THE TWO FACES OF HIGH DENSITY LIPOPROTEIN:

OXIDIZED HDL ELICITS PRO-ATHEROGENIC RESPONSE IN HUMAN MONOCYTE-MACROPHAGES

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A THESIS PRESENTED BY

SOUMYARANI V.S.

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IN PARTIAL FULFILMENT OF THE REQIUREMENTS
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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CERTIFICATE

I, **Soumyarani V.S.** hereby certify that I had personally carried out the work depicted in the thesis entitled, **"The two faces of high density lipoprotein: oxidized HDL elicits pro-atherogenic response in Human monocyte-macrophages".** No part of the thesis has been submitted for the award of the any other degree or diploma prior to the date.

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CERTIFICATE

This is to certify that Soumyarani V.S. in the Department of Biochemistry of this Institute has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The thesis entitled, **"**The two faces of high density lipoprotein: oxidized HDL elicits pro-atherogenic response in human monocyte-macrophages**"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Signature

Date

The thesis entitled

THE TWO FACES OF HIGH DENSITY LIPOPROTEIN: OXIDIZED HDL ELICITS PRO-ATHEROGENIC RESPONSE IN HUMAN MONOCYTE-MACROPHAGES

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Somyarani V.S.

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ABBREVIATIONS

ABCA1	-	ATP –binding cassette transporter A1
ABCG1	-	ATP –binding cassette transporter G1
ABCG4	-	ATP –binding cassette transporter G4
AP-1	-	Activation protein 1
ATP	-	Adenosine triphosphate
BHT	-	Butylated hydroxy toluene
CAD	-	Coronary artery disease
CD36	-	Cluster of differentiation 36
CETP	-	Cholesterol ester transfer protein
CRP	-	C-reactive protein
$CuSO_4$	-	Copper sulphate
DCFH-DA	-	2,7 Dichlorodihydro fluoresceine diacetate
DPI	-	Diphenylene iodonium chloride
ECM	-	Extracellular matrix
ELISA	-	Enzyme-linked immunosorbent assay
ERK1/2	-	Extracellular signal-regulated kinase1/2

FC	-	Free cholesterol
GPx	-	Glutathione peroxidase
HDL	-	High-density lipoprotein
HDL-C	-	High-density lipoprotein cholesterol
HL	-	Hepatic lipase
HPETE	-	Hydroperoxy eicosatetra enoic acid
HPODE	-	Hydroperoxy octadecadienoic acid
hsCRP	-	high sensitivity C- Reactive protein
IL-1	-	Interleukin-1
INF-γ	-	Interferon gamma
JNK	-	C-Jun N-terminal kinase
LCAT	-	Lecithin cholesterol acetyl transferase
LDL	-	Low-density lipoprotein
LDL-C	-	Low- density lipoprotein cholesterol
Lp-PLA_2	-	Lipoprotein associated phospholipase A_2
LXR	-	Liver X receptor
MAPK	-	Mitogen activated protein kinase
MCP-1	-	Monocyte chemoattractant protein -1
M SF	-	Macrophage colony-stimulating factor
MetS	-	Metabolic syndrome
MMP	-	Matrix metalloproteinase
MPO	-	Myeloperoxidase
NAC	-	N-aetyl cysteine
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NADPH oxidase	-	Nicotinamide adenine dinucleotide phosphate oxidase
NFKB	-	Nuclear factor kappa-light chain-enhancer of activated B cells

nHDL	-	native high density lipoprotein
NO	-	Nitric oxide
OxHDL	-	Oxidised-high density lipoprotein
p38 MAPK	-	p38 Mitogen activated protein kinase
PAF-AH	-	Platelet activating factor acetyl hydrolase
PGI_2	-	Prostaglandin I_2
PKC	-	Protein kinase C
PL	-	Phospholipid
PLTP	-	Phospholipid transfer protein
PON	-	Paraoxonase
PPAR	-	Peroxisome proliferator-activated receptor
RCT	-	Reverse cholesterol transport
ROS	-	Reactive oxygen species
RT-PCR	-	Reverse transcription polymerase chain reaction
RXR	-	Retinoid X receptor
SAA	-	Serum amyloid A
sdLDL	-	small dense-low density lipoprotein
SMC	-	Smooth muscle cell
SOD	-	Superoxide dismutase
SR-B1	-	Scavenger receptor B1
TG	-	Triglyceride
TIMPs	-	Tissue inhibitor of metalloproteinases
TNF-α	-	Tumor necrosis factor- alpha
TXA_2	-	Thromboxane A_2
VCAM-1	-	Vascular cell adhesion molecule -1
vWF	-	von Willebrand factor

Synopsis

Study background

Coronary artery disease (CAD) is the leading cause of death worldwide. Its prevalence in Kerala is much higher than other parts of India and is affecting younger people also. Coronary artery atherosclerosis is the principal cause of coronary artery disease. Various reasons have been cited for this phenomenon. Genetic factors may underlie CAD in some people, though most often CAD is an acquired condition which is the direct consequence of lifestyle factors such as cigarette smoking, eating habits and physical inactivity. CAD develops [over decades] when atherosclerotic plaque infiltrates the arterial intima and accumulates into deposits called atheromas. These factors converge to restrict blood flow to the heart and deprive segments of the heart of adequate oxygenation. The result is cardiac ischemia which may be asymptomatic or may cause chest pain, known as angina pectoris.

High-density lipoprotein (HDL) is considered to be cardioprotective in nature. Current thought regarding HDL and cardiovascular protection focus almost exclusively on serum HDL-cholesterol concentration as a determinant of cardiovascular disease risk. Although HDL possess many features that contribute to the association between elevated HDL-cholesterol and protection from atherosclerosis, this lipoprotein is known to undergo modification in certain individuals or circumstances to become pro-atherogenic. It is hypothesized that high prevalence of dysfunctional HDL could be a contributing factor to the excessive risk of CAD in our subjects. However, the pro-atherogenic pathways exerted by modified HDL remain poorly understood.

Objectives

The major focus of the current study is identification of the prevalence of dysfunctional HDL in apparently healthy subjects, and elucidation of its functional consequences. We also studied how the intrinsic function of monocytes, the key cell type involved in the development of atherosclerotic lesion, might be influenced by its interaction with modified HDL particles. To stimulate the efflux of cholesterol from macrophage-foam cells as well as to initiate other anti-atherogenic functions in endothelium, HDL should make contact with these cells. Therefore it is important to determine the effect of modified HDL on monocyte- macrophage functions relevant to atherogenesis.

Experimental approach

In order to study the functionality, HDL fractions were isolated from blood samples collected from apparently healthy volunteers by ultracentrifugation. The purity of isolated HDL was checked with polyacrylamide gel electrophoresis and dot blot analysis using specific antibodies [against apo A1 and apo B]. HDL functionality was measured in terms of its ability to inhibit LDL oxidation induced by copper ions. The oxidation kinetics was measured as change in conjugated dienes formation using a UV spectrophotometer at 234nm.

For in vitro experiments, HDL particles were isolated from human blood samples by ultracentrifugation and subjected to oxidation with $CuSO_4$. The extent of oxidation was quantitated by measurement of lipid peroxides. Human peripheral blood mononuclear cells were isolated and cultured under standard conditions. Cells were treated with native and oxHDL at varying concentrations for different time intervals and used for several analyses. Intracellular reactive oxygen species (ROS) production was assessed based on ROS-mediated DCFH fluorescence of the cells. The release of TNF-α and matrix

metalloproteinases (MMPs) was quantitated using ELISA kit and gelatine zymography respectively. RT-PCR analysis was employed for examining the gene expression and immunocyto chemistry was performed for protein expression analysis. Various inhibitors were used for checking the involvement of signaling pathways.

Major findings of the study

Antioxidant capacity of HDL

It was observed that unlike LDL, HDL was able to resist oxidation induced by copper sulphate at a higher concentration of 10μM showing its resistance to oxidation due to its antioxidant capacity. Further co-incubation of HDL isolated from healthy subjects [having normal lipid profile] with LDL at equal protein concentration showed maximum inhibitory capacity against LDL oxidation [~70 %]. This functional property (antioxidant capacity) of HDL was found to be varying among subjects having same HDL-cholesterol concentration. HDL showed less inhibitory capacity against LDL oxidation [less than 40%] in few healthy subjects and also in those with metabolic syndrome [having more than three of the risk factors for heart disease such as dyslipidemia - high cholesterol and/or triglycerides, low HDL-cholesterol; high blood pressure and obesity]. These subjects with less antioxidant capacity of HDL showed higher oxidative stress and inflammation as evidenced by greater concentration of serum lipid peroxides, protein carbonyls and hsCRP. Generally HDL is considered to be an antiatherogenic particle. However, the functional assay of HDL particles indicates that all HDLs are not same in terms of antioxidant capacity. Although the exact reason for this functional deficiency in HDL is not known, these subjects were found to have systemic oxidative stress and inflammation.

