is regarded as the starting point for some other risk factors such as dyslipidaemia, hypertension, insulin resistance and glucose intolerance (Wilson *et al*., 1999). A Study by McGill *et al* demonstrated that obesity is linked to increased atherosclerosis in young adult men (McGill *et*

al., 2002). The link between obesity and CVD is of growing concern due to the increase in the prevalence of obesity in developed society. Other studies have shown that depletion of visceral fat reduces risk factors associated with obesity such as impaired glucose metabolism, hypertension and dyslipidaemia hence preventing CVD (Shimano, 2009). Other factors such as endothelial dysfunction, inflammation, inflammasome activation, and adipokine imbalance have been proposed to link obesity to atherosclerosis (Lovren *et al.*, 2015).

1.2.5.8 Sedentary lifestyle and lack of exercise

Lack of physical exercise is a predisposing factor to CHD, aerobic exercise is associated with a lower risk of CVD. It has been revealed that individuals who expend more energy weekly are less likely to develop CHD (Thompson *et al*., 2003). Exercising regularly promotes anti-atherosclerotic properties such as increased HDL cholesterol concentration, less body fat, lower level of blood pressure and glucose, decreased LDL cholesterol and higher sensitivity to insulin (Assmann *et al*., 1999). Large population studies showed that physical inactivity doubles the risk of CVD compared to physically active individuals (Powell *et al*., 1987, Wannamethee *et al*., 1998). Increased level of physical fitness is inversely correlated with CHD and starting an exercise regimen has been suggested to reduce the risk of CVD. (Paffenbarger *et al.*, 1993, Wannamethee *et al*., 1998, Schroeder *et al*., 2007).The possible mechanistic events that might enhance reduced susceptibility to CVD was suggested to include but not limited to the release of less inflammatory mediators from adipose tissue, decreased thrombotic events, stabilised vulnerable plaques and enhancement of the endothelial function (Bowles and Laughlin, 2011).

1.2.5.9 Other factors

Small dense LDL, which is one of the phenotypes of LDL known as pattern B with a high proportion of small dense LDL particles, has been demonstrated to be linked with increased CAD (Austin *et al.*, 1988, Krauss, 2010, Arai *et al*., 2013). *In vitro* studies have shown that dense LDL had increased susceptibility to oxidation hence promotes increased atherogenic

effects (de Graaf *et al*., 1991). There is evidence on the possible link between accumulation Lp(a), the variant form of LDL (which contains additional apo (a) attached to apoB-100) and the level of fibrinogen with increased events of CHD (Thompson *et al*., 1995). The similarity in the sequence of its protein and that of plasminogen and relation to fibrinolysis suggest its important role in thrombosis (Gerald and Daphne, 2012). The evidence that Lp(a) is a cause of CAD was strengthened by Clarke *et al.*, who discovered an association between isoforms of Lp(a) and the development of CAD (Clarke *et al.*, 2009). Atherogenic properties of Lp(a) may be due to its similarity to LDL and the transport of cholesterol to areas with vessel injury and its high propensity to aggregate (Hajjar and Nachman, 1996).

Homocysteine produced as an intermediate product in the metabolism of sulphur containing proteins. Elevation of plasma homocysteine caused by homocystinuria is associated with atherosclerosis and premature thrombosis. Gerhard and Duell showed that an elevated homocysteine level is an independent risk factor for atherosclerosis. However, the contribution of homocysteine to the disease is still under investigation (Gerhard and Duell, 1999). Hyperhomocysteinaemia has been linked to endothelial dysfunction in humans (Tawakol *et al*., 1997, Woo *et al.*, 1997). The clinical trial of homocysteine lowering showed no effect (Armitage *et al*., 2010). Other studies have found high levels of homocysteine mediating vascular damage such as promoting mitogenesis of vascular smooth muscle cells (Tsai *et al.*, 1996), causing damage to endothelial cells (Starkebaum and Harlan, 1986), increased platelet aggregation (Durand *et al*., 1997).

C-reactive protein (CRP) and fibrinogen have also been identified as a novel risk factor for atherosclerosis (Ridker *et al*., 2001). Inflammation which regulates the acute phase proteins

such as serum amyloid A, fibrinogen, and CRP, have a vital role in atherosclerosis (Gabay and Kushner, 1999, Libby *et al*., 2002). Some population and clinical studies have demonstrated the association between these inflammatory mediators and increased cardiovascular events (Ridker *et al.*, 1998, Koenig *et al*., 1999). The relevance of the functional effects of CRP as a possible causal factor in atherosclerosis was discussed by Paffen and deMatt (Paffen and deMaat, 2006)

These factors can work independently or together to increase susceptibility to the risk of atherosclerosis. The interactions between the associated risk factors make an investigation into causal factors difficult, hence most common forms of CHD occur as a result of genetic susceptibility, aging, and environmental factors. Of note is the important link between LDL and most forms of the disease.

1.3 Lipoproteins and Atherosclerosis

1.3.1 Introduction to lipoproteins

Lipoproteins are formed from the combination of lipids and protein. Lipids are insoluble in aqueous solution. The combination with protein is necessary for the transportation of lipid through the blood stream to tissues. In general, lipoproteins are spherical particles in which the outer component is made up of polar proteins, phospholipids and non-esterified cholesterol (free cholesterol). This surrounds nonpolar molecules triacylglycerols (TG), free cholesterol and cholesteryl esters. The protein parts are often called apolipoproteins, which aside from their structural roles facilitate the cellular uptake of lipoproteins by serving as ligands for the lipoprotein receptors.

Lipoproteins differ in their composition, size, electrophoretic mobility and density. They are classified into chylomicrons, very low density lipoproteins (VLDL), low density lipoprotein (LDL) and high density lipoproteins (HDL). VLDL is synthesized in the liver and transport TG produced endogenously in the liver to peripheral tissues for energy needs, they contain apoB, apoC-II and apoE (apoC-II and apoE are acquired after secretion). LDL is mostly derived from delipidated VLDL when it forms VLDL remnants that can be degraded further to form LDL, but a small portion is directly released from the liver. It transfers cholesterol from the liver to peripheral tissues. It contains only ApoB-100 proteins. HDL is the main transport form of cholesterol from peripheral tissues to the liver. They contain apoA1 with apoA2, apoC and apoE (Goldstein and Brown, 1977, Gerald and Daphne, 2012).

The function of HDL and LDL in cholesterol transport gives them clinical significance in atherosclerosis, the major component of arterial lesion is cholesterol-enriched cells. Elevated level of serum HDL is negatively correlated with incidence of CVD. The HDL is said to be protective and anti-atherogenic in nature, although this has not been proven. However, LDL is positively correlated with the incidence of CVD and pro atherogenic in nature (Gerald and Daphne, 2012).

1.3.2 Lipid Metabolism

1.3.2.1 Endogenous lipid pathway

Lipoproteins can be synthesised from triglycerides and cholesterol in the hepatocytes. In the liver triglycerides and cholesterol are attached to apo B to form VLDL. It is formed through lipidation of ApoB-100, these VLDL rich in triglyceride enters the plasma to deliver triacylglycerol to muscle and adipose tissue through lipoprotein lipase activity (Feingold and Grunfeld, 2000). VLDL is degraded by lipoprotein lipase found on endothelial cells to form VLDL remnants or intermediate density lipoprotein (IDL) leading to the detachment

of Apo C while ApoE remains attached to the IDL particle (Murdoch and Breckenridge, 1996). The IDL in circulation can be taken up by the liver through the interaction of ApoE/ApoB-100 with the IDL receptor (Mahley and Ji, 1999) or can be further degraded to form triglycerides and fatty acids by hepatic lipase (Zambon *et al*., 2003) leaving the IDL remnant known as LDL behind. The metabolism of LDL is discussed in section 1.3.4.

1.3.2.2 Exogenous lipid pathway

The exogenous lipid pathway begins in the intestine where lipase hydrolyses dietary triglycerides into monoacylglycerol and free fatty acids emulsified with cholesterol, fat soluble vitamins, plant sterol and bile acids to form micelles which are then transported into the intestinal cells. The uptake of plant sterol and cholesterol is mediated by Niemann-Pick C1-like protein (Feingold and Grunfeld, 2000). The enterocytes convert the fatty acids and monoacylglycerol back to triglycerides. ApoB-48 and triglycerides are incorporated into chylomicrons through microsomal transfer protein (MTP) (Hussain, 2014). Chylomicrons bypass the liver circulation entering the blood stream via the lymph where they acquire Apo C's and Apo E from HDL(Ramasamy, 2013). ApoC2 activates lipoprotein lipase present in the endothelium of capillaries, which removes triglycerides from the core to produce a chylomicron remnant. The chylomicron remnant releases its surface material to HDL leading to the formation of a smaller size chylomicron which passes through the capillary endothelium in the liver and then taken up through the binding of ApoE to the LDL receptor family.

1.3.2.3 Reverse cholesterol transport

HDL mediates the transportation of cholesterol from extrahepatic tissues back to the liver for it to be excreted in bile, this process is known as reverse cholesterol transport. The formation of nascent HDL occurs in the hepatocytes and enterocytes through the addition of free cholesterol and phospholipids to ApoA1 by the ATP-binding cassette transporter A1 (ABCA1) (Kiss *et al*., 2003, Hussain, 2014). The phospholipid transfer protein transfers phospholipid to

HDL from chylomicrons when they become hydrolysed to chylomicron remnants (Huuskonen *et al*., 2001). The phospholipid-rich HDL becomes matured by ABCA1 adding free cholesterol to them (Ge *et al.*, 2013). Most of the cholesterol is then converted to cholesteryl ester by lecithin-cholesterol acyltransferase (LCAT) bound to HDL and the esterification causes them to relocate to the core of the HDL particle. The cholesteryl ester transfer protein (CETP) exchanges the esters with triglycerides derived from other classes of lipoprotein (Chajek and Fielding, 1978). Alternatively, the cholesteryl esters and cholesterol can be released in the liver by binding scavenger receptor class B1 (SR-B1) (Leiva *et al.*, 2011).

1.3.3 Low density lipoprotein (LDL)

LDL is a small particle, which may vary in diameter in diameter between 20-25 nm and molecular weight of about 2 to 3.5 million Daltons. The density ranges between 1.019 g/ml – 1.063 g/ml, the variation in size is due to the extent of metabolism by hepatic lipase (Goldstein, 1977). Generally, the LDL particle (Fig 1.2) is made up of 75% lipids, mainly cholesteryl esters and 25% protein. It has an outer monolayer made up of phospholipid, free cholesterol and ApoB-100 protein (Steinberg, 2002). It contains one molecule of Apo B and on average about 185 molecules of TG, 700 molecules of phospholipids (PL), mainly phosphatidylcholine, 1600 molecules of cholesteryl esters and 600 molecules of non-esterified cholesterol (free cholesterol) (Steinberg, 1997b). It also contains a certain amount of antioxidants, such as α- and γ-tocopherol, β-carotene and lycopene (Jessup *et al*., 2004, Siess, 2006). LDL has long been identified as a causal factor in atherosclerosis. Elevated LDL level in plasma was first recognised to be strongly associated with atherosclerosis by Gofman and colleagues in 1950 (Gofman *et al*., 1950).

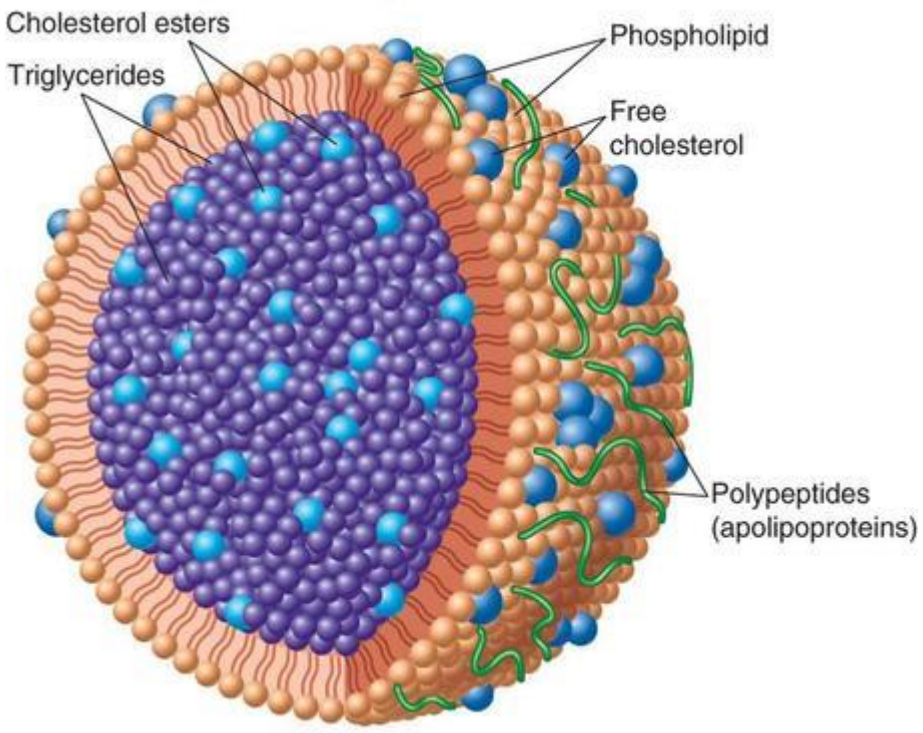


Figure 1.2 Structure of LDL

Source:(Rausch and Kortleever, 2011)

1.3.4 LDL metabolism

The uptake of LDL is dependent on LDL receptor-mediated endocytosis. The LDL receptor was discovered by Brown *et al*. in 1973 from the study on cultured human cells (Brown *et al*., 1973). The most abundant LDL receptors are present in the hepatic cells, but they are present in most other cells as well. The receptors are located in clathrin-coated pits in specialized regions. The binding of LDL to its receptor is through the apoB-100 region of the molecule. The process by which LDL binds its receptor and LDL cholesterol uptake in cells is a highly regulated event. The apo-B-100 protein binds to the receptor, the LDL receptor complex is internalized via endocytosis. The endocytotic vesicle formed fuses with a lysosome. The content of LDL along with its apoB-100 content are hydrolyzed by enzymes present in

lysosomes (hydrolases), to form free cholesterol and amino acids. The LDL receptor is recycled back to the surface of the cell where it can bind more LDL particles (Brown *et al*., 1973, Goldstein and Brown, 1977, Goldstein and Brown, 1979).

1.3.5 Role of LDL in atherosclerosis

Lipoproteins, particularly LDL are a common feature of the hypothesis on atherogenesis. Individuals with high concentrations of LDL due to genetic inheritance or lifestyle are more susceptible to heart attack. Genetic, epidemiological and clinical studies have provided some evidence in support of the association between LDL and atherosclerosis. The most convincing link of LDL to was seen in familial hypercholesterolaemia (FH). This is a genetic disease and if homozygous and untreated, the patient's death from coronary atherosclerosis occurs in the second decade of life due to the accumulation of LDL arising from the absence of the LDL receptor (Goldstein and Brown, 1973).

Autosomal dominant hypercholesterolaemia (ADH) is also a genetic disease associated with increased levels of LDL cholesterol and CVD. The mutation of three genes (ApoB, LDL receptor and proprotein convertase subtilisin-kexin type 9 (PCSK9)) is associated with ADH (Marduel *et al*., 2010). PCSK9 is important in the regulation of LDL-cholesterol, it interacts with LDL receptors in the liver and targets the LDL receptors for degradation in lysosomes and regulates LDL concentration in plasma. Inactivation of PCSK9 extends the life span of the LDL receptors and leads to decreased LDL cholesterol plasma concentration and reduced CHD (Farnier, 2014, Tavori *et al*., 2015, Shapiro and Fazio, 2017). The effect of PCSK9 on LDL receptor led to the development of active drugs to lower plasma LDL cholesterol thereby reducing CVD.

Several epidemiological studies have supported the proatherogenic role of the Apo B-100 containing lipoprotein (Kannel *et al*., 1984) and the fact that clinical studies have shown that lipid-lowering drugs, such as statin therapy, decrease the risk of CVD is an important indication of the association between LDL and CVD (Serruys *et al*., 2002, Baigent *et al*., 2010). It was anticipated that LDL will show an atherogenic effect *in vitro* because of its role as a major cholesterol transporter to tissues and the fact that early lesion of atherosclerosis is characterised by cholesterol-laden macrophages. This was opposed by the failure of LDL to increase cholesterol accumulation in macrophages despite the high concentration of LDL incubated with the cells (Goldstein *et al*., 1979b, Fogelman *et al*., 1980). *In vitro* studies demonstrated that macrophage uptake of LDL is too low to cause the sufficient accumulation found *in vivo* and these led to the suggestion that LDL may be modified *in vivo* to a form that is rapidly taken up through a different receptor (Goldstein and Brown, 1979). The down regulation of the LDL receptor by the increased intracellular amount of cholesterol via the sterol regulatory element binding protein 2 (SREBP2) might be the reason to the inability to increase the cholesterol level of macrophages by way of LDL receptor (Steinberg *et al*., 1989, Steinberg, 1997a). The LDL is likely to undergo changes that can affect its usual pathways to cells and alter their interaction with surface receptors on the cells (Chait, 1987).

1.4 Oxidative Modification of LDL and Atherosclerosis

1.4.1 LDL modification hypothesis

The hypothesis originated in 1979 when Goldstein and Brown discovered that patients with homologous FH who lack functional LDL receptors were still able to accumulate foam cells like hypercholesterolaemic patients that possess normal LDL receptor (Goldstein *et al*., 1979b). They hypothesized that LDL might be altered prior to the uptake. To test the hypothesis, they

tried modifying LDL to identify the forms of LDL that will lead to accumulation of cholesterol in macrophages. Acetylated LDL (acetyl-LDL) provided higher uptake of LDL and the uptake was mediated by receptors identified and characterised as scavenger receptor A (SRA) in macrophages (Brown and Goldstein, 1983). Henriksen *et al*., discovered that incubation of endothelial cells with LDL for 24 hours led to modification of LDL (Henriksen *et al.*, 1981) to a form that was recognized by peritoneal macrophages via the acetylated receptor similar to that which was previously described by Goldstein and colleagues (Goldstein *et al*., 1979b). Later studies showed that LDL was oxidatively modified by endothelial cells and other cell types (Steinbrecher *et al*., 1984, Witztum, 1993) and these findings led to the proposal of the LDL modification hypothesis.

The LDL oxidation hypothesis proposed that oxidative modification is involved in the onset of atherosclerosis and also contributes to the progression of the disease. The presence of oxLDL in circulation is a known marker of atherosclerosis, hence a risk factor for the development of CVD (Stocker and Keaney, 2004). There is compelling evidence that atherosclerotic lesions of both animal models and humans contain oxLDL. Small amounts of oxidised LDL can be observed immunologically in plasma, using specific monoclonal antibodies such as FOH1a/DLH3 that can bind to the epitopes of oxidised lipids bound to apo B-100 (Itabe *et al*., 1994). The amount of detectable oxLDL was significantly elevated in diabetes, renal and coronary heart disease (Itabe, 2003, Tsimikas, 2006, Maiolino *et al*., 2013). The use of antibodies staining against oxLDL has demonstrated the presence of oxLDL in atherosclerotic lesions and not in the arterial wall in hypercholesterolaemic rabbits (Boyd *et al*., 1989), humans (Ylä-Herttuala *et al*., 1989).

Evidence of oxLDL as the main culprit in atherogenesis, rather than native LDL has been well demonstrated in several studies which showed that modified forms of LDL such as oxidised and acetylated form are insatiably taken up by macrophages through the scavenger receptors. The principal receptors responsible for the binding and uptake of oxLDL are the scavenger receptor class A and CD36 (Collot-Teixeira *et al*., 2007). Unlike the LDL receptor, the scavenger receptors are not downregulated by the presence of oxLDL hence the substantial accumulation of cholesterol in macrophages leading to the formation of foam cells (Witztum and Steinberg, 1991, Yoshida *et a*l., 1998). There are very strong line of evidence, that the *in vivo* modification of LDL increases its ability to induce inflammatory response and promote atherosclerosis, this is supported by the accumulation of oxidised LDL (oxLDL) in animal models with atherosclerosis (Steinberg *et al*., 1989, Harats *et al*., 2000) and the presence of oxidised lipids in both chemical and immunological analysis of atherosclerotic plaques in humans (Kritharides *et al*., 1998). LDL oxidation leads to the particles of LDL having immunogenic epitopes promoting the formation of antibodies against them, these antibodies have been found in many advanced atherosclerotic lesions of patients (Erkkila *et al.*, 2000, Inoue *et al*., 2001). The main indication of this hypothesis is peroxidation of LDL promotes atherosclerosis.

1.4.2 Proatherogenic effects of oxidised LDL

Many lines of evidence support the role of oxLDL in foam cell formation and lesion advancement. There are strong indications, that the *in vivo* modification of LDL increases its ability to induce inflammatory response and promote atherosclerosis, this is supported by the accumulation of oxidised LDL (oxLDL) in animal models with atherosclerosis (Ylä-Herttuala *et al*., 1989) and the presence of oxLDL oxidised lipids in both chemical and immunological analysis of atherosclerotic plaques in humans (Kritharides *et al*., 1998). They induce

inflammatory responses by activating genes for the synthesis of cellular adhesion molecules and chemotactic proteins (Boullier *et al*., 2001). Extensively oxidised LDL can also inhibit the outward movement of macrophages from the arteries (Quinn *et al*., 1987), hence they remain in the arteries and further accumulate or undergo apoptosis in them (Tabas, 2005).

OxLDLs are cytotoxic to many cells, Chisolm *et al*. revealed that oxidised LDL was cytotoxic to the cells of the arterial wall, and thereby proposed that oxidised LDL may be significant for atherogenesis (Hessler *et al*., 1979). The ECs are susceptible to LDL toxicity, the accumulation of oxLDL can thus lead to endothelial dysfunction (Pech *et al.*, 1992) and promote a further inflammatory response. The ability of LDL to cause cell death or injure cells is a general result of oxidation irrespective of the mode of oxidation. The potency of OxLDL to kill cells is often attributed to the degree of oxidation, although moderately oxidised LDL can also kill cells (Morel *et al*., 1983, Hessler *et al*., 1983, Siow *et al*., 1999b). Two decades later Chisolm and colleagues identified the oxidised cholesterol content (mainly cholesterol hydroperoxides) as a major factor responsible for the injurious nature of oxidised LDL (Colles *et al*., 2001b). Katouah *et al* recently showed that oxidised LDL triggered cell death in human macrophages via upregulation of intracellular production of reactive oxygen species (ROS) and inhibition of the major catabolic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and aconitase (Katouah *et al*., 2015). Berliner and others reported that minimally oxidised LDL increased the binding of monocytes to endothelial cells through the upregulation of production of the differentiating factor macrophage-colony stimulating factor (M-CSF) and the chemokine monocyte chemotactic factor (MCP-1) (Cushing *et al*., 1990, Berliner *et al*., 1990). OxLDL upregulates the production of metalloproteinases, major factors in thrombosis due to their contribution to destabilising fibrous cap in advanced lesions (Rajavashisth *et al*., 1999). The association of oxLDL with peripheral, coronary, preclinical atherosclerosis and vulnerable

plaques has been demonstrated in humans (Ylä-Herttuala *et al.*, 1989). All this evidence put together signifies the potential importance of oxLDL as a biomarker for atherosclerosis and its contribution to atherogenesis.

Despite the presence of small amounts of oxidatively modified LDL in plasma and its isolation from the atherosclerotic lesion of animals and man and its proatherogenic nature, the mechanism by which LDL is oxidatively modified *in vivo* is still largely debated. Most studies on the process are done *in vitro*. Understanding how LDL is oxidised is critical in inhibiting the process and its contribution to the onset and progression of the disease.

1.4.3 Oxidation of LDL

Oxidised LDL has been broadly classified into minimally oxidised LDL (MM-LDL) and fully or extensively modified LDL (oxLDL). The MM-LDL is less modified and can still be recognized by the LDL receptor. However, it is capable of increasing M-CSF and MCP-1 release by endothelial cells (Liao *et al*., 1991). The extensively oxidised OxLDL has lost its recognition by the LDL receptor but can be recognized by scavenger receptors such as SRAI/II and CD36 on vascular cells (Levitan *et al.*, 2010). MM-LDL and OxLDL differ from each other in terms of what they contain and their effect in the body, both types differ from native LDL. Modification of LDL includes aggregation, lipolysis, proteolysis and oxidation. LDL is readily denatured, aggregated and easily prone to oxidation. LDL can be extensively aggregated by vortexing for few seconds (Khoo *et al*., 1988)

The oxidation of LDL is often complex as both the protein and the lipid component can be modified. It was originally described as LDL containing modified protein product. Evidence

from previous research suggested the formation of hydroperoxides of proteins prior to the formation of lipid hydroperoxides and the modified protein might play a role in the oxidation of lipids (Gebicki *et al.*, 2000, Gieseg *et al.*, 2000). However, the evidence implicating oxLDL in atherosclerosis has been attributed mainly to the lipid component. LDL with oxidised protein without changes to its lipid component is yet to be identified whereas oxidation of LDL can occur with little or no change to the apoB-100 (Parthasarathy *et al.*, 2010). Hence oxLDL has been defined as LDL containing the products of lipid oxidation such as the peroxides and their catabolic products (Levitan *et al*., 2010). A recent review by Davies, however, suggests that depending on the concentration and reactivity protein is the main target for biological oxidants although this has been linked to the pathology of some human diseases the link to causality of the diseases is yet to be confirmed (Davies, 2016).

A number of lipid peroxidation products arise from the peroxidation of lipids (Esterbauer et al., 1992). Lipid peroxidation is the process whereby initiators of oxidation or radicals abstract an electron from lipids. Radicals are highly reactive chemical species that can exist independently and possess one or more lone pair electron (Aruoma, 1999, Sparrow *et al.*, 1992). The lipid peroxidation reaction occurs in three major overlapping steps: initiation, propagation, and termination steps. In the first step, a lipid radical is produced. At the propagation stage, the unstable lipid radical produced readily reacts with molecular oxygen to create a peroxyl-lipid radical, another unstable species that reacts with another lipid to yield a lipid radical and lipid-peroxide. The reaction proceeds as the next lipid radical reacts in a similar manner. The reaction of the radical with a non-radical continues to yield another radical (chain reaction mechanism). The termination reaction occurs when radicals react with each other to form a non-radical (Schafer *et al*., 2000).

L-H + oxidant$^{\cdot}$ $\longrightarrow$ L$^{\cdot}$ + oxidant-H (initiation)

$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$

$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$ (propagation)

$L^{\cdot} + L^{\cdot} \longrightarrow$ Non radical products (NRPs) (termination)

$L^{\cdot} + LOO^{\cdot} \longrightarrow$ NRPs

Equation adopted from Schafer *et al*, (Schafer *et al.*, 2000) L-H = lipids**,** $L^{\cdot}$ = lipid radical, LOOH=hydroperoxide, $LOO^{\cdot}$ = peroxyl radical

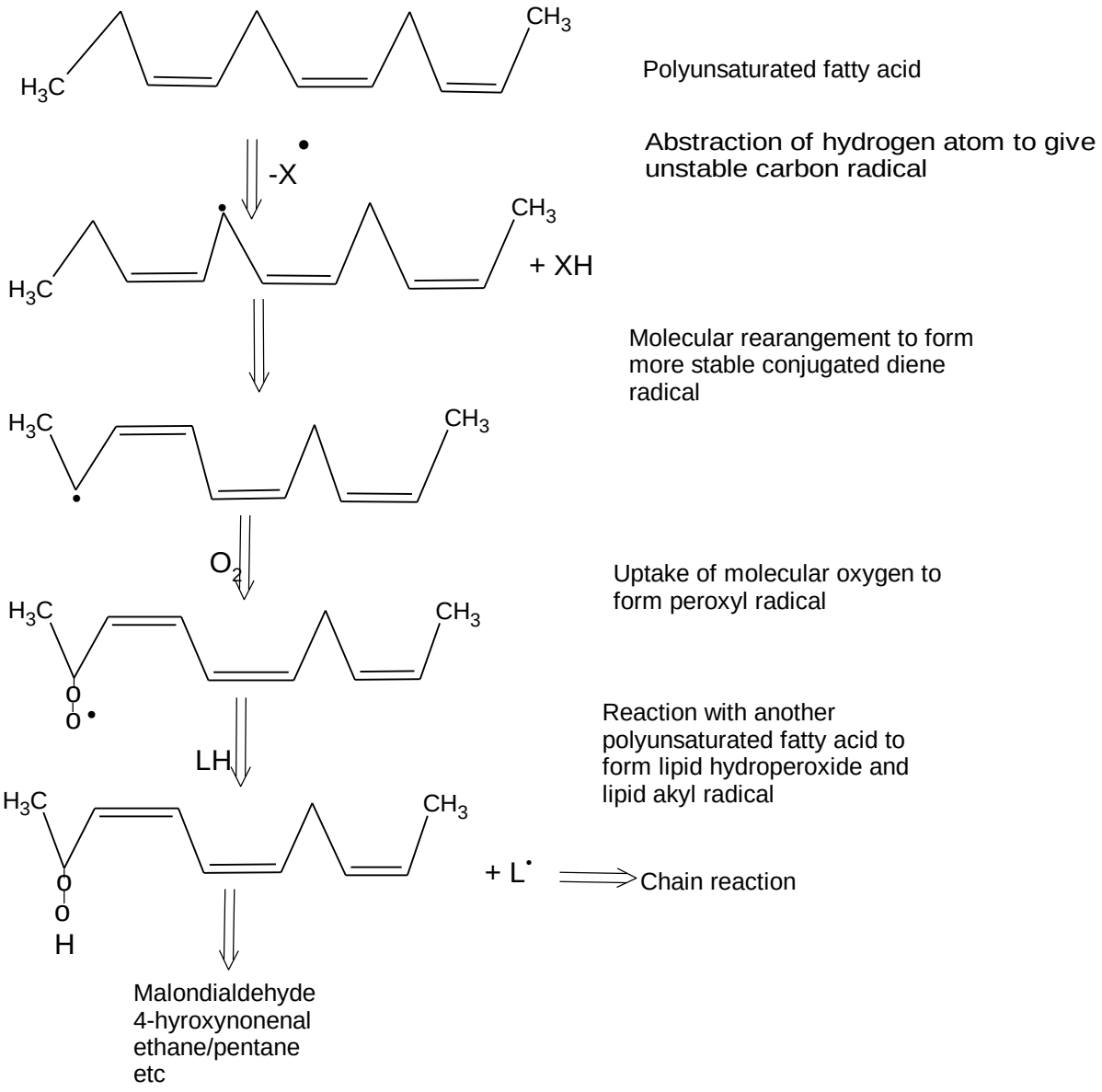


Fig 1.3: Schematic representation of peroxidation reaction in polyunsaturated fatty acid

Diagram created with ChemSketch software. Modified from (Young and McEneny, 2001)

The major component susceptible to oxidation is the polyunsaturated fatty acids (PUFA) of the lipids present in LDL (the mechanism involved is illustrated in figure 1.3). PUFAs are more susceptible to oxidation because they possess multiple bonds. The autoxidation of PUFA leads to the rearrangements of double bonds and the formation of conjugated dienes (a product containing two conjugated carbon double bonds) and their products. The carbon centred conjugated radicals have very short lifetimes. The rearrangement of bonds and reaction with molecular oxygen give rise to hydroperoxides the first stable products that are likely to be conjugated dienes. For example, the oxidation of linoleic acid leads to formation of well-known conjugated hydroperoxides, hydroxyoctadecanoic acids (HPODE) such as 9-HPODE and 13-HPODE which can further be transformed by isomerisation or chain cleavage to for further oxidation products or reacts with a lipid radical to promote the propagation step (Schneider, 2009). The chain reactions lead to formation of different products of oxidation.

Products of lipid modification include oxidised free fatty acids (FA), sphingolipid, and phospholipid products, oxysterols, cholesteryl ester oxidised on the fatty acyl moiety and short chain aldehydes. Further oxidation gives rise to hydroxenonenal (HNE) one of the most abundant aldehydes found in oxLDL (Levitan *et al.*, 2010). The amino acids present in lipoproteins can react with aldehydes to form adducts. HNE can readily react with cysteine and histidine residues (Uchida and Stadtman, 1992). Malondialdehyde (MDA) is also an important aldehyde product of lipid peroxidation and are able to react with lysine residues of apoB. MDA-modiodified LDL correlated with LDL-cholesterol in plasma of patients with CHD (Viigimaa *et al*., 2010).

The oxidation of the core of rich in cholesteryl esters leads to formation of more lipid hydroperoxides which are further degraded into aldehydes (Leitinger, 2003). Cholesteryl ester hydroperoxides components have been suggested to enhance pro-inflammatory response in macrophages and identified as major constituents of LDL that are moderately oxidised (Harkewicz *et al*., 2008). Phospholipids within LDL are targets of lipid peroxidation from attack by oxidant or free radicals. The oxidation of phospholipids and the derived oxidation products depends on the type of oxidant involved in the initiation process, the length of fatty acyl group attached and the link that is present between the glycerol backbone and the fatty acyl group (Spickett *et al*., 2011, Reis and Spickett, 2012). Phosphatidylcholine derivative 1-palmitoyl-2-(5-oxo-pentanoyl)-3-glycero-PC (POVPC) and other derivatives similar in structure were found in atherosclerotic plaques and minimally oxidised LDL (Watson, 1997, Hoff *et al*., 2003). The role of oxidised phospholipids products in atherosclerosis was reviewed by Lee and colleagues (Lee *et al*., 2012), they suggested that the effects of oxidised phospholipid could be attributed to different signalling pathways and gene regulation among other things. Oxidised phospholipid products obtained from phospholipid oxidation mediated by HOCL was shown to have anti proliferative effects, cytotoxic and increased levels of reactive oxygen species in endothelial cells (Robaszkiewicz *et al*., 2014). The oxidised phospholipids products can be detected by electrospray mass spectrometry which can detect oxidation products using the fragmentation patterns and can detect the phospholipid head groups, the chlorine and hydroperoxide contents of the oxidised phospholipids (Reis and Spickett, 2012). Ceroid is also a product of LDL oxidation that has been found in atherosclerotic lesion as a product of LDL oxidation. It contains complexes of protein and polymerized insoluble lipid (Mitchinson, 1982).

1.4.4 Reactive oxygen species and atherosclerosis

The possible role of reactive oxygen species (ROS) in atherogenesis has been examined over the last three or four decades and this has often been linked to the LDL oxidation theory. There is also evidence which suggests that certain risk factors of atherosclerosis, such as aging, smoking diabetes mellitus and arterial hypertension, leads to increased production of ROS from endothelial cells, adventitial cells and smooth muscle cells (Ohara *et al*., 1993, Gozin *et al*., 1998, Vogiatzi *et al*., 2009). The main origin of ROS and oxidants in atherosclerotic vessels are macrophages and smooth muscle cells. Several studies have demonstrated that oxLDL stimulates the production of ROS in macrophages, VSMCs and endothelial cells (Hsieh *et al*., 2001, van Aalst *et al*., 2004). It has also been suggested that ROS derived from macrophages are possible regulators of matrix metalloproteinase which can then lead to plaque instability (Rajagopalan *et al.*, 1996). The role of oxidative stress has been evident in humans with observed significantly lower levels of malondialdehyde in healthy patients as compared to patients with myocardial infarction and unstable angina (Dubois-rande *et al*., 1994). Superoxide anion was suggested to participate in LDL oxidation in human monocytes and enhanced formation of cytotoxic modified LDL (Cathcart *et al*., 1989). High levels of extracellular and intracellular reactive nitrogen and oxygen species might play a vital role in development of atherosclerosis through vascular homeostasis.

1.4.5 Potential Mechanisms of LDL modification

Several mechanisms have been proposed for the oxidation of LDL. Most of the oxidative enzymes have been implicated in the oxidation by cells and also the non-enzymatic oxidation by transition metals and other catalysts. *In vitro* oxidations of LDL have been studied extensively using peroxidative enzymes and redox active transition metals (Yoshida and Kisugi, 2010).

1.4.5.1 Myeloperoxidase

Myeloperoxidase (MPO), an enzyme linked to oxidative stress and inflammation can generate hypochlorous acid (HOCl) and other chlorinated biological molecules from hydrogen peroxide and chloride. 3-Chlorotyrosine a product of MPO-catalysed reaction was shown to be significantly increased in LDL that was extracted from atherosclerotic lesions (Himmelfarb *et al*., 2001). The previous study by Carr *et al* suggests that MPO may contribute to modification of LDL due to its ability to bind to LDL in the presence of robust antioxidants such as albumin, urate, and ascorbate (Carr *et al*., 2000a). The modification of LDL by MPO mainly transforms the apolipoprotein moiety through tyrosines (nitrotyrosine, chlorotyrosine, and dityrosine). The study by Spickett and colleagues, however, suggested that the modification of the lipid component is largely determined by pre-existing hydroperoxides and pH (Spickett *et al*., 2000, Zouaoui Boudjeltia *et al*., 2004). The fact that macrophages and neutrophils can express MPO, generates H_2O_2 suggest that MPO mediated system might be involved in physiological oxidation of LDL (Schindhelm *et al.*, 2009). MPO-oxidised LDL can trigger an inflammatory response in endothelial cells and macrophages by inducing TNF-α and IL-8 hence promoting atherosclerosis (Delporte *et al*., 2013).

1.4.5.2 Lipoxygenase

Lipooxygenase (LOX), is one of the oxidative enzymes in cells that directly oxidises PUFA. *In vitro*, LOX has been shown to oxidize LDL, through direct oxidation and indirect oxidation (non-enzymatically) by the production of reaction products that can further promote the peroxidation of the lipids (Heydeck *et al*., 2001). The mRNA of the protein and the protein itself has been found to be present in the human atherosclerotic tissues. A slight increase in the expression of the protein in rabbit resulted in an increased level of antigenic determinants for oxidation products (Yla-Herttuala *et al*., 1990, Yla-Herttuala *et al*., 1995). LOX with the antigenic determinant of oxLDL has also been identified in human atherosclerotic lesion. All

these suggest that the enzyme might have a role in the physiological oxidation of LDL and the onset of atherosclerosis.

The relevance of LOX was supported when Cyrus *et al.*, showed that apoE deficient mice lacking the genes for 12/15 LPO enzyme showed decreased lipid peroxidation product in plasma and the urinary concentration of isoprostanes had a positive correlation with the extent of the disease and enzyme activity (Cyrus, 1999). Lipoxygenase might be able to contribute to LDL oxidation by reacting with its lipid component directly or stimulating the production of ROS which can oxidise LDL (Delporte *et al*., 2013). In contrast, overexpression of LOX in both Watanabe-heritable hyperlipidaemia (WHHL) and cholesterol-fed rabbits were protected against atherosclerosis (Shen *et al.*, 1996). Also, the knockout of LOX in apoE deficient mice did not reduce atherosclerosis (Merched *et al*., 2008). This contradictory evidence suggests that LOX can protect or promote atherosclerosis. The dual role of LOX makes its role in the oxidation of LDL *in vivo* controversial.

1.4.4.3 Reactive nitrogen species

Reactive nitrogen species (RNS) have also been implicated in the oxidative modification process. Nitric oxide (NO) produced by ECs of the artery is a potent antioxidant possessing a number of anti-atherogenic properties such as reducing the vascular pressure and preventing the adherence of platelets and leukocyte to the endothelium (Heinecke, 1998). However, in the presence of superoxide anion ($O_2^{\cdot -}$), NO can be pro-atherogenic. It generates peroxynitrite ($ONOO^-$).

$$NO + O_2^{\cdot -} \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH$$

$$ONOOH \longrightarrow NO_2 + OH^{\cdot}$$

Peroxynitrite is a strong oxidant that can nitrate the protein and lipid component of LDL to a form that is not recognised by the LDL receptor but can be recognized by the scavenger receptor (Darley *et al.*, 1992, Graham *et al.*, 1993). 3-Nitrotyrosine a product of the protein nitrating effect of $ONOO^-$ has been shown to be present in atherosclerosis (Graham *et al.*, 1993, Beckman *et al*., 1994). Hence, peroxynitrite may have a role in the *in vivo* oxidation of LDL.

1.4.5.4 Glycation of LDL

The non-enzymatic addition of carbohydrate molecules to LDL can produce a pro-atherogenic form of LDL. Lysine, a major amino acid component of the apoB-100 component of LDL, can undergo glycation leading to the formation of glycated lysine-LDL (Younis *et al*., 2008). There is a higher proportion of glucose bound lysine in apoB-100 in diabetics (Tames *et al.*, 1992). Chronic hyperglycaemia has been implicated in increased lipoprotein oxidation. Glucose contribution to peroxidation of lipids in LDL is mediated through a superoxide radical pathway (Kawamura *et al*., 1994). Moreover, LDL isolated from diabetics patients has been proven to be more susceptible to oxidation (Colas *et al.*, 2010). Glycated LDL has been proven to be more susceptible to oxidation than native LDL (Sobal *et al*., 2000). A recent study by Younis *et al* suggests that THP-1 derived macrophages incubated with glycated LDL had more cholesterol accumulation compared to control cells incubated with non-glycated LDL (Younis *et al*., 2009). This evidence put together suggest that glycation of LDL speeds up its oxidation and promotes its atherogenicity and therefore may be important in atherosclerosis.

1.4.5.5 Transition metals

LDL has been found to undergo oxidative modification when incubated with vascular cells (smooth muscle cells (Heinecke *et al*., 1984), endothelial cells(Steinbrecher *et al*., 1984) and macrophages (Leake and Rankin, 1990), but this often than not occurs in the presence of

transition metals in the medium. Copper or iron is required in cell-mediated oxidation and contributes to oxidation even when present in micromolar concentration. Iron has been demonstrated to catalyse the oxidation of LDL by smooth muscle cells (Heinecke et al., 1986), macrophages (Henriksen *et al.*, 1983) and endothelial cells (Morel *et al*., 1983). The ability of metal chelating agents in the medium to stop the oxidation of LDL suggests the presence of metal ion might be necessary for the process of LDL oxidation within cells (Steinbrecher *al* et., 1984). Free metal ions can oxidize LDL even in the absence of cells when present in high concentrations. Oxidation of LDL initiated by copper and iron are the most studied models in vitro. Copper-mediated LDL oxidation is, however, the most widely used method for oxidation of LDL in vitro. The oxidation of LDL by copper often with the incubation of 2 to 25 µM concentrations of copper in the culture medium or phosphate Saline buffer or MOPS buffer with LDL concentrations between 50 g protein/ml and 2 mg protein/ml. The Kinetics of oxidation of LDL by metal ions is often measured by the continuous change in diene adsorption at 234 nm (Esterbauer *et al*., 1989b). Our Laboratory has previously described a rapid method to generate copper-oxidised LDL rich in hydroperoxides or oxysterols (Gerry *et al*., 2008). The products of oxidation by copper at pH 7.4 is well characterised (Esterbauer *et al*., 1990b, Brown *et al*., 1996).

Presence of free iron in the body is hazardous due to its ability to transport electrons and interconvert from iron (II) to iron (III). Iron has a deleterious effect in lipid peroxidation as it has the capacity to both initiate and increases LPO. It can mediate this effect in several ways. Iron can initiate lipid peroxidation by the formation of hydroxyl radicals or directly decomposing lipid hydroperoxides.

$$\mathbf{Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^{-} + LO^{\cdot}}$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\cdot} + H^{+}$$

Ferrous Iron can react with hydrogen peroxide (H_2O_2) to form a hydroxyl ion and hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$$

The hydroxyl radical is a powerful oxidant, highly reactive and reacts directly where it is formed. Iron can also react directly with molecular oxygen (O_2) to form superoxide anion.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -}$$

The superoxide itself, is a less reactive and powerful oxidant which has the capacity of generating more of ferrous iron (Fe^{2+}) by acting as a reducing agent for ferric iron (Fe^{3+}), hence, more of the $OH^{\cdot}$ radical is generated from the Fe^{2+} produced (Schafer *et al.,* 2000). The role of iron in initiating lipid peroxidation in liposomes has been explored (Tang *et al*., 2000, Mozuraityte *et al*., 2008). The characterisation of iron-mediated oxidation and the role of iron in mediating lipid peroxidation within lysosome is now generating attention within our laboratory.

1.5 Lysosomal oxidation of LDL

1.5.1 General structure and function of lysosomes

Lysosomes are single membrane bounded spherical organelles discovered by Christian de Duve in 1955 (De Duve *et al*., 1955). The lysosomes are important constituents found in nearly all eukaryotic cells, where they are present in the cytosol as dense bodies. They in shape and size depending on the type of cells, they appear spherical in shape or sometimes tubular. Their size in most cells exceeds 1 µm, although in cells such as macrophages their diameter can be

more than several microns. The size increase in macrophages is likely to be due to the accumulation of materials that are undigested. The lysosomes are separated from the cytosolic environment by a phospholipid- bilayer 7-8 nm in diameter (Saftig and Klumperman, 2009, Appelqvist *et al*., 2013).

The lysosomes are made up of many hydrolytic enzymes that can digest damaged or unwanted materials. The enzymes present in lysosomes possess the ability to degrade polysaccharides, lipids, proteins, RNA, and DNA. Genetic mutation of the genes encoding the lysosomal enzymes which are responsible for a different genetic disease in humans which are classified as lysosomal storage disorders (Pu *et al*., 2016). The inside of lysosomes is acidic in nature (about pH 4.5) (Mindell, 2012). ATP- dependent proton pumps on the membrane of the organelle, the vacuolar H+- ATPase, maintain the pH of this compartment. The H+- ATPase pump creates and preserves the acidity of the lumen of lysosomes by utilising free energy derived from the hydrolysis of ATP to pump protons into the lumen and the "counterion flux" made up secondary ion movements is used to prevent the creation of membrane potential that could arise from proton accumulation. The maintenance of its characteristic acidic pH is important for the activities of lysosomal enzymes most of which are active at pH of about 4.5-5.0 but no the cytosolic neutral pH of about 7.2 (Ohkuma *et al.*, 1982, Mindell, 2012, Ishida *et al*., 2013). Lysosome was initially identified as mere waste bags in cells. Advances in science have over time allowed them to be classified as important organelles that are associated with a large number of cellular events. Lysosomes play a major role in the digestion of extracellular materials taken up by endocytosis, lysosomes are also involved in the degradation of cellular contents through autophagy. In addition to endocytosis and autophagy, lysosomes are also utilised by a phagocytic cell such as macrophages to degrade materials that are phagocytosed including cellular debris, long lived cells, and pathogens such as bacteria. They are believed to

be vital regulators of cellular homeostasis, they are involved in cholesterol homeostasis as proteins required for cholesterol efflux from endolysosomes. Niemann-Pick disease type C $_1$ (NPC1) and NPC $_2$ are located in lysosomes. NPC$_1$ is found in lysosomes and endosomes while NPC $_2$ is found in the lumen of lysosomes (Appelqvist *et al.*, 2013). Lysosomes also play avital role in mediating cell death, although this has been suggested to be induced under certain pathological conditions (Kreuzaler *et al*., 2011). The role of lysosomes in the pathogenesis of disorders such as lysosomal storage disorders, neurodegenerative disorders (Bellettato and Scarpa, 2010) and cardiovascular diseases (Lutgens *et al.*, 2007) is becoming more evident. These findings might create new therapeutic approaches to these diseases.

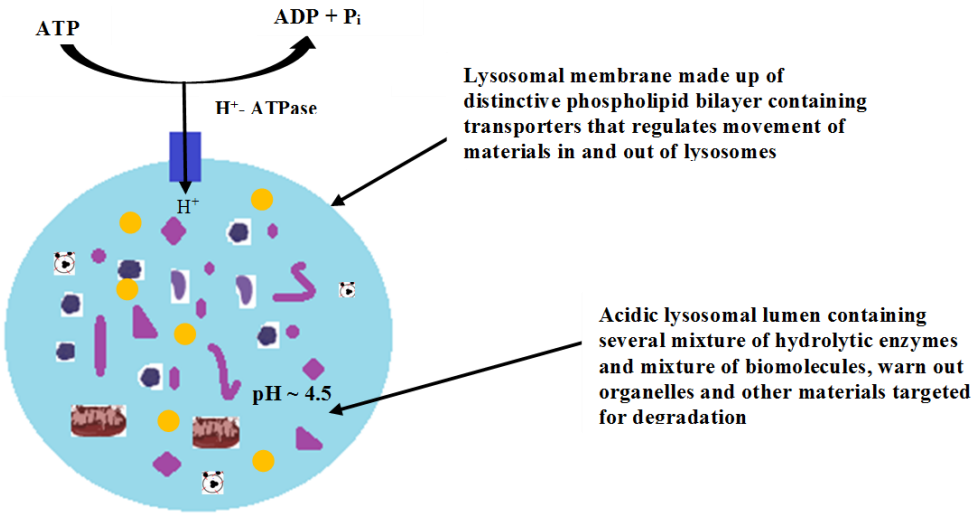


Figure 1.4: Lysosome structure

Pictorial representation of the lysosome depicting various lysosomal enzymes, biomolecules such as lipids and proteins, warn out organelles such as mitochondrial and other materials targetted for degradation.

1.5.2 Lysosomes as a source of redox active iron

Some studies have demonstrated the presence of redox active iron in lysosomes (Yuan *et al.*, 1996, Yu *et al*., 2003, Zhang and Lemasters, 2013). Lysosomes and endosomes are major sites for accumulation of chelatable iron (Zhang and Lemasters, 2013). Petrat *et al*. detected higher amount of redox active iron was present in lysosomes compared other organelles. The study showed that lysosomes contained as much as 16 µM iron compared to 9.2 µM and 7.3 µM concentrations of iron found in mitochondria and cytosol, respectively (Petrat *et al*., 2001).

The presence of redox-active iron ferrous/ferric stored in nonheme form of resident macrophages in different tissue was demonstrated by Meguro *et al* (2005). They suggested that most ferrous mediated reactions were localised in the phagolysosomes(pH 5.3) of iron-loaded and normal rats which implies that the acidic pH may facilitate the release of ferrous iron from hemosiderin and the insolvent of lysosomes (Meguro e*t al*., 2005). The lysosome is the site for intracellular recycling of damaged, worn-out or old proteins and organelles through autophagy (Kurz *et al*., 2007). Autophagocytosed iron-rich proteins deliver iron to lysosomes (Yu *et al*., 2003). Degradation of ferritin in lysosomes could also account for some of this iron (Radisky and Kaplan, 1998, Yu *et al.*, 2003, Kidane *et al*., 2006). The lysosomal compartment is important in macrophages as they are scavenger cells. The use of ESR combined with increased lysosomal pH to prevent proteolysis in lysosomes or lysosomotropic iron chelators showed that most intracellular labile iron is derived from lysosomes (Persson *et al.*, 2003, Yu *et al*., 2003). The presence of this redox-active iron makes the lysosomes susceptible to Fenton type reaction, promotes the associated peroxidative process of materials as they are degraded and when exposed to oxidative stress the resultant iron centred radicals or hydroxyl radicals could destabilise the lysosomal membrane (Öllinger and Brunk, 1995, Persson *et al*., 2001b, Persson *et al*., 2001a).

1.5.3 Lysosomal storage disorder and atherosclerosis

The lysosomes of macrophages are crucial to the onset and progression of atherosclerosis due to the role they play in the degradation of lipids and the regulation of signalling pathways. A defective lysosomal function has been identified as a characteristic of atherosclerotic plaque development (Sergin *et al*., 2015). Lysosomal storage disorders are caused by defects in lysosomal membrane protein and lysosomal enzymes which cause impaired functions in lysosomes and accumulation of undigested materials in the lysosomes (Schultz *et al*., 2011). The majority of the excess cholesterol contained in atherosclerotic foam cell macrophages are found in lysosomes that are swollen as a result of accumulating the cholesterol (Jerome, 2010). The study by Bobryshev et al confirms that changes in lysosomal function have a role in the onset of atherosclerosis with consequential changes in CD68 antigen in lysosomes in fatty streak present in the lesion (Bobryshev *et al*., 2013). Accumulation of cholesterol in lysosomes has recently been linked to coronary atherosclerosis in mice (Xu *et al.*, 2016).

1.5.4 Evidence for LDL oxidation by iron in lysosomes

LDL was previously generally believed to be oxidised within the interstitial fluid of the arterial wall and then bound to the scavenger receptors on macrophages, but this belief is challenged by the robust presence of antioxidants, such as ascorbate, urate, and albumin, in the extracellular fluid (Levitan *et al*., 2010). The fact that serum or interstitial fluid at low level have been shown to be protective against LDL oxidation mediated by copper or iron *in vitro* (Dabbagh and Frei, 1995, Patterson and Leake, 1998, Namazi, 2009, Rodriguez *et al.*, 2009). Wen and Leake were the first to demonstrate the oxidation of LDL within the lysosomes of macrophages. They showed that LDL aggregated by vortexing was rapidly taken up by both mouse macrophage–like cell lines (J774) and human monocyte-derived macrophages (HMDM), then oxidised within the cells. Droplets of lipid were absent in control macrophages while large amount of lipid droplets were found in the cells incubated with aggregated LDL.

Co-incubation with fluorescent dextran revealed the dextran-labelled lysosomes as the main site for the lipids with the presence of cholesteryl linoleate and arachidonate, which were absent in control cells. About a 40% rise in free cholesterol was observed compared to control cells. Ceroid was formed in the lysosomes of both HMDM and J774 cells. Confirmatory experiments with acetylated LDL internalized into lysosomes of cells and incubated without access to LDL in the extracellular space still produced increased levels of oxysterols, which implies that they were mainly oxidised intracellularly (Wen and Leake, 2007).

Wen and Leake also demonstrated the importance of iron in the lysosomal oxidation, as LDL oxidation was inhibited in cells by desferrioxamine (an iron chelator). In a cell free system, ferrous sulphate extensively oxidised LDL at pH 4.5 (similar to the pH of lysosomes) but not at the pH of plasma (pH 7.4), although pH exerted the opposite effect with copper sulphate (Wen and Leake, 2007). In 2012, the group further demonstrated the implication of extensive oxidation of LDL by iron at lysosomal pH in atherosclerosis. Satchel and Leake again showed the effects of iron in the two oxidation states (Fe^{2+} and Fe^{3+}), iron chelating agents, antioxidants on the oxidation of LDL and the chemical changes mediated by iron at lysosomal pH. To check if the oxidation was dependent on the presence of iron, iron chelating agent was added at different phases during oxidation and it was suggested that iron was involved in the three phases of oxidation (Satchell and Leake, 2012).

The oxidation of LDL was described by Esterbauer in 1989. LDL is said to undergo three phases of oxidation the lag phase (during which there is little or no increase in formation of dienes), the propagation phase (which involves a rapid increase in the formation of dienes) and the decomposition phase when lipid hydroperoxides breaks down to form other oxidation

products (Esterbauer *et al.*, 1989a). In 2012, Satchel and Leake demonstrated four phases involved in oxidation mediated by iron at lysosomal pH (lag, rapid oxidation, slow oxidation, aggregation and sedimentation). This is because at acidic pH oxidised LDL undergoes extensive aggregation and the aggregates then sediment towards the bottom of the cuvettes. The characterisation of the product of oxidation by iron at lysosomal pH revealed increased amounts of cholesteryl ester hydroperoxides and 7-ketocholesterol confirming that LDL oxidised at this pH might be atherogenic in nature (Satchell and Leake, 2012).

1.5.5 LDL aggregation and lysosomal LDL oxidation

Maor *et al.* demonstrated that inhibition of both LDL aggregation and oxidation is important in reducing aortic lesion in apoE deficient mice (Maor *et al*., 1997). There is evidence supporting the involvement of aggregated LDL in the pathogenesis of atherosclerosis and their existence of LDL aggregates in arterial (Hoff and Morton, 1985, Hoff and O'Neil, 1991, Steinbrecher and Lougheed, 1992) atherosclerotic lesions (Lu and Gursky, 2013). The core of LDL containing non-polar lipids and hydrophobic in nature surrounded by a monolayer containing apoB, free cholesterol (unesterified). Hence, understanding the involvement of the different constituents of the LDL particle in aggregation is important. Changes in the structure of the apoB component and the lipid component of LDL leads to its aggregation and enhanced lipid droplet formation. These lead to increased uptake by macrophages and promotes an inflammatory response with subsequent formation of the atherosclerotic lesion. LDL aggregation is considered to begin with, the rearrangement of the surface components (Lu and Gursky, 2013).

Modification of LDL by the enzymatic or non-enzymatic process can lead to aggregation and fusion of LDL (Oorni *et al.*, 2000). Treatment of LDL with hydrolytic enzymes and substances

that are pro-oxidative can promote LDL aggregation (Oorni *et al*., 2000), lipolysis, proteolysis, glycation, oxidation, prolonged storage and other biochemical modification such as acetylation can mediate LDL aggregation. Proteolytic modification of the surface ApoB can cause conformational changes in the functional groups of lipid and protein component which can influence the interaction between different LDL particles and cause them to aggregate (Lu and Gursky, 2013). The oxidation of LDL induces different conformational changes of the lipid and protein component of LDL and these changes often leads to the aggregation of LDL (Maor *et al*., 1997, Xu and Lin, 2001). The lipid peroxides products such as aldehydes can form adducts with protein. The reactions between aldehydes and proteins have been demonstrated to induce LDL aggregation (Hoff and O'Neil, 1991). The degradation of the hydrophobic part of ApoB and its redistribution to the surface can lead to instability in the structure of LDL which can result in aggregation of LDL(Oorni *et al.*, 2000). Lipoprotein lipase and/or sphingomyelinase (SMase) can mediate aggregation of LDL (Tabas, 1999). Phospholipase C (PLC) hydrolysis of phospholipids causes the rearrangement of non-polar diacylglycerol from LDL surface to the core and the release of phosphocholine and these can rearrangement of the hydrophobic content can promote LDL aggregation (Lu and Gursky, 2013). Aggregation of LDL is mostly found in electronegative LDL, phospholipase A_2 (PLA_2) and PLC has been linked to the observation of electronegative LDL in human plasma (Bancells *et al*., 2010, Sanchez-Quesada *et al*., 2012).

