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Ralefatane, Maile George

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DETERMINING THE *IN VITRO* LEVEL OF FOAM CELL FORMATION IN DIFFERENT SOUTH AFRICAN POPULATIONS

Maile George Ralefatane

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg 2018

DECLARATION

I, Maile George Ralefatane declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Signed by 

04th Day of OCTOBER 2018 in WITS MEDICAL SCHOOL

DEDICATION

I am dedicating this project to **Almighty God** and my family especially my late mother **Mpapedi Grace Ralefatane** who once told me that "Academic excellence is not a race to be won but a height to be attained,"

"Doing a lot more does not mean you getting a lot more done, don't confuse movement with progress"

Denzel Washington

CONFERENCE CONTRIBUTIONS

Society of Endocrinology, Metabolism and Diabetes of South Africa (51st SEMDSA Congress), 2016 April (Cape Town, RSA)

- Maile G Ralefatane, Eleanor Cave and Nigel J Crowther. Development of a method for studying the *in vitro* formation of foam cells from human monocytes (Poster Presentation)

University of the Witwatersrand Faculty of health sciences research day & postgraduate expo, 2016 September (Johannesburg, RSA)

- Maile G Ralefatane, Eleanor Cave and Nigel J Crowther. Development of a method for studying the *in vitro* formation of foam cells from human monocytes (Poster Presentation)

Society of Endocrinology, Metabolism and Diabetes of South Africa (52nd SEMDSA Congress), 2017 April (Johannesburg, RSA)

- Maile G Ralefatane, Eleanor Cave and Nigel J Crowther. Comparing the *in vitro* level of foam cell formation in young and older adults (Oral Presentation)

23rd International Congress of Clinical Chemistry and Laboratory Medicine (IFCC Worldlab), 2017 October (Durban, RSA)

- Maile G Ralefatane, Eleanor Cave and Nigel J Crowther. Comparing the *in vitro* level of foam cell formation in young and older adults (Poster Presentation)

ABSTRACT

Aim: A critical event in atherosclerosis is the accumulation of oxidised (ox) LDL-laden foam cells (FCs) derived from macrophages within the arterial wall. Macrophages are transformed to FCs by the uptake of oxLDL by scavenger receptors i.e. MSR-1, CD36 and CD68. Atherosclerosis differs in prevalence across genders (more common in males) and ethnic groups (less common in African than Asian-Indian or European populations). Therefore, the aim of this study was to compare FC formation across genders and ethnicities to determine if gender and ethnic differences in the prevalence of atherosclerotic diseases are mirrored by similar differences in the level of *in vitro* FC formation.

Methods: Forty one subjects were recruited into 2 groups; a younger group (n=11; 7 African, 4 European) and an older group (n=30; ten from each of the 3 ethnic groups; 15 males and 15 females). Anthropometry and fasting lipid and glucose levels were measured. Monocytes were isolated from whole blood and converted to macrophages and FCs using standard cell culture procedures. The level of FC formation was determined by measuring intra-cellular lipid accumulation (ICLA) using the lipid-specific dye Oil red O. Macrophage expression of scavenger receptors (MSR-1, CD36, CD68) was measured by quantitative rtPCR.

Results: The ICLA in macrophages exposed to oxLDL (measured as a % of ICLA in unexposed cells) was significantly higher in the older (151.1 ± 27.9%) than the younger group (99.9 ± 22.1%; p=0.0001). No differences in macrophage ICLA were observed between ethnic groups (African, 156.8 ± 28.1%; European, 155.9 ± 57.9%; Indian, 136.8 ± 22.2%; p=0.42) or genders (males, 158.8 ± 48.5%; females; 140.9 ± 25.3%; p=0.24). The expression of all scavenger receptors changed minimally during foam cell formation and did not reflect the gender and ethnic differences in the prevalence of atherosclerosis. Baseline (before addition of oxLDL) macrophage expression levels of CD36, CD68 and MSR1 correlated positively with

serum LDL-C ($p<0.05$ for MSR1 and CD36) and triglyceride levels ($p=0.005$; CD68 only), and negatively with HDL-C ($p=0.003$; MSR1 only).

Conclusions: This study shows that older subjects have a higher level of foam cell formation, which may relate to their higher level of atherosclerosis. Gender and ethnic differences in foam cell formation were not observed; whilst expression levels of scavenger receptors did not mimic the gender and ethnic differences observed in the prevalence of atherosclerosis. This suggests that *in vitro* foam cell formation does not mirror *in vivo* formation or that gender and ethnic differences in the prevalence of atherosclerosis are not due to similar differences in foam cell formation. The small effect of oxLDL on scavenger receptor expression suggests that scavenger receptor levels may increase before foam cell formation occurs i.e. during the transformation from monocytes to macrophages. The relationships observed between serum lipid levels and basal scavenger receptor expressions are novel and may reflect an ability of serum lipids to modulate scavenger receptor expression in macrophages even before exposure to oxLDL.

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- Lastly the participants for their willingness to participate in this study.

LIST OF ABBREVIATIONS

ACAT-1: Acyl-CoA: cholesterol acyltransferase-1

BMI: body mass index

CAD: coronary artery disease

CE: cholesteryl ester

CETP: cholesteryl ester transfer protein

CVD: cardiovascular disease

ECM: extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FCs: foam cells

FPP: farnesyl pyrophosphate

GPP: geranyl pyrophosphate

HDL: high density lipoprotein

HDL-C: high density lipoprotein cholesterol

HL: hepatic lipase

HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA

ICAM-1: intercellular adhesion molecule-1

ICLA: intra-cellular lipid accumulation

IDL: intermediate density lipoprotein

IFN- γ: interferon-gamma

IL: interleukin

IPP: isopentenyl pyrophosphate

LAMP: lysosome-associated membrane protein

LCAT: lecithin cholesterol acyltransferase

LDL: low density lipoprotein

LDL-C: low density lipoprotein cholesterol

LPL: lipoprotein lipase

MCP-1: monocyte chemotactic protein-1

M-CSF: macrophage colony-stimulating factor

MSR1: Macrophage scavenger receptor-1

OxLDL: oxidized low density lipoprotein

OxPL: oxidized phospholipids

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PSGL-1: P-selectin glycoprotein ligand-1

RPMI: Roswell Park Memorial Institute

SRs: Scavenger receptors

SST: separator serum tube

TGF-β1: transforming growth factor-β1

TNF-α: tumor necrosis factor-α

VCAM-1: Vascular cell-adhesion molecule-1

VLA-4: very late antigen- 4

VLDL: very low density lipoprotein

VSMC: vascular smooth muscle cells

WHO: World Health Organization

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CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

Cardiovascular diseases (CVDs) are defined as a group of disorders that involve narrowed or blocked blood vessels that can lead to coronary heart disease or stroke (World Health Organisation, 2015). In 2015, CVD was reported to be the disease responsible for more deaths globally than any other illness (World Health Organisation, 2015). CVD was responsible for approximately 31% of global deaths (comprising of approximately 17.7 million people). Coronary heart disease (narrowing of the blood vessels which supply the heart muscle) and stroke (blockage of the blood vessels supplying the brain) were the primary contributors to CVD related mortality (with deaths of 7.4 and 6.7 million, respectively), (World Health Organisation, 2015). Extrapolating from current data, the WHO predicted by 2030, the number of CVD related deaths will have increased by a further 5.9 million (World Health Organisation, 2015). Atherosclerosis is the major cause of stroke, coronary heart disease and myocardial infarction and in western society (Lusis, 2000). Cholesterol plays an important role in the aetiology of the atherosclerotic plaque, and its metabolism will therefore be described in the following sections.

1.2. ATHEROSCLEROSIS AND CHOLESTEROL

Atherosclerosis is defined as "a chronic inflammatory disease characterized by lipid and cholesterol accumulation within the walls of the arteries" (Lusis, 2000). Cholesterol is located in the cellular plasma membrane, where it functions in both lipid raft assembly, lipid raft function and membrane fluidity (Simons and Ikonen, 2000), however, it is the excess of cholesterol in the blood which contributes to the development of atherosclerotic lesions (Fenyo and Gafencu, 2013).

1.2.1. Cholesterol

Cholesterol is a 27-carbon tetracyclic molecule which can either be synthesised *de novo*, or absorbed from the diet (Ikonen, 2006). It is then either transported through the circulation in lipoprotein particles, stored in the cells in the form of cholesteryl esters or metabolised (Lusis et al., 2004). The lipoprotein particles can be subdivided into two broad groups namely low density lipoprotein (LDL) and high density lipoprotein (HDL) (Ikonen, 2006).

1.2.2. Cholesterol synthesis

Cholesterol synthesis takes place primarily in endoplasmic reticulum of the liver, however it begins with the transport of mitochondrial acetyl-CoA to the cytosol (Ikonen, 2006). In the cytosol both acetyl-CoA and Acetoacetyl-CoA are converted through HMG-CoA synthase action to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), (Ikonen, 2006). HMG-CoA reductase then catalyses the conversion of HMG-CoA to mevalonate. Mevalonate is converted to the isoprene-based molecule, isopentenyl pyrophosphate (IPP), catalysed by mevalonate kinase, and phosphomevalonate kinase (Ikonen, 2006). Isopentenyl pyrophosphate condenses with geranyl pyrophosphate (GPP) to generate farnesyl pyrophosphate (FPP) and this reaction is catalysed by farnesyl diphosphate synthase (Szkopińska and Plochocka, 2005). The enzyme

squalene synthase catalyses the synthesis of squalene from FPP (Pandit et al., 2000). Squalene then undergoes cyclization in a 2-step process to yield lanosterol. The first cyclization reaction results in the production of squalene-2, 3-epoxide, a reaction that is catalysed by squalene epoxidase. Squalene-2, 3-epoxide is then converted to lanosterol through the action of the enzyme lanosterol synthase (Wang et al., 2009, Trapani et al., 2012). Finally lanosterol is converted to cholesterol with the reaction catalysed by delta (24)-sterol reductase (Wang et al., 2009) (Figure 1.1).

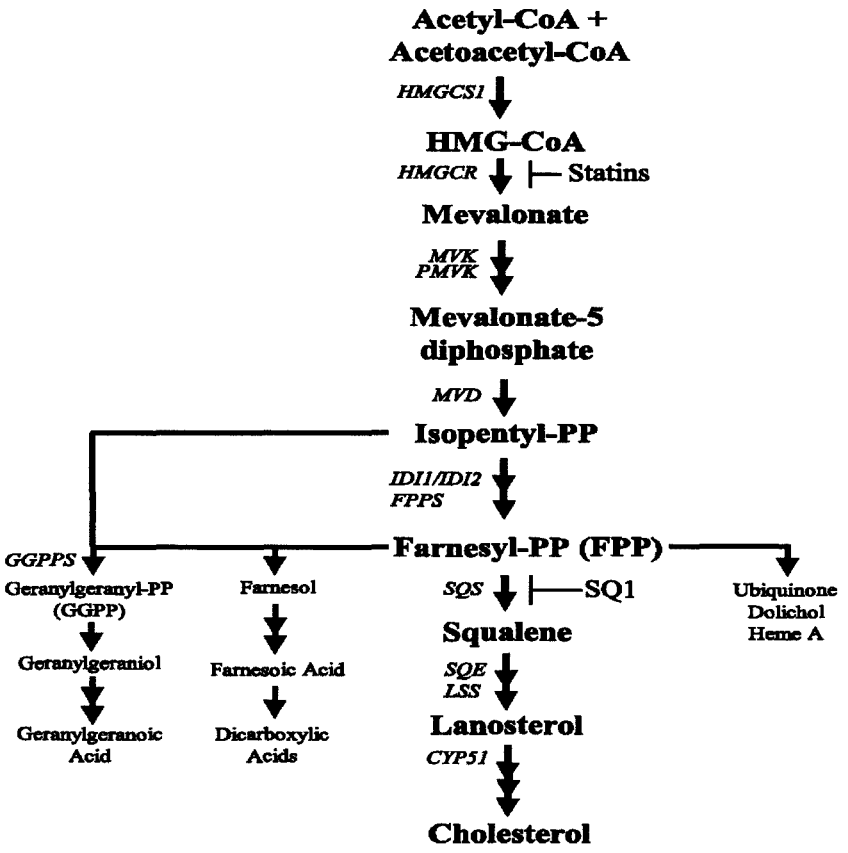


Figure 1.1. Pathway of cholesterol synthesis. *The synthesis of cholesterol is divided into five steps. It begins with the conversion of acetyl-CoA/acetoacetyl-CoA to HMG-CoA. The HMG-CoA is converted to mevalonate and mevalonate is converted to IPP. IPP is then converted to squalene and finally squalene is converted to cholesterol. The rate limiting step in cholesterol synthesis is the conversion of acetyl-CoA to HMG-CoA (catalysed by HMG-CoA synthase), (Rondini et al., 2016).*

1.2.3. Cholesterol transport

Cholesterol is either transported to tissues of the body for use, or stored intracellularly (Ikonen, 2006). Prior to intracellular storage, cholesterol is converted into cholesteryl esters by Acyl-CoA: cholesterol acyltransferase-1 (ACAT-1) (Chang et al., 2001). For cholesterol to be transported in the blood, it needs to be converted to cholesteryl esters and be associated with lipoproteins. There are different types of lipoproteins which are involved in cholesterol transport, namely, very low density lipoprotein (VLDL), chylomicron, low density lipoprotein (LDL), and high density lipoprotein (HDL) (Ikonen, 2006). Cholesterol which is taken in through the diet, is absorbed by the small intestine, packaged into chylomicrons and transported to the tissues. LDL is the most important lipoprotein in the circulation and functions to carry cholesterol from the liver to peripheral tissues (Ikonen, 2006). Reverse cholesterol transport is mediated by HDL, and is the process whereby unwanted cholesterol is trafficked from the peripheral tissues back to the liver, where it is then excreted (van der Velde, 2010) (Figure 1.2).

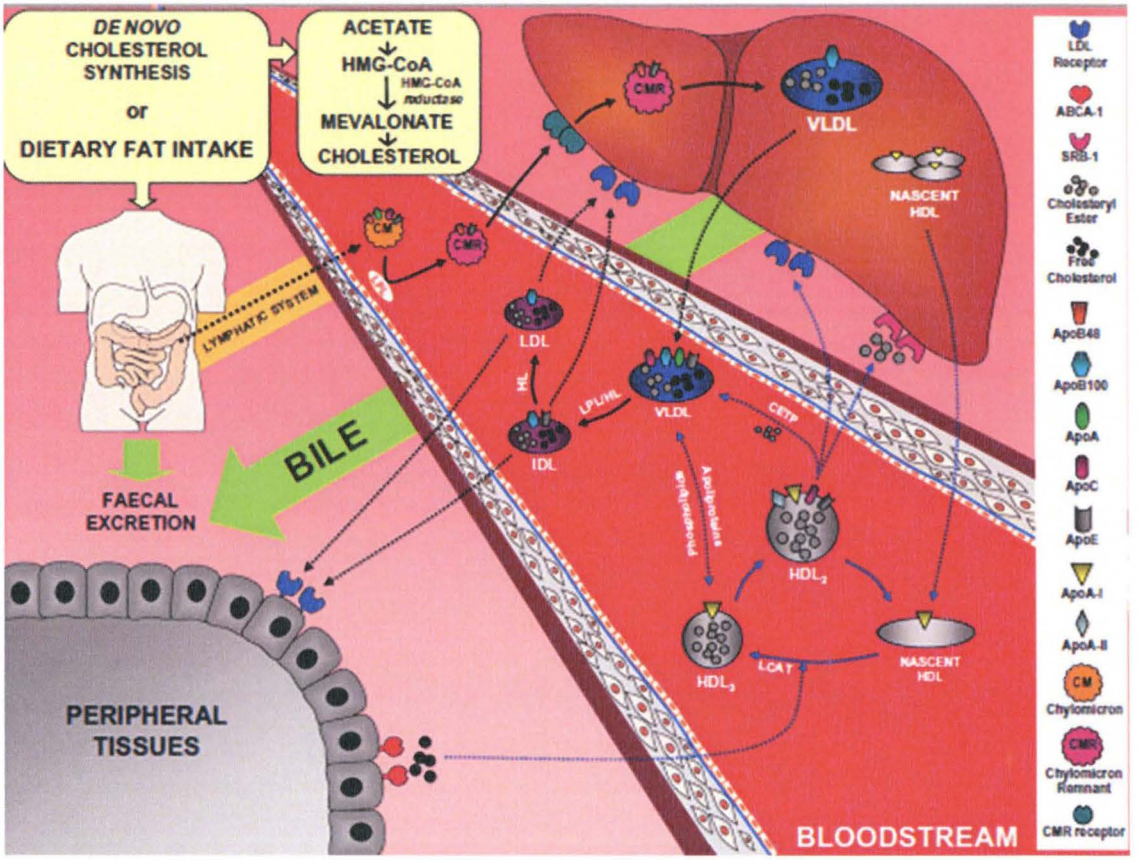


Figure 1.2. Lipoprotein mediated cholesterol transport. *Dietary lipids are absorbed in the small intestine where they are packaged into chylomicrons. The chylomicrons are transported to the blood stream by the lymph; in the blood triacylglycerol is hydrolysed (by lipoprotein lipase (LPL)) to non-esterified fatty acids (FAs) and 2-monoacyl glycerol. Following triacylglycerol hydrolysis the new particle is called a chylomicron remnant. Chylomicron remnant receptors in the liver take up the chylomicron remnants, and hepatocytes then package cholesterol and cholesteryl esters with apo B-100, to form VLDL. After VLDL is transported into the circulation, it acquires HDL derived apo-lipoproteins and is thus converted into IDL through LPL and hepatic lipase (HL) mediated hydrolysis of triacylglycerol. Further triacylglycerol hydrolysis by the actions of HL results in IDL being converted into LDL. The apo B-100 on IDL and LDL attach to LDL receptors on cell membranes (following transport through junctions between capillary endothelial cells). Synthesis of cholesterol-free HDL within the liver facilitates the return of cholesterol from peripheral tissues to the liver. Thus, within the circulation free cholesterol from hepatic cells, chylomicrons VLDL obtained by nascent HDL and are therefore converted to HDL3. Lecithin cholesterol acyltransferase (LCAT) esterifies cholesterol and the resultant cholesteryl esters are transferred to remnant lipoproteins by cholesteryl ester transfer protein (CETP) in exchange for triglyceride. The liver then removes the remnant particles from circulation and it is then excreted in bile. (McLaren et al., 2011).*

1.3. PATHOLOGY OF ATHEROSCLEROSIS

1.3.1. Elevated plasma LDL cholesterol concentrations and atherosclerosis

Factors believed to cause endothelial dysfunction during atherosclerosis include elevated levels of modified LDLs, free radicals, hypertension, smoking, genetic factors, homocysteinemia and infections (Ross, 1999, Libby et al., 2002, Packard and Libby, 2008).