Invitro experiments: Influenec of oxHDL on monocytes/macrophage function

HDL particle can undergo structural alterations during conditions like acute phase response and oxidative stress and transform into dysfunctional form. Although dysfunctional HDL has been implicated in the pathogenesis of atherosclerosis, the underlying pathways remain poorly understood. It has been proposed that HDL loses its cardioprotective ability through oxidative modifications by reactive oxygen species (ROS) and promote atherogenesis. However the pro-atherogenic pathways undergone by oxidized HDL remain poorly understood. Since monocytes play a crucial role in atherogenesis, the current study was aimed to investigate the influence of both native and oxidized HDL (oxHDL) on monocytes/macrophages functions relevant to atherogenesis. HDL particles were isolated from human blood samples by ultracentrifugation and subjected to in vitro oxidation with $CuSO_4$. The extent of oxidation was quantitated by measurement of lipid peroxides. Human peripheral blood mononuclear cells were isolated and cultured under standard conditions. Cells were treated with native and oxHDL at varying concentrations for different time intervals and used for several analyses. Intracellular ROS production was assessed based on ROS-mediated DCFH fluorescence of the cells. The release of inflammatory markers- TNF-α and matrix metalloproteinases (MMPs), was quantitated using ELISA kit and gelatine zymography respectively. Treatment of cells with oxidized HDL enhanced the production of ROS in a concentration- dependent way, while native HDL had no such effect. Further, the release of TNF-α, MMP-9 and MMP-2 was found to be remarkably higher in cells incubated with oxHDL than that of native HDL. These findings demonstrate that oxidative modification of HDL induces pro-inflammatory response and oxidative stress in human monocytes/macrophages.

Matrix proteolysis is a common feature of several physiological and pathological possesses. Metalloproteinases participate in extracellular matrix (ECM) remodeling and regulatory signaling during chronic inflammatory state such as

atherosclerosis formation. Studies on human atherosclerotic plaques have revealed that lesion that tend to rupture are rich in activated monocytes and have a thin fibrous cap, implicating macrophages as a key regulator of atherosclerotic plaque stability. However, the mediators of MMP upregulation in inflammatory states are not well established. The inflammatory cytokines and oxidized lipids can induce MMP activity in the cells. The current study demonstrates for the first time that oxHDL can induce MMP-9 secretion in monocytes/macrophages. Several lines of evidence support the potential role of MMPs in human atherosclerosis and plaque disruption. This study suggests that unlike native HDL and mildly oxHDL, oxHDL may promote matrix degradation by enhancing the release of MMP-9 and MMP-2 from arterial monocytes/macrophages, favoring atherosclerotic plaque destabilization and rupture.

Role of signaling pathways-:MAP Kinases

We have further examined the possible involvement of stress kinases on oxHDL-induced MMP secretion from monocytes/macrophages, using specific inhibitors against MAP kinases, such as p38, JNK, ERK1/2. The results showed that blocking ERK1/2 and JNK -MAPK pathways significantly inhibited the secretion of MMP-9. In contrast, when cells were exposed to p38- inhibitor, oxHDL- still induced MMP-9 secretion from monocytes/macrophages and thus indicating the involvement of ERK1/2 and JNK -MAPK signaling in oxHDL-mediated upregulation of MMP-9 pathway. A basal level of MMPs are important for cell structure and survival , but increased expression of MMPs could contribute to tissue damage. In addition, experiments were also performed to delineate the role of the transcription factor-NF-kB, using specific inhibitor and immunostaining of p65 nuclear translocation.The results indicated that activation of NF-kB was not essential for oxHDL-induced MMP-9 release from monocytes/macrophages and thereby suggesting the possible involvement of other transcription factor-AP-1, that need clarification.

Role of oxidative stress and NADPH oxidase

Oxidative stress and inflammation play major role in atherogenesis. Oxidative stress is defined as the imbalance redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased production of ROS. There are several potential sources of ROS in most cells. Current study proves that oxHDL can induce ROS formation in monocytes/macrophages that mediate various signaling pathways leading to macrophage inflammatory response. Because of the apparent importance of ROS in vascular disease, there has been substantial interest in the enzymatic sources that contribute to production of free radicals in vascular tissues. The next objective of the present study was to delineate the redox signaling pathways induced by oxHDL in monocytes-macrophages. Since NADPH oxidases appear to be especially important for redox signaling, inhibitor for this oxidative enzyme was used to assess its role in oxHDL- mediated ROS formation. Exposure of monocytes/macrophages to oxHDL triggered the release of ROS, which was blocked by pre-treatment of cells with ***Diphenyleneiodonium*** [DPI.], an inhibitor of NADPH oxidase. Pre-treatment of cells with DPI effectively suppressed the production of intracellular ROS and MMP-9 indicating that activation of NADPH oxidase plays a significant role in ROS formation. Further the antioxidants-N-acetyl cysteine (NAC) and butylated hydroxy toluene (BHT) could effectively inhibit the ROS production and also the release of MMP-9 in these cells when stimulated with oxHDL and thus confirming the pro-oxidative property of oxHDL. All these results reveal that oxHDL-mediated MMP-9 expression in monocytes/macrophages, is dependent on the formation of ROS which is induced by NADPH oxidase system as well as the activation of ERK1/2 and JNK- MAPK pathways.

Effect of oxHDL on genes responsible for lipid homeostasis

Macrophages have important roles in both lipid metabolism and inflammation and are central to the pathogenesis of atherosclerosis. Liver X receptors (LXRs) are key

transcriptional regulators of genes involved in lipid homeostasis and inflammation and are determinants of atherosclerosis susceptibility. ATP-binding cassette transporters (ABC) – ABCA1 and ABCG1 are membrane cholesterol transporters and have been implicated to mediate cholesterol efflux from cells to apolipoprotein A-I, the major protein constituent of HDL and mature HDL respectively. LXR is the major regulator of ABCA1, ABCG1, and apoE. Since LXR signaling is critical for lipid homeostasis its role in oxHDL-mediated monocytes/macrophages function was examined. Treatment of cells with oxHDL showed an enhanced gene expression of LXR alpha and ABCG1 and supressed expression of CD36 as evidenced by RT-PCR analyses while treatment with native HDL showed only basal level expression of the above genes. This suggests that oxHDL-induced oxidative stress and lipid accumulation in monocytes/macrophages might act as an adaptive stimuli for lipid homeostasis in cells as evidenced by enhanced expression of LXR and ABCG1 for cholesterol efflux and supressed expression of CD36 [a strong receptor of modified lipids] to inhibit further lipid uptake. Similar response was observed in the protein expression levels of ABCG1 and CD36. LXR activation represents a mechanism to prevent macrophage foam cell formation. However, adequate cholesterol efflux through ABCG1 to the acceptor, i.e oxHDL, might not be taking place and thus resulting in lipid accumulation in these cells. In consistence with other reports current findings also demonstrated that treatment of monocytes/macrophages with oxHDL increased the neutral lipid accumulation suggesting its pro-atherogenic role. Lipid and inflammatory pathways induced in activated macrophages are central to the pathogenesis of inflammatory diseases including atherosclerosis.

HDL modifications can be achieved by different means such as non-enzymatic modifications due to the presence of free metal ions in atherosclerotic plaque, cell -associated inflammatory enzymes, association with acute phase protein and metabolic

modifications that can lose its anti-atherogenic activities. Copper and iron may be important modulators of lipid peroxidation. In our study a mild oxidative condition in HDL does not elicit significant inflammatory response suggesting that low degree of oxidation in HDL may not be deleterious while extreme oxidation induces deleterious effects leading to intracellular lipid deposition. Macrophages in the intima and media express scavenger receptors that bind oxidized lipids and form foam cells. The exact cellular mechanisms associated with these effects of oxHDL remains to be elucidated. It is possible that the oxidative stress induced by oxHDL in monocytes/macrophages can lead to pro-inflammatory response. This response in the artery wall can recruit other cell types also and can contribute to the development of complex lesions. The oxidative stress exerted by oxHDL could also be cytotoxic and promote cell death.

Oxidation, particularly oxidative modification of low-density lipoproteins (LDL) within the artery wall and its subsequent unregulated uptake by macrophages, has been postulated to be an important event in disease development . A range of reactive species can oxidize lipoproteins, including HDL in vitro, but the nature of oxidants under in vivo condition is controversial. Recent studies have demonstrated the presence of elevated levels of specific protein oxidation products in advanced human lesions and identified metal ions [high iron and copper] as possible catalysts for these species . These findings are consistent with the hypothesis that high iron and copper levels may contribute as an independent factor for atherosclerosis, a multifactorial disease, and its sequelae. Although dysfunctional HDL is implicated in the pathogenesis of cardiovascular disease, the underlying pathways of its formation and its effect on cellular environment remains poorly understood. Current study demonstrates that copper-mediated oxidation caused accumulation of MDA in HDL, thereby converting it into a pro-inflammatory particle that stimulates the production of ROS, TNF-α, MMP-9 and MMP-2 through the activation of NADPH oxidase,

in cultured monocytes/macrophages- a possible way of modified HDL-mediated pro-atherogenic properties. Although MMP-9 plays a significant role in the pathology of atherosclerosis, the signaling required for MMP-9 up-regulation has yet to be fully elucidated. This work adds to this area of literature indicating that ROS produced by oxHDL can induce the expression of MMP-9. In fact, MMP-9 emerges as a potential mediator of the pro-atherosclerotic actions of phagocytic NADPH oxidase in both symptomatic and asymptomatic subjects. The research findings on oxHDL-induced ROS and MMP-9 activity in mo.mac have implications for potential therapies that aim to reduce the amount of MMP-9 or inflammatory response in patients.

Conclusion

. In conclusion this study reveals that human HDL exhibits variable functional ability and the levels of HDL-C are insufficient to predict the functional heterogeneity of HDL. Further, this study demonstrates that following in vitro oxidative modification, HDL loses its atheroprotective functions and exerts pro-inflammatory response by releasing TNF-α and MMP-9 as well as promotes oxidative stress in human monocytes/macrophages. However, monocytes/macrophages exhibited no such responses with mildly oxHDL and native HDL.The generation of oxHDL in vivo might therefore be regarded as possibly atherogenic.