Pentikainem and colleagues showed that copper ion induced LDL oxidation, vortexing and treatment with SMase produced aggregated LDL (Pentikainen *et al*., 1996). Sphingomyelinase enzyme can promote LDL aggregation *in vivo*, up to 50 times increase in ceramide, the product SMase hydrolysis of sphingomyelin was observe in LDL derived from atherosclerotic lesion compared to LDL isolated from plasm of healthy individuals (Schissel *et al*., 1996). Ceramide

induced LDL aggregation might be as a result of its non-polar nature which can cause the disruption of existing balance between the polar surface and the hydrophobic core (Lu and Gursky, 2013).

Recent work in our laboratory demonstrated the lysosomal oxidation of sphingomyelinase-aggregated LDL (SMase-LDL) (Ahmad and Leake, 2018). Sphingomyelinase-aggregated LDL is a better candidate of modified LDL likely to be present in disease state compared vortexed or acetylated LDL. Findings from our laboratory have demonstrated that SMase-LDL can be oxidised to a great extent in in the lysosomes, as the presence of a large amount of ceroid was observed in the lysosomes after seven days of incubation with SMase-LDL. The recent HPLC analysis of *in vitro* SMase-LDL oxidised by iron at also revealed its ability to generate atherogenic products such as oxysterol and lipid hydroperoxides. Modification of LDL by sphingomyelinase also contributed to increased lipid peroxidation in lysosomes (Wen *et al*., 2015, Ahmad, 2016, Ahmad and Leake, 2019)

1.6 Iron and atherosclerosis

Iron was first linked to heart diseases by Sullivan in 1981. The iron hypothesis proposed that sustained deficiency of iron protected against ischaemic heart disease while iron overload, on the other hand, promoted CVD. The theory was based on the gender difference in the risk of CVD, higher risk observed in males and the fact that post-menopausal women showed reduced protection. Based on the Framingham study (Kannel *et al*., 1976), Sullivan argued that the lower risk observed in pre-menopausal women was not the effect of oestrogen but rather due to regular loss of iron due to menstrual bleeding (Sullivan, 1981, Sullivan, 1989). The

hypothesis was supported by the failure of oestrogen replacement therapy to protect against coronary events in post-menopausal women (Hulley *et al*., 1998).

After over 30years of its proposal, the iron hypothesis remains a subject of debate. While some epidemiological studies have found an association between iron and CVD, others have found no association (Moore *et al.*, 1995, Eichner *et al.*, 1998). Depletion of iron has been shown to reduce the risk of MI, and other events associated with CVD, as this was demonstrated by Facchini and Saylor with the use of phlebotomy bimonthly or monthly to achieve the depleted iron state (Facchini and Saylor, 2002). There may be no consistent relationship between plasma iron and human atherosclerosis because in an inflammatory state, iron sequestered in macrophages and the plasma concentration falls (Ganz, 2005). The most common measure of iron status is ferritin, the main iron storing protein which is directly proportional to tissue iron stores (You and Wang, 2005). Kiechl *et al*. assessed the relationship between body iron stores and carotid atherosclerosis and identified the plasma concentration of the iron storing protein ferritin as the major indication of CAD. They found that increased levels of ferritin correlated with increased CAD and hypercholesterolemia (Kiechl *et al*., 1994). Salonen *et al* reported the correlation between the level of ferritin in serum and risk of MI, men with higher ferritin levels had a two-fold increased risk compare to other men (Salonen *et al*., 1995).

The link between iron and atherosclerosis was demonstrated in animal studies. Lee et al confirmed the association between the deposition of iron and the progression of atherosclerotic lesion in ApoE deficient mice (Lee *et al*., 1999). Other studies have reported no correlation between iron and CVD. Sempos and colleagues argued that there was no correlation between the marker of iron and the onset of CAD (Sempos *et al*., 1994), although this study did not

measure the level of serum ferritin. More *et al* found no association between the thickening of the intima and serum ferritin (Moore *et al*., 1995). The role of iron in atherosclerosis continues to be a controversial topic, hence there is a need for more researchers to focus on this relationship.

1.6.1 Iron in atherosclerotic plaques

The atherosclerotic lesion environment has been demonstrated to contain copper and iron which can mediate lipid peroxidation and free radical reactions (Smith *et al*., 1992, Lamb *et al*., 1995). A study by Pang et al demonstrated deposition of iron is important in the formation of advanced atherosclerotic lesion (Pang *et al.*, 1996). Lee *et al*. discovered the presence of both iron and ceroid in foam cells present in atherosclerotic lesion (Lee *et al*., 1998), their results demonstrated a close association between deposition of iron in human aortic wall and progression of atherosclerosis. The deposits of ceroid and iron were more prominent in advanced atherosclerotic lesion suggesting iron can mediate oxidative reactions in atherosclerosis (Lee *et al*., 1998).

Another study by Lee *et al* in 1999 used an animal model, apoE deficient mice, to investigate the role of iron deposition in atherosclerosis. Iron deposition was observed in intermediate lesions and the deposition increased as the lesions became advanced lesions. They also demonstrated that iron restriction in the diet reduced the susceptibility of LDL to *in vitro* oxidation and decreased oxidative stress (Lee *et al*., 1999). Yuan *et al.* previously showed the occurrence of iron in early atherosclerotic lesions using Perl's method and the more sensitive modified sulphide silver method. The sulphide silver method showed that the majority of the foam cells contained iron, while few were found to contain iron with Perl's method. More iron was seen for macrophages that had engulfed erythrocytes with both methods (Yuan *et al.*,

1996). Stadler and his colleagues' quantified copper and iron in carotid lesions compared with normal controls using electron paramagnetic resonance (EPR) and inductively coupled mass spectroscopy (ICPMS). The results showed elevated iron and copper in carotid lesions compared to the controls (Stadler *et al*., 2004). This further confirms the presence of transition metals, particularly iron and copper, in atherosclerotic plaques.

1.6.2 Iron metabolism

Iron is an important element as it forms a crucial part of many redox enzymes utilised in metabolic processes. It serves as a component of haemoglobin and myoglobin (oxygen transport proteins). Iron is an important cofactor to many enzymes required for DNA repair and synthesis (Puig *et al*., 2017). The majority of iron is contained in the haemoglobin of erythrocytes and developing erythroid cells. Notable amounts are contained in macrophages and myoglobin while excess iron in the body is stored in the liver. The body loses iron through general bleeding, menstruation, sloughing of epithelial cells of the intestine, and desquamation of the skin. (Nemeth and Ganz, 2006, Schmidt, 2015).

Since humans do not have an effective iron excreting mechanism, body iron must be closely regulated. Iron is absorbed in the small intestine by enterocytes and then transported into cells in need of supply (Munoz *et al*., 2009). Following the release to the bloodstream, iron binds to transferrin (a plasma glycoprotein that has the capacity to bind two atoms of iron). The iron-transferrin complex binds to the transferrin receptor and it is then endocytosed. In the acidic compartment of the late endosomes, iron is released from the complex and transported into the cytoplasmic pool of labile iron by divalent metal transporter 1 (DMT1). Iron is then transported to ferritin (an iron binding protein) for storage or to sites where macromolecules containing iron are synthesized mainly mitochondria (Kurz *et al*., 2011).

Transferrin-iron in plasma and extracellular fluid are kept at a stable concentration by systemic iron regulation. The systemic iron regulation maintains the flow of iron into plasma by controlling major iron sources such as iron released from hepatocytes. Macrophage and hepatocytes iron are stored in intracellularly in ferritin and can be mobilised in situations where there is high demand for iron. Excess iron and deficiency of iron can lead to damages to cells and dysfunctional organs. Hence regulation of iron is essential. Hepcidin is a major regulator of iron homeostasis in the whole body; it is predominantly synthesised in the liver before been released into circulation (Collins *et al*., 2008). It can also be synthesised in other tissue such as kidney (Kulaksiz *et al*., 2005), adipose tissue (Bekri *et al.*, 2006) and the heart (Qian *et al*., 2007, Merle et al., 2007). Although hepcidin is mainly secreted by hepatocytes (De Domenico *et al*., 2008), other cells such as myeloid cells (Peyssonnaux *et al*., 2006), alveolar and splenic macrophages (Liu *et al*., 2005), monocytes (Theurl *et al.*, 2008) and adipocytes (Bekri *et al.*, 2006) also synthesise hepcidin but at very low rate compared to hepatocytes. Hepcidin has the ability to regulate the systemic metabolism of iron. The synthesis of hepcidin is directly linked to inflammation and increased level of iron but inversely related to erythropoiesis. It regulates plasma iron by binding to ferroportin, the iron exporter which is highly expressed on macrophages and duodenal enterocytes. Hepcidin has been demonstrated to inhibit iron efflux into plasma from hepatocytes, enterocytes and macrophages by binding ferroportin and leads to conformational changes that cause both molecules to be endocytosed and targeted for degradation. Hepcidin is therefore considered to regulate iron absorption and recycling (Nemeth *et al*., 2004b, Knutson *et al*., 2005, Collins *et al*., 2008).

1.6.3 Iron binding proteins and LDL oxidation

The iron binding proteins may have a role to play in the availability of iron under certain conditions. Studies have suggested the release of iron from ferritin (Minotti and Aust, 1987), and transferrin (Lamb and Leake, 1994c) and the iron released from transferrin oxidised LDL

in vitro. Moreover, iron containing proteins such as myoglobin (Rodriguez-Malaver *et al*., 1997) and haemoglobin (Paganga *et al*., 1992) and copper containing protein such as caeruloplasmin (Lamb and Leake, 1994a) have also oxidised LDL *in vitro* (Yoshida and Kisugi, 2010). Ferryl myoglobin oxidised LDL as shown by increased electrophoretic mobility of LDL (Rice-Evans *et al*., 1993). Since the proposal of the iron hypothesis by Sullivan, the relationship between the iron storing proteins and atherosclerosis continues to be a topic largely debated in the literature. Hence, the role of ferritin as the major iron store should be further evaluated.

1.7 Ferritin

Ferritin (Fig.1.5) is the main protein for the intracellular storage of iron found in almost all organisms. It is a protein of molecular weight of about 500 kDa with an inside diameter of 7-8 nm and 12-13 nm on the outside.

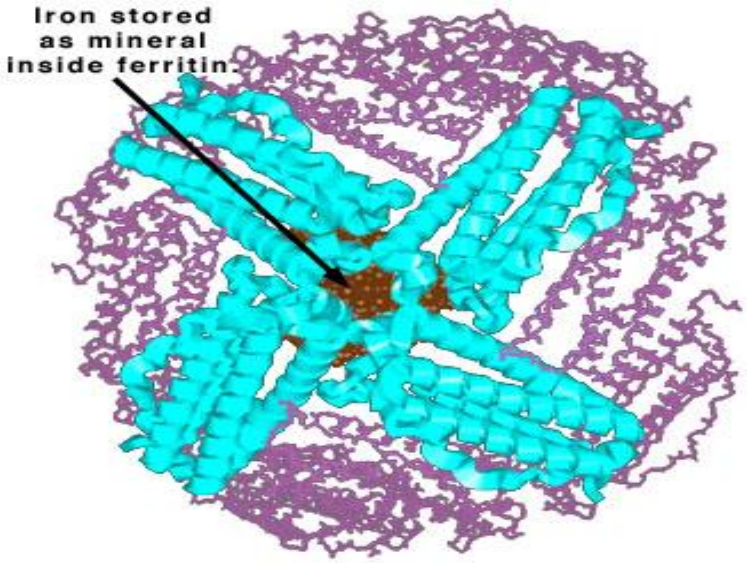


Figure 1.5: Three-dimensional representation of ferritin
(Casiday and Frey, online)

Ferritin is made up of 24 polypeptide chains with light (L) subunits and heavy (H) subunits with a molecular weight of 19 kDa and 21 kDa, respectively (Theil, 1987, Zarjou *et al*., 2010). It is considered the main iron storage protein because of its large cavity for iron storage. The H subunit plays a major role in the binding and release of intracellular iron, iron transport and sequestration because of its ease in the binding and release of iron as compared to L subunit. It possesses a ferroxidase activity which converts Fe (II) to Fe (III) for storage while the Light subunit is involved in mineralization storage for a long term and the nucleation using iron atoms (Rucker *et al*., 1996, Arosio *et al*., 2017).

Ferritin is found mainly in organs that store iron, such as the spleen and liver. It is also found in organs that have low iron levels such as pancreas and heart (Kohgo *et al.*, 1980, Bomford *et al*., 1981). Ferritin is mainly located in the cytosol but has also been seen in animal mitochondria, plant plastids, nucleus, insect endoplasmic reticulum and circulating plasma (Arosio *et al*., 2017). Ferritin synthesis is regulated by iron (decreased synthesis in the presence of low iron and vice versa) and oxidative stress. Cytokines regulate ferritin at the transcriptional and translational level particular TNFα and interleukin 1α have been demonstrated to stimulate the synthesis of the heavy chain of ferritin in human muscle cells, mouse adipocytes and other types of cells (Wei *et al.*, 1990, Tsuji *et al*., 1991). This suggests that the level of ferritin may be upregulated in inflammation.

Since the proposal of the iron hypothesis, many studies have measured the relationship between ferritin as an iron store and atherosclerosis. The evidence remains controversial, however, recent molecular studies in humans and rabbits revealed elevated mRNA for both L and H chains in atherosclerotic lesion compared to normal arteries(Pang *et al*., 1996). Direct evidence

obtained with proteomics by measuring the level of ferritin in CAD, found higher level of ferritin in diseased arteries (You *et al*., 2003). The role of ferritin in lysosomal oxidation is still in its infancy and can be further explored for its implication in the onset and progression of atherosclerosis.

1.7.1 Iron incorporation into ferritin

The ability of iron to induce oxidative damage is prevented by storing it in a form that reduces the concentrations of redox-active iron and at the same time ensure its availability for biochemical reactions when needed (Bouton *et al*., 1996, Rudeck *et al.*, 2000). Ferritin binds iron to inhibit its catalytical activity and protect cells from iron-mediated damage. However, damage to ferritin can lead to ferritin losing its physiological role hence (Grune *et al*., 1997, Rudeck *et al*., 2000), malfunction and releasing iron-to mediate toxic reactions.

Incubation with iron chelators and reducing agent can remove iron from ferritin while the addition of iron in the presence of oxygen can reform the core. This event may be similar to what happens *in vivo*. Fe (II) is delivered through the hydrophilic channel to the centre where it forms a diferric-peroxo complex after an encounter with O_2 or H_2O_2. This complex rapidly forms two ferric ions and H_2O_2, which is used locally for iron oxidation and hence no free radical is formed. The oxidised iron then relocates into the cavity where hydrolysis and nucleation occur (Theil *et al.*, 2013, Honarmand Ebrahimi *et al*., 2015).

1.7.2 Control of cellular iron availability by ferritin

Evidence suggests that cellular activities are affected by cellular iron availability controlled by the ferroxidase activity of ferritin. Accumulation of iron through ferritin activity has been shown to be important in atherosclerosis. Four types of cells are involved in plaque formation which includes macrophages, lymphocytes, smooth muscle cells and endothelial cells. The main cause of plaque formation is the alteration of endothelial cell function. Endothelial

dysfunction has been linked with the uptake of iron by endothelial cells (Rooyakkers *et al*., 2002). Accumulation of iron leads to alteration of ECs structurally and activates the recruitment of monocytes (Kamanna *et al*., 2012). Iron overload in macrophages causes polarisation of macrophages to pro inflammatory M1 macrophages which expresses higher amounts of ferritin and reduced iron turn-over (Cairo *et al*., 2011).

1.8 The use of Antioxidants in atherosclerosis

Generally, antioxidants delay or inhibit LDL oxidation *in vitro* and in cultured cells. They exert varying effects depending on the means of LDL oxidation and the properties of the antioxidant. The risk of atherosclerosis might be alleviated by inhibiting the process of oxidation of LDL independently of lowering blood cholesterol level. Nutritional and pharmacologic antioxidants have been suggested to play a role in the prevention of atherosclerosis. Studies on animals as well as cultured cells showed that probucol, a drug with antioxidant and cholesterol lowering properties, prevented the oxidative modification of LDL (Parthasarathy *et al*., 1986). The results from probucol experiments remain controversial: Bird and colleagues showed that probucol did not decrease atherosclerosis but rather increased atherosclerosis even in the presence of vitamin E in LDL receptor deficient mice. Another study by Choy *et al*. demonstrated a positive effect of probucol in decreasing apoE-deficient mice, which was attributed to the anti-inflammatory properties of probucol (Choy *et al*., 2005). However, results from the human clinical trial suggest that probucol did not significantly reduce atherosclerosis (Walldius *et al.,* 1994). The use of probucol in western countries was withdrawn, as its efficacy in atherosclerotic cardiovascular diseases is challenged by the failure of the clinical trial in humans and the arrival of more potent lipid lowering drugs such as statins. However, Yamashita and others

have designed a prospective study yet to be concluded to examine the efficacy and safety of probucol in preventing secondary cardiovascular diseases (Yamashita *et al.*, 2016). Recent evidence from our laboratory has shown that probucol offered no protection for initial oxidation of LDL by iron ions at lysosomal pH (Ahmad and Leake, 2018), which might be the reason for the failure in human trials.

Antioxidants such as β-Carotene, ascorbate, and α-tocopherol are important antioxidants, as their levels can be considerably increased without causing side effects in the system. β-Carotene is a hydrocarbon carotenoid derived from plants with the capacity to trap free radicals (Burton and Ingold, 1984, Krinsky, 1989). β-Carotene has been demonstrated to inhibit LDL oxidation mediated by human monocyte macrophages and copper ions (Jialal *et al.*, 1991). The role of β-carotene in inhibiting LDL oxidation is controversial. A study by Gaziano and colleagues demonstrated that *in vitro* and *in vivo* supplementation with β-carotene was not protective against LDL oxidation (Gaziano *et al.*, 1995).

Vitamin C has been established as a cofactor for enzymes and antioxidants, but the mechanisms by which this vitamin can improve chronic disease state is less understood. Vitamin C has been demonstrated to reduce the adhesion of monocytes to the endothelium (Weber *et al.*, 1996) and also improve the production of nitric oxide by endothelium (Kashiba-Iwatsuki *et al.*, 1996), these effects may in turn decrease atherosclerosis. Previous work by Horsley *et al* showed that the oxidised product of vitamin C, dehydroascorbate switches from antioxidant to pro-oxidant during oxidation of LDL by copper (Horsley *et al.*, 2007). Clinical trials result showed no protective effect of vitamin C against CVD (Cook *et al.*, 2007, Sesso *et al.*, 2008). The trials by cook and Sesso *et al.* also included vitamin E but showed no effect.

α-Tocopherol is considered the main antioxidant contained in LDL particles with each LDL particle containing about five to nine molecules of vitamin (Esterbauer *et al*., 1990a). The α-tocopherol are believed to protect LDL from oxidants (Meydani, 2001) as it is considered as the most important and most active antioxidant extracted in LDL (Esterbauer *et al.*, 1990a), which can break the chain reactions of free radical. It can exert this effect by scavenging alkoxyl radicals and peroxyl radicals formed from LDL lipids (Sies *et al.*, 1992, Liebler, 1993) and prevent them from promoting the chain reactions of lipid peroxidation. Supplementation with vitamin E has been shown to decrease atherosclerotic lesions in apolipoprotein E-deficient mice (Peluzio *et al.*, 2001) and prevented diet induced atherosclerosis in rabbits (Schwenke *et al.*, 2002). *In vitro* studies have demonstrated a complex role for α-tocopherol in the oxidation of LDL. Some studies have shown that increased α-tocopherol content in LDL made them less susceptible to oxidation by copper ions (Esterbauer *et al.*, 1991b) and macrophages (Jessup *et al*., 1990). Other studies have shown that Vitamin E could be a pro-oxidant and mediate LDL peroxidation, which can be caused by α-tocopherol radical (α-Toc$^{\cdot}$) reacting with a lipid group (LH) in LDL (Bowry and Stocker, 1993, Kontush *et al.*, 1996). Evidence from some small clinical trials showed that vitamin E could protect against CVD (Stephens *et al*., 1996, Boaz *et al*., 2000). The beneficial role of vitamin E in preventing CVD is often challenged by the lack of protection seen in large clinical trials against CVD, such as the HOPE (Yusuf *et al*., 2000, Investigators, 2005) and GISSI (Jiala*l et al.*, 1999, Collins *et al*., 2002). Additional negative evidence was equally obtained from a randomised trial with a low dose of aspirin in combination with vitamin E (de Gaetano, 2001).

Prevention and management of cardiovascular diseases have focussed largely on lifestyle changes and reducing risk factors. Drugs that can effectively lower high blood pressure and cholesterol concentration have emerged. Despite the better understanding of the pathogenesis of atherosclerosis, many challenges remain in the treatment of the disease. The recent lysosomal oxidation hypothesis raises further questions on the mechanisms of the lysosomal oxidation *in vivo*, the key players in the oxidation and the possibility of a novel therapy for atherosclerosis from antioxidants that can accumulate in the lysosomes.

1.9 Thesis hypothesis and aims

LDL oxidation by ferritin in the lysosomes of macrophages is important in the pathogenesis of atherosclerosis. The overall hypothesis of this study was that ferritin as the major iron-storing protein can mediate LDL oxidation at lysosomal pH and within cells to contribute to the progression of atherosclerosis and some antioxidants can ameliorate this effect. The concept of lysosomal LDL oxidation by catalytically active iron was recently proposed and there is a need to explore the relevant sources of iron for this oxidation *in vivo*. The iron binding proteins may serve as a source of iron under certain conditions. Some studies have demonstrated the release of iron from ferritin and transferrin. Iron released from transferrin has contributed to oxidising LDL *in vitro*. Moreover, iron-containing proteins such as myoglobin and haemoglobin and copper-containing protein such as caeruloplasmin have also oxidised LDL *in vitro*. Since the proposal of the iron hypothesis of atherosclerosis by Sullivan, the relationship between the iron-storing proteins and atherosclerosis continues to be a topic actively debated in the literature. Hence the role of ferritin as the major iron store can be further evaluated. This thesis addressed the following aims:

Aim 1: The first aim of this thesis was to investigate the role of ferritin on LDL oxidation at lysosomal pH by exploring the mechanisms by which ferritin can catalyse LDL oxidation in

the lysosomes. monitoring its effect on the formation of lipid peroxidation products (conjugated dienes, cholesteryl ester hydroperoxides and 7-ketocholesterol) at lysosomal pH *in vitro*. The ability of ferritin to release catalytically active iron during the oxidation of LDL was monitored. The levels of these oxidised lipids formed in the presence ferritin might enable us to predict the potential atherogenicity of LDL oxidised by ferritin.

Aim 2: The degradation of many intracellular and extracellular proteins occurs in the lysosomes. Autophagocytosed ferritin would be degraded in lysosomes. There is evidence suggesting the release of iron from ferritin after different treatment such as proteases. The effects of degradation of ferritin or LDL on the oxidation of LDL by ferritin under the lysosomal pH condition *in vitro* were assessed. This was important to explore in the lysosomal oxidation theory, as both LDL and ferritin are targeted for degradation in lysosomes. It is possible that degradation of ferritin, apoB and lipid component (mainly cholesterol esters) component and of LDL by lysosomal enzymes will affect the rate of oxidation of LDL by ferritin at lysosomal pH

Aim 3: It was relevant to explore the role of antioxidants in inhibiting lysosomal LDL oxidation mediated by ferritin as the oxidative modification theory of atherogenesis is constantly been challenged by failure of some previously used antioxidants to protect against CVD in large clinical trials. Antioxidants which can accumulate in lysosomes might be a more potent candidate in inhibiting LDL oxidation. The ability of antioxidants in to protect LDL against oxidation by ferritin *in vitro* was assessed using the water-soluble antioxidants cysteamine (a lysosomotropic antioxidant) and vitamin C, the lipophilic antioxidants α-tocopherol and N, N'-diphenyl-p-phenylenediamine (DPPD) and the amphipathic compound, Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl).

Aim 4: Some evidence supports the role of oxLDL in foam cell formation in atherosclerotic lesion advancement. There were strong indications that the *in vivo* modification of LDL increases its ability to induce an inflammatory response and promote atherosclerosis. In addition, there is ample evidence in support of the proatherogenic effects of oxLDL and its ability to induce cellular damage. Lysosomes possess the ability to process ferritin by autophagy. Understanding the role of ferritin in lysosomal oxidation is still in its infancy and can be further explored for its implication in the onset and progression of atherosclerosis. It was important to understand the effects ferritin-oxidised LDL will have at the intracellular level. Our final aim was to investigate the effects of oxidation of LDL by ferritin in lysosomes on macrophage function. The link between upregulation of iron in macrophages by hepcidin and lysosomal LDL oxidation was explored. We tested the ability of ferritin to increase lipid peroxidation in lysosomes. The relevance of ferritin-oxidised LDL on the intracellular generation of reactive oxygen species and induction of apoptosis was explored. We assessed the effect of lysosomal LDL oxidation by ferritin on cellular respiration in cultured human macrophages.

Chapter 2 - General materials and methods

2.0 Materials and Methods

2.1 Chemicals

All laboratory reagents obtained were of analytical grade. Ferritin and all antioxidants used were prepared in the appropriate buffer for the particular experiment just before use. General laboratory reagents and the companies that supplied them are listed in appendix 1, the lysosomal lipid peroxidation probe (Foam-LPO) was a gift from Dr Yi Xiao, Dalian University of Technology, China.

2.1.1 Solutions

Cryopreservation medium

The medium was prepared with Dimethyl sulphoxide (DMSO), foetal calf serum and RPMI-1640 (10/40/50, v/v/v).

Lipid hydroperoxide assay (colour reagent)

Colour reagent was prepared with sodium azide NaN_3 (150 μM), potassium phosphate monobasic KH_2PO_4 (163 mM), dipotassium hydrogen orthophosphate K_2HPO_4 (37 mM), potassium iodide KI (120 mM), ammonium molybdate $(NH_4)_2MoO_4$ (10 μM), Trition X-100 (2g/L) and Benzakonium chloride $C_6H_5CH_2N(CH_3)_2RCl$ (0.1g/L). The pH was then adjusted to pH 6.0.

DAPI

Ultrapure water (2 ml) was added to 10 mg/ml stock to give 5 mg/ml. 1 μg/ml solution was prepared in fluorescence mounting medium (DAKO S3023).

Buffer for dialysing LDL

10 Litres of dialysis buffer (140mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 and 100 μM EDTA) were prepared by addition of 81.83 g NaCl, 14.42g $Na_2HPO_4.2H_2O$, 2.964 $NaH_2PO_4.2H_2O$ and 372.2 mg EDTA. It was made up to 10 litres and the pH adjusted to 7.4.

Dihydroethidium (DHE)

DHE (10 mg) was dissolved in 1585 µL Dimethyl sulphoxide (DMSO) to make 20 mM stock solution.

Flow cytometer buffer

5mM EDTA with 0.6% BSA in PBS.

High-density solution (HDS).

1L of HDS (2.97 M KBr, 2.62 M NaCl and 297 µM EDTA) was prepared by the addition of 354 g KBR, NaCl 153g, EDTA.$Na_2.2H_2O$ 110.5mg and then made up with distilled water to 1 litre and pH was adjusted to pH 7.4.

1.063 g/ml NaCl/KBr/EDTA solution

$V_{HDS} = V_{LDS} (D_{REQ} - D_{CURR}) / D_{HDS} - D_{REQ})$

Vol of HDS = $\frac{0.057 \times 2{,}000 \text{ ml of LDS}}{\text{The density of HDS} - 1.063}$

The volume of HDS obtained was added to 2 litre LDS and the pH was adjusted to pH 7.4 and the density was checked with calibrated 100ml flask at 4 ^{o}C to ensure it was 1.063 g/ml. Adjustment where made where necessary.

HPLC oxidation mobile phase

HPLC grade acetonitrile, isopropanol and water at 44/54/2 % by volume. To make 1 litre 440 ml acetonitrile, 540 ml isopropanol and 20 ml water were mixed.

Stabilising buffer for incubating LDL with sphingomyelinase

100 ml of stabilising buffer was prepared with HEPES 8 0ml of 150 mM/L NaCl, 10 ml of 50 mM HEPES and 10 ml of 100 mM $MgCl_2$ and then pH to 7.4.

Low density solution (LDS)

10 litres of LDS with density 1.006 g/ml (150mM NaCl and 297 µM EDTA) was prepared by adding 87.68 g NaCl, 110.5 mg EDTA and then made up to 10 litres. The pH was adjusted to pH 7.4.

1.019 g/ml NaCl/KBr/EDTA solution

$V_{HDS} = V_{LDS} (D_{REQ} - D_{CURR}) / D_{HDS} - D_{REQ}$

$$\text{Vol of HDS} = \frac{0.013 \times 2{,}000 \text{ ml of LDS}}{\text{The density of HDS} - 1.019}$$

The volume of HDS obtained was added to 2 litre LDS and the pH was adjusted to pH 7.4 and the density was checked with a calibrated 100 ml flask at 4 ºC to ensure it was 1.019 g/ml.

Lowry A

Lowry A solution (0.2 M Na_2CO_3, 0.1 M NaOH, 5.7 mM sodium tartrate and 35 mM SDS) was prepared with 5 g Na_2CO_3, 1 g NaOH, 0.328 g sodium tartrate and 2.523 g SDS made up to final volume (250 ml) with water.

Lowry B

To make 250 ml, 0.16 M $CuSO_4$, 6.384 g was dissolved in ultra-pure water and made up to 250 ml.

MOPS buffer pH7.4

NaCl, 8.8 g (150 mM) and 2.09 g 3-N-[Morpholinopropane] sulphonic acid (MOPS) (10 mM) with pre-washed Chelex-100 (1 g) was made up to 1 litre and adjusted to pH 7.4, mixed overnight with stirring. The Chelex was removed by filtration and the pH was readjusted. Chelex-100 is used to remove transition metals that might be present.

Oil Red O stain

500mg of Oil Red O powder was dissolved in 100 ml of 99% Isopropanol solution and placed in a warm water bath to dissolve. To prepare working solution from this stock solution, 3 parts of Oil red O stock was mixed with 2 parts ultra-pure water (depending on volume needed for

each experiment). This was then incubated at room temperature for 10 minutes and filtered using a 0.45 µm Minisart filter. The working solution was used within two hours of preparation.

Paraformaldehyde cell fixation solution (4%)

4g of paraformaldehyde was dissolved in 60ml PBS and kept in a warm water bath at 37 ^{o}C in a fume hood overnight. The pH was then adjusted to pH 7.4 and the volume was made up to 100 ml.

PBS (Phosphate buffer saline) 1X

NaCl, 8.0 g (137 mM), 0.20 g KCl (2.7 mM), 0.23 g NaH_2PO_4 (1.9 mM), 0.12 g Na_2HPO_4 (0.8 mM) was dissolve in 1 litre and the pH was adjusted to pH 7.4

Reducing sample treatment buffer 6X RSTB

SDS (12%), 30% B mercaptoethanol, 30 % stacking gel buffer, 30% glycerol) 50 ml was prepared with 6 g SDS, 15 ml B mercaptoethanol, 15 ml stacking gel buffer, 15 ml glycerol and 5 ml ultra-pure water. A trace of bromophenol was added.

Resolving gel buffer

1.5M Tris-HCl (500 ml) pH 8.8 was prepared with 181.5 g of Tris and made up with ultra-pure water and pH was adjusted with only HCl to pH 8.8.

RIPA lysis buffer

Lysis buffer was prepared with 150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 8.0, 1.0 % Triton X-100, 0.5 % Sodium deoxycholate and SDS (0.1 %).

Sodium chloride/sodium acetate buffer pH 4.5

NaCl, 8.8 g (150 mM) and 0.82g (10 mM) Sodium acetate with pre-washed Chelex-100 (1g) was made up to 1L and adjusted to pH4.5 and mixed overnight with stirring. The Chelex was removed by filtration and the pH was readjusted.

Stacking gel buffer

500 ml 0.5 M Tris-HCl pH 6.8 was prepared with 30g of Tris and made up with ultra-pure water and pH was adjusted with only HCl to pH 6.8.

2.1.2 Laboratory equipment

Name	Manufacturer
A1 inverted epifluorescent microscope	Carl Zeiss
Axioscope epifluorescent microscope	Carl Zeiss
Atomic absorption spectrophotometer (AAS)	Analytik Jena
BD Accuri™ C6 flow cytometer	BD Biosciences
Countess II FL automated cell counter	Invitrogen
Evos XL cell imaging system	ThermoFisher
Inductively coupled plasma mass spectrophotometer (ICP-MS)	ThermoFisher
Lambda-2 6-cell spectrophotometer	PerkinElmer
Lambda Bio 40 8-cell spectrophotometer	PerkinElmer
Lambda 35 8-cell spectrophotometer	PerkinElmer
Libra S22 UV/Vis Spectrophotometer	Biochrom
Optima ™ XPN ultracentrifuge	Beckman Coulter
PerkinElmer 200 HPLC system	PerkinElmer
Agilent 1200 HPLC	Agilent
Speed Vac	ThermoFisher
U genius image capturing machine	Syngene
Seahorse XFp Analyzer	Agilent

2.2 Preparation of LDL

2.2.1 Ethics and blood collection

Permission was received from the University of Reading Research Ethics Committee to take blood from volunteers on 2 February 2012 (Project number 12/7; Isolating low density lipoprotein and monocytes from human blood for studies of atherosclerosis). Blood was usually taken from one to three healthy volunteers. Blood (250 ml) was collected from each donor into 50ml syringes containing (3 mM EDTA at pH 7.4 to inhibit the oxidation of LDL and prevent blood clotting).

2.2.2 Isolation of LDL

Native LDL (d=1.019-1.063g/ml) was isolated from normal human blood (after an overnight fast) according to the method previously used in our laboratory (Wilkins and Leake, 1994). LDL isolated by sequential ultracentrifugation of the plasma. Blood collected as described in section 2.2.1 were transferred into 50 ml tubes, the tubes were inverted to mix and placed on ice. The blood samples were centrifuged at 1500 g for 30 min at 4 ˚C. The plasma was removed from the upper layer and its volume measured. The density was adjusted to 1.019g/ml by adding HDS (prepared from 297 µM EDTA, 2.62 M NaCl and 2.97 M KBr). The volume of HDS to be added was calculated as follows:

V_{HDS} = Vplasma (D_{REQ} -D_{CURR})/ D_{ADD} -D_{REQ}) where V_{HDS} =Volume of high density solution HDS to be added to the pooled plasma, Vplasma =Volume of plasma obtained from blood collected, D_{REQ} = final density required which is 1.019 mg/l, D_{CURR} = the current density of pooled plasma which is 1.006 mg/l and D_{ADD} = the density of the HDS solution prepared.

$$\text{Vol of HDS} = \frac{0.013 \times \text{volume of plasma}}{\text{The density of HDS} - 1.019}$$

The calculated volume of HDS was added to the plasma transferred into the dialysis tubing and then placed in 2 litres of 1.019 g/ml NaCl/KBR/EDTA solution (prepared as described in

section 2.1.1), dialysed for 2 hours at 4 ^{o}C with stirring. The plasma was transferred into 35 ml ultracentrifuge tubes and centrifuged at 115,000 x g (40,000 rpm) at 4^{o}C for 18 hours. The tubes were sliced at the upper region to obtain a coloured solution at the bottom. The clear solution in the upper region containing VLDL and IDL was discarded. The bottom fraction containing HDL, LDL and other plasma proteins was collected, the gelatinous material at the bottom was drawn with a Pasteur pipette and all gelatinous material were re-suspended. The volume was measured and the density was adjusted to approximately 1.063 g/ml by addition of HDS using the following equation to determine the amount required:

$$\text{Vol of HDS} = \frac{0.044 \times \text{volume of plasma}}{\text{The density of HDS} - 1.063}$$

To adjust the density to exactly 1.063g/ml the fraction was transferred into dialysis tubing and dialysed against 1 litre of 1.063 g/ml solution (prepared as described in section 2.1.1), for 2hours at 4^{o} C with stirring. After dialysis, the plasma was transferred to ultracentrifuge tubes and centrifuged for 18 hours at 115,000 x g (40,000rpm) at 4^{o}C. After 18 hours of centrifugation, the tubes where sliced below the upper golden layer containing the LDL, the bottom fraction containing HDL was discarded. The LDL fraction was transferred to a dialysis tube and dialysed for 2 hours against 1 litre of 1.063g/ml solution at 4^{o}C with stirring. The solution was then centrifuged for another 18 hours at 115,000 x g (40,000rpm) at 4^{o}C. After 18 hours the ultracentrifuge tubes were sliced below the LDL fraction at the upper layer and the lower layer was discarded.

The obtained LDL fraction was dialysed against NaCl/phosphate/EDTA (40 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 and 100 µM EDTA) (pH 7.4) overnight with several changes. The LDL was filter sterilised using a Millipore filter of pore size (0.45 µm) and stored in the dark at 4 ^{o}C. The LDL protein was then assayed to determine the concentration. The LDL

was stored at 4 ºC in the dark room in the presence of 100 µM EDTA and used within one month of isolation.

2.2.2 Enrichment of LDL with α-tocopherol

The α-tocopherol content of native LDL was increased as described by Esterbauer *et al* (Esterbauer *et al.*, 1991a). The plasma of blood collected from healthy volunteers was obtained by centrifugation at 1500 g for 30 min at 4 ºC. The pooled plasma was then incubated for 3 h at 37 ºC with 1% (v/v) DMSO containing 100 mM α-tocopherol (final concentration 1mM), to allow the α-tocopherol to diffuse into the LDL particles or 1% (v/v) DMSO as a control. LDL was then isolated by ultracentrifugation as described in section 2.2.1.

2.2.3 Lowry protein assay

The concentration of LDL protein was determined by a modified Lowry assay for protein determination previously described by Markwell *et al* (Markwell *et al.*, 1978). Standard masses (0, 20, 40, 60, 80, 100 µg) of bovine serum albumin (BSA) were prepared from a stock solution of BSA (400 µg/ml) in triplicates and made up to 500 µl with ultra-pure water. Sample (125 µl) was added to assay tubes in triplicates and also made up to 500 µl with ultra-pure water. Freshly prepared Lowry A solution (1.5ml) was added to each assay tubes and placed at room temperature for ten minutes. Lowry B reagent (150 µl) was added to each assay tube and incubated at 55ºC for 5mins. The assay tubes were then allowed to cool to room temperature, the absorbance at 650nm was measured. The assay is based on the ability of the peptide bonds present in both BSA and apoB-100 of LDL to form a complex with copper ions in Lowry A reagent. The complex formed then reduces the Lowry B (phosphomolybdic-phosphotungstic Folin reagent) to a blue chromophore that absorbs light at 650nm. The LDL protein contained in the sample was then determined against the standards (BSA). Usually about 2.5mg to 4mg of LDL protein/ml was obtained.

2.2.4 Preparation of sphingomyelinase aggregated LDL

Native LDL was diluted to 2 mg protein/ml with LDL stabilising buffer containing (NaCl 150 mM, $MgCl_2$ 100 mM and HEPES 50 mM, pH 7.4). Sphingomyelinase from *Bacillus cereus* (Sigma-S9396-50UN) was added at 10 mU/ml and incubation at 37 ^{o}C was started in a water bath (Yabu *et al.*, 2008, Walters and Wrenn, 2010). The attenuance (absorbance plus light scattering) was measured at 430 nm as well as 680 nm was measured at 0 h and every hour afterwards. At each time point LDL diluted to 100 μg protein/ml was measured in cuvettes containing PBS. Using PBS as reference the sample was read at 430 and 680 nm simultaneously. The process was stopped when the absorbance at 430 and 680 nm reached about 0.09 and 0.013 respectively. From previous confirmatory experiments using dynamic light scattering (Wen *et al.*, submitted). It was indicated that the average LDL particle size at this stage would have increased from about 25 nm to about 200 nm. After suitable aggregation was achieved the incubation was stopped and sphingomyelinase aggregated LDL (SMase-LDL) was dialysed against 2 L of the buffer used for dialysing LDL (40 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 and 100 μM EDTA) (pH 7.4) overnight in a cold room with stirring. The SMase-LDL was collected and sterilised with a 0.45 μm Minisart filter and SMase-LDL protein concentration was assessed by the Lowry method described in section 2.2.3. The aggregation was confirmed by comparing light scattering at 680 nm by comparing SMase-LDL to control LDL (Fig 2.1).

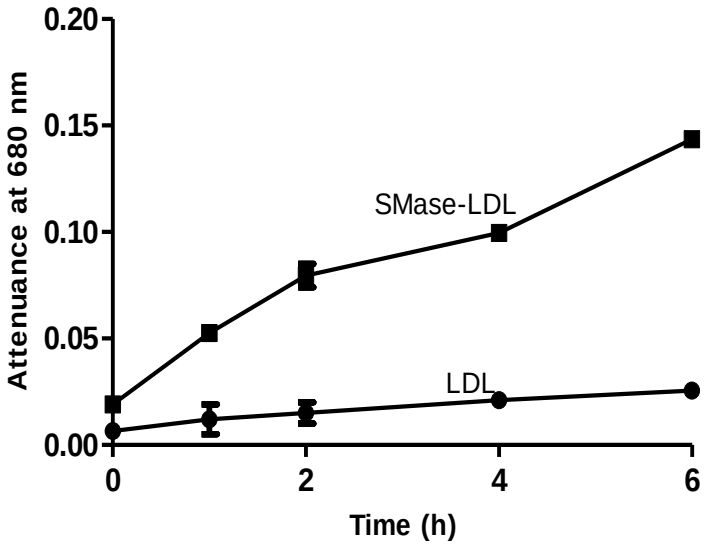


Figure 2.1 Measurement of aggregation in SMase-LDL compared to native LDL

The attenuance at 680 nm of native LDL and SMase-LDL (100 µg protein /ml) out of 2 mg LDL protein/ml incubated with or without sphingomyelinase (10 mU/ml) at 37 ^{o}C was compared at different time intervals for up to 6 h.

2.2.4 Preparation of lipoprotein-deficient Serum (LPDS)

To prepare lipoprotein-deficient serum 1 ml was assayed for protein. To every 120 ml of serum 47.71 g potassium bromide was added to adjust the density to 1.25 g/ml stirred by hand then transferred to 35ml ultra clear centrifuge tubes and centrifuged at 40,000 rpm at 10 ^{o}C for 48 h. After 48 h the tubes were sliced at the upper layer to remove lipoproteins at the top. The remaining serum was then dialysed against 10 litres of dialysis buffer at 4 ^{o}C, the buffer was changed several times overnight. It was then dialysed against 10 litres of PBS containing calcium and magnesium with two changes for 3 h. The protein was assayed again and readjusted to the original protein concentration by adding PBS then filtered through a 0.22 µM filter.

2.3 Spectrophotometric measurement of LDL oxidation by ferritin at lysosomal pH

Native LDL (50 µg protein/ml) was oxidised with freshly prepared ferritin (0.05µM, 0.1µM and 0.2µM) in Chelex-100 treated NaCl/ sodium acetate buffer (150 mM NaCl, 10 mM acetic acid) (pH 4.5) at 37 ˚C. The Chelex-100, pre-washed in ultra-pure water was added to NaCl/sodium acetate buffer at 0.1% (w/v, 1 g/l), to remove soluble transition metal binding activity (Van Reyk *et al.*, 1995). The Chelex-100 treated NaCl/ sodium acetate buffer pH 4.5 left at 4 ^{o}C overnight with stirring. The Chelex-100 was then removed by filtration prior to re-adjusting pH to 4.5. The formation of conjugated diene was measured according to the method previously used by Esterbauer to continuously monitor LDL oxidation (Esterbauer *et al.*, 1989b). Freshly dissolved LDL (50 µg protein/ml) and the different concentrations of freshly diluted ferritin was placed in 10 mm path length quartz cuvette in duplicates against reference cuvettes that lacked LDL. Ferritin was added last in all experiments. To measure the formation of conjugated dienes, the attenuance (absorbance plus UV scattering) at 234nm was measured at 37^{o}C for 1200 minutes at a one-minute interval in a dual Lambda Bio 40 8-cell spectrophotometer with UV software. The attenuance at zero was subtracted from all time points.

2.3.1 Comparison of oxidation of LDL by ferritin at pH 4.5 and pH 7.4

The effect of changes in pH on the oxidation of LDL by ferritin was assessed. Native LDL (50 µg protein/ml) was oxidised with 0.1 µM or 0.2 µM ferritin at 37˚C using NaCl/ sodium acetate buffer (pH 4.5) and MOPS buffer (pH 7.4) both pre-treated with Chelex - 100. The formation of conjugated dienes was monitored.

2.3.2 Measurement of LDL aggregation in the presence of ferritin

Native LDL (50 µg protein/ml) was oxidised with freshly prepared ferritin (0.1µM) in Chelex - 100 treated NaCl/ sodium acetate buffer (pH 4.5) at 37˚C in capped quartz cuvettes. The

method previously used by Khoo *et al* was adopted (Khoo *et al*., 1988). Light scattering by LDL oxidised with freshly prepared ferritin was compared to LDL or ferritin by itself. The change in attenuance at 680 nm was monitored every minute at 37˚C against reference cuvettes that lacked LDL. The attenuance was measured for 1200 minutes.

2.3.3 Atomic absorption spectrophotometric quantification of iron in ferritin and apoferritin

The amount of iron in horse spleen ferritin was determined using flame atomic absorption spectrophotometry (F-AAS). To digest the sample, 1 ml of 5 M nitric acid was added to ferritin (15 µl of 53 mg/ml protein) or apoferritin (35 µl of 25mg/ml) was prepared in triplicate in test tubes and heated for 2 h at 75^{o} C. The samples were dissolved in 50 ml and 10 ml of water, respectively. Standard concentrations of iron (0, 1, 2, 3, 4, 5 mg/l) were prepared from a stock solution of iron (Fe^{3+}) (10 mg/l) and treated the same way as the sample. Samples of the nitric acid digest were assayed directly in an atomic absorbance spectrophotometer (AAS) (Analytik JENA AAS NovAA) using an air/acetylene flame. Absorbance was measured at 248.3nm.

2.3.4 Comparison of iron released from ferritin at pH 4.5 and 7.4 using iron chelator

To determine the amount of iron released from ferritin at pH 4.5 and 7.4. Native LDL (50 µg protein/ml) was oxidised with freshly prepared ferritin (0.1 µM) in Chelex-treated NaCl/ sodium acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) in 15ml tubes, in triplicate. These were incubated at 37˚C. Aliquots of 1ml were taken at intervals up to 24 hours. Bathophenanthroline (BP) (30 µl of 10mM) was added at different time points and absorbance was measured immediately at 535nm using the Biochrom Libra S22 spectrophotometer.

2.3.5 Comparison of iron released from ferritin by the ultrafiltration method

All solutions were prepared prior to use. Ferritin was incubated in triplicate at 0.1 µM in Chelex-treated NaCl/ sodium acetate buffer (pH 4.5) in 15ml tubes at 37˚C for 24 h, then

filtered with 30,000 Mr cut off microcentrifuge filter tubes at 9000g for 25 min. The quantity of iron in the filtrate was determined using AAS.

2.3.6 The effect of DTPA and EDTA on LDL oxidation by ferritin

The effect of iron chelators on LDL oxidation by ferritin was tested. Native LDL (50 µg protein /ml) was oxidised with 0.1 µM ferritin in the absence or presence of 100 µM EDTA or DTPA. The formation of conjugated dienes was continuously monitored by measuring attenuance at 234nm for 1200 minutes at one minute interval.

2.3.7 Effect of antioxidants on LDL oxidation by ferritin

The effect of antioxidants (tempol, α-tocopherol, ascorbate, DPPD and cysteamine) on oxidation of LDL by ferritin was tested. Native LDL (50 µg protein/ml) was oxidised with 0.1µM of ferritin without or with enrichment with α-tocopherol or in the presence of tempol (10 µM) or different concentrations of ascorbate (10-100 µM), DPPD (5-10 µM) or cysteamine (5µM-10mM) in Chelex - 100 treated NaCl/sodium acetate buffer (pH 4.5) at 37˚C. The samples were placed against appropriate reference cuvette that lacked LDL. The formation of conjugated dienes was monitored by measuring attenuance at 234nm for 1200 minutes at one-minute intervals.

2.3.8 Effect of cysteamine on iron released by ferritin

Iron released from ferritin in the presence of cysteamine was assessed. Native LDL 50µg protein/ml) was oxidised with freshly prepared ferritin (0.1 µM) in Chelex-treated NaCl/acetate buffer (pH 4.5) in the presence or absence of cysteamine in 15ml tubes. A fraction of 1ml was taken at intervals up to 24 h. BP (30 µl of 10mM) was added at different time points, absorbance was measured immediately at 535nm using the Biochrom Libra S22 spectrophotometer.

2.4 Measurement of LDL oxidation by reverse-phase HPLC analysis of lipid species

Reverse phase HPLC was used to analyse LDL oxidised by ferritin following a method previously used by Kritharides *et al*. (Kritharides *et al.*, 1993). The method of preparation of samples and analysis is described below. PerkinElmer 200 and Agilent 1200 system were used, data analysis was done using Totalchrom workstation and Chemstation software, respectively. The column used on both machines was a C18 reverse phase column, 250 mm x 4.6 mm, 5 µm particle size with 5 µm guard column. Reverse phase HPLC uses Stationary phase that is hydrophobic (non–polar) and an aqueous mobile phase (polar). The oxidised Lipid products were detected at 234 nm.

2.4.1 LDL oxidation with ferritin for HPLC analysis

Native LDL was incubated with or without freshly dissolved ferritin (0.1µM) at 37˚C using NaCl/Na acetate buffer (pH 4.5) in 15ml polypropylene tubes. At different time points for up to 48 h, the oxidation was stopped at each time point by adding BHT in ethanol stock concentration at 2 mM (final concentration of 80 µM) and EDTA (final concentration 4mM). The lipids were then extracted as described in the next section.

2.4.2 Extraction of lipids from the oxidised sample for HPLC analysis

Methanol (1ml) was added to each oxidised sample (80 µg LDL protein) and then vortexed for 10 seconds. Hexane (3ml) was added and the samples were vortexed for 30 seconds in order to transfer the hydrophobic fraction into hexane. The sample was then centrifuged at 1500 x g at room temperature for 15 min, 2ml of the upper hexane layer was then transferred to a 5 ml propylene tube and dried in SpeedVac (ThermoFisher). The dried samples were dissolved in 200µl of a relevant mobile phase and analysed.

2.4.3 HPLC Measurement

Each sample (20 µl) was injected into a reverse-phase HPLC and C18 column (described in section 2.4). The Oxidised products 7-keto cholesterol (7-Keto) and cholesteryl linoleate hydroperoxide (CLOOH) were detected at 234 nm using a mobile phase as described in section 2.4.3.1. Example chromatograms are shown in figures 2.2 & 2.3. Oxidised products were quantified by comparing the peak area with those of known concentrations of commercially available standards.

2.4.3.1 Conditions for analysis of targeted lipid species

The targeted lipid species were 7-ketocholesterol and cholesteryl linoleate hydroperoxide. Mobile phase was prepared with 2% water, 44% acetonitrile, 54% isopropanol (by vol.) and oxidised lipids were detected at a wavelength of 234 nm at a flow rate of 1ml/min for 15 minutes.

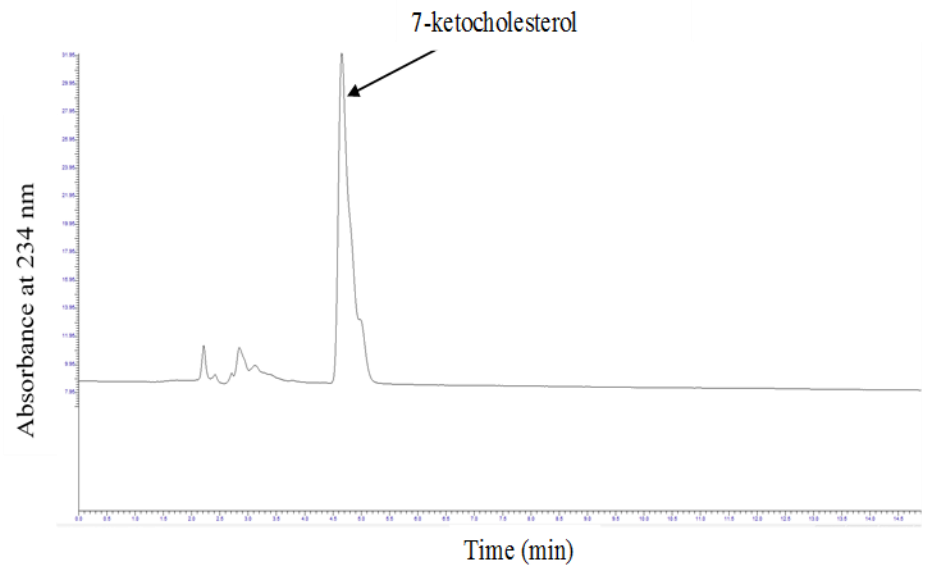


Figure 2.2: Example chromatogram showing 7-ketocholesterol

Mobile phase as described in section 2.4.3.1 was used with a flow rate of 1 ml/min and detected at a wavelength of 234 nm in 7-ketocholesterol standard.

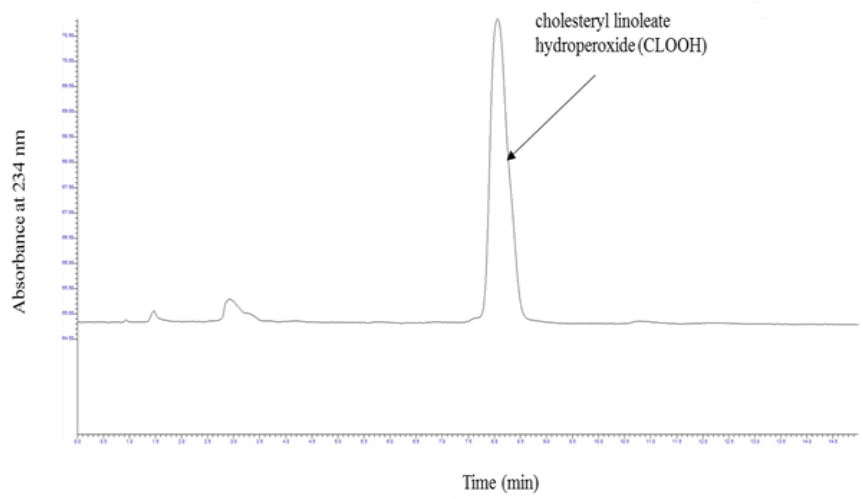


Figure 2.3: Example chromatogram showing cholesteryl linoleate hydroperoxide

Mobile phase as described in section 2.4.3.1 was used with a flow rate of 1 ml/min and detected at a wavelength of 234 nm in the cholesteryl linoleate hydroperoxide standard.

2.5 Measurement of LDL oxidation by determination of total lipid hydroperoxide content by the tri-iodide assay

The method previously described by El-Saadani et al(el-Saadani et al., 1989) was adapted to measure the total lipid hydroperoxides in LDL oxidised by ferritin at lysosomal pH. The samples of LDL used for assay had a final mass of 25 µg LDL protein per tube. Standard masses of H_2O_2 (0, 5, 10, 15, 20, 25, 50 nmol) was prepared from a stock solution of H_2O_2 (100 µM). LDL samples oxidised with ferritin as described in section 2.4.1 were made up in 15 ml polypropylene tubes (25 µg protein/ml). Colour reagent (composition described in section 2.1.1) was then added (1 ml) to standards of H_2O_2 and LDL samples and vortexed. The samples were then placed in the dark for 1 hour at room temperature. The samples were read in a spectrophotometer at 365 nm. The total lipid hydroperoxides were determined in nmol/mg LDL protein. This assay is based on the ability of peroxides in standards and sample to oxidise iodide ions in the colour reagent to form molecular iodine which then reacts with excess iodide ions present in the colour reagent to form the tri-iodide chromophore (I_3^-) which absorbs ultraviolet radiation at 365 nm.

2.6 Proteolytic degradation of ferritin

2.6.1 Enzyme digestions

2.6.1.1 Incubation of ferritin with cathepsin D

Enzyme digestions were carried out at 37^{o}C, the protein solution was placed in NaCl/sodium acetate buffer (pH 4.5). Ferritin (2mg/ml) was incubated with or without 6.2 µg/ml cathepsin D and incubated for 24 h.