Accumulation and retention of LDL and lipoprotein remnants in the sub-endothelial intima are the major cause of atherosclerosis (Lusis, 2000, Tabas et al., 2007). The retention of LDL with oxidative modification at the sub-endothelium represents the initial event in the atherogenesis, the presence of the modified LDL results is the activation of blood inflammatory cells and subsequent infiltration at the sub-endothelium (Pirillo et al., 2013). The major transport of cholesterol to body tissues is LDL, and increased LDL-cholesterol is known to be a risk factor for CVD (Trpkovic et al., 2014). When the plasma levels of LDL are elevated, the LDL retention takes place more at the areas of arterial branching (Lusis, 2000).

Following transport of LDL to the artery wall (which is an antioxidant-poor environment) as described in Figure 1.3, LDL becomes susceptible to self-aggregation, leading to the formation of lipid deposits which are trapped in the extracellular matrix and exposed to oxidative stress (Dhaliwal and Steinbrecher, 1999, Kaplan and Aviram, 1999). The arterial endothelium is activated to secrete immuno-regulatory peptides by an initial immune response directed to the LDL trapped in the intima (Tabas, 2010), and this in turn triggers a chronic inflammatory response. Monocytes are then recruited to the lesion site and induce proliferation of monocyte-derived macrophages, which contribute to the inflammatory response (Hansson, 2001, Gui et al., 2012).

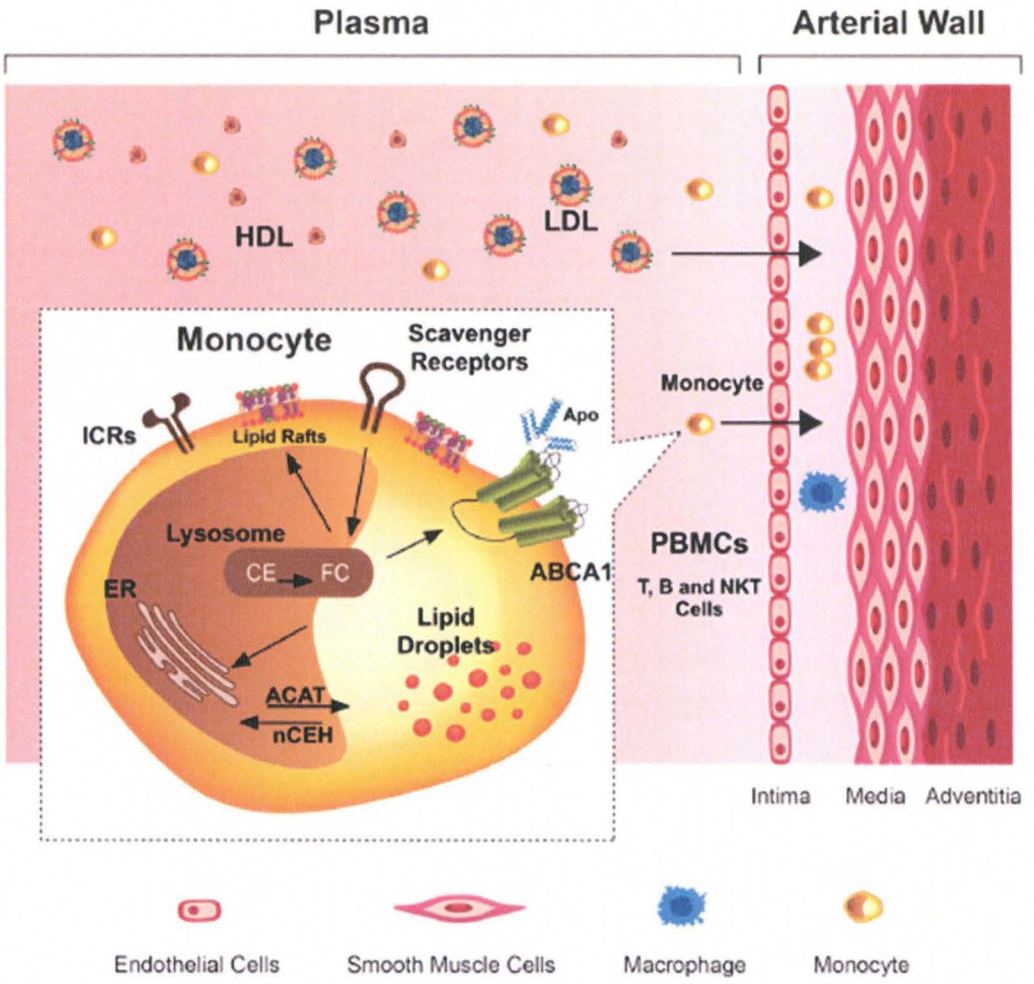


Figure 1.2. Increased plasma LDL cholesterol concentrations influence foam cell formation. *Increased plasma LDL concentrations together with low HDL concentrations result in peripheral blood mononuclear cells (yellow) becoming cholesterol enriched. These monocytes adhere to damaged endothelium, and subsequently move into the artery intima where they become macrophages (blue). Immune cells (T, B and Natural killer T cells) are then recruited to the intima where they multiply. This causes an infusion of LDL into the intima, the LDL then aggregates and becomes oxidized. During normal levels of cholesterol influx in monocytes, there is hydrolysis of lipoprotein cholesteryl ester (CE) in the lysosome to free cholesterol (FC). Free cholesterol then moves to the endoplasmic reticulum where ACAT re-esterifies it to form CE. The CE are then stored in cytoplasmic lipids droplets. The FC can also accumulate in lipid rafts in the plasma membrane from where it can be transported to the liver via lipoprotein particles. When cholesterol influx rates are excessive, FC cannot be esterified, and lipid raft FC cannot be eliminated and this leads to high intra-cellular levels of FC and more immune cell activation causing chronic inflammatory states, and the formation of foam cells (Sorci-Thomas and Thomas, 2016).*

1.3.2. Recruitment and entry of monocytes into arterial walls

Monocytes are bone marrow-derived circulating leukocytes (Geissmann et al., 2003). Typically, monocytes act to phagocytose pathogens or toxins which are digested and then presented as antigens on the cell surface. In addition, monocytes secrete chemokines, and proliferate in response to infection and injury (Chiu and Bharat, 2016), however more is known about their recruitment into the intima during atherogenesis (Bobryshev, 2006). During the development of atherosclerosis, in the intima, LDL is exposed to oxidative agents that cause its conversion into oxidised LDL (oxLDL). The accumulation of oxLDL in the sub-endothelial intima causes the release of endothelial-derived chemokines such as monocyte chemotactic protein-1 (MCP-1), which attract circulating monocytes to the site of activation (Li and Glass, 2002).

Monocytes adhere to endothelial adhesion molecules such as P-selectin glycoprotein ligand-1 (PSGL-1) and endothelial selectins (Mestas and Ley, 2008). Then overlapping of E-selectin with P-selectin support 'rolling' of monocytes across the endothelial surface (Kumar et al., 2001). Vascular cell-adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and very late antigen- 4 (VLA-4) slow rolling of the monocytes by causing tighter adhesion (Galkina and Ley, 2007). Finally, monocyte migration is facilitated by endothelial-expressed chemokines (MCP-1 and macrophage colony-stimulating factor (M-CSF)) and cytokines (interleukin-8 (IL- 8) and tumour necrosis factor-α (TNF-α)), which mediate trans-endothelial diapedesis at cell–cell junctions (Bobryshev, 2006, Weber et al., 2007) (Figure 1.4).

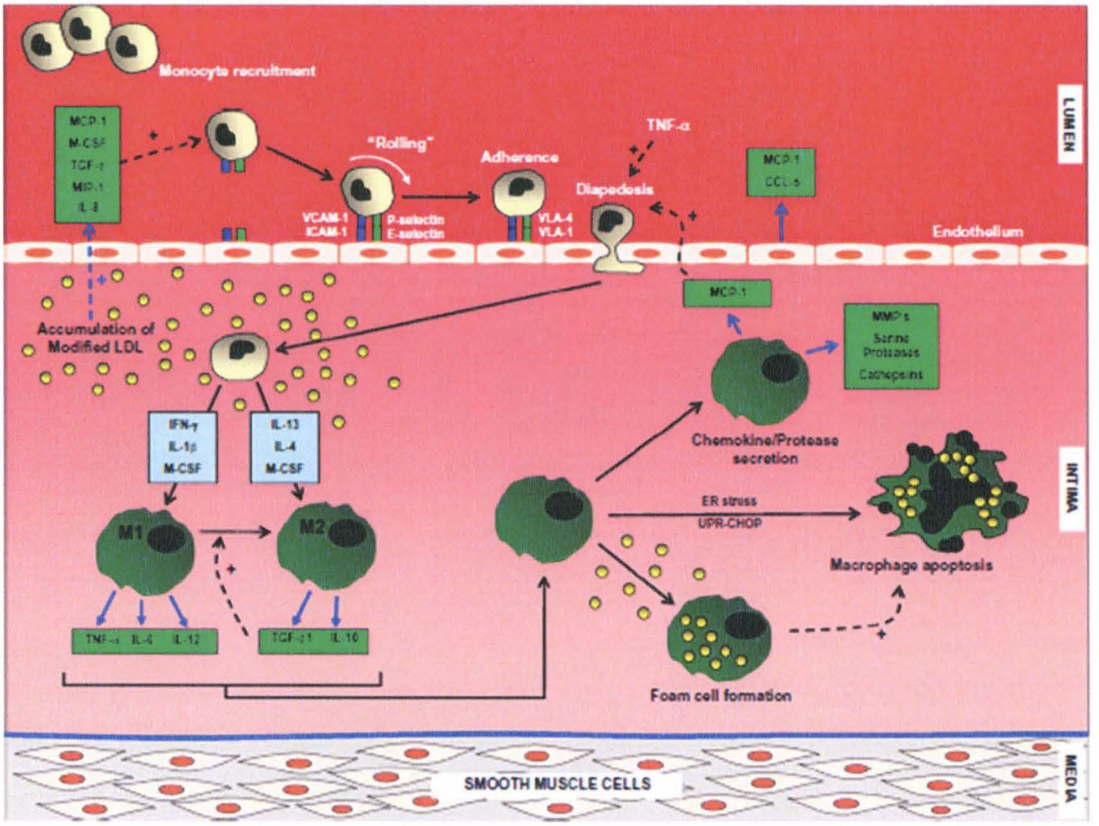


Figure 1.4. Monocyte recruitment and macrophage function. *The attraction of monocytes to the site of activation (endothelium) is due to chemokines such as MCP-1, which are released by endothelium through its activation by accumulation of modified LDL in the sub-endothelial intima. This is followed by rolling and adhesion of monocytes to endothelial adhesion markers such as VCAM-1 and P-selectin, and finally monocytes migrate through endothelium by trans-endothelial diapedesis. Within the sub-endothelial intima monocytes differentiate into two subsets of macrophages, M1 and M2. Their production is dependent on the presence of specific cytokines, with M1 differentiation activated by T_h1 cytokines such as IFN-γ and IL-1β and M2 differentiation activated by T_h2 cytokines such as IL-4 and IL-13. During atherosclerosis macrophages ingest native and modified LDL particles to become cholesterol-rich foam cells. In advanced atherosclerotic lesions macrophages undergo apoptosis, where free cholesterol accumulation in foam cells activates apoptotic signals such as the ER stress pathway (McLaren et al., 2011).*

1.3.3. Monocyte differentiation into M1 and M2 subsets of macrophages

Monocytes differentiate into macrophages, within the arterial intima, through a process regulated by M-CSF. Macrophages are phagocytic cells, residing in all tissues, which are involved in the removal of cellular debris generated by tissue remodelling and cellular necrosis. They are derived from bone marrow myeloid progenitor cells, and they play a key role in the inflammatory response (Mosser and Edwards, 2008). Monocytes differentiate into either M1 or M2 macrophages depending on the type of chemokines to which the monocyte is exposed (Shimada, 2009). T-helper 1 (T_h1) cytokines, IFN-γ and IL-1β, play a key role in producing M1 macrophages and the T_h2 cytokines, IL-4 and IL-13, are important for M2 macrophage production (Shimada, 2009, Martinez et al., 2009, Tabas, 2010).

These two subsets of macrophages found within atherosclerotic lesions, namely M1 and M2. Interestingly, these macrophages have opposing roles in the inflammatory process. The M1 macrophages promote inflammation by secreting pro-inflammatory cytokines (IL-2, IL-23, IL-6, IL-1, and TNF-α), while M2 macrophages resolve inflammation through the secretion of anti-inflammatory cytokines (IL-10 and transforming growth factor-β1 (TGF-β1)). TGF-β1 is known to mediate the conversion of M1 macrophages into M2 macrophages. TGF-β1 is further known to enhance efferocytosis (Shimada, 2009, Martinez et al., 2009, Tabas, 2010) (Figure 1.4). It is believed that an imbalance in the M1: M2 ratio contributes to the development of the plaque and impaired resolution of the inflammatory state, as a result of increased levels of the T_h2 cytokines (Tabas, 2010).

1.3.4. Macrophage function and foam cell formation

Within the context of atherosclerosis, macrophages recruited to the atherogenic lesion become cholesterol-rich "foam cells" through the ingestion of trapped modified LDL or lipoprotein remnants within the intima (Li and Glass, 2002, Bobryshev, 2006). These lipid dense macrophages are termed foam cells (FCs) due to the foamy appearance they exhibit under a microscope (Mallat, 2014). The rate-limiting step in the development of fatty streaks (visible lesion in atherosclerosis) is the formation of FCs and the tendency to form FCs have been reported to differ among individuals (Asmis and Jelk, 2000).

Numerous studies have shown that oxidatively modified low density lipoprotein (oxLDL) can induce macrophage differentiation and FC formation (Dhaliwal and Steinbrecher, 1999, Kaplan and Aviram, 1999, Jessup and Kritharides, 2000). The formation of oxLDL primarily occurs within extracellular space under conditions of oxidative stress. However the continuation of oxidation process is facilitated by degradation of LDL particles by lysosomal enzymes within macrophages (Dhaliwal and Steinbrecher, 1999, Kaplan and Aviram, 1999, Jessup and Kritharides, 2000) (see Figure 1.3). The receptor-mediated ingestion of oxLDL transforms macrophages into foam cells (Dhaliwal and Steinbrecher, 1999, Kaplan and Aviram, 1999, Jessup and Kritharides, 2000). The sub-endothelial accumulation of foam cells leads to the formation of fatty streaks, a marker of early atherosclerosis (Trpkovic et al., 2014).

OxLDL promotes platelet activation, the activation and dysfunction of endothelial cells as well as vascular smooth muscle cell (VSMC) migration and proliferation (Trpkovic et al., 2014).

1.3.5. Macrophage scavenger receptor expression

Scavenger receptors (SRs) are membrane-bound receptors that bind and internalize modified LDL. Identification of these SRs in macrophages was first described in the 1970s by Brown and Goldstein (Prabhudas et al., 2014). Macrophages express several SRs and these cell surface receptors are responsible for internalisation of oxLDL. Scavenger receptors, CD36, MSR1, and CD68 are the main receptors involved in the binding and uptake of oxLDL into macrophages (Matloubian et al., 2000).

The CD36 SR is an 88-kDa transmembrane glycoprotein receptor that belongs to the class B SR family (Febbraio et al., 2001). It is encoded by the human *Cd36* gene, which is localised on chromosome 7 and consists of 15 exons (Silverstein et al., 2010, Liani et al., 2012). The gene has an extracellular domain flanked by two transmembrane and two cytoplasmic domains (Hoosdally et al., 2009). It binds many different ligands including thrombospondin-1, oxidized phospholipids (oxPL), oxLDL, and long-chain fatty acids (Silverstein and Febbraio, 2009). However it has been shown that CD36 possesses specific and high affinity binding for oxLDL (Handberg et al., 2012). The oxLDL-binding region of CD36 is located between amino acids 155 and 183 (Navazo et al., 1996). The CD36 SR is expressed on various cell types including monocytes and macrophages and its expression levels are increased by a fat-rich diet, inflammation and oxidative stress (Silverstein et al., 2010, Liani et al., 2012).

Macrophage scavenger receptor-1 (MSR1) is a 77-kDa cell surface glycoprotein and is a member of the class A SR family (Dai et al., 2012). The human and murine *MSR1* genes are located on chromosome 8. It is highly expressed in macrophages but is also present on vascular smooth muscle and endothelial tissues and it mediates the uptake of oxLDL into macrophages (Mietus-Snyder et al., 1997). Inhibition of MSR1 in macrophages significantly decrease foam cell formation and atherosclerosis (Dai et al., 2012).

The CD68 molecule is a 110-kDa glycoprotein that is a member of the class D SR family (Song et al., 2011). The *CD68* gene is located on human chromosome 17 and it is highly expressed by monocytes and tissue macrophages (Li et al., 1998, Yoshida et al., 1998b). This receptor contains three domains which include N-proximal mucin-like domain, lysosome-associated membrane protein (LAMP) domain and a short 12-residue cytoplasmic domain. (Holness and Simmons, 1993). The CD68 SR is able to bind oxLDL, lectins, selectins and further initiates phagocytosis (Ramprasad et al., 1996, da SILVA and Gordon, 1999).

The MSR1 and CD36 molecules have been identified as the SRs responsible for the uptake of modified lipoproteins into macrophages. The interaction of CD68 with modified lipoproteins is still controversial and their relevance is still unclear (Kunjathoor et al., 2002). Studies have reported that blocking both MSR1 and CD36 significantly reduces the uptake of oxLDL (Sugano et al., 2001, Kunjathoor et al., 2002), while another study reported that reduction in CD68 levels caused little change in oxLDL uptake (De Beer et al., 2003).

1.3.6. Smooth muscle cell migration and plaque progression

Development of fatty streaks into plaques leads to the production of lesions that are covered with a fibrous cap which is composed primarily of elastin, collagen and VSMC (Katsuda and Kaji, 2003). The fibrous cap surrounds a necrotic core containing modified LDL, cholesterol and apoptotic/necrotic cells. These cells are unable to survive in the nutrient sparse environment found in the lesion. The formation of the fibrous cap is dependent on the migration and proliferation of VSMC, and this is highly regulated by elastin, collagen and cholesterol released by resident macrophages, endothelial cells and T-cells (Newby, 2006) (Figure 1.5).

Interaction between T-cells and CD40, which is expressed on macrophages play a key role in the migration of VSMC (Lusis, 2000). Activation of CD40 promotes macrophages to release

chemokines such as IL-8 and the inflammatory cytokines IL-1 and interferon-γ (IFN- γ). This causes VSMC to change from its phenotype from contractile to an active, synthetic state, allowing their migration into the intima (Schonbeck et al., 2000, Rudijanto, 2007). The activation of CD40 also causes the release of extracellular matrix (ECM) degrading proteases which alter the ECM allowing for VSMC migration and proliferation to take place (Newby and Zaltsman, 1999, Lusis, 2000). As the fibrous cap develops, the lesion becomes more complex, stabilises with an enlarged necrotic core. The equilibrium level between degradation and synthesis of ECM determines the stability of the plaque. Therefore when the equilibrium is lost, the plaque stability is threatened and the risk of rupture and thrombosis increases. The plaque stability is also determined by the level of VSMC apoptosis due to ECM degrading enzymes released by apoptotic cells (Newby, 2006).