INTRODUCTION

Coronary heart disease (CHD) is now the leading cause of death worldwide. The incidence of CHD is still on the increase, especially in developing countries, such as India, and has become a major burden upon public health. Its prevalence in Kerala , particularly among the urban population, is much higher than other parts of India, and is affecting more subjects at the young age, below 40 years. Moreover significant number of women is affected with CHD in their reproductive age. CHD that manifests at a younger age can have devastating consequences for an individual, the family, and society. Prevention of these deaths in young people is a nation's moral responsibility. Although a large proportion of CHD is preventable, they continue to increase mainly because preventive measures are inadequate. Clearly, these factors indicate the importance of developing novel therapeutic approaches that can further improve CHD management/ prevention. High-density lipoprotein (HDL) metabolism represents a major target for the development of therapies intended to reduce the risk of atherosclerotic-cardiovascular disease. The current study is focused on the importance of HDL functionality in its atheroprotective nature and its contribution to atherosclerosis.

The underlying pathology of most clinical manifestations of CHD is atherosclerosis, a slowly progressive process that generally begins in childhood and manifests clinically in middle to late adulthood. Various reasons have been cited for this phenomenon. Genetic factors may underlie CHD in some people, though most

often CHD is an acquired condition which is the direct consequence of lifestyle factors such as cigarette smoking, eating habits and physical inactivity. CHD develops when atherosclerotic plaque infiltrates the arterial intima and accumulates into deposits called atheromas. These factors converge to restrict blood flow to the heart and deprive segments of the heart of adequate oxygenation. The result is cardiac ischemia which may be asymptomatic or may cause chest pain, known as angina pectoris.

1.1. Lipid lowering therapy in the prevention of CHD: The most effective means of preventing CHD is to prevent atherosclerosis. Several variables have been taken into consideration to determine CHD risk. Observational studies over many decades have shown a close, direct relationship between dyslipidemia and coronary heart disease risk (Lloyd-Jones et al. 2004; Neaton et al. 1992). The only blood lipid biomarkers currently recommended for use in cardiovascular risk prediction by The Adult Treatment Panel(2001) are low-density lipoproteins-cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], and triglycerides (TG) *(*ATP- III report (The Adult Treatment Panel III (ATP III). The last decades of clinical study were concentrated on lipid lowering therapy for the prevention of atherosclerosis and increasing the so-called athero-protective HDL-C levels. Although statin therapy [3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor] has been remarkably successful, its use has not eliminated CHD and there exists significant 'residual' cardiovascular risk in patients (Mark & Tyan, 2010). It has become increasingly apparent that a large proportion of cardiovascular events occur in subjects with normal levels of LDL-C and HDL-C (Navab et al. 2005; Ansell et al. 2003). This may reflect inadequate control of disease progress due to our incomplete

understanding of the disease mechanisms. This has led to heightened interest in HDL, as a potential target for therapy.

Recently, studies using recombinant apolipoprotein AI liposomes have shown that direct infusion can effectively reduce established atheromatous plaques in animals and in coronary patients (Chiesa & Sirtori, 2003 ; Nissen et al. 2007). Further attention has been turned to raise HDL -C with drugs, such as cholesteryl ester transfer protein [CETP] inhibitor [Torcetrapib], niacin and several genetic variants of Apo A1, but the clinical outcomes are disappointing and no benefit has been demonstrated. From these observations it is obvious that the relationship between HDL-C and CHD is more complicated than the originally thought. Future research need to be concentrated in studies aimed at better understanding of the different biological functions of HDL (functional abnormality in HDL resulting in specific loss of atheroprotective function) as well as the proteins and receptors with which HDL interacts, in order to identify the exact relationship between HDL and CHD, which might pave the way for future pharmacotherapeutic research.

1.2. Pathogenesis of Atherosclerosis: Marchand introduced the term "atherosclerosis" describing the association of fatty degeneration and vessel stiffening (Aschoff, 1933). This process affects medium and large-sized arteries and is characterized by patchy intramural thickening of the subintima that encroaches on the arterial lumen. The earliest visible lesion of atherosclerosis is the fatty streak, which is due to an accumulation of lipid-laden foam cells in the intimal layer of the artery. With time, the fatty streak evolves into a fibrous plaque, the hallmark of established atherosclerosis. Ultimately the lesion may evolve to contain large amounts of lipid; if

it becomes unstable, denudation of overlying endothelium or plaque rupture may result in thrombotic occlusion of the overlying artery.

Atherosclerosis is no longer considered a disorder due to abnormalities in lipid metabolism. In fact, the inciting event of atherosclerosis is likely an inflammatory insult that occurs decades before the disease becomes clinically apparent. It is a complex multifactorial progressive disease characterized by increased number of infiltrated inflammatory cells and deposition of modified proteins and lipids in the vascular wall (Lusis, 2000).

1.3. Inflammation, endothelial perturbation and LDL- modification in atherosclerosis

The atherosclerotic process is characterized, in its earliest stages, by perturbations in endothelial function. It is likely initiated when endothelial cells over-express adhesion molecules in response to turbulent flow in the setting of an unfavorable serum lipid profile. When the arterial endothelium encounters certain bacterial products or risk factors, these cells augment the expression of adhesion molecules [vascular cell adhesion molecule,VCAM-1] that promote the sticking of blood leukocytes [monocytes and T-cells] to the inner surface of the arterial wall; subsequent release of monocyte chemo-attractant protein-1 (MCP-1) by leukocytes magnifies the inflammatory cascade. Increased cellular adhesion and associated endothelial dysfunction then sets the stage for the recruitment of inflammatory cells, release of cytokines and recruitment of lipid into the atherosclerotic plaque. It is now widely accepted that the earliest stages of the development of atherothrombosis are mediated, in large part, by the inflammatory cascade (Crowther, 2005).

Once within the media three fates can befall the LDL; it may move back into the bloodstream (a hallmark of lesional regression and a process that may be facilitated by some lipid lowering strategies), it may become oxidized (through action of free radicals or direct activity of leukocytes) or it may be taken up by monocytes-macrophages which ultimately become foam cells. Oxidized LDL is particularly atherogenic and is chemotactic for monocyte-macrophages. Outcomes of their activation include recruitment and proliferation of smooth muscle cells [SMC], further LDL oxidation, recruitment of additional monocyte/foam cells and additional impairment of endothelial function. Monocytes directly interacting with human endothelial cells increase monocyte MMP production several fold, allowing for the subsequent infiltration of leukocytes through the endothelial layer. SMCs migrate from the tunica media into the intima via degradation of the extracellular matrix mediated by MMP9 and other proteinases (Ketelhuth et al. 2010). In the intima, SMCs proliferate under the influence of various growth factors and secrete extracellular matrix proteins. This process causes the lesion to evolve from a lipid-rich plaque to a fibrotic and, ultimately, a calcified plaque that may create a stenosis (Crowther, 2005).

The gradually enlarging plaque may precipitate chronic stable angina due to flow-limiting epicardial coronary disease. Acute myocardial infarction is usually due to acute thrombotic occlusion of an epicardial vessel. It occurs as a consequence of sudden disruption of the atherosclerotic-plaque associated with spontaneous fissuring or rupture when exposed to high shear stress at sites of stenosis and arterial branching. However, myocardial infarction often occurs within vessels with relatively unremarkable narrowing. Some plaques grow at a much greater rate than would be

predicted by simple lipid accumulation and expansion of the components of the fibrous plaque. Cholesterol accumulation within such plaques is due to both passive transfer of LDL from the circulation and scavenging of red blood cell membranes deposited during intraplaque hemorrhage (Miller et al. 2003; Packard & Libby. 2008). Plaque hemorrhage is likely attributable to bleeding from fragile microvessels that proliferate within the plaque itself, presumably in response to local angiogenic stimuli (Kockx et al. 2003).

It has become clear in recent years that clinical manifestation of atherosclerosis is the consequence of sudden lesion disruption and subsequent thrombosis. Human plaque analysis has revealed that MMP9 is catalytically active and may thus contribute to the dysregulation of extracellular matrix that leads to plaque rupture during the complication of atherothrombosis (Galis et al. 1994). Further evidence suggests that local over expression of MMP9, [by enhanced inflammatory response] promotes intravascular thrombus formation through increased tissue factor expression and associated activation of the coagulation cascade (de Nooijer et al. 2004). These data support an important role for MMP-9 both in atherosclerosis and plaque rupture and indicate that MMP inhibition could be a suitable preventive therapy. What we are missing currently are safe and specific MMP inhibitors. Considering inflammation as a crucial contributing factor in these basic processes, an alternative approach directed at understanding the contribution of HDL in such situation [regulating MMP expression] is of great importance.