2.6.1.2 Incubation of ferritin with cathepsin D and B

Ferritin (500 µg protein/ml) was incubated with or without 5 µg/ml cathepsin D and 5 µg/ml cathepsin B in Nacl/sodium acetate buffer pH 4.5 at 37ºC for 24 h.

2.6.1.3 Incubation of LDL with cathepsin D

The intact LDL (500 µg protein/ml) was incubated in the presence or absence of cathepsin D (5 µg/ml) in NaCl/sodium acetate buffer (pH 4.4) at 37˚C for 24 h.

2.6.1.3 Co-Incubation of LDL with cholesteryl esterase.

LDL (50 µg protein /ml) was co-incubated with 0.0625 unit/ml of cholesterol esterase from *Pseudomonas sp* which is active at pH 4.5. In another experiment, LDL (50 µg protein /ml) was co-incubated with 1.25 µg/ml cathepsin D, 1.25 µg/ml cathepsin B and 0.0625 unit of cholesteryl esterase/ml.

2.6.2 Electrophoresis

The intact ferritin, LDL and digested proteins were evaluated by gel electrophoresis under reducing condition using 6X RSTB. The treatment with SDS and β-mercaptoethanol contained in the RSTB was used to produce a linear structure of the proteins. The SDS denatures the secondary and tertiary structures while the β-mercaptoethanol reduces and breaks the disulphide bonds. The SDS confers a uniform negative charge on the proteins by masking the native charge of the protein, hence enable them to migrate based on size. Ferritin contains heavy and light chains (21 kDa and 19 kDa). Samples (15 µg) were loaded per well in 15% polyacrylamide gels for ferritin and 5% polyacrylamide gel for LDL along with molecular weight markers and the gel was left to run at 150 volts for 1 h. The protein bands were located by staining with Coomassie blue for 1 h and then de-stained for several hours.

2.6.3 Effect of proteases and cholesterol esterase on LDL oxidation by ferritin

The effect of enzyme digestion on the oxidation of LDL by ferritin was tested. Native LDL (50 µg protein/ml) was oxidised with 0.1 µM ferritin only or ferritin that was pre-incubated

with cathepsin D only or with the addition of cathepsin B or co-incubated with cholesterol esterase from Pseudomonas sp. Oxidation of LDL degraded with cathepsin D by ferritin was also compared to intact LDL in Chelex - 100 treated NaCl/sodium acetate buffer (pH 4.5) at 37˚C. Control samples not treated with enzymes were compared to samples treated with enzymes. The samples were placed against appropriate reference cuvettes that lacked LDL. The formation of conjugated dienes was monitored by measuring attenuance at 234nm for 1200 minutes at one-minute intervals.

2.7 Cell culture

THP-1 cells originally obtained from the blood of a one-year-old leukaemic patient were bought from European Collection of Authenticated Cell Cultures (EACC). They are monocytic cell line which can be differentiated into macrophages with phorbol 12-myristate 12-acetate (PMA). The THP-1 monocyte was maintained in RPMI 1640 medium modified with L-glutamine, phenol red, HEPES, sodium pyruvate with low sodium bicarbonate and high glucose. The medium obtained from Life Technologies, UK was supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), antibiotics (50 UI penicillin, 50 µg streptomycin and 0.95 µg amphotericin/ml). Cells were cultured in T75 flasks with filter caps using 20 ml media. Cells were cultured under humidified air (95%) / 5% CO_2 at 37ºC until confluent. Cells were subcultured usually every three to four days. The cell suspension was drawn out of a T75 flask into 50ml tubes and centrifuged at 500 x g for 5 min at room temperature. The supernatant was discarded and cells re-suspended in 5 to 7ml media, counted and re-seeded at 3 x 10^5/ml in 20ml culture medium in the new flasks.

NB: All media used in the experimental procedures were supplemented with 10% FCS and antibiotics (50UI penicillin, 50 µg streptomycin and 0.95 µg amphotericin/ml), except stated otherwise.

2.7.1 Cryopreservation of THP-1 monocytes.

Cells were collected as described above and centrifuged at 500 x g for 5 min at room temperature. The supernatant was removed, and the pellet was re-suspended in an ice-cold cryopreservation medium containing RPMI/FCS/DMSO (5/4/1 by vol.) using 1 ml for each confluent T75 flask. The cells were then kept in cryovials packed in polystyrene containers to ensure slow cooling then transferred to a -80 ^{o}C freezer overnight. Vials were moved to liquid nitrogen to store for a longer period. When needed vials were taken out, rapidly thawed in a water bath at 37 ^{o}C. Each 1 ml content was transferred to 50 ml tubes and washed with RPMI by centrifuging twice at room temperature, then seeded in T75 flask with medium containing 20% FCS (volume in flask was 20 ml) then transferred to an incubator left in standing position for about 3 hours then placed in a normal position for five days after which they are split into media containing 10% FBS at 3 X 10^5 cells/ml in 20ml culture medium.

2.7.2 Conversion of THP-1 cells to THP-1 derived macrophages

The THP-1 cells were cultured inT75 flask at 3 x 10^5 cells per ml and allowed to reach the density of 8 x 10^5 cells per ml before changing media. The viability of cells was maintained by not allowing the density to exceed 1 x 10^6 cells per ml. Depending on the experiment to be carried out, THP-1 cells were differentiated in tissue culture plates (6-, 12-, 24- or 96-well or) using phorbol 12-myristate 12-acetate (PMA) (25ng/ml). After 72 h cells were washed with sterile PBS and re-suspended in fresh media. Cells were then rested for 48 h before experiments were carried out.

2.8 Isolation of human monocyte-derived macrophages

Human monocytes were isolated from blood collected of healthy volunteers(as described in section2.2.1) by density gradient centrifugation with a Lymphoprep^{TM} solution (AXIS-SHIELD PoC AS, Oslo, Norway) (Firth *et al*., 2007). Erythrocytes and granulocytes have a

density greater than 1.077g/ml, while monocytes have a density of less than 1.077g/ml. Lymphoprep™ has a density of around 1.077g/ml and it, therefore, allows monocytes to be maintained at the interface between Lymphoprep™ and the buffer in which the cells were suspended while erythrocytes and granulocytes sediments.

The blood collected from donors were transferred to 50ml centrifuge tubes and centrifuged at 1500g for 30 minutes at 4 ^{o}C. The plasma was carefully removed leaving about 1 cm of plasma above the buffy coat. Using a sterile Pasteur pipette the buffy coat was removed into new 50ml Falcon tubes. About 15ml buffy coat was obtained (containing red blood cells which were removed in the next step). Then 20 ml of PBS (at room temperature was added. The mixture of buffy coat and PBS was gently underlaid with 15 ml of Lymphoprep™. The solution was then centrifuged at 1000g at room temperature for 20 minutes with the brake off and soft start. The red blood cells were visible at the bottom and the white monocyte/lymphocyte floating half way down the tube was collected (approximately 15 ml per tube). PBS (at room temperature) was added to each tube to a final volume of 45 ml and tubes were inverted to mix and then centrifuged at 500g at room temperature for 15 minutes (with the break on at the end). The supernatant was taken off and the pellet re-suspended in 50 ml PBS and then centrifuged at 500g for 10 minutes. This process was repeated 3 times to ensure cells were fully washed. The cells concentration was adjusted to 5 x 10^6 / ml in serum-free RPMI (containing antibiotics) and then plated at 6 ml in 6 well suspension plates. Heat-inactivated human serum was added at 5 µl /ml to each well and plates were incubated at 37 ^{o}C / 5 % CO_2 for 40 hours to allow T cell death and platelet adhesion while monocytes remain in suspension and viable.

The cell suspension from each well was collected into tubes pipetting thoroughly within the wells to collect resting monocytes whilst avoiding detachment of platelets. The cell suspension was centrifuged at 500 g for 15 minutes at room temperature. The supernatant was removed and re-suspended in RPMI media containing 10% heat-inactivated foetal calf serum and antibiotics (50 UI, 50 µg streptomycin and 0.95 µg amphotericin/ml). The cells were seeded at 3 x 10^6 / ml in 12 well adherent plates and GM-CSF (25 ng/ml) was added to the 1ml of suspension in each well (to differentiate them into M1 (pro-inflammatory) macrophages) and then transferred to an incubator at 37 ºC / 5 % CO_2. The cells' gross morphology was examined with a microscope to confirm they were monocytes. The media was changed every 3 days and monocytes were allowed to differentiate into macrophages after 7 days. The cells were plated according to the requirements of the particular experiments.

2.9 Determination of the effect of hepcidin on intracellular iron in macrophages

2.9.1 Treatment of THP-1 derived macrophage cells with hepcidin.

THP-1 derived macrophages (1.2 x 10^5) differentiated with PMA (25ng/ml) and rested for 48 h were plated in 24 well tissue culture plates using three media to vary the media concentration of iron (RPMI-1640, DMEM and F10 supplemented with 10% FCS and antibiotics). For each media cells were treated with or without hepcidin (1000 nM) and media was replaced after 24 h with media containing 10% LPDS with or without hepcidin (100nM) every 2 days. The protein content and iron concentration were measured after seven days.

2.9.2 Determination of intracellular protein concentrations

2.9.2.1 Preparation of cell lysate

The medium was removed, the plates were then kept on ice. Cells were then washed with cold PBS. 500 µl RIPA lysis buffer with protease inhibitors was added to each well and then swirled to distribute buffer. The cells were gently scraped and transferred into tubes. The cells were then incubated on ice for 15 minutes. The lysate was then sonicated and centrifuged at 13,000 g for 5 minutes and the supernatant collected.

2.9.2.2.Determination of protein content with Bio-Rad DC™ protein assay kit.

BSA standards (0, 5, 25, 50, 100 µg) was prepared and the protein concentration in samples was measured using a Bio-Rad DC™ protein assay kit. This assay is based on a similar principle with Lowry assay in which the protein content of the sample reacts with copper tartrate and then the copper treated protein subsequently reduces the Folin reagent. This reduction produces reduced species with chararteristic blue colour which absorbs maximally at 750 nm. Reagent A was added to the reagent S ratio (200 µl: 4 µl). Reagent A (5000 µl) and 100 µl of reagent S. This mixture (200 µl) was then added to each standard and samples. Reagent B (800 µl) was then added, each tube was vortexed and left for 15 minutes at room temperature. The absorbance was measured at 750 nm. The protein contained in the cell lysates were then determined in comparison with the BSA standards.

2.9.3 Inductively coupled plasma mass spectrometry (ICP-MS) for measuring total iron in macrophages

THP-1 macrophage-like cells treated with or without hepcidin as described in section 2.9.1 were transferred to 15ml tubes and centrifuged at 500 g for 10 min. Cell pellets were washed with PBS. The cell pellets were then digested with nitric acid, equivalent to 2% of the final volume, and incubated at 75^{o}C for 2 h. It was then diluted to 10ml with ultra-pure water. The concentration of iron in samples were measured with ICP-MS (Flores *et al*., 2015). The use of

ICP-MS is an established method for the analysis of multiple elements, very sensitive and able to detect as low as the ultra-trace concentration of varying elements. The sample is introduced to the system true the liquid-phase nebulizer. The major analytical feature of the system is the production of ions in the inductively coupled plasma (ICP) section of the machine. The ICP mass spectra show the amount of ions measured in every second.

2.10 Measurement of lysosomal lipid peroxidation in THP-1 cells and human monocyte-derived macrophages.

The local lipid peroxidation (LPO) in lysosomes of macrophages was measured with the novel lysosome-targeted probe Foam-LPO which was recently synthesised by Zhang and colleagues (Zhang *et al*., 2015). The Foam-LPO is a fluorescent probe, which possesses an alkaline tertiary amino group which enables it to localise in the acidic environment of lysosomes (becomes protonated and accumulates in the lysosome). Its fluorophore structure acts as a signal of lipid peroxidation and the conjugated diene group present in the BODIPY fluorophore are degraded in response to lipid peroxidation leading to a fluorescence shift from 586 nm to 512 nm which can be measured by two-way flow cytometry.

THP-1 macrophages prepared as described in section 2.7.2 were plated at 5 x 10^5 cells per well were treated with pre-warmed RPMI–1640 with 10% human serum (2ml per well) alone or with ferritin (100 µg/ml) for 24 h in an incubator at 37 ^oC / 5 % CO_2. The cells were then treated with or without LDL (200 µg protein/ml) for 24 h to have the following four conditions: no LDL, LDL only, and LDL with ferritin and ferritin only. After the total 48 h treatment for ferritin and LDL treatment, the cells were washed three times with PBS (pre-warmed to 37 ^oC) and then added fresh media. The adhered macrophages were scraped into a clear 96 well round

bottom microplate (Greiner CellStar®) and centrifuged at 500 g at room temperature for 5 min. The cells were then re-suspended in 200 µl media (no serum) containing 2 µM Foam-LPO (a kind gift from Dr Xiao, Dalian University China) for 15 min at room temperature in the dark. The cells were then centrifuged at 500 g for 5 min at 4 ^{o}C and the supernatant was discarded and the cells re-suspended in PBS and centrifuged at 500 g for 5 min at 4 ^{o}C. Washing with PBS was repeated once and then the cells were re-suspended in flow cytometer buffer.

The cells were analysed with a Becton Dickinson flow cytometer (BD Biosciences C6 flow cytometer). FlowJo software was used to determine the mean fluorescence intensity of the two channels and the ratio of green channel (FL1) to the red channel (FL2) was used as a measure of LPO in lysosomes.

The experiment was also carried out using the same procedure with sphingomyelinase-aggregated LDL with THP-1 cells or human monocytes-derived macrophages (HMDM) isolated as described in section 2.8.

2.11 Measurement of intracellular lipid and ceroid levels after treatment with ferritin

2.11.1 Preparation of sterilised coverslips

Prior to the start of experiments sterile coverslips (18 x 18 mm) were cleaned in acid (65% nitric acid left overnight on a rocker at low rpm overnight well labelled. Coverslips were then washed in ultra-pure water several times. The coverslips were then dried in the safety cabinet to dry and then placed in a container and autoclaved. Prior to each experiment–requiring coverslips, the cover slips were exposed to UV light to further sterilise.

2.11.2 Measurement of intracellular lipids and intralysosomal ceroid

THP-1 cells differentiated with PMA (25ng/ml) were plated at 45,000 on sterilised coverslips in two 6-well tissue cultured plates. In each plate, three wells were pre-treated with 100 µg ferritin for 24 h. Cells were washed with PBS and RPMI-1640 media (2ml) was added to two wells (RPMI only and RPMI + preloaded ferritin), native LDL (200 µg protein/ml) was added to next two wells (native LDL only and native LDL + preloaded ferritin), the last two wells received 200 µg protein/ml SMase-LDL (SMase-LDL only and SMase-LDL + preloaded ferritin) and the treatment was left for 24 h. The cells were then washed with PBS and 2ml of RPMI-1640 containing 10% (v/v) lipoprotein deficient serum and antibiotics (50UI, 50 µg streptomycin and 0.95 µg amphotericin/ml). This was replaced every two days for each well for seven d the cells in one plate were stained for intracellular lipids and the other for ceroid.

2.11.3 Oil Red O Staining for intracellular lipids

The tissue culture plate was transferred to the fume hood, the media was then replaced with 2ml of PBS. The PBS was then replaced with 2ml of 4% (w/v) paraformaldehyde and then incubated for 15 minutes. Paraformaldehyde was removed into a waste container. Cells were gently rinsed with distilled water. Cells were washed with PBS and after washing, 1.5 ml Oil Red O (0.5% w/v) working solution was added to each well and then incubated for 10 minutes. After 10 minutes. Oil Red O was removed and cells were gently rinsed with water until the distilled water rinsed clear. Coverslips were then mounted on glass slides with cells facing downwards on a drop of mounting media. The intracellular lipids were visualised using light microscopy with Axiovision software and analysed with Image J software.

2.11.4 Oil Red O Staining for Ceroid

At the end of treatment, cells were washed two times with PBS and then fixed by adding 2ml 4% paraformaldehyde for 15 minutes. The cells were gently rinsed with 2ml water and then washed with twice with 2ml PBS. The cells on coverslips were transferred to a glass staining rack and 2ml of xylene was added to each well and incubated for 5 minutes. Each well was then rinsed with 60% (v/v) isopropanol. 2ml ethanol was then added and incubated for another 5 minutes. Oil Red O (1.5 ml) was added and incubated for 15 minutes. The Oil Red O was removed and then rinsed with distilled water several times until it appeared clear. The ceroid content was then visualised with light microscopy (Axioscop 2, Carl Zeiss Ltd) and images were captured with Axiovision software and analysed with Image J software.

2.12 Determination of intracellular reactive oxygen species formation induced by ferritin-oxidised LDL

THP-1 cells (1.5 x 10^5/ml) was added to PMA (25 ng/ml) with pre-warmed RPMI– 640 media containing 10% (v/v) heat-inactivated foetal calf serum and antibiotics (50UI, 50 µg streptomycin and 0.95 µg amphotericin/ml). The cell suspension (300 µl) (4.5 x 10^4) of THP-1 cells was placed in tissue culture-treated coverslips in 6-well tissue culture plates for 72 h with humidified air (95%) / 5% CO_2 at 37ºC After 72 h the cells were washed with PBS and rested in pre-warmed RPMI – 1640 for 48 h. (coverslips were prepared as described in section 2.11.1)

Prior to treating cells, oxidised LDL was prepared by incubating (500 µg LDL protein/ml) with 1 µM ferritin at 37˚C for 24 h NaCl/sodium acetate buffer (pH 4.5). LDL (500 µg protein/ml),

ferritin (1 µM) and buffer (equivalent volume), each in separate tubes were also incubated at 37˚C for 24 h.

The THP-1 cells on coverslips were then incubated with pre-warmed RPMI–1640 media alone (2 ml per well) or containing either NaCl/ sodium acetate buffer or LDL (50 µg protein/ml) or ferritin (0.2 µM) or oxidised LDL (50 µg protein/ml). After incubation for 24 h with humidified air (95%) / 5% CO_2. The cells were washed twice with PBS and incubated with 10 µM dihydroethidium (DHE) in PBS (this working solution was prepared on the day of the experiment from 20mM (10 mg in 1585 µl DMSO). Placed in a non-CO_2 incubator in the dark for 30 min. The cells were then washed twice with PBS and the cells were mounted with fluorescence mounting media containing DAPI. Images were captured using Axioimager. The intensity of the DHE stain was determined using ImageJ software

2.13 Determination of the effect of oxidation of LDL by ferritin on cellular respiration/metabolism using a Seahorse analyser.

The Seahorse XF analyser is an important tool in the measurement of cellular energy metabolism. The Seahorse can be used to determine the energy metabolism in live cells by monitoring the oxygen uptake and the pH in real time.

THP-1 cells were differentiated in T25 flasks using PMA (25ng/ml) at 3 x 10^5 cells/ ml for 72 h with humidified air (95%) / 5% CO_2 at 37^{o}C with pre-warmed RPMI – 1640 media containing 10% (v/v) heat-inactivated foetal calf serum and antibiotics (50UI, 50 µg streptomycin and 0.95 µg amphotericin/ml). After 72 h the cells were washed and rested in pre-warmed RPMI-

1640 for 48 h prior to treatment with LDL. After a 24 h rest period, the cells were detached from the flask using Accutase and transferred to an Agilent Seahorse XFp cell culture mini plate to rest for another 24 h. The mini-plate has 8 wells labelled A to H. The wells A and H always served as blanks with no cells, just media, while wells 6 x 10^4 cells were plated in 200 µl for wells B to G.

On the day before the assay, the Agilent cartridge was hydrated with calibrant by adding 200 µl calibrant in the wells A-H and 400 µl PBS into the moat on the side. The sensor cartridge was then placed on to the plate submerging the sensors in the calibrant and then incubated in a non CO_2 incubator at 37˚C for 24 h. The cells in Agilent plate was treated as follow, 3 wells E, F and G were pre-treated with ferritin (0.2µM) for 24 h. After treatment with ferritin, the cells in position B got RPMI media only, C and D got native LDL (100 µg protein/ml), position E got only the pre-incubated ferritin while F and G got native LDL (100 µg protein/ml) in addition to pre-incubated ferritin.

On the day of the assay, 10 ml of XF base media was prepared, glucose (10 mM), pyruvate (1 mM) and Glutamine (2 mM) were added. The pH of the media was then adjusted to pH 7.4 and warmed up to 37˚C and filtered with a 0.2 µm Minisart filter. The cells in the Agilent mini plates were washed with XF base media freshly prepared by taking 180 µl of the RPMI-1640 from treated cells and replacing it with180 µl XF base media. This step was repeated 2 times using XF base media and wells A to H were then made up to a final volume of 180 µl. The cells in the Agilent plate was then placed in the non-CO_2 incubator at 37˚C for 1 hour.

The Agilent Seahorse XFp analyser was turned on to allow it warm up to 37˚C. A stressor mix was immediately prepared. This was prepared in accordance with Agilent Seahorse XFp cell energy phenotype kit. XF base media (120 µl) was added to 60 µl oligomycin (2 µM/well) and 120 µl FCCP (2 µM/well). The calibrated cartridge was brought out of the incubator after 24 h, 20 µl of the stressor mix was added to ports A of all the 8 positions of the hydrated cartridge (given 10 µM oligomycin and 20 µM FCCP in each of the port A). The energy phenotype test was selected on the Seahorse XFp analyser. The number of cycles for base line and after oligomycin injection was set at five cycles each. The cartridge plate already loaded with the stressor mix in each port A was then placed in the instrument for calibration. The calibration process takes approximately 20 minutes. The bottom plate of the cartridge was then replaced with the cells in the Agilent tissue culture plate that had stayed in the non CO_2 incubator for 1 h. The plate was placed without the lid. The energy phenotype test was then carried measuring the oxygen consumption rate (OCR) and the extracellular acid Rate (ECAR).

2.14 Determination of apoptosis in macrophages treated with ferritin-oxidised LDL

Phosphatidylserine in healthy cells is mainly located on the cytosolic side of the plasma membrane. The externalisation of phosphatidylserine in cells to the surface of the cells is an early indication of apoptosis (Martin *et al.*, 1995). Annexin V binds to externalised phosphatidylserine and fluorescent annexin V can be used to detect apoptotic cells. Apoptosis was measured using the commercially available kit (FITC Annexin V Apoptosis Detection Kit with PI, BioLegend, San Diego, CA, USA) and flow cytometry. The kit contains fluorescein isothiocyanate (FITC) - annexin V conjugate which can be used to identify apoptotic cells. Propidium Iodide (PI) was added to separate the necrotic cells from apoptotic cells. PI has the

capacity to enter cells and stain the DNA of cells undergoing necrosis. Cells that possess increased levels of PI labelling are necrotic, cells with increased levels of fluorescent annexin V labelling are apoptotic and cells having high levels of both annexin V and PI are going through secondary necrosis.

Prior to treating cells, oxidised LDL was prepared by incubating (1 mg LDL protein/ml) with 2 µM ferritin at 37˚C for 24 h in NaCl/sodium acetate buffer (pH 4.5). LDL (1 mg protein/ml), ferritin (2 µM) and buffer (equivalent volume), each in separate tubes were also incubated at 37˚C for 24 h.

THP-1 cells were differentiated in 12-well tissue culture plates at 5 x 10^5 cells per well using PMA (25ng/ml) as described in section 2.7.1. After 72 h the cells were washed and rested in pre-warmed RPMI – 1640 for 48 h prior to treatment with LDL. The cells were then incubated with pre-warmed RPMI – 1640 media alone (2 ml per well) or containing either NaCl/sodium acetate buffer or LDL (100 µg protein/ml) or ferritin (0.2 µM) or oxidised LDL (100 µg protein/ml). After incubation for 48 h with humidified air (95%) / 5% CO_2 at 37 ^oC, the medium was removed from the cells and kept for each well. To detach cells, Accutase (750 µl) was added and then placed in an incubator for 10 min. The removed media was then added accordingly to each well (750 µl). Each well was then collected into an Eppendorf tube and centrifuged at 100g for 5min. The pellet was washed twice with cell staining buffer. The cells were re-suspended in annexin V binding buffer (800 µl), then 250 µl was transferred to brown Eppendorf tube for protection from light. PI (2.5 µg) and (FITC)-annexin V (0.5 µg) was added and then incubated for 15 min at room temperature in the dark. The volume was made up to 500 µl and analysed immediately using a Becton Dickinson FACScan flow cytometer (BD Biosciences C6 flow cytometer). The percentage of healthy cells, necrotic, apoptotic and

secondary necrotic cells was determined using the BD Biosciences C6 flow cytometer software.

2.15 Statistical analysis

The data are presented as the mean plus or minus standard error of the mean (SEM) of the number of independent experiments indicated. Data were statistically analysed using a Student's paired t test, one way analysis of variance (ANOVA) followed by Tukey's post hoc test but when comparing multiple parameters, we made use of two-way ANOVA and a Bonferroni post hoc test to determine the significance of the criterion tested. GraphPad prism 5 software was used for all statistical analysis carried out. For all hypothesis we tested, 0.05 was considered as the significance level. Hence, a P value < 0.05 was considered a statistical significance.

Chapter 3 - Mechanisms of oxidation of LDL by ferritin at lysosomal pH

3.0 Background and rational

Research over the last few decades has proposed oxidised LDL to be an important marker in the onset and progression of atherosclerosis. The Oxidised LDL hypothesis emphasised the fact that oxidative modification of LDL promotes its atherogenic nature. Oxidised LDL instigates many pro-atherogenic effects ranging from induction of formation of lipid-laden macrophages (foam cells) to the promotion of inflammatory responses hence activating endothelial cells and promoting smooth cells proliferation. The mechanism by which LDL is oxidised *in vivo*, the site of oxidation and when LDL is oxidised is continuously deliberated amongst researchers (Itabe *et al.*, 2011).

Many mechanisms have been proposed for oxidation of LDL ranging from oxidation by cells, which are dependent on the availability of free metal ions to free metal ion–independent oxidation mediated by enzymes such as lipoxygenase and myeloperoxidase (Yoshida and Kisugi, 2010). The oxidation of LDL by free metal ions is the most studied model of LDL oxidation *in vitro* and the oxidation of LDL by cells of the arterial walls indeed requires the presence of certain concentrations of copper or iron (Leake and Rankin, 1990, Leeuwenburgh *et al.*, 1997). Transition metal ions have been confirmed to catalyse the oxidation of DNA, protein, and lipids. Increased levels of transition metals have also been found in animal models and human atherosclerotic plaques (Stadler *et al.*, 2004, Stanley *et al.*, 2006). Hence, this supports the suggestion that metal ions contribute to the onset and progression of atherosclerosis.

The iron hypothesis of atherosclerosis proposed that men and post-menopausal women are more susceptible to heart diseases due to elevated levels of body iron compared to premenopausal women with lower levels of stored iron as a result of monthly bleeding (Sullivan, 1981). Over the last three decades, several researchers have measured the correlation

between iron levels in the body and atherosclerosis. Despite the continuous research in this field, this hypothesis is continuously debated and remains controversial (Munoz-Bravo *et al*., 2013, Aursulesei *et al.*, 2014). Sullivan, however, subsequently argued that this gender difference in the incidence of heart diseases is linked to body iron stores and not the oestrogen levels (Sullivan, 1989, Sullivan, 1992, Sullivan, 2003, sullivan, 2005, Sullivan, 2007). Further to this other studies have demonstrated an association between iron deposition and the progression of atherosclerosis (Lee *et al.*, 1998, Stadler *et al*., 2004).

Iron is important in maintaining the integrity of cells and its function by mediating various metabolic systems through enzymes that are dependent on iron. Approximately all iron in the human body is bound to molecules. The existence of iron in bound form is highly advantageous to the body, as iron is capable of participating in redox reactions that are harmful to the body. It has the ability to exist in several oxidation states ranging from Fe^{2+} to Fe^{6+}. However, the transfer of one electron between ferric ion (Fe^{3+}) and ferrous iron (Fe^{2+}) has been implicated in iron–dependent free radical reactions, which have been linked to cell injury and several pathophysiological reactions (Ramakrishna *et al*., 2003, Yuan and Li, 2008).

The redox-active iron pool, also referred to as labile iron pool (LIP), is present in small amounts in cells but it is an important part of certain metabolic pathways and Fenton reaction which may contribute to lipid peroxidation and pathological process. The LIP is not only found in the cytosol but also present in the nucleus, mitochondria and lysosomes (Kruszewski, 2003, Lv and Shang, 2018). Lysosomes possess a higher concentration of the LIP, as it was discovered to be about 16 μM in endothelial cells of rat liver (Petrat *et al*., 2001). LIP has been connected to atherogenesis in several studies. The iron storing and regulatory proteins such as transferrin, ferritin and hepcidin have been associated with atherosclerosis (Yuan and Li, 2008).

Human atherosclerotic lesions have been shown to contain ferritin and lysosomal iron in the lipid laden cells present at this site (Yuan *et al.*, 1996, Lee *et al.*, 1998, Yuan, 1999). Lysosomes are one of the few sites in the body where redox-active iron is present; the source of iron in lysosomes could be from the decomposition of endocytosed or autophagocytosed iron containing proteins such as ferritin or mitochondrial metalloproteins. The iron present in these molecules can be released by the iron transporter DMT1 (Kurz *et al.*, 2007, Lv and Shang, 2018). Ferritin as a protein plays a key role in the storage of iron in the body. In recent times ferritin is now becoming an evidently important factor in the development of iron storage and transport diseases, cancer, diabetes, neuropsychiatric disorders and atherosclerosis (Goswami *et al.*, 2008).

The ferritin molecule is the main protein for the intracellular storage of iron present in almost all organisms. The hollow protein weighs about 500 kDa and contains 24 polypeptide chains made up of the light (L) subunits and heavy (H) subunits with molecular weights of 19kDa and 21kDa, respectively, with capacity for storing up to 4500 iron atoms. The ratio of these two species varies in different tissues, however, ferritin rich in L subunits is mainly found in pancreas, the heart, the liver and spleen (Bomford *et al.*, 1981, Rucker *et al.*, 1996, Goswami *et al.*, 2008, Arosio *et al.*, 2017). Although there are conflicting data from epidemiological studies on the role of ferritin in CVD, some studies have ascertained the association between serum ferritin levels and increased susceptibility to myocardial infarction and coronary artery diseases (Salonen *et al.*, 1992, Kiechl *et al.*, 1994, Olesnevich *et al.*, 2012).

Iron regulatory/binding proteins such as ferritin and hepcidin have been linked to the inflammatory response induced pathogenesis of atherosclerosis (Yuan and Li, 2008). Deposits of cholesterol crystals derived from LDL oxidation in lysosomes have been demonstrated to activate NLRP3 inflammasome and lead to these organelles rupturing in macrophages, hence

contributing to the destabilisation of lysosomes and promoting atherosclerosis (Duewell *et al*., 2010). Our laboratory demonstrated and identified lysosomes in macrophages as a site for the oxidation of LDL and this is mainly mediated by iron (Wen and Leake, 2007). Oxidation of polyunsaturated fatty acids and cholesterol in low density lipoprotein gives rise to fatty acid hydroperoxides and derivatives of oxidised cholesterol. Cholesteryl esters and cholesterol are the major lipids in LDL particle. The core containing cholesteryl esters is fluid while the outer monolayer containing cholesterol is more rigid and this might help to explain why the formation of cholesteryl ester hydroperoxide is more rapid compared to phospholipid hydroperoxide in the outer monolayer (Noguchi *et al*., 1998).

Decomposition of the hydroperoxides leads to the formation of aldehydes, apart from PUFA and cholesterol content of LDL the apoB content can also be chemically modified (Thomas *et al.*, 1994). The oxidation products and quantity obtained depends on the conditions used for oxidation. Oxidation by copper leads to fragmentation of apoB, loss of amino acids from apoB, generation of lipid hydroperoxides and aldehydes (Steinberg *et al.*, 1989, Esterbauer *et al.*, 1992). Oxidation by iron at lysosomal pH leads to formation of the oxidised product of cholesterol and hydroperoxides (Satchell and Leake, 2012, Ahmad, 2016) and loss of tryptophan from apoB (Ahmad, 2016, Ahmad and Leake, 2018). *In vitro* oxidation of LDL can be catalysed by iron and this oxidation has been proven to be much faster at acidic pH similar to the pH of lysosomes. The mechanisms by which iron contributes to LDL oxidation in lysosomes and at lysosomal pH has been previously explored in our laboratory (Morgan and Leake, 1993, Morgan and Leake, 1995, Wen and Leake, 2007, Satchell and Leake, 2012, Ahmad and Leake, 2018)

We have now postulated that ferritin could substitute for iron and oxidise LDL at lysosomal pH. This chapter aims to explore the mechanisms of LDL oxidation by ferritin at lysosomal pH while addressing the following objectives.

3.1 Objectives

i) To investigate the effect of ferritin on LDL oxidation at lysosomal pH (pH 4.5), compare the oxidation of LDL at pH 4.5 to pH 7.4 and to evaluate the effect of pH changes on LDL oxidation by ferritin.

ii) To investigate the possibility of ferritin as a source redox- iron at lysosomal pH 4.5

iii) To determine the effects of iron released from ferritin on oxidation of LDL by ferritin by testing the effects of iron chelators on this oxidation.

v) To explore the effect of apoferritin, the protein component of ferritin, on LDL oxidation at lysosomal pH.

Vii) To characterise the oxidised products of LDL oxidation by ferritin at lysosomal pH.

3.2 Methods

LDL was isolated as described in sections 2.2.1 and 2.2. Formation of conjugated dienes, kinetics of oxidation of LDL oxidation by ferritin and aggregation of LDL by ferritin was measured as described in (sections 2.3, 2.3.1, and 2.3.2). The amount of iron in ferritin and apoferritin was measured by AAS (section 2.3.2). Iron released from ferritin at varying pH was measured using the iron chelator bathophenanthroline disulphonic acid and by ultrafiltration followed by AAS (sections 2.3.4 and 2.3.5, respectively). Effect of iron chelators was also determined spectrometrically, as described in section 2.3.6. The formation of oxidised lipids,

mainly 7-ketocholesterol and cholesteryl linoleate hydroperoxide, in LDL oxidised by ferritin was measured by HPLC as described in sections 2.4, 2.4.1, 2.4.2, 2.4.3 and 2.4.3.1. The total hydroperoxides in the oxidised LDL were also measured colourimetrically using the tri-iodide assay (section 2.5).

3.3 Results

3.3.1 Spectrophotometric measurement of LDL oxidation by ferritin at lysosomal pH

In order to confirm the ability of ferritin to oxidize LDL at lysosomal pH, LDL (50µg protein/ml) was oxidised in NaCl/sodium acetate buffer (pH 4.5) at 37 ^{o}C with varying concentrations of ferritin (0.05 µM, 0.1 µM and 0.2 µM). We monitored the kinetics of oxidation by measuring the formation of conjugated dienes at 234 nm.

All concentrations of ferritin (0.05 µM - 0.2 µM), used in this experiment oxidised LDL effectively (Fig 3.1A). The kinetics of oxidation was somewhat similar to what was previously observed for ferrous iron at pH 4.5 (lag, rapid, slow oxidation, aggregation and sedimentation phases) (Satchell, 2008). The lag phase of oxidation was not as pronounced in LDL oxidised by ferritin and was sometimes not present. The conjugated diene formation began at an almost constant rate for the three concentrations of ferritin. The slow phase was observed at the three concentrations, but this was observed more at 0.1µM and very little or no slow phase was seen at 0.2 µM. At the highest concentration of ferritin, (0.2 µM) aggregation (when the aggregated LDL scatters UV radiation) began as early as the first 300 minutes and the LDL began to sediment below the beam of UV in the spectrophotometer at about 580 minutes, while the presence of 0.1 µM and 0.05 µM caused aggregation later.

The attenuance at 200 min was compared with the control and to each other (Figure 3.1B). Statistical analysis showed the control (LDL only) was significantly different ($p<0.001$) as

compared to the addition of the three concentrations of ferritin (0.05 µM, 0.1 µM, and 0.2 µM). The attenuance at 200 min was used for statistical analysis because at this time the LDL is in the middle of the oxidation phase and not yet in the aggregation phase. This time point should be accurate because there is quite a lot of attenuance but this is a long way from the aggregation phase as shown by fig. 3.2 and by previous research (Satchell and Leake, 2012). The maximum attenuance in experiments was not measured because at the stage there would be a "race" between aggregation and sedimentation of the LDL particles. The lag phases were also not considered, as this would be shortened by storage of the stock LDL at 4 ^{o}C. This present study was focused on the events at the oxidation phase, however, measuring for a longer time gives a picture of the events in the aggregation phase as this might provide evidence for further research.

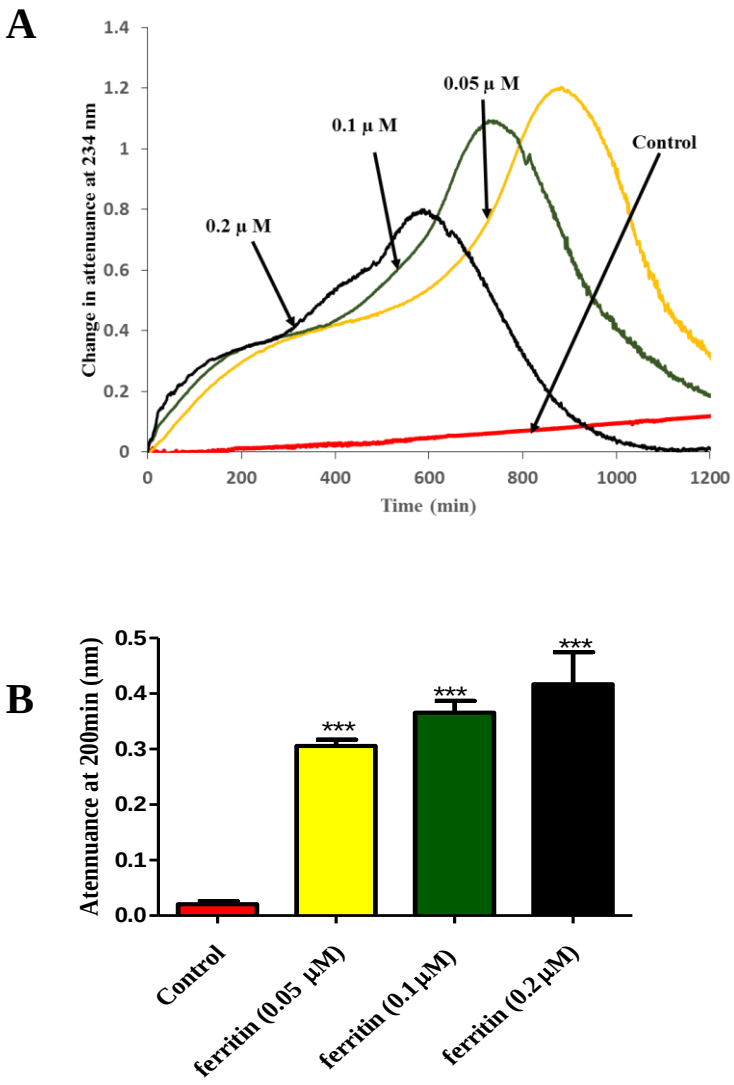


Figure 3.1: The oxidation of LDL by different concentrations of ferritin

LDL (50µg protein/ml) in NaCl/ sodium acetate buffer (pH 4.5) was incubated in the absence (red line) or presence of varying concentrations of ferritin, 0.05 µM (yellow line), 0.1 µM (green line) and 0.2 µM (black line) at 37ºC in capped cuvettes. The formation of conjugated dienes was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL (A). This is a representative of three independent experiments. The increase in attenuance at 200 min in the presence or absence of ferritin in four independent experiments. Mean and SEM were obtained for each concentration and compared to control (n=4). The means were compared with one-way ANOVA followed by a post hoc Tukey test ($P < 0.001$) (B).

3.3.2 Measurement of LDL aggregation during oxidation by ferritin

Aggregation of LDL has been shown to occur alongside its oxidation and also enhance its formation of lipid-laden macrophages (Maor *et al.*, 1997). It was of interest to investigate whether the oxidation of LDL by ferritin leads to its aggregation. To test the effect of ferritin on LDL aggregation. LDL (50μg protein/ml) and ferritin (0.1 μM) was incubated in NaCl/sodium acetate buffer (pH 4.5) at 37^{o}C in the presence or absence of each other. The aggregation was then continuously measured at 680 nm. The extent of LDL oxidation by ferritin was directly correlated with its rate of aggregation while the LDL and ferritin on their own were not aggregated

The increase in attenuance at 800 min, a time at which LDL was expected to have been fully oxidised, was compared and there was a significant difference when LDL was incubated with ferritin ($P<0.001$). There was very little aggregation during the oxidation phases.

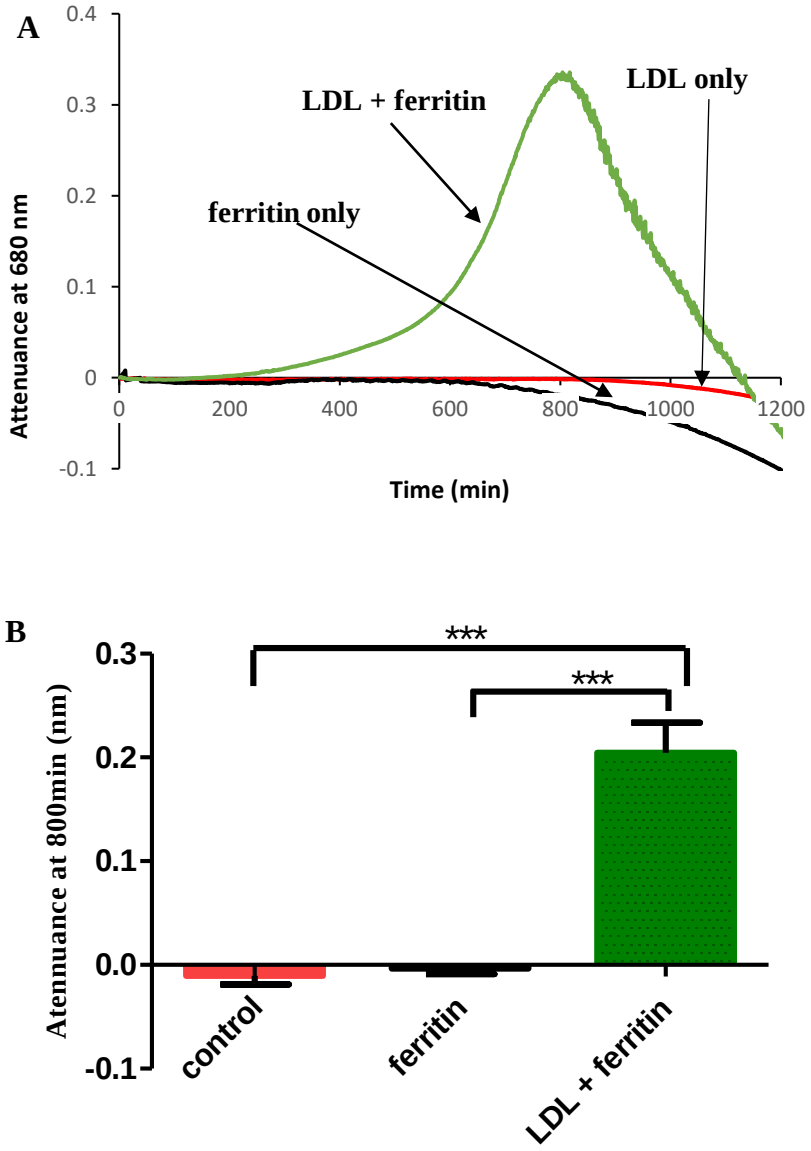


Figure 3.2: Effect of LDL oxidation by ferritin on LDL aggregation

Native LDL (50µg protein/ml), ferritin only (0.1 µM) and LDL (50µg protein/ml) plus ferritin (0.1 µM) was incubated in NaCl/sodium acetate buffer (pH 4.5) at 37^{o}C in capped cuvettes (A), the aggregation of samples was monitored by measuring attenuance at 680 nm against appropriate reference. This is a representative of three independent experiments. The means were compared with one-way ANOVA followed by a post hoc Tukey test ($P < 0.0001$). Attenuance at 800 min was significantly higher at in oxidised LDL compared to control (B).

3.3.3 Comparison of oxidation of LDL by Ferritin at pH 4.5 and pH 7.4

The effect of pH on the ability of ferritin to oxidise LDL was tested. LDL (50µg protein/ml) was incubated with 0.1 and 0.2 µM ferritin at pH 7.4 (MOPS buffer) was compared to pH 4.5 (NaCl/sodium acetate buffer) (Fig 3. 3). The formation of conjugated dienes was monitored continuously. The result showed that the oxidation of LDL by ferritin is slower at pH 7.4 for both concentrations as compared to pH 4.5 where LDL was effectively oxidised. The rate of oxidation observed at both pH values was similar to that of the oxidation of LDL by ferrous sulphate described by Satchell and Leake (Satchell and Leake, 2012): unlike the rapid oxidation observed at pH 4.5, the oxidation at pH 7.4 only rose to attenuance of about 0.2 throughout the course of the experiment. The increase in attenuance at 200 min was compared for each concentration (0.1 and 0.2 µM) at the different pH (4.5 and 7.4) (Fig 3.3B). The statistical analysis of both concentrations showed a significant difference between incubation with ferritin at pH 4.5 and pH 7.4. The attenuance was higher at 0.2 µM showing that the effect was also concentration dependent.

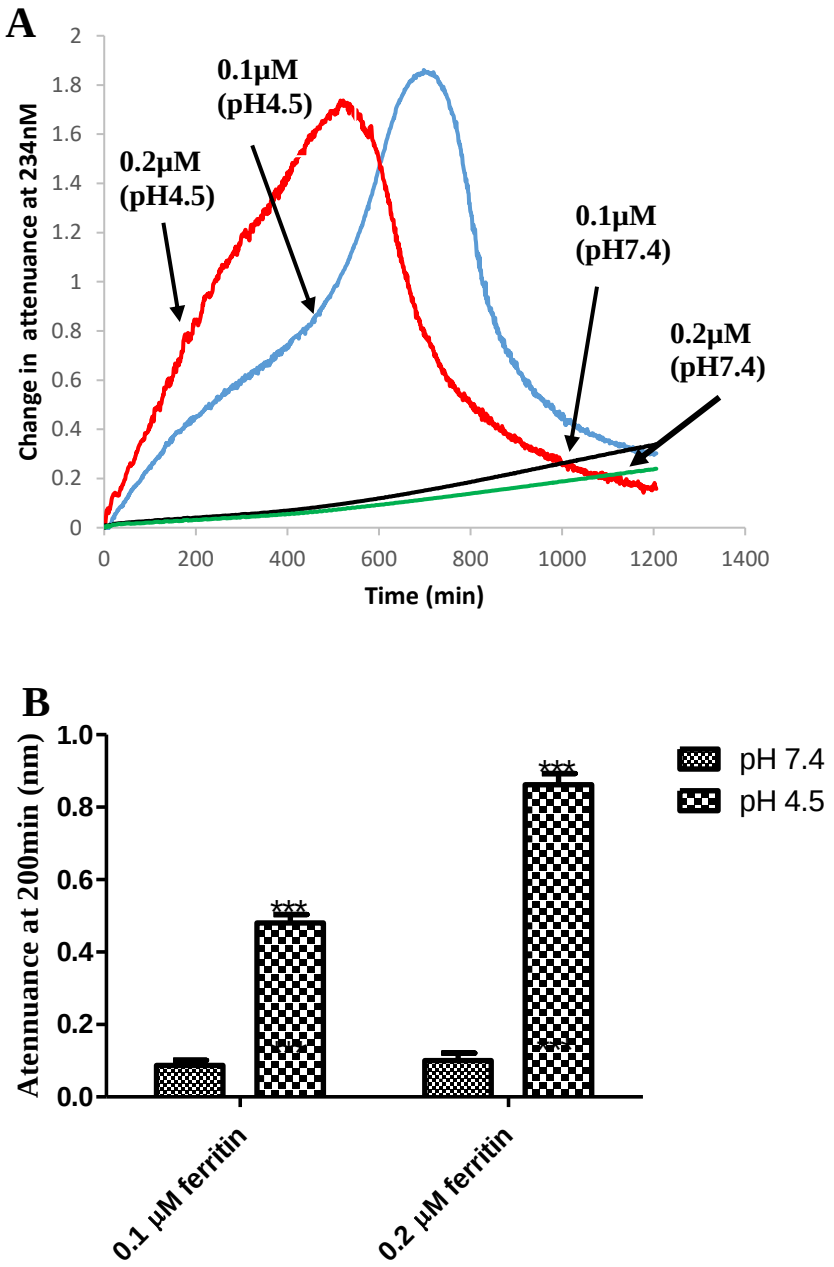


Figure 3.3: Comparison of oxidation of LDL by ferritin at pH 4.5 and pH 7.4

LDL (50µg protein/ml) was incubated with 0.1 µM or 0.2µM in NaCl/sodium acetate buffer (pH 4.5) and MOPS buffer (pH 7.4) at 37^{o}C in capped cuvettes (A), the formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments. The means were compared with two-way ANOVA followed by Bonferroni post-tests ($P < 0.0001$). Attenuance at 200 min at pH 4.5 was compared to pH 7.4 (B).

3.3.4 Determination of the quantity of iron contained in ferritin.

Ferritin has the capacity to bind up to 4500 atoms of iron per particle. In order to determine the level of saturation of the horse spleen ferritin used, the amount of iron present was estimated using FAAS at 248.3nm. The concentration of iron was obtained in comparison with known standards of iron ($Fe^{3+)}$. Iron concentration in ferritin was estimated from the linear trendline of the standard plot (Fig 3.4). Ferritin particles were estimated to contain 1001 atoms of Fe /particles. (The molecular weight of ferritin obtained from Sigma is 440kDa.) The amount of iron contained in ferritin concentrations (0.05, 0.1, and 0.2 µM) added was estimated to be 50 µM, 100 µM and 200 µM (see appendix 2 for calculation of iron concentration).

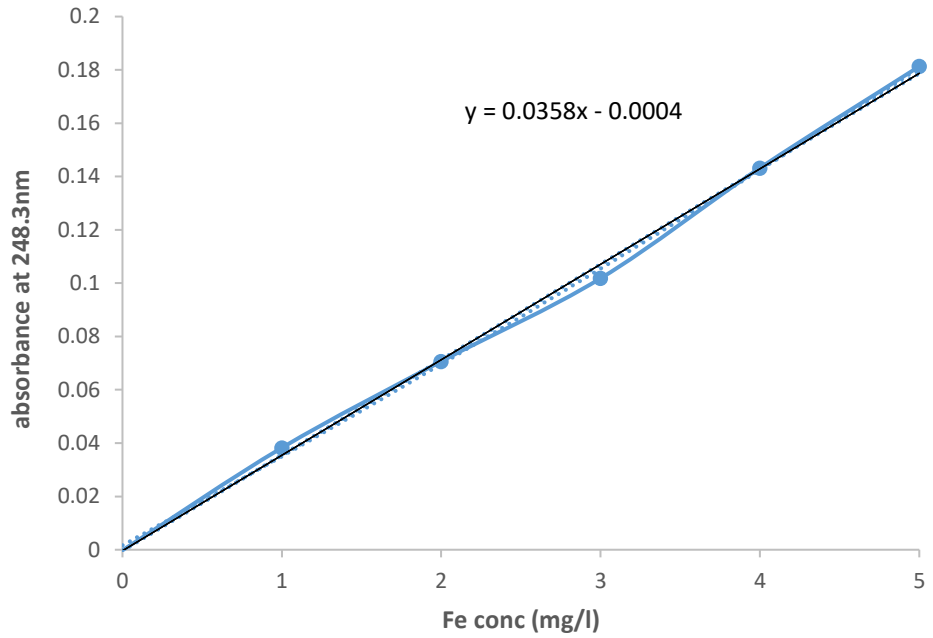


Figure 3.4: Standard plot of iron determination in ferritin assay by atomic absorption spectroscopy

The blue line represents the standard plots obtained from the standards 0-5mg/L. The black line represents the trendline for the equation used to extrapolate the quantity of ferritin.

3.3.5 Measurement of iron released from ferritin at pH 4.5 versus pH 7.4 using the iron chelator bathophenanthroline (BP)

To examine whether more iron released from ferritin at acidic pH was responsible for the faster oxidation observed at pH 4.5 compared to pH 7.4, we measured the amount of iron released at lysosomal pH was compared to what was released at interstitial or plasma. Ferritin (0.1 µM) was incubated at 37 ˚C in NaCl/sodium acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) in 15 ml tubes, in triplicate. Bathophenanthroline (BP) (30 µl of 10 mM) was added at different time points (up to 24 h). Absorbance was measured at 535 nm (Figure 3.5). The amount of ferrous complex released was higher at pH 4.5 compared to pH 7.4 at all the time points that are taken. At 0 hr, more ferrous complex was detected at pH 4.5 (5.49 ± 0.17µM) compared to 0.68 ± 0.07 µM at pH 7.4. The initial rapid release is either due to iron that had already come out of ferritin during its storage or to iron still inside the ferritin which BP was able to bind. Over time, the concentration increased to about 15.1 ± 0.1 µM and 1.32 ± 0.1 µ M respectively.

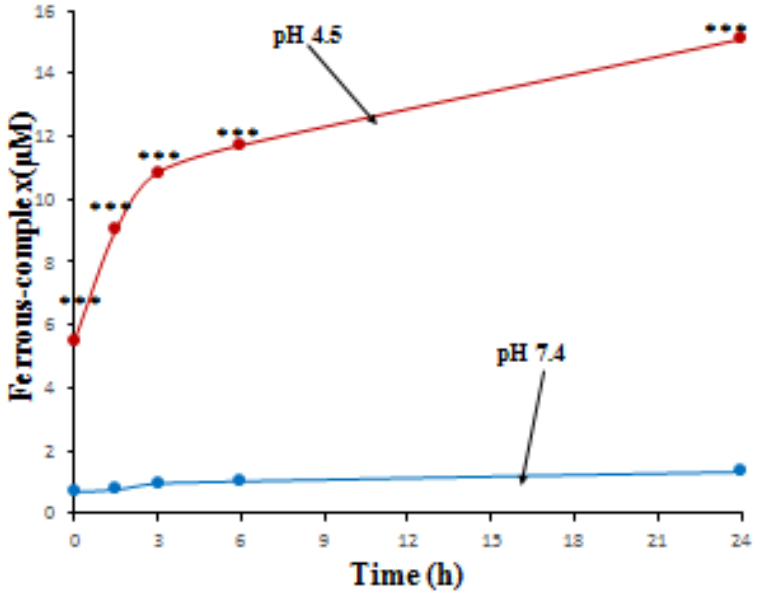


Figure 3.5: Ferrous complex formation at pH 7.4 and pH 4.5

Ferritin (0.1 μM) was incubated at 37˚C in NaCl/Na acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) in 15ml tubes, in triplicate. One millilitre was taken out and BP was added at different time points (0, 1.5, 3, 6, and 24hrs), left for 5mins and absorbance was measured at 535nm. Mean ± SEM of iron concentration released at pH4.5 was compared to pH 7.4 with two-way ANOVA followed by Bonferroni post tests. *** indicates P <0.001.

3.3.6 Ultrafiltration method for determination of iron released

In order to confirm the iron released from ferritin was not due to the effect of the iron chelator BP, a confirmatory experiment was carried out by the method previously used to determine the amount of iron released from transferrin (Lamb and Leake, 1994). Ferritin (0.1 µM) was incubated at 37 ˚C in NaCl/sodium acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) for 24hrs, then filtered with 30,000Mr cut off microcentrifuge filter tubes and then assayed for iron using AAS. The amount of iron was determined from the standard plot of iron was 12. ± 2.3 µM at pH 4.5 as compared to 3.29 ± 0.67 µM released at pH7.4 (Fig 3.6).

Although the AAS measured total iron concentration while the iron chelator measured ferrous iron, it was suspected that the lower concentration observed with AAS might be as a result of some of the iron retained in the microcentrifuge. The iron released, as measured AAS after 24 h incubation and ultrafiltration were also significantly higher at pH 4.5 ($P < 0.05$).

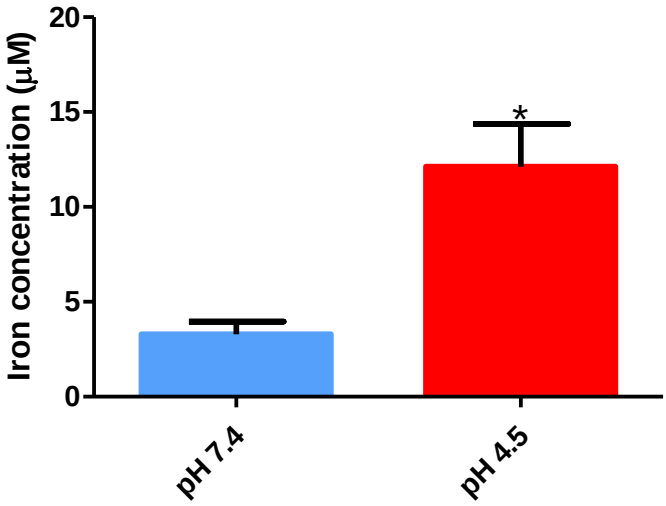


Figure 3.6: Iron released from ferritin at pH 4.5 measured by ultrafiltration and AAS

Iron released from 0.1 µM ferritin incubated in pH 4.5 and pH 7.4 is shown. Mean and SEM were obtained for each pH. A paired student's t-test comparism (n=3) showed there was a significant difference ($P < 0.05$).