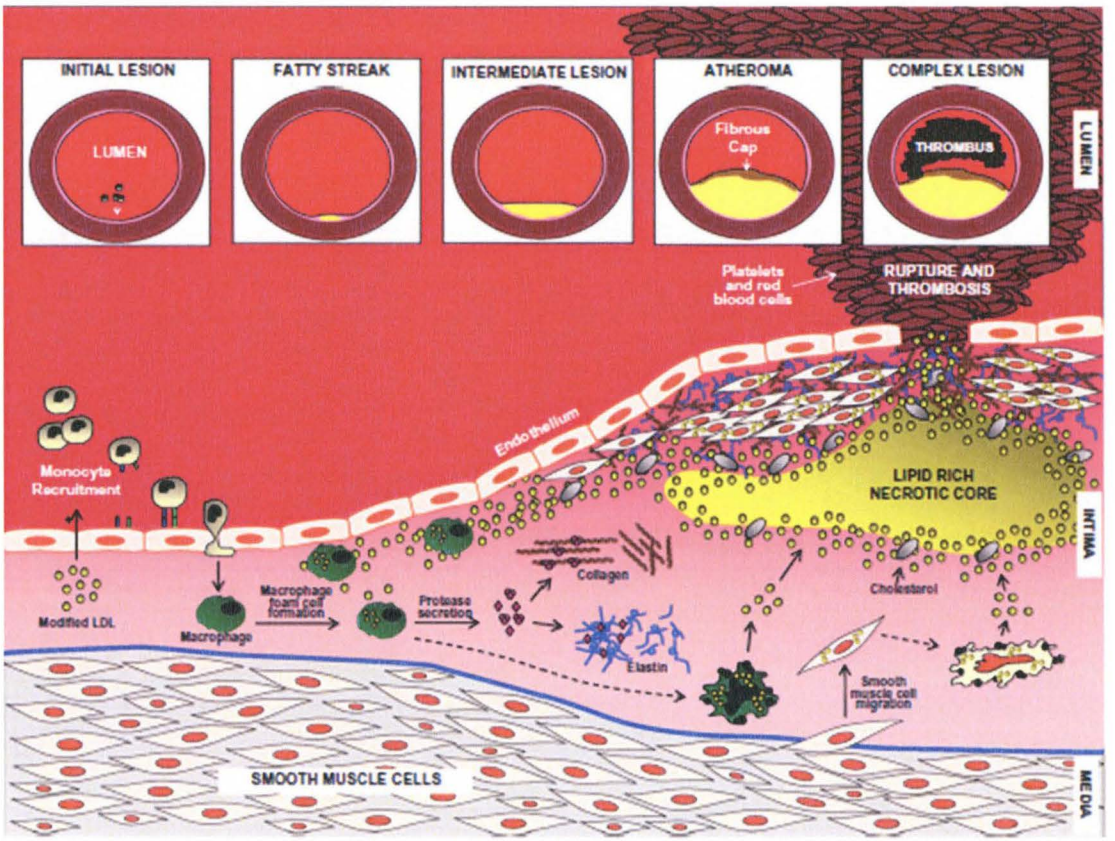


Figure 1.5. The development of an atherosclerotic lesion. *Ingestion of modified LDL and conversion into lipid-laden foam cells by resident or differentiated macrophages form fatty streaks at the site of the initial lesion. Collagen and elastin degradation with secretion of foam cell protease results in the formation of a fibrous cab. The release of cellular content from apoptotic foam cells and smooth muscle cells results in LDL and cholesterol accumulation , thus forming an intermediate lesion. Advanced atherosclerotic lesion results from increased inflammation and necrotic enviroment within the atherosclerotic plaque. This causes the fibrous cap to be thickened , and leading to deterioration of the stability of the fibrous cab and thus rupture, resulting in the release of interal contents into the circulation ,causing thrombosis and blockage of the artery (McLaren et al., 2011).*

1.4. RELATIONSHIP OF ETHINICITY, AGE AND GENDER WITH CVD IN SUB-SAHARAN POPULATIONS

In sub-Saharan Africa, despite populations having the youngest global CVD-induced age of death (Moran et al., 2013), individuals of African descent display an athero-protective lipid profile compared to European and Indian populations (Cappuccio, 1997). Indeed, within the sub–Saharan African population, there is a lower level of cardiac disease attributed to dyslipidemia, with the majority of cardiac events attributed to hypertension (Sliwa et al., 2012).

Each popululation (ethnic) group has a distinct risk profiles for the development of cardiac events (Sliwa et al., 2012). South Africa has a multi-ethnic population, consisting primarily of those of African, European and Indian decent. Within South Africa cholesterol-attributable mortality is highest in Indian subjects (22.2%), followed by European (20.5%) and mixed-ancestry (8.8%) population groups. The attributable mortality was lowest in the black African group, accounting for only 1.8% of all deaths (Norman et al., 2007). Another later study in 2014 reported that black African race group had the lowest prevalence of abnormal serum total-cholesterol (24.9%), and LDL-cholesterol (26.4%) than whites and indians (Shisana et al., 2014). The prevalence of coronary artery disease (CAD) in South Africa have been reported to be highest in indian and lowest in African subjects (Walker et al., 1993, Naran et al., 2008).

In addition, CAD is reported to be two to three times more prevalent in men than in women (Edmunds and Lip, 2000), but displays a more prominent increase in women older than 50 years (Mosca et al., 2004). Atherosclerotic disease is more prevalent in older subjects, in males and in those of Asian Indian ancestry however there is no data comparing FC formation across these different groups (Herrington et al., 2016). This data indicates that there is ethnic variation in the development of atherosclerotic disease, and this warrants further investigation.

1.5. STUDY AIMS AND OBJECTIVES

The hypothesis to be tested in this study is that ethnic and gender differences in the risk for atherosclerotic disease are related to ethnic and gender differences in the level of FC formation. Therefore, the aim of this study is to compare FC formation across genders and different South African population groups (Black, Indian and White).

The objectives of the study are as follows:

- Measure *in vitro* FC formation in human monocytes.
- Compare FC formation across genders.
- Compare FC formation across different South African population groups.
- Use regression models to characterise the main determinants of FC formation and scavenger receptor expression.

CHAPTER 2: MATERIALS AND METHODS

2.1. PARTICIPANT RECRUITMENT

Forty one participants were recruited into 2 groups; a younger group (section 2.1.1) and an older group (section 2.1.2). Information on socio-demographic status (education level and occupation) and anthropometric measurements (weight, height, and hip and waist circumferences) were collected for all participants. Body mass index (BMI) was calculated using weight and height (BMI = Weight (kg)/ height^2 (m)). The participant's ethnicity was self-reported. Informed consent (refer appendix A) was obtained from all participants.

2.1.1. Younger group

Eleven Participants aged between 20 and 25 years were recruited to participate in the study. A 45ml aliquot of blood in Ethylenediaminetetraacetic acid (EDTA) tubes (Lasec, Midrand, SA) was collected from each participant for peripheral blood mononuclear cell (PBMCs) isolation. The ethnicity of the participants was Black South African (n=7) and White South African (n=4).

2.1.2. Older group

Thirty participants (≥40 years) volunteered to participate in the study. Ten participants were recruited from each ethnic group (Black South African, White South African and South African Indian). The 3 groups were matched for gender, with 5 males and 5 females in each of the 3 groups. Seven blood tubes (5 x 9ml EDTA – for PBMC isolation; 1x Separator Serum Tube (SST) – for fasting lipograms; and 1x potassium oxalate tube – for fasting glucose) were collected from each participant.

2.1.3. Exclusion criteria

Subjects receiving therapy for high lipid levels, diabetic subjects or those having a history of cardiac problems were excluded from the study.

2.2. DETERMINING FOAM CELL FORMATION DIFFERENCES BETWEEN OLDER AND YOUNGER GROUPS

To determine whether there were age related differences in the ability to form foam cells, we isolated PBMCs (section 2.5) from all 11 of the younger group and 11 of the older subjects who were matched with the younger group in terms of gender and ethnicity. The monocytes were then differentiated into macrophages (section 2.5.4) and induced to form foam cells (section 2.5.5). Foam cell formation was determined through calculating the relative lipid uptake using Oil red O (Sigma, St louis, Missouri, USA) (section 2.6) and compared between the groups. Intra-cellular lipid levels were calculated in both macrophages and foam cells.

2.3. COMPARING FOAM CELL FORMATION BETWEEN DIFFERENT ETHNIC GROUPS

Differences in foam cell formation across ethnicity were compared only in the older group (10 participants from each ethnicity). The PBMCs were prepared (section 2.5), converted to macrophages (section 2.5.4) and induced to accumulate lipids (section 2.5.5) and foam cell formation was determined through calculating the relative lipid uptake using Oil red O (section 2.6) and compared between the ethnicities.

2.4. MEASUREMENT OF FASTING GLUCOSE AND LIPID LEVELS

Both Separator Serum Tubes (SST) (for fasting lipograms) and potassium oxalate tubes (for fasting glucose) were centrifuged at 2360 x *g* for 10 min using an Allegra® X-22R centrifuge (Beckman Coulter, Brea, California, USA). Fasting lipograms and glucose were measured on the Cobas® 8100 (Roche, Basel, Switzerland) auto-analyzer in the routine Chemical Pathology Laboratory, of National Health Laboratory Service (NHLS) based at Charlotte Maxeke Johannesburg Academic Hospital.

2.5. MONOCYTE ISOLATION

Human monocytes were isolated using a modified method based on that described by de Almeida and colleagues (de Almeida et al., 2000). Briefly, 45ml of whole blood in an EDTA Vacutainer tubes was centrifuged at 2360 x *g* for 10 min using an Allegra® X-22R centrifuge. Following centrifugation, the buffy coat layer was collected and diluted in a 1:1 ratio with sterile phosphate buffered saline (PBS [pH 7.2]; Lonza, Basel, Switzerland) in a 50 ml conical tube. The buffy coat-PBS mixture was then layered over an equal volume of Histopaque®-1077 (density 1.077 g/ml, [Sigma]) and centrifuged at 400 x *g* for 30 min without brakes using the Allegra® X-22R centrifuge. The PBMCs, located at the interface of the two phases, were harvested and washed three times in 10 ml warm sterile PBS through centrifugation at 300 x *g* for 10 min. The supernatant was discarded and the pellet (containing the PBMCs) was re-suspended in 1.6 ml Roswell Park Memorial Institute (RPMI) medium (Lonza) supplemented with 10% fetal bovine serum (FBS) (Capricorn Scientific ,Ebsdorfergrund, Germany), 25mM HEPES, 2mM L-glutamine, and 100U/ml Pen/Strep (Lonza). The medium was warmed to 37°C to prevent cell death. The method used for assessment of the purity of the monocytes is described in section 2.5.3. The major problem encountered during isolation was that when

using both Histopaque and Percoll for isolation, the monocyte purity was very low (below 60%), but when using Histopaque only the purity was higher (70-100%).

2.5.1. Cell counting

Re-suspended cells (10µl) were placed into a 1.5ml microcentrifuge tube together with 40µl of 0.4% (w/v) Trypan Blue solution (Sigma). Cells were counted on a haemocytometer (Spencer, Chicago, USA). Briefly, a cover slip was mounted over the haemocytometer counting chamber and the cell/trypan blue mixture was added to the chamber. Cells were visualised under the brightfield using the 10x objective on a Nikon TMS microscope (Nikon, Tokyo, Japan). Three random fields were counted for each sample, the count averaged and multiplied by the dilution factor (5X) and 10^4 to give the number of cells per ml. The cells stained with trypan blue were not included in the count.

2.5.2. PBMC culture

Cells were plated into a 6 well cell culture plate (Nest Biotechnology, Wuxi, China) at a concentration of 2 x 10^6 per well in growth medium (RPMI supplemented with 10% fetal bovine serum (FBS), 25mM HEPES, 2mM L-glutamine, and 100U/ml Pen/Strep). The cells were maintained in an incubator at 37°C and 5% CO_2 in humidified air. After 24 hours of culture the monocytes adhered to the culture plate and non-adherent lymphocytes were removed by rinsing the culture plate with 2 ml growth medium.

2.5.3. Monocyte/macrophage purity

After 24 hours of culture, one well (containing a coverslip) was used for determining the monocyte purity. Briefly cells were fixed with 2ml 4% (w/v) paraformaldehyde (Sigma) (refer appendix C2) for 10 min and then permeabilized with 2ml Tris buffered saline (TBS) plus 0.025% (v/v) Triton X-100 (British Drug Houses (BDH), Poole, UK) (refer appendix C4) for

5 minutes and then incubated in 1ml 1% bovine serum albumin (BSA) (Merck, Darmstadt, Germany) / 10% (w/v) FBS in TBS (refer appendix C4) for 2 h at room temperature to block non-specific protein-protein interactions. The cells were then incubated with the primary antibody diluted in TBS with 1% (v/v) BSA (1/100 dilution, ab22506, 1μg/ml [Abcam, Cambridge, UK]) (refer appendix C4) overnight at 4°C. The primary antibody binds to calprotectin, an intracytoplasmic protein comprising of a 12kD alpha chain and a 14kD beta chain expressed by monocytes and macrophages. After an overnight incubation the cells were rinsed twice for 5 minutes with 2ml TBS/0.025% Triton with gentle agitation. The secondary antibody (goat anti-mouse IgG-complexed with Alexa Fluor® 594 (Invitrogen Molecular probes, California, USA)) was diluted 1:200 in TBS with 1% (v/v) BSA (refer appendix C4), applied to the cells and incubated for 1 h at room temperature in the dark (to avoid photobleaching). The coverslip containing the stained cells was then mounted onto a slide using UltraCruz mounting medium (Santa Cruz, Texas, USA) containing 4', 6-diamidino-2-phenylindole (DAPI), which stains the nuclei. A negative control, incubated with the secondary antibody only was included to rule out any non-specific binding.

Slides were visualised using 20 x objectives on an Olympus XC50 fluorescent microscope (Olympus, Tokyo, Japan). The purity of the monocytes was determined by viewing a random section on the cover slip and counting a hundred cells. Both stained and unstained cells were counted, and then the percentage of stained cells was calculated. The excitation and emission wavelengths of the fluorophore (Alexa) were 590 nm and 617 nm whereas, DAPI which stains the nuclei was viewed at an excitation and emission wavelengths of 358 nm and 461 nm respectively.

2.5.4. Macrophage differentiation

After 24 hours, monocytes were differentiated into macrophages by incubation with 1ml growth media supplemented with macrophage colony-stimulating factor (M-CSF (100 ng/ml) [Biolegend, California, USA]) for 5 days under standard conditions. Macrophage differentiation was confirmed through microscopic detection of morphological changes (elongated or stellate morphology) (van Tits et al., 2011).

2.5.5. Foam cell formation

To induce foam cell formation, macrophages were exposed to copper oxidized human low density lipoprotein ((Cu-oxLDL) [Cellbiolabs, San Diego, USA]) at a concentration of 3.0 µg/ml for 48 hours at 37°C, in 5% CO_2. Foam cell formation was confirmed through morphological examination of the oxLDL exposed macrophage. Cells were classified as foam cells if they exhibited a 'foamy' appearance caused by intracellular lipid accumulation (Van Tits et al., 2011).

2.6. OIL RED O STAINING

Intracellular lipid accumulation was determined using the Oil Red O staining procedure. The same procedure was used for staining lipids in cells grown on cover slips and visualised using a microscope and mounted camera (see section 2.7). Neutral lipid droplets have the ability to bind Oil Red O stain, thus staining the lipids red and allowing for lipid visualisation and quantification. Briefly, medium was removed from the culture plate and cells were rinsed twice with 2ml PBS. Cells were fixed in 4% formaldehyde for 10 minutes at room temperature, and then rinsed once with 2ml PBS. Five (5) ml Oil Red O stain (w/v) (refer appendix C1) solution was added to the cells, and the lipids allowed to stain for 1h 30minutes at room temperature. The stain was removed and the cells rinsed with 2ml of PBS for 2 minutes. Lipids were then visualised as described in section 2.7, or lipid content quantified as described in section 2.8.

2.7. VISUALISATION OF LIPIDS

The coverslip containing cells stained for lipids with Oil Red O were mounted onto a slide using UltraCruz mounting medium and the slide was visualised using 100 x objectives on Olympus XC50 fluorescent microscope (Olympus) under bright field. The XC50 Color Camera was used for recording images.

2.8. LIPID EXTRACTION AND MEASUREMENT

Lipid bound Oil Red O stain was extracted for 10 minutes by the addition of 500µl absolute isopropanol (BDH). The extracted stain was used for spectrophotometric quantification.

In order to perform a relative quantification, the ratio of lipids to cells was determined. To determine relative cell number, the nuclei were stained with crystal violet (Merck) following the extraction of Oil Red O. Briefly, the cells were submerged in 2 ml of 0.01% (w/v) solution of crystal violet (refer appendix C3) in water for 5 minutes. The cells were then washed three times with 1ml PBS and the stain was extracted for 5 minutes using 500µl of absolute ethanol (Merck).

The absorbance of the Oil Red O and crystal violet extracts were measured using a plate reader (Powerwave XS (Biotek, Vermont, USA)) at 510 nm for Oil Red O and 570 nm for crystal violet. Absolute isopropanol and absolute ethanol were used as blanks for the Oil Red O and crystal violet respectively. Using the absorbance readings as markers of lipid content and cell numbers, the ratio of lipids to cells (lipids content [OD at 510nm]/number of cells [OD at 570nm]) was then calculated.

2.9. RNA ISOLATION

The RNA was extracted from macrophages that had been exposed to oxLDL and from macrophages that had not been exposed to oxLDL using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly cells were detached from the plate using cell scrapers (Nest Biotechnology) and 500µl of growth medium was added to the wells and mixed by pipetting up and down. The cell suspension was transferred into a 1.5ml RNAse-free polypropylene centrifuge tube and centrifuged using Mikro 200 centrifuge (Labotec, Midrand, RSA) at 300 x *g* for 5 minutes at room temperature. The supernatant was completely aspirated and the cell pellet was loosened thoroughly by flicking the tube. Cells were disrupted by the addition of 350µl of RLT buffer (lysing of cells) supplied in the kit and homogenized through pipetting up and down. One volume (350µl) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Up to 700µl of the lysate was transferred onto an RNeasy mini column placed into a 2ml collection tube and centrifuged at 8000 x *g* for 15 seconds. Flow through was discarded and 700µl of wash buffer RW1 was added to the column and centrifuged for 15 seconds at 8000 x *g*. The flow through was discarded and 500µl of buffer RPE was added to the column and the tube was centrifuged at 8000 x *g* for 15 seconds. The flow through was discarded and another 500µl of buffer RPE (removes traces of salt from the column) was added to the column and the tube was centrifuged for 2 minutes at 8000 x *g* to wash the spin column membrane. The RNeasy mini column was placed into a new collection tube and centrifuged for one minute at 14,000 x *g* to eliminate any possible carry over of buffer RPE. The column was placed in a new 1.5ml collection tube and 30µl of RNAse-free water was added directly to the RNeasy spin column membrane. The tube was centrifuged for 1 minute at 8000 x *g* to elute RNA. The RNA containing eluate was stored at – 80°C until needed.