1.4. HDL and Atherosclerosis

HDL has well-established protective influence against atherosclerotic-CHD and is an attractive target for antiatherogenic drug therapy (Marchesi and Sirtori. 2006; Nicholls and Nissen. 2007, Kapur et al. 2008). Classically, the protective functions of HDL particles have been attributed to their capacity to facilitate cholesterol efflux from peripheral tissues and notably macrophage-foam cells, and to transfer such cholesterol to the liver in the process of reverse cholesterol transport (RCT) (Lewis. 2006). This process may minimize the accumulation of foam cells in the artery wall. HDL has additional properties that may also be antiatherogenic. HDL is an effective antioxidant, and has the capacity to inhibit the oxidative modification of LDL in a process that reduces the atherogenicity of LDL. Indeed, HDL may afford protection from vascular disease by exerting additional effects that include anti-inflammatory, antiapoptotic, antithrombotic, and vasodilatory functions (Assman and Gotto. 2004; van Lenten.et al. 2001). By virtue of their ability to inhibit the expression of adhesion molecules in endothelial cells, they reduce the recruitment of blood monocytes into the artery wall. HDL antioxidative properties are related to enzyme including paraoxonase, platelet activating factor acetyl hydrolase (PAF-AH), glutathione selenoperoxidase, as well as protection of HDL apolipoproteins against oxidative stress; such apolipoproteins include apoA-I, apoA-II, and apoA-IV (Kontush and Chapman. 2010). Apo A1, the major apoprotein of HDL is directly involved in reverse cholesterol transport process and it also have antiinflammatory and anti-oxidative actions. New antiatherogenic roles of HDL are currently emerging, which are related to endothelial cell turnover and function (Lesnik and Chapman. 2006). All these protective mechanism of HDL arise due to the concerted action of HDL-

associated anti-oxidative/antiinflammatory enzymes and proteins. These properties are of particular interest as they provide indirect confirmation of the latest theories of atherogenesis, which are centered on the effect of lipoproteins and inflammation in the vascular wall.

HDL structure is complex because of the existence of subclasses of particles and because of the remodeling that is induced by interaction with lipases, lipid transfer proteins and HDL receptors on cell surfaces. Recent studies, however, have recognized that the physical heterogeneity of HDLs is associated with multiple functions that involve both the protein and the lipid components of these particles (Phillips. 2013). The physiological functions of HDL influence the cardiovascular system favorably, unless it is modified pathologically.

1.4.1. Functionally Defective High-Density Lipoprotein: new insights

Current thought regarding HDL and cardiovascular protection focus HDL heterogeneity and functionality as determinants of cardiovascular disease risk. HDL is known to undergo dramatic modification in structure and composition as a result of the concerted actions of the acute-phase response and inflammation (Khovidhunkit et al. 2004). Evidence shows that pathological processes associated with systemic inflammation including CHD, arthritis, systemic lupus erythematous disease are characterized by the presence of functionally altered HDL(Charles-Schoeman et al. 2009; McMahon et al. 2006). As a result, HDL particles progressively lose normal biological activities and acquire altered properties. Such altered HDL particles have been termed “dysfunctional HDL” (Navab et al. 2001). The abnormality in HDL function raises the possibility of an indirect proatherogenic effect of these particles.

Failure in cholesterol efflux capacity of HDL can result in enhanced accumulation of cholesterol in the arterial wall and reduced RCT flux, leading to proatherogenic environment in the vascular wall. Similarly a deficiency in the anti-oxidative and antiinflammatory properties of HDL may also result a pro-inflammatory response and accelerated atherosclerosis. Thus, HDL particles can exhibit complex, and sometimes contradictory roles in vascular biology. However, the precise role of functionally modified HDL in atherogenesis has not yet been fully elucidated.

1.5. Significance of the study

We currently understand atherogenesis as a complex interaction of risk factors including cells of the artery wall and the blood and molecular messages that they exchange. Most atherosclerotic lesions are clinically silent and may exist for years with out any meaningful sequelae. It is only when atherosclerotic-lesions become active, myocardial infarction occurs. It is now obvious that inflammatory activation, rather than the degree of stenosis, renders the plaque rupture and precipitates thrombosis and resulting tissue ischemia. HDL is the only atheroprotective lipoprotein and the physiological functions of HDL influence the cardiovascular system in multiple favorable ways, except when HDL is modified pathologically. Given the pathophysiological implications of the detrimental effect of inflammation on HDL function, it is clearly important to understand the mechanism that may involve. Functional heterogeneity of HDL is an area of recent interest, where much remains to be determined regarding the functional alteration in HDL and its relation to atherogenesis.

There is substantial evidence indicating that exacerbated oxidative stress is relevant for the development of atherosclerosis and its associated complications (Heinecke, 1998; Navab et al. 2004; Bonomini et al. 2008; Lakshmi et al. 2009; Madamanchi et al. 2005). Another process integral to atherosclerosis is inflammation and this concept is firmly established (Ross. 1999; Libby. 2002). According to the oxidative modification hypothesis of atherosclerosis , oxidized -LDL is a key element in atherogenesis and it induces atherosclerosis by triggering an inflammatory cascade within the vascular wall.The validity of this statement has been confirmed in numerable studies (Steinberg. 2009). An emerging consensus also underscores the importance in vascular disease of oxidative events in addition to LDL oxidation. These include the production of reactive oxygen and nitrogen species by vascular cells (Beckman and Koppel. 1996; Bedark and Krausw. 2007; Guzik et al. 2000; Sorescu et al. 2002; Stocker and Kaeney. 2004). The specific role that reactive species play in the inflammation of vascular disease, however, is not yet clear. Generally, these pro-atherogenic processes can be effectively inhibited or delayed by the anti-oxidative and anti-inflammatory properties of HDL, which in turn depend on the preservation of HDL's structural integrity and composition. However, these HDL functions can also be compromised by oxidative stress. Recent research suggests that dysfunctional HDL production may be paradoxically proatherogenic (Ansell et al, 2004; Pennathur et al. 2004). There are reports for the in vivo oxidative modification of HDL in humans (Nakajima et al. 2000). Oxidized form of ApoA-1 has also been detected in advanced atherosclerotic plaques (Zheng et al. 2004). It is proposed that reactive oxygen species (ROS), myeloperoxidases, and metal ions can induce dysfunctionality in HDL by oxidative modifications. The role of ROS in inducing

oxidative modification to HDL particle, altering its anti-oxidative/anti-inflammatory properties and promoting proinflammatory response in arterial cells is relatively an unexplored area.

Our current understanding of HDL metabolism suggests that a new HDL hypothesis needs to be formulated, pointing to the importance of the functionality of HDL particles that are capable of reducing the risk of CHD. It is hypothesized that dysfunctionality in HDL could be a contributing factor to the excessive risk of CHD in subjects. However, the proatherogenic pathways exerted by functionally modified HDL remain poorly understood. Hence there is a need to define the quality of HDL by identifying its functional properties. Further, inhibiting the formation of defective HDL would be expected to improve its function and provide cardiac protection for which various factors that regulate HDL function; interaction of HDL with arterial cells and its contribution to atherogenesis need to be investigated. Detailed investigation is essential to assess the antagonistic effects of HDL and its consequences for the atherosclerotic process. Understanding these cellular mechanisms mediated by functionally modified- HDL might provide an important link between oxidative stress and inflammation in the pathogenesis of atherosclerosis and contribute to the precise understanding of the relationship between HDL and CHD.

The thesis is an attempt to identify functional abnormality, if any , in circulating HDL in subjects, in terms of its antioxidative property to inhibit LDL oxidation as well as to elucidate the possible role of oxidatively -modified HDL [induced by in vitro oxidation] in promoting inflammatory response in

monocytes/macrophages, the key cell types involved in atherogenesis and to delineate the associated molecular mechanism using standard cell culture system and molecular laboratory techniques.

This study demonstrates that all HDL are not same in quality i.e.antioxidant-atheroprotective property. Blood levels of HDL-C do not predict the functional heterogeneity of HDL and point out the need for functional assay of HDL for better predicting the cardiovascular risk. Following in vitro oxidative modification, HDL loses its atheroprotective functions and exerts pro-inflammatory response by releasing TNF-α and MMP-9 as well as promotes oxidative stress [ROS] in human monocytes-macrophages., thus providing evidence for the pro-atherogenic role of functionally altered HDL. However, monocytes-macrophages exhibited no such responses with native HDL and mildly oxHDL. The generation of functionally altered HDL in vivo might therefore be regarded as possibly atherogenic. These findings open a window for the development of appropriate therapy to enhance HDL's atheroprotective function as a preventive approach for the treatment of common metabolic diseases featuring dyslipidemia, inflammation, and premature atherosclerosis.

Objectives

1. Identification of the prevalence of dysfunctional HDL in subjects.
2. Invitro induction of dysfunctionality in HDL using an oxidative system and its functional characterization.
3. Investigation of the effect of oxidatively modified HDL on macrophage functions relevant to atherosclerosis, i.e. macrophage inflammatory response.
4. Delineating the role of NADPH Oxidase, Nuclear transcription factor-Liver X receptor (LXR), surface receptors- Adenosine triphosphate binding cassette (ABC) transporters and CD36 in oxidized HDL- induced monocyte-macrophage functions.

REVIEW OF LITERATURE

2.1. Coronary heart disease

Coronary heart disease (CHD) is the most prevalent form of cardiovascular disease (CVD) and the leading cause of death globally; more people die annually from CVDs than from any other cause. It affects people at younger ages, especially in countries like India, and has become a major burden upon public health. Approximately 3.8 million men and 3.4 million women worldwide die each year from CHD (World Health Organization, 2004). According to present trends in the United States, half of healthy 40-year-old males will develop CAD in the future, and one in three healthy 40-year-old women (Rosamond et al. 2007). This shows that vascular diseases will continue to impose a substantial burden on health care resources throughout the next generation.

CHD is a disease of the heart in which the inner endothelial lining or walls of one or more of its coronary arteries become partially or completely narrowed by a long-term accumulation of atheromatous plaque which reduces the flow of blood to the heart muscle, and increases the risk of cardiac events such as chest pain (angina pectoris) and heart attack (myocardial infarction). Such atherosclerotic plaques result from the progressive accumulation of cholesterol and diverse lipids in native and oxidized forms, calcium, extracellular matrix material and inflammatory cells. Atherosclerosis is the principal cause of majority of coronary artey disease, but coronary disease can be due to other causes, such as coronary vasospasm (Williams

et al. 1998) where the stenosis is caused by spasm of the blood vessels of the

heart.