3.3.6 Effect of iron chelators on LDL oxidation by ferritin at pH 4.5

In order to assess whether iron released by ferritin contributed to LDL oxidation by ferritin, we tested the effects of the iron chelators DTPA and EDTA on the formation of lipid peroxidation products (conjugated dienes). LDL (50µg LDL protein/ml) was oxidised with 0.1µM ferritin in NaCl/sodium acetate buffer (pH 4.5) in the presence or absence of EDTA or DTPA (100 µM) (Fig. 3.6). Formation of conjugated dienes was monitored. It was expected that the presence of iron chelators would inhibit entirely the oxidation of LDL at all time points as it did with ferrous iron (Satchell and Leake, 2012). The presence of iron chelators did not completely inhibit the initial oxidation of LDL by ferritin but only appeared to reduce the formation of conjugated dienes at a later stage.

The attenuance at 200 min was compared for control oxidised LDL and LDL oxidised by ferritin in the presence of EDTA and DTPA using one-way ANOVA followed by Tukey post hoc test. The oxidation of LDL by ferritin in the presence of iron chelators were statistically different from the control.

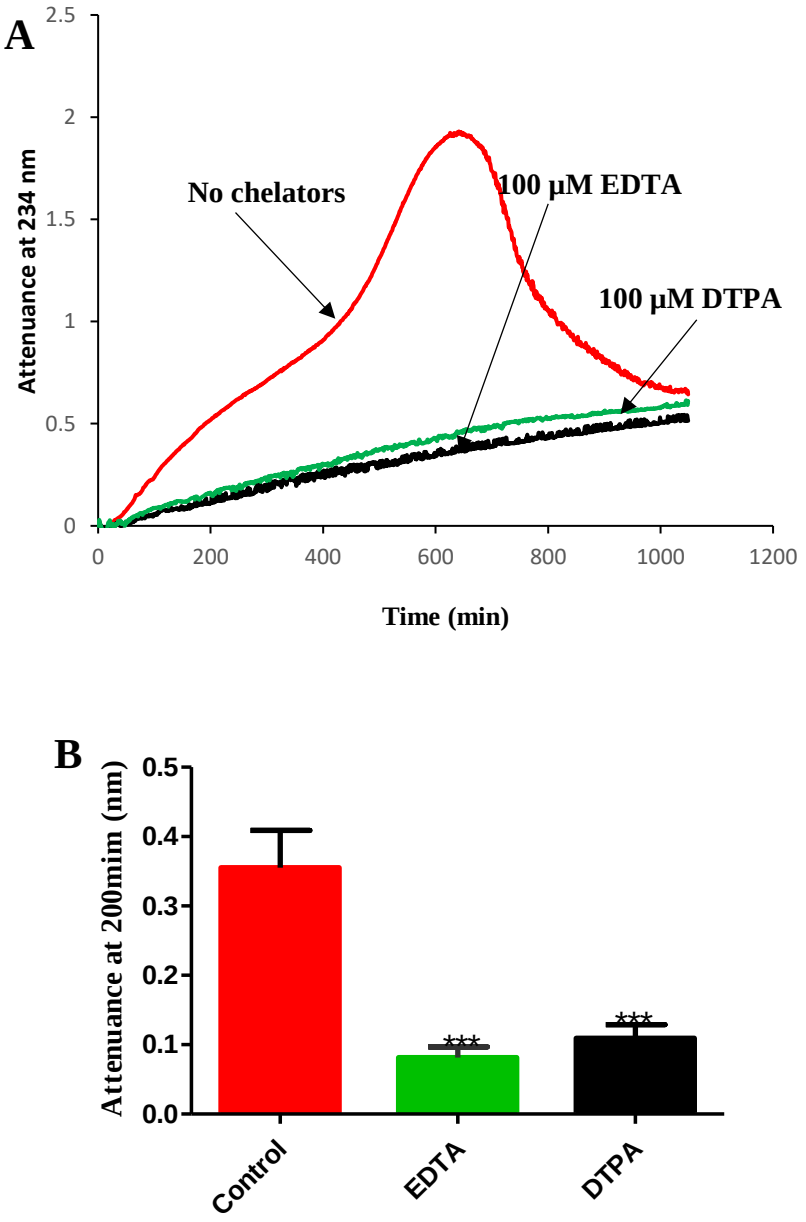


Figure 3.6: Effects of EDTA and DTPA on oxidation of LDL by ferritin at lysosomal pH

LDL (50µg protein/ml) was oxidized with ferritin (0.1µM) in NaCl/sodium acetate buffer (pH 4.5) at 37 ºC in the absence (red line) or presence of iron chelators, 100 µM EDTA (black line) and 100 µM DTPA (green line). The formation of conjugated dienes was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments (A). Mean and SEM of increase in attenuance at 200 min in the presence or absence of iron chelators were obtained for each concentration and compared to control with one-way ANOVA (n=3) followed by a post hoc Tukey test. *** indicates $P < 0.001$ (B).

3.3.7 Oxidation of LDL in the presence of apoferritin at lysosomal pH

The effect of Apoferritin, the protein component of the iron storage protein ferritin, on LDL oxidation at lysosomal pH was explored. LDL (50µg protein/ml) was oxidised in NaCl/sodium acetate buffer (pH 4.5) at 37ºC with varying concentrations of apoferritin (0.05 µM, 0.1 µM and 0.2 µM). The course of oxidation was monitored by measuring the formation of conjugated dienes at 234 nm (Fig 3.7). There was little or no oxidation of LDL in the presence of 0.05 µM apoferritin. At 0.1 µM, there was a slow increase of attenuance, but the kinetics of oxidation were somewhat different from what was observed with ferritin. At 0.2 µM, the kinetics were somewhat similar to what was described for $FeS0_4$ at pH 4.5 (lag, rapid, slow oxidation and aggregation phases).

The increase in attenuance at 200 min was compared (Fig 3.7B). Statistical analysis showed the control (LDL only) was not significantly different as compared to 0.05 µM and 0.1 µM but significantly different from 0.2 µM apoferritin. Due to the effect observed at higher concentrations of apoferritin we thought it would be necessary to quantify the amount of residual iron present in apoferritin. The amount of iron was determined by AAS (assuming a Mr of 443,000) and was 17 atoms of Fe per apoferritin particle which is only 1.7% of the total Fe per ferritin particle.

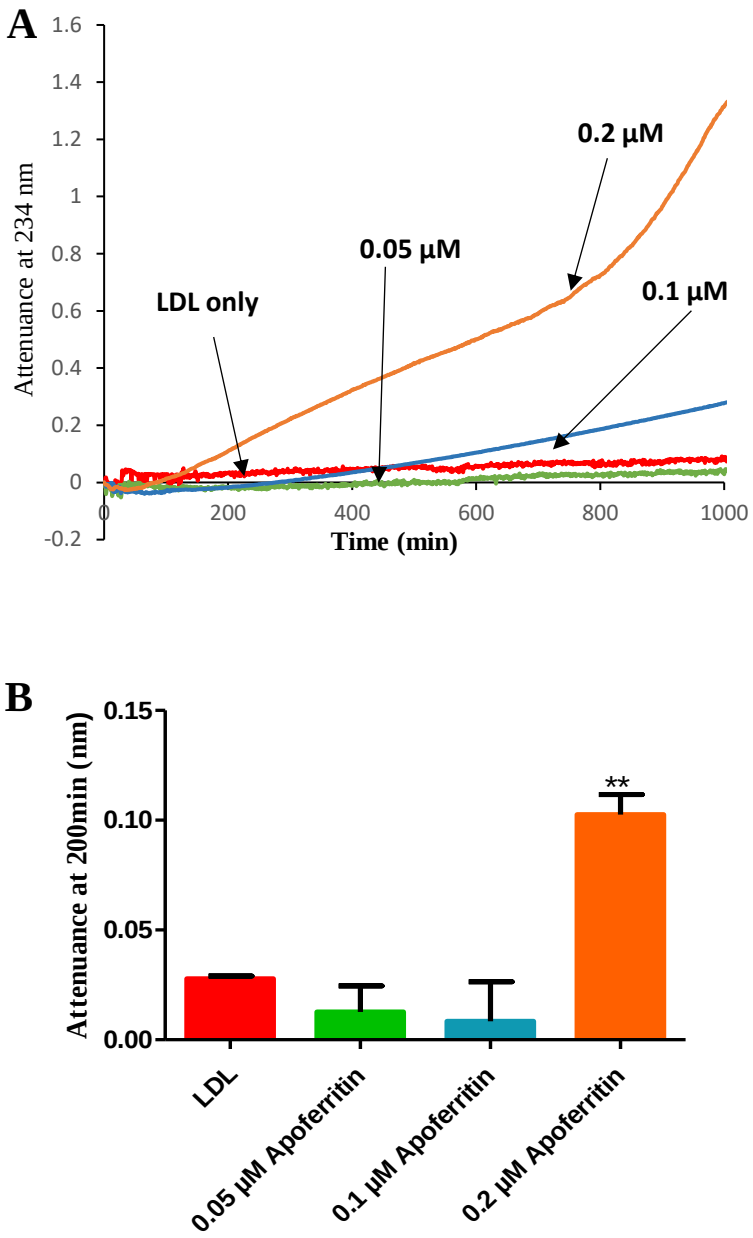


Figure 3.7: Oxidation of LDL by apoferritin at lysosomal pH

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated in the absence (red line) or presence of varying concentrations of apoferritin, 0.05 µM (green line), 0.1 µM (blue line) and 0.2 µM (orange line) at 37 ºC in capped cuvettes. The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments (A). The mean and SEM of attenuance at 200 min were obtained for each concentration and compared (n=3) by one-way ANOVA followed by a Tukey post hoc test. Increase in attenuance for 0.2 µM was compared control. ** indicates P<0.01(B).

3.3.8 Formation of oxidised lipids by LDL oxidised by ferritin at lysosomal pH

In order to confirm the atherogenic nature of LDL oxidised by ferritin, it was necessary to determine the formation of oxidised lipid products after LDL (50µg protein/ml) has been incubated in the presence or absence of ferritin (0.1µM) in NaCl/sodium acetate buffer (pH 4.5) at 37ºC. The oxidised lipids present mainly 7-ketocholesterol and cholesteryl linoleate hydroperoxides were measured by reverse HPLC. The total hydroperoxides formed were measured by a tri-iodide assay. These parameters were measured after the reaction was stopped at varying time points up to 48 h using EDTA and BHT. Longer time points were taken for measurement of oxidised lipids due to the much slower formation of 7-ketocholesterol compared to hydroperoxides formed as shown by this present study and other previous research from our group (Satchell, 2012, Ahmad, 2018).

The levels of 7-ketocholesterol and cholesteryl linoleate hydroperoxide (CLOOH) increased in the presence of ferritin while very little or none was formed in the control LDL. After 48 h of incubation, 7-ketocholesterol increased from 0 to 3.9 ± 1.0 nmol/mg of LDL protein; however, the 7-ketocholesterol formed was highest at 12 h with the value 4.0 ± 2.2 nmol/mg of LDL protein. The level of CLOOH formed in LDL oxidised by ferritin increased to 237 ± 118 nmol/mg of LDL protein and none was observed in control LDL at all time points. Total hydroperoxide formed increased with time in the presence of ferritin up to 1513 ± 204 nmol/mg of LDL protein. The comparison between native LDL only and LDL plus ferritin were determined by two-way ANOVA followed by Bonferroni's post-test.

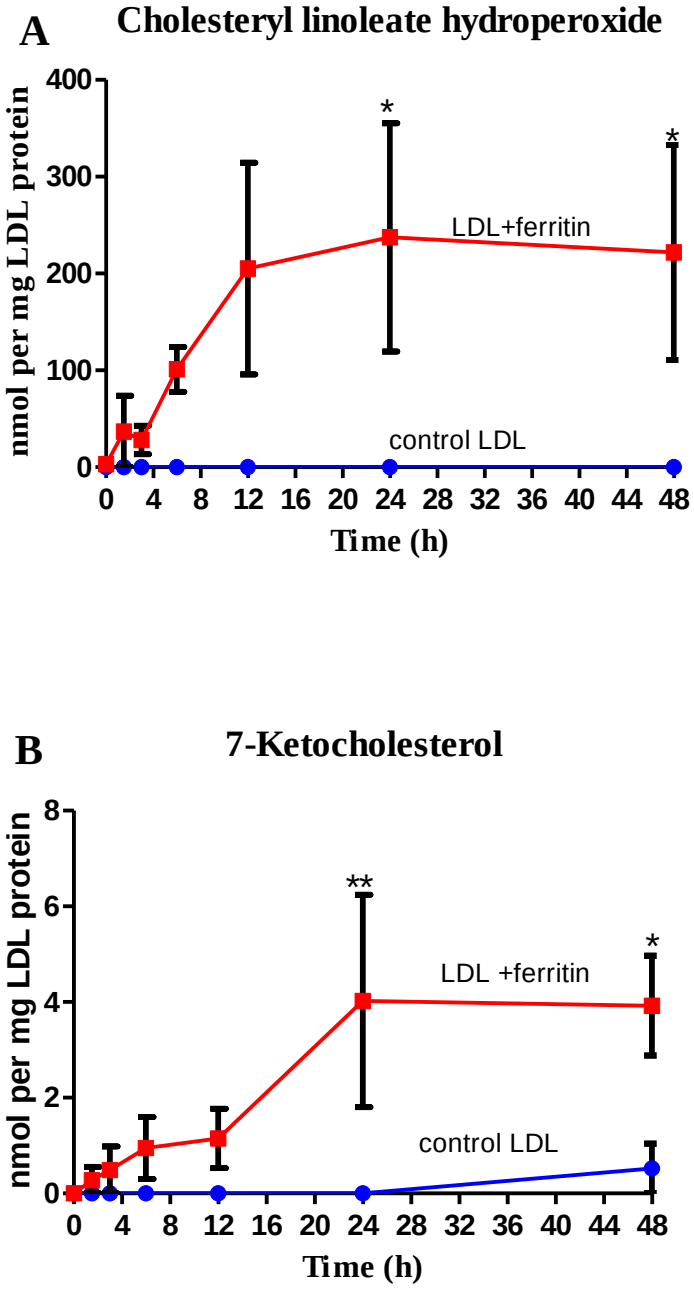


Figure 3.8: **Oxidised lipids formed from oxidation of LDL by ferritin at pH 4.5**

LDL (50 µg protein/ml) was incubated in the presence or absence of ferritin (0.1 µM) in sodium acetate buffer (pH 4.5) at 37 ºC. At varying time points up to 48 h, the oxidation was stopped by addition of EDTA (4 mM) and BHT (80 µM). The samples were analysed for (A) 7-ketocholesterol or (B) cholesteryl linoleate hydroperoxide (CLOOH) by reverse-phase HPLC. Graphs are representative of three independent experiments. Difference between control LDL and LDL oxidised by ferritin at each time point were determined by two-way ANOVA followed by Bonferroni's post-test (* indicates $p < 0.05$. ** indicates $P < 0.01$.

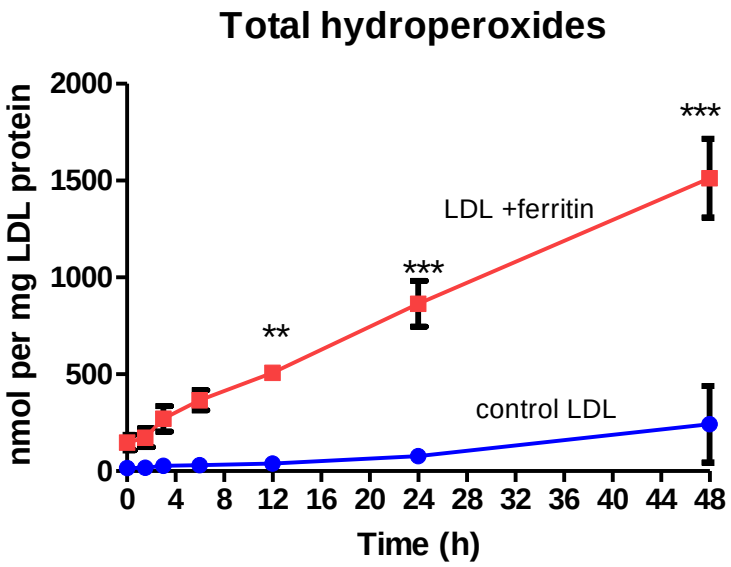


Figure 3.9: **Total hydroperoxides formed by LDL oxidised by ferritin at pH 4.5**

LDL (50µg protein/ml) was incubated in the presence or absence of ferritin (0.1µM) in sodium acetate buffer (pH 4.5) at 37ºC. At varying time points up to 48 h, the oxidation was stopped by addition of EDTA (4mM) and BHT (80 µM). The samples were then analysed for total hydroperoxides by a tri-iodide assay. The graph represents three independent experiments. Differences between control LDL and LDL oxidised by ferritin at each time point were determined by two-way ANOVA followed by Bonferroni post-tests. ** indicates P<0.01,*** indicates p<0.001.

3.4 Discussion

The mechanisms by which LDL is oxidised by iron in the lysosomes is still in its infancy (Wen and Leake, 2007). Upregulation of synthesis of the H and L subunits of ferritin was found in macrophages and endothelial cells of atherosclerotic lesions (Pang *et al.*, 1996, You *et al.*, 2003). The presence of redox-active iron in lysosomes has been previously demonstrated in some studies (Yuan *et al.*, 1996, Petrat *et al*., 2001) and this has been associated with ferritin degradation (Sibille *et al*., 1989, Radisky and Kaplan, 1998, Yu *et al*., 2003, Lv and Shang, 2018). Increased levels of iron (Casey *et al*., 1988) and cytokines (Miller *et al.*, 1991) are present in atherosclerotic lesions and both of this can upregulate the synthesis of ferritin. There are however suggestions that iron containing proteins might have a role to play as a source of iron in lysosomal oxidation (Leake, 1997, Satchell, 2008).

In this present study, it was demonstrated that ferritin can catalyse the oxidation of LDL at pH 4.5 which is an approximate pH of lysosomes. All concentrations of ferritin used (0.05 µM, 0.1 µM and 0.2 µM) were effective in oxidising LDL. The kinetics of oxidation by ferritin, however, differs from that of ferrous sulphate or ferric chloride earlier described (Satchell and Leake, 2012) as the lag phase was often times not observed at the early stage of oxidation by ferritin. However, the aggregation and sedimentation phases were also observed. As earlier discussed by Satchell and Leake, aggregation of LDL at acidic pH might occur as a result of the presence of net positive charge and repels each other at pH4.5, oxidation of LDL might reduce the net positive charge and leads to aggregation of LDL particles (Satchell and Leake, 2012).

Aggregation of LDL occurring alongside its oxidation and often times promotes its pro-atherogenic effects (Maor *et al*., 1997, Jayaraman *et al.*, 2011). Our data in the present study

show that LDL oxidised by ferritin aggregates at lysosomal pH, which further implies that LDL oxidation by ferritin may promote fusion or binding together of these particles and enhance its atherogenic effects.

A previous study by Kidane and his colleagues has demonstrated that the acidity of lysosomes and the activity of proteases are necessary for the release of iron from ferritin (Kidane *et al*., 2006). Although proteases would not have been present in our system, the increased oxidation by ferritin at lysosomal pH might be due to this pH enhancing the release of iron from the core of ferritin. Previous work from our laboratory has shown the release of iron from other iron binding proteins such as transferrin increased oxidation of LDL (Lamb and Leake, 1994c). It was thought that iron released from ferritin at this pH might be responsible for the higher oxidative effect observed at lysosomal pH (pH 4.5) compared to normal interstitial fluid or plasma (pH 7.4).

In this present study the oxidation of LDL in the presence of 0.1 µM and 0.2 µM ferritin was faster at the pH approximately similar to that of lysosomes pH 4.5, Fully saturated ferritin has the capacity to store up to 4500 atoms of iron. The determination of the amount of iron showed that ferritin from equine spleen used in this experiment contained about 1001 atoms of iron hence (0.05 µM, 0.1 µM and 0.2 µM) used in this present study contained 50 µM, 100 µM and 200 µM of iron respectively (Section 3.3.4). The presence of 5 µM ferrous sulphate has been shown to catalyse the oxidation of LDL and oxidation of LDL by iron was previously demonstrated to be much slower at pH 7.4, the pH of normal interstitial fluid or plasma compared to acidic pH (Wen and Leake, 2007, Satchell and Leake, 2012). The link between rapid oxidation at lysosomal pH and the release of iron at this pH was investigated by measuring the amount of ferrous iron (Fe^{2+}) that could be released at lysosomal pH (pH 4.5)

compared to normal interstitial fluid or plasma (pH 7.4). The levels of ferrous complex formed were significantly higher at lysosomal pH at all-time points as measured using the iron chelator bathophenathroline (BP). This iron chelator does not detect ferric iron (Fe^{3+}), but only measures ferrous iron (Fe^{2+}) (Lynch and Frei, 1995). It should be noted that ferritin was incubated in the absence of BP and samples were taken at various times and BP added to them and the absorbance quickly measured. The iron chelator was therefore not binding iron and sequestering it out of the ferritin during the 24 h incubation. About 5% of the iron contained in ferritin was released at lysosomal pH at 0 h compared to 0.7% released at pH 7.4. The immediate formation of ferrous complex may be due to loosely bound iron present in the ferritin core which had been released during the storage of the ferritin or iron still associated with the ferritin particles but loosely bound which the iron chelator BP might be able to quickly complex with. It is unclear why more iron is released at acidic pH, but it might be speculated that this is due to the solubility of iron at acidic pH and the fact that acidic pH might enhance the opening of the pores of ferritin through which iron can be released (Jin *et al*., 2001). The amino acids present in the pores of ferritin are highly conserved to tightly regulate the opening and closing of the pores. The localised unfolding of these sites can increase the amount of iron that exits the pores (Takagi *et al*., 1998).

An iron chelator independent technique (ultrafiltration followed by AAS) also revealed that more iron was released from ferritin at lysosomal pH as this was significantly higher compared to pH 7.4. The form of the oxidation state of iron in lysosomes has been previously debated (Collins *et al.*, 1991) and some researchers have argued that both forms ferric (Fe^{3+}) and ferrous (Fe^{2+}) might be present (Meguro *et al.*, 2005). However, a study by Terman and Kurz (Terman and Kurz, 2013) suggested that the ferrous form (Fe^{2+}) might be favoured in lysosomal

conditions. We have shown in this present study that ferrous iron is spontaneously released from ferritin at acidic pH, a similar condition to lysosomal conditions.

EDTA has a good chelating capacity for iron (Rizvi *et al*., 2011). DTPA has the capacity to bind all the coordination sites of both ferric and ferrous iron and has a much higher binding affinity for iron than does EDTA (Tang *et al*., 1997), hence it might be able to inhibit iron-mediated lipid peroxidation. The deleterious effect of lysosomal iron can be prevented by iron chelators present within lysosomes or entering the lysosomes through endocytosis, autophagy or permeating the membrane (Terman and Kurz, 2013). Wen and Leake also demonstrated the importance of iron in the lysosomal oxidation in macrophages, as LDL oxidation was inhibited in cells by desferrioxamine (an iron chelator) (Wen and Leake, 2007). Addition of iron chelators EDTA and DTPA showed inhibition of LDL oxidation by ferritin. It was expected that DTPA would prevent the formation of conjugated dienes completely as previously observed with ferrous iron (Satchell and Leake, 2012) but this was not the case for either DTPA or EDTA. It is proposed that, as well as iron release from the ferritin core, there might be formation of reactive oxygen species in the ferritin particles which can contribute to oxidation of LDL by ferritin. The nature of these reactive oxygen species and the mechanism of their formation requires a lot more research.

Ferrous iron released from ferritin might generate superoxide radical ($O_2^{\cdot -}$) (eqn 1) by reacting with molecular oxygen. The superoxide radical ($O_2^{\cdot -}$) produced can be protonated at acidic pH to form the hydroperoxyl radical ($HO_2^{\cdot}$) (eqn 2). Hydroperoxyl radicals are much more reactive than superoxide radicals in oxidising LDL (Bedwell *et al.*, 1989). They are also less hydrophilic and might enter the LDL particles and abstract hydrogen atoms from bisallylic methylene groups in lipids of LDL (eqn 3 and 4).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -} \quad \text{eqn 1}$$

$$O_2^{\cdot -} + H^+ \rightarrow HO_2^{\cdot} \quad pK_a\ 4.8 \text{ eqn 2}$$

$$HO_2^{\cdot} + LH \rightarrow H_2O_2 + L^{\cdot} \quad \text{eqn 3}$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad \text{eqn 4}$$

Apoferritin, the protein component of ferritin, oxidised LDL at a concentration of 0.2 µM but there was no significant difference at 0.1µM and 0.05 µM. The kinetics of oxidation were different from what was observed with ferritin. The formation of conjugated dienes was prolonged at the highest concentration. It is unclear what was responsible for this observation. A lag phase characteristic of $FeSO_4$ oxidation was observed although the kinetics of oxidation was not what was observed with $FeSO_4$. Quantification of residual iron contained in apoferritin revealed the presence of 3.4 µM iron in 0.2 µM. Ferrous or ferric iron at 3.4 µM would give faster oxidation of LDL (Satchell and Leake, 2012) than that shown by apoferritin (0.2 µM), suggesting that not all iron present in apoferritin was catalytically active.

Cholesterol oxidation products oxysterols (27-carbon products) have been demonstrated to possess several biological activities that can lead to initiation and progression of atherosclerosis (Brown and Jessup, 1999). 7-keto cholesterol is the main oxysterol found in atherosclerotic lesion (Lyons and Brown, 1999). The animal study by Lyons and Brown (1999) reported that 7-ketocholesterol is more atherogenic compared to cholesterol. It is cytotoxic and can induce apoptotic vascular cells (Lyons and Brown, 1999). Cholesteryl linoleate is found in abundance in LDL and quantification of its hydroperoxide is a good indicator of LDL oxidation. This present study indicated that 7-keto cholesterol, cholesteryl linoleate and total hydroperoxides were generated in LDL oxidised by ferritin. The high levels of these products may promote atherogenic properties to the oxidised LDL. Previously reported levels of lipid hydroperoxides formed by ferrous iron oxidised tend to decrease slowly between 24 h and 48 (Satchell and

Leake, 2012) but this was not the case for LDL oxidised by ferritin. The reason for this difference is unclear. Total lipid hydroperoxides continued to increase after 24 h, whereas CLOOH did not either because phospholipids oxidised later than CL or because CLOOH became oxidised further and eluted from the HPLC column at a different time and were not detected.

Work presented in this chapter indicates that ferritin could oxidise LDL at lysosomal pH. This oxidation was mediated mainly by redox-active iron, as iron was released from ferritin at acidic pH and iron chelators slowed down this oxidation. The fact that LDL oxidised by ferritin aggregates suggest that it may enhance lipid accumulation in macrophage lysosomes and promote lysosomal engorgement which may lead to lysosomal dysfunction. The atherogenicity of LDL oxidised in this manner was also suggested by its ability to form oxidised lipids products which have been shown to confer atherogenic properties on LDL. Further research into the role of lysosomal LDL oxidation by ferritin in atherosclerosis is required and the work presented here might form the basis for further research in this area.

Chapter 4: Degradation of ferritin and LDL in lysosomes: implications for lysosomal LDL oxidation by ferritin.

4.0 Background and rational

The majority of extracellular and intracellular proteins are degraded partly or fully in the lysosomes (Jackson and Hewitt, 2016). The classification of lysosomes as the main location for intracellular proteolysis has been established as far back as the early 1960s (De Duve, 1963). Autophagy is the main process through which long lived or damaged proteins and organelles are turned over and reutilised. Proteasomes (multicatalytic proteinases), which also play a key role in protein turnover, are also degraded through autophagy. Many long lived ferruginous proteins are turned over by autophagy which makes lysosome a source of low mass redox-active iron (Kurz *et al.*, 2008).

The most effective way to prevent the deleterious effects of increased labile iron cations is through storage in ferritin (Bou-Abdallah, 2010). Evidence of decrease in lysosomal degradation of intracellular ferritin and iron release has been found in cells after treatments with compounds that inhibit lysosomal activity but not with compounds that inhibit proteasome (Kidane *et al*., 2006, Arosio *et al.*, 2009). This implies that lysosomal autophagy and degradation by proteases play a key role in the degradation of ferritin. Radisky and Kaplan demonstrated that degradation of ferritin in lysosomes is a means of mobilising iron from the core of ferritin (Radisky and Kaplan, 1998). However, De Domenico and colleagues have suggested that mobilisation of iron is not dependent on the degradation of ferritin as iron can be mobilized in the absence of degradation (De Domenico *et al*., 2006). Other studies have shown the degradation of ferritin via the proteasomal degradation pathway (Cozzi *et al*., 2006). Prior to their degradation low saturated ferritin with iron-binding capacity may temporarily protect against iron-mediated oxidative stress. Saturated ferritin, on the other hand, can be auto phagocytosed and degraded in the lysosomes leading to the presence of more redox active iron and susceptibility to oxidative stress (Kurz *et al*., 2011).

The lysosomal compartment contains a wide range of hydrolytic enzymes which are very important mediators of recycling of biomolecules such as proteins, phospholipids and polysaccharides. Endopeptidases are mainly believed to initialise the cleavage of proteins and they act in synergy with exopeptidases to break down the resulting peptides to amino acids (van der Westhuyzen *et al*., 1980). The cathepsins are evidently the main categories of the hydrolytic enzymes. They are classified as aspartic proteases (e.g. cathepsins D and E), cysteine proteases (e.g. cathepsins B and C) and serine carboxypeptidases (e.g. cathepsin G) with an optimum pH of around 5 (Turk *et al*., 2002, Kuester *et al*., 2008, Kurz *et al*., 2011). However, the participation of cathepsin D (Dean, 1975) and cathepsin B (Hopgood *et al*., 1977, Libby and Goldberg, 1978) in general degradation of intracellular proteins has been shown previously, using inhibitors that are specific to these enzymes, such as pepstatin and leupeptin, respectively.

Low density lipoprotein is internalized by receptor-mediated endocytosis, after which it is delivered to the lysosomes. The apoB-100 component of LDL is degraded in lysosomes and inhibitors of lysosomal function, such as chloroquine, have been demonstrated to prevent the degradation of apoB-100 (Stein *et al*., 1977). Van der Westhuyzen and colleagues demonstrated that the degradation of apoB-100 component of LDL in cells cultured from arterial smooth muscle cells or human fibroblast and adult bovine aortic smooth muscle cells was initiated by cathepsin D. The enzyme then acted in synergy with other enzymes which included cathepsin B to complete the degradation process (van der Westhuyzen et al., 1980). This was supported by work by Leake and Peters which also suggested that the synergistic action of both cathepsins D and B might have degraded the apoB-100 component of LDL, but the majority of the degradation of LDL protein smooth muscle cells cultured from aorta of was carried out by cathepsin D (Leake and Peters, 1981). Recent *in vitro* study by Linke and others

have shown that cathepsins V, S, L, K, F and B degraded the protein component of LDL under acidic pH condition (pH 5.5) with cathepsin S having activity at pH 7.4. (Linke *et al*., 2006). The core of LDL consists mostly of cholesteryl esters and cholesteryl esterase has the capacity to hydrolyse cholesteryl esters. Chao and colleagues hydrolysed the cholesteryl ester component of LDL using cholesteryl esterase and also suggested that the particle derived from this hydrolysis were chemically similar to the unesterified cholesterol-rich LDL found in atherosclerotic lesions (Chao *et al*., 1992). Many forms of enzymatic modification of LDL such as apoB-100 proteolysis, lipolysis, which includes phospholipid hydrolysis, hydrolysis of cholesteryl esters and hydrolysis of sphingomyelin, can lead to aggregation and fusion of LDL into lipid droplets which have been linked to the development of atherosclerosis (Lu and Gursky, 2013).

Although the autophagic process is rapid and effective, it can still give room for some peroxidation catalysed by redox active iron to occur within the lysosomes. These can lead to oxidative modification of some materials autophagocytosed (Brunk and Terman, 2002). In view of the recent emergence of the lysosomal oxidation theory, it was hypothesised that during the process of degradation of both LDL and ferritin in lysosomes there might be conformational changes in ferritin and/or LDL that could enhance ferritin mediated LDL oxidation. The overall aim of this chapter was to modify ferritin and LDL with enzymes that possess activity at lysosomal pH and explore the effects of these modifications might have on oxidation of LDL by ferritin at lysosomal pH. The effects of major lysosomal endoproteases cathepsin D, cathepsin B and the lipolytic enzyme cholesteryl esterase were explored.

4.1 Objectives

i) To proteolytically modify ferritin with cathepsins at lysosomal pH

ii) To proteolytically modify of LDL with cathepsins at lysosomal pH

iii) To compare the oxidation of LDL by intact ferritin and enzyme-treated ferritin

iv) To test the effects of modification of apoB content of LDL by cathepsins on oxidation of LDL by ferritin at lysosomal pH.

v) To investigate the effect of cholesteryl esters degradation by cholesteryl esterase on LDL oxidation by ferritin.

4.2 Methods:

The method for this session is described in section 2.6. The digestion of ferritin and LDL with cathepsin D was described in section 2.6.1.1 and digestion of ferritin with cathepsins B and D was described in section 2.6.1.2. The co-incubation with cholesteryl esterase and proteases (cathepsins B and D) was described in section 2.6.1.3. The method for evaluation by SDS-PAGE after digestion was described in section 2.6.2 while the effects of the proteases and cholesteryl esterase on LDL oxidation was described in section 2.6.3.

4.3 Results

4.3.1 Proteolytic degradation of ferritin by cathepsins *in vitro* (pH 4.5)

As mentioned earlier, there is evidence from previous researchers suggesting the degradation of ferritin occurs in lysosomes. To test the impact of the major lysosomal endoprotease on degradation of ferritin at lysosomal pH, intact ferritin (2 mg/ml) was treated with or without cathepsin D (6.2 µg/ml) and incubated in Chelex - 100 treated NaCl/sodium acetate buffer (pH 4.5) at 37 ˚C for 24 h. After incubation, the ferritin samples were evaluated by polyacrylamide gel electrophoresis under reducing conditions. Figure 4.1A shows the pattern obtained with or without treatment of ferritin protein with cathepsin D. The heavy and light chains of 21 kDa and 19 kDa were observed without treatment, but a less intense band was observed for the light

chain. No lower Mr degradation products were observed after treatment with cathepsin D. Analysis of the density of the heavy chain bands was carried out using image J software to compare the band intensity with or without enzymes. The result showed band intensity of 96.5 ± 17.0 in untreated ferritin to 136.9 ± 14.9 band intensity of ferritin pre-treated with cathepsin D, there was no significant different between the ferritin treated with cathepsin and the control ($P > 0.05$). Ferritin modification in the presence of cathepsin B, another main endoprotease present in lysosome was tested with increased the enzymes to protein ratio. Ferritin (500 µg) was treated with or without both cathepsin D (5 µg) and cathepsin B (5 µg) and incubated in Chelex-100 treated NaCl/sodium acetate buffer (pH 4.5) at 37˚C for 24 h. After incubation the ferritin samples were evaluated by polyacrylamide gel electrophoresis under reducing condition (Figure 4.1B). No lower molecular weight products were again observed.

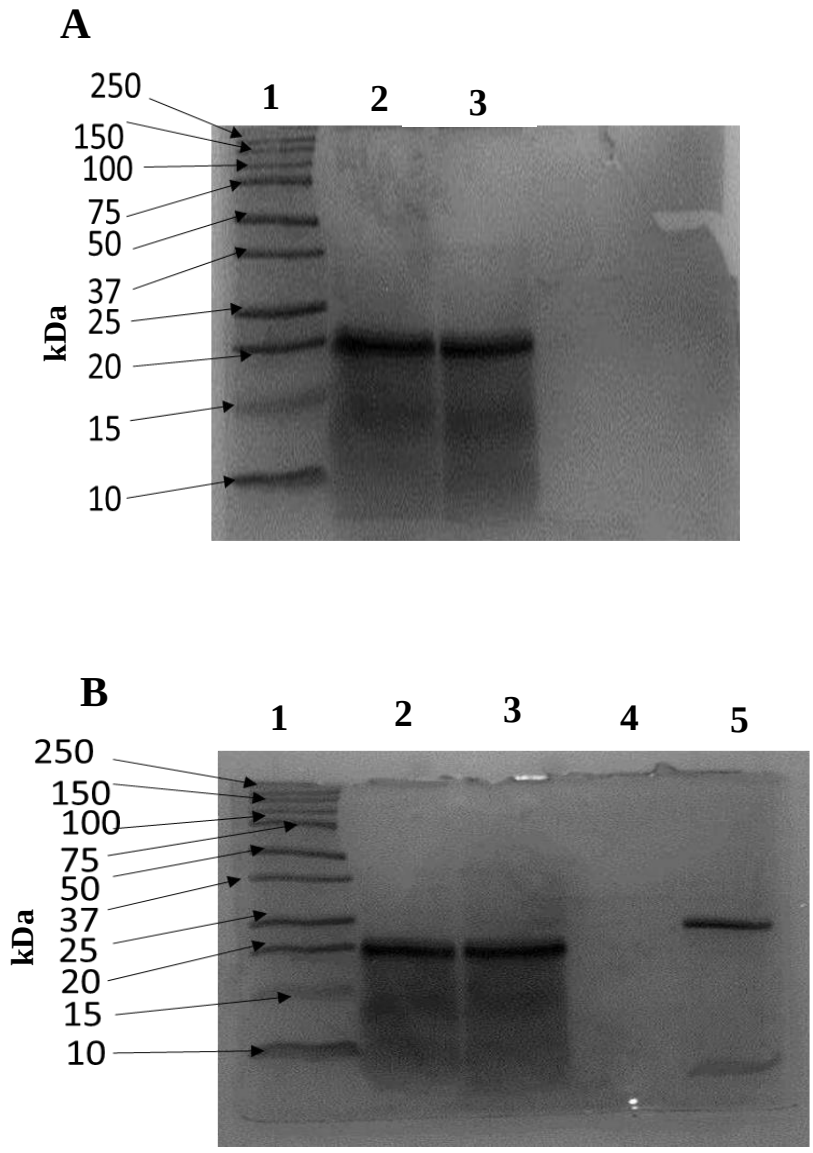


Figure 4.1: SDS-PAGE of ferritin treated with cathepsins

Ferritin (2 mg/ml) incubated with or without cathepsin D (6.2 µg/ml) at 37 ^{o}C for 24 h, lane 1 contains molecular weight markers, lane 2 contains ferritin only, lane 3 contains ferritin plus cathepsin D (A). Ferritin incubated with or without cathepsin B and D at 37 ^{o}C for 24 h, lane 1 contains molecular weight markers, lane 2 contains ferritin only, lane 3 contains ferritin pre-incubated with cathepsin B and cathepsin D at weight ratio 100:1:1 (Ferritin (500 µg/ml), Cathepsin D (5 µg/ml) and cathepsin B (5 µg/ml) and lane 5 contains cathepsin B only (B). All samples were treated with reducing sample treatment buffer (RSTB) and about 15 µg in terms of ferritin protein was loaded onto gels with stacking gel of 4% and resolving gel gradient ratio of 15%. It was run for one hour at 150 V before staining with Coomassie blue to detect bands and de-stained. The pictures of the gels were captured using a U Genius image capturing machine.

4.3.2 Effects of treatment of ferritin with proteases on LDL oxidation by ferritin at lysosomal pH

Previous work with caeruloplasmin which contains the majority of copper ions in the plasma has shown that proteolytically modified caeruloplasmin was not able to enhance oxidation of LDL by SMC compared to the intact caeruloplasmin molecule (Mukhopadhyay *et al.*, 1996). It was of interest to test whether proteolytic modification of ferritin affects its ability to oxidise LDL at lysosomal pH (Figure 4.2). Ferritin (2 mg/ml) was incubated with or without cathepsin D (6.2 µg/ml) for 24 h in NaCl/sodium acetate buffer (pH 4.5) at 37 ˚C and then used the ferritin (0.1 µM) to oxidise native LDL (50 µg protein/ml). The formation of conjugated dienes every minute interval at 234 nm for up to 1200 minutes were measured. The results show that treatment of ferritin with cathepsin D, did not affect its ability to catalyse LDL oxidation. The oxidation followed a similar pattern at the early phase of oxidation. In ferritin pre-incubated with cathepsin D, the aggregation phase began later and the peak attenuance was higher compared to untreated ferritin. The attenuance at 200 min was compared to control. The statistical analysis using Student's paired t test showed there was no statistically significant difference to control ($P > 0.05$).

Following up with this result, the effects of a decreased protein enzyme ratio and addition of cathepsin B, another lysosomal protease to the system were tested. We incubated ferritin (500 µg) with or without cathepsin D (5 µg) and cathepsin B (5 µg) for 24 h in NaCl/sodium acetate buffer (pH 4.5) at 37˚C. After incubation, the ferritin (0.1 µM) with or without enzymes were used to oxidise native LDL (50 µg protein/ml) in capped cuvettes. Cathepsin D ad B (0.44 µg/ml each) were added to freshly prepared to ferritin (0.1 µM) and native LDL (50 µg protein/ml, as an additional control (Fig. 4.3A). The attenuance at 200 min was compared

using one way ANOVA followed by Tukey post hoc test. The attenuance of the control at this time was 0.070 ± 0.021 compared to 0.100 ± 0.038 observed in ferritin pre-treated with cathepsin D and B (Fig. 4.3B). However, the treatments were not statistically significantly different when compared to control or each other ($P > 0.05$), but results suggest that ferritin is able to catalyse LDL oxidation even after treatment with enzymes.

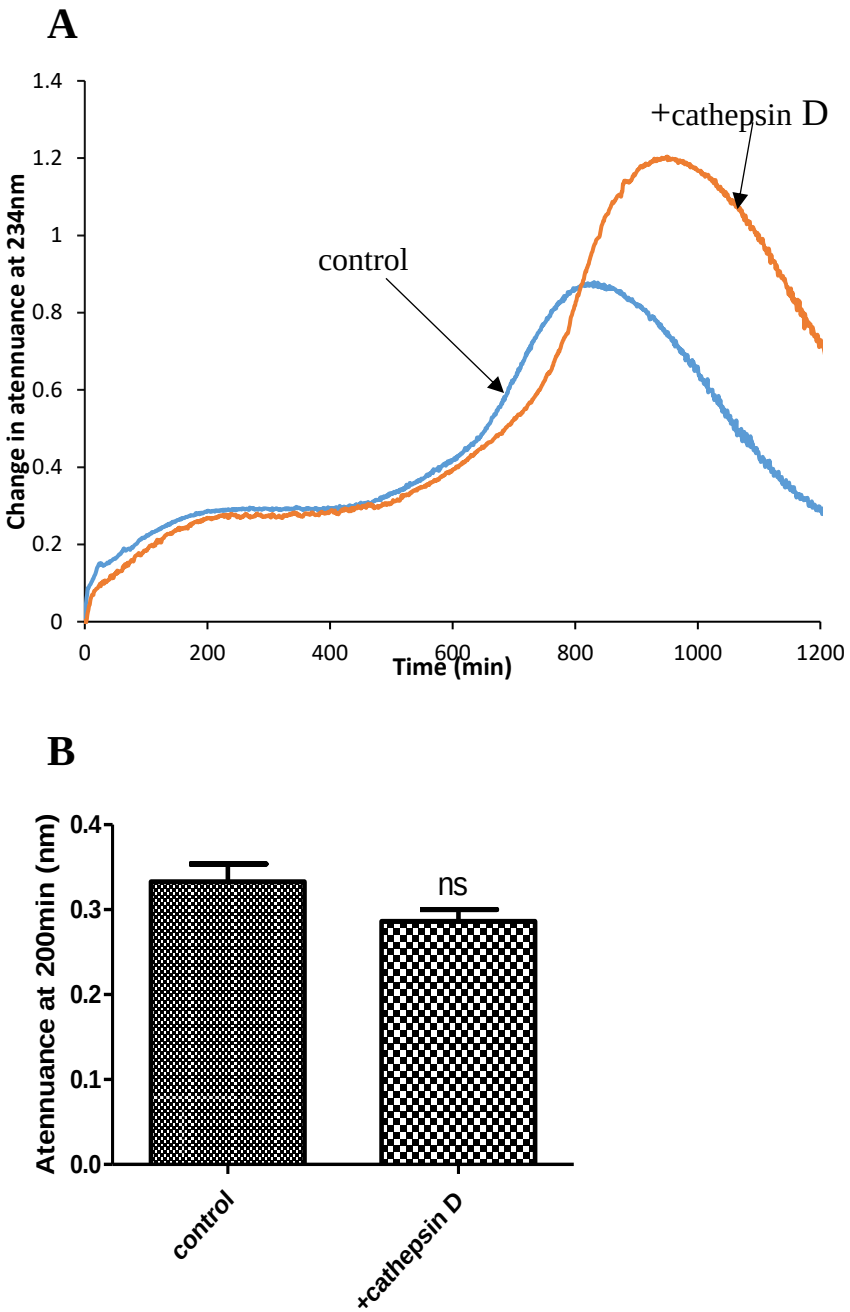


Figure 4.2: Effects of pre-treatment of ferritin with cathepsin D on LDL oxidation by ferritin

Native LDL (50 µg protein/ml) was oxidised with ferritin (pre-incubated alone or with cathepsin D) (0.1 µM), in NaCl/ sodium acetate buffer (pH 4.5) at 37 ^{o}C in capped cuvettes. The formation of conjugated dienes was monitored by attenuance at 234 nm against appropriate reference cuvettes that lacked LDL (A). This is a representative of three independent experiments. The increase in attenuance at 200 min compared with the controls using Student's paired t test (n=3) ($P > 0.05$). ns indicates not significant (A).

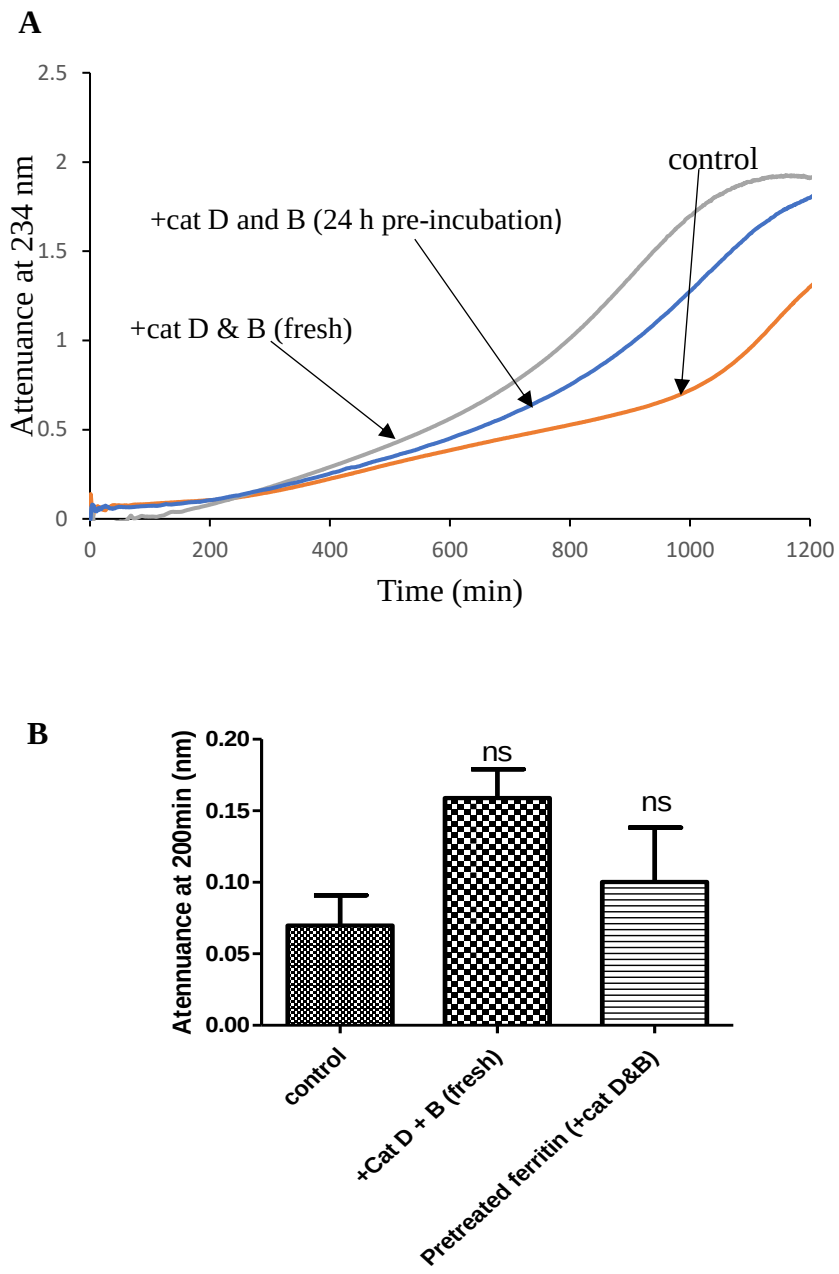


Figure 4.3: Effect of treatment of ferritin with cathepsin D and B on LDL oxidation by ferritin

Native LDL (50 µg protein/ml) was oxidised with ferritin pre-incubated alone or with cathepsin D and B (0.1 µM), and freshly prepared cathepsin D and B (0.44 µg) and ferritin (0.1 µM) in NaCl/ sodium acetate buffer (pH 4.5) at 37 ^{o}C in capped cuvettes. The formation of conjugated dienes was monitored at attenuance of 234 nm against appropriate reference cuvettes that lacked LDL (A). This is a representative of three independent experiments. Mean ± SEM increase in attenuance at 200 min was compared with one way ANOVA (n = 3) followed by Tukey post hoc test ($P > 0.05$). ns indicates not significant (B).

4.3.3 Proteolytic degradation of LDL by cathepsin D

The intact LDL (500 µg protein/ml) was incubated in Chelex - 100 treated NaCl/sodium acetate buffer (pH 4.4) at 37˚C for 24 h in the presence or absence of cathepsin D (5 µg/ml). After incubation, the extent of degradation of the samples was evaluated using polyacrylamide gel electrophoresis under reducing condition. Figure 4.4 shows the pattern obtained with or without treatment of LDL with cathepsin D. The Mr weight of around 250 kDa was observed in LDL without cathepsin D, whereas the M_r of apoB-100 is 513 kDa. This discrepancy might be explained by the migration of very large proteins into SDS-PAGE gels might not always be consistent. There were many degradation products in the presence of cathepsin D. This *in vitro* result was as expected, as previous work has shown the importance of cathepsin D in LDL degradation by arterial smooth muscle cells (Leake and Peters, 1981).

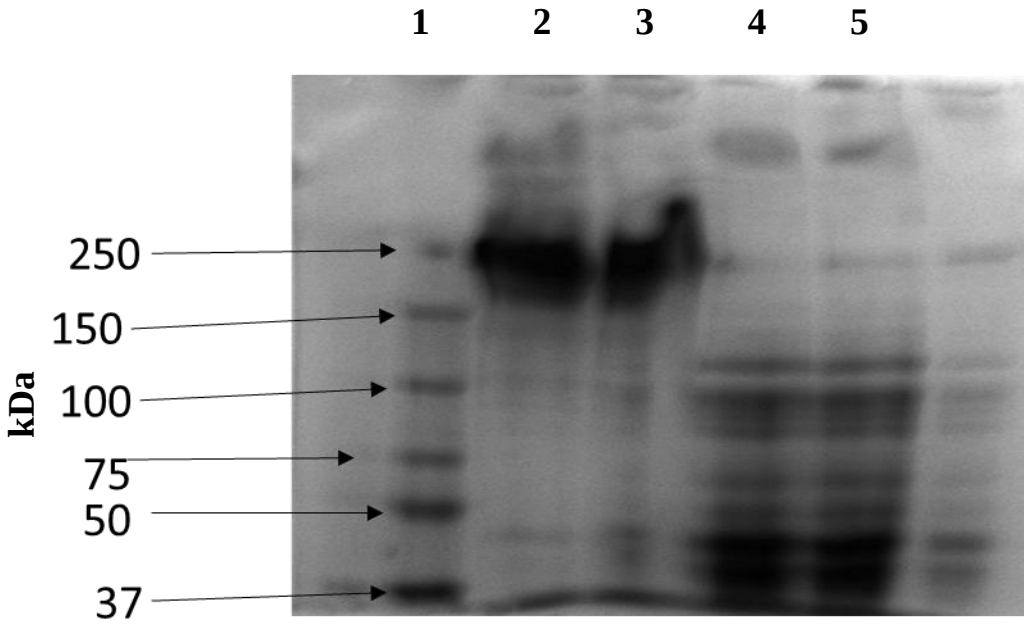


Figure 4.4: SDS-PAGE of LDL treated with cathepsin D

LDL (500 µg/ml) incubated with or without cathepsin D 5 µg /ml) at 37 ^{o}C for 24hrs, lane 1 contains molecular weight marker, lane 2 and 3 contains LDL only, lane 4 and 5 contains LDL plus cathepsin D. All samples were diluted to 15 µg, treated with reducing sample treatment buffer (RSTB) and loaded onto gels with stacking gel of 4% to resolving gel of 5%. The gel was run for one hour at 150 V before staining with Coomassie blue to detect bands and de-stained. The picture of the gel was captured using a U Genius image capturing machine.

4.3.4 Effect of treatment of LDL with proteases on LDL oxidation by ferritin at lysosomal pH

It was of interest to test whether proteolytic modification of LDL can affect its oxidation by ferritin at lysosomal pH, as LDL will be degraded in lysosomes at the same time as it is being oxidised (Figure 4.5). LDL (500 µg protein/ml) was incubated with or without cathepsin D and B (5 µg/ml each) for 24 h in NaCl/sodium acetate buffer (pH 4.5) at 37˚C. The pre-treated LDL (50 µg protein/ml) was oxidised with ferritin (0.1 µM). LDL not pre-treated with enzymes (50 µg protein/ml) was also oxidised with ferritin (0.1 µM) in the presence or absence of freshly added cathepsin D and B (0.5 µg/ml each) to the cuvettes; this was equivalent to the concentration of enzymes that would have present in the pre incubated LDL. This was done because the cathepsins would have been active in the cuvettes at pH 4.5. The formation of conjugated dienes was measured at every one minute interval at 234 nm for 1200 minutes. The result shows that proteolytic degradation of LDL increased the attenuance at 234 nm compared to the control. The rise in attenuance was more prominent in proteolysed LDL with the aggregation phase observed earlier compared to the untreated LDL and the LDL freshly treated with the enzymes in the test cuvettes.

The increase in attenuance at 200 min was compared with one-way ANOVA followed by a Tukey post hoc test (n = 4) (Fig 4.5B). Oxidation of previously proteolysed LDL was significantly different from control LDL ($P < 0.01$). However, the freshly treated LDL was not statistically different from control.

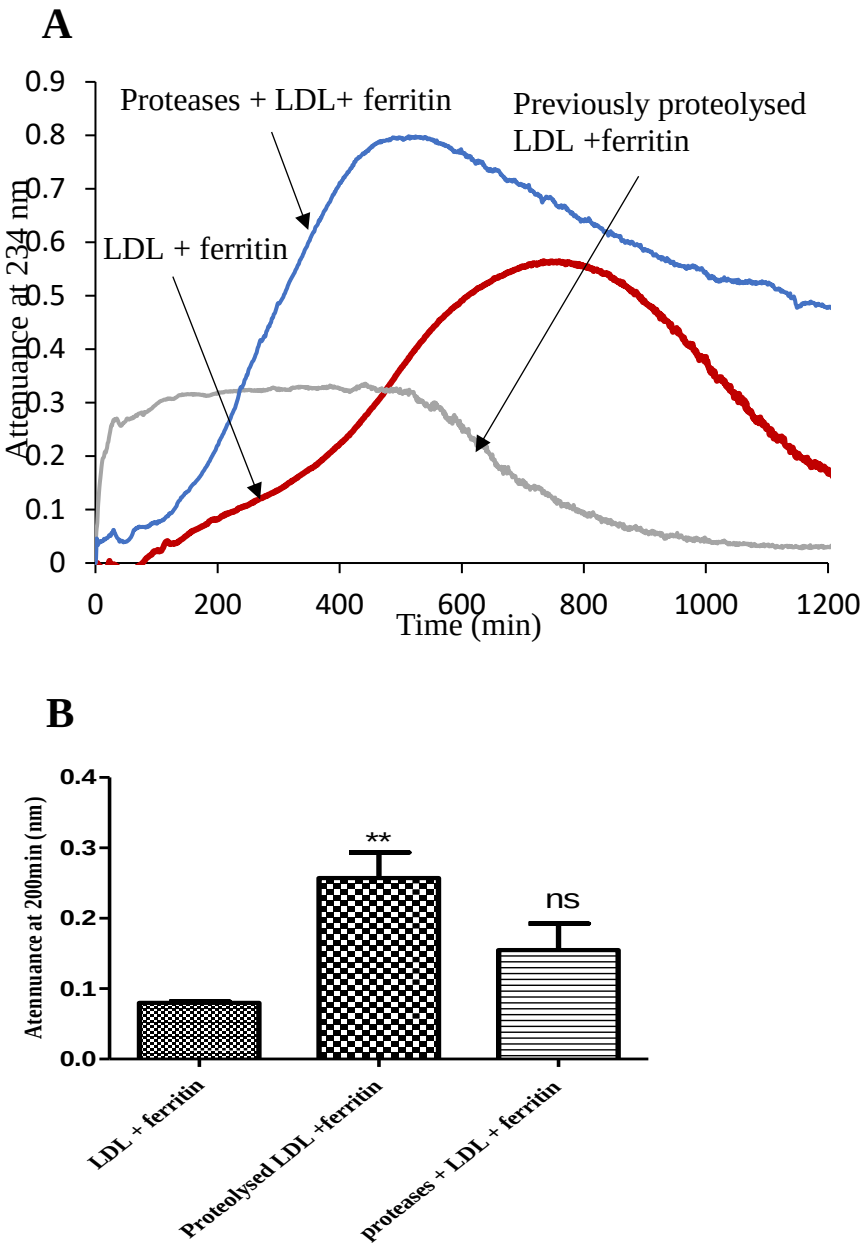


Figure 4.5: Effects of treatment of LDL with cathepsin D and B on LDL oxidation by ferritin

LDL (500 µg protein/ml) was incubated with or without cathepsin D and B (5 µg/ml each) for 24 h in NaCl/sodium acetate buffer (pH 4.5) at 37˚C. LDL pre-treated with cathepsin D and B (50 µg protein/ml) was then oxidised with ferritin (0.1 µM), in NaCl/ sodium acetate buffer (pH 4.5) at 37 ºC. LDL with freshly added cathepsin D and B to the cuvettes (0.5 µg/ml), the concentrations they would have been present in the cuvettes due to carry over, was also included. The formation of conjugated dienes was monitored at attenuance of 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of four independent experiments (A). The increase in attenuance at 200 min compared with one-way ANOVA followed by Tukey post hoc test (n = 4). ** indicates $P < 0.01$ and ns indicates not significant (B).