2.9.1. DNAse treatment

Amplification Grade DNase I (Sigma) was used to eliminate DNA from RNA template prior to applications, such as cDNA synthesis and polymerase chain reaction (PCR). Currently there is no RNA isolation procedure that removes 100% of the DNA. All RNA samples should be digested with DNase I before RT-PCR because PCR can detect even a single molecule of DNA (Mutimer et al., 1998, Huang et al., 1996).

Briefly, 6 µl RNA (3 µg/µl) was added to RNase-free water to create a final volume of 8µl. The diluted RNA was then added to 1µl of 10X Reaction Buffer and 1µl of DNase I (Sigma). The suspension was mixed gently and incubated for 15 minutes at room temperature. Following the incubation, 1µl of Stop Solution was added to the solution (to bind calcium and magnesium ions and to inactivate the DNase I) and the mixture was incubated for 10 minutes at 70°C to denature the DNase I. The RNA template was chilled on ice prior to reverse transcription.

2.9.2. RNA quantification

The concentration of total RNA isolated was determined on the NanoDrop® 1000 spectrophotometer (Thermo Scientific, Massachusetts, USA,) using RNase-free water as a blank. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of 2.0 is generally accepted as good for RNA (NanoDrop ND1000 Full-spectrum UV/Vis spectrophotometer User's Manual 2005, NanoDrop technologies Inc.Wilmington, DE USA).

2.9.3. cDNA synthesis

Complementary DNA (cDNA) was synthesized from DNAse treated RNA (see section 2.9.1) using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). The procedure is summarised below:

RNA, Oligo (dT)18 primer and water were mixed together (Table 1) centrifuged at 10 000 x g for 1 minute using a Mikro 200 centrifuge (Labotec) and incubated at 65°C for 5 minutes. The RNA was then transferred to ice and the reverse transcriptase reaction was set up by adding the reagents in the order shown in table 2. The reaction was mixed, and reverse transcription was allowed to proceed at 42°C for 60 minutes. The reaction was terminated by heating at 70 °C for 5 minutes.

Table 2.1. Components of cDNA synthesis during first incubation

Reagents	**Volume (µl)**
Template RNA (2.5 µg)	6
Oligo $(dT)_{18}$ primer (0.5 µg/µl)	1
Water (nuclease - free)	5
Total volume	**12**

Table 2.2. Components used during second incubation of cDNA synthesis

Reagents	Volume (µl)
5X Reaction Buffer	4
RiboLock RNase Inhibitor (20 U/µL)	1
10 mM dNTP Mix	2
RevertAid RT (200 U/µL)	1
Total volume	**20**

To determine whether there was DNA contamination, an RT negative template control was included. This control RT negative reaction contains every reagent for the reverse transcription except the reverse transcriptase enzyme (RevertAid RT).

2.10. QUANTITATIVE REAL TIME PCR

2.10.1. Primer design and synthesis

The primers for scavenger receptors and reference gene were obtained from PrimerBank (Table 3) under their accession numbers. Primers were synthesized by Inqaba Biotec (Pretoria, RSA). Synthesized lyophilized primers were reconstituted with RNA/DNAse-free water to give a stock solution of 100µM. The amplicons were sequenced at Inqaba Biotec to confirm their identity (refer to appendix E).

Table 2.3. PCR primers for the amplification of scavenger receptors (CD36, CD68 and MSRI) and beta actin genes in macrophages.

Genes	Primer sequence	bases	Tm (°C)	Amplicon size
CD36	F 5' - AAGCCAGGTATTGCAGTTCTTT -3'	22	60.2	
	R 5' - GCATTTGCTGATGTCTAGCACA- 3'	22	61.2	220
CD68	F 5'- GGAAATGCCACGGTTCATCCA- 3'	21	63.0	
	R 5'- TGGGGTTCAGTACAGAGATGC - 3'	21	61.2	247
MSRI	F 5'- GCAGTGGGATCACTTTCACAA- 3'	21	60.5	
	R 5'- AGCTGTCATTGAGCGAGCATC- 3'	21	62.8	85
Beta actin	F 5'-CATGTACGTTGCTATCCAGGC-3'	21	60.8	
	R5'- CTCCTTAATGTCACGCACGAT-3'	21	60.2	250

2.10.2. Real Time PCR

Real-Time PCR was performed using Maxima SYBR® Green master mix (Thermo Scientific). The SYBR Green is a fluorescent intercalating dye which binds to the double stranded DNA (dsDNA) and emits a fluorescent signal upon binding (Longo et al., 1990). The excitation and emission of SYBR Green are at 494 nm and 521 nm, respectively. As SYBR green binds all dsDNA, multiplexing is impossible; therefore, the PCR reactions for each of the four genes were set up in a separate tube using their specific primer sets (Table 3). All reactions contained the same cDNA template preparation to determine whether there are differences in gene expression across ethnicities'. The PCR reaction was set up as described in table 4. The PCR reaction was gently mixed. The PCR mix was centrifuged to remove any bubbles as they interfere with fluorescence detection.

Table 2.4. PCR components for the real time PCR detection of scavenger receptor (CD36, CD68 and MSRI) and beta actin genes in macrophages using StepOnePlus Real time PCR system

Reagents	**Volume (µl)**
Maxima SYBR green qPCR master mix	12.5
10 µM forward primer (CD36/CD68/MSRI/b-actin)	0.4
10 µM reverse primer (CD36/CD68/MSRI/b-actin)	0.4
Distilled water	8.7
cDNA template (1 µg)	3
Total volume/tube	**25**

The quantification of the levels of expression of the scavenger receptors genes were carried out using StepOnePlus Real time PCR system (Applied Biosystems, Foster City, California, USA) using the conditions described in Table 5.

Table 2.5. Thermal cycling conditions

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 sec	45
Annealing	55	30 sec	
Extension	72	1 min	

The negative template control (NTC) contained all the reagents of the PCR except the cDNA template. Relative gene expressions were calculated using the ΔΔCt relative quantification method with beta (β)-actin used as the normalization control. Our choice of reference gene was based on normalisation controls previously used in the literature (De Villiers et al., 1994, Waldo et al., 2008) and the melt curve analysis for β-actin is shown in appendix D. Briefly, to determine differences in expression levels between genes, the crossing point (threshold) of the reference gene was compared with the crossing point of the target gene whereby the ratio of the cycle at which the reference gene crosses the threshold and the cycle at which the target gene crosses the threshold was calculated.

Melting curve analysis (refer appendix D) was performed to assess whether the intercalating dye (SYBR green) had produced single, specific products. Briefly, SYBR green fluoresces

only when bound to double-stranded DNA (dsDNA). It does not fluoresce in the presence of single-stranded DNA (ssDNA). When the amplification cycles (refer appendix D) are completed, the thermal cycler produces the melt curve. As the thermal cycler continue with the measurement of fluorescence ,the temperature of the sample increases. Therefore the dsDNA denatures as the temperature increases becoming single stranded, and thus the SYBR green dye detach, resuting in the decrease of the fluorescence. The melt curve is produced , by measuremering the change in fluorescence with inreasing temperature. (Dwight et al., 2011).

2.11. STATISTICAL ANALYSIS

Data are presented as means ± SD, and as median (interquartile range) in text and tables. Data that were not normally distributed were log transformed to normality before being analysed. Comparisons were made across the 3 ethnic groups using ANOVA with *post hoc* analysis using Tukey's test while comparisons across genders were made using Student's unpaired t-test. Wilcoxon matched pairs test was used to compare baseline lipid uptake and scavenger receptors expression between macrophages treated with oxLDL and macrophages without oxLDL treatment.

Multivariable regression analysis was performed to find the main determinants of macrophage lipid uptake and scavenger receptor expression levels. Thus, univariate correlations of macrophage lipid uptake and scavenger receptor levels (dependent variables) with other study variables (independent variables) were assessed with Spearman's correlation. Independent variables that correlated with $p<0.2$ were then included in multivariable regression models and backward stepwise removal of these variables was undertaken until only those with $p<0.05$ remained in the model. A $p<0.05$ was considered significant. Statistical analyses were performed using Statistica software version 13.0.

2.12. ETHICS

Ethical clearance for this study was obtained from the University of Witwatersrand Human Research Ethics Committee (Medical); ethics clearance certificate no: M161057 (see appendix B).

CHAPTER 3: RESULTS

3.1. LIPID ACCUMULATION IN MACROPHAGES FROM PARTICIPANTS <25 YEARS-OF-AGE

We performed 41 isolations of monocytes and the results on the purity of the monocytes isolated are as follows: 90.7 ± 11.5% purity (range, 60-100%). Initial experiments were performed on monocytes isolated from subjects below the age of 25 years (n=11). Following exposure of macrophages to oxLDL to induce foam cell formation, intra-cellular lipid accumulation (expressed as a % of that in cells not exposed to oxLDL) of these cells was not significantly different to that seen in unexposed macrophages (99.9 ± 22.1% vs 100%; p=0.65), suggesting no foam cell formation (Table 3.1).

Table 3.1. Data from experiments on subjects below the age of 25

Variables	Levels
Subject numbers	11
Males (%)	45.4
Africans: Europeans (%)	63.6 : 36.4
Age (years)	23.3 ± 1.35
BMI (kg/m^2)	22.6 (20.5, 28.0)
Waist (cm)	78.4 ± 13.1
Hip (cm)	96.7 ± 6.6
Macrophage ICLA (%)	99.9 ± 22.1

Data given as mean ± SD or median (interquartile range); ICLA is intra-cellular lipid accumulation

3.2. COMPARISON OF LIPID ACCUMULATION IN MACROPHAGES FROM SUBJECTS <25 YEARS AND SUBJECTS >40 YEARS, AND DETERMINANTS OF LIPID UPTAKE

As macrophages from participants <25 years-of-age failed to accumulate lipids in the presence of oxLDL, the ability of macrophages isolated from participant >40 years-of-age (n=11) to accumulate lipids was assessed and compared to the data collected on the younger cohort (Table 3.2).

The participants <25 years and >40 years did not differ significantly in terms of BMI, however, waist and hip circumferences were significantly higher (waist, p=0.04; hip, p=0.01) in participants above 40 years. Macrophage intra-cellular lipid accumulation (ICLA), used as a measure of foam cell formation, was significantly higher in the older than the younger group (p=0.0001) (Table 3.2).

Table 3.2. Data comparison between age groups

Variables	Age <25 years	Age >40 years
Subject numbers	11	11
Males (%)	45.4	45.4
Africans: Europeans (%)	63.6 : 36.4	63.6 : 36.4
Age (years)	23.3 ± 1.35	47.6 ± 7.4***
BMI (kg/m^2)	22.6 (20.5; 28.0)	27.7 (22.3; 30.7)
Waist (cm)	78.4 ± 13.1	90.4 ± 12.1*
Hip (cm)	96.7 ± 6.6	105.8 ± 8.9*
Macrophage ICLA (%)	99.9 ± 22.1	151.1 ± 27.9***

Data given as mean ± SD or median (interquartile range); *p<0.05, ***p<0.0005 vs age<25 years

In order to find out which variables were the main determinants of ICLA in the macrophages isolated from the young and older subjects, univariate correlations were analysed to determine the variables that associated with lipid accumulation in the combined group (Table 3.3). It was found that age (p=0.002), BMI (p=0.02), waist (p=0.02) and hip (p=0.03) circumferences were all positively associated with ICLA.

Table 3.3. Univariate correlations of macrophage intra-cellular lipid accumulation

Variables	p-value	r
Age (Years)	**0.002**	.617
BMI (kg/m^2)	**0.023**	.482
Waist (cm)	**0.019**	.495
Hip (cm)	**0.034**	.453

Bolded p-values indicating significant difference; N=22

Variables from the univariate analyses shown in Table 3.3 which correlated with ICLA at p<0.2 i.e. age, BMI, waist and hip were incorporated into a backward, stepwise multivariate linear regression model to identify the main determinants of lipid accumulation (Table 3.4). This regression model suggests that age is the main predictor of macrophage ICLA (p=0.002)

Table 3.4. Multivariate linear regression model for intra-cellular lipid accumulation

Dependent variable	Independent varables with β-coefficient (p-value)	Adjusted R^2 (p-value)
Macrophage ICLA (%)	Age; 0.62 (0.002)	0.35 (<0.002)

N=22

3.3. CHARACTERISTICS OF THE OLDER POPULATION

As age was the main predictor of macrophage ICLA, and no macrophage lipid accumulation was observed in younger subjects we altered our inclusion criteria to include only participants aged 40 years and above. All subsequent analyses within this dissertation were performed on the data obtained from the older subject group. The participants were grouped according to their ethnicity (Africans, Europeans and Indians). Equal numbers of each ethnicity were recruited and participants were marched for gender and age. Table 3.5 shows the data comparison across ethnicity. Africans had a significantly higher BMI than Europeans (p=0.03). Hip circumference was significantly higher in Africans as compared to Europeans (p=0.01) and Indians (p=0.004). Waist circumference was also higher in Africans than in Europeans and Indians however, the differences were not statistically significant (p=0.12). No differences were observed in terms of fasting lipograms (total cholesterol, HDL-C, LDL-C and triglycerides) and glucose between the ethnic groups.

Table 3.5. Data comparison across ethnic groups

Variables	Africans	Europeans	Indians
Subject numbers	10	10	10
Males (%)	50.0	50.0	50.0
Age (years)	46.9 ± 5.1	53.8 ± 10.6	54.4 ± 9.8
BMI (kg/m^2)	30.2 (27.8, 40.4)	24.1 (20.8, 25.9)*	26.9 (21.1, 29.0)
Waist (cm)	96.6 ± 9.1	88.7 ± 10.6	85.2 ± 15.7
Hip (cm)	115.5 ± 14.9	100.6 ± 6.6*	98.7 ± 9.0**
Cholesterol (mmol/L)	4.6 ± 0.7	5.1 ± 0.6	5.2 ± 0.8
HDL-C (mmol/L)	1.4 (1.0, 1.7)	1.5 (1.3, 1.9)	1.5 (1.1, 1.6)
LDL-C (mmol/L)	3.0 ± 0.6	3.5 ± 0.5	3.5 ± 0.9
Triglycerides (mmol/L)	0.9 (0.7, 1.1)	0.7 (0.6, 1.2)	0.9 (0.7, 1.1)
Glucose (mmol/L)	4.7 ± 0.8	5.1 ± 0.6	5.3 ± 1.0

Data given as mean ± SD or median (interquartile range); *p<0.05, **p<0.005 vs African.

When females were compared with males (Table 3.6), waist circumference (p=0.008) was significantly higher in males than females. In addition, males had higher LDL-C (p=0.02) and glucose (p=0.02), whereas HDL-C (p=0.0006) was significantly higher in females. No differences in terms of BMI and hip circumference were observed across genders.

Table 3.6. Data comparison across genders

Variables	Females	Males
Subject numbers	15	15
Africans: Europeans: Indians (%)	33.3 : 33.3 : 33.3	33.3 : 33.3 : 33.3
Age (years)	52.5 ± 8.1	50.9 ± 10.4
BMI (kg/m^2)	26.0 (21.4, 30.7)	27.7 (22.3, 29.8)
Waist (cm)	84.2 ± 13.3	96.1 ± 9.0*
Hip (cm)	107.1 ± 16.3	102.8 ± 8.3
Cholesterol (mmol/L)	4.9 ± 0.6	5.0 ± 0.8
HDL-C (mmol/L)	1.6 (1.5, 1.9)	1.1 (0.9, 1.5)**
LDL-C (mmol/L)	3.0 ± 0.6	3.6 ± 0.7*
Triglycerides (mmol/L)	0.7 (0.5, 0.9)	1.0 (0.8, 1.2)
Glucose (mmol/L)	4.7 ± 0.5	5.4 ± 1.0*

Data given as mean ± SD or median (interquartile range); *p<0.05, **p<0.005 vs females

3.4. MACROPHAGE INTRA-CELLULAR LIPID ACCUMULATION ACROSS ETHNICITY AND GENDERS

Macrophage ICLA in cells exposed to oxLDL was compared with the baseline ICLA (set at 100%) in macrophages not exposed to oxLDL, in all ethnic groups. Macrophages exposed to oxLDL had higher ICLA compared to baseline and these differences were significant for all ethnicities (African, p=0.005; European, p=0.005; Indian, p=0.007) as shown in Figure 3.1. However, no differences in macrophage ICLA were observed between ethnic groups (African, 156.8 ± 28.1%; European, 155.9 ± 57.9%; Indian, 136.8 ± 22.2%; p=0.42). When ICLA in

macrophages exposed to oxLDL were compared with non-exposed cells in males and females, significant differences were observed in both genders (males, p=0.0006; females, p=0.0009) as shown in Figure 3.2. However, there was no significant differences in ICLA between genders (males; 158.8 ± 48.5%, females; 140.9 ± 25.3%, p=0.24).

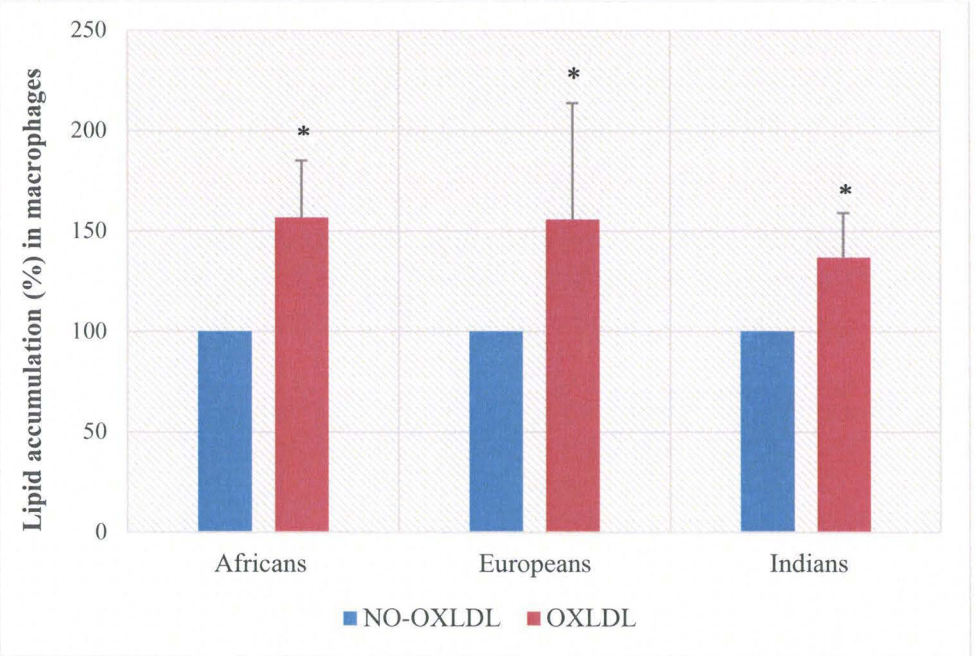


Figure 3.1. Macrophage intra-cellular lipid accumulation (ICLA) comparison across ethnicities. No differences in ICLA were observed between ethnicities, but all ethnic groups had higher ICLA in macrophages exposed to oxLDL when compared to non-exposed cells (where ICLA was set at 100%). Data given as mean ± SD; *p<0.05 vs No oxLDL. ANOVA and Wilcoxon matched pairs tests were used.