2.2. Atherosclerosis and Coronary heart disease [CHD]

2.2.1. Historical perspective

Atherosclerosis represents the pathological process that typically underlies several important vascular disorders including coronary artery disease, cerebrovascular disease and diseases of the aorta and peripheral arterial circulation (Allam et al. 2011). Atherosclerosis is an ancient disease that was detected in Egyptian mummies. Several sixteenth century anatomists, Andreas Vesalius and Gabriele Falloppio described aneurysms of the aorta and peripheral arteries, but the pathophysiology was unknown (Fye, 2005). Swiss physiologist Albrecht von Haller [1757 monograph] made several important observations on the cardiovascular system and described progressive atherosclerotic changes in the arteries of the elderly. In the monograph on aneurysm [Sull Aneurisca 1804] Antonio Scarpa concluded that most common and important antecedent to aneurysm formation was an ulcerated atheromatous lesion, a localized disease of the arterial wall (Fye, 2005). Further attention in vascular disease was focused on the pathophysiology of atherosclerosis. London Surgeon Joseph Hodgson published in his "Treatise on the disease of arteries and veins" (1815) claimed that inflammation was the underlying cause of atheromatous arteries. He identified atheromatous material between the intima and media (Fye, 2005). In 1858, pathologist, Rudolf Virchow, made pioneering observations on thrombosis and embolism, using microscopic study

of blood vessels. He concluded from his research that atherosclerotic lesions were located within the intimal layer (Thompson et al. 2013).

The Leipzig pathologist Marchand in 1904 first used the term atherosclerosis, which since has been widely adopted, instead of arteriosclerosis, to designate the degenerative process of the intimal layer of the arteries (Aschoff. 1933). During the final decades of the nineteenth century, several theories were advanced to explain the pathophysiology of the various forms of arterial disease. Atherosclerosis is now recognized as a chronic inflammatory disease of arterial blood vessels (Ross, 1999; Libby, 2002).

2.3. Prevalence, incidence, and mortality of coronary heart disease

The prevalence, incidence and mortality statistics related to CHD reveal that more Americans are failing to practice good heart health (Garko, 2012). It is estimated that 7% of American adults 20 years of age and older have CHD. Out of the total population of people diagnosed with CHD, 8.3% are males and 6.1% are females. The average age of experiencing a first heart attack is 64.5 years for men and 70.3 years for women. According to Roger et al (2011) the incidence of CHD in women falls behind men by 10 years for total CHD and lags behind by 20 years for more catastrophic clinical events such as MI and sudden death. When compared to other deadly degenerative diseases CHD ranks as the single leading cause of death of American males and females. It is responsible for one out of six deaths in the United States (Roger et al. 2011).

2.4. Trends of coronary heart disease in India

The prevalence of cardiovascular disease is very high in India and south Asia.CHD is forecasted to be the most common cause of death globally, including India, by 2020 **(**Yusuf et al**.** 2001**).** In 2003, the prevalence of CHD in India was estimated to be 3-4% in rural areas and 8-10% in urban areas with a total of 29.8 million affected according to population-based cross-sectional surveys. It was found that CHD is more common in urban than rural areas of India. Unadjusted CHD rates have ranged from 1.6% to 7.4% in rural populations and 10% to 13.2% in urban populations (Gupta et al. 2008). CHD affects Indians with greater frequency and at a younger age than counterparts in developed countries, as well as many other developing countries. Its prevalence appears to be worsening in India. Age-standardized CVD death rates in people 30-69 years old are 180 per 100,000 in Britain, 280 per 100,000 in China, and 405 per 100,000 in India. Also, 50% of CHD-related deaths in India occur in people <70 years of age, whereas only 22% of CHD-related deaths in Western countries occur in this age group (Gaziano et al. 2006). As a result, the Indian subcontinent suffers from a tremendous loss of productive working years due to CVD deaths. The huge burden of CHD in the Indian subcontinent is the consequence of the high prevalence of CHD risk factors.

2.5. Coronary heart disease-Kerala Scenario

Kerala has the highest life expectancy, the lowest infant mortality rate, and maternal mortality rate. This social transition also has unfortunately led to the highest prevalence of CHD among all Indian states with a rural prevalence of 7.5% and urban

prevalence of 12% (www.csikerala.org). The prevalence of heart disease in rural Kerala is 7%, which is nearly double that of north India. Moreover, lifestyle diseases-CHD, diabetes, high blood pressure, and obesity, are paradoxically high and result in very high mortality and morbidity. The age-adjusted CHD mortality rates per 100,000 are 382 for men and 128 for women in Kerala. These CHD rates in Kerala are higher than those of industrialized countries and 3 to 6 times higher than Japanese and rural Chinese (Soman et al. 2011). CHD in Kerala is premature and resulting in death at a very young age. Approximately 60% of CHD deaths in men and 40% of CHD deaths in women occur before the age of 65 years. The high rates of premature CHD in Kerala also result in a high economic burden. This warrant prompt attention for prevention and control of CHD in India particularly Kerala.

2.6. Risk factors for coronary heart disease

The aetiology of CHD is multifactorial. It is the result of interaction between genetic, lifestyle and environmental factors. The most important behavioral risk factors of heart disease are unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol. Behavioral risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease. The effects of unhealthy diet and physical inactivity may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, and overweight and obesity (WHO, 2013). There are also a number of underlying determinants of CHDs. These are a reflection of the major forces driving social, economic and cultural change – globalization, urbanization, and population ageing. Other determinants of CHDs include poverty, stress and hereditary factors.

Over the last 50 years, a number of clinical and laboratory variables have proven predictive of the incidence of cardiovascular disease and thus qualify as cardiovascular disease risk factors. According to the INTERHEART study, which enrolled 29,972 subjects in 52 countries worldwide, the most strongly predictive cardiovascular risk factors for myocardial infarction were dyslipidemia, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, less consumption of fruits/vegetables, intake of alcohol, and lack of regular physical activity (Yusuf et al. 2004). Collectively, these factors accounted for most (≥90%) of the risk of myocardial infarction in both sexes and at all ages in all regions. Among the more recently recognized markers of CHD risk, are resting heart rate and the metabolic syndrome (Borer, 2008; Fox et al. 2008).

In addition, a number of more recently identified and less well-known factors have received intense investigation over the past few years. These include both lipid [small, dense low-density lipoprotein particles (sdLDL), oxidized low-density lipoprotein, and apolipoprotein B] and nonlipid variables, such as metabolic factors (eg, impaired fasting glucose), thrombogenic/haemostatic factors (eg, fibrinogen), and inflammatory markers [high sensitivity C-reactive protein] (hsCRP). Efficient preventive strategies are needed and urgent measures should be taken to control the associated risk factors (Borer, 2008; Fox et al . 2008).

Atherogenic dyslipidemia, a highly prominent cardiovascular risk factor, is intimately associated with premature atherosclerosis. Among factors other than low-density lipoprotein-cholesterol [LDL-C] that are associated with dyslipidemia, a low level of high-density lipoprotein-cholesterol (HDL-C <40 mg/dl) is recognized (Gotto

& Brinton, 2004) and is an independent risk factor for coronary heart disease. Moreover low HDL-C is characteristic of atherogenic dyslipidemia and increased CHD risk in patients with metabolic diseases such as type 2 diabetes and metabolic syndrome (MetS). The metabolic syndrome encompasses a range of cardiovascular risk factors.

The last decades of clinical study were concentrated on lipid lowering therapy for the prevention of atherosclerosis and increasing the so-called athero-protective HDL-C levels. Although statin therapy [3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitor] has been remarkably successful, its use has not eliminated CHD and there exists significant 'residual' cardiovascular risk in patients (Fruchart et al. 2008). This has led to heightened interest in HDL, as a potential target for therapy.

Studies using recombinant apolipoprotein AI liposomes have shown that direct infusion can effectively reduce established atheromatous plaques in animals and in coronary patients (Sirtori, 2006). Further attention has been turned to raise HDL -C with drugs, such as cholesteryl ester transfer protein [CETP] inhibitor [Torcetrapib], niacin and several genetic variants, but the clinical outcomes are disappointing and no benefit has been demonstrated. Evolving knowledge that a significant number of cardiovascular events occur in subjects with normal levels of both HDL-C and LDL-C (Navab et al. 2005; Castelli et al. 1986) has fueled a search for additional biomarkers with better predictive value.

As a result, other HDL-associated factors have been investigated, including the quality and function of HDL in contradistinction to the level of HDL-C.

Regarding their quality, HDL particles are highly heterogeneous and contain varying levels of antioxidants and pro-oxidants, which results in variation in HDL function. A number of studies have provided evidence that HDL undergoes post-translational changes that can affect its atheroprotective profile. The atheroprotective functions are lost in the post-translational dependent dysfunctional plasma HDL of subjects with systemic inflammation, coronary heart disease, diabetes, and chronic renal disease. The emerging notion that particle quality has more predictive power than quantity has stimulated further exploration of the HDL proteome (Shao et al. 2008 ; Scanu & Edelstein, 2008). The wealth of these new findings on HDL has opened new areas of exploration.