4.3.5 Effect of co-incubation with cholesteryl esterase on LDL oxidation by ferritin at lysosomal pH.

It was of interest to test the effect of modifying the lipid component of LDL with a lipolytic enzyme on its oxidation by ferritin at lysosomal pH, as this will mimic what might happen in lysosomes. Previous work by Kyger *et al*. has demonstrated that bovine and human pancreatic cholesteryl esterase had optimum catalytic pH from pH 5.5 to 6.5 in the presence of taurocholate. Their data also showed some taurocholate independent activity of the enzyme in the synthetic activities (Kyger *et al.,* 1990). Work by Chao *et al.* revealed the dependence of hydrolysis of lipoprotein cholesteryl esters by cholesteryl esterase from *Candida cylindracea* on the treatment of LDL with trypsin as the cholesteryl ester hydrolysis was only possible after treatment with trypsin (Chao *et al.*, 1992). In this present study, cholesteryl esterase from *Pseudomonas sp* was used as the lipolytic enzyme. Preliminary data showed increased cholesterol content in LDL from the degradation of cholesteryl esters into fatty acids and cholesterol, in the presence of cholesteryl esterase with or without trypsin. LDL (50 µg protein/ml) treated with cholesteryl esterase (0.0625 unit/ml) in the presence or absence of trypsin (1.25 µg/ml) was incubated in NaCl/sodium acetate buffer (pH 4.5) at 37˚C for 2 h. The cholesterol content was measured by reverse phase HPLC at 210 nm. The data showed increased cholesterol content from 775 ± 91.5 nmol/mg LDL protein to 1454 ± 65.0 nmol/mg LDL protein in LDL plus cholesteryl esterase and 2360 ± 220.5 nmol/mg LDL protein in LDL plus cholesteryl esterase and trypsin. The result showed that cholesteryl esterase from *Pseudomonas sp* can hydrolyse cholesteryl esters in the presence or absence of trypsin (unpublished data).

To test the effect of hydrolysis with cholesteryl esterase on LDL oxidation by ferritin at lysosomal pH, we incubated LDL (50 µg protein/ml) with or without cholesteryl esterase (CE)

from *Pseudomonas sp* (0.0625 unit/ml) in NaCl/sodium acetate buffer (pH 4.5) at 37˚C with ferritin (0.1 μM). The results show that co-incubation with cholesteryl esterase increased the formation of conjugated dienes by LDL compared to control (Fig 4.6B). The early phase of oxidation was increased, and the aggregation phase occurred earlier compared to the control. The increase in attenuance at 200 min was compared with a paired Student's t test (n = 3). The oxidation of LDL was significantly increased ($P<0.001$) (Fig. 4.6B)

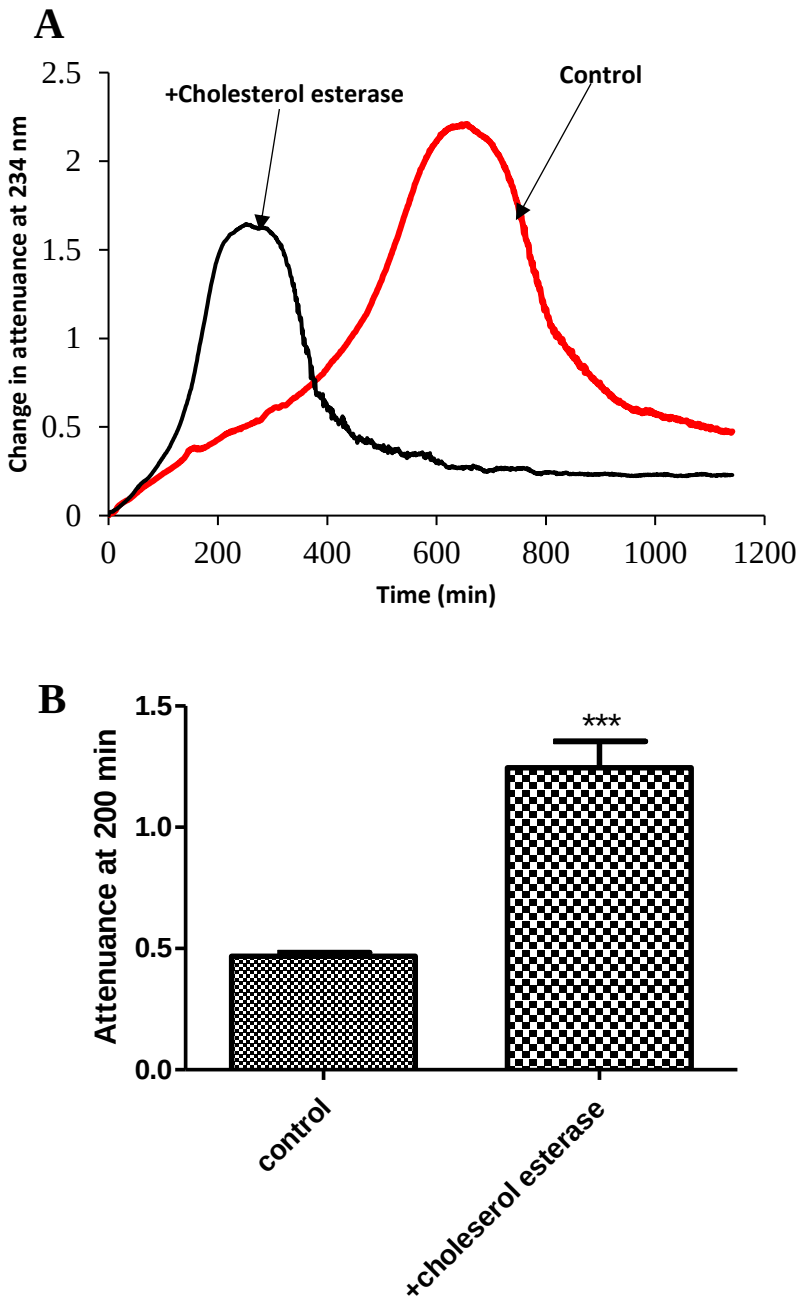


Figure 4.6: Effects co-incubation with cholesteryl esterase on LDL oxidation by ferritin

LDL (50 µg protein/ml) was incubated with or without cholesteryl esterase (0.0625 unit/ml) and was oxidised with ferritin (0.1 µM) in NaCl/ sodium acetate buffer (pH 4.5) at 37 ºC. The formation of conjugated dienes was monitored at attenuance of 234 nm against appropriate reference cuvettes that lacked LDL (A). This is a representative of three independent experiments. The increase in attenuance at 200 min compared with paired Student's t test (n = 3). *** indicates $P < 0.001$ (B).

4.3.6 Effects of co-incubation with cholesteryl esterase and proteases on LDL oxidation by ferritin at lysosomal pH.

The lysosomal compartment contains both proteolytic and lipolytic enzymes along with other hydrolases. The multienzynme composition of the lysosomal compartment allows its rapid, efficient and coordinated degradation of targetted substrates. Degradation of macromolecules often involves multiple enzymes working together in a stepwise manner (Bonten *et al.*, 2014). It was of interest to test the effect of modifying a part of lipid component (cholesteryl esters) and the protein component (apoB-100) of LDL concurrently on its oxidation by ferritin at lysosomal pH (Figure 4.7). LDL (50 µg protein/ml) was co-incubated with or without cholesteryl esterase (0.0625 unit/ml) and cathepsin D and B (1.25 µg/ml) in NaCl/sodium acetate buffer (pH 4.5) at 37˚C, with ferritin (0.1 µM). The formation of conjugated dienes was measured for every one minute interval at 234 nm for up to 1200 minutes. The results show that co-incubation with cholesteryl esterase and proteases cathepsin D and B increased the formation of conjugated dienes by LDL compared to control.

The increase in attenuance at 200 min was compared with a paired Student's t test ($n = 3$). The oxidation of LDL was significantly increased by the presence of cholesteryl esterase and the proteases $P < 0.01$ (Fig. 4.7B).

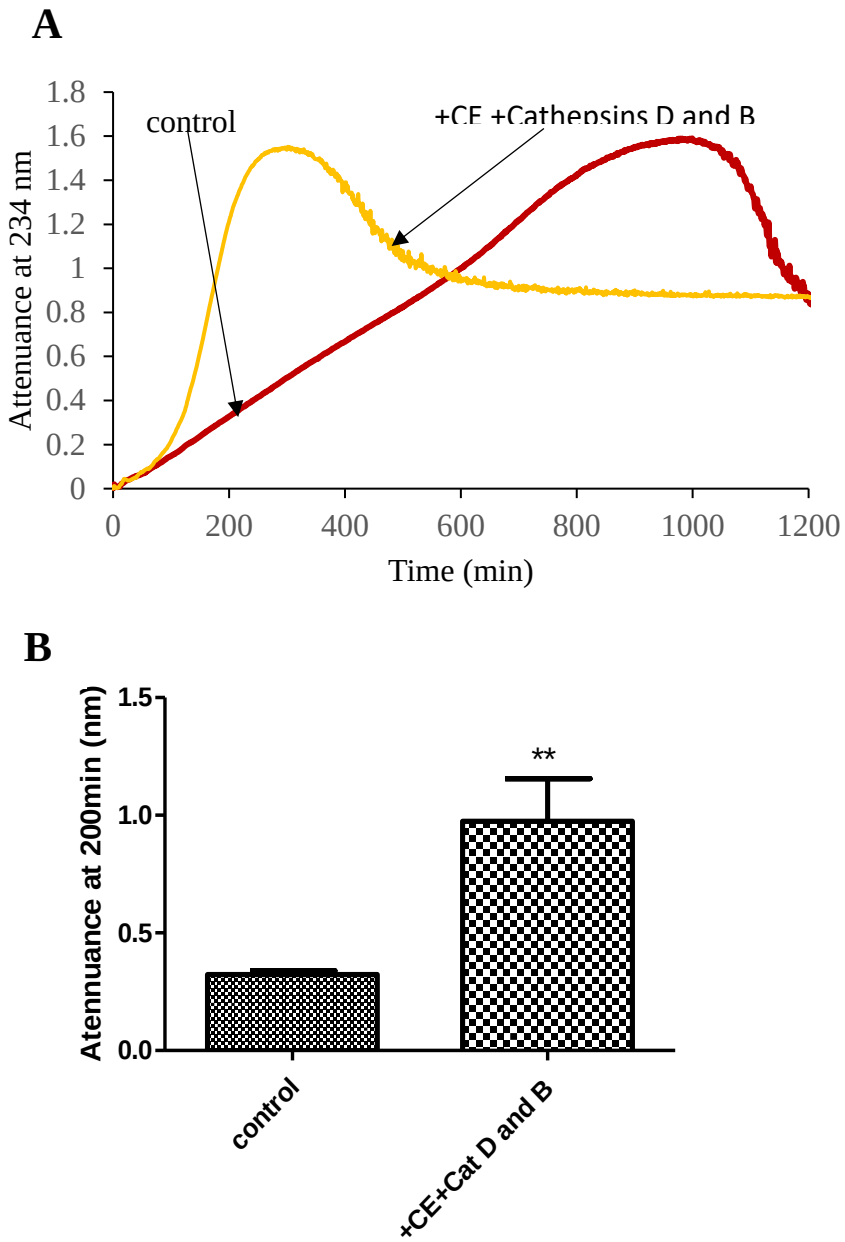


Figure 4.7: Effects of co-incubation with cholesteryl esterase and proteases on LDL oxidation by ferritin

LDL (50 µg protein/ml) was incubated with or without cholesteryl esterase (0.0625 unit/ml), cathepsin D and B (1.25 µg/ml each) with ferritin (0.1 µM) in NaCl/ sodium acetate buffer (pH 4.5) at 37^{o}C. The formation of conjugated dienes was monitored by measuring attenuance of 234 nm against appropriate reference cuvettes that lacked LDL (A). This is a representative of three independent experiments. Figure 4.7B shows the increase in attenuance at 200 min compared with paired Student's t test (n = 3). ** indicates $P < 0.01$ (B).

4.4 Discussion

Existing evidence regarding the mechanisms for degradation of ferritin and the link to iron mobilisation is controversial. However, some have suggested that lysosomal enrichment with ferritin stabilise cells after autophagocytosis by binding low mass iron in the lysosomes (Garner *et al.*, 1997, Persson *et al*., 2001a). Others have argued that there is possibility for the release of iron from ferritin in lysosomes during ferritin degradation (Roberts and Bomford, 1988, Radisky and Kaplan, 1998, Zhang *et al*., 2010). Lysosomal proteases such as cathepsins D and B, have been demonstrated to be involved in the degradation of the apoB-100 component of LDL (van der Westhuyzen *et al.*, 1980, Leake and Peters, 1981, Linke *et al*., 2006) and ferritin (Richter, 1986, Kidane *et al*., 2006, Laskar *et al*., 2012). This evidence put together suggests the degradation of ferritin and LDL under lysosomal conditions using enzymes *in vitro* and within lysosomes in cells. The recent lysosomal LDL oxidation theory has provided data suggesting the lysosomal oxidation of LDL mediated by iron in macrophages *in vitro* to the onset and progression of atherosclerosis (Wen and Leake, 2007, Satchell and Leake, 2012, Ahmad and Leake, 2018, Ahmad and Leake, 2019). Work from the previous chapter of this thesis demonstrated that ferritin can catalyse LDL oxidation at lysosomal pH and also showed that there is enhanced release of iron from ferritin under the lysosomal acidic condition in the absence of proteases. This study explored the effects of proteases and lipolytic enzymes on the oxidation of LDL by ferritin at lysosomal pH.

The present study showed that intact ferritin and ferritin pre-treated with cathepsin D can catalyse the oxidation of LDL in a similar manner, not surprisingly. However, pre-incubation of ferritin with cathepsin had no effect on the rate of oxidation compared to control. It was tested whether pre-incubating ferritin with more proteolytic enzyme (with lower protein to enzyme ratio) would catalyse the oxidation of LDL by ferritin more effectively, but there was

no significant effect. The results of SDS-PAGE analysis of ferritin after incubation with proteolytic enzymes showed that ferritin is somewhat resistant to degradation by cathepsin D and B at pH 4.5, although exposure of apoB-100 in LDL to similar condition with ferritin caused extensive degradation of apoB-100. Chrichton has previously demonstrated the synergistic digestion of ferritin and apoferritin using cathepsin D and pepsin at pH 3.0 (Crichton, 1971), which is a more acidic pH compared to what was used in this study. However, their findings indicated that ferritin was less susceptible to digestion compared to apoferritin. The capacity of lysosomes to degrade endogenous and extracellular biomolecules is of great importance in handling the excess lipid and cytotoxic materials that are present in atherosclerotic plaque. The paramount role of lysosomes in degradation has been well acknowledged (Sergin *et al*., 2015). From our results, it is suspected that ferritin degradation may take a longer time and require synergistic actions of several proteolytic enzymes which were not included in this present study but can be explored in the future.

These experiments have shown that ferritin showed no observable degradation products after treatment with cathepsins D and B but it catalysed LDL oxidation after treatment with enzymes. This implies that it may stay longer in the lysosomes to oxidise LDL. The fact that ferritin was still able to catalyse LDL oxidation without complete degradation supports the spontaneous release of iron at lysosomal pH which catalyses LDL (demonstrated in chapter 3). The question of introducing more lysosomal enzymes is, however, relevant because autophagocytosed ferritin would be eventually be degraded in lysosomes. It would be of interest to know if it would be more or less effective in oxidising LDL during its life time in lysosomes before it is degraded entirely.

Enzymatically modified LDL has been produced *in vitro* with the use of both proteases and cholesteryl ester hydrolase (Bhakdi *et al*., 1995, Chellan *et al*., 2016). Preliminary data from our laboratory showed that LDL pre-incubated at pH 7.4 and 4.5 was degraded by trypsin and cathepsin D, respectively (unpublished data). The proteolytic modification of LDL in this present study was carried out with cathepsin D at lysosomal pH (pH 4.5). The enzyme cathepsin D has been identified as the key player in proteolytic degradation of LDL (Leake and Peters, 1981). The result from this present *in vitro* experiment was consistent with our previous work (unpublished data) and the result from others which showed that lysosomal proteases degraded apoB-100 at pH 5.5 (Linke *et al*., 2006). Although cathepsin D is not a cysteine protease and was not among the cathepsins used by Link *et al.*, however, low molecular weight fragments were observed in LDL pre-treated with cathepsin D at pH 4.5. Previous studies have demonstrated the importance of cathepsin D and B in the degradation of LDL in arterial smooth muscle cells (van der Westhuyzen *et al.*, 1980, Leake and Peters, 1981).

Enzymatically modified LDL has been demonstrated to be present in atherosclerotic lesions (Torzewski *et al*., 1998, Hakala *et al.*, 2003) and cathepsins are highly synthesised in atherosclerotic plaques (Hakala *et al.*, 2003, Sukhova *et al*., 2003, Oorni *et al*., 2004) which could act on LDL in lysosomes. Cathepsin B, D and X has been suggested to play a key role in the development of atherosclerosis (Zhao and Herrington, 2016). It was reported here that treatment of LDL with cathepsin D and B prior to oxidising with ferritin, increased modification of LDL at lysosomal pH but also its aggregation. Further work will be required to see if this increased modification is due to lipid peroxidation forming conjugated dienes or it is aggregation causing UV scattering. Measuring oxidised lipids by HPLC should clarify this question. Previous studies suggest that degrading apoB-100 in LDL leads to aggregation and fusion of lipoprotein in complex lipid aggregates that increase as the atherosclerotic lesions

progress (Piha *et al.*, 1995, Camejo, 2003). This present finding might imply that pre-treatment with cathepsins promote the formation of aggregated lipids and enhance atherogenicity in lysosomal LDL oxidised by ferritin. The important role of cathepsins, mainly the cysteine proteases, in atherosclerosis was earlier suggested by Liu *et al.* (Liu *et al*., 2004). This was later supported with evidence from reviews by Lutgens and others, whose work suggested that potential inhibitors of cathepsins might be beneficial in the treatment of atherosclerosis (Lutgens *et al*., 2007, Sjöberg and Shi, 2011).

High concentrations of cholesteryl ester hydrolase have been detected in atherosclerotic lesions by immunostaining (Sakurada *et al*., 1976, Hakala *et al*., 2003). The purified enzyme had an optimum pH of 4.5 -5.0 and 7.0 -7.5 (Sakurada *et al*., 1976) which includes the pH of lysosomes and that of plasma, respectively. The co-incubation of LDL with cholesteryl esterase from *Pseudomonas sp* and ferritin (Figure 4.6) showed a significant ($P < 0.001$) increase in the formation of conjugated dienes compared to incubation with ferritin alone. This breakdown of cholesteryl esters may contribute to more lipid peroxidation in lysosomal LDL oxidation, possibly by giving ferritin iron more access to the PUFA formed than to cholesteryl esters.

Hydrolysing cholesteryl esters and degrading the apoB-100 component of LDL at the same time produced LDL that had a similar chemical structure and biological activity to LDL derived from atherosclerotic lesions (Bhakdi *et al.*, 1995). The lysosomal compartment is highly dynamic with multiple enzymes acting in synergy to degrade biomolecules. These enzymes working together may have positive and negative consequence in relation to disease development (Bonten *et al*., 2014). The effect of the joint action of proteases (cathepsins B and D) and cholesteryl esterase (figure 4.7) on LDL oxidation by ferritin showed increased formation of conjugated dienes ($P < 0.01$). This suggests that the oxidation of LDL in lysosomes might speed up as the LDL is degraded in these organelles.

The findings in this chapter put together show that LDL oxidation by ferritin can proceed in the presence of lysosomal enzymes and might actually be increased by them. The degradation of the protein and lipid component of LDL particles enhanced its oxidation by ferritin at lysosomal pH. The results suggest that, as LDL is degraded in lysosomes its oxidation by ferritin might be speeded up. The results also show the remarkable resistance of ferritin (unlike apoB-100 in LDL) to proteolysis by major lysosomal endoproteases cathepsin B and D. The next chapter explores the role of antioxidants in the prevention of LDL oxidation by ferritin at lysosomal pH.

Chapter 5: The role of antioxidants in preventing LDL oxidation by ferritin

5.0 Background and rational

Atherosclerosis is a complex disease involving many factors. The oxidative modification of LDL and a chronic inflammatory response has been suggested as key events in the development of atherosclerotic lesions. LDL oxidation is believed by many to be closely linked with atherosclerosis with relevance in all stages of atherosclerosis (Nilsson *et al.*, 1992, Itabe *et al*., 2011). The fact that low concentrations of antioxidant compounds can delay or inhibit the oxidation of oxidisable substrates (Halliwell, 1990) makes antioxidants one of the promising candidates for prevention and treatment of atherosclerosis. Nutrients, such as and phenolic component of red wine (Teissedre *et al.*, 1996), vitamins C (Retsky et al., 1993) and E (Jessup *et al*., 1990) can reduce the susceptibility of LDL to oxidation *in vitro*. Plasma thiols such as homocysteine, cysteine and reduced glutathione have been demonstrated to inhibit LDL oxidation *in vitro* (Lynch *et al.*, 2000).

The LDL isolated from humans contains a number of lipid-soluble antioxidants which includes several carotenoids and oxycarotenoids, ubiquinol-10, γ-tocopherol and α-tocopherol (Esterbauer *et al.*, 1992). Out of these antioxidants, α-tocopherol (Figure 5.1A) is believed to be the most important and abundant antioxidant present in human LDL (Esterbauer *et al*., 1990a, Meydani, 2001). It possesses the ability to limit the production of lipid peroxidation products via its chain breaking action by scavenging alkoxyl and peroxyl radicals (Sies *et al.*, 1992, Liebler, 1993). The α-tocopherol radical produced is stabilised by delocalisation of unpaired electron around its ring. LDL isolated from people who had previously received vitamin E as a supplement was less susceptible to oxidation induced by macrophages or copper (Jessup *et al*., 1990, Dieber-Rotheneder *et al*., 1991) and lipid peroxidation increases after α-tocopherol consumed during LDL oxidation (Esterbauer *et al.*, 1987, Jessup *et al.*, 1990).

However, this was contradicted by arguments from other researchers who reported that the decrease in susceptibility of LDL to oxidation does not correspond to the α-tocopherol content of LDL (Stocker *et al*., 1991, Bowry *et al.*, 1992) and the pro-oxidant activity of vitamin E *in vivo* and *in vitro* especially in conditions of low oxidative stress, was demonstrated by Bowry and others (Bowry and Stocker, 1993, Neuzil *et al*., 1997). Other studies have suggested that the activity of α-tocopherol as an antioxidant *in vivo* can be upheld by activities of other antioxidants, such as ascorbate, found within atherosclerotic lesion (Suarna *et al.*, 1995) and ubiquinol-10 which can both convert the pro-oxidant radical form of α-tocopherol back to the antioxidant α-tocopherol (Stocker *et al*., 1991, Neuzil *et al*., 1997, Stocker, 1999). Ubiquinol-10 and vitamin C (Retsky et al., 1993) prevents the formation of lipid peroxidation products in LDL containing vitamin E (Bowry *et al*., 1992).

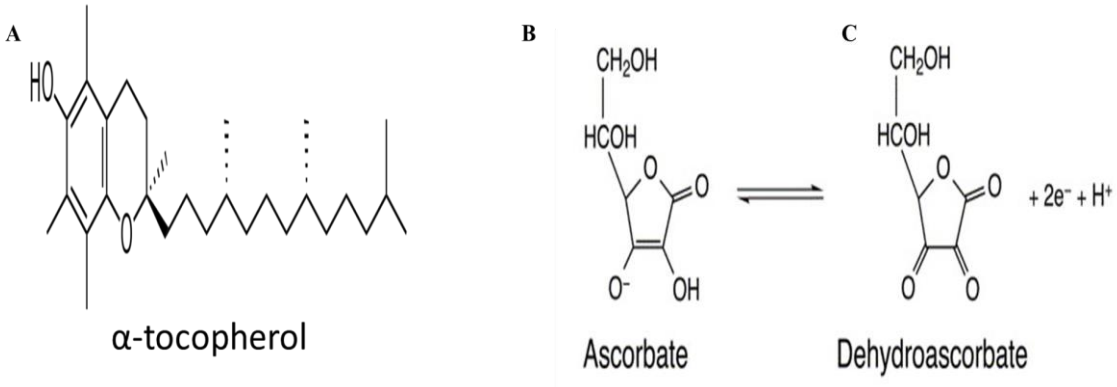


Figure 5.1: The structures of α-tocopherol, ascorbate and dehydroascorbate

Vitamin C (ascorbate) (Figure 5.1B) is considered to be a main water-soluble antioxidant which also possesses a chain breaking activity like the lipid-soluble vitamin E. Ascorbic acid possess a strong reducing ability and has been demonstrated to act as antioxidant *in vitro* and *in vivo* (Bendich *et al*., 1986, Vinson and Jang, 2001). Vitamin C preserved LDL and human plasma lipids from oxidant-induced peroxidative damage and was suggested to be more potent in preventing peroxidative events than endogenous antioxidants present in LDL and plasma (Frei

et al., 1989, Frei, 1991) and Jialal *et al*. previously reported that vitamin C is a more potent antioxidant than vitamin E, in terms of inhibition of oxidative modification of LDL (Jialal et al., 1990). Vitamin C protected against homocysteine and ferric iron induced LDL oxidation (Alul *et al.*, 2003). Horsley *et al* reported the pro-oxidant and antioxidant behaviour of oxidised form of vitamin C, dehydroascorbate (Fig. 5.1C) (Horsley *et al.*, 2007). They showed that dehydroascorbate can act as an antioxidant with fresh LDL or a pro-oxidant with mildly-oxidised LDL in the presence of copper. This finding corroborates previous findings by Stait and Leake that dehydroascorbate and ascorbate increase the oxidation of partially oxidised LDL and enhance its uptake by macrophages (Stait and Leake, 1994, Stait and Leake, 1996).

The role of vitamin E and C in protecting LDL against oxidation remains controversial. The role of antioxidants in preventing atherosclerosis is continuously challenged by the lack of protection against CVD in large human clinical trials with vitamin E, vitamin C or β-carotene (Jialal *et al*., 1999, Yusuf *et al.*, 2000, de Gaetano, 2001, Lonn *et al.*, 2002, Collins *et al*., 2002) and (Lee *et al*., 2004, Cook *et al*., 2007, Sesso *et al*., 2008). Although some small trials with vitamin E (Stephens *et al*., 1996, Boaz *et al*., 2000) and vitamin C (Khaw *et al*., 2001, Osganian *et al.*, 2003) showed protection against CVD but there are no substantial data to support a protective role for vitamin E and C from human trials. A recent review by Al-Khudairy and colleagues suggested that there is no evidence for the reduction of risk of CVD by vitamin C in healthy subjects (Al-Khudairy *et al*., 2017). The lack of strong evidence of protection from the clinical trials and the usually positive evidence on protection of fresh LDL against oxidation makes the role of vitamin E and C in lysosomal LDL oxidation a subject of interest in this present study.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) (Fig 5.2) is a potent antioxidant with super oxide (O_2^-) radical scavenging, superoxide dismutase mimetic property and has the ability to scavenge other reactive oxygen species (Wilcox and Pearlman, 2008). Tempol has been previously reported to reduce lipid peroxidation and blood pressure in animal models (Schnackenberg *et al.*, 1998, Schnackenberg and Wilcox, 1999) with the antihypertensive effect been related to its *in vitro* ability to dismutate superoxide (Patel *et al*., 2006). Tempol is considered to have the most powerful effects amongst nitroxides in preserving cells from the deleterious effects of reactive oxygen species (Krishna *et al.*, 1998, Li *et al.*, 2006). The superoxide mimetic property and the free radical scavenging capacity might be of value in protecting LDL from oxidation by ferritin.

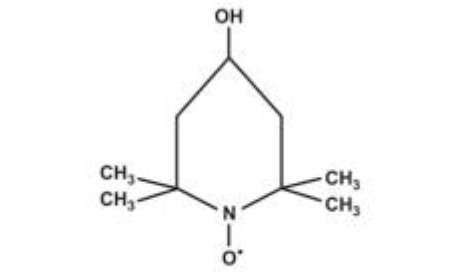


Figure 5.2: Structure of tempol

N, N′- Diphenyl 1, 4-phenylenediamine (DPPD) (Figure 5.3A) is an orally active compound with antioxidant properties. Previous studies have revealed that DPPD reduced the progression of atherosclerosis in rabbits (Sparrow *et al*., 1992) and apoE deficient mice (Tangirala *et al.*, 1995) fed high cholesterol diets without affecting the cholesterol concentration in plasma.

DPPD has previously been shown to protect LDL from oxidation by endothelial cells (Sparrow *et al.*, 1992) and copper-mediated oxidation (Tangirala *et al*., 1995).

Chloroquine a weak base with the ability to increase the pH of lysosome and concentrate in lysosomes inhibited the oxidation of LDL (Wen and Leake, 2007). Antioxidants that possess the ability to concentrate in the lysosomes might be protective against lysosomal LDL oxidation. Cysteamine (Figure 5.3B) is present normally in low concentrations in plasma because it is produced endogenously from the degradation of coenzyme A. It can upregulate the synthesis of glutathione, protecting cells from oxidative damage (Wilmer *et al.*, 2011). Cysteamine is currently used in the treatment of cystinosis, a disease characterised by the accumulation of cystine in lysosomes due to mutation in the genes for the lysosomal cystine transporter (cystinosin). The disease leads to progressive dysfunction of multiple organs due to accumulation of cystine in all the cells in the body. Cysteamine exports cysteine out of the lysosome via the lysine transport system by the formation of the mixed disulphide of cysteamine and cysteine. Cysteamine, which was introduced in 1976, remains the only drug for the treatment of cystinosis (Gahl *et al*., 2002, Napolitano *et al.*, 2015).

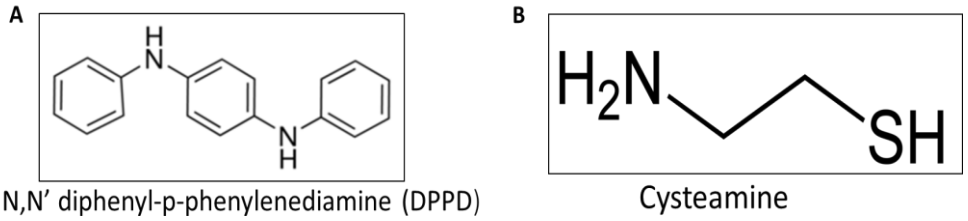


Figure 5.3: Structures of DPPD and cysteamine

The usual view of the oxidative modification process of LDL is challenged by the mostly negative effects seen with large clinical trials against CVD (Steinberg and Witztum, 2002) and the fact that low concentrations of serum or interstitial fluid inhibit LDL oxidation (Leake and Rankin, 1990, Dabbagh and Frei, 1995). These challenges can be accounted for by the recently proposed lysosomal theory which suggests that non-oxidatively modified LDL can be rapidly taken up by macrophages and then extensively oxidised within the lysosomes (Wen and Leake, 2007). A recent study from our laboratory showed the ability of DPPD and cysteamine to inhibit LDL oxidation mediated by iron at lysosomal pH (Ahmad and Leake, 2018).

In view of all the controversy surrounding the use of antioxidants in prevention and treatment of atherosclerotic cardiovascular disease, the identification of lysosomal LDL oxidation is a candidate for reinvigorating the LDL oxidation hypothesis. There is a need for identification of appropriate antioxidants that can be beneficial for the treatment of atherosclerosis. Chapter three and four of this thesis have shown that ferritin can mediate LDL oxidation at lysosomal pH. It was hypothesised that antioxidants might play a role in protecting LDL from oxidation by ferritin. Hence, this chapter explores the role of antioxidants in preventing LDL oxidation by ferritin using the major physiological lipid and water soluble vitamins (vitamin E and C) with antioxidant properties, the amphipathic compound tempol, the hydrophobic compound DPPD and the water soluble lysosomotropic compound cysteamine.

5.1 Objectives

i) To investigate the role of vitamin E (α-tocopherol) and vitamin C(ascorbate and dehydroascorbate) in LDL oxidation mediated by ferritin at lysosomal pH.

ii) To evaluate the effects of existing lipid hydroperoxides and pH on antioxidant effects of ascorbate

iii) To explore the ability of tempol to scavenge superoxide radical during LDL oxidation by ferritin and prevent oxidation.

iv) To evaluate the effects of DPPD and cysteamine on LDL oxidation by ferritin at lysosomal pH.

5.2 Methods

LDL was isolated as described in sections 2.2.1 and 2.2. Formation of conjugated dienes, kinetics of oxidation of LDL oxidation by ferritin in the presence or absence of antioxidants was monitored as described in section 2.3.7. The release of iron from ferritin in the presence of cysteamine at varying time points was measured using the iron chelator bathophenanthroline disulphonic acid (sections 2.3.8).

5.3 Results

5.3.1 Effect of α-tocopherol in LDL oxidation mediated by ferritin

The enrichment of LDL with α-tocopherol inhibited LDL oxidation by copper sulphate (5 µM) at pH 7.4 (Dieber-Rotheneder *et al.*, 1991, Satchell and Leake, 2012, Alboaklah, 2018b). In order to test the effect of vitamin E on LDL oxidation by ferritin, we enriched LDL with α-tocopherol by incubating human plasma with α-tocopherol dissolved in DMSO for 3 h and then isolating LDL by ultracentrifugation. The α-tocopherol content was measured as previously described by Satchell (Satchell and Leake, 2012). The α-tocopherol content in LDL increased from 15 ± 0.4 to 26 ± 0.8 nmol/mg protein (Alboaklah, 2018b), increasing the average number of α-tocopherol molecules per LDL particle from about 8 to 13. Control LDL or α-tocopherol enriched LDL (50 µg protein/ml) was oxidised with ferritin (0.1 µM) at pH 7.4 (MOPS buffer) and pH 4.5 (NaCl/sodium acetate buffer) (Figure 5.4) at 37 ^{0}C. The formation of conjugated

dienes was monitored continuously at 234 nm. The mean attenuance at 200 min at acidic pH was slightly increased from 0.204 ± 0.12 in control compared to 0.260 ± 0.12 in α-tocopherol enriched LDL. The increase in formation of conjugated dienes by α-tocopherol was not statistically significant compared to control LDL at either pH 4.5 or pH 7.4.

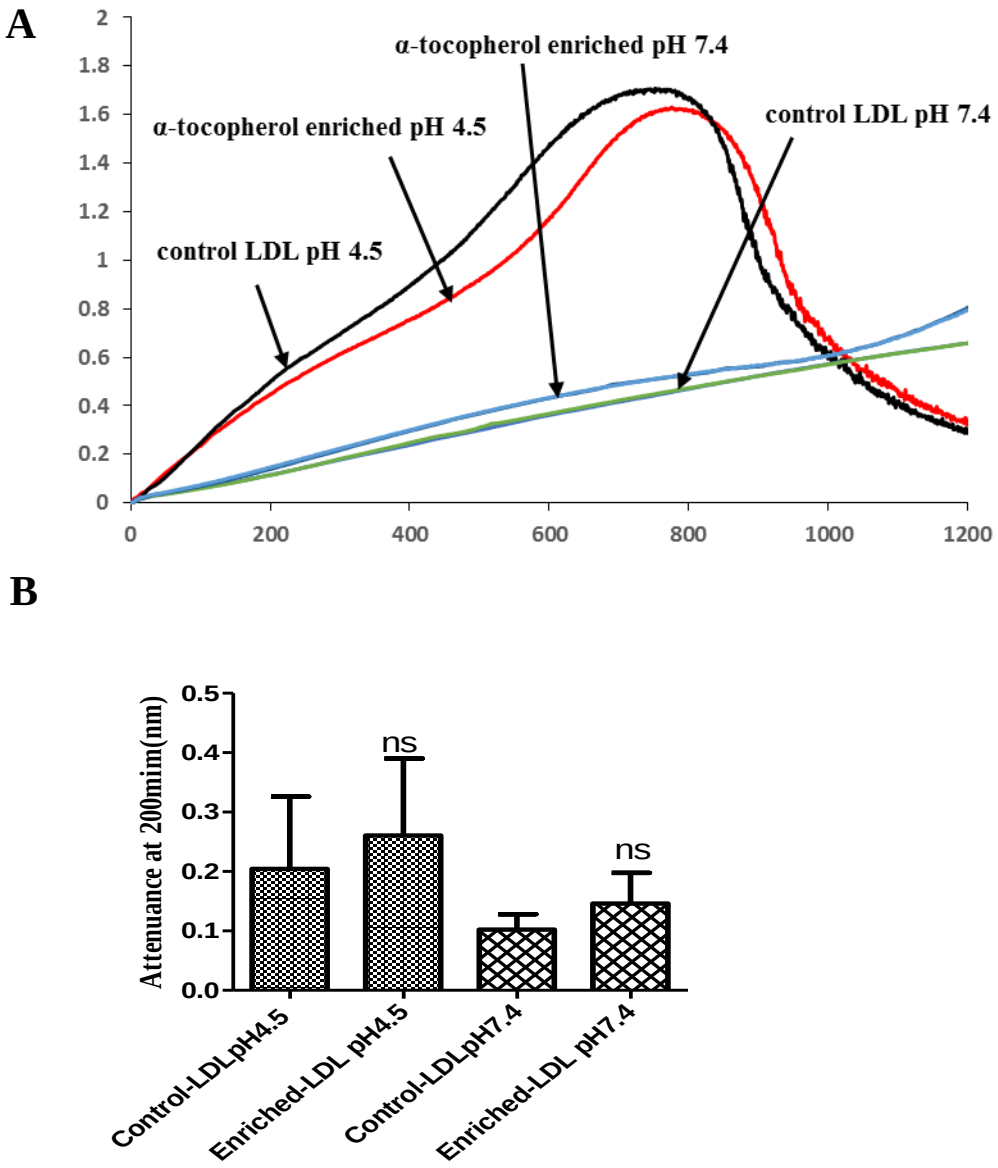


Figure 5.4. The effect of α-tocopherol on LDL oxidation by ferritin at pH 7.4 and pH 4.5

Control LDL or LDL enriched with α-tocopherol (50 µg protein/ml) was incubated with ferritin (0.1 µM) at pH 4.5 or pH 7.4 and the formation of conjugated dienes was monitored at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments (A). The mean and SEM of attenuance at 200 min were obtained for each concentration and compared by one-way ANOVA followed by a Tukey post hoc test. (n=4) ($p>0.05$), ns indicates not significant (B).

5.3.2 Effects of vitamin C (ascorbate and dehydroascorbate) on LDL oxidation by ferritin at lysosomal pH.

According to a 2015 study, the recommended vitamin C intake is an amount that can make up for the metabolic loss of vitamin C and guarantees a fasting plasma level of 50 µM of ascorbate (German Nutrition, 2015). The effect of varying concentrations of vitamin C (ascorbate and the oxidised form dehydroascorbate) on LDL oxidation mediated by ferritin were evaluated. LDL (50 µg protein/ml) was incubated with ferritin (0.1 µM) in the presence or absence of varying concentrations (10 - 100 µM) of ascorbate or dehydroascorbate in NaCl/sodium acetate buffer at 37 ^{o}C. The formation of conjugated dienes was monitored at 234 nm at one minute intervals for about 1200 minutes. Ascorbate inhibited the initial phase of oxidation of LDL in a concentration-dependent manner, however, ascorbate exhibited a pro-oxidant effect at a later stage (Fig 5.5). The oxidised form (dehydroascorbate) does not offer any protection towards LDL oxidised by ferritin; it rather acted as a pro-oxidant increasing the oxidation of LDL by ferritin in a concentration-dependent manner. The pro-oxidant effect was seen from early phase with rapid oxidation and early observation of aggregation and sedimentation phase (Fig. 5.6)

Statistical analysis of the increase in attenuance at 200 min with one way ANOVA (n = 4) followed by Tukey post hoc test showed that the two higher concentrations (30 µM and 100 µM) of ascorbate was significantly different from control with 100 µM exhibiting a stronger statistical effect (Figure 5.5). The pro-oxidant effects of higher concentrations of dehydroascorbate were significantly different from control ($p < 0.001$) (Fig. 5.5). The effects of varying concentrations dehydroascorbate on the early oxidation of LDL was the opposite of that for ascorbate.

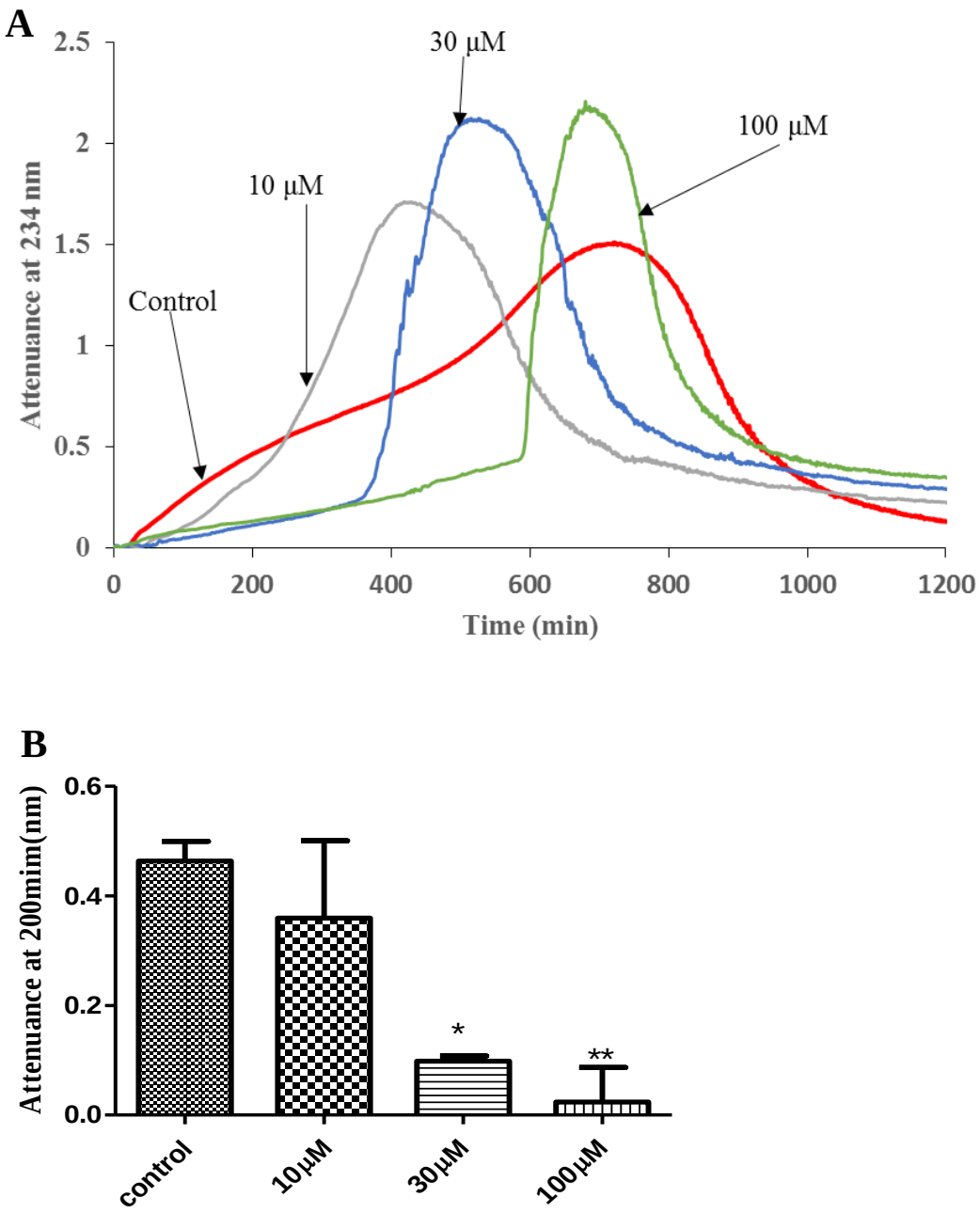


Figure 5.5: The effect of ascorbate on LDL oxidation by ferritin at lysosomal pH

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with ferritin(0.1 µM) in the absence (red line) or presence of varying concentrations of ascorbate 10 µM (grey line), 30 µM (blue line) and 100 µM (green line) at 37^{o}C in capped cuvettes. The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of four independent experiments (Fig. 5.5A). The mean and SEM of attenuance at 200 min were obtained for each concentration and compared (n=4) by one-way ANOVA followed by a Tukey post hoc test. * indicates $P < 0.05$ and ** indicates $P < 0.01$ (Fig. 5.5B).

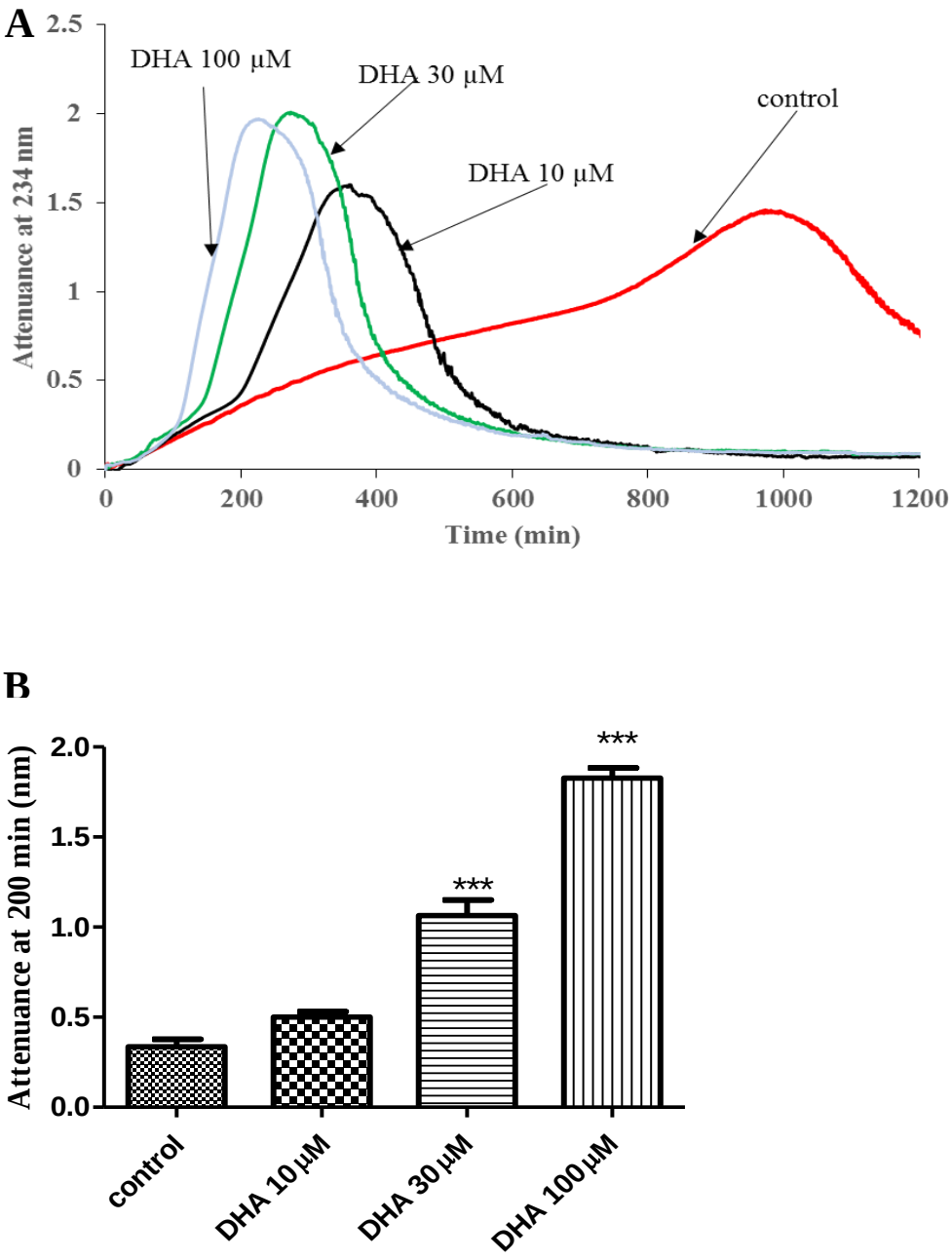


Figure 5.6: The effect of dehydroascorbate on LDL oxidation by ferritin at lysosomal pH

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with ferritin (0.1 µM) in the absence (red line) or presence of varying concentrations of dehydroascorbate 10 µM (black line), 30 µM (green line) and 100 µM (blue line) at 37°C in capped cuvettes. The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of four independent experiments (A). The mean and SEM of attenuance at 200 min were obtained for each concentration and compared to the control by one-way ANOVA (n = 4) followed by a Tukey post hoc test. *** indicates $P < 0.001$ (B).

5.3.3 Effects of existing oxidised lipids and pH on antioxidant effects of ascorbate

A Previous study has demonstrated that the antioxidant activity of ascorbate and dehydroascorbate on copper-mediated oxidation of LDL was lost when LDL is partially oxidised (Stait and Leake, 1996). The effects of the presence of existing oxidised lipids on antioxidant/pro-oxidant activities of ascorbate on LDL oxidised by ferritin was tested. LDL (50 µg protein/ml) was incubated with ferritin (0.1 µM) in the presence or absence of 30 µM ascorbate added at three different time points (0, 100 and 200 minutes) in NaCl/sodium acetate buffer at 37 ^{0}C. The formation of conjugated dienes was monitored continuously at 234 nm at one minute intervals for about 1200 minutes (Figure 5.7A). The results showed that the presence of lipid hydroperoxides or other products of oxidation led to ascorbate exhibiting an immediate and rapid pro-oxidant effect on LDL. The increase in attenuance at 200 min were compared with one way ANOVA (n = 3) followed by Tukey post hoc test showed that ascorbate significantly decreased the attenuance at pH 7.4 compared to control ($p< 0.001$) (Fig 5.7B).

Ascorbate has been shown to inhibit oxidation of fresh LDL by macrophages (Stait and Leake, 1994) and copper (Stait and Leake, 1996). The effect of pH on the antioxidant activity of ascorbate towards fresh LDL oxidised by 5 µM $CuSO_4$ was tested. LDL (50 µg protein/ml) was incubated with $CuSO_4$ (5 µM) in in NaCl/sodium acetate buffer (pH4.5) or MOPS buffer (pH 7.4) in the presence or absence of 30 µM ascorbate at 37 ^{0}C. The formation of conjugated dienes was monitored at 234 nm at one minute intervals for about 1200 minutes (Figure 5.8A). Ascorbate effectively delayed the formation of conjugated dienes in LDL oxidised with $CuSO_4$ at pH 7.4, but after a lag time of about 100 min increased the rate of oxidation of LDL oxidised with $CuSO_4$ at pH 4.5. Statistical analysis of the increase in attenuance at 200 min with one

way ANOVA (n = 3) followed by Tukey post hoc test showed that ascorbate significantly decreased the attenuance at pH 7.4 compared to control ($p < 0.001$) (Fig 5.8B).

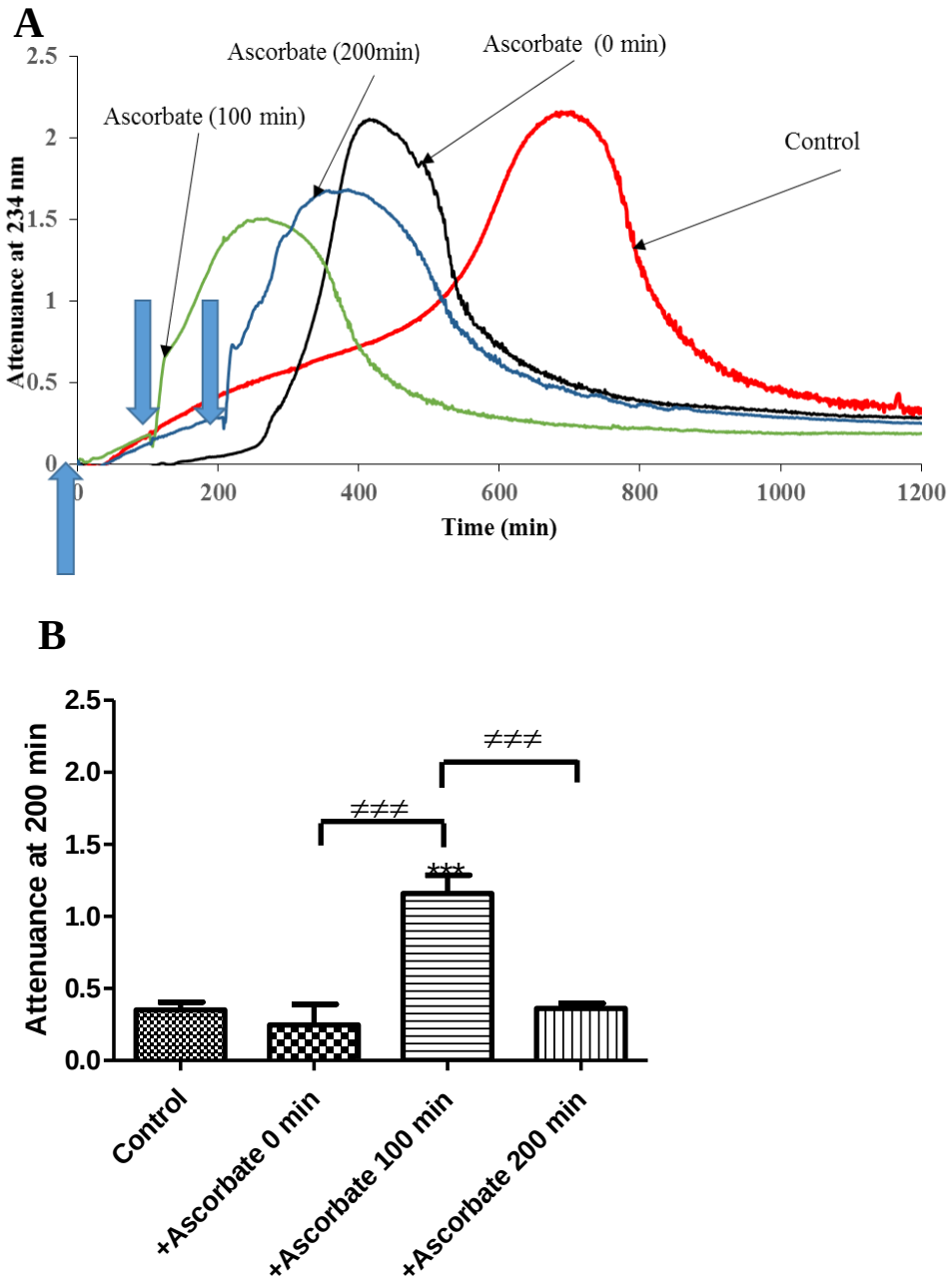


Figure 5.7. The effect of existing oxidised lipids on the effect of ascorbate on LDL oxidation by ferritin at lysosomal pH

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with ferritin (0.1 µM) in the absence (red line) or presence of 30 µM ascorbate added at different time points, namely 0 min (black line), 100 min (green line) and 200min (blue line) at 37^{o}C in capped cuvettes. The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments (A). The mean and SEM of attenuance at 200 min were obtained for each concentration and compared by one-way ANOVA (n = 3) followed by a Tukey post hoc test. *** indicates P < 0.001. ### indicates P< 0.001 to shown comparison (B).