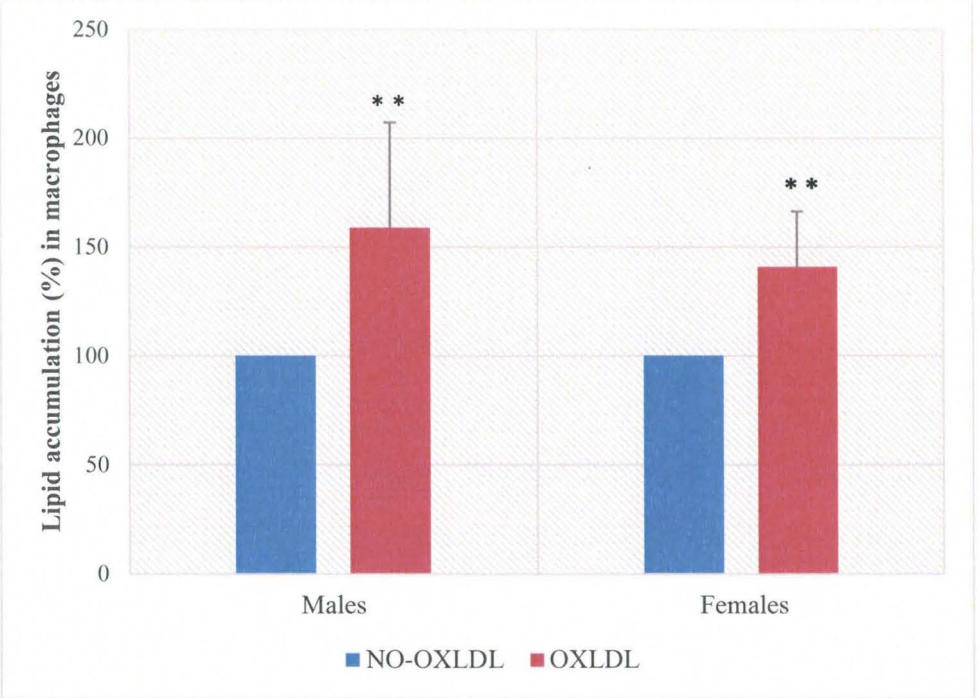


Figure 3.2. Macrophage intracellular lipid accumulation (ICLA) comparison across genders. There was no significant difference in ICLA across genders; however both males and females had increased ICLA in macrophages exposed to oxLDL when compared to non-exposed cells (where ICLA was set at 100%). Data given as mean ± SD; **$p<0.005$ vs No oxLDL. Student's unpaired t-test and Wilcoxon matched pairs test were used.

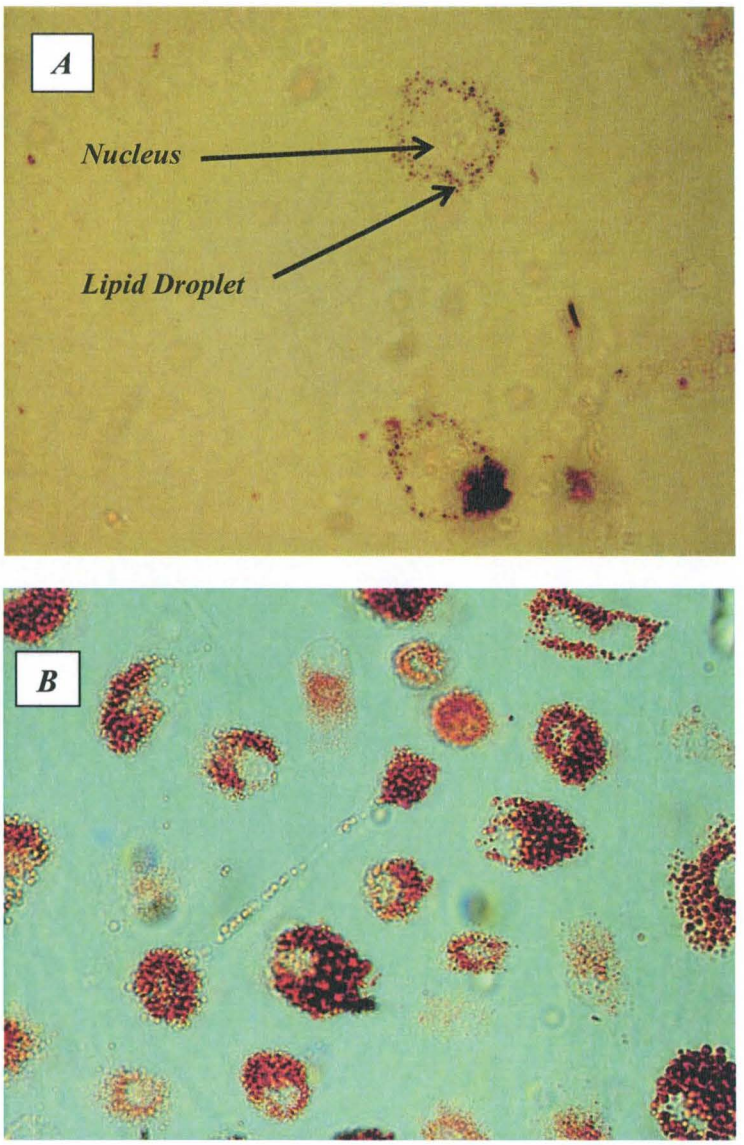


Figure 3.3. Macrophage ICLA before and after exposure to oxLDL. Macrophages were induced to accumulate cholesterol through exposure to oxLDL for 48 hours. Foam cell formation was assessed through microscopic morphological examination (100 X magnification). Increased lipid accumulation (measured through Oil Red O staining) and a foamy appearance were indicative of foam cell formation (B) when compared to macrophages not exposed to ox-LDL (A).

3.5. SCAVENGER RECEPTOR EXPRESSION LEVELS ACROSS ETHNICITY AND GENDERS

3.5.1 CD36

Quantification of all three scavenger receptors, MSR-1, CD36 and CD68 was successfully performed using real-time PCR, which indicated that we had amplification for all scavenger receptors. Clear, single peaks on the melting curve plot indicated precise amplification with no unspecific products (refer appendix D). The gene expression levels of scavenger receptors (CD36, CD68 and MSR1) in macrophages exposed to oxLDL were expressed as a % of the expression level in cells that were not exposed (this level was set at 100%). The CD36 expression levels in the presence of oxLDL were compared across ethnic groups. African subjects had a significantly higher level of CD36 expression levels as compared to Europeans ($p=0.007$) and Indians ($p=0.03$; see Figure 3.4 (A)). Macrophages isolated from African participants and exposed to oxLDL showed a significantly greater CD36 gene expression level than non-exposed cells ($p=0.008$). Europeans had lower CD36 gene expression levels in cells exposed to oxLDL than non-exposed cells, and the difference was significant ($p=0.03$). A similar trend was observed in macrophages from Indian subjects but the difference was not significant ($p=0.28$). There was no significant trend observed when comparing CD36 gene expression levels in the presence of oxLDL across genders ($p=0.29$), as shown in figure 3.4 (B). There was also no significant effect of oxLDL on CD36 expression.

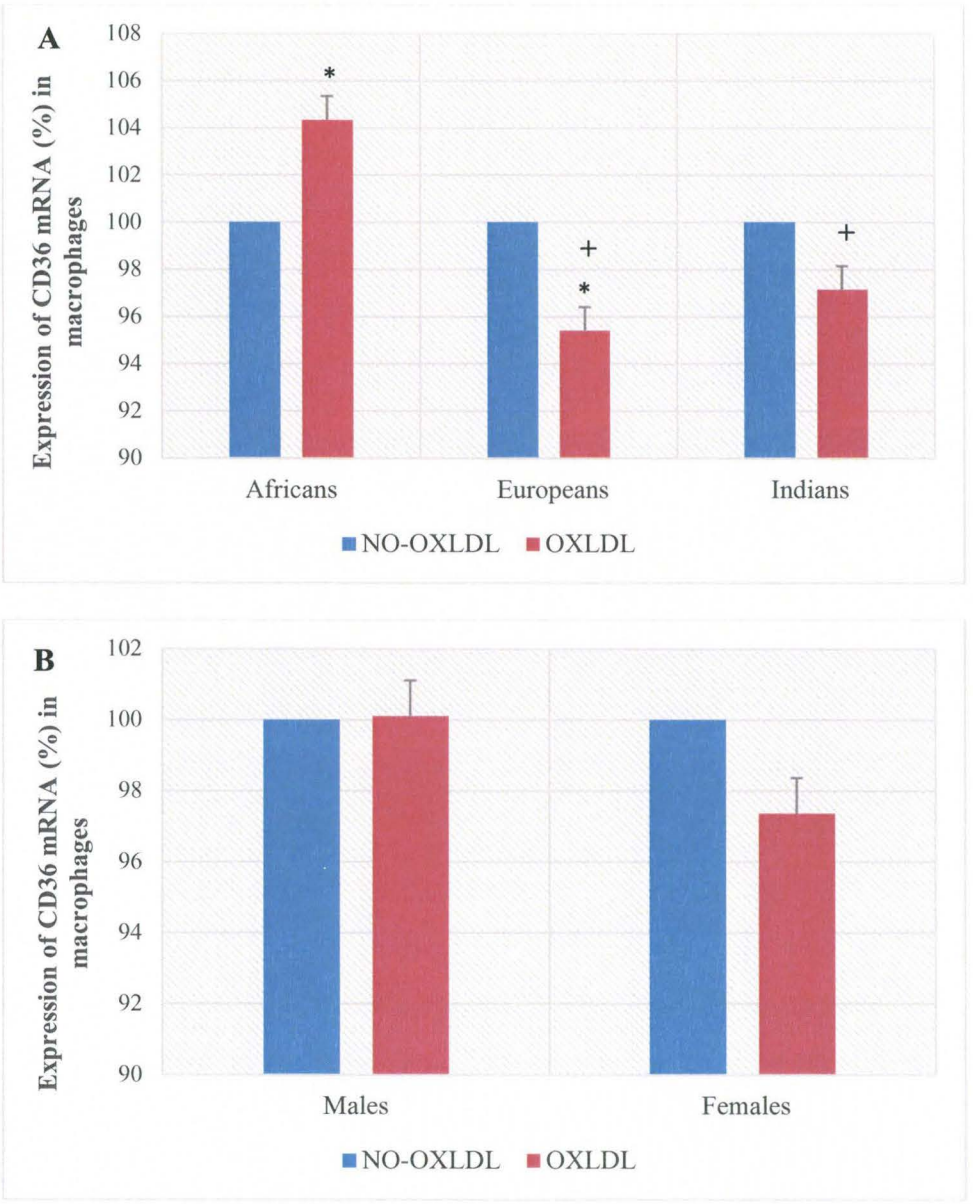


Figure 3.4. Expression levels of CD36 across ethnicities (A) and genders (B). Africans had higher CD36 expression in the presence of oxLDL than Europeans and Indians. Africans had higher CD36 expression levels in macrophages exposed to oxLDL than unexposed cells. Europeans had a lower CD36 expression level in macrophages exposed to oxLDL when compared to unexposed cells (A). No differences in CD36 expression in the presence of oxLDL were observed across genders (B). Data given as mean ± SD; *$p<0.05$ vs No oxLDL, +$p<0.05$ vs Africans. ANOVA, Student's unpaired t-test and Wilcoxon matched pairs test were used.

3.5.2 CD68

The three ethnic groups did not differ significantly in terms of macrophage CD68 gene expression in the presence of oxLDL ($p=0.81$). Also, within each ethnic group there was no significant effect of oxLDL exposure on CD36 expression levels (Figure 3.5 (A)). There was no significant difference in macrophage CD68 gene expressions in the presence of oxLDL between genders ($p=0.31$) (Figure 3.5 B) and within genders oxLDL had no effect on CD68 expression.

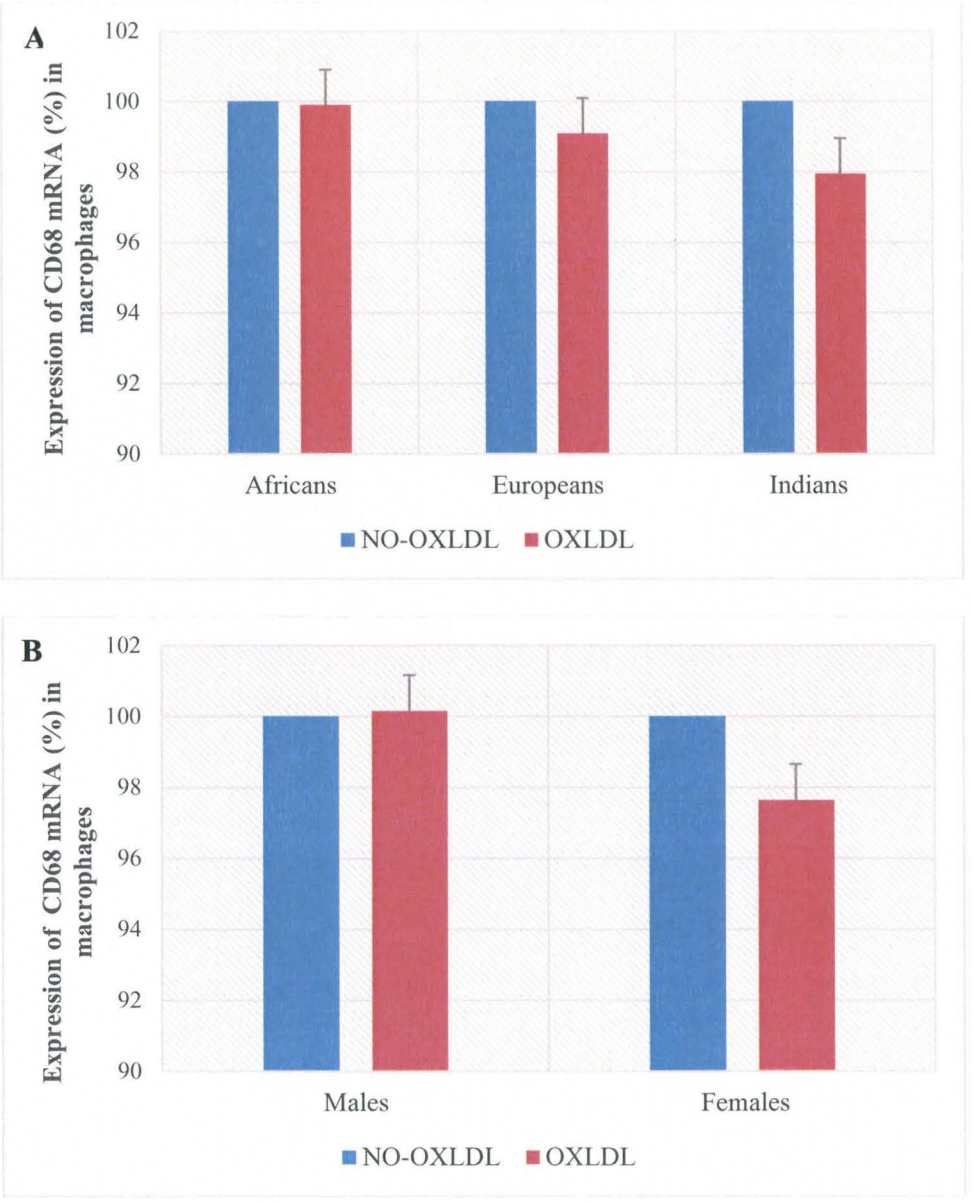


Figure 3.5. Macrophage CD68 expression levels across ethnicities (A) and genders (B). The CD68 expression levels in the presence of oxLDL did not differ between ethnicities (A) or genders (B). Data given as mean ± SD. ANOVA, Student's unpaired t-test and Wilcoxon matched pairs test were used.

3.5.3 MSR1

Figure 3.6 (A) shows the comparison of macrophage MSR1 gene expressions levels in the presence of oxLDL across ethnic groups. Indian subjects had signficantly lower MSR1 gene expression as compared to African subjects (p=0.02) but no differences when compared to Europeans (p=0.84). Within each ethnic group, oxLDL had no significant effect on MSR1 expression. No differences in MSR1 gene expressions levels in the presence of oxLDL were observed between genders (p=0.10) (Figure 3.6 (B)). Furthermore, within each gender there was no significant effect of oxLDL on macrophage MSR1 gene expression.