2.7. Pathogenesis of Atherosclerosis

2.7.1. Normal structure of the arterial wall

The artery wall is a three-layered structure: intima, media and adventitia. The innermost layer is the tunica intima, which consists of an endothelial tube of longitudinally arranged endothelial cells and their basal lamina. The second layer of the vessel, the tunica media is composed of multiple concentric layers of circularly arranged, smooth muscle cells and extracellular matrix, and serves the contractile and elastic functions of the vessel. The tunica adventitia, the outermost layer of the vessel, is relatively thin connective tissue layer. The vasa vasorum serve to nourish the vessel (Tegos et al. 2001).

Arteries are classified into three types according to their size: large or elastic arteries; medium (or muscular or distributive) arteries; and small arteries or arterioles,

which are less than 0.5 mm in diameter. A characteristic feature of arteries, regardless of size, is a well-defined lumen, rounded or oval, maintained by the muscularity of the vessel wall. Elastic arteries are large, thick-walled vessels near the heart, such as the aorta and its major branches. Their large-diameter lumen allows them to serve as low-resistance conduits. Endothelial cells have very distinct and unique functions that are paramount to vascular biology.

2.7.2. Endothelium and its function

The endothelium has emerged as the key regulator of vascular homeostasis, in that it has not merely a barrier function but also acts as an active signal transducer of physical and chemical signals by production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation (Deanfield et al. 2007). The endothelium has the ability to act in both sensory (sense changes in blood flow and blood pressure, as well as inflammatory and hormonal signals from the bloodstream) and effector capacities (release a variety of vasoactive, anti-inflammatory. and thromboregulatory substances). Normal functions of endothelial cells include mediation of coagulation, platelet adhesion, immune function and control of volume and electrolyte content of the intravascular and extravascular spaces**.**Thus, the endothelium may be considered the ‘‘gatekeeper’’ of the vascular wall.

2.7.3. Pathophysiology

Marchand introduced the term “atherosclerosis” describing the association of fatty degeneration and vessel stiffening (Aschoff , 1933). The term atherosclerosis is

derived from the Greek "athero," meaning gruel, or wax, corresponding to the necrotic core area at the base of the atherosclerotic plaque, and "sclerosis" for hardening, or induration, referring to the fibrous cap of the plaque's luminal edge. Atherosclerosis is a complex inflammatory and fibroproliferative disease affecting large and medium sized muscular and elastic arteries and is characterized by patchy intramural thickening of the subintima that encroaches on the arterial lumen and preventing blood to flow freely. Ultimately the lesion may evolve to contain large amounts of lipid; if it becomes unstable, denudation of overlying endothelium, or plaque rupture, may result in thrombotic occlusion of the overlying artery and finally leads to myocardial infarction [heart attack]. Atherosclerosis actually starts at the very beginning of life with the appearance of soft, fatty streaks along the inner walls of the coronary arteries (McGill et al. 2000), but it remains asymptomatic for several years or in some cases for the whole life.

Atherosclerotic lesions develop as a result of inflammatory stimuli, subsequent release of various cytokines, proliferation of smooth muscle cells, synthesis of connective tissue matrix, and accumulation of macrophages and lipid. Typically, red blood cells, LDL, HDL, monocytes, and platelets course easily through healthy coronary arteries. However, when there is damage to the endothelial cells lining the arteries, the immune system’s inflammation response is triggered to repair the damage. Several factors, including viruses, bacteria, excessive oxidized-LDL, hypertension, homocysteine, tobacco smoke toxins, industrial chemical toxins, alcohol, refined sugar, excess saturated and trans-fats, insulin, excess refined, processed carbohydrates have been mentioned as causes of inflammation or at least risk factors for it. It is hypothesized that when the inflammatory agent damages the

endothelium lining of the coronary artery the immune system responds and sends white blood cells to repair the injured site.

Endothelial dysfunction is a systemic pathological state of the endothelium and can be broadly defined as an imbalance between vasodilating and vasoconstricting substances produced by (or acting on) the endothelium. The increased expression of adhesion molecules, inflammatory cytokines after an endothelial injury promotes recruitment of circulating monocytes to the endothelial layer and their subsequent migration into the sub-endothelial space, where they differentiate into macrophages (Galkina & Ley, 2007; Valgimigli, Merli et al. 2003). Endothelial dysfunction is an important early event in the pathogenesis of atherosclerosis, contributing to plaque initiation and progression.

Current evidence suggests that certain chemo-attractant chemokines, such as macrophage chemo-attractant protein-1 (MCP-1), direct the migration of leukocytes into the intima. Once mononuclear leukocytes collect in the intima, they typically accumulate lipid and become macrophage foam cells, the hallmark of the early atheromatous precursor, the fatty streak. Because it is a large-sized transporter of cholesterol, the LDL becomes trapped in the inner most layer of the arterial wall. Some of the trapped LDL then becomes oxidized by free radicals producing an inflammation response (Yla et al. 1989). The monocyte--macrophages consume the oxidized-LDL and grow into foam cells, which in turn become oxidized, thereby, promoting more inflammatory response to resolve cellular damage. The immune system starts out to repair arterial damage. However, it ends up setting into motion a process that results in plaque development in the artery. In the attempt to repair the

oxidative damage, there is proliferation of smooth muscle cells lining the arterial wall. The smooth muscle cells and macrophages produce connective tissue, all of which becomes mixed with the foam cells resulting in the scar-like tissue of fibro-lipid plaque to form along the arterial wall. The result is a subendothelial fibrous plaque and these advanced lesions have usually a fibrous cap made up of smooth muscle cells, collagen fibrils and proteoglycan. Beneath the fibrous cap lies a core that contains intact foam cells, cellular debris, extracellular lipids, cholesterol and cholesteryl esters, calcium deposits and components of blood. Subsequent thrombus formation and ulceration of atherosclerotic lesion result in the appearance of the complicated lesion. The complicated lesion of atherosclerosis is the clinically significant end-point for the formation of a plaque, characterized by-thrombosis, neovascularisation, thinning, calcification and ulceration. The net result of these changes is the occlusion of the blood vessel and the formation of emboli, both of which end up producing ischemia in the tissues supplied by the atherosclerotic or otherwise occluded blood vessels (Osterud & Bjorklid, 2003).

Thrombotic complications: The thrombotic complication of atherosclerotic lesion, [atherothrombosis] can cause acute heart attack, stroke, and critical limb ischemia[gangrene]. Plaque disruption takes two major forms: (i) a superficial erosion of the intimal surface and (ii) a rupture of the plaque's fibrous cap. The accumulated macrophages can continually secrete proteolytic enzymes such as matrix metallo proteinases. These enzymes are matrix degrading in nature and can break down the fibrous cap, generating a vulnerable plaque that is prone to rupture (Ketelhuth and Back. 2010). Rupture of the fibrous cap exposes the pro-thrombotic necrotic core to the circulation and results in thrombus formation and occlusion of the artery. Besides

thrombotic plaque rupture, plaque erosion is responsible for thrombotic coronary sudden death. In this case, thrombus is formed on a denuded endothelial plaque surface (Ketelhuth and Back. 2010).

All stages of atherosclerosis—from initiation and growth to complication of the plaque—are considered an inflammatory response to injury mediated by specific cytokines.

2.8. Current theories of atherosclerosis - Inflammation and oxidative stress

Atherosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, calcium, and cellular debris within the intima of the vessel wall (Gotlieb. 2007). This buildup results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow, and diminished oxygen supply to target organs.

There are a variety of hypotheses that describe the process of atherosclerosis. While there is good post-mortem evidence of what components comprises a plaque, and now better data on the risk factors for developing atherosclerosis, the actual mechanisms of how the plaque is formed is severely limited by our inability to actually observe this process *in vivo.* An incompletely understood interaction exists between the critical cellular elements—endothelial cells, smooth muscle cells, platelets, and leucocytes—of the atherosclerotic lesion. Vasomotor function, the thrombogenicity of the blood vessel wall, the state of activation of the coagulation

cascade, the fibrinolytic system, smooth muscle cell migration and proliferation and cellular inflammation are complex and interrelated biologic processes that contribute to atherogenesis and the clinical manifestations of atherosclerosis. As a result there are several different theories that describe the mechanism of atherogenesis. Some of these theories are complimentary and some are antagonistic to each other. Although the pathophysiological mechanisms underlying atherosclerosis are not completely understood, it is widely recognized that both inflammation and oxidative stress play important roles in all of the phases of atherosclerosis evolution (Cipollone et al. 2007).

Recently, inflammatory and immunological mechanisms have been increasingly implicated in the pathogenesis of this disease. It is now well recognized that atherosclerotic lesions contain activated, immunocompetent cells, including T lymphocytes and monocyte/macrophages. Inflammatory and/or immune mechanisms appear to be particularly active when plaques are activated and rupture and they may therefore cause acute coronary syndromes or stroke. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis (Libby. 2002). These new findings provide important links between risk factors and the mechanisms of atherogenesis.

2.8.1. Inflammation

Inflammation is a protective reaction against a variety of exogenous (microbial, chemical, physical) or endogenous (immunological, neurological) disturbances, which is characterized by the accumulation of specific subsets of

leukocytes to sites of infection or tissue damage, and their subsequent activation (Sullivan et al. 2000). The attraction of leukocytes to tissues is essential for inflammation and the host response to infection. Depending on the cause, inflammation can resolve rapidly or develop into a complex process. There are two classes of inflammation: acute inflammation, which is of short duration and is characteristically accompanied by plasma fluid exudates and neutrophils accumulation; and chronic inflammation which involves other leukocyte types such as monocytes/macrophages, T cells, eosinophils, basophils, mast cells and dendritic cells (Mach. 2005). This class of inflammation is of longer duration and is characterized by dense cellular infiltrates. Emerging evidence supports involvement an implication of chronic inflammation as the crucial cornerstone of atherogenesis.