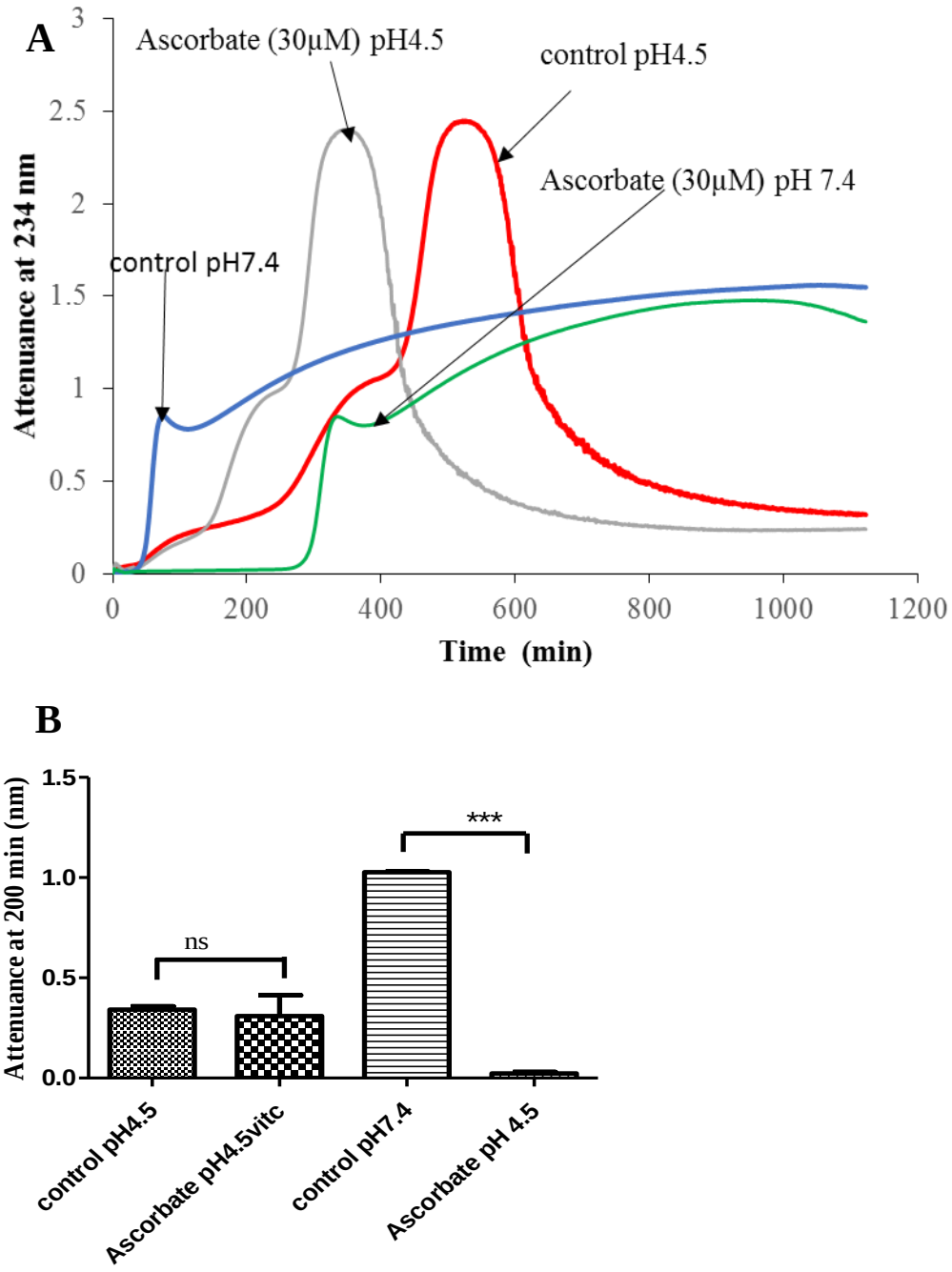


Figure 5.8. The effect pH on ascorbate protecting LDL from copper-mediated LDL oxidation

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) or MOPS buffer (pH 7.4) was incubated with 5 µM $CuSO_4$ in the absence or presence 30 µM ascorbate at 37^{o}C in capped cuvettes. The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference cuvettes that lacked LDL. This is a representative of three independent experiments (A). The mean and SEM of attenuance at 200 min were obtained for each experiment and compared by one-way ANOVA (n = 3) followed by a Tukey post hoc test. *** indicates $P < 0.001$, ns indicates not significant (B).

5.3.4 Effects of tempol on LDL oxidation by ferritin at lysosomal pH.

Work from chapter three of this thesis suggested that oxidation of LDL by ferritin might be mediated by highly reactive hydroperoxyl radicals which are formed by protonation of superoxide radicals and this reaction is highly favoured at acidic pH. Hence, the superoxide dismutase mimetic property of tempol is of interest as this could prevent the formation of hydroperoxyl radicals and prevent LDL oxidation by ferritin. Previous work from our laboratory (unpublished data) demonstrated that tempol inhibited all phases of oxidation completely in LDL oxidised by 5 μM $FeSO_4$ but not the lag phase. This effect was concentration dependent with concentration as low as 10 μM exhibiting this effect.

LDL (50 μg protein/ml) was incubated with ferritin (0.1 μM) in the presence or absence of 10 μM tempol in NaCl/sodium acetate buffer (pH 4.5) at 37^{o}C. The formation of conjugated dienes was monitored at 234 nm at one minute intervals for about 1200 minutes (Figure 5.9A). The reactive oxygen species scavenging compound, tempol (10 μM) exhibited no effect on the early stage of oxidation of LDL by ferritin but inhibited the formation of conjugated dienes later on (Fig 5.9). The inhibition observed at later times was sometimes a partial inhibition, as shown in Fig. 5.9A, or sometimes more pronounced as shown in Fig 5.9B. Statistical analysis of mean ± SEM increase in attenuance at 200 min with student t test (n = 6) showed the effect of tempol on LDL oxidation by ferritin was not statistically significant ($P > 0.05$) (Figure 5.9C).

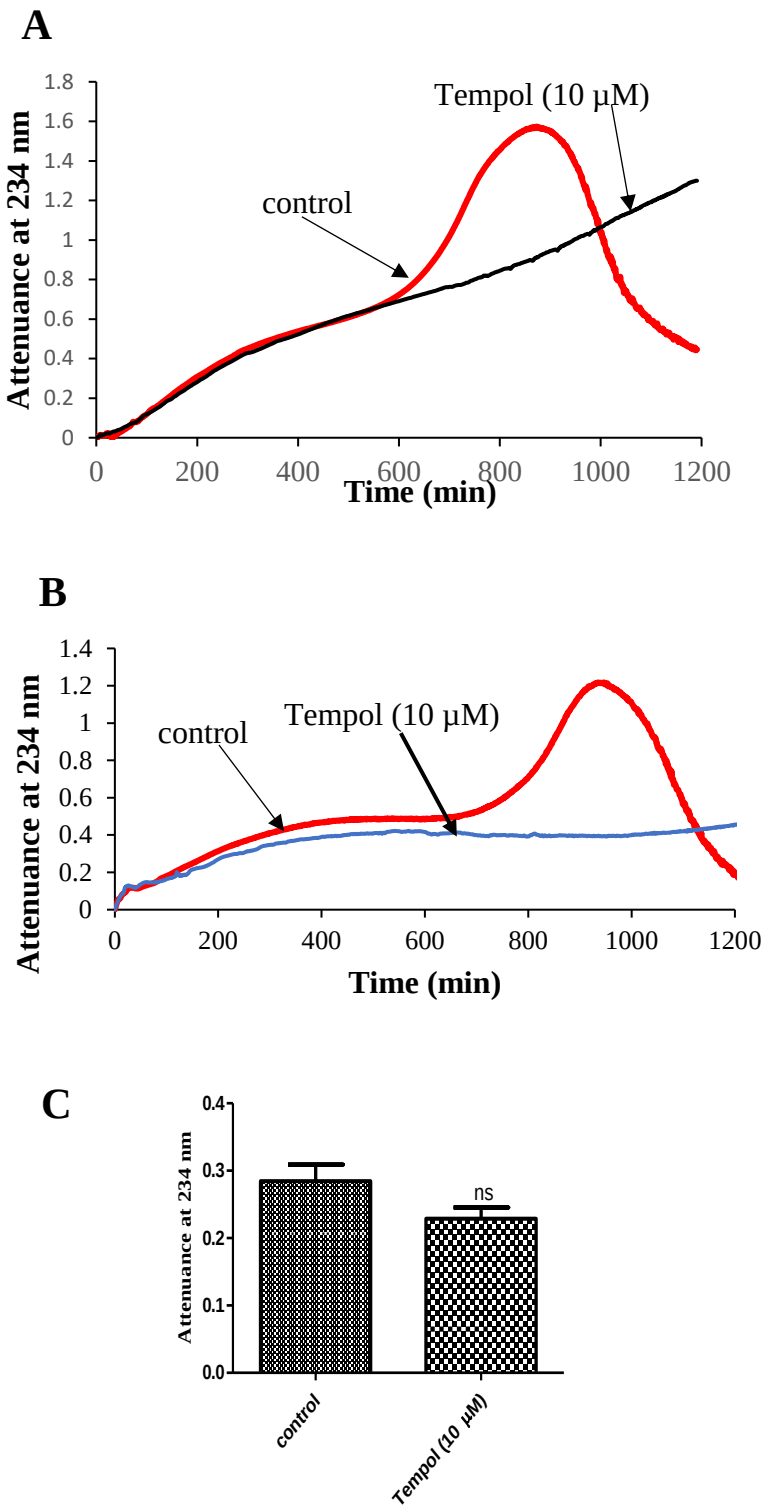


Fig. 5.9: The effect of tempol on LDL oxidation by ferritin at lysosomal pH

LDL (50µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with ferritin (0.1 µM) at 37 ^{0}C in the absence or presence of tempol (10 µM). These results are representative of six independent experiments (A and B). The mean and SEM of attenuance at 200 min were obtained for each experiment and compared by paired Students t test (n = 6). ns indicates not significant (C).

5.3.5 Effects of DPPD on LDL oxidation by ferritin at lysosomal pH.

DPPD has been shown to promote LDL resistance to copper-mediated oxidation (Tangirala *et al*., 1995). We tested the effect of DPPD on LDL oxidised by ferritin at lysosomal pH. Native LDL (50µg protein/ml) was oxidised with 0.1µM ferritin in NaCl/sodium acetate buffer (pH 4.5) in the presence or absence of DPPD (5 µM or 10 µM). DPPD was dissolved in ethanol hence ethanol was added (1% v/v) in the absence of DPPD to check if it can influence LDL oxidation. Formation of conjugated dienes was monitored (Fig. 5.10). The presence of DPPD greatly inhibited the formation of conjugated dienes. Ethanol had little effect on the oxidation of LDL by ferritin.

The rise in attenuance at 200 min for three independent experiments were compared using one-way ANOVA followed by Tukey post hoc test (Fig. 5.10B). The oxidation of LDL by ferritin in the presence of 5 µM and 10 µM are statistically different from the control ($P<0.001$). However, the addition of 1% (v/v) ethanol exhibited a little effect on LDL oxidation.

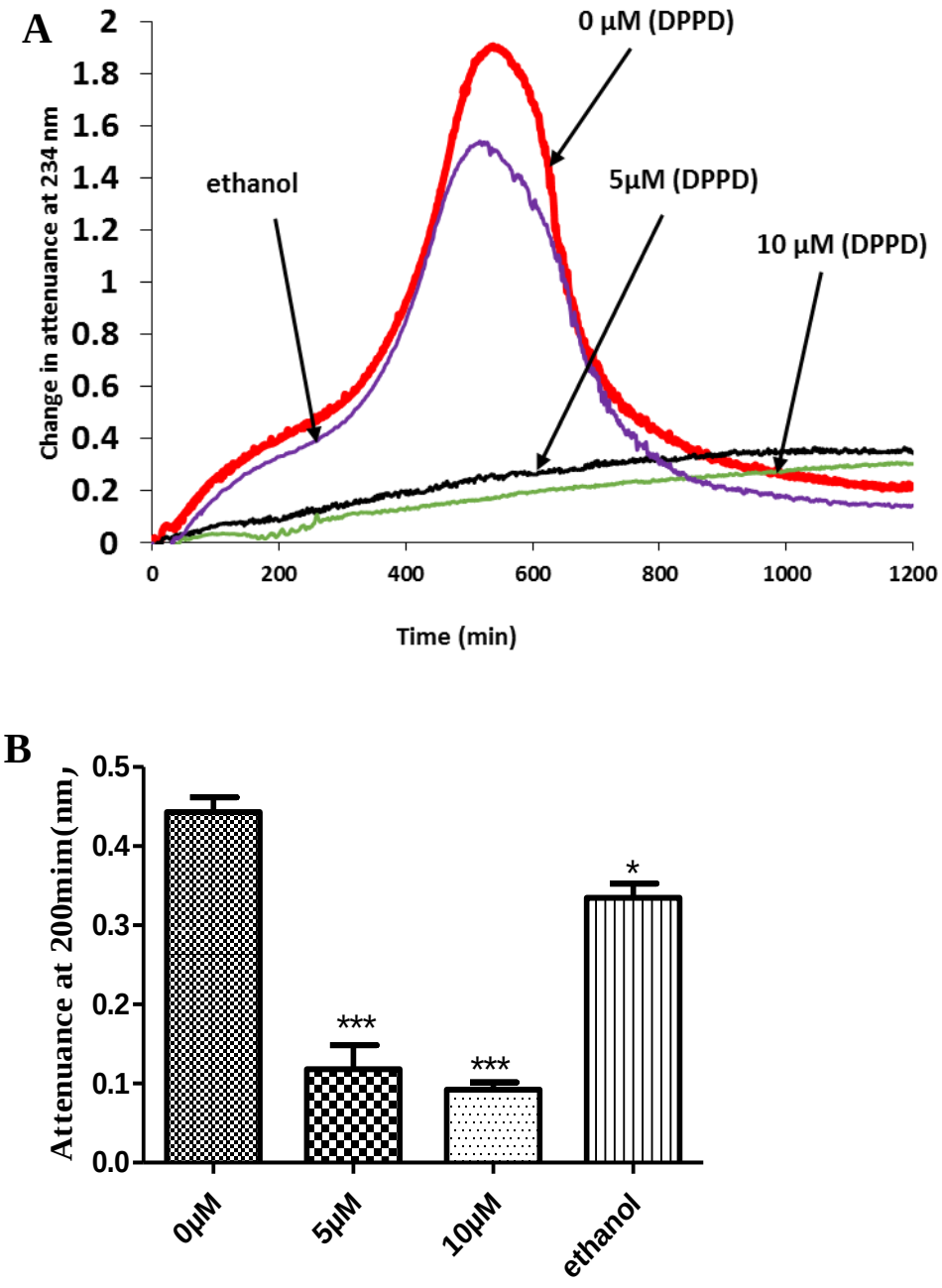


Figure 5.10: The effect of DPPD on LDL oxidation by ferritin

LDL (50µg protein/ml) was oxidized with ferritin 0.1µM in NaCl/sodium acetate buffer (pH 4.5) at 37ºC in the absence or presence of DPPD 5 µM, 10 µM and ethanol (1% v/v). The formation of conjugated diene was monitored by measuring attenuance at 234 nm against appropriate reference that lacked LDL. This is a representative of three independent experiments (A). The rise in attenuance at 200 min in the presence or absence of cysteamine (5, 10µM) and ethanol (1% v/v) in three independent experiments was determined. Mean and SEM were obtained for each concentration and compared with one-way ANOVA (n=3) followed by Tukey's post hoc test. *** indicates P <0.001 and * indicates P <0.05 (B).

5.3.6 Effect of cysteamine on LDL oxidation mediated by ferritin at lysosomal pH

5.3.6.1 Effect of lower concentrations of cysteamine on LDL oxidation by ferritin at lysosomal pH

It has been proposed that the ability of cysteamine to concentrate in the lysosomes might be of benefit in inhibition of LDL oxidation. It was of interest to see if cysteamine will exert on inhibitory effect on the oxidation of LDL by ferritin.

LDL (50µg LDL protein/ml) was oxidised with 0.1µM ferritin in NaCl/sodium acetate buffer (pH 4.5) in the presence or absence of cysteamine using varying concentrations of cysteamine (5 µM – 1000 µM). Formation of conjugated dienes was monitored. Cysteamine had a complex, but consistent, the effect on the rate of LDL oxidation. The presence of cysteamine slowed down the early oxidation by ferritin in a concentration-dependent manner (Fig. 5.11A). At later time points, cysteamine increased the rate of oxidation of LDL compared to the control LDL with the greatest effect seen with 25-100 µM cysteamine. All cysteamine concentrations decreased the time to maximum aggregation, except 1,000 µM. The rise in attenuance at 200 min for three independent experiments were compared using one-way ANOVA followed by Tukey post hoc test. The oxidation of LDL by ferritin in the presence of 250 µM and 1000 µM were statistically different from the control (Fig. 5.11B).

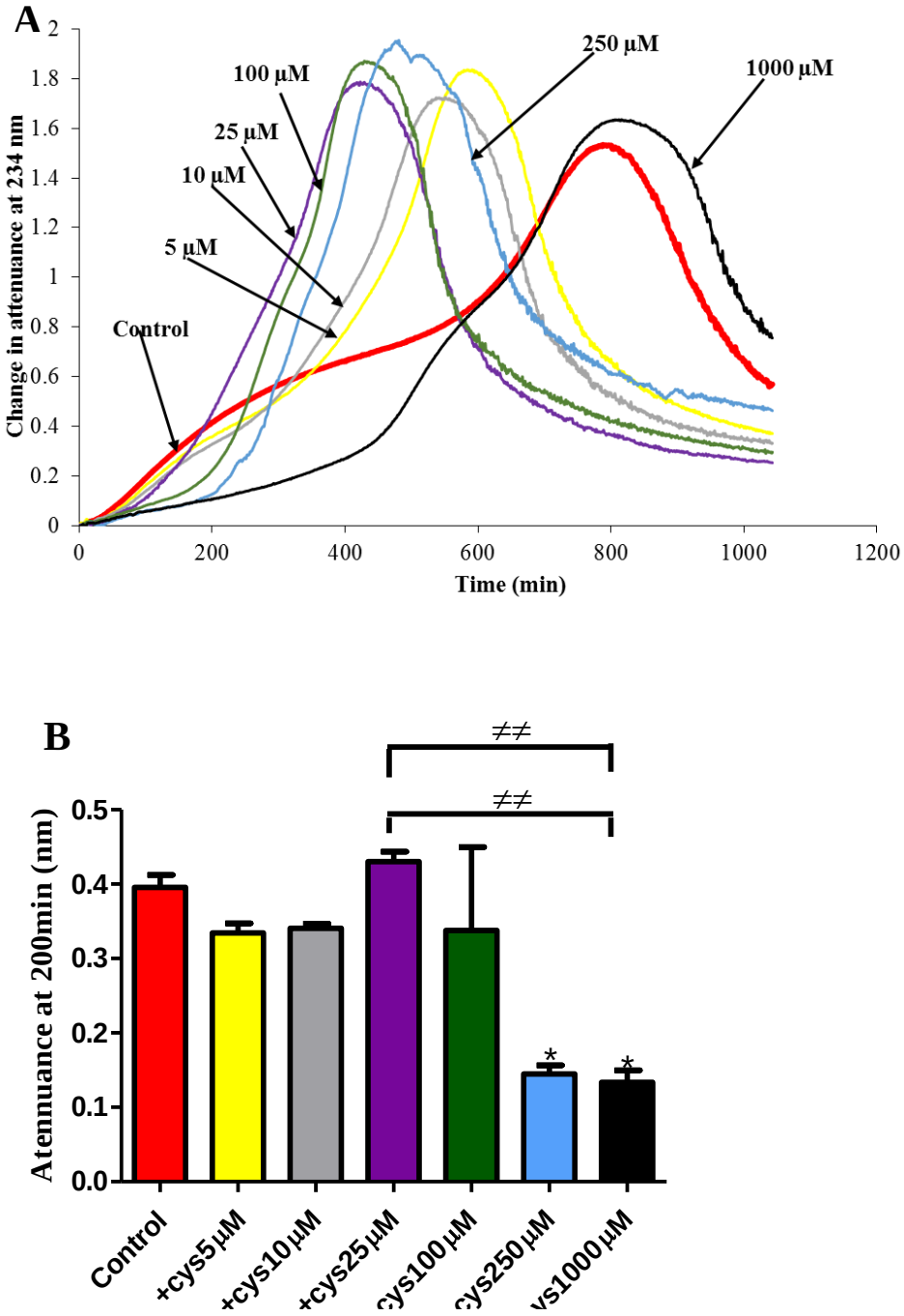


Fig 5.11: The effect of cysteamine on LDL oxidation by ferritin.

LDL (50µg protein/ml) was oxidised with ferritin (0.1µM) in NaCl/sodium acetate buffer (pH 4.5) at 37ºC in the absence or presence of cysteamine (at various concentration (5 - 1000 µM). The formation of conjugated dienes was monitored by measuring attenuance at 234 nm against appropriate references that lacked LDL. This is representative of three independent experiments (A). The rise in attenuance at 200 min in the presence or absence of cysteamine (5 - 1000 µM) in three independent experiments was determined. Mean and SEM were obtained for each concentration and compared (n=3) ANOVA followed by Tukey's post hoc test. * indicates P <0.05 compared to control. ≠≠ indicates P <0.01 to shown comparison (B).

5.3.6.2 Effect of higher concentrations of cysteamine on LDL oxidation by ferritin at lysosomal pH

Up to 1.1.95g/m^2/day of cysteamine is recommended to patients with nephropathic cystinosis over the age of 12 and weight above 50kg divided over a period of four times (Besouw *et al*., 2011, Besouw and Levtchenko, 2014). The concentration of cysteamine in plasma was estimated to be about 40 µM. It should accumulate up in several orders of magnitude in lysosomes (Pisoni *et al*., 1995), hence millimolar concentrations would be expected to accumulate in the lysosomes.

LDL (50µg LDL protein/ml) was oxidised with 0.1µM ferritin in NaCl/sodium acetate buffer (pH 4.5) in the presence or absence of cysteamine (1, 3 and 10mM). Formation of conjugated dienes was monitored (Fig. 5.12). The presence of higher concentration of cysteamine inhibited the formation of conjugated dienes to a large extent. The rise in attenuance at 200 min for three independent experiments were compared using one-way ANOVA followed by Tukey post hoc test. The oxidation of LDL by ferritin in the presence of 1mM, 3mM and 10 mM are statistically different from the control (Fig. 5.11B). However, there was no significant different between the two higher concentrations as compared to each.

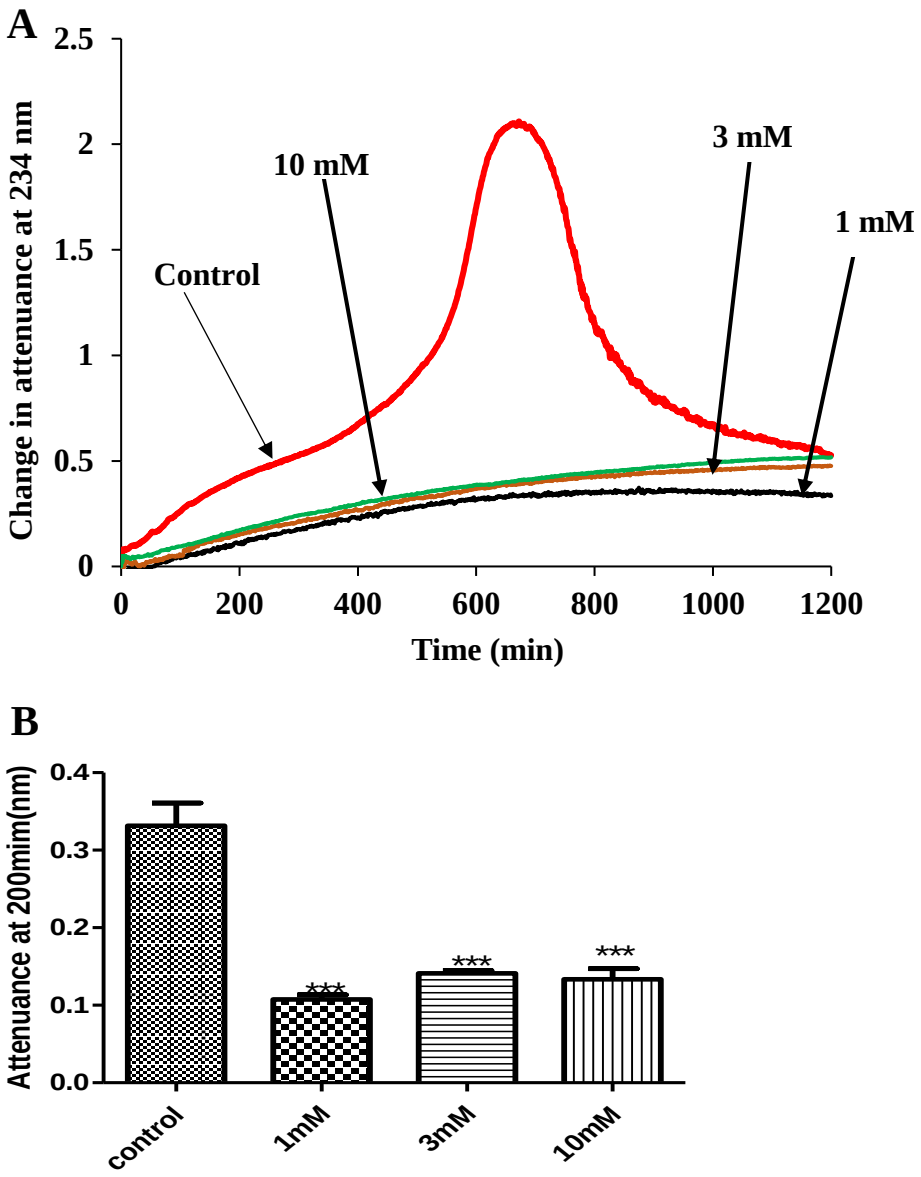


Fig 5.12: The effect of higher concentrations of cysteamine on LDL oxidation by ferritin at lysosomal pH.

LDL (50µg protein/ml) was oxidised with ferritin 0.1µM in NaCl/sodium acetate buffer (pH 4.5) at 37^{o}C in the absence, 0 µM (red line) or presence of cysteamine 1mM (black line), 3mM (orange line) and 10 mM (green line). The formation of conjugated dienes was monitored by measuring attenuance at 234nm against appropriate reference that lacked LDL. This is a representative of three independent experiments (A). The rise in attenuance at 200 min in the presence or absence of cysteamine (1, 3 and 10mM) in three independent experiments was determined. Mean and SEM were obtained for each concentration and compared by ANOVA followed by Tukeys post hoc test. *** indicates P <0.001 (B).

5.3.7 Effect of cysteamine on ferrous complex formation by ferritin

Iron released from ferritin in the presence of cysteamine was assessed to explore the pro-oxidant mechanism of cysteamine. Ferritin (0.1 µM) was incubated in Falcon tubes (15ml) in Chelex-treated NaCl/sodium acetate buffer (pH 4.5) at 37 ^{o}C in the presence or absence of 25 µM or 1mM cysteamine. A fraction of 1ml was taken at intervals up to 24 h. BP (30 µl of 10mM) was added at different time points and absorbance was measured at 535nm. More ferrous complex was formed in the presence of cysteamine (Fig. 5.13).

The ferrous complex formed were compared for each time point, using two-way ANOVA followed by Bonferoni post-test. The mean ferrous complex formed by ferritin in the presence of cysteamine 25 µM and 1 mM (A and B) were significantly higher over time when compared to the controls.

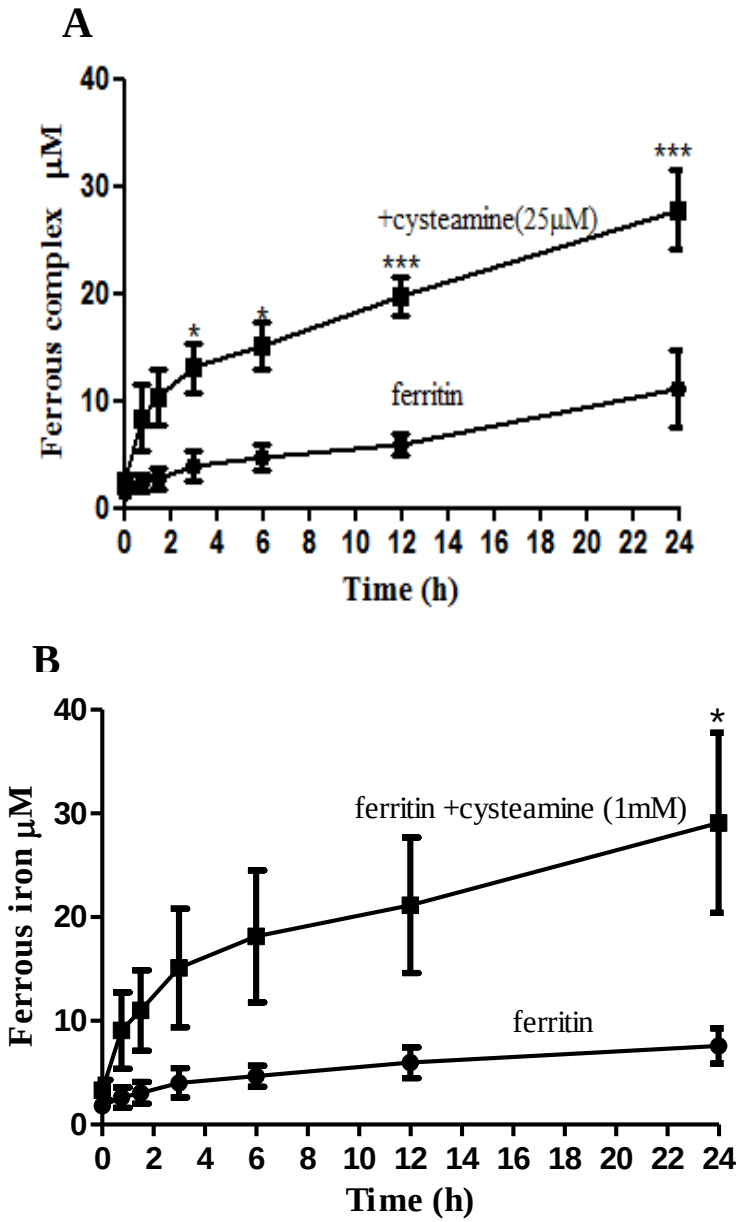


Fig. 5.13: The effect of cysteamine on ferrous complex formation by ferritin at pH 4.5.

Ferritin (0.1 µM) was incubated at 37 ˚C in NaCl/Na acetate buffer (pH 4.5) in the absence or presence of cysteamine (25 µM) (A) or 1 mM (B). At different times, a sample (1 ml) was taken and the ferrous chelator bathophenanthroline was added, left for 5 min and the absorbance was measured at 535 nm. These results were obtained from mean ± SEM of three independent experiments. The means were compared with two-way ANOVA followed by Bonferoni post-test. *** indicates P <0.001 and * indicates P <0.05.

5.4 Discussion

The Oxidative modification hypothesis argues that oxidised LDL is an early event in atherosclerosis and oxidised LDL promotes progression of atherosclerosis. Oxidised LDL incites many atherogenic events (Steinberg *et al*., 1989). This hypothesis was supported by the protective effects observed with some antioxidants in animal models reviewed by Steinberg in 2009 (Steinberg, 2009). However, the relevence of this hypothesis is continually questioned due to emerging evidence of lack of antioxidant protection against CVD in human trials (Steinberg and Witztum, 2002). These suggest that there is a need for more understanding of the mechanism of LDL oxidation, identification of more appropriate antioxidants. Our laboratory has shown that lysosomal oxidation of LDL might account for this challenge (Wen and Leake, 2007) and demonstrated that some antioxidants inhibit the oxidation of LDL by $FeSO_4$ less at lysosomal pH (Ahmad and Leake, 2018) than they do at interstitial fluid or plasma pH. This present study examined the effects of antioxidants on LDL oxidation by ferritin.

Our laboratory has shown previously that α-tocopherol enrichment of LDL inhibits LDL oxidation by copper ions at pH 7.4, but it did not effectively inhibit LDL oxidation by ferrous iron at lysosomal pH and had an initial pro-oxidative effect (Satchell, 2008, Satchell and Leake, 2012, Alboaklah, 2018a). In this present study, enrichment of LDL with α-tocopherol did not decrease the rate of oxidation of LDL by ferritin at pH 4.5 or pH 7.4. There was a tendency of α-tocopherol enrichment of LDL to increase the oxidation of LDL by ferritin at pH 4.5, but this was not statistically significant. The lack of antioxidant effect might be due to ferric ions released from ferritin (or ferrous ions released from ferritin subsequently oxidised to ferric ions) converting α-tocopherol to α-tocopheroxyl radicals, which are not entirely stable and can

abstract a hydrogen atom from the bisallylic groups of polyunsaturated fatty acids present in lipids in LDL increasing lipid peroxidation (equation 1 and 2 (Bowry *et al*., 1992).

α-toc OH + Fe^{3+} → α-toc $O^{\cdot}$ + H^{+} + Fe^{2+} eqn 1

α-toc $O^{\cdot}$ + LH → α-toc OH + $L^{\cdot}$ eqn 2

This pro-oxidant effect of α-tocopherol might negate the antioxidant effect of α-tocopherol (equation 3) leading to little net effect of this compound.

α-toc OH + $LOO^{\cdot}$ → **α-toc OH + LOOH eqn 3**

This increased oxidation effects observed in ferritin oxidation of LDL enriched with α-tocopherol supports previous evidence from our laboratory that vitamin E loses its antioxidant potential against Cu^{2+} and Fe^{3+} at lysosomal pH (Alboaklah, 2018a).

The role of ascorbate in atherosclerosis remains controversial, whilst some studies have demonstrated an anti-atherogenic effect for ascorbate supplementation in cholesterol fed-animals (Verlangieri *et al.*, 1977, Beetens *et al*., 1984, Lynch *et al.*, 1996). Some other studies have found no beneficial effect against atherosclerosis in animal models (Morel *et al*., 1994). Antioxidant and pro-oxidant effects of ascorbate against cell-mediated and cell-free LDL oxidation systems has been previously demonstrated (Stait and Leake, 1994, Stait and Leake, 1996). It was demonstrated that ascorbate exhibited a concentration-dependent antioxidant and pro-oxidant effects on LDL oxidation by ferritin at pH 4.5. It can be proposed that the antioxidant effect observed may be due to ascorbate regenerating α-tocopherol from α-tocopheroxyl radicals (equation 4). The ascorbyl radical formed during this process can be converted back to its stable form by dismutation, hence ascorbate may be acting as a co-antioxidant for α-tocopherol (Carr *et al*., 2000b). Ascorbate might possibly be binding the ferrous iron released from ferritin and preventing it from mediating lipid peroxidation reaction.

α-toc $O^{\cdot}$ + ASC H_2 → α-toc OH + $ASCH^{\cdot}$ eqn 4

However, antioxidants that regenerate α-tocopherol may not be the most appropriate antioxidants for preventing lysosomal LDL oxidation. They may not be efficient in protecting LDL as their antioxidant capacity might be slowed down or be lost completely when endogenous α-tocopherol in LDL is depleted.

The antioxidant activity of ascorbate was lost over time or in the presence of midly oxidised LDL. It can be suggested that the pro-oxidant effect of ascorbate may be due to (1) reduction of ferric iron to ferrous iron, the ferrous iron may then mediate the formation of hydroperoxyl radical as discussed in chapter 3. (2) The pro-oxidant effect in the presence of midly oxidised LDL may be due to the released ferrous iron rapidly reacting with the pre-existing lipid hydroperoxides (ferric iron reacts slowly with lipid hydroperoxides equation 5 to 7).

$$\textbf{Ferritin-Fe}^{3+} \textbf{ + ASC H}_2 \rightarrow \textbf{Ferrritin- Fe}^{2+} \textbf{ + ASCH}^{\cdot} \textbf{ + H}^{+} \quad \textbf{eqn 5}$$

$$\textbf{Fe}^{2+} \textbf{ + LOOH} \rightarrow \textbf{LO}^{\cdot} \textbf{ + OH}^{-} \textbf{ + Fe}^{3+} \quad \textbf{fast eqn 6}$$

$$\textbf{Fe}^{3+} \textbf{ + LOOH} \rightarrow \textbf{LOO}^{\cdot} \textbf{ + OH}^{-} \textbf{ + Fe}^{2+} \quad \textbf{slow eqn 7}$$

Dehydroascorbate did not inhibit LDL oxidation by ferritin at lysosomal pH. A previous study has demonstrated that dehydroascorbate increased the oxidation of copper mediated LDL oxidation in fresh LDL and midly oxidly oxidisded LDL in a concentration-dependent manner (Stait and Leake, 1996). Similarly, in this present study dehydroascorbate promotes the oxidation of LDL by ferritin in a concentration-depentent manner. Varying plasma concentration of dehydroascorbate has been reported with 29 µM siting at the top of the list (Sinclair *et al*., 1991). Dehydroascorbate at 30 µM concentration significantly increased LDL oxidation by ferritin at pH 4.5 ($P<0.001$). As previously discussed by Horsley *et al*, this pro-oxidative effect of dehydroascorbate might be as a result of an indirect effect of its irreversible delactonisation product (2,3-diketo-L-gulonic acid) and reversible reduction products (ascorbic acid and erythroascorbate) (Horsley *et al*., 2007). Dehydroascorbate is capable of producing a

wide range of degradation product which may, in turn, produce free radicals that can mediate lipid peroxidation in the lipids of LDL (Takagi *et al*., 1988, Deutsch *et al.*, 1994). As expected, ascorbate effectively inhibited LDL oxidation by 5 µM $CuSO_4$ at pH 7.4 but had no antioxidant effect on oxidation of copper at lysosomal pH. The effect of ascorbate on fresh LDL oxidation by $CuSO_4$ at pH 7.4 was similar to a previous observation by Jialal *et al.* and others (Jialal *et al*., 1990, Stait and Leake, 1996). Similarly to result observed with α-tocoperol, ascorbate loses its antioxidant activity at lysosomal pH and this might be as a result of reducton of Cu^{2+} to Cu^{+} by ascorbate (equation 7 and 8). The Cu^{+} formed can rapidly react with lipid hydroperoxides (Stait and Leake, 1996).

$$\mathbf{ASC\ H_2 + Cu^{2+} \rightarrow ASCH^{\cdot} + Cu^{+} + H^{+}} \quad \textbf{eqn 8}$$

$$\mathbf{Cu^{+} + LOOH \rightarrow LO^{\cdot} + OH^{-} + Cu^{2+}} \quad \textbf{eqn 9}$$

The balance between the antioxidant and pro-oxidant effects of ascorbate (and dehydroascorbate) appears to be altered as the pH changes. These findings put together may account for the failure of vitamin E and C to decrease cardiovascular disease in large human clinical trials (Yusuf *et al*., 2000, Collins e*t al.*, 2002, Sesso *et al*., 2008).

Tempol is suggested to be the most potent nitroxide compound in scavenging reactive oxygen species and possesses superoxide dismutase activity (Li *et al*., 2006, Wilcox and Pearlman, 2008). Tempol completely decreases the later phases of LDL oxidation by ferrous and ferric iron in a concentration-dependent manner but could not inhibit the core oxidation of the cholesteryl ester rich core during the lag phase of ferrous/ferric oxidation of LDL (unpublished data). Surprisingly, tempol did not prevent the initial phase of LDL oxidation by ferritin at lysosomal pH but inhibited the later phase of oxidation partially and sometimes totally.

The amphipathic property of tempol might enhance its accumulation in the outer monolayer of LDL containing phospholipid, rather than in the core of LDL containing mainly cholesteryl

ester. It was suspected that LDL oxidation by iron at lysosomal pH begins in the core of LDL and then later proceeds in its monolayer (Ahmad and Leake, 2018). Tempol might lack the capacity to quickly scavenge highly reactive hydroperoxyl radicals effectively enough to prevent them from mediating oxidation of the hydrophobic core of LDL, but it might be able to prevent the oxidation of the phospholipids surface monolayer by scavenging alkoxyl or peroxyl radicals formed by the phospholipids in the monolayer. Recent work from our laboratory demonstrated that tempol completely inhibited LDL oxidation by copper at pH 7.4 but loses its antioxidant capacity on the initial phase of LDL oxidation in the presence of iron at pH 4.5 (Alboaklah, 2018a).

DPPD has been demonstrated to effectively scavenge the peroxyl radical (Tangirala *et al.*, 1995). In this present study, DPPD concentrations as low as 5µM and 10 µM were able to reduce the formation of conjugated dienes in LDL oxidised by ferritin to a large extent ($P<0.05$). Others have shown DPPD to effectively inhibit oxidation of LDL by copper and endothelial cells (Sparrow *et al.*, 1992). Its hydrophobic nature is of advantage, as it can possibly scavenge radicals formed within the core of LDL. The ability of DPPD to inhibit LDL oxidation mediated by ferrous iron (Ahmad and Leake, 2018) and ferritin at lysosomal pH, might explain while it protected animal models against atherosclerosis. The use of DPPD in humans is, however, prevented by its mutagenic nature (Sofuni *et al.*, 1990).

A recent study from our laboratory has shown that cysteamine effectively inhibited LDL oxidation by ferrous iron at pH 4.5 (Ahmad and Leake, 2018). This present study showed that 5 µM -1000 µM cysteamine protected LDL oxidation but not completely, as a later pro-oxidant effect was observed. All concentrations slowed down the oxidation for about 200 min. The

antioxidant effect increased with the concentration of cysteamine, the two upper concentrations were significantly different from others and each other ($P<0.05$).

Cysteamine contains a thiol group and thiols have previously been seen to act as promoters (Heinecke *et al.*, 1993) and inhibitors of LDL oxidation (Patterson *et al.*, 2003b). Pro-oxidant and antioxidant effects might depend on cysteamine concentrations in differing ways and this may explain the complex kinetics. From low to moderate concentrations of cysteamine, the pro-oxidant effect observed at later times increased, but with higher concentrations (3 mM and over) an antioxidant effect was seen at all times. The complexity of the effect of cysteamine was concentration and time dependent. The antioxidant effect observed in the presence of cysteamine might be due to the lower availability of iron, as cysteamine might possibly be able to bind to and inactivate released iron. It might also be scavenging the peroxyl radical ($HO_2^{\cdot}$) and superoxide radical ($O_2^{\cdot -}$) formed as oxidation products (equation 10 and 11 below).

<u>Proposed mechanisms for antioxidants effects of cysteamine</u>:

$$HO_2^{\cdot} + NH_2CH_2CH_2SH \rightarrow H_2O_2 + NH_2CH_2CH_2S^{\cdot} \quad \textbf{eqn 10}$$

$$O_2^{\cdot -} + NH_2CH_2CH_2SH + H^+ \rightarrow H_2O_2 + NH_2CH_2CH_2S^{\cdot} \quad \textbf{eqn 11}$$

The pro-oxidant effect observed might be due to cysteamine removing iron from ferritin, as it might reduce ferric ion (Fe^{3+}) in ferritin to ferrous ion (Fe^{2+}) which might diffuse out of the pores in the surface of the ferritin particle. The Fe^{2+} might mediate the generation of lipid peroxides, peroxyl radicals ($HO_2^{\cdot}$) and superoxide radicals ($O_2^{\cdot -}$) (as described in chapter 3, section 3.4).

<u>Proposed mechanisms for prooxidants effects of cysteamine</u>:

$NH_2CH_2CH_2SH + Fe^{3+} \rightarrow NH_2CH_2CH_2S^{\cdot} + H^{+} + Fe^{2+}$ eqn 12

It was thought that the concentration-dependent inhibition observed might mean further increases in cysteamine concentration will exert a near complete inhibitory effect. Plasma concentration of about 40µM are found in cystinosis patients (Dohil *et al.*, 2006). Cysteamine is expected to accumulate up to two or three order of magnitude in lysosomes due to proton trapping and so lysosomal concentration might be at least several millimolar. Higher concentration of cysteamine (1 mM, 3 mM and 10 mM) inhibited the oxidation of LDL to a large extent ($P<0.05$). Both low and high concentrations of cysteamine were confirmed to release iron from ferritin at lysosomal pH. The antioxidant capacity of cysteamine is more pronounced as more cysteamine is available to scavenge free radicals or possibly bind and inactivate released iron. The concentrations at which cysteamine inhibited LDL oxidation by ferritin is consistent with the concentrations already used in the treatment of the rare lysosomal storage disorder cystinosis. It is interesting to note that there was an inverse correlation between the years of cysteamine intake by patients with cystinosis and arterial calcification (Ueda *et al*., 2006). The potency of cysteamine to reduce atherosclerosis might be supported by the ability of cysteamine to reduce ceroid formation within lysosomes (Ahmad and Leake, 2018, Wen *et al*., submitted) and significantly reduced atherosclerotic lesions in mice (Wen *et al.*, submitted).

Work presented in this chapter suggests that the failure of some antioxidants to show protection against cardiovascular disease in human trials does not disprove the LDL oxidation hypothesis of atherosclerosis. The study, however, implies that the lysosomotropic antioxidant, cysteamine might be a more appropriate antioxidant in inhibiting LDL and reducing events of atherosclerosis. The role of ferritin and antioxidants in lysosomal oxidation has been explored

so far at lysosomal pH, there is however need to further explore the impact of LDL oxidised by ferritin within lysosomes on macrophages and atherosclerosis. This is addressed in the next chapter of this thesis.

Chapter 6: The effects of lysosomal oxidation of LDL by ferritin in macrophages

6.0 Background and rational

Macrophages play a key role in the onset and progression of atherosclerosis and they regulate cellular immune response and metabolism of lipids (Ross, 1999, Lusis, 2000). As one of the major types of cells found in atherosclerotic plaque, they have great influence on inflammation, accumulation of lipids, formation of the necrotic core and degradation of extracellular matrix (Legein *et al.,* 2013). The accumulation of lipid-laden macrophages in the walls of the artery is considered an early indication of an atherosclerotic lesion. Lipid-loaded macrophages (foam cells) triggers the mediators of inflammation such as cytokines and are able to recruit other cell types to contribute to the advancement of the lesions (Shibata and Glass, 2009).

The uptake of VLDL, LDL and oxidised LDL by macrophages occur through phagocytosis, micropinocytosis, receptor-mediated endocytosis and pathways mediated by scavenger receptors. Lipids ingested by macrophages are digested in lysosomes (Tabas and Bornfeldt, 2016, Remmerie and Scott, 2018). The prevailing concept of oxidative modification is that LDL is modified in the arterial wall and then recognised by pattern recognition receptors on immune cells. The type of pattern recognition receptors on macrophages are scavenger receptors, they were suggested to recognise and take up modified LDL. Different family members of this receptor have been identified, such as scavenger receptor A (SRA), scavenger receptor class B member 1(SRB1), CD36, lectin-type oxidised LDL receptor 1 (LOX1) and others which can all bind oxidised LDL and promote foam cell formation (Moore and Freeman, 2006). However, SRA and CD36 have been identified as the major receptors responsible for mediating uptake and degradation of acetylated and oxidised LDL in macrophages (Kunjathoor *et al*., 2002). The foam cell formation in apoE knockout mice deficient in CD36 and SRA was reduced, but not completely prevented which suggests there are other mechanisms for the formation of foam cells *in vivo* (Manning-Tobin *et al*., 2009), suggesting there might be an

additional mechanism for macrophage transformation to foam cells. The *in vivo* relevance of this oxidation paradigm is challenged by the failure of antioxidants to be effective against CVD in large clinical trials (Steinberg and Witztum, 2002). Many types of LDL oxidation strongly inhibited by serum or interstitial fluid (Leake and Rankin, 1990, Dabbagh and Frei, 1995, Patterson *et al*., 2003a). These findings have led to the consideration of alternate hypotheses for foam cell formation.

Modification of LDL by various proteases and lipases present in the arterial intima can promote its uptake by macrophages. Phospholipase A_2 (Oorni and Kovanen, 2009) and sphingomyelinases (Xu and Tabas, 1991) have been shown to modify LDL to enhance its uptake. These events may contribute to foam cell formation, initiation and progression of atherosclerosis. Our laboratory proposed an alternate mechanism for oxidation of LDL, demonstrated that LDL is oxidised within lysosomes of macrophages (Wen and Leake, 2007) and suggested that LDL is aggregated by sphingomyelinase (SMase) or other means and rapidly taken up by macrophages and oxidised within lysosomes (Wen *et al.*, 2015). Sphingomyelinase has been shown to be present extracellularly in atherosclerotic lesion (Marathe *et al*., 1999). Lysosomal oxidation of SMase-LDL mediated by iron has been shown to increase secretion of pro-inflammatory cytokines (Ahmad and Leake, 2019). The pro-inflammatory cytokines produced by foam cells lead to vascular inflammation and lipoprotein accumulation (Libby, 2002). The link between inflammation and plaque formation has been supported by numerous studies (Libby, 2002, Libby *et al*., 2009, Libby, 2012). Inflammation promotes atherosclerosis and eventually causes complications linked to thrombosis, which eventually leads to the clinical events, thrombotic stroke, myocardial infarction (MI) and death arising from CVD (Libby, 2002, Libby *et al*., 2009).

Hepcidin, the systemic iron regulatory hormone, has been demonstrated to be elevated during inflammation (Nemeth *et al*., 2004a, Wrighting and Andrews, 2006). Following up on the iron hypothesis of atherosclerosis discussed in chapter one of this thesis, hepcidin has been proposed to be linked to increased risk of atherosclerotic cardiovascular disease by reducing the exit of iron from macrophages and increasing their transformation to foam cells (Sullivan, 2007). Hepcidin levels and hepcidin/ferritin ratio has been shown to be associated with increased atherosclerosis in post-menopausal women (Galesloot *et al*., 2014). The intracellular amount of ferritin is controlled by the intracellular labile iron pool and chelatable iron-induced translation of heavy and light chain mRNAs (Hentze *et al*., 1987, Casey *et al.*, 1988, Gray *et al*., 1993). The pathways of ferritin regulation have been linked with inflammation, as the heavy chain is transcriptionally induced by cytokines. Interleukin (IL-1), IL-6 and TNF-α upregulate the post-transcriptional synthesis of ferritin (Muntane-Relat *et al*., 1995, Tran *et al*., 1997). Thus, during the inflammatory process and when iron is trapped in macrophages by hepcidin, ferritin levels might also be increased. It will be interesting to understand how this is linked to lysosomal LDL oxidation by ferritin.

Lipid peroxidation has been proposed to be a major contributor to the pathogenesis of atherosclerosis. Lipid peroxidation, a free radical-mediated chain reaction propagation of oxidation of unsaturated fatty acids, might promote atherosclerosis by inducing transformation of macrophages into foam cells (Esterbauer *et al.*, 1993, Chisolm and Steinberg, 2000, Young and McEneny, 2001). Ferritin has been demonstrated in a previous chapter to oxidise LDL at lysosomal pH, hence it is relevant to explore the effect of ferritin on lipid peroxidation within lysosomes of macrophages. Reactive oxygen species (ROS) have been shown to contribute to oxidation of LDL. ROS mediation of lipid oxidation is highly dependent on superoxide anion formation which can form other reactive species such as hydroperoxyl radicals, lipid peroxides

and peroxynitrites that can further contribute to the pathogenesis of atherosclerosis (Madamanchi *et al*., 2005, Leopold and Loscalzo, 2008). Oxidative stress has been proposed to be important in the onset of atherosclerosis (Witztum and Berliner, 1998) and ROS derived from macrophages are capable of regulating matrix metalloproteinases and might contribute to plaque instability (Rajagopalan *et al.*, 1996).

Macrophages undergo strong metabolic changes after activation which can, in turn, control the inflammatory responses. In atherosclerosis alterations in macrophage metabolism is an important factor that can dictate the function of macrophages and the progression of the disease. The mechanism of metabolism in macrophages can be modified to meet immediate cellular demands, such as proliferation, production of cytokines and phagocytosis (Liu *et al*., 2016, Lachmandas *et al*., 2016). The transformation of macrophages to classically activated phenotypes or the alternatively activated phenotypes requires a change in metabolic processes from anabolic to the catabolic mechanism, respectively. The inflammatory phenotype M1 macrophages, which are classically activated, utilises anabolic metabolism to make up for the higher rate of glycolysis and the increased need for macromolecular building blocks. The ATP quickly generated from glycolysis serves as a source of main intermediates utilised in pentose phosphate pathway (PPP), which is essential for nucleotide and NADPH synthesis and other events. The change in metabolic pathways continues to promote ongoing and future inflammatory responses (Koelwyn *et al.*, 2018). Understanding the immunometabolic pathways in atherosclerosis and how they can be regulated might represent a novel therapeutic target in treating atherosclerosis. It was of interest to understand the effect oxidation of LDL by ferritin within macrophages can have on their energy phenotype.

Macrophages that accumulate in atherosclerotic plaques become less able to migrate, which further contributes to inflammation and progression to a more advanced plaque. In the complex plaque, macrophages continue the production of proteases that can degrade the matrix and the macrophages eventually die by necrosis or apoptosis (Randolph, 2008, Moore *et al*., 2013). Apoptotic cell death occurs in all stages of atherosclerotic lesion development, however, apoptosis might have a negative impact on atherosclerotic plaque stability (Kockx, 1998, Kockx *et al*., 1998). LDL oxidised by copper at pH 7.4 has been shown to induce apoptosis in HMDM (Wintergerst *et al*., 2000). Oxidised LDL has also been shown to induce apoptosis in cultured cardiomyocytes from neonatal rats (Wang *et al*., 2016). Apoptosis mainly occurs in the region of the lesion where the large lipid core is found and the majority of cells that die by apoptosis are macrophages (Hegyi *et al*., 2001). The cytotoxicity of oxidised LDL towards HMDM has been attributed to its ability to induce apoptosis in these cells (Hardwick *et al*., 1996). Necrotic cells are characterised by loss of integrity of the cell membrane that allows an influx of water, Ca^{2+} and Na^{2+}, which leads to swelling of cytoplasm followed by the release of the cellular content to extracellular space and result in inflammation (Roos *et al*., 2004, Tabas, 2005).

Studies by Jerome and others have previously shown that different forms of modified lipids can disrupt the function of lysosomes and result in intralysosomal accumulation of lipids (Griffin *et al*., 2005, Cox *et al*., 2007, Jerome, 2010). Following up on this Emanuel *et al*. later demonstrated that exposure of macrophages to cholesterol crystals and oxLDL led to lysosomal dysfunction which is characterised by increased pH, decreased function of lysosomal enzymes and loss of lysosomal membrane integrity (Emanuel *et al*., 2014). The oxidised LDL used previously to demonstrate pro-atherogenic effects of oxidised LDL has mostly been produced by incubating with copper ions at pH 7.4. Work presented in this thesis and previously from

our laboratory have demonstrated that iron is important in mediating LDL oxidation within macrophage lysosomes (Wen and Leake, 2007, Satchell and Leake, 2012, Ahmad and Leake, 2018), lysosomal LDL oxidation promotes formation of ceroid in lysosomes of macrophages (Wen and Leake, 2007, Ahmad and Leake, 2018), promote cellular senescence and disrupts lysosomal function by altering their pH (Ahmad and Leake, 2019). If lysosomal dysfunction occurs in macrophages, the lysosomes might release their content into the cytosol, which might induce apoptosis and once cells die it contents might be released extracellularly to affect neighbouring macrophages. Further elucidation of the role of ferritin in oxidising LDL within lysosomes of macrophage might provide a novel insight into the role of ferritin-oxidised LDL in atherosclerosis. It was hypothesised that lysosomal oxidation of LDL in macrophages can modulate macrophage function and promote atherosclerosis. Hence, possible effects of lysosomal oxidation of LDL by ferritin on macrophage function as regards atherosclerosis was explored.

6.1 Objectives

i) To examine the role of hepcidin in influencing iron/ferritin levels in macrophages might play a role in lysosomal LDL oxidation.

ii) To Test the effects of ferritin on lysosomal lipid peroxidation in macrophages.

iii) To evaluate the effect of lysosomal ferritin-oxidised LDL on intracellular lipids levels and ceroid formation macrophages.

iv) To test if ferritin-oxidised LDL can mediate oxidative stress by upregulation of intracellular levels of reactive oxygen species

v) To examine the effects of ferritin-oxidised LDL on macrophages cellular respiration/ metabolism.

vi) To test the effects of ferritin-oxidised LDL on apoptotic cell death of macrophages.

6.2 Methods

The methods used for culturing THP-1 cells was described in section 2.7 and the isolation of HMDM was described in section 2.8. The effect of hepcidin on intracellular iron levels was tested as described in section 2.9. Lysosomal lipid peroxidation was measured with the novel lysosomal targettable probe (Foam-LPO) as described in section 2.10. The intracellular lipids and ceroid were measured using Oil Red O staining, as described in section 2.11. Intracellular reactive oxygen species were measured with the fluorescent probe dihydroethidium (DHE), as described in section 2.12. The effect of LDL oxidised by ferritin on respiration/metabolism was determined with Seahorse analyser, as described in section 2.13. The induction of apoptosis in macrophages was measured using propidium iodide and annexin V, as described in section 2.14.