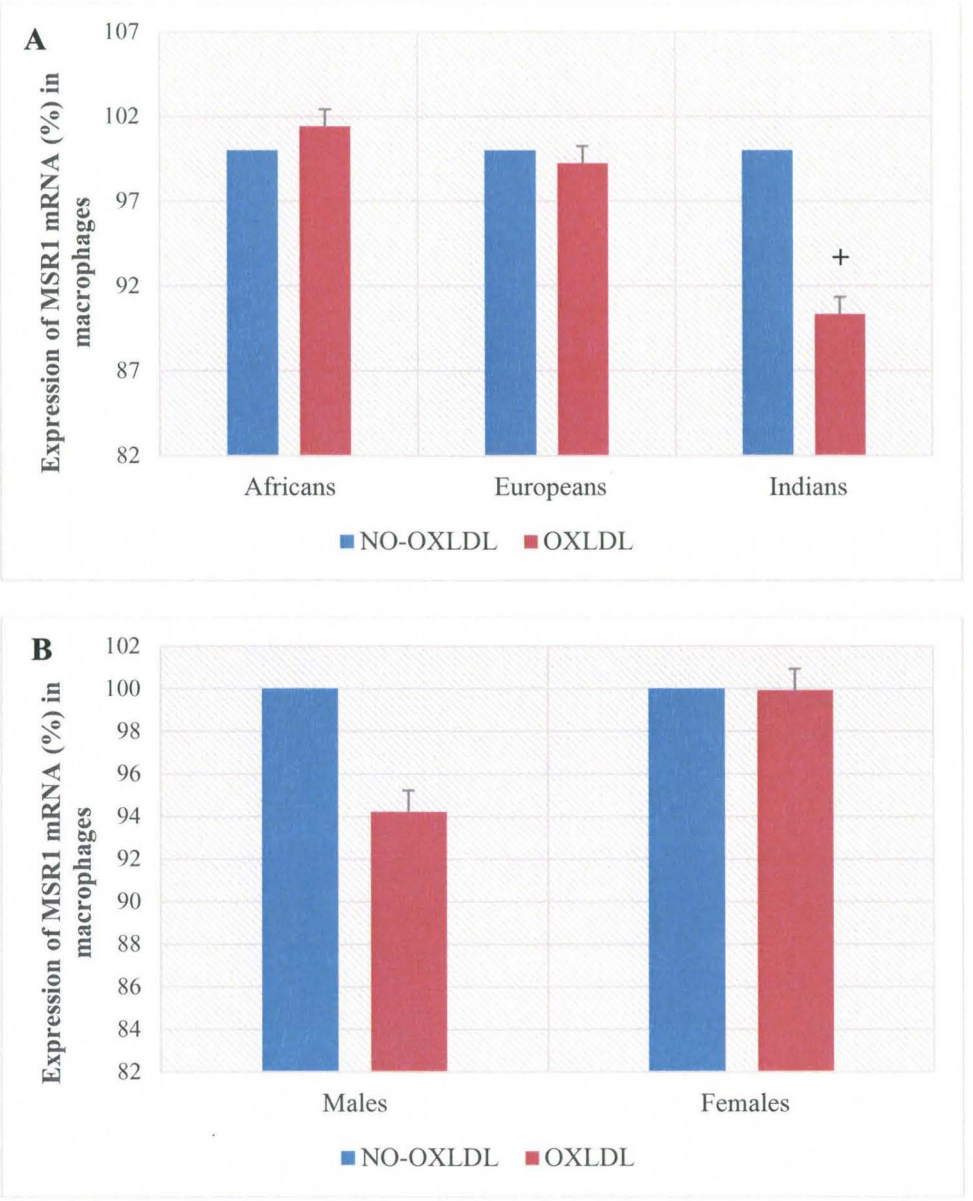


Figure 3.6. Macrophage MSR1 gene expression levels across ethnicities (A) and genders (B). Indians had lower MSR1 gene expression levels in the presence of oxLDL than African subjects but not Europeans (A). Males and females did not differ in terms of MSR1 expression levels in the presence of oxLDL (B). Data given as mean ± SD; +$p<0.05$ vs Africans. ANOVA, Student's unpaired t-test and Wilcoxon matched pairs test were used.

3.6. DETERMINANTS OF MACROPHAGE INTRA-CELLULAR LIPID ACCUMULATION IN SUBJECTS OLDER THAN 40 YEARS-OF-AGE

Univariate correlations showed that age ($p=0.04$) and baseline macrophage intra-cellular lipid levels ($p=0.04$) were significantly and negatively associated with oxLDL-stimulated ICLA (Table 3.7). Baseline macrophage lipid levels were calculated using Oil red O staining at the stage before incubation with oxLDL.

No other variables were significantly associated with ICLA, but oxLDL-associated CD36 gene expression levels did correlate with ICLA at $p<0.20$, and therefore this variable, along with age and baseline lipid levels were included in a backward, stepwise multivariable linear regression analysis, the results of which are shown in Table 3.8. This model demonstrates that only baseline macrophage intra-cellular lipid levels correlated significantly with oxLDL-stimulated ICLA.

Table 3.7. Univariate correlations of macrophage intra-cellular lipid accumulation in subjects >40 years of age

Variables	p-value	R
Age (years)	**0.04**	-.375
BMI (kg/m^2)	0.38	.166
Waist (cm)	0.77	.055
Hip (cm)	0.97	.007
Baseline macrophage lipid levels	**0.04**	-.378
Cholesterol (mmol/L)	0.28	-.202
HDL-C (mmol/L)	0.64	-.090
LDL-C (mmol/L)	0.46	-.139
Triglycerides (mmol/L)	0.30	.195
Glucose (mmol/L)	0.82	.043
CD36 expression (%)	0.14	.278
CD68 expression (%)	0.50	.365
MSR1 expression (%)	0.29	.205

Table 3.8. Multivariate regression models for macrophage intra-cellular lipid accumulation in subjects >40 years-of-age

Dependent variable	Independent variables with β-coefficient (p-value)	Adjusted R^2 (p-value)
Macrophage ICLA (%)	Baseline macrophage lipid level; -0.38 (0.04)	0.11 (0.04)

3.7. FACTORS CORRELATED WITH MACROPHAGE SCAVENGER RECEPTOR EXPRESSION LEVELS

In section 3.7 and 3.8 macrophage scavenger receptor gene expression levels in the presence of oxLDL are expressed as a % of gene expression in the absence of oxLDL, as in the previous sections of this dissertation, and are referred to as % gene expression. Baseline scavenger receptor gene expression is the expression level at the stage before incubation with oxLDL.

Univariate correlations of scavenger receptor % gene expression are shown in Table 3.9. Percentage CD36 expression associated positively with % expression of CD68 (p=0.004) and MSR1 (p=0.05), and negatively associated with baseline CD36 (p=0.001) and baseline MSR1 expression (p=0.03). Percentage gene expression of CD68 was positively associated with % expression of MSR1 (p=0.04) and negatively associated with baseline CD68 expression (p=0.002). Percentage MSR1 gene expression was negatively associated with LDL-C (p=0.03), glucose (p=0.03) and baseline MSR1 gene expression (p=0.0006), and positively associated with HDL-C (p=0.006).

Table 3.9. Univariate correlations for percentage gene expression of scavenger receptors

Variables	CD36 mRNA (%)	CD68 mRNA (%)	MSR1 mRNA (%)
	r-values		
Age (years)	-0.331	-0.219	-0.207
BMI (kg/m2)	0.336	0.003	0.003
Waist (cm)	0.173	-0.025	-0.276
Hip (cm)	0.246	-0.109	0.011
Cholesterol (mmol/L)	-0.088	0.217	-0.038
HDL-C (mmol/L)	-0.105	0.128	0.510*
LDL-C (mmol/L)	-0.062	0.091	-0.404*
Triglycerides (mmol/L)	-0.039	-0.137	-0.118
Glucose (mmol/L)	-0.089	0.013	-0.411*
CD36 mRNA (B)	-0.595**	-0.264	-0.204
CD68 mRNA (B)	-0.298	-0.547**	0.062
MSR1 mRNA (B)	-0.415*	-0.261	-0.707**
CD68 mRNA (%)	0.519**	_	0.386*
MSR1 mRNA (%)	0.376*	0.386*	_

*p<0.05, **p<0.005

Multivariate regression analyses were performed to identify the main determinants of % gene expression of scavenger receptors from among the factors shown in Table 3.9. Thus, variables from the univariate analyses displayed in Table 3.9 and with $p<0.20$ were included in backward, stepwise multivariable linear regression models and the results are shown in Table 3.10. With regard to % gene expression of CD36 this correlated negatively with European ethnicity ($p=0.04$) and baseline CD36 expression ($p=0.002$), and positively with % CD68 gene expression ($p=0.0001$) and baseline CD68 expression ($p=0.003$). Percentage gene expression levels of CD68 correlated negatively with the baseline CD68 expression ($p<0.0005$) and correlated positively with % gene expression of MSR1 ($p=0.004$). Percentage MSR1 gene expression correlated negatively with Indian ethnicity ($p=0.0006$) and correlated positively with HDL-C ($p=0.0006$).

Table 3.10. Multivariate regression models for % expression of scavenger receptors

Dependent variable	Independent variables with β-coefficient (p-value)	Adjusted R^2 (p-value)
CD36 % expression (n=28)	Europeans; -0.36 (0.04) CD68 %; 0.7 (<0.0005) Basal CD36; -0.58 (0.002) Basal CD68; 0.62 (0.003)	0.60 (<0.0005)
CD68 % expression (n=28)	Basal CD68; -0.63 (<0.0005) MSR1 %; 0.42 (0.004)	0.51 (<0.0005)
MSR1 % expression (n=28)	Indians; -0.53 (0.0006) HDL-C; 0.53 (0.0006)	0.51 (<0.0005)

3.8. FACTORS CORRELATED WITH THE BASELINE LEVELS OF MACROPHAGE SCAVENGER RECEPTOR GENE EXPRESSION

Table 3.11 outlines the factors that are associated with the baseline scavenger receptor expression in macrophages. Univariate correlation analysis of baseline CD36 gene expression demonstrated positive associations with age ($p=0.01$), baseline CD68 expression ($p=0.0006$) and baseline MSR1 expression ($p=0.02$). Baseline CD68 gene expression associated positively with serum triglycerides ($p=0.04$). Baseline MSR1 gene expression associated positively with serum LDL-C ($p=0.001$) and plasma glucose ($p=0.005$).

Table 3.11. Univariate correlations for baseline scavenger receptor expression

	Baseline CD36 (n=29)	**Baseline CD68** (n=29)	**Baseline MSR1** (n=28)
Variables		**r-value**	
Age (Years)	.455*	.130	.256
BMI (kg/m^2)	-.260	-.084	-.266
Waist (cm)	.027	.119	-.026
Hip (cm)	-.239	.030	-.334
Cholesterol (mmol/L)	.291	-.039	.345
HDL-C (mmol/L)	-.010	-.117	-.273
LDL-C (mmol/L)	.333	-.023	.575**
Triglycerides (mmol/L)	.112	.377*	-.022
Glucose (mmol/L)	.105	-.117	.516*
Baseline CD36 expression	_	.607**	.424*
Baseline CD68 expression	.607**	_	.072
Baseline MSR1 expression	.424*	.072	_

*p<0.05, **p<0.005

Using the data from Table 3.11, backward, stepwise linear multivariate regression analysis of baseline gene expression was performed, and the results are shown in Table 3.12. Baseline CD36 scavenger receptor expression correlated with age (p=0.02), LDL (p=0.03) and baseline CD68 expression (p<0.0005). Baseline CD68 expression correlated positively with triglycerides (p=0.005) and European ethnicity (p=0.0006). Baseline expression of MSR-1 correlated positively with LDL (p=0.01) and negatively with HDL (p=0.003) and African ethnicity (p<0.0005).

Table 3.12. Multivariate regression models for baseline scavenger receptor expression

Dependent variable	Independent variables with β-coefficient (p-value)	Adjusted R^2 (p-value)
Baseline CD36 (n=28)	Age; 0.33 (0.02) LDL-C; 0.29 (0.03) Baseline CD68; 0.57 (<0.0005)	0.55 (<0.0005)
Baseline CD68 (n=28)	Europeans; 0.56 (0.0006) Triglycerides; 0.45 (0.005)	0.41 (<0.0005)
Baseline MSR1 (n=28)	Africans; -0.73 (0.0005) HDL-C; -0.33 (0.003) LDL-C; 0.27 (0.01)	0.77 (<0.0005)

CHAPTER 4: DISCUSSION

4.1. SUMMARY OF RESULTS

In our study, only macrophages from participants above the age of 40 accumulated lipids after exposure to oxLDL. The study found no differences in intra-cellular lipid accumulation (ICLA) of macrophages, when compared across genders or ethnic groups (Africans, Europeans and Indians) of the donor. Males had significantly higher waist circumference, LDL-C and glucose, whereas females had significantly higher HDL-C. The baseline level of macrophage intracellular lipid content was the main determinant of ICLA in subjects above 40 years of age, whereas age was the principal determinant of the difference in macrophage ICLA when comparing subjects over the age of 40 years with subjects who were less than 25 years-of-age.

The African subjects had a higher level of CD36 % gene expression in the presence of oxLDL when compared to Europeans and Indians. African participants also showed a significantly higher level of CD36 % gene expression in the presence of oxLDL when compared to gene expression in the absence of oxLDL, whereas macrophages isolated from Europeans had a significantly lower level of CD36 gene expression in the presence of oxLDL when compared to unexposed macrophages. The % level of CD68 gene expression in the presence of oxLDL did not differ across ethnicities and nor was it different within each ethnic group from the level observed in macrophages that were not exposed to oxLDL. The MSR1 % gene expression level was signficantly lower in Indian compared to African subjects but not different when compared to Europeans. Within each ethnic group, % MSR1 gene expression in macrophages exposed to oxLDL was not significantly different to that observed in unexposed macrophages. Our study did not show any differences in scavenger receptor gene expressions levels in the presence of oxLDL between genders.

Multivariable regression models demonstrated that European and Indian ethnicity, baseline CD36 and baseline CD68 gene expression levels and HDL-C serum levels were the main determinants of scavenger receptors % gene expression levels in the presence of oxLDL. Age, LDL-C, HDL-C, triglycerides, African and European ethnicity were shown to be the main determinants of baseline scavenger receptor gene expression levels.

4.2. DIFFERENCES IN MACROPHAGE INTRA-CELLULAR LIPID ACCUMULATION ACROSS AGE GROUPS

Atherosclerotic diseases are known to be the major causes of mortality and morbidity in the world. Accumulation of oxidized low-density lipoprotein (oxLDL)-laden foam cells derived from macrophages is the critical event in the development of atherogenesis (Yu et al., 2013). Using an *in vitro* model of foam cell formation, macrophages from older subjects were shown to form foam cells more readily than those from younger subjects. In our study, ICLA of macrophages was used as a measure of foam cell formation, a method that has also been used in previous studies (Mori et al., 2001, Xu et al., 2010).

This is consistent with the increased prevalence of atherosclerotic diseases in older individuals (Herrington et al., 2016). In the current study, in multivariable models, we observed that higher macrophage ICLA was positively associated with age. This suggests that age is the main determinant of macrophage lipid uptake and explains the difference in ICLA between the older and younger groups. Age is a major independent risk factor for the development of atherosclerosis (Angelovich et al., 2016). Our findings are supported by results from a similar *ex vivo* study, where older men were shown to form foam cells more readily than those from younger men (Angelovich et al., 2016). Therefore, the increased formation of foam cells in

older subjects may be one of the factors that explain the increased prevalence of atherosclerotic disease with aging.

4.3. COMPARISON OF RISK FACTORS FOR ATHEROSCLEROSIS AND LEVEL OF MACROPHAGE INTRA-CELLULAR LIPID ACCUMULATION ACROSS GENDERS AND ETHNICITY

Low density lipoproteins and high density lipoproteins are the major carriers of cholesterol in the circulation (Kresanov et al., 2013). Elevated plasma cholesterol and LDL cholesterol together with low levels of HDL cholesterol have been identified as independent risk factors for atherosclerosis (Kresanov et al., 2013). The risk profiles of each ethnic group for the development of cardiac events are unique. A study performed in South Africa by Sliwa *et al* reported that individuals of African descent have the lowest LDL-C and triglycerides levels whilst subjects of Indian ancestry have the highest LDL-C and triglycerides levels, with HDL-C levels not differing across ethnic groups (Sliwa et al., 2012). Furthermore, Norman and colleagues found that cholesterol-attributable mortality is highest in Indian subjects (22.2%), followed by European (20.5%) and mixed-ancestry (8.8%) population groups. Cholesterol attributable mortality was lowest in the black African group, accounting for only 1.8% (Norman et al., 2007). Within the present study triglyceride, total cholesterol, LDL-C and HDL-C serum levels did not differ across ethnicity, probably due to the small sample size. However, it must be noted that the main purpose of this study was not to assess differences in lipid levels across ethnic groups.

In our study males had significantly higher waist circumference, LDL-C and glucose levels than females whereas the latter gender had significantly higher HDL-C levels than males. The HDL-C findings in our study are in accordance with a previous study, where HDL-C levels

were significantly higher in healthy females compared with the males (Marhoum et al., 2013). Another study by Li et *al* reported that women have significantly higher values of HDL-cholesterol, and lower values of triglyceride than men (Li et al., 1996) which is in accordance with our findings. The observed finding of elevated LDL-C in males compared to females in our study may be associated with the higher incidence of atherosclerosis in men than women, with male gender known to be an independent risk factor for atherosclerosis (McCrohon et al., 2000). Increased oestrogen levels in women has been shown to increase HDL by increasing hepatic clearance of LDL, thus lowering the LDL levels (Marhoum et al., 2013). Therefore differences in the sex steroid levels between genders might partially explain the differences of LDL-C and HDL-C across genders in our study. The current study demonstrated that waist circumference was higher in males than females, despite similar BMI measurements. This is supported by many other studies which show that waist circumference is greater in males than females due to the higher levels of visceral fat in males (Stevens et al., 2010).

In our study, using macrophages isolated from older subjects, we observed no differences in macrophage ICLA between ethnic groups, but we did observe that macrophages exposed to oxLDL had higher ICLA compared to unexposed macrophages and these differences were significant for all ethnicities. The observed high levels of lipids in macrophages exposed to oxLDL is supported by a study showing that incubation of macrophages with oxLDL results in lipid accumulation and transformation into foam cells (Yoshida et al., 1998a). Also, *in vitro* studies have shown that incubating macrophages with oxLDL results in the lysosomal accumulation of cholesteryl esters (CEs) and free cholesterol (Yancey and Jerome, 2001, Ullery-Ricewick et al., 2009). Males and females also did not differ in terms of ICLA. The lack of both ethnic and gender differences in ICLA may be a result of the limited sample size, which is one of the main drawbacks of this study.

Using multivariable models, we observed that lower ICLA in macrophages exposed to oxLDL was associated with a higher level of lipid accumulation in the macrophages immediately before incubation with oxLDL. This demonstrates that the higher the level of intra-cellular lipids within macrophages the lower will be the accumulation of oxLDL within the intra-cellular stores. This association may reflect the finite storage capacity for lipids within the macrophage, with higher baseline intra-cellular lipid levels restricting further accumulation of oxLDL within the cell. This is a novel finding that requires further investigation.

4.4. COMPARISON OF SCAVENGER RECEPTOR GENE EXPRESSION LEVELS ACROSS GENDERS AND ETHNICITY

Scavenger receptors (MSR1, CD36 and CD68) have been shown to be responsible for the uptake of modified LDL leading to foam cell formation (Matloubian et al., 2000). The MSR1 and CD36 receptors have been identified as those primarily responsible for the uptake of modified lipoproteins into macrophages in most studies, however the interaction of CD68 with modified lipoproteins is still controversial and their relevance is still unclear (Kunjathoor et al., 2002) but however a study by Tsukamoto reported that CD68 may play a crucial role in foam cell formation through retention of oxLDL macrophages (Tsukamoto et al., 2002). Thus, in an *in vitro* study by Kunjathoor *et al* it was reported that scavenger receptors CD36 and MSR1 are responsible for over 90% of oxidized LDL uptake by macrophages (Kunjathoor et al., 2002). Another study showed a significant role for CD36 and CD68, but not MSR1 in FC formation during oxLDL treatment of the macrophage-like THP-1 cell line (Tsukamoto et al., 2002). A study by van Tits *et al* showed high expression of CD36 and MSR1 in human M2 macrophages with CD36 highly expressed and reached levels 2-fold higher than MSR1 (van Tits et al., 2011).