2.8.2. Inflammation and Atherosclerosis

From a pathological viewpoint, all stages of the atherosclerotic plaque might be considered to be an inflammatory response to injury. The normal endothelium does not in general support binding of white blood cells. However, when the endothelial monolayer becomes injured or activated, it expresses adhesion molecules that bind cognate ligands on leukocytes. Once resident in the arterial wall, the blood-derived inflammatory cells participate in and perpetuate a local inflammatory response. The macrophages express scavenger receptors for modified lipoproteins, permitting them to ingest lipid and become foam cells. Fatty streaks have focal increases in the content of lipoproteins within regions of the intima, where they associate with components of the ECM such as proteoglycans, slowing their egress. This retention sequesters lipoproteins within the intima, isolating them from plasma

antioxidants, thus favoring their oxidative modification (Kruth, 2002; Packard & Libby, 2008). Oxidatively modified LDL particles comprise an incompletely defined mixture, because both the lipid and protein moieties can undergo oxidative modification. Constituents of such modified lipoprotein particles can induce a local inflammatory response (Miller et al. 2003; Packard & Libby, 2008).

Chemoattractant factors, which include monocyte chemoattractant protein-1 (MCP-1) produced by vascular wall cells in response to modified lipoproteins, direct the migration and diapedesis of adherent monocytes (Boring et al. 1998; Packard & Libby, 2008). Monocytic cells, directly interacting with human ECs, increase several fold monocyte matrix metalloproteinase (MMP)-9 production, allowing for the subsequent infiltration of leukocytes through the endothelial layer and its associated basement membrane (Amorino & Hoover, 1998; Packard & Libby, 2008). T lymphocytes encounter signals that cause them to elaborate inflammatory cytokines, such as interferon- γ, interleukins, or tumor necrosis factor-α [TNF-α], which in turn can stimulate macrophages as well as vascular endothelial cells and SMCs. Proinflammatory cytokines provide a chemotactic stimulus to the adherent leukocytes, directing their migration into the intima. M-CSF stimulation also increases macrophage expression of scavenger receptors, members of the pattern-recognition receptor superfamily, which engulf modified lipoproteins through receptor-mediated endocytosis. Accumulation of cholesteryl esters in the cytoplasm converts macrophages into foam cells, i.e., lipid-laden macrophages characteristic of early-stage atherosclerosis. In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and

cytokines, including TNF-α and IL-1. Recent evidence supports selective recruitment of a proinflammatory subset of monocytes to nascent atheroma in mice (Packard & Libby, 2008).

A number of proinflammatory cytokines have been shown to participate in atherosclerotic plaque development, growth and rupture (Dabek, 2010; Libby, 2002). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) seems to be a crucial transcription factor in the cross-talk among cytokines, adhesion molecules and growth factors. (Dabek, 2010). In atherogenesis, NF-kB regulates the expression of cyclooxygenases, lipooxygenases, cytokines, chemokines (i.e., MCP-1) and adhesion molecules (Dabek, 2010; Kutuk & Basaga, 2003). As stated, atherosclerosis is an inflammatory reaction of the arterial wall. The factors IL-1, TNF-α, IL- 6, IL-12 and interferon γ are involved in this reaction and their expression is co- regulated by NF-kB (Dabek, 2010).

Inflammatory processes not only promote initiation and evolution of atheroma, but also contribute decisively to precipitating acute thrombotic complications of atheroma. Most coronary arterial thrombi that cause fatal acute myocardial infarction arise because of a physical disruption of the atherosclerotic plaque. The activated macrophage abundant in atheroma can produce proteolytic enzymes capable of degrading the collagen that lends strength to the plaque's protective fibrous cap, rendering that cap thin, weak, and susceptible to rupture. Macrophages produce tissue factor, the major procoagulant and trigger to thrombosis found in plaques.Thus, when the plaque ruptures, the tissue factor induced by the inflammatory signaling triggers the thrombus that causes most acute complications of atherosclerosis (Glass, 2001;

Lusis, 2000; Libby, 2002). Intracellular matrix degradation is an important process in both plaque development and rupture. The vital factors involved include MMPs, particularly those that are able to break down the vascular base membrane.

2.8.3. Matrix metalloproteinases [MMPs] in atherosclerosis

Far from being a static structure, extra cellular matrix [ECM] is a dynamic interactive milieu undergoing continuous remodeling that critically influences cell functions. The activity of proteolytic enzymes is the rate-limiting step in ECM degradation. It is now well established that among numerous other proteinases, the matrix metalloproteinases (MMPs) play the major role in the degradation of ECM components. MMPs are specialized enzymes involved in processes such as wound healing, but there is growing interest focusing on a pathological role for MMPs in vascular disease states (Jones et al. 2003). MMPs are a family of zinc-dependent proteases produced by a variety of cell types, including endothelial, smooth muscle cells (SMC), and monocytes. MMPs are a group of endopeptidases with capacity to cleave components of extracellular matrix, such as collagen and elastin. This enzyme family consists of a zinc ion centered catalytic site co-ordinated by three histidine residues (Gomis-Ruth et al. 1993). The ability to modify the tissues is important for several aspects of normal and abnormal physiology. The main physiological function of these proteases was originally ascribed to the modulation and regulation of ECM turnover by direct proteolytic degradation of the ECM proteins (e.g., collagen, proteoglycans and fibronectin (Woessner, 1991). They are also involved in the biological activation of other proteins such as cytokines, growth factors and chemokines.

Approximately 20 different MMPs are identified, and they can be subdivided into different groups according to which components of the extracellular matrix they degrade. They are classified into subgroups based upon substrate specificity and/or structure, including collagenases (MMP-1,-8,-13,-18), stromelysins (MMP-3,-10,-11), gelatinases (MMP-2, -9), and the membrane-type MMPs (MT-MMPs). In this nomenclature, the membrane-anchored MMPs (MMP-14,-15, -16, -17, -24 and -25) are considered as a separate class (Sternlicht & Werb, 2001). MMPs are secreted in a latent proform and require activation for proteolytic activity. The activation of proMMPs can occur through several mechanisms. The most important mechanism is proteolytic removal of the pro-domain by other group of enzymes such as endopeptidases or plasmin and other serine proteases, or even other MMPs. A cysteine switch mechanism is involved in the regulation of MMP activation. This can happen by chemical reactions with ROS or by mercury containing compounds such as 4-aminophenylmer curicacetate (APMA) or denaturing surfactants such as sodium dodecyl sulfate (SDS). MMPs are inhibited by the general protease inhibitor- alpha 2-macroglobulin, and a small family of natural inhibitors known as tissue inhibitor of mettallo proteinase (TIMPs) (Nagase et al. 2006).

Gelatinase A [MMP-2] is ubiquitously expressed as a 72-kDa proenzyme [64-kDa active enzyme] and is capable of cleaving gelatin, type I, IV and V collagens, elastin and vitronectin. Gelatinase B [MMP-9] is expressed as a 92-kDa proenzyme, which can be activated to the 83-kDa enzyme. While a considerable overlap exists in the substrates degraded by MMP-2 and -9, MMP-9 is incapable of direct proteolysis of collagen 1. Through their ability to degrade collagen in the vascular basal membranes, the gelatinases are involved in neovascularisation, tumor metastasis and

can also facilitate migration of inflammatory cells by direct degradation of the basement membrane (Nagase, 1998). MMP-9 plays an important role in migration of immune cells, release of growth factors, promoting angiogenesis. It is also important in the remodelling of endometrial tissue that occurs during the menstrual cycle and in bone development. Contrary to MMP-2, which is expressed ubiquitously under physiological conditions, MMP-9 is only present constitutively in neutrophils, where it is stored in granules to be rapidly released after stimulation.

The activity of MMPs is normally low in healthy tissue, but the increased expression and activity of several MMPs in a range of pathological processes, such as inflammation and ventricular remodelling after myocardial infarction, might indicate that they play a role in the pathophysiology and progression of atherosclerotic disease (Agewall, 2006). The activity of MMPs is tightly regulated at gene transcription level and is also regulated by their secretion in an inactive zymogen form that requires extracellular activation and co-secretion of the tissue inhibitors of metalloproteinases (TIMPs). In healthy humans, MMP-2 and the inhibitory TIMP-1 and TIMP-2 are expressed across the vessel wall. Furthermore, focally increased expression of several MMPs and presence of MMP activity have been observed in diseased human arteries and in association with arterial morphological changes in experimental models of atherosclerosis (Brown, 1995). MMP activity may contribute to the pathogenesis of atherosclerosis by facilitating migration of vascular smooth muscle cells through the internal elastic lamina into the intima of the vessel wall, where they proliferate and contribute to plaque formation. However, MMP activity may also diminish plaque volume by degrading extracellular matrix in the intima.

MMP activity is tightly regulated at both intracellular and extracellular levels. Growth factors, cytokines, hormones, and tumor promoters regulate MMP expression at the transcriptional level, while heparin, transforming growth factor-β (TGB-β), and corticosteroids have an inhibitory effect. Extracellular activation of the latent proenzymes represents a second level of control. The major physiological activator of MMPs is plasmin, which activates pro-MMPs to active MMPs. A further level of control of MMP activity involves the binding of MMPs to specific tissue inhibitor of metalloproteinases (TIMPs) (Nagase et al. 2006). At least four members of TIMP gene family are known: TIMP-1,-2,-3, and -4, all sharing structural features. TIMPs inhibit MMPs by binding irreversible to the active form of the enzyme. Overall, proteolytic activity depends on the relative concentration of the active enzymes and their inhibitors. MMP expression may also be regulated by cell–cell contact or interaction of cells with ECM components (Biswas, Zhang et al. 1995).