6.3 Results

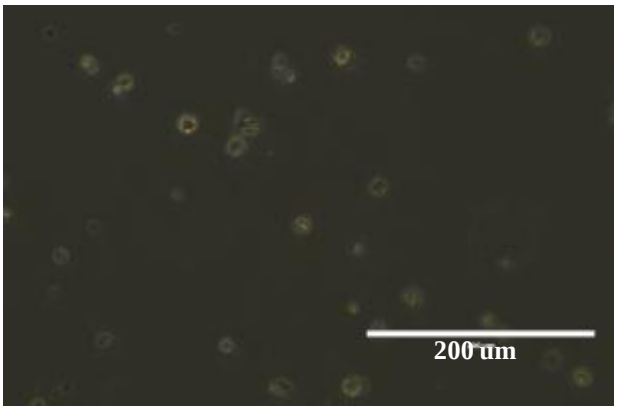
6.3.1 Effect of hepcidin on intracellular iron in macrophages

Hepcidin has been associated with decreasing body iron stores and promoting iron sequestration in macrophages during inflammation and infection (Ganz and Nemeth, 2009). The link between inflammation and sequestration in macrophages of iron on oxidation of LDL in macrophages was therefore explored. THP-1 macrophage-like cells (1.2 x 105) derived from THP-1 monocytes (Fig 6.1) were treated for 24 h with or without hepcidin (1000 nM) with RPMI-1640 (which does not contain added iron), DMEM (which contains 2.5 µM ferric iron) and F10 (which contains 3 µM added ferrous iron) supplemented with 10% (v/v) FCS. The media was replaced after 24 h with media supplemented with 10% (v/v) LPDS containing hepcidin (100 nM) or media only. This was added every 2 days. The protein content per well and iron concentration was measured after seven days.

The protein content of cells treated with hepcidin was not significantly different from control cells for all media treatments (Fig. 6.2). Surprisingly, the iron content of THP-1 macrophages was not increased by hepcidin treatment (Fig. 6.3). Work by Agoro and Mura (Agoro and Mura, 2016) suggested that the link between inflammation and hepcidin is dependent on macrophage polarization. Hence polarisation of THP-1 cells might make a difference in the experiments designed to increase macrophage iron levels by hepcidin. It would be interesting to address this in future work.

A

THP-1 monocytes



B

THP1 macrophages

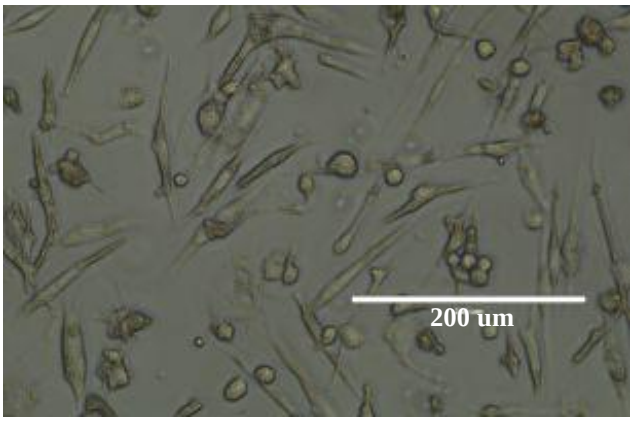


Figure 6.1: Untreated THP1 monocytes and macrophages
Thp1 monocytic cell line (A) and THP1 converted to macrophages after treatment with 25ng/ml PMA for 72 h (B). The images were captured with an Evos XL cell imaging system using a 20x objective.

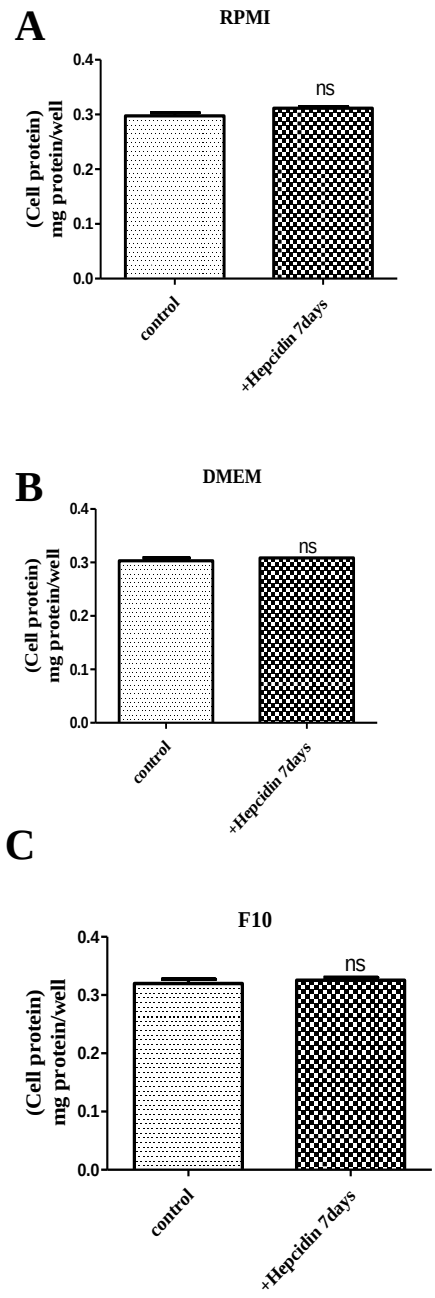


Figure 6.2: Intracellular protein content of THP1 macrophages treated with hepcidin

THP-1 cells (1.2 x 10^5) were treated with or without hepcidin (1000 nM) in RPMI-1640 (A), DMEM (B) or F10 (C) with 10% (v/v) FCS. The media was replaced after 24 h with media containing 10% (v/v) LPDS with or without hepcidin (100 nM), added every 2 days. The protein content of cells per well was measured with a Bio-Rad DC™ protein assay kit. The protein content of cells treated with hepcidin was not different compared to control cells using paired Student's t test (n=3). ns indicates not significant.

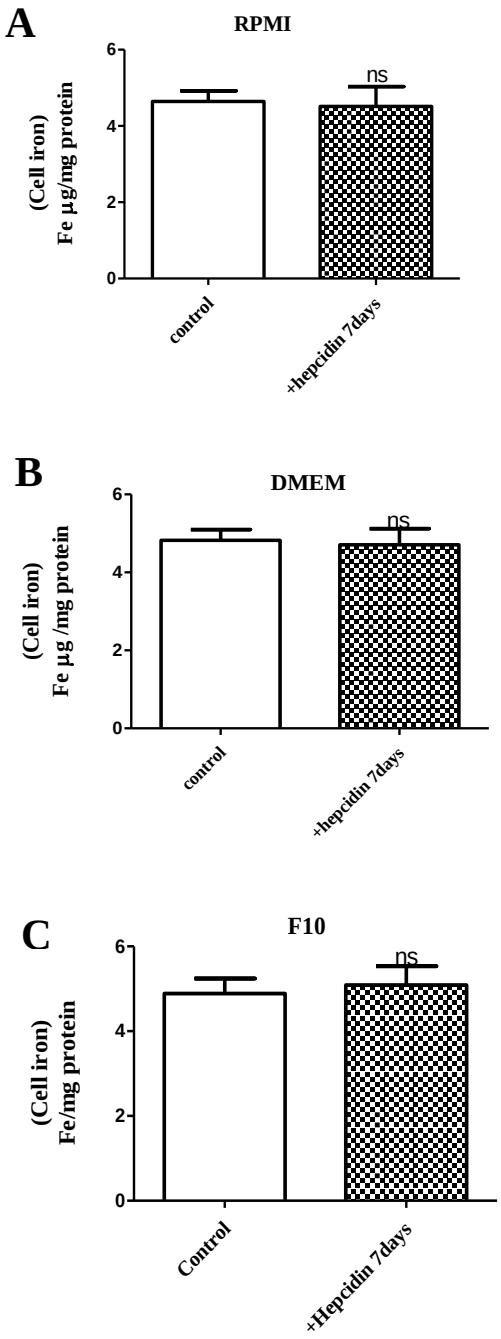


Figure 6.3: Intracellular iron content of THP-1 macrophages treated with hepcidin

THP-1 cells (1.2 x 10^5) were treated with or without hepcidin (1000 nM) in RPMI-1640 (A), DMEM (B) and F10 (C) with 10% (v/v) FCS. The media was replaced after 24 h with media containing 10% (v/v) LPDS with or without hepcidin (100 nM), added every 2 days. The iron content of cells was measured with ICP-MS. There was no significant different between iron levels in cells treated with hepcidin compared to control cells, as compared with paired Student's t test (n=3). ns indicates not significant.

6.3.2 Effects of ferritin on lipid peroxidation within lysosomes of macrophages

Lipid peroxidation mediated by free radicals has been linked to in vivo oxidative stress and associated with the lipid modification hypothesis of atherosclerosis (Niki *et al.*, 2005). In a previous chapter, it was demonstrated that ferritin increased the formation of conjugated dienes and total lipid hydroperoxides, both of which are products of lipid peroxidation in LDL, at lysosomal pH. It was of interest to test the effect of ferritin on lipid peroxidation within the lysosomes of cultured macrophages in order to determine if ferritin contributes to increasing lysosomal LDL oxidation.

THP-1 macrophages (5×10^5 cells/well) treated RPMI–1640 with 10% human serum (2ml per well) alone or with ferritin (100 µg protein/ml in terms of total protein and this is equivalent to 0.2 µM) for 24 h. The cells were then treated with or without LDL (200 µg protein/ml) for 24 h to give the following condition (no LDL, native LDL only, LDL with ferritin and ferritin only). After the 48 h treatment with ferritin and LDL, the intralysosomal lipid peroxidation was measured by two-way flow cytometry (using the green (FL1) and the red (FL2 channel), following treatment with Foam-LPO (2 µM) for 15 min in the dark. The lysosomal targettable probe is flexibly linked to a diene-fluorophore, the Foam-LPO is intercalated in the lipid accumulation and can detect the local lipid peroxidation within lysosomes. Foam-LPO detects the peroxidation by spectral shifting. Hence, lysosomal lipid peroxidation was quantified by the ratio of the intensity of the fluorescent green channel to that of the red channel (FL1/FL2). The analysis was carried using flow Jo software version 10.

The results showed that ferritin with or without native LDL significantly increased lipid peroxidation ($P < 0.001$) (Figs. 6.4 & 6.5). A significant difference was seen with ferritin alone as well as ferritin plus LDL. Ferritin plus LDL was significantly different ($P < 0.001$) from

native LDL only and control ($p < 0.05$). While treatment with LDL only was not significantly different compared to control.

LDL aggregated with sphingomyelinase has been demonstrated to be present in atherosclerotic lesions (Marathe *et al*., 1999) and rapidly taken up by macrophages and oxidised in lysosomes (Wen *et al*., 2015, Ahmad and Leake, 2019). The mechanism of SMase aggregation of LDL was described in detail in section 1.5.5 and measured in fig. 2.1. THP1- cells and HMDM cells were treated with or without ferritin (0.2 µM) for 24 h. We then incubated with or without SMase-LDL (200 µg protein/ml), respectively. We analysed for lipid peroxidation using the lysosomal targeted probe. The results showed that lysosomal lipid peroxidation increased in the presence of ferritin in THP-1 macrophages ($P < 0.001$) and HMDM ($P<0.01$) (Fig. 6.6). The results show that ferritin with or without SMase-LDL can mediate lysosomal lipid peroxidation in macrophages.

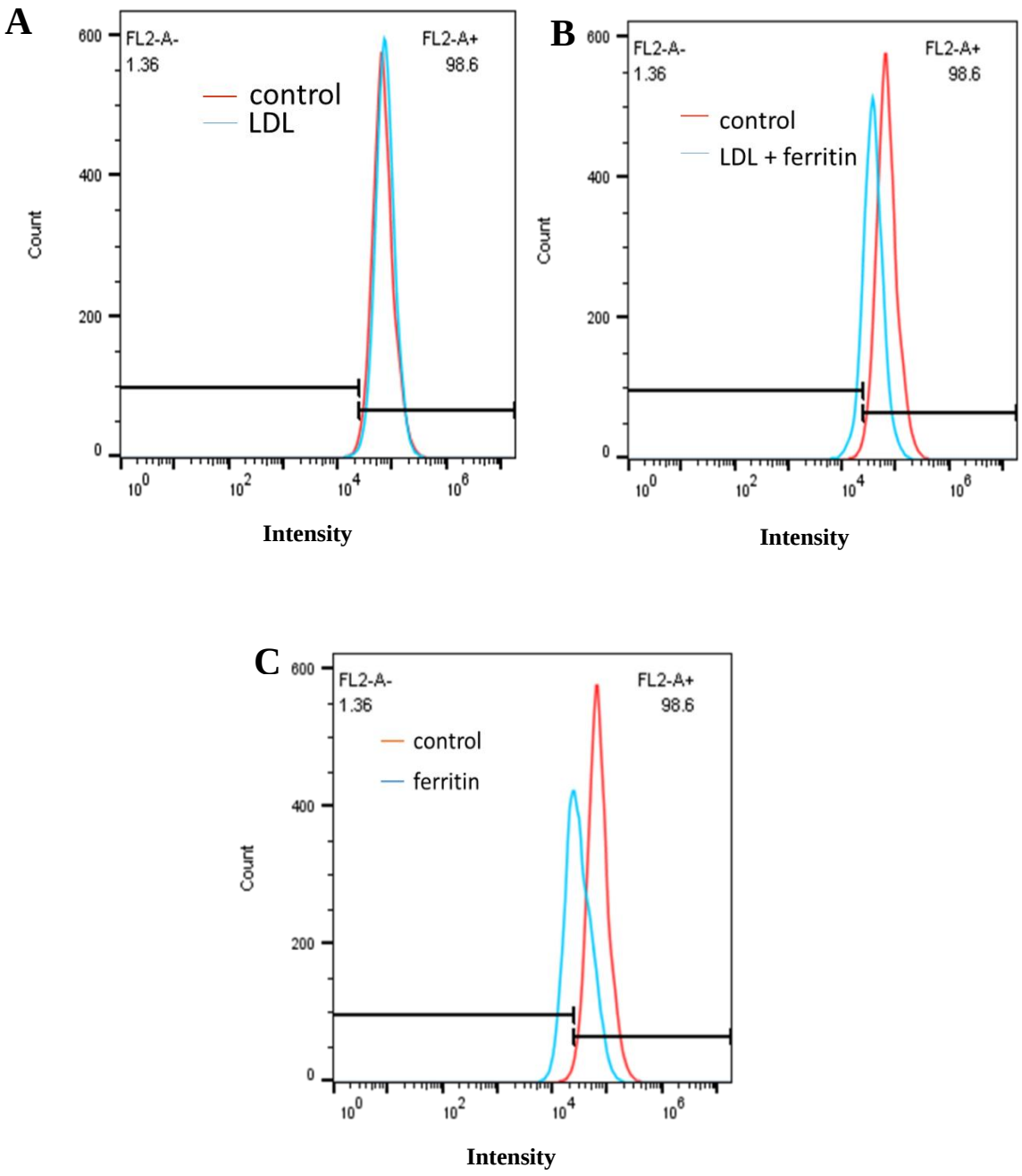


Figure 6.4: Two-way flow cytometry analysis of lipid peroxidation in THP-1 macrophages

THP1-cells were treated with or without ferritin (0.2 µM) for 24 h and then incubated in the presence or absence of native LDL (200 µg protein/ml) for 24 h. THP1-cells were harvested and incubated with the lysosomal lipid peroxidation probe, Foam-LPO (2 µM) for 15 min and assayed by flow cytometry. The ratio of FL1/FL2 corresponds to the level of lipid peroxidation as detected by the Foam-LPO (A) Flow cytometry results for untreated macrophages (control cells) and macrophages treated with native LDL. (B) Flow cytometry results for control cells and macrophages pre-treated with ferritin and then treated with native LDL. (C) Flow cytometry results for control cells and macrophages pre-treated with ferritin. The image shown is a representative of three independent experiments.

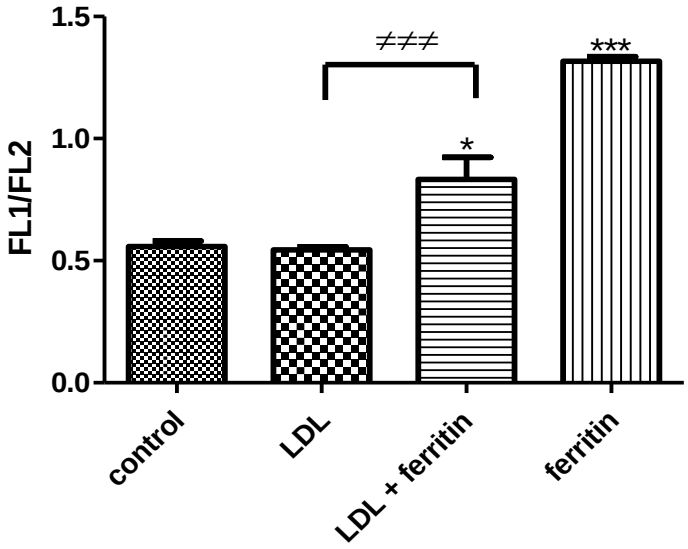


Figure 6.5: Measurement of lipid peroxiation in THP-1 macrophages in the presence of native LDL and ferritin.

THP1-cells were treated with or without ferritin for 24 h and then treated with or without native LDL (200 µg protein/ml) for 24 h. THP1-cells were then incubated with the lysosomal lipid perorixidation probe, Foam-LPO (2 µM) for 15 min. The lysosomal lipid peroxidation was determined by two-way flow cytometry and quantified by the ratio of intensity of fluorescence of the green channel compared to that of the red channel (FL1/FL2) using Flow Jo software. The ratio of intensity was compared for the treated cells and control cells using one-way ANOVA followed by a Tukey's post hoc test (n=3). * indicates $P < 0.05$ and *** indicates $P<0.001$ compared to control cells. ≠≠≠ indicates $P < 0.001$ for the indicated comparison.

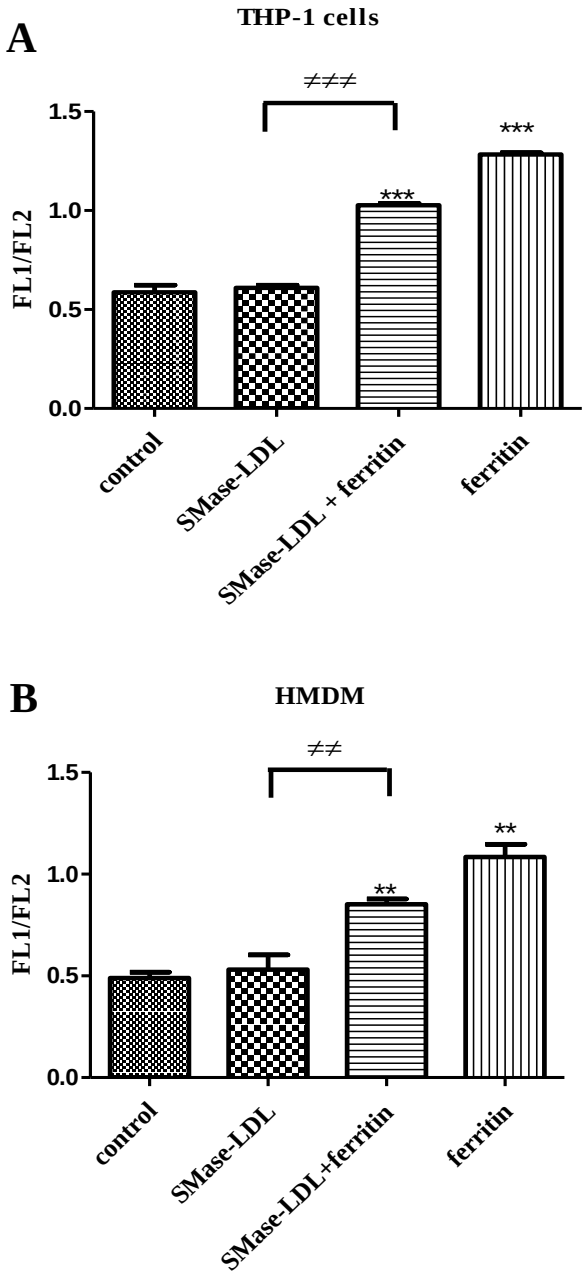


Figure 6.6: Measurement of lipid peroxiation in THP-1 macrophages and HMDM in the presence of SMase-LDL and ferritin.

THP1-cells (A) or HMDM cells (B) were treated with or without ferritin for 24 h and then treated with or without SMase-LDL (200 µg protein/ml) 24 h. THP1-cells were then incubated with the lysosomal lipid perorixidation probe, Foam-LPO (2 µM) for 15 min. The lysosomal lipid peroxidation was determined with a two-way flow cytometry and quantified by the ratio of intensity of fluorescence of green channel to that of red channel (FL1/FL2) using Flow Jo software. The ratio of intensity was compared for the treated cells and control cells using one-way ANOVA followed by a Tukey's post hoc test (n=3). * indicates $P < 0.05$ and *** indicates $P<0.001$ compared to control cells. ## indicates $P < 0.01$ and ### indicates $P < 0.001$ for the shown comparison.

6.3.3 Effects of ferritin on intracellular lipids and ceroid

Ceroid is an advanced product of oxidation of lipids which consists of complex polymerised insoluble lipids and proteins and has been demonstrated to be present both intracellularly and extracellularly in atherosclerotic lesions (Mitchinson *et al*., 1985, Haka *et al*., 2011). Both intracellular lipids and ceroid can be detected using Oil Red O, however, detection of ceroid requires removal of other lipids using organic solvents using ethanol and xylene as previously described by Ball *et al*. (Ball *et al*., 1988). Wen and Leake (2007) have previously demonstrated ceroid formation in lysosomes of HMDM and J774 cells treated with aggregated LDL (Wen and Leake, 2007). Having established that ferritin might upregulate lipid peroxidation in lysosomes of human THP1 macrophages and HMDM, it was of interest to examine the effects of ferritin on intracellular lipids and the formation of the advanced product of oxidation, ceroid.

THP-1 cells (45,000) were plated on sterilised coverslips in two 6-well tissue cultured plates. In each plate, three wells were pre-treated with 100 µg protein/ml (0.2 µM) ferritin for 24 h. Cells were washed with PBS and incubated for 24 h with RPMI only (2 ml), native LDL (200 µg protein/ml) or SMase-LDL (200 µg protein/ml). After the 24 h treatment, the cells were then washed with PBS and the medium was replaced with medium containing 10% (v/v) lipoprotein deficient serum. This was replaced every two days for each plate for seven days and the cells in one plate were stained for intracellular lipids and the other for ceroid. The intracellular lipids and ceroid were measured by calculating the mean integrated density of at least 50 cells expressed as the percentage decrease compared to the cells treated with SMase-LDL and ferritin.

The levels of intracellular lipids with native LDL or SMase-LDL were significantly increased in the presence of ferritin (Fig. 6.7 & 6.8). The intracellular lipid significantly increased with

native LDL to 80 ± 5 % (of that with SMase-LDL and ferritin) in the presence of ferritin compared 63 ± 3% with native LDL only. The intracellular lipid levels with SMase-LDL addition was also increased in the presence of ferritin compared to when cells were treated with SMase-LDL only ($P < 0.001$). Similarly, the levels of ceroid in the cells were significantly higher in the presence of ferritin (Fig. 6.9 & 6.10). SMase-LDL treatment alone showed significantly lower amount of ceroid compared to SMase-LDL with ferritin ($P < 0.001$). The ceroid levels in cells treated with SMase-LDL was significantly greater compared to native LDL ($P< 0.05$). The percentage of ceroid in cells treated with or without native LDL was also increased with the presence of ferritin ($P < 0.05$). The increase in the ceroid compared to the controls (no LDL) was much greater than the increase in the intracellular lipids compared to controls (no LDL). Overall these results show that ferritin can be a source of iron in lysosomal LDL oxidation in macrophages and might contribute to the significant increase in the advance lipid oxidation product ceroid found in atherosclerotic lesions. Experiments were normalised with cell numbers.

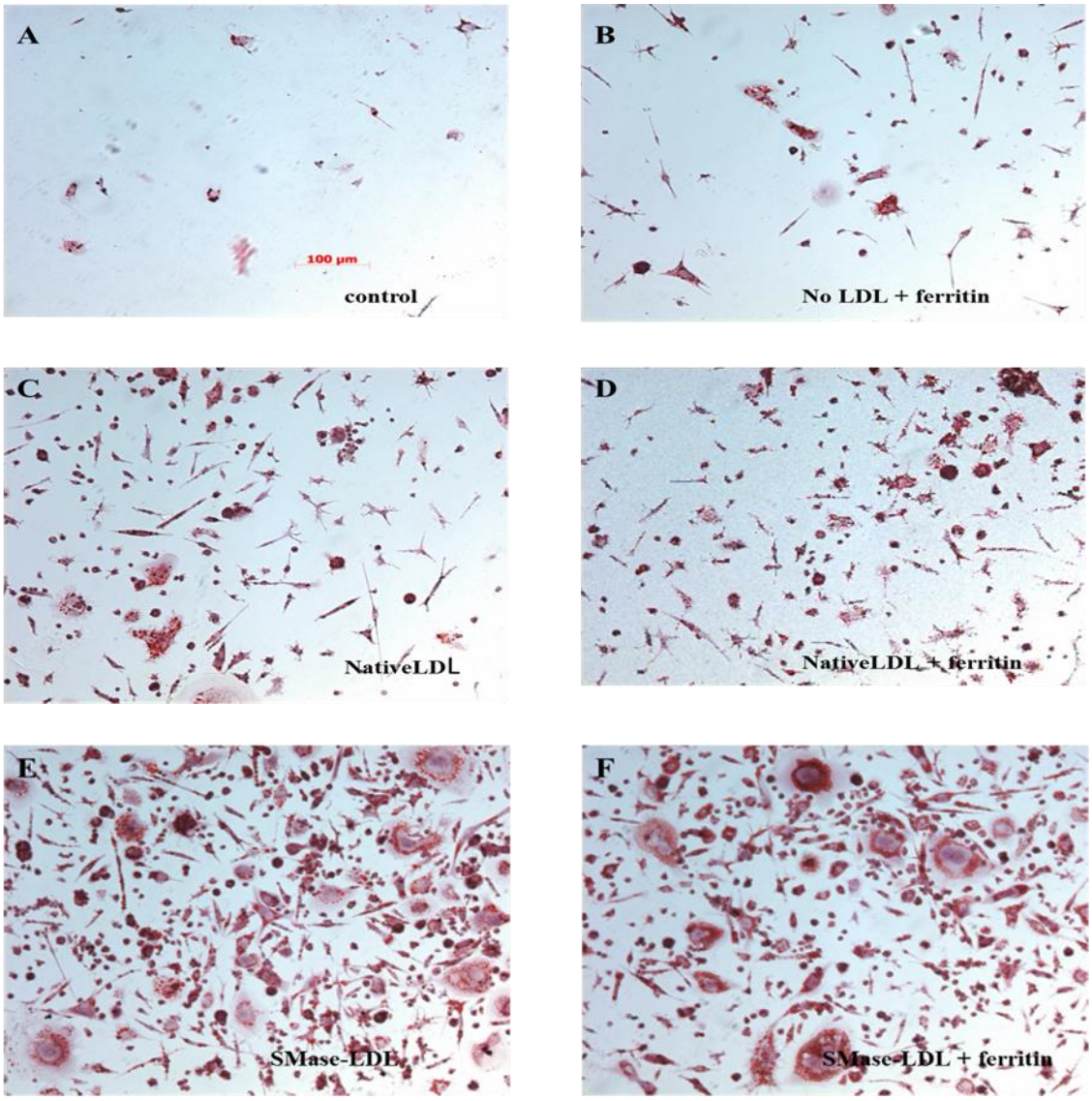


Figure 6.7: Detection of intracellular lipids in THP-1 macrophages

THP-1 cells (4.5 X 10^4) cultured on sterilised coverslips were incubated with or without 100 µg protein /ml (0.2 µM) ferritin for 24 h. Cells were washed with PBS and treated with RPMI only (2 ml) or with native LDL (200 µg protein/ml) or with or without SMase-LDL (200 µg protein/ml) and the treatment was left for another 24 h. After seven days incubation in RPMI medium containing LPDS, the treated cells were stained for intracellular lipids using Oil Red O and images was taken with light microscopy. Scale bar is 100 µm. This is a representative of three independent experiment.

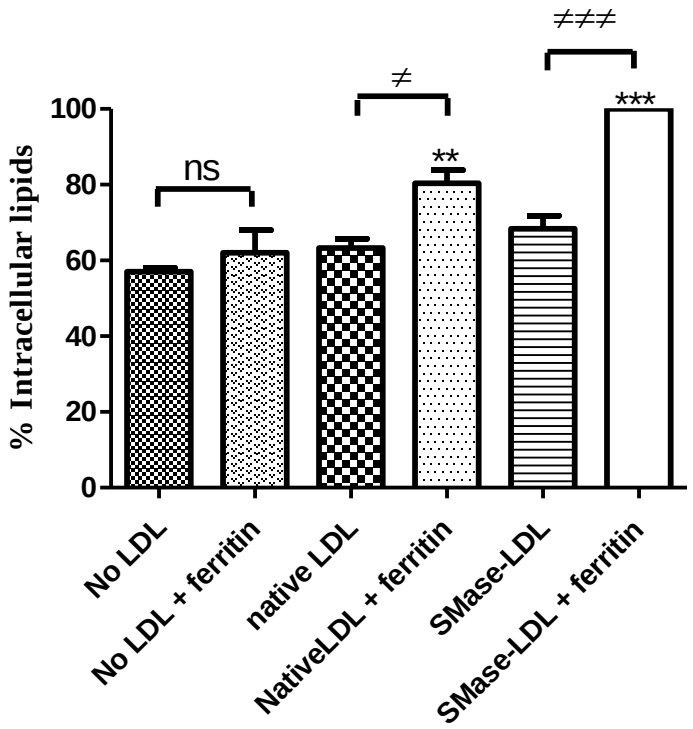


Figure 6.8: Measurement of percentage intracellular lipids in THP-1 macrophages

THP-1 cells (4.5 X 10^4) cultured on sterilised coverslips were incubated with or without 100 µg protein /ml (0.2 µM) ferritin for 24 h. Cells were washed with PBS and treated with RPMI only (2 ml) or with native LDL (200 µg protein/ml) or with SMase-LDL (200 µg protein/ml) and the treatment was left for another 24 h. After seven days incubation in RPMI medium containing LPDS, the treated cells were stained for intracellular lipids using Oil Red O. The levels of intracellular lipids was quantified with ImageJ by measuring the mean intensity of at least 50 cells for each treatment and presented as percentage intracellular lipids in relation to cells treated with ferritin and SMase-LDL. Treated cell were compared by one way ANOVA (n=3 independent experiments). ** indicates $P < 0.01$) and *** indicates $P<0.001$ compared to SMase-LDL plus ferritin. ≠ indicates $P < 0.05$ for the indicated comparison.

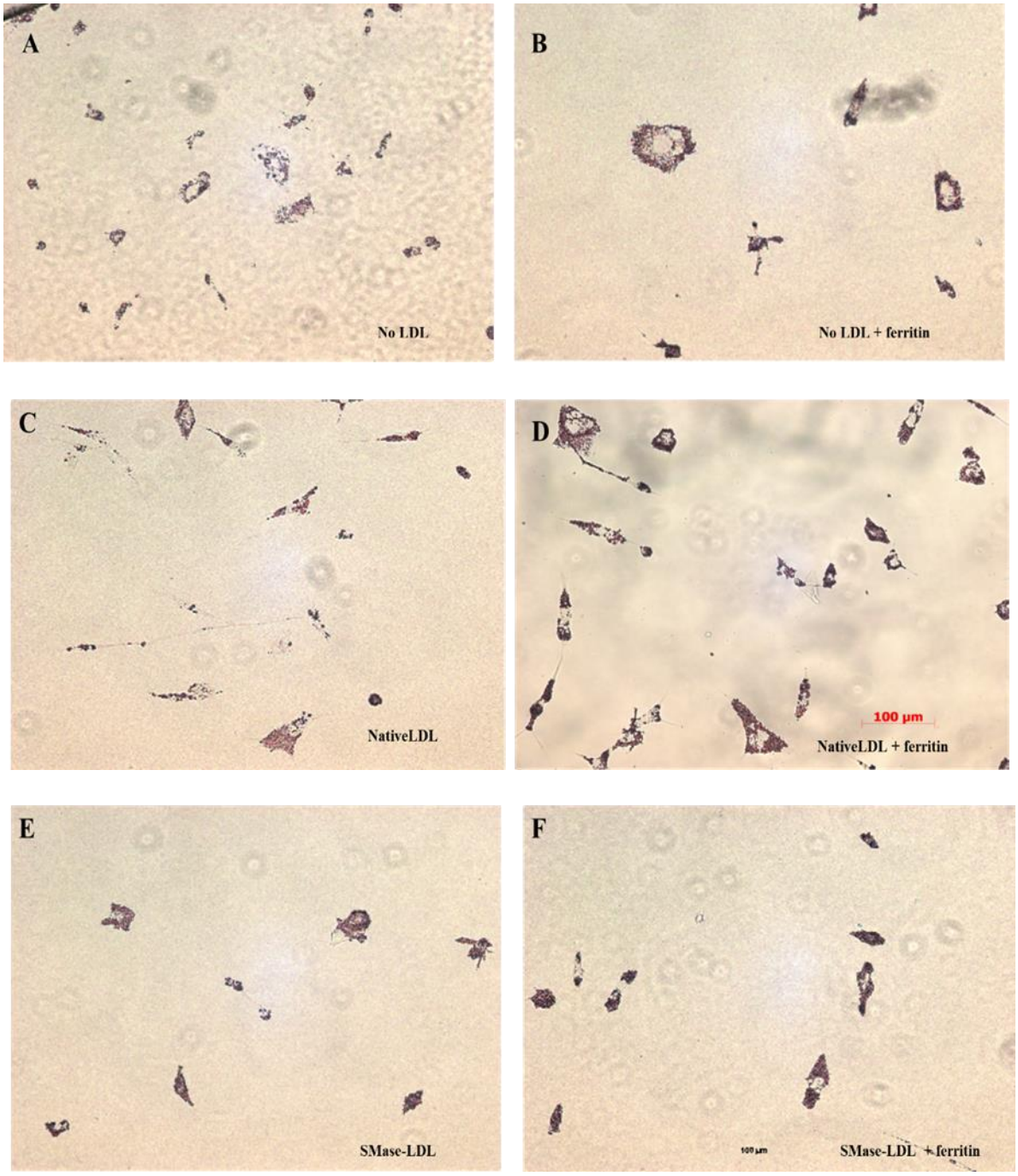


Figure 6.9: Detection of ceroid in lysosomes of THP-1 macrophages

THP-1 cells (4.5 X 10^4) cultured on sterilised coverslips were incubated with or without 100 µg protein /ml (0.2 µM) ferritin for 24 h. Cells were washed with PBS and treated with RPMI only (2 ml) or with native LDL (200 µg protein/ml) or with SMase-LDL (200 µg protein/ml) and the treatment was left for another 24 h. After seven days incubation in RPMI medium containing LPDS, soluble lipids were removed with organic solvents (ethanol and xylene) and the treated cells were stained for ceroid using Oil Red O and images were taken by light microscopy. Scale bar is 100 µm. This is representative of three independent experiments.

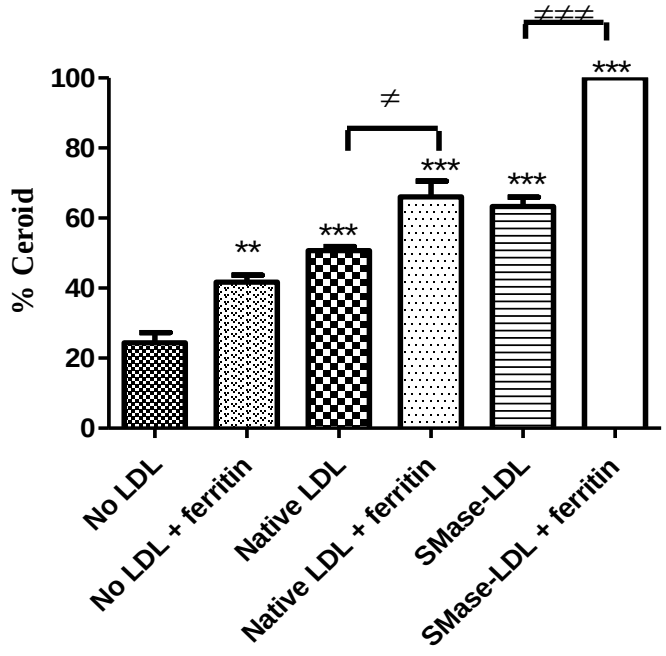


Figure 6.10: Measurement of percentage ceroid in THP-1 macrophages

THP-1 cells (4.5 X 10^4) cultured on sterilised coverslips were pre-treated with or without 100 µg protein /ml (0.2 µM) ferritin for 24 h. Cells were washed with PBS and treated with RPMI only (2 ml) or with native LDL (200 µg protein/ml) or with SMase-LDL (200 µg protein/ml) and the treatment was left for another 24 h. After seven days incubation in RPMI medium containing LPDS, soluble lipids were removed with organic solvents and the treated cells were stained for ceroid using Oil Red O. The levels of ceroid was quantified with ImageJ by measuring the mean intensity of at least 50 cells for each treatment and presented as percentage ceroid in relation to cells treated with ferritin and SMase-LDL. Treated cell were compared by one way ANOVA (n=3 independent experiments). * indicates $P < 0.05$) and *** indicates $P<0.001$. ≠ indicates $P < 0.05$ and ≠≠ indicates $P < 0.01$ for the shown comparison.

6.3.4 Effects of ferritin-oxidised LDL on intracellular ROS in macrophages

Intracellular reactive oxygen species generated in macrophages have been suggested to play a key role in atherogenesis by mediating signalling pathways that can contribute to the onset and progression of atherosclerotic lesion (Singh and Jialal, 2006). We measured the intracellular levels of reactive oxygen species in macrophages using the fluorescent probe, DHE which has been frequently used as the probe to measure the levels of intracellular superoxide (Wardman, 2007). THP-1 cells were incubated with pre-warmed RPMI – 1640 media alone (2 ml per well) or containing either NaCl/sodium acetate buffer (10% as a vehicle control for the oxidised LDL) or ferritin (0.2 µM) or LDL (50 µg protein/ml) or ferritin-oxidised LDL (50 µg protein/ml). The cells were also treated with these conditions in the presence or absence of cysteamine (40 µM). The cells were then washed twice with PBS and mounted with fluorescence mounting media containing DAPI. Images were captured using fluorescent microscopy and intensity of DHE staining was quantified using ImageJ software and compared with one-way ANOVA (n=3) followed by Tukey post hoc test.

The results showed that the intracellular level of reactive oxygen species was increased significantly in cells treated with oxidised LDL (Fig. 6.11), the cells treated with LDL only and ferritin only was not significantly different from the controls. This indicates that ferritin-oxidised LDL can promote the generation of reactive oxygen species in macrophages. The effect of the antioxidant cysteamine on intracellular reactive oxygen species was tested. Cysteamine (40 µM) significantly reduced intracellular levels of ROS in cells (Fig. 6.12). Cells treated with cysteamine had significantly less amount of reactive oxygen species compared to the controls.

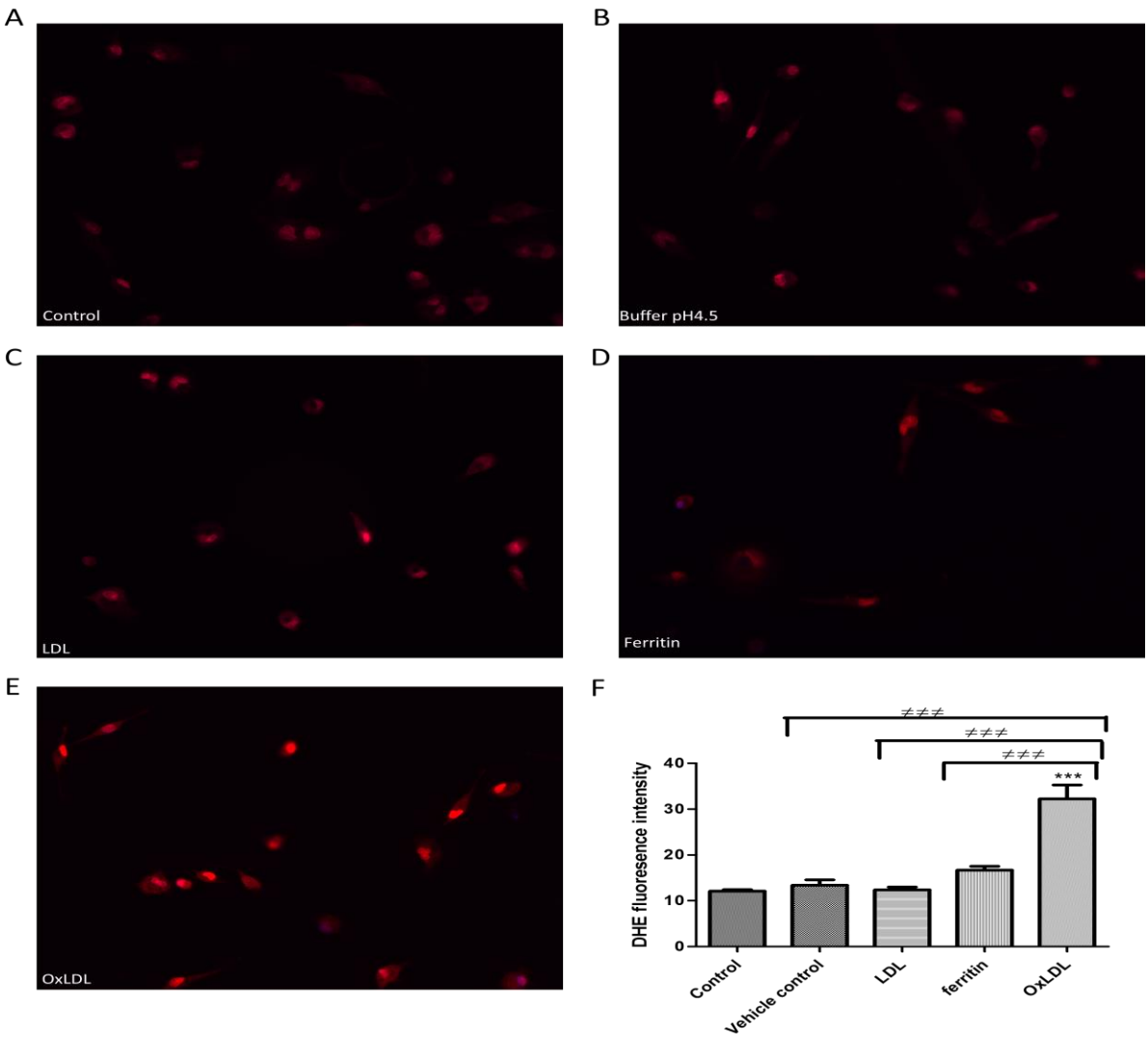


Figure 6.11: Effect of ferritin-oxidised LDL on THP-1 cells intracellular ROS

THP-1 macrophages (4.5 x10^4) were plated on cover slips in 6 well sterile tissue culture plates. The cells were incubated in RPMI medium (containing 10% (v/v) FCS) (A), with sodium acetate buffer pH4.5 (10% (v/v) as a vehicle control) (B), with native LDL (50 µg protein/ml) (C), ferritin (0.1 µM) (D) or ferritin-oxidised LDL (50 µg protein /ml) for 24 h (E). The cells were then washed with PBS and incubated with 10 µM DHE for 30 min in the dark at 37 ^oC in a non CO_2 incubator, washed with PBS and mounted using fluorescence mounting medium containing DAPI. The images were captured using an Axioimager epifluorescent microscope using a 20x objective. (F) Shows the increase in fluorescence intensity of DHE after treatments (n=3 independent experiments). *** indicates P<0.001 compared to the control cells. ### indicates P < 0.001 for the shown comparison

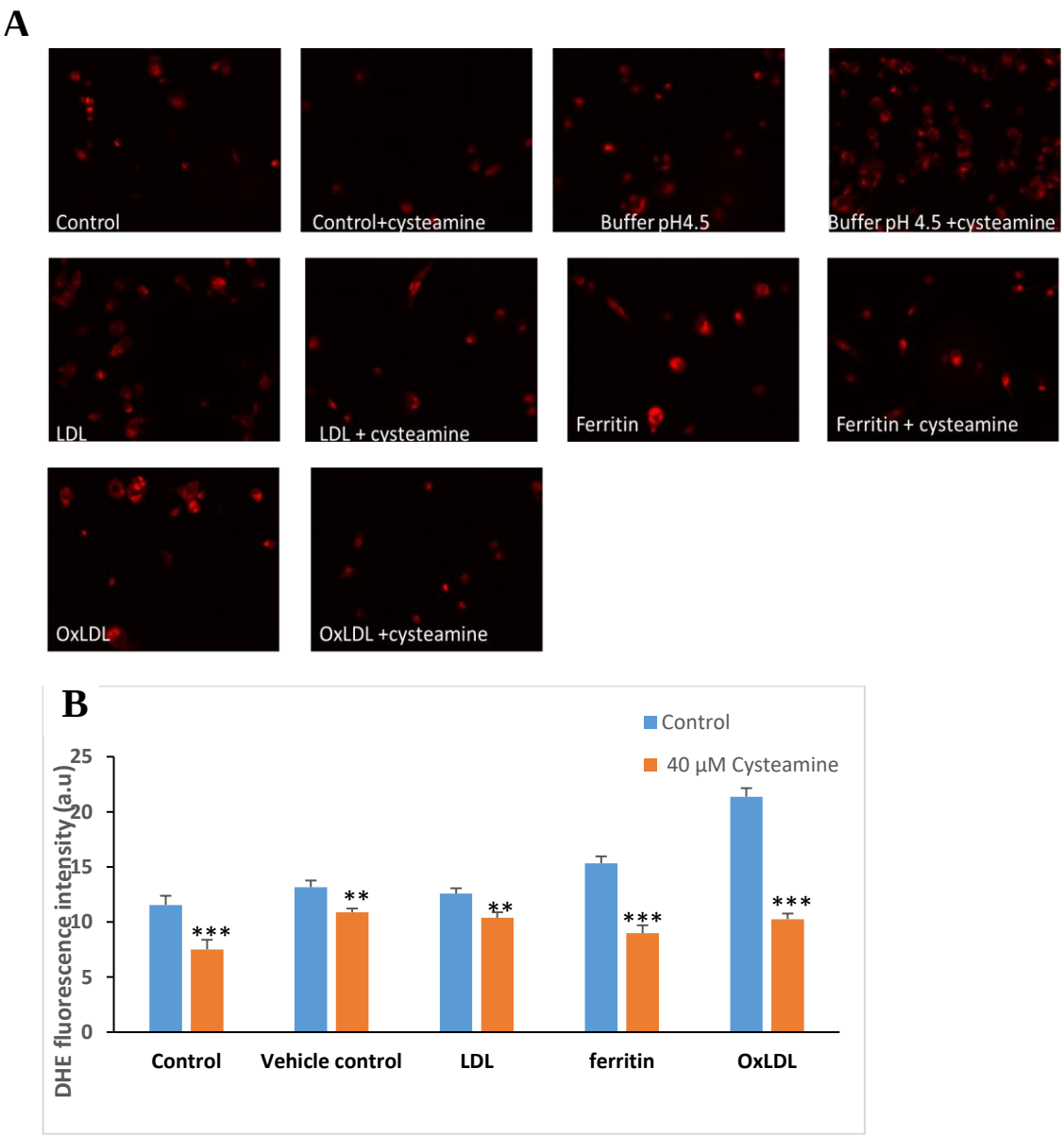


Figure 6.12: Effect of cysteamine on ROS formation induced by ferritin-oxidised LDL

THP-1 macrophages (4.5 x10^4) were plated on cover slips in 6 well sterile tissue culture plates. The cells were incubated in RPMI medium (containing 10% (v/v) FCS), with sodium acetate buffer pH 4.5 (10% (v/v) as a vehicle control), with native LDL (50 µg protein/ml), ferritin (0.1 µM) or ferritin-oxidised LDL (50 µg protein/ml) for 24 h. All conditions were incubated with or without cysteamine (40 µM). The cells were then washed with PBS and incubated with 10 µM DHE for 30 min in the dark at 37 ^oC in a non CO_2 incubator, washed with PBS and mounted using fluorescence mounting medium containing DAPI. The images were captured using an Axioimager epifluorescent microscope using a 20x objective. The fluorescence intensity of DHE was compared by one way ANOVA (n=3 independent experiments). ** indicates P < 0.01) and *** indicates P<0.001 compared to the cells without cysteamine.

6.3.5 Effect of ferritin oxidation of LDL on cellular respiration/metabolism in THP1-cells

The metabolic energy phenotype was measured using Agilent Seahorse XF technology. Glycolysis has been suggested to be important in fuelling inflammation and the progression of atherosclerosis (Groh *et al.*, 2018). The effects of LDL oxidation by ferritin on the metabolic pathways in macrophages were tested. THP-1 cells (6×10^4) in Seahorse tissue culture wells were incubated with or without ferritin (0.2 μM) for 24 h. They were then incubated for 24 h in RPMI medium alone or with native LDL (100 μg protein/ml). The metabolic and energy phenotype was then determined by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Difference between control and treated cells were determined by two-way ANOVA followed by Bonferroni's post-test.

The oxygen consumption rate in cells treated with LDL and ferritin showed a significant increase ($P < 0.001$), but no significant increase was observed with LDL only (Fig. 6.13). The treatment with LDL and ferritin caused significant increase in the OCR ($P < 0.001$) in all measurements made after the addition of the stressor mix oligomycin and FCCP compared to control cells and a decreased oxygen uptake over time. The ECAR followed a similar pattern, the extracellular acidification rate significantly increased in the presence of ferritin and LDL. The results suggest that the cells were more energetic and metabolically activated after treatment hence becoming more glycolytic (Fig. 6.14).

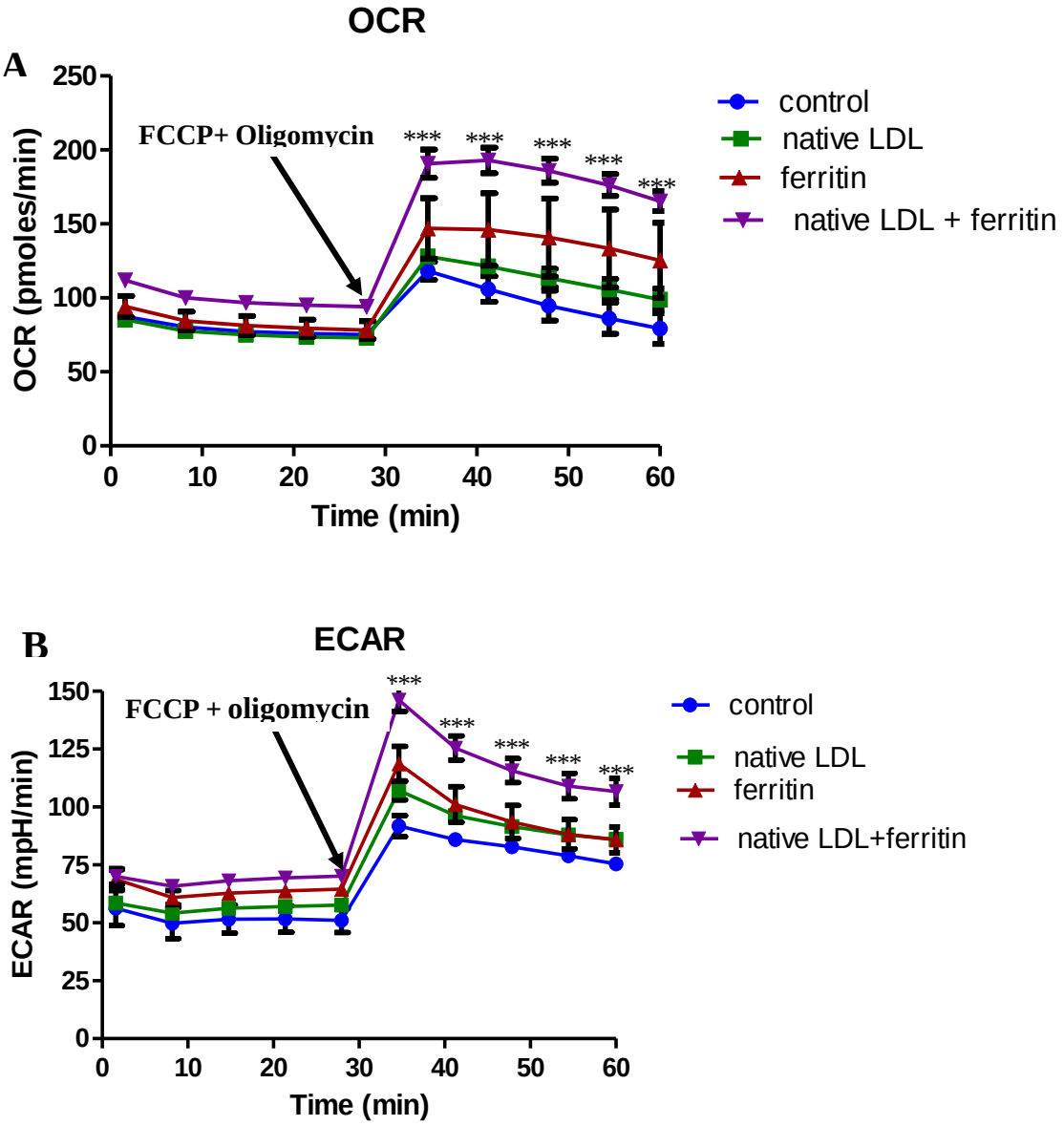


Figure 6.13: Time course of effect of ferritin and LDL on metabolism of macrophages

THP-1 macrophages (6 x10^4 per well) were cultured in sterile Seahorse tissue culture plates with ferritin (0.2 μM) for 24 h. They were then incubated in RPMI medium alone or native LDL (100 μg protein/ml) for 24 h. The cells were then washed with XF base media containing glucose, glutamine and pyruvate as fuels and analysed to determine the metabolic phenotype measuring the OCR and ECAR. The OCR and ECAR over time were compared for the treated cells and control using two-way ANOVA followed by a Bonferroni's post-test (n=4). *** indicates $P < 0.001$.

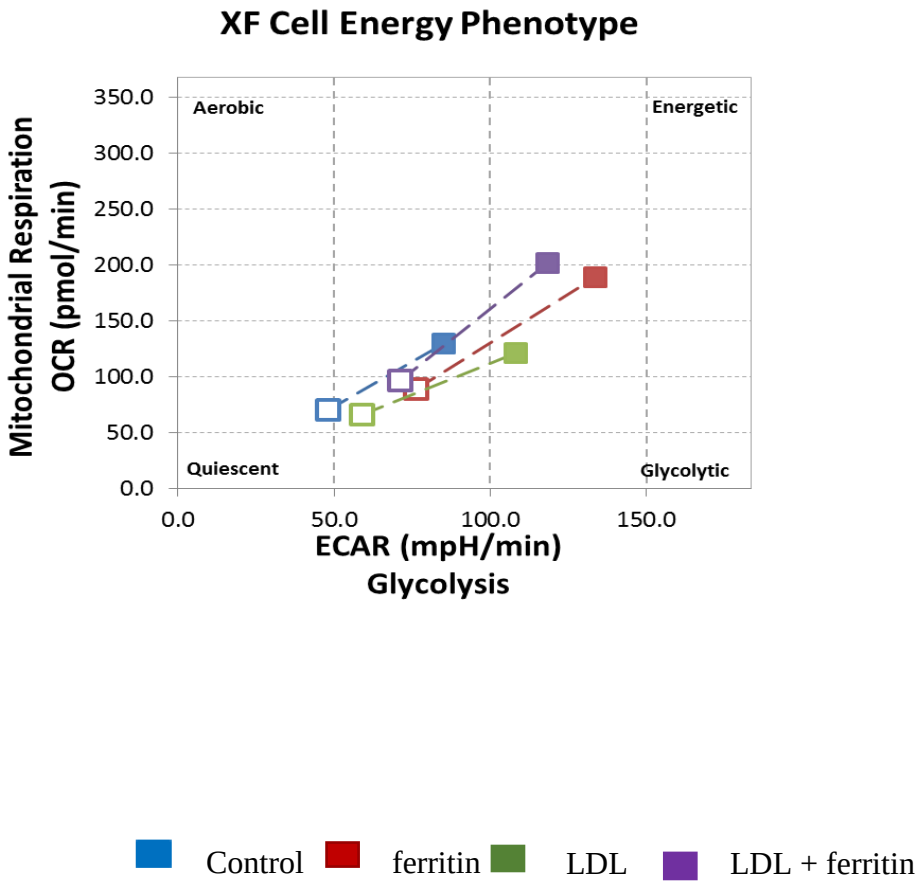


Figure 6.14: Effects of LDL and ferritin on metabolism of macrophages

THP-1 macrophages (6 x10^4 per well) were grown in 8 well Seahorse sterile tissue culture plates. The control cells were incubated with or without ferritin (0.2 μM) for 24 h. The cells were the incubated in RPMI medium or with native LDL (100 μg protein/ml) for 24 h. The cells were then washed and incubated with XF base media containing glucose, glutamine and pyruvate as fuels. The cells were then analysed to determine their metabolic phenotype. The cells were stressed with oligomycin, FCCP, and the energy phenotype before (unfilled squares) and after (filled in squares) the stressor mix were measured. The result was analysed with Seahorse bioscience Wave software. This is a representative of four independent experiments. OCR is oxygen consumption rate and ECAR is extracellular acidification rate.