The CD36 receptor has been proven to possess specific and high affinity binding to oxLDL and its expression is increased following exposure to oxLDL (Tsukamoto et al., 2002), but in our study following exposure of macrophages to oxLDL, CD36 % expression levels showed a significant increase in Africans when compared to unexposed cells, whilst in Europeans the % expression levels of CD36 was significantly lower when compared to the unexposed cells. However, comparison of data from the current study with that from the Tsukamoto et al (2002) report must be interpreted with care as the latter study used the THP-1 monocyte cell line rather than primary cultured human monocytes. Within the present study, MSR1 % expression levels were significantly higher in Africans as compared to Indians, but no ethnic differences were observed in the % expression levels of CD68. The lower % expression levels of CD36 in European subjects and MSR1 in Indian subjects were confirmed by the multiple regression analyses, which demonstrate that these effects occurred independently of other possible confounding variables. The reason for these ethnic differences in scavenger receptor expression levels is not known. They do not reflect ethnic differences in the prevalence of atherosclerotic cardiac disease suggesting either that the level of foam cell formation is not a major contributor to the risk of atherosclerosis or that *in vitro* measures of foam cell formation do not reflect the situation *in vivo*. It is also possible that the gene expression levels of scavenger receptors in foam cells do not reflect the protein levels of these receptors or the level of these receptors present on the cell surface. No differences in scavenger receptor % expression levels were observed across genders. Again, this does not reflect the sex differences observed for atherosclerotic disease, and the argument advanced for the discrepancy between scavenger receptor expression and atherosclerosis risk across ethnicities may also be used to explain the similar gender discrepancy.

Interestingly in our study, baseline scavenger receptor expression levels correlated with lipid profiles, ethnicity and age. Thus, baseline CD36 expression levels correlated positively with

LDL-C, CD68 baseline expression and age. Baseline CD68 expression correlated positively with triglycerides and European ethnicity, whilst MSR1 baseline expression correlated positively with LDL-C, and negatively with HDL-C and African ethnicity. The positive association of serum LDL levels with baseline scavenger receptor gene expression is a novel finding and is supported by a study showing that LDL upregulates the expression of macrophage scavenger receptors in a murine macrophage cell line (Han and Nicholson, 1998). Furthermore, high levels of plasma LDL has the ability to activate signal transduction and gene expression in macrophages (Lusis and Navab, 1993), through stimulation of phosphoinositide turnover and also elevates cytosolic calcium levels in vascular smooth muscle cells (Bochkov et al., 1993). The current study however was able to show a direct association between serum LDL levels and scavenger receptor expression in cultured human macrophages. In addition serum triglyceride levels correlated positively with basal CD68 gene expression, whilst serum HDL levels correlated negatively with basal MSR1 gene expression. These associations mirror the effect of these lipid molecules on the risk of atherosclerosis and suggest that serum lipids may modulate atherosclerotic plaque formation by modifying the expression levels of scavenger receptors in macrophages even before exposure to oxLDL.

The current study investigated the expression of scavenger receptors during the conversion of macrophages to foam cells, a process induced by exposure to oxLDL. Previous studies have demonstrated that the differentiation of monocytes to macrophages is characterised by increased scavenger receptor expression (Geng and Hansson, 1992). A study by Geng and colleagues also found that differentiation of monocytes to macrophages was associated with an increase in MSR1 gene expression (Geng et al., 1994). Another study reported increased expression of CD36, CD68 and MSR1 mRNA during differentiation of monocytes to macrophages in the human monocyte cell line, THP-1 (Tsukamoto et al., 2002). In our study we did not measure the level of scavenger receptor expression in monocytes, and therefore the

increase in scavenger receptors might have occurred during the monocyte to macrophage conversion rather than during foam cell formation. It is interesting to note that very little change was noted in scavenger receptor expression levels after treatment of macrophages with oxLDL, the exceptions being a small rise in CD36 expression in African subjects and a minor decrease in CD36 expression in European subjects. These data suggest that the major changes in scavenger receptor expression may occur during the differentiation of monocytes to macrophages rather than during foam cell formation.

4.5. CONCLUSIONS

Our study shows that older subjects have a higher level of foam cell formation than younger subjects. These findings may partially explain the increased prevalence of atherosclerotic disease observed with aging. Our data also shows that foam cell formation does not differ between genders or ethnic groups, suggesting that ethnic and gender differences in the risk of atherosclerotic disease are not due to parallel differences in foam cell formation. The expression of scavenger receptors changed minimally during foam cell formation suggesting that the up-regulation of receptor gene expression may occur before foam cell formation. The baseline levels of scavenger receptor gene expression in macrophages correlated positively with LDL-C, and triglycerides, and negatively with HDL-C. These results suggest that systemic levels of lipoproteins may be able to modify foam cell formation via modulation of scavenger receptor expression in macrophages or monocytes even before exposure to oxLDL in the arterial intima.

4.6. LIMITATIONS AND FUTURE STUDIES

Our findings were limited by the relatively small sample size however, the sample size was still sufficient to observe differences in lipid accumulation across age groups and significant associations in regression models. The other limitation was the lack of data on lipid levels or scavenger receptor expression in the monocytes. Therefore, future studies would involve increasing the sample size in each ethnic group and gender and measuring lipid levels and scavenger receptor gene expression in monocytes, macrophages and foam cells. These studies should also analyse the intra-cellular levels of the scavenger receptor proteins using Western blots and the sub-cellular localisation of the scavenger receptors and their movement from internal membranes to plasma membrane during foam cell formation. Other future work will involve a comparison of foam cell formation in lean and obese subjects and whether adipokines secreted by adipose tissue have an influence on foam cell formation. We initially wanted to determine whether alkaline phosphatase was involved in lipid accumulation within foam cells, however we were unable to detect any alkaline phosphatase gene expression in the macrophages or foam cells. Future studies will involve confirming these findings using alkaline phosphatase activity assessments and re-designing the alkaline phosphatase primers.

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APPENDIX A

PATIENT INFORMATION

Study title: Determining differential rates of foam cells formation in multi-ethnic South Africans: a potential role for tissue non-specific alkaline phosphatase.

1. INTRODUCTION

Hello, my name is _____. I am working in the Department of Chemical Pathology. You are invited to consider participating in a research study. Your participation in this study is entirely voluntary.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks and precautions as well as your right to withdraw from the study at any time.

This information leaflet is to help you decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.

If you have any questions, please don't hesitate to ask me...

2. PURPOSE OF THE STUDY

Atherosclerosis, is where your artery wall thickens because certain cells from your blood (called macrophages), accumulate in the artery wall. The cells found in the artery wall change into cells called 'foam cells' because they have lots of fat inside them and when you look at them under a microscope they look foamy. Having atherosclerosis affects you by stopping your blood from flowing properly, causing your tissues to die. Different individuals have different tendencies to develop atherosclerosis. This seems to be different for different ethnic groups in South Africa, with Indian people having more chance of developing atherosclerosis and black people being most protected. We want to see if differences exist between the macrophages of people of different ethnicities to change into foam cells. We also know that in fat cells, an enzyme called alkaline phosphatase is needed to make fats accumulate in cells, so we would also like to see if this enzyme is involved in changing the macrophages in your blood into foam cells.

3. LENGTH OF STUDY AND NUMBER OF PARTICIPANTS

Approximately 60 participants will be recruited to participate in the study (20 from each ethnic group – Black, White, Indian). The total amount of time required for your participation will be approximately half an hour and you will not be expected to return for a follow up.

4. PROCEDURES

If you agree to take part in this study, you will first be asked questions about yourself, including if you have any illnesses.

Nine tubes of blood will be collected from a vein in your arm, this will be equal to 45mls (the same amount as three tablespoons). We will also take your hip and waist measurements, together with your weight and height.

5. WILL ANY OF THESE STUDY PROCEDURES RESULT DISCOMFORT OR INCONVENIENCE?

Drawing of blood may cause some discomfort associated with pain, bruising or bleeding at the puncture site. The procedure will be performed by a trained nurse

6. BENEFITS

There will be no immediate benefit to you. However your participation in this study may result in increased understanding of why some people develop foam cells when others do not. It may also allow us to discover new ways to stop people from getting atherosclerosis.

7. RIGHTS AS A PARTICIPANT IN THIS STUDY.

Voluntarity

Your participation in the study is entirely voluntary and you may decline to participate.

Withdrawal

You may withdraw at any time. If you withdraw from the study, your samples will be destroyed.

8. FINANCIAL ARRANGEMENTS

You will not be required to pay for any of the study related procedures.

9. REIMBURSMENT FOR STUDY PARTICIPATION

You will not be paid to participate in the study.

10. ETHICAL APPROVAL

This study has been approved by the Human Ethics Committee of the University of the Witwatersrand, clearance number...

Please feel free to contact the Chair of the Human Research Ethics Committee with any questions or complaints: Prof Cleaton-Jones Tel: 011 717 1234

11. SOURCES OF ADDITIONAL INFORMATION

If at any time after you feel you have questions regarding this study, please do not hesitate to contact the principal investigator, Dr. Nitien Naran, at this number 0846060201

12. CONFIDENTIALITY

All information obtained during the course of the study including personal data and research data will be kept strictly confidential. Data that may be reported in scientific or medical journals will not include any information that identifies you as a participant in this study.

Any information uncovered regarding your test results or state of health as a result of your participation in this study will only be made known to your treating doctors. You will be informed of any findings of importance to your health during the participation in this study

I hereby confirm that the researcher has explained the nature, conduct, benefits and risks of this research project.

- I have also received, read and understood the above written information regarding the study.
- I am aware that the results of the study, including personal details regarding my age, sex and diagnosis will anonymously be processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researchers involved in this study.
- I may at any stage, without prejudice, withdraw my consent and participation in this study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

PARTICIPANT

Printed Name	Signature/Name or Thumbprint	Date

WITNESS (if applicable)

Printed Name	Signature/Name or Thumbprint	Date

Researcher Name	**Signature**	**Date**

APPENDIX B

R14/49 Mr Maile George Ralefatane et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M161057

NAME: (Principal Investigator)	Mr Maile George Ralefatane et al
DEPARTMENT:	Chemical Pathology
PROJECT TITLE:	Determining Differential Rates of Foam Cells Formation in Multi-Ethic South Africans: A Potential Role for Tissue Non-Specific Alkaline Phosphatase
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Substudy under Primary Study M140736 Dr Nitien Naran et al
SUPERVISOR:	Prof Nigel Crowther
APPROVED BY:	Professor P Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/10/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in October and will therefore be due in the month of October each year.

Principal Investigator Signature Date 2017/05/14

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX C

Appendix C1

Preparation of Oil red O stain

- Stock solution was prepared by weighing out 0.6 g of oil red o powder and adding it 200 ml of 99% isopropanol.
- To stain the cells, 3 parts (30 ml) of oil red o stock solution was mixed with 2 parts (20 ml) of deionized water and the solution was allowed to sit at room temperature for 10 minutes.
- A piece of filter paper was placed in a funnel and the funnel was placed in a 50 ml conical tube and therefore the oil red o working solution was filtered completely through the filter funnel.
- The working solution was only stable for no longer than 2 hours.

Appendix C2

Preparation of 4% paraformaldehyde

- Ninety (90) ml of distilled water was heated to 60 °C.
- Four (4) g (100 ml final volume 4% = 4 g) of paraformaldehyde was weighed and paraformaldehyde (4g) was added to water and stirred at 60 °C using magnetic stirrer.
- One drop of 2 N NaOH was added and the solution became clear (2 N NaOH is the same as 2 M NaOH, therefore to make 100 ml; 8 g of NaOH was dissolved in 100 ml of H2O).
- 10 ml of 10X PBS was added and the solution was allowed to reach room temperature.
- The pH was adjusted to 7.4 with 1N HCl.
- The solution was filtered using a 0.2 µm syringe filter and cool to 4 °C for use.

Appendix C3

Preparation of Crystal violet

- To prepare 0, 1 M citric, firstly 1.9212g of citric was weighed and secondly it was dissolved in 100 mL distilled water.
- To prepare 0.1 M citric acid containing 0.01% (w/v) crystal violet, 0.005g crystal violet was weighed and dissolved in 50mL of the 0.1M citric acid prepared in previous step.

Appendix C4

Preparing Tris-buffered saline (TBS) (1X)

- To prepare 1X TBS, 6.05 g Tris and 8.76 g NaCl were dissolved in 800 mL of H2O. pH was adjusted to 7.5 with 1 M HCl and make volume up to 1 L with H2O. TBS was stable at 4°C for 3 month.

TBS plus 0.025% Triton X-100

- For 50 ml TBS: 12.5 µl of Triton X-100 was added to 50 ml TBS to make TBS plus 0.025% Triton X-100

10% fetal bovine serum with 1% BSA in TBS

- For 50 ml: 10% FBS = 5 ml in 50 ml and 1 % BSA = 0.5 g in 50 ml
- Therefore 5 ml of FBS and 0.5 g of BSA were added in 50 ml TBS

Primary antibody diluted in TBS with 1% BSA

- TBS with 1% BSA: For 50 ml solution, 0.5 g of BSA was dissolved in 50 ml TBS

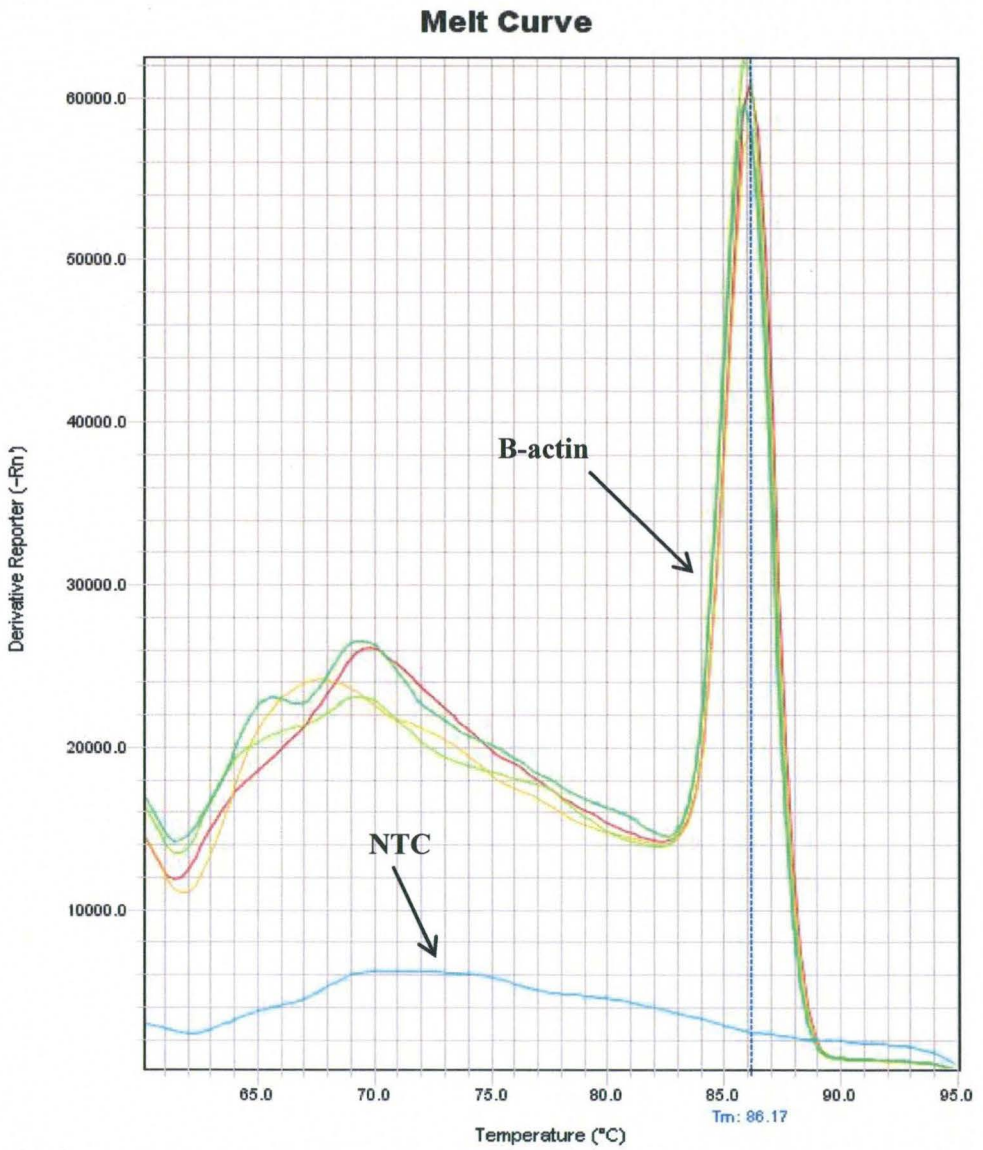
- For 1/100 dilution: 1 µl of primary antibody diluted in 99 µl of TBS with 1% BSA

Secondary biotinylated antibody diluted in TBS with 1% BSA

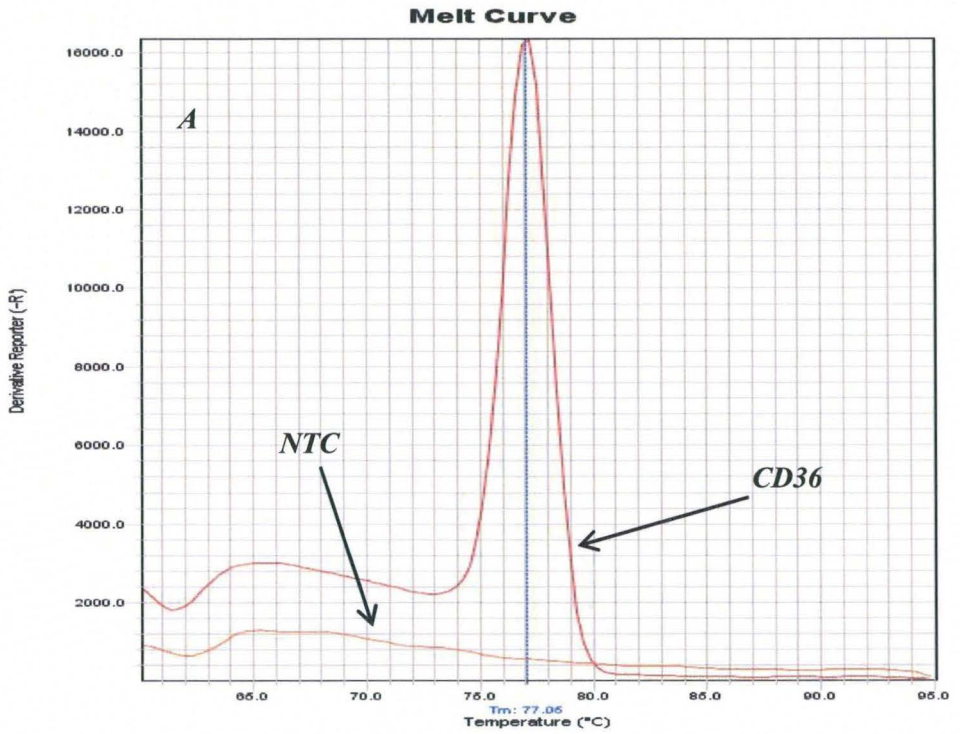
- For 1/200 dilution: 1 µl of secondary antibody diluted in 199 µl of TBS with 1% BSA

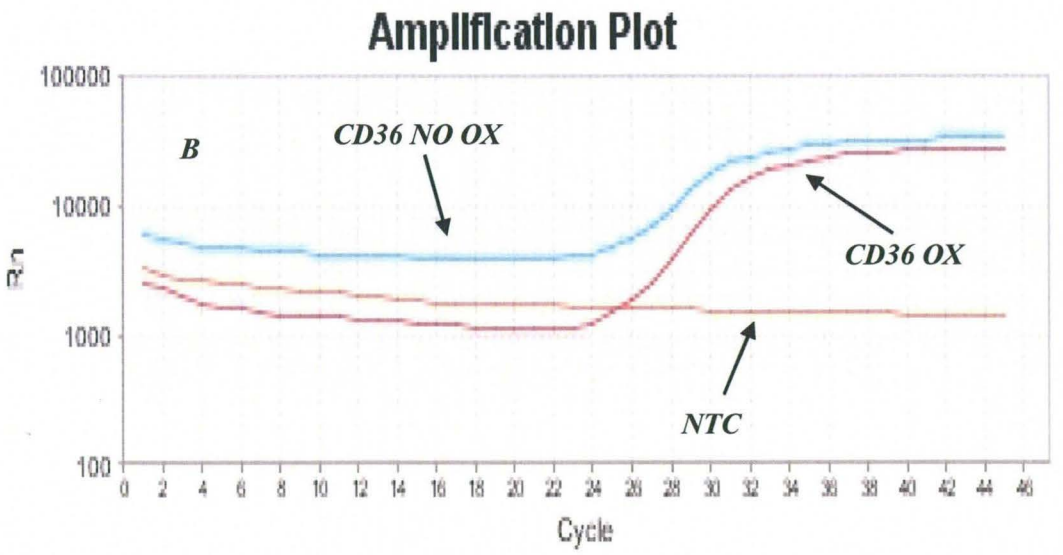
APPENDIX D

Melting curve of beta- actin in macrophages. NTCs are negative controls (A & B).

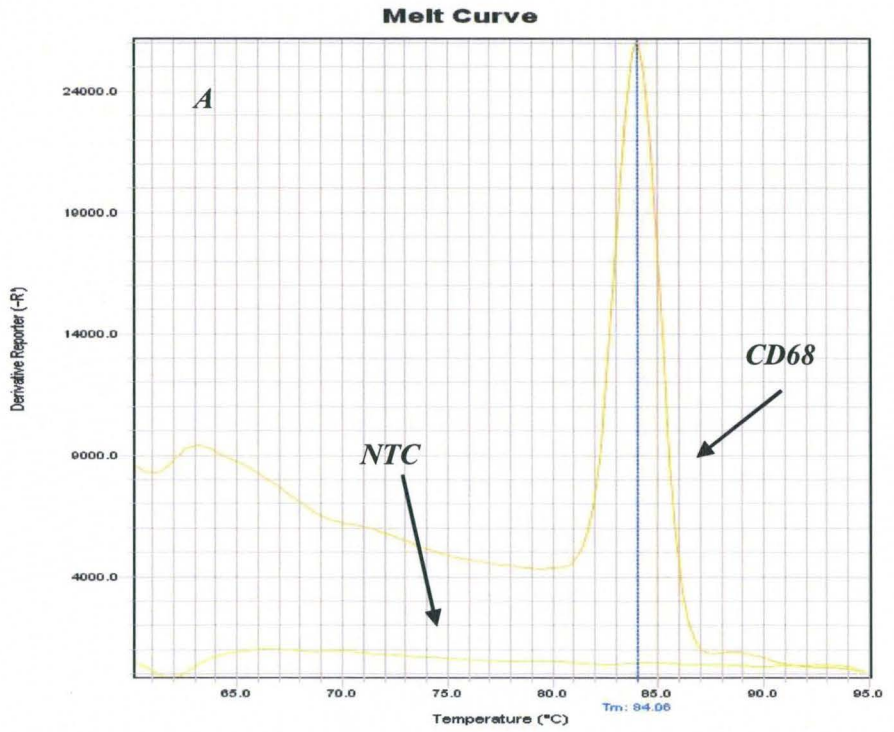


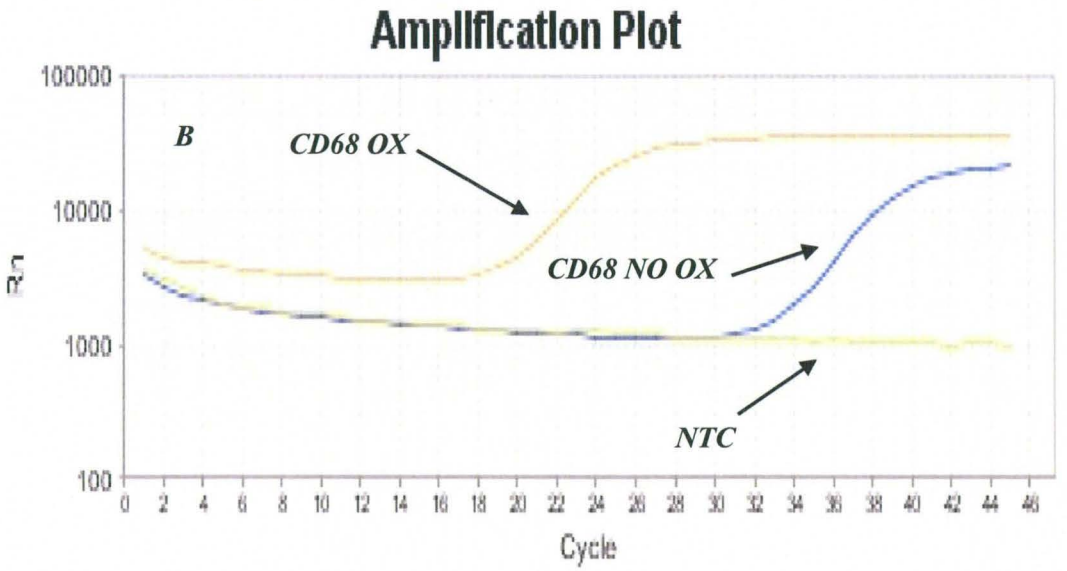
Real time PCR amplification plot (B) and melting curve of CD36 (A) on macrophages. Specific amplication gene is shown by single melting curve of their PCR product. NTCs are negative controls.



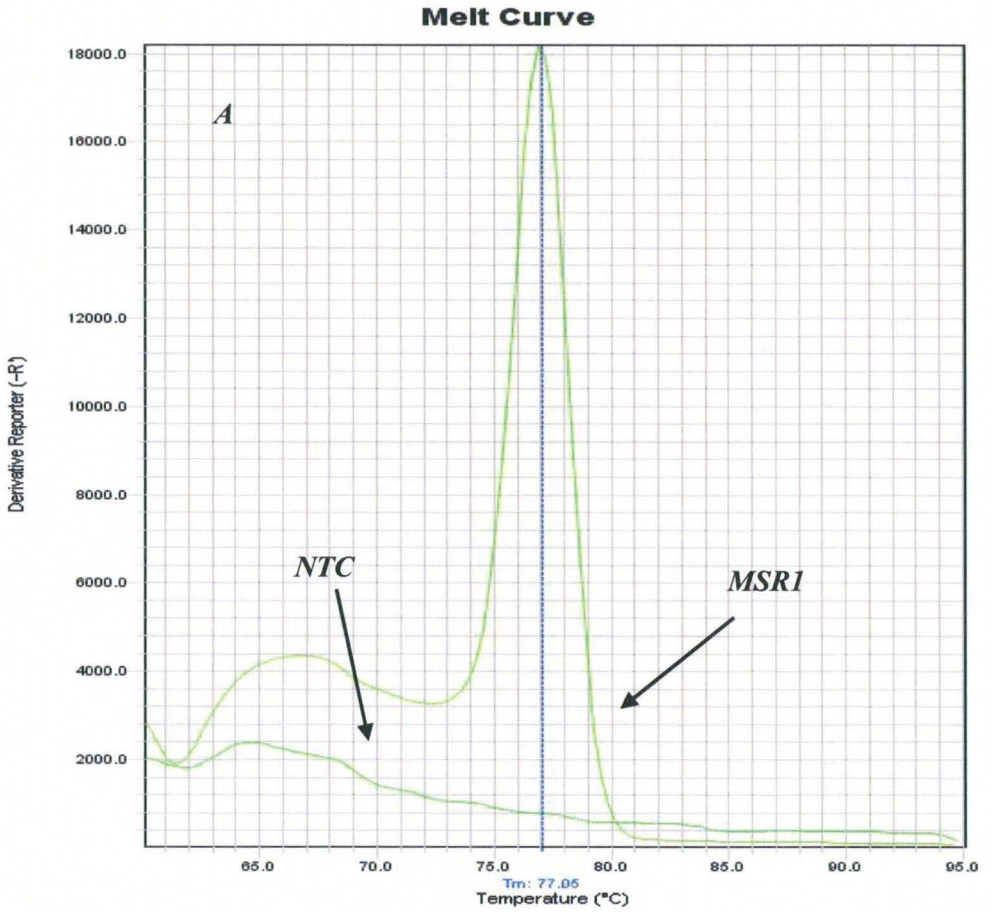


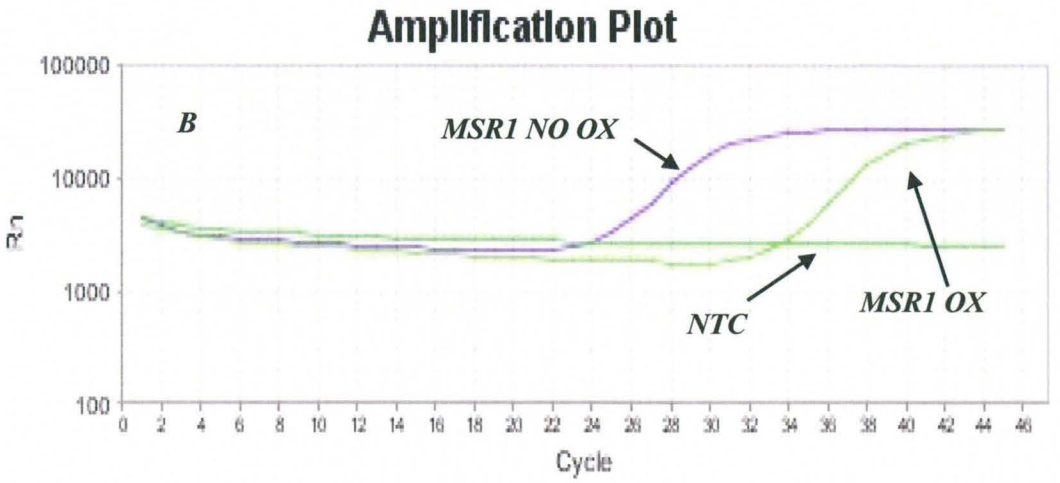
Real time PCR amplification plot (B) and melting curve of CD68 (A) on macrophages. Specific amplication gene is shown by single melting curve of their PCR product. NTCs are negative controls.





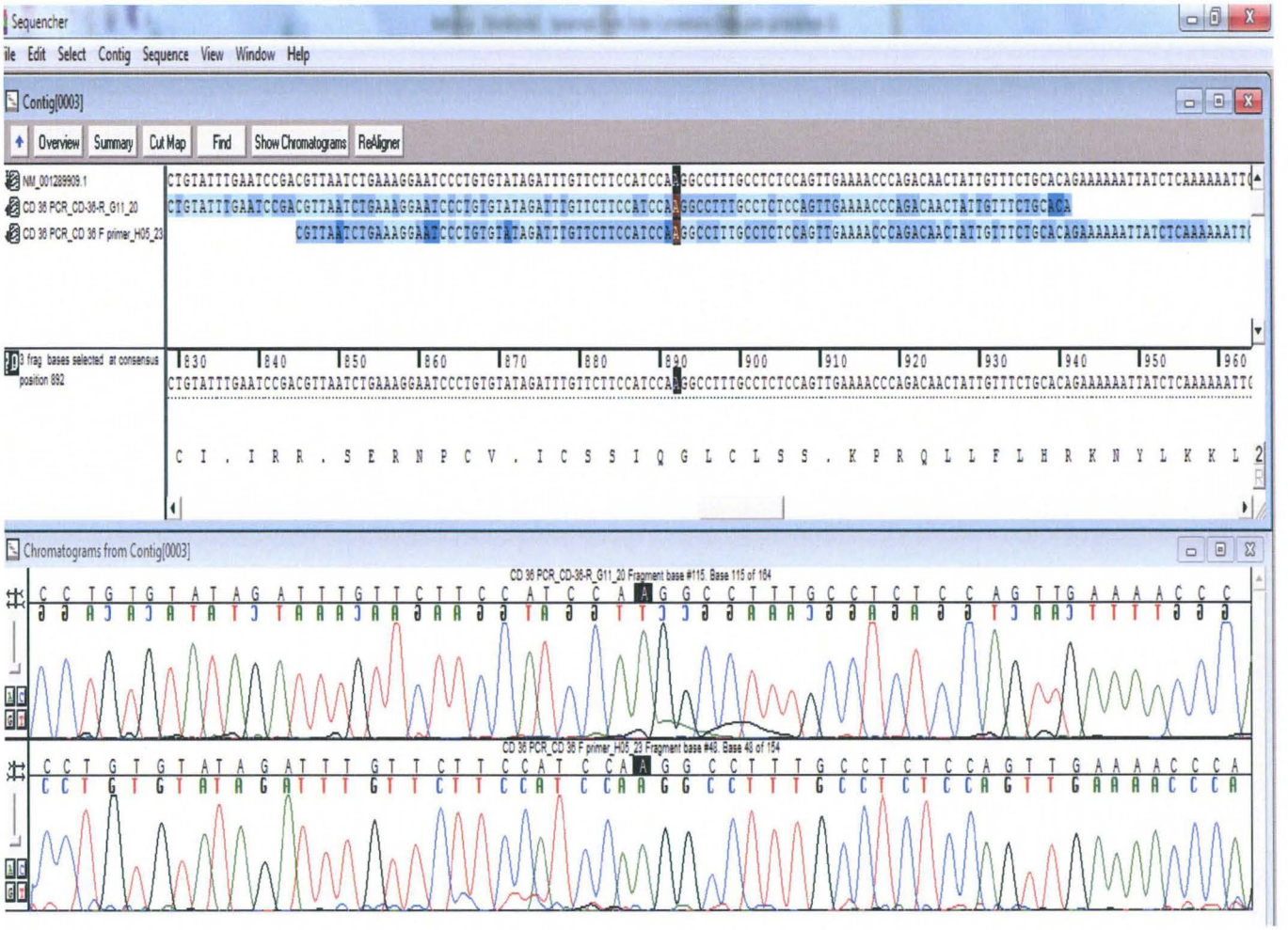
Real time PCR amplification plot (B) and melting curve of MSR1 (A) on macrophages. Specific amplication gene is shown by single melting curve of their PCR product. NTCs are negative controls



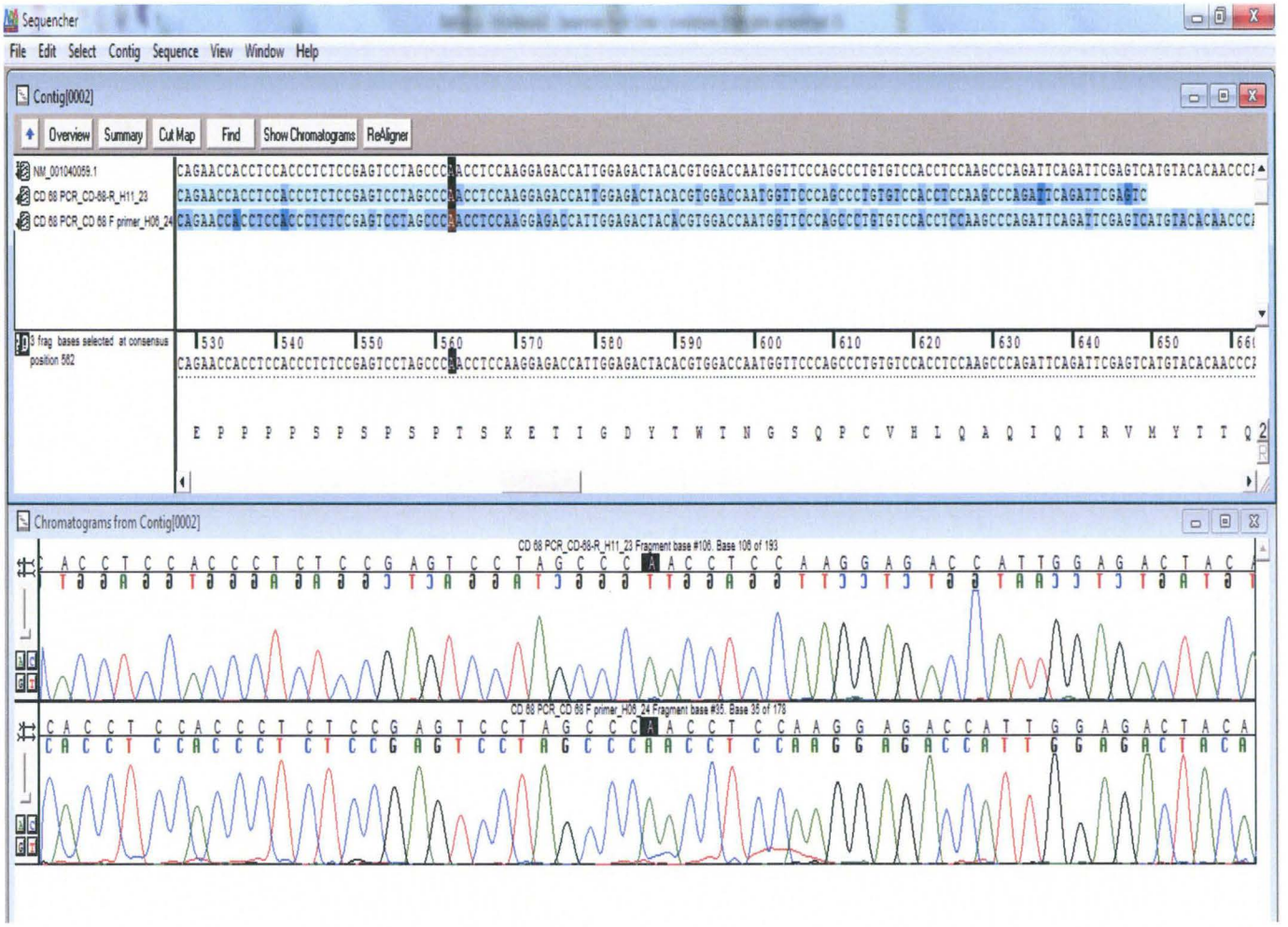


APPENDIX E

Consensus sequence of the CD36 gene and a portion of its chromatogram below



Consensus sequence of the CD68 gene and a portion of its chromatogram below



Consensus sequence of the MSR1 gene and a portion of its chromatogram below