6.3.6 Effect of ferritin-oxidised LDL on macrophages cell death

The ability of LDL oxidised by ferritin to induce cell death in THP-1 macrophages was tested. THP-1 cells (5 x 10^5 cells per well) were cultured in RPMI-1640 containing 10% FCS (v/v) either alone or in the presence of NaCl/sodium acetate buffer (pH 4.5) (10%) or native LDL (100 µg protein/ml) or ferritin alone (0.2 µM) or ferritin-oxidised LDL (100 µg protein/ml). After 48 hours of incubation the proportion of cells that were live or undergoing apoptosis, necrosis or secondary necrosis were assayed using flow cytometry.

The externalisation of phosphatidylserine, which is a measure of apoptosis, was determined by the binding of FITC-labelled annexin V. The permeability of the plasma membrane to propidium iodide was used as a measure of necrosis. Significantly lower levels of live cells were detected in cells treated with ferritin and ferritin-oxidised LDL. The percentage of cells that were alive, apoptotic, necrotic or secondary necrotic was determined by the measurement of the levels of annexin V and PI they contained (Figures 6.15 & 6.16) using flow cytometer. Higher levels of apoptotic cells were observed in cells treated with ferritin and oxidised LDL. The levels of apoptotic cells present in ferritin and ferritin-oxidised LDL treated cells were significantly higher compared to control cells and cells treated with LDL ($P < 0.001$). However, the level of necrotic cells observed was very low in all treatments and there was no statistically significant difference compared to control cells. The levels of secondary necrotic cells (apoptosis followed by permeabilisation of the plasma membrane) was significantly higher in cells treated with ferritin-oxidised LDL compared to control ($P < 0.01$) and cells treated with LDL alone ($P < 0.01$). A proportion of the toxic effects observed was due to the presence of ferritin itself. However, the toxic effect was enhanced in the presence of ferritin-oxidised LDL (which would have contained ferritin).

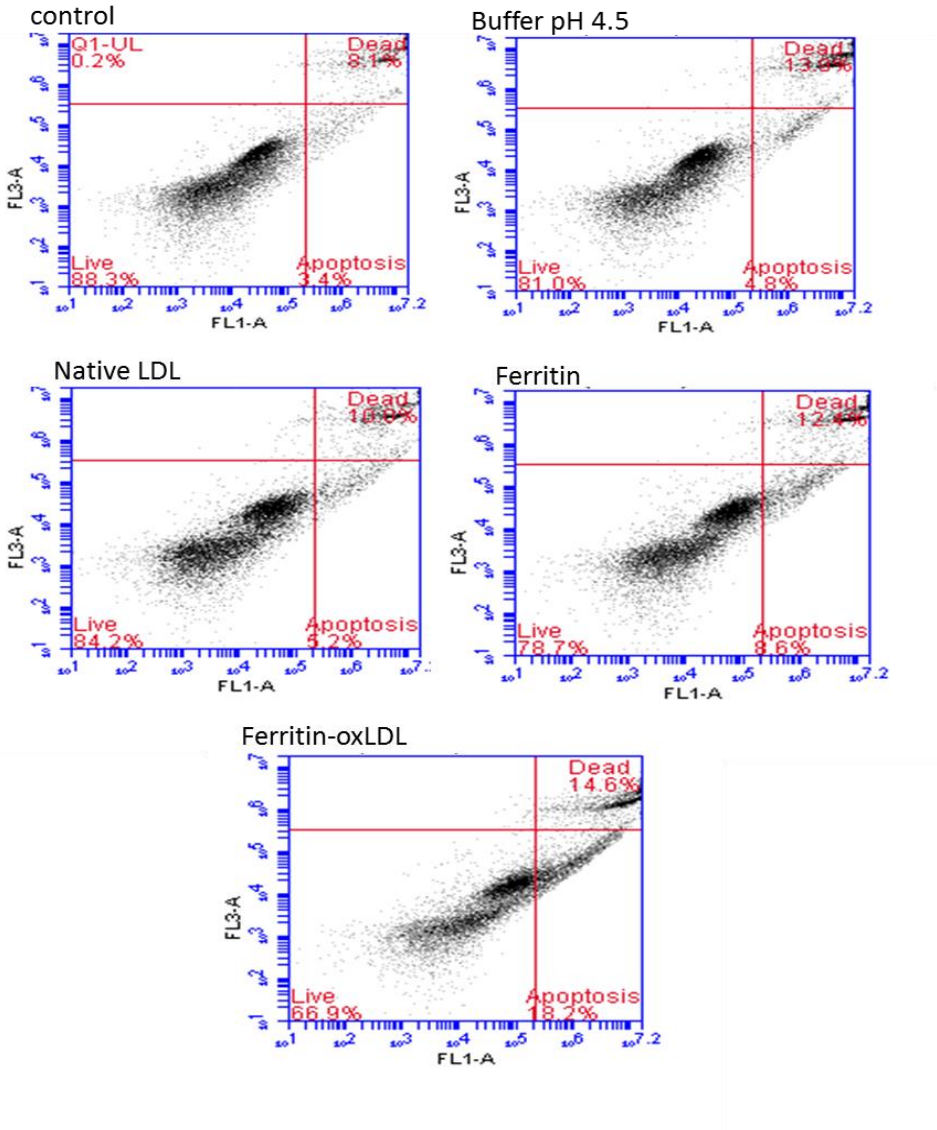


Figure 6.15: Flow cytometry analysis of ThP1 macrophages cell death

Ferritin-oxidised LDL prepared by incubating (1 mg LDL protein/ml) with 2 µM ferritin at 37˚C for 24 h in NaCl/sodium acetate buffer (pH 4.5). THP-1 cells (5 x 10^5 cells per well) were then cultured in RPMI-1640 containing 10% FCS (v/v) either alone or in the presence of NaCl/sodium acetate buffer (pH 4.5) (10%) or native LDL (100 µg protein/ml) or ferritin (0.2 µM) alone or ferritin-oxidised LDL (100 µg protein/ml). After incubation for 48 h the cells were harvested and assayed by flow cytometry. The results were analysed using the BD Biosciences C6 flow cytometer software. The abscissa shows annexin V binding and the ordinate shows the PI content. The data shown is a representative of three independent experiments.

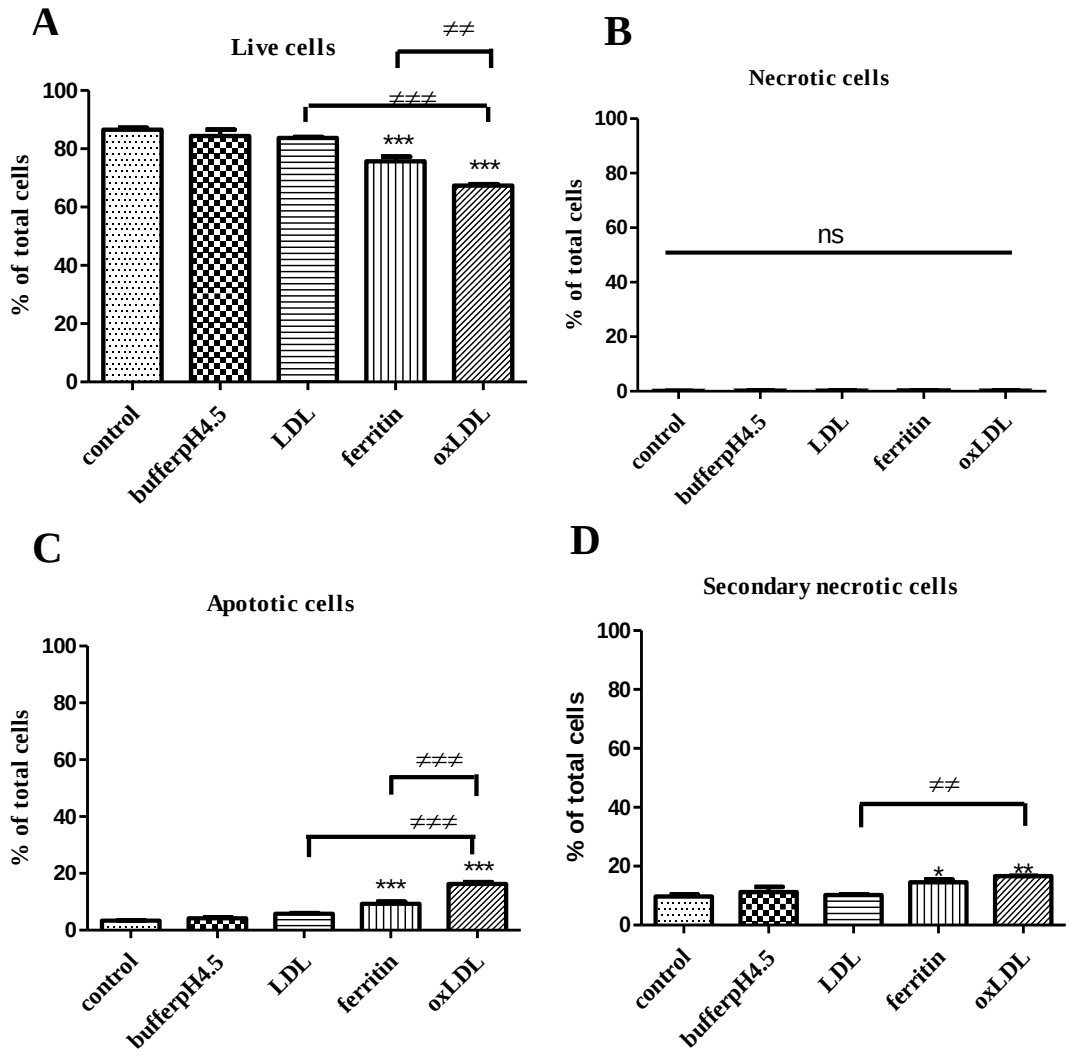


Figure 6.16: Effects of ferritin-oxidised LDL on apoptosis in THP-1 macrophages

Ferritin-oxidised LDL prepared by incubating (1 mg LDL protein/ml) with 2 µM ferritin at 37˚C for 24 h in NaCl/sodium acetate buffer (pH 4.5). THP-1 ells (5 x 10^5 cells per well) were then cultured in RPMI-1640 containing 10% FCS (v/v) either alone or in the presence of NaCl/sodium acetate buffer (pH 4.5) (10%) or native LDL (100 µg protein/ml) or ferritin (0.2 µM) alone or ferritin-oxidised LDL (100 µg protein/ml). After incubation for 48 h the cells were harvested and assayed by flow cytometry. The apoptotc cells were measured by externalisation of phosphatidyl serine determined by the FITC-labelled annexin V binding and necrosis was measured by membrane permeability to propidium iodide (PI). The percentage of live cells (A), necrotic cells (B), apoptotic cells (C) and secondary necrotic cells (D) was compared by a one-way ANOVA followed by a Tukey's post hoc test (n=3). * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$. ≠≠ indicates $P < 0.01$ and ≠≠≠ indicates $P < 0.001$ for the shown comparison.

6.4 Discussion

Macrophages are well recognised to play an important role in all stages of atherosclerosis, as they constitute major cellular components of early and advanced atherosclerotic lesions (Glass and Witztum, 2001). The sustained recruitment of macrophages and continuous pro-inflammatory mediators in the lesion supports the growth of the lesion and instability of the plaque. The balance between macrophages maintaining their function, survival, apoptosis and clerance from the lesion environment is a major determining factor of the progression and the fate of atherosclerotic lesions (Tano *et al.*, 2012). This thesis reported in previous chapters of that ferritin is a possible candidate for lysosomal LDL oxidation at lysosomal pH. In this present chapter, we investigated the role of ferritin in promoting the oxidation of LDL in the lysosomes of macrophages and the possible effects of this oxidation on macrophage function and atherosclerosis.

Studies investigating the role of body iron stores in CVD have remained inconclusive (Moore *et al*., 1995, Meyers *et al*., 2002, Ganz, 2005, Peffer *et al*., 2013). This inconclusive evidence might because the association is not driven by plasma iron levels but driven by the distribution of iron in macrophages, hepatocytes and enterocytes, as determined by serum hepcidin levels (Galesloot *et al*., 2015). An attempt was made to upregulate intracellular iron levels and ferritin levels through the addition of hepcidin up to 1000 nM. The result showed that hepcidin did not significantly increase the iron levels in THP-1 macrophages. The reason for this observation is unclear, as hepcidin has been previously demonstrated to decrease iron concentration in blood but increase intracellullar levels of iron in macrophages by binding to and downregulating the iron transporter ferroportin 1 (FPN1). FPN1 is known as the sole iron transporter responsible for the outflow of iron from cells. The decrease in the quantity of FPN1 in hepatic and splenic

macrophages by hepcidin decreases the ability of macrophage iron efflux (Zhao *et al.*, 2013). Contrary to our result, a previous study by Chung *et al*. showed that exposure of THP-1 macrophages to 300 and 1000 nM hepcidin decreased FPN1 expression and caused a decrease in duodenal iron and serum iron. However, they did not measure the intracellular iron levels in THP-1 macrophages. They reported that the efflux of iron from Caco-2 cells was significantly reduced by addition of hepcidin, although in addition to hepcidin the Caco-2 cells received 10 μM iron in form of $FeCl_3$ and the THP-1 macrophages used was transformed with twice the amount of PMA used in our experiment (Chung *et al.*, 2009). RPMI 1640 (which does not contain added iron) was used, but also F10 and DMEM media (which contain iron) could not increase the iron levels inside the macrophages. Work by Agoro and Mura also suggested that macrophage polarisation plays a role in the relationship between hepcidin and intracellular macrophage iron levels (Agoro and Mura, 2016). Hence in the future experiment, the introduction of an external source of iron and tranformation of THP-1 macrophages to the M1 phenotype would be considered.

Macrophages treated with SMase-LDL have recently been demonstrated to exhibit increased lysosomal lipid peroxidation (Ahmad and Leake, 2019). Lipid peroxidation in LDL increases the atherogenicity of LDL (Steinbrecher, 1991), hence the role of ferritin in lysosomal lipid peroxidation were determined. The cells were pre-incubated with ferritin to allow it to be be endocytosed and delivered to the lysosomes and then followed up with treatment with either native LDL or SMase-LDL. Cells that were pretreated with ferritin had a significant increase in levels of lysosomal lipid peroxidation, as shown by the lysosomally targetted lipid peroxidation probe Foam-LPO. The increase in lysosomal lipid peroxdation suggests that ferritin has the potential to promote the oxidation of LDL inside the inflammatory cells, macrophages. Our laboratory has shown that SMase-LDL is rapidly endocytosed by

macrophages and it was mainly deposted in the lysosomes where it was oxidised by iron (Wen *et al.*, 2015, Wen *et al*., submitted). Aggregated forms of LDL are notable features of atherosclerotic lesions and they are powerful inducers of foam cell formation in atherosclerotic lesions (Hoff and Morton, 1985, Guyton and Klemp, 1996, Tabas, 1999). LDL aggregated by sphingomyelinase is one of the most feasible mechanisms for LDL aggregation *in vivo* (Pentikiiinen *et al*., 1996). The addition of SMase-LDL and ferritin also promoted increased lipid peroxidation in the same pattern in both THP-1 macrophages and HMDM. The Foam-LPO itself consists of a conjugated diene group which can undergo oxidation in lysosomes and can cause spectral shifting (Zhang *et al*., 2015). The increased green to red ratio also signifies more potential for lipid peroxidation in the presence of ferritin with or without SMase-LDL. The competition between the probe and Smase-LDL for oxidation by ferritin might be responsible for the slightly lower peroxidation when SMase-LDL was present. These experiments support the possibility that ferritin might catalyse lipid peroxidation in lysosomes.

Our laboratory has previously shown that cultured THP-1 (Ahmad and Leake, 2019) J774 cells and HMDM (Wen and Leake, 2007) treated with aggregated LDL formed increased ceroid in their lysosomes and this can be attributed to iron-catalysed oxidation. In this present study, our results showed that ceroid was increased considerably in the presence of ferritin and and was increased further when SMase-LDL was added, much more than the intracellular lipids were increased. Potentially importantly, this suggests that ferritin may be a key factor in releasing catalytically active iron that can mediate LDL oxidation in lysosomes of macrophages.

Studies have shown that oxLDL stimulates the intracellular production of ROS in macrophages, VSMCs and endothelial cells (Hsieh *et al*., 2001, van Aalst *et al.*, 2004,

Zmijewski *et al*., 2005). The superoxide radical is one of the major reactive oxygen species, and work presented in chapters three and five of this thesis suggested that the superoxide radical and its protonated form, hydroperoxyl radicals, might be involved in lysososomal LDL oxidation by ferritin. Here it was shown that ferritin-oxidised LDL can induce increased formation of reactive oxygen species in THP-1 macrophages. Increased levels of reactive oxygen species can be prevented by antioxidants that can scavenge free radicals. Cysteamine demonstrated strong ability to reduce the formation of conjugated dienes in LDL oxidised with ferritin. Cysteamine significantly reduced the formation of reactive oxygen species in THP-1 cells treated with ferritin-oxidised LDL. In this context, it is interesting that Vendrov *et al* showed that limiting the production of superoxide in macrophages/monocytes or other cells of the vessel wall caused decreased atherogenesis (Vendrov *et al*., 2007).

Oxidised LDL has been demonstrated to induce an inflammatory response in macrophages (Li *et al*., 2010, Lara-Guzmán *et al.*, 2018). Induction of a pro-inflammatory reasponse in macrophages is characterised by a shift in metabolic pathways to glycolysis leading to decreased uptake of oxygen (Fukuzumi *et al*., 1996, Rodriguez-Prados *et al*., 2010) and reduction in oxidative phosphorylation (Haschemi *et al*., 2012, Freemerman *et al.*, 2014). Metabolic pathways have a central role in the function of immune cells and many studies have utilised lipolysaccharide (LPS) to understand the effects of inflammation on metabolic pathways in macrophages. Shirai *et al.* explored the effects of stimulating monocytes on their metabolic pathways. LPS and IFNγ-stimulated monocytes from healthy individuals had a lower oxygen consumption rate (OCR) and extracellular acidificaion rate (ECAR) compared to monocytes isolated from atherosclerotic patients which demonstrated increased glycolytic flux, (Shirai *et al*., 2016). Treatment of macrophages with LPS and IFNγ is well known to produce the pro- inflammatory macrophages (M1 macrophages) and treatment of bone marrow-derived

macrophages with LPS and IFNγ significantly upregulated glycolytic pathways significantly (Liu *et al*., 2016). This present study showed that treatment of THP-1 macrophages with ferittin, especially in combination with LDL, which can promote lysosomal oxidation of LDL, significantly increased their oxygen uptake ($P < 0.001$) and also the ECAR was signifuicantly increased ($P < 0.001$) Overall these results showed that there was increased glycolysis and aerobic capacity in the macrophages and the macrophages became more metabolically activated. This might enhance the macrophages' pro-inflammatory response. In addition, it might acidify the extracellular space and further promote atherosclerosis (Leake, 1997).

Cell death caused by oxidised lipoprotein is believed to be important in lesion progression. Different oxidised lipid products of LDL have been demonstrated to be cytotoxic to cells, lipid hydroperoxides (Siow *et al*., 1999a) as well as oxysterols (Colles *et al*., 2001a), have been demonstrated to be cytotoxic to cells. Pevious work by Gerry and Leake (Gerry and Leake, 2008) has demonstrated that oxysterol-rich LDL was more cytotoxic to macrophages than LDL with high levels of lipid hydroperoxides. The most potent cytotoxic substance out of these two products of LDL oxidation remains controversial as previous work by Siow et al showed that lipid hydroperoxide-rich LDL was more toxic than hydroperoxide-rich LDL to smooth muscle cells (Siow *et al*., 1999a). Other products of LDL oxidation, such as aldehydes, have also been shown to be cytotoxic (Esterbauer *et al*., 1991c). Oxidised phospholipids have also been shown to be cytotoxic to macrophages (Stemmer *et al*., 2012) and fibroblasts (Colles and Chisolm, 2000). Work presented in this chapter has demostrated that ferritin–oxidised LDL can induce apoptosis, but not primary necrosis, in cultered THP-1 macrophages. The toxicity of ferritin-oxidised LDL was not unexpected. Ferritin oxidation of LDL promoted the formation of 7-ketocholesterol, CLOOH and total hydroperoxides and all these oxidised lipid products have been demostrated to be cytotoxic. Ferritin-oxidised LDL also increased the level of ROS in

cells and this might possibly mediate induction of apoptosis in cells. The role of ROS in prommoting apoptosis was previously reviewed by Simon *et al*. (Simon *et al*., 2000).

Data presented in this chapter demonstrated that ferritin can also mediate some of the effects observed with ferritin-oxidised LDL, such as apoptosis and lipid peroxidation. It was speculated that the iron-rich protein, ferritin, can be endocytosed by macrophages and delivered to the lysosomes where it can release its iron to catalyse redox reactions and mediate lysosomal lipid peroxidation. The continuous oxidation process in the lysosomes mediated by iron released from ferritin might lead to destabilisation of these organnelles and cell death. Recent work from our laboratory has demonstrated that lysosomal LDL oxidation mediated by iron can lead to increased lysosomal pH (Ahmad and Leake, 2019), which might cause loss of lysosomal function (Ohkuma and Poole, 1978). There is also evidence that lysosomal destabilisation and loss of funtion mediated by iron-catalysed redox reactions less can cause enzymes to leak and trigger apoptotic cell death (Guicciardi *et al*., 2004, Aits and Jäättelä, 2013).

Work presented in this chapter lends support to the ability of ferritin to oxidise LDL within the lysosomes of macrophages and possible promotion of atherogenesis. Further experiments are required to further explore the upregulation of iron and ferritin by hepcidin within macrophages in relation to lysosomal LDL oxidation. However, there is evidence that ferritin promotes lysosomal lipid peroxidation, causes increased formation of the advanced lipid oxidation product~~s~~ ceroid and activates macrophages making them more metabolically active and glycolytic. Ferritin-oxidised LDL was also shown to increase the intracellular generation

of reactive oxygen species in macropohages and promote apototic cell death. Overall, all these events have pro-atherogenic consequences.

Chapter 7: General Discussion

7.0 General Discussion

Modification of LDL is important in the onset and advancement of atherosclerotic lesions (Jialal and Devaraj, 1996). Oxidised LDL exerts effects on many *in vitro* activities of vascular cells, which includes proliferation, inflammatory response, migration and cell death. The majority of these effects are consistent with its enhanced pro-atherogenic nature (Berliner and Heinecke, 1996, Steinberg, 1997b, Mehta and Li, 2005). Several mechanisms have been postulated for the oxidation of LDL *in vivo* (Yoshida and Kisugi, 2010, Maiolino *et al*., 2013). The precise mechanisms and site of modification of LDL *in vivo* remain debatable. Jerome suggested that accumulation of lipids in the lysosomes is an important component in atherosclerosis (Jerome, 2006). Some studies have suggested a link between interference of autophagy and increased atherosclerosis (Ouimet *et al*., 2011, Razani *et al*., 2012) and the enhancement of autophagic-lysosomal biogenesis has been suggested as a plausible mechanism for reducing atherosclerotic cardiovascular diseases (Sergin *et al*., 2017). The lysosomes are indispensable intracellular organelles responsible for the degradation of both intracellular and extracellular cargo including lipoproteins (Sergin *et al*., 2015). The lysosomal compartment is suggested to be rich in redox active iron since many macromolecules derived from heterophagy or autophagy might be degraded to release iron into lysosomes, hence it is suggested to have the highest amount of redox active iron (Petrat *et al*., 2001, Kurz *et al*., 2007, Lv and Shang, 2018). These events make lysosomes a possible site for LDL oxidation.

As mentioned earlier in this thesis, our laboratory (Wen and Leake, 2007) was first to identify lysosomes as a site for LDL oxidation and proposed iron as the possible mediator of the oxidation LDL in lysosomes. Studies have suggested the presence of transition metals (Lamb *et al*., 1995) and iron in particular in atherosclerotic lesions (Yuan *et al*., 1996, Lee *et al*., 1998, Stadler *et al*., 2004). Since most of the transition metals *in vivo* are tightly bound to prosthetic

groups or sequestered by proteins, the question of how the free copper or iron ions can be available in significant enough amounts to mediate LDL oxidation is a debated topic (Halliwell and Gutteridge, 1990, Mukhopadhyay and Fox, 1998). Our laboratory (Satchell and Leake, 2012, Ahmad and Leake, 2018) later explored the mechanisms by which Fe^{2+} and Fe^{3+} can mediate lysosomal LDL oxidation with respect to the possible treatment of the disease with antioxidants. Previous work from Balla *et al.* and others (Balla *et a*l., 1991, Abdalla *et al*., 1993, Lamb and Leake, 1994c, Lamb and Leake, 1994b, Mukhopadhyay *et al.*, 1996, Rodriguez-Malaver *et al*., 1997, Grinshtein *et al*., 2003) have identified some metal-ion binding proteins that can possibly contribute to oxidation of LDL. However, the exact role of iron binding proteins in lysosomal LDL oxidation is yet to be explored in great detail and if there is a pathophysiological role for the main iron storage protein ferritin in lysosomal LDL oxidation *in vivo*.

Work presented in this thesis has demonstrated that ferritin is a candidate for oxidation of LDL. It was shown for the first time the novel contributions ferritin might have as a major iron-storage protein to the oxidation of LDL at lysosomal pH and within lysosomes of macrophages. The present study utilised different independent methods to assess the ability of ferritin to promote formation of products of oxidation in human LDL. The method for continuously measuring conjugated dienes formation in LDL established by Esterbauer and colleagues three decades ago (Esterbauer *et al*., 1989b), is still widely accepted and applied in the research community to access the kinetics of LDL oxidation. The experiments were conducted with LDL were incubated with 0.1 μM ferritin in terms of total protein content (which was measured to contain iron centration of 100 μM). The rate of oxidation as measured by the formation of conjugated dienes over time was different from the $FeSO_4$-induced oxidation, in the sense that there were less distinctive rapid and slow phases of oxidation. There was extensive oxidation

of LDL by ferritin at pH 4.5, but very little or no oxidation was observed at normal interstitial fluid or plasma pH (pH 7.4). It was expected that ferritin oxidation may progress more in the lysosomes rather than interstitial fluid or plasma because of substantial antioxidant protection that is present in arterial intima (Leake and Rankin, 1990, Dabbagh and Frei, 1995). Our data showed that ferritin spontaneously released iron at lysosomal pH without requiring the activity of lysosomal protease, in contrast to what was earlier suggested by Kidane and colleagues (Kidane *et al.*, 2006). As much as 15 µM iron was demonstrated to be released form ferritin over 24 h which is similar to the 16 µM concentration that Petrat *et al* found in lysosomes of endothelial cells from rat Liver (Petrat *et al*., 2001). The iron chelators EDTA and DTPA slowed down the oxidation of LDL by ferritin at lysosomal pH significantly, thus the ferritin-mediated LDL oxidation is mainly due to the iron released from ferritin at pH 4.5.

The data obtained from HPLC and tri-iodide assays lend support to the ability of ferritin to oxidise LDL at lysosomal pH. It revealed that ferritin promotes formation of oxidised lipids in LDL (7-ketocholesterol and cholesteryl linoleate hydroperoxide). Elevated levels of 7-ketocholesterol were observed in patients with CAD was compared to patients with normal arteries. The increase in 7-ketocholesterol correlated with the events of myocardial infarction and increased C-reactive proteins providing a link between 7-ketocholesterol levels and inflammation (Hitsumoto *et al*., 2009) and increased atherosclerotic cardiovascular events (Hitsumoto *et al*., 2009, Song *et al*., 2017). Walter *et al.*, also showed that elevated lipid hydroperoxides were a predictive factor for cardiovascular events and this prediction was independent of inflammatory markers and other risk factors of CVD (Walter *et al*., 2008). Hence oxidised products from LDL oxidation by ferritin at lysosomal could potentially promote atherosclerotic cardiovascular events.

Lysosomes with a pH between 4.5 and 5.0 (Mindell, 2012) consist of over sixty types of hydrolytic enzymes (Xu and Ren, 2015) and lysosomal enzymes such as cathepsins might possibly influence the progression of CVD by contributing to inflammation and apoptosis (Lutgens *et al*., 2007). The effects certain lysosomal proteases might have on lysosomal LDL oxidation by ferritin were demonstrated. The results obtained showed that oxidation of LDL by ferritin occurred in the presence of the proteolytic enzymes cathepsins B and D. However, there was no significant difference between oxidation mediated by ferritin pre-treated with cathepsins. This observation might be attributed to the lack of degradation products observed in ferritin after treatments with cathepsins B and D when analysed with SDS-PAGE. The ApoB-100 component of LDL was evidently susceptible to degradation by cathepsin D, showing that the cathepsin D was active. Ferritin is highly resistant to proteolysis by the major endoproteases cathepsins D and B and thus might survive in lysosomes for long periods of time. Treatment with cathepsins B and D increased LDL oxidation by ferritin, which suggests that the lysosomal oxidation of LDL by ferritin might accelerate during the lysosomal degradation of LDL.

We described the impact of treating LDL with a lipolytic enzyme on its rate of oxidation. Co-incubation of LDL with cholesteryl esterase from *Pseudomonas sp* increased the rate of the initial phase of oxidation. Cholesteryl esterase might degrade the core cholesteryl esters into fatty acids and cholesterol allowing free radicals generated from ferritin-iron more access to PUFA such as Linoleic acid and arachidonic acid and hence increased conjugated diene formation. Lysosomal enzymes released by human macrophages modified LDL in vitro (Hakala *et al.*, 2003). The modification of LDL by processes such as proteolysis, lipolysis and oxidation carried out *in vitro* has helped in producing LDL particles similar to those found in atherosclerotic lesions and thus contributed to more understanding of the disease. The results

presented here demonstrated that ferritin might continually oxidise LDL even after modification by enzymes in lysosomes.

Due to the novel suggested lysosomal LDL oxidation mechanisms involving redox active iron and ferritin-iron, there is a need to reconsider the choice of appropriate antioxidants in the treatment of atherosclerosis. Antioxidants that can be targeted to the lysosomes and able to prevent the oxidation in the core of LDL appear to be more appropriate. The failure of human antioxidant clinical trials that mainly used α-tocopherol (Yusuf *et al*., 2000, de Gaetano, 2001, Collins *et al*., 2002, Cook *et al*., 2007, Sesso *et al*., 2008) did not mean that modified LDL lacks pro-atherogenic properties. It rather suggests the need for a better understanding of the mechanisms involved and a better approach with selection of appropriate antioxidants to reduce atherogenic events caused by oxidised LDL. Work presented in this thesis and previously (Ahmad and Leake, 2018) suggested that the free radicals superoxide and its protonated form, $HO_2^{\cdot}$ are involved in ferritin and $FeSO_4$ oxidation of LDL. Hence the antioxidant of choice must also possess strong ability to scavenge superoxide and the highly reactive $HO_2^{\cdot}$. The question of why the antioxidants used have failed in large clinical trials of CVD persists. It should be noted that we showed the inability of enrichment of LDL with α-tocopherol to prevent LDL oxidation by ferritin at lysosomal pH. This supported previous findings from our laboratory have shown that α-tocopherol has both pro-oxidant and antioxidant effects on LDL oxidation by $FeSO_4$ (Satchell and Leake, 2012, Alboaklah, 2018b). This might help to explain why the large antioxidant trials using α-tocopherol did not succeed. It was also shown that vitamin C did not effectively inhibit the oxidation of LDL by ferritin at lysosomal pH. The data showed that the antioxidant properties of ascorbate were lost over time in the presence of lipid hydroperoxides. Ascorbate was not a strong inhibitor of oxidation at pH 4.5 compared to pH 7.4, where it completely inhibited oxidation by $CuSO_4$. We found that the reduced form of

vitamin C, dehydroascorbate was a pro-oxidant and increased oxidation of LDL by ferritin. Hence these vitamins might not be the most efficient choice of antioxidants in preventing lysosomal LDL oxidation by ferritin.

Tempol has superoxide dismutase mimetic properties (Luo *et al*., 2009) and reduced the later oxidation phase of LDL, but notably had no effect on the early phase of LDL oxidation. $HO_2^{\cdot}$ is a strong oxidant which can initiate lipid peroxidation even in the esterified cholesterol core of LDL, due to its ability to pass through the phospholipid monolayer of LDL. The amphipathic nature of tempol might prevent it from entering the hydrophobic core of LDL. This might allow the initial oxidation of the esterified cholesterol rich core of LDL by hydroperoxyl radicals to proceed uninhibited. Tempol, being amphiphilic, might enter the phospholipid monolayer of LDL and inhibit the later oxidation of LDL here. Like ascorbate, tempol was able to completely inhibit LDL oxidation mediated by copper at pH 7.4 but loses this ability at lysosomal pH (Alboaklah, 2018a). Hence antioxidants that are able to maintain their activities at lysosomal pH might be important in reducing the development of atherosclerosis.

DPPD and cysteamine showed more potential in preventing LDL oxidation. However, the use of DPPD in humans is prevented due to its mutagenic nature (Sofuni *et al.*, 1990). But drugs with analogous antioxidant properties to DPPD and not mutagenic can be synthesised, which might be a plausible treatment for atherosclerosis. Antioxidants that can concentrate in reasonable concentrations in lysosomes would, however, be more beneficial. Cysteamine is drug well tolerated by humans and it is currently in use for treatment of cystinosis (Gahl *et al*., 2002). Although cysteamine had complex effects on LDL oxidation by ferritin at lower concentrations, the concentrations equivalent to what would be present in lysosomes were highly effective in inhibiting LDL oxidation by ferritin at lysosomal pH. Cysteamine has the

ability to scavenge superoxide radical (Sunman *et al*., 1993). Cysteamine has now been demonstrated as a possible treatment for atherosclerosis. The study revealed the inhibitory effects of cysteamine on LDL oxidation in the lysosomal environment. Cysteamine also reduced atherosclerotic lesions in mice lacking LDL receptors fed a high fat diet by 33% (Wen *et al.*, submitted). Recent work from our laboratory showed that cysteamine decreased ceroid formation in macrophages incubated with sphingomyelinase-aggregated LDL (Ahmad and Leake, 2018).

It is noteworthy that lysosomes are of significant importance in biomedicine as lysosomal enzymes and lysosomal alterations are linked to a number of human diseases (Lübke *et al*., 2009). The overexpression of lysosomal proteins and lysosomal acid lipase was demonstrated to promote atherosclerosis (Zschenker *et al*., 2006). The animal models and human atherosclerotic lesions contain many macrophage foam cells with lipid-engorged lysosomes (Jerome and Yancey, 2003, Jerome *et al*., 2008). It was important to answer the question of the role lysosomal ferritin-mediated LDL oxidation might play in the lysosomes of macrophages and the progression of atherosclerosis. We attempted to investigate how existing inflammation can increase the iron and ferritin levels in macrophages can influence lysosomal LDL oxidation. Nevertheless, this investigation was unsuccessful, as surprisingly we were unable to upregulate intracellular iron with hepcidin treatment.

Atherosclerosis is a direct example of a disease amplified by a lipid peroxidation process and the transformation of macrophages to lipid-laden cells is well acknowledged as the starting phase of atherosclerosis (Esterbauer *et al*., 1993). Elevated lipid peroxides were found in atherosclerotic tissue compared to the normal human aorta (Piotrowski *et al*., 1990). It was established that the presence of ferritin increased intracellular lipid levels, lysosomal lipid

peroxidation and percentage ceroid formation in lysosomes of macrophages. These three events have major implications for the onset and progression of atherosclerotic lesions. Our laboratory has previously demonstrated that J774 mouse macrophages and HMDM treated with additional iron or ferritin generated more 7-ketocholesterol (Wen and Leake, 2007). Sphingomyelinase aggregation of LDL produces ceramide, which has been suggested to enhance uptake through endocytosis by causing invagination of the cellular membrane (Holopainen *et al.*, 2000). SMase-LDL is increasingly taken up rapidly by macrophages (Wen *et al*., 2015). Wen and Leake treated macrophages with acetylated LDL and ferritin. It is interesting, to see ferritin also promotes lipid oxidation and ceroid in human THP-1 macrophages treated with the more pathophysiological SMase-LDL and native LDL.

White *et al* earlier suggested that development of atherosclerosis involves peroxynitrite formation from nitric oxide and superoxide (White *et al*., 1994). Superoxide or its protonated form the hydroperoxyl radical is proposed to oxidise LDL in lysosomes (Ahmad and Leake, 2019). Measuring intracellular levels of superoxide was therefore relevant to atherosclerosis. We used the fluorescent probe DHE, as it has high affinity for superoxide (Wardman, 2007) (but also has a non-specific affinity for peroxynitrite and hydroxyl radicals) (Gomes *et al*., 2005, Kalyanaraman *et al.*, 2012). We reported that LDL oxidised by ferritin at lysosomal pH enhances the formation of reactive oxygen species in THP1- macrophages. The potency of cysteamine in protecting the cells from increased reactive oxygen species was demonstrated, as there was significant reduction of ROS in the presence of cysteamine.

It was also reported here that ferritin and ferritin-oxidised LDL produced *in vitro* induced apoptosis and secondary necrosis in cultured macrophages. These findings imply that, if cells have extensively oxidised LDL in lysosomes and the oxidised LDL is released, possibly due to

lysosomal destabilisation or cell death, the released oxidised LDL might increase oxidative stress or apoptosis in those cells or neighbouring cells. There is evidence that intralysosomal redox reactions can cause leakage from lysosomes and trigger apoptosis and necrosis of cells (Brunk *et al*., 2001, Guicciardi *et al.*, 2004). Hence, it would be appropriate to suggest that ferritin-mediated oxidation of LDL is a possible redox-iron driven reaction that can occur in lysosomes and trigger apoptosis.

The role of apoptosis in atherosclerosis depends on the disease stage. The occurrence of apoptosis in early lesions is likely to be beneficial, as phagocytic events may still occur efficiently to clear foam cells before they induce an inflammatory response. However in advanced lesions, the clearing of apoptotic cells is less efficient and they might contribute to inflammation and necrotic core formation (Tabas, 2005, Schrijvers *et al*., 2005). Cells might release their content, such as prothrombotic molecules and matrix metalloproteinases, which can cause thrombogenic events and degradation of ECM (Mallat *et al*., 1999, Mallat and Tedgui, 2000, Kenagy e*t al*., 2011). Consequently, they can promote plaque vulnerability and make them more prone to rupture (Tabas, 2005). Thus, apoptosis induced by ferritin-oxidised LDL might be detrimental to atherosclerosis. It was suspected that the lysosomal oxidation of LDL partly by ferritin might contribute to atherosclerosis and in advanced lesions to apoptosis would contribute to plaque instability.

Previous studies have shown that activated macrophages show enhanced glycolysis along with decreased oxygen uptake. Monocytes stimulated with LPS switched towards a more glycolytic pathway which was suggested to be due to an increase in hypoxia inducible factor (HIF- 1α) enhanced by NF-κB. HIF-1α causes an increase in uptake of glucose by upregulating the synthesis of GLUT-1 glucose transporter (Tawakol *et al*., 2015, Groh *et al*., 2018). The study

reports that the extracellular acidification rate and oxygen consumption rate were significantly increased in cells treated with ferritin and LDL. The treatment of THP1- macrophages with LDL and ferritin appears to be driving cells towards a more energetic state (with greater rather than less oxygen consumption). This metabolic switch is attributed to the pro-inflammatory phenotype (Koelwyn *et al*., 2018). It would be relevant in the future to observe the effects lipopolysaccharide (LPS) might have on the effects observed. Ahmad and Leake (Ahmad and Leake, 2019) have recently shown that native LDL and SMase-aggregated LDL increased LPS-induced secretion of pro-inflammatory cytokines. Hence LDL oxidation by ferritin in lysosomes of macrophages might also elicit a pro-inflammatory effect and contribute to acidifying the extracellular space. Inflammation (Libby, 2012) can promote atherogenesis and acidification might also do so (Leake, 1997). Figure 7.1 shows a schematic diagram of the possible events involving lysosomal LDL oxidation by ferritin can contribute to atherogenesis.

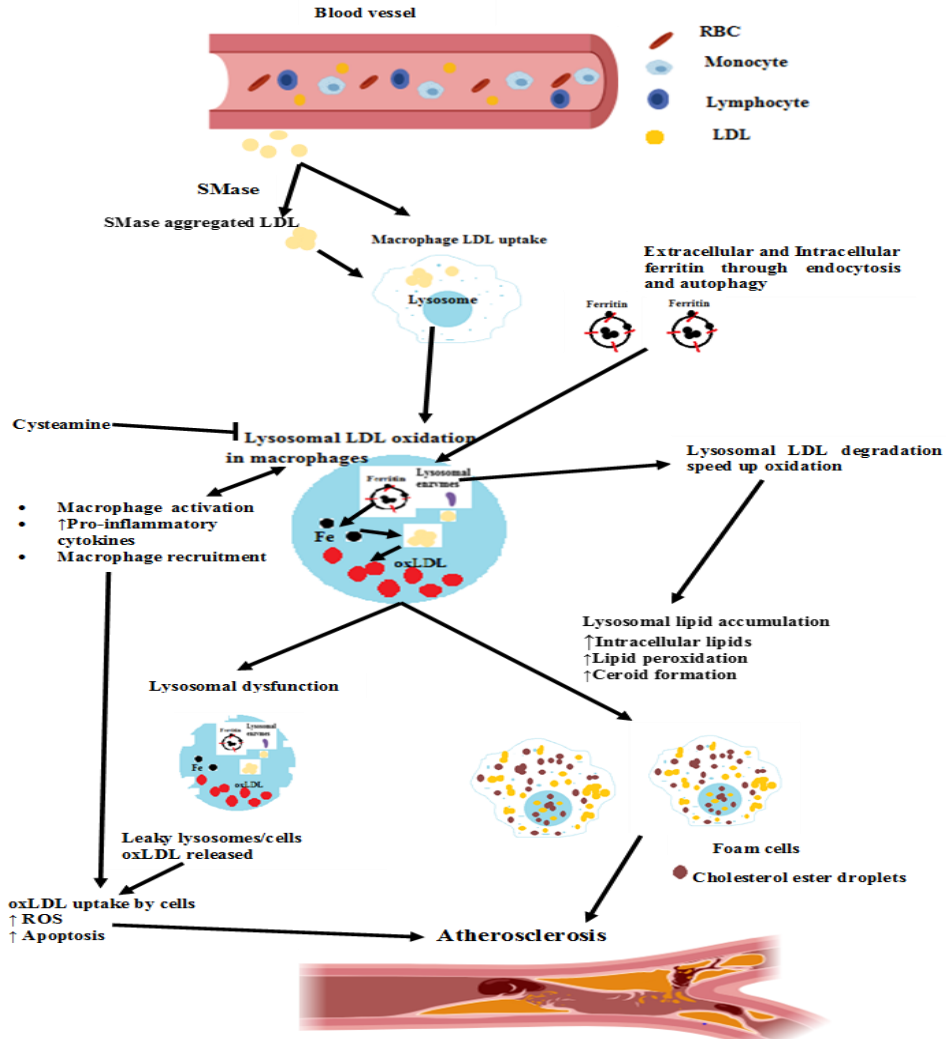


Figure 7.1: The role of Lysosomal LDL oxidation by ferritin in macrophages and atherosclerosis.

Lysosomal LDL oxidation induced by iron released from ferritin might play an important role in atherosclerosis. LDL or aggregated LDL (such as SMase-LDL) are taken up by macrophages and targetted to lysosomes for degradation. Extracellular and intracellular ferritin is also delivered to the cells through endocytosis and autophagy. The acidic environment of lysosomes can enhance the release of redox active iron, which can oxidise LDL. This process leads to increased intracellular lipids, increased lysosomal lipid peroxidation and lysosomal ceroid accumulation that transforms the macrophages to foam cells. The accumulation of lipids in the lysosomes can cause lysosomal dysfunction and cause the contents to leak, which can then release oxidised LDL into the extracellular space. The lysosomal LDL oxidation can promote release of pro-inflammatory cytokines such as TNF-α, IL-6 and MCP-1 causing more macrophages to be recruited which can take up oxidised LDL. The oxidised LDL can promote ROS generation and increased macrophage cell death. The lysosomal dysfunction can also lead to the inability cells to clear dead cells by phagocytosis and promote the activation of the inflammasome upregulating IL-1β; these can contribute to the pro inflammatory response. All these events might promote atherogenesis.

7.1 Critical evaluation of the present study

7.1.1 Summary of main findings

Atherosclerosis is a multifactorial disease, with complex events involved in the pathogenesis. This thesis provides answers to some important questions in relation to the onset, progression and considerations for treatment of atherosclerosis. The main findings of the present research work can be summarised as follows:

- Ferritin effectively promotes LDL oxidation at lysosomal pH as shown by increased conjugated diene formation and higher amount of oxidised lipids in the presence of ferritin.

- The oxidation was much faster at lysosomal compared to interstitial fluid or plasma pH, which was demonstrated to be as a result of more iron release from ferritin at lysosomal pH. The contribution of ferritin-iron to the oxidation of LDL was further demonstrated by the ability of the iron chelators EDTA and DTPA to inhibit this oxidation.

- Oxidation of LDL by ferritin proceeded in the presence of lysosomal cathepsins B and D. The proteolysis and lipolysis of LDL enhanced its susceptibility to oxidation by ferritin. Ferritin showed remarkable resistance to proteolytic degradation by cathepsin B and D, hence it may take longer to be degraded in the lysosome and have a longer time to oxidise LDL.

- LDL oxidation by ferritin was not inhibited by α–tocopherol enrichment of LDL or dehydroascorbate. Ascorbate or had both pro-oxidant and antioxidant effects on LDL oxidation by ferritin.

- Tempol slowed down the later, but not the early phase of oxidation of LDL by ferritin, possibly by scavenging lipid radicals in the phospholipid monolayer of LDL. The lysosomotropic antioxidant, cysteamine was shown to be the most appropriate antioxidant in preventing oxidative modifications of LDL by ferritin at lysosomal pH.

- Incubation of macrophages with ferritin promoted intralysosomal lipid peroxidation in human macrophages. The presence of ferritin increased intracellular lipids and intralysosomal ceroid formation in human THP-1 macrophages.

- Ferritin-oxidised LDL induced increased intracellular formation of reactive oxygen species that might further contribute to the pathogenesis of atherosclerosis and cysteamine significantly reduced the generation of reactive oxygen species in human THP-1 macrophages.

- Macrophages treated with ferritin and LDL became activated and glycolytic compared to untreated THP1-macrophages. Ferritin–oxidised LDL induced apoptosis in human THP1-macrophages.

7.1.2 Limitations of the study

Although it was established that ferritin caused increased lipid peroxidation in THP-1 macrophages and human monocytes derived macrophages in a similar way, it would be of advantage to also conduct all other experiments in HMDM as well. However, this could not be achieved due to time and financial constraints. We could not increase iron and ferritin levels in macrophages to try to increase lysosomal LDL oxidation and did not do *in vivo* work (there is no mouse knockout model for ferritin, as it is embryonically lethal if homozygous).

7.2 Possible future work

Building on previous research from our Laboratory, the findings presented in this thesis offers some new insights into the lysosomal LDL oxidation theory. The role of ferritin in human diseases is currently generating interest within the cardiovascular research community. This presents the opportunity to undertake further work within this topical area, which would add more value to this field of research. Further investigation could include:

- Characterise phospholipid oxidation products in LDL oxidised by ferritin by liquid chromatography-electrospray ionisation mass spectrometry.
- Characterise the oxidised lipids in LDL pre-treated with enzymes (cathepsin D and B) prior to oxidation by ferritin and test the possible effects of LDL oxidation by ferritin on LDL degradation and explore the effects of other lysosomal proteases on oxidation of LDL by ferritin.
- LPS and IFN-γ stimulated macrophages could be treated with hepcidin in the presence or absence of iron. The upregulation of iron could be measured using ICP-MS and increase in ferritin levels determined with western blot for ferritin heavy and light chains. The effect of these on lysosomal LDL oxidation could be monitored by ceroid detection.
- As part of another study, the plasma concentrations of ferritin and iron could be compared to those in macrophages of the LDL receptor knock out mice and also stain for ferritin in the atherosclerotic lesions with or without the novel anti-atherosclerotic drug cysteamine.

The results of these studies might further demonstrate the link between ferritin and CVD and provide evidence for increased ferritin levels as a well-defined biomarker in the incidence of atherosclerosis and development of CVD.

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Appendices

Appendix 1: List of chemicals and suppliers

Chemicals	Supplier
Acetic Acid	Sigma-Aldrich Ltd
Acetonitrile (HPLC grade)	Sigma-Aldrich Ltd
Apoferritin from equine spleen (443kDa)	Sigma-Aldrich Ltd
Ammonium molybdate	Sigma-Aldrich Ltd
Ammonium persulphate	Fisher Scientific Ltd
Amphotericin B	Sigma-Aldrich Ltd
Apoptosis Kit	Biolegend
Bathophenanthrolinedisulfonic acid	Sigma-Aldrich Ltd
Bio-Rad DC^{TM} protein assay	Bio-Rad
Bovine serum albumin (BSA)	Invitrogen Ltd
Benzalkonium chloride	Sigma-Aldrich Ltd
Beta-hydroxytoluene	Sigma-Aldrich Ltd
Beta-Mercaptoethanol	Fisher Scientific Ltd
Butanol	Sigma-Aldrich Ltd
Cathepsin B (43 kDa) (Native human cathepsin B)	Abcam
Cathepsin D (45 kDa)(from human liver)	Sigma-Aldrich Ltd
Cell staining buffer	Biolegend
Chelex-100	Sigma-Aldrich Ltd
Cholesterol esterase from Pseudomonas sp.	Sigma-Aldrich Ltd
Cholesterol linoleate hydroperoxide	Cayman chemicals

Coomassie brilliant blue R	BDH Chemicals Ltd
Copper sulphate	Fisher Scientific Ltd
Cysteamine hydrochloride	Sigma-Aldrich Ltd
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Fisher Scientific Ltd
Diethylenetriaminepentaacetate (DTPA)	Sigma-Aldrich Ltd
Dihydroethidium	Sigma-Aldrich Ltd
Dipotassium hydrogen orthophosphate	Fisher Scientific Ltd
Disodium hydrogen orthophosphate	Fisher Scientific Ltd
Dulbecco's modified Eagle's medium	Invitrogen Ltd
Ethanol	Sigma-Aldrich Ltd
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sigma-Aldrich Ltd
F10 medium	Invitrogen Ltd
Ferric chloride	Sigma-Aldrich Ltd
Ferrous sulphate	Sigma-Aldrich Ltd
Ferritin from equine spleen (440kDa)	Sigma-Aldrich Ltd
Fetal calf serum (FCS)	Sigma-Aldrich Ltd
Fluorescence mounting medium	Global Science
Folin and Ciocalteu phenol reagents	Fisher Scientific Ltd
Glycerol	Fisher Scientific Ltd
Glycine	Fisher Scientific Ltd
GM-CSF	Sigma-Aldrich Ltd
Hexane (HPLC grade)	Sigma-Aldrich Ltd
Human Hepcidin-25 peptide	Abcam
Human serum	Sigma-Aldrich Ltd
Hydrochloric acid	Fisher Scientific Ltd
Hydrogen peroxide	Fisher Scientific Ltd
Isopropanol (HPLC grade)	Fisher Scientific Ltd

7-Ketocholesterol	Sigma-Aldrich Ltd
Magnesium chloride	Sigma-Aldrich Ltd
Methanol (HPLC grade)	Sigma-Aldrich Ltd
3-N-[Morpholinopropane] sulphonic acid	Fisher Scientific Ltd
N,N′-Diphenyl,1,4-phenylenediamine (DPPD)	Sigma-Aldrich Ltd
N, N,N,N’-tetraacetylenediamine	Sigma-Aldrich Ltd
Paraformaldehyde	Sigma-Aldrich Ltd
Penicillin/streptomycin	Sigma-Aldrich Ltd
Potassium acetate	Sigma-Aldrich Ltd
Potassium bromide	Fisher Scientific Ltd
Potassium chloride	Fisher Scientific Ltd
Potassium dihydrogen orthophosphate	Fisher Scientific Ltd
Potassium hydroxide	Fisher Scientific Ltd
Potassium iodide	Sigma-Aldrich Ltd
Potassium sodium tartrate	Fisher Scientific Ltd
RPMI	Invitrogen Ltd
Sodium acetate	Sigma-Aldrich Ltd
Sodium azide	Sigma-Aldrich Ltd
Sodium carbonate	Fisher Scientific Ltd
Sodium chloride	Fisher Scientific Ltd
Sodium deoxycholate	Sigma-Aldrich Ltd
Sodium dihydrogen orthophosphate	Fisher Scientific Ltd
Sodium hydrogen carbonate	Fisher Scientific Ltd
Sodium hydroxide	Fisher Scientific Ltd
Sodium dodecyl sulphate	Sigma-Aldrich Ltd
Sphingomyelinase from Bacillus cereus	Sigma-Aldrich Ltd
Tris-HCl	Fisher Scientific Ltd

Triton X-100	Sigma-Aldrich Ltd

Appendix 2: Quantity of iron contained in ferritin.

Ferritin 15 µl of 53 mg protein/ml obtained from Sigma digested in 1ml nitric acid (5M) and made up to 50 ml was analysed using AAS.

Atomic weight of Fe is 55.84

1M Fe is 55.84 g/l

Top standard (5 mg/l) = 89.54 µM

Linear trend line equation is **Y= 0.0358x - 0.0004**

0.0358x - 0.0004 = Y

0.0358x = Y + 0.0004

x = (Y + 0.0004) / 0.0358

Where Y=0.07186 (mean absorbance of sample)

x = (0.07186 + 0.0004) / 0.0358 = 2.018mg/l

5 mg/l = 89.54 µM

2.018 mg/l = 36.14 µM

36.14 µM = 36.14 µmol/l

1 l contains 36.14 µmol

50 ml of ferritin contains 1.807 µmol Fe

15 µl of ferritin solution has 1.807 µmol Fe

1 ml of ferritin solution = (1000 x 1.807) /15 = 120.5 µmol Fe

1 ml of ferritin has 120.5 µmol

The ferritin concentration obtained from Sigma is 53mg protein/ml

53mg has 120.5 µmol Fe

1 g protein = 120.5 x 1000 / 53 =2,274 µmol Fe

= 2.274 mmol

Molecular weight of ferritin obtained from Sigma is 440kDA

440,000g protein has 440,000 x 2.274 mmol Fe =1,000,560 mmol Fe = 1,001 mol Fe

Therefore 1,001 atoms of Fe per ferritin particle.

Concentration of iron in ferritin in the spectrophotometer

1 M ferritin = 1001 M Fe

1 µM ferritin = 1001 µM Fe
0.05 µM ferritin contains 50.05 µM Fe
0.1 µM ferritin contains 100.1 µM Fe
0.2 µM ferritin contains 200.2 µM Fe

Appendix 3: Quantity of iron contained in Apoferritin.

Apoferritin 35.5 µl of 25 mg protein/ml obtained from Sigma digested in 1ml nitric acid (5M) and made up to 10 ml mark was analysed using AAS.

Atomic weight of Fe is 55.84

Top standard (5 mg/l) = 89.54 µM

Linear trend line equation is **Y= 0.0509x - 0.0031**

0.0509x - 0.0031 = Y

0.0509x = Y + 0.0031

x = (Y + 0.0031)/ 0.0509

Where Y=0.00646 (mean absorbance of sample)

x = 0.00646 + 0.0031 / 0.0509= 0.188mg/l

5 mg/l = 89.54 µM

0.188 mg/l = 3.36µM

3.36 µM = 3.36µmol/l

1 l contains 3.36 µmol

10 ml of apoferritin nitric digest contains 0.0336 µmol

35.5 µl of apoferritin has 0.0336 µmol

1 ml of apoferritin = 1000 x 0.0336 /35.5 = 0.9465µmol

1 ml of apoferritin has 0.9465 µmol

The apoferritin concentration obtained from Sigma is 25mg protein/ml

25mg apoferritin has 0.9465 µmol Fe

1 g protein = contains 0.9465 x 1000 / 25 = 37.86µmol Fe

= 0.03786mmol Fe

1g of protein has 0.03786 mmol of Fe

M Wt of apoferritin= 443kDA

443,000g =443,000 x 0.03786 mmol Fe =16,772 mmol Fe = 17 mol Fe

Therefore 17 atoms of Fe per apoferritin particle

1 M apoferritin = 17 M Fe

1 µM apoferritin = 17 µM Fe
0.2 µM apoferritin contains 3.4 µM Fe
0.1 µM apoferritin contains 1.7 µM Fe
0.05 µM apoferritin contains 0.85 µM Fe