Enhanced matrix breakdown has been attributed primarily to MMPs that are expressed in atherosclerotic plaques by inflammatory cells. In the latter stages of atherosclerosis, thrombotic complications often result from disruption of the atherosclerotic plaque due to rupture of the fibrous cap or superficial erosion of the endothelium. Both of these processes largely depend on excessive ECM breakdown (Woessner, 1991). MMPs may also be activated by thrombin in atherosclerotic plaques. In atherosclerotic plaques, thrombin could promote plaque instability by increasing the local matrix-degrading activity of MMPs. As acute plaque disruption leads to local thrombin production at the site of vascular injury, this may facilitate proteolytic activation of MMP, which may start a vicious circle with platelet aggregation and further generation of thrombin and then more MMP activation.

Neovascularization within the plaque may also play a role in promoting plaque destabilization. Intraplaque angiogenesis is influenced by MMP activity through interactions between integrins and proteinases. Finally, aneurysm formation represents an extreme stage of arterial remodeling due to increased ECM breakdown mediated by MMP-2 and- 9. Thus MMPs are a complex group of endopeptidases with important roles in cardiovascular pathophysiology (Loftus et al. 2002; Galis et al.1994). There is growing evidence that MMPs are involved in all stages of the atherosclerosis process, from the initial lesion to plaque rupture. Immuno – cytochemistry, zymography, and in situ hybridization studies have demonstrated an increased expression of different MMPs in human atherosclerotic plaques (Loftus et al. 2002). Another work by Loftus et al. (2000) have shown an increase of MMP-9 in unstable carotid plaque. Furthermore, a significant increase in circulating MMP-9 levels was observed in patients undergoing carotid endarterectomy with evidence of ongoing spontaneous embolization (Molloy et al. 2004). Because of the relevance of MMPs in atherosclerosis, it is not surprising that MMP inhibition represents a potential therapeutic strategy aimed at stabilizing plaques by reducing ECM degradation and restoring the MMP/TIMP equilibrium. Macrophages are the major source of MMPs in atherosclerotic rupture prone area (Loftus et al. 2002; Gough et al. 2006). Inhibiting the MMP expression is a new therapeutic way for the stabilization of the plaque (Crisby et al. 2001; Libby & Aikawa, 2002). Potential methods for MMP inhibition include increasing TIMP levels, administration of inhibitors of MMP activity, and reducing MMP production. Thus, MMPs represent an attractive target to prevent plaque destabilization. Different therapies, including antioxidant vitamins and statins, can contribute to prevent matrix degradation in atherosclerosis.

2.8.4. Oxidative stress and atherosclerosis

Oxidative stress is increasingly being recognized as a potentially important contributor to atherogenesis. Oxidative stress can be defined as an "imbalance between oxidant and antioxidant factors in favor of pro-oxidants and is central to the pathophysiology of atherosclerosis.

2.**8.4.1. Reactive Oxygen Species and Vascular Oxidative Stress**

Reactive oxygen species (ROS) are produced during normal aerobic processes in the body and are highly reactive with other biological molecules. Low levels of ROS play a pivotal role in numerous biochemical processes such as intracellular messaging, cell differentiation, apoptosis, and microorganism defense (Rodella & Favero, 2013). The production of ROS is under tight control in healthy cells, but overproduction during metabolic dysfunction leads to cellular injury. Thus, ROS are indispensable in the normal physiological processes in the body. Excessive production of reactive oxygen species (ROS) during oxidative stress, out stripping endogenous anti-oxidant defence mechanisms, has been implicated in processes in which they oxidize and damage DNA, protein, carbohydrates and lipids. Each of these responses, when uncontrolled, contributes to vascular diseases.

The endothelium maintains vascular homeostasis via the release of numerous substances, including nitric oxide [NO], prostaglandins, hyperpolarizing factors, endothelin and ROS. Of these NO, a volatile gas, is the predominant stimulus for vasodilatation in large- and medium-sized arteries. NO is a free radical formed by the oxidation of the guanidine nitrogen terminal of L-arginine. CHD risk factors such as

hypertension, diabetes, dyslipidemia, smoking, physical inactivity, and obesity increase production of vascular ROS, which results in a reduction of bioavailable nitric oxide and ultimately endothelial dysfunction and endothelial cell activation. ROS appears to mediate the inflammatory pathways that participate in the development and progression of atherosclerosis.

In the vasculature , all the layers produce ROS, including tunica intima, media and adventitia. ROS include superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitric oxide (NO), and peroxynitrite (ONOO-) (Lakshmi et al. 2009). The major vascular ROS is O_2-, which inactivates NO, the main vascular relaxing factor, thus impairing relaxation (Cai & Harrison, 2000; Kojda & Harrison, 1999). Dismutation of O_2- by superoxide dismutase (SOD) produces H_2O_2, a more stable ROS, which, in turn, is converted to water by catalase and glutathione peroxidase. H_2O_2 and other peroxides appear to be important in the regulation of growth-related signaling in VSMCs and inflammatory responses in vascular lesions (Irani, 2000; Li et al. 1997). ROS have detrimental effects on vascular function through several mechanisms.

High levels of O_2^-, the consequent accumulation of H_2O_2 and diminished NO bioavailability play a critical role in the modulation of vascular remodeling. Finally, ONOO-, resulting from the reaction between O_2^- and NO, constitutes a strong oxidant molecule, which is able to oxidize proteins, lipids and nucleic acids and then causes cell damage (Beckman & Koppenol, 1996; Fortuño et al. 2005; Bonomini et al. 2008).

ROS may contribute to LDL oxidation, inflammation, local monocyte chemoattractant protein production, upregulation of adhesion molecules and macrophages recruitment, endothelial dysfunction, platelet aggregation, extracellular matrix remodeling through collagen degradation, thus playing a central role in the development and progression of atherosclerosis and eventually in plaque rupture (Lakshmi et al. 2009). Under normal conditions the antioxidant defense system within the body can easily handle free radicals that are produced. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage cell.

There are several potential sources of ROS production. In cardiovascular disease the sources include xanthine oxidase, cyclooxygenase, lipooxygenase, mitochondrial respiration, cytochrome P450, uncoupled nitric oxide synthase (NOS) and NAD(P)H oxidase. However, the predominant ROS-producing enzyme in the VSMCs and in the myocardium is NADPH oxidase, that plays a pivotal role in the atherogenesis.

2.8.4.2. NADPH oxidase - a major source of ROS in the vasculature

Despite the presence of multiple ROS sources, studies in the last decade have indicated that a major source of ROS involved in redox signaling is a family of NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (NOX). NOXs are expressed in endothelial, adventitial, and smooth muscle cells of the vasculature (Bedard & Krause, 2007). The general opinion is that NOXs do have an important regulatory role on the vasculature, including an effect on vascular tone and blood pressure regulation. Unlike the neutrophil oxidase, the enzyme in cardiovascular cells

continuously generates intracellular ROS at a low level even in the absence of cell stimulation (Bedard & Krause, 2007). The physiological generation of ROS can occur as a byproduct of other biological reactions. ROS generation as a byproduct occurs with mitochondria, peroxisomes, cytochrome *P*-450, and other cellular elements.

It has long been known that phagocytes, including neutrophils, monocytes, and macrophages, contain a plasma membrane-bound, multi component oxidase that utilizes electrons derived from NADPH to reduce molecular oxygen to O_2^-. The superoxide anion radicals (O_2^-) can be dismutated to hydrogen peroxide (H_2O_2), either spontaneously or by the antioxidant enzyme superoxide dismutase, and H_2O_2 may subsequently be converted into a variety of active oxygen species, such as singlet oxygen and hydroxyl radicals. Over the last years, six homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Together with the phagocyte NADPH oxidase itself (NOX2/gp91^{phox}), the homologs are now referred to as the NOX family of NADPH oxidases (Bedard & Krause, 2007). The classical NADPH oxidase complex comprises a membrane-bound cytochrome b558 (composed of one gp91^{phox} and one p22^{phox} subunit) which forms the catalytic core of the enzyme, and four cytosolic regulatory subunits (p47^{phox}, p67^{phox}, p40^{phox} and Rac) which translocate to the cytochrome b558 to activate the enzyme.

The NOXs are thought to exert their effects in a number of ways. Signaling is thought to be regulated directly by the oxidation or reduction of signaling proteins, like protein tyrosine kinases and phosphatases, and mitogen-activated protein kinases,

and by activating transcription factors, such as nuclear factor-κB and activator protein. There is abundant evidence for the regulation of gene expression by ROS (Van Heerebeek, 2002; Griendling, 2000). NOX-derived ROS are involved in the regulation of expression and/or activation of matrix metalloproteinases.

NADPH oxidase and atherosclerosis: An increase in oxidative stress appears to be a major mechanism underlying the development of vascular endothelial dysfunction in a wide range of cardiovascular diseases. In the last decade, numerous studies have found that major sources of ROS responsible for this increased oxidative stress are vascular NADPH oxidases. A large body of work published over the last decade indicates that NOX enzymes play important roles in the pathophysiology of many cardiovascular diseases (Azumi et al. 2002; Brandes. 2010). Judkins et al (2010) have demonstrated a role for Nox2-NADPH oxidase in vascular ROS production, reduced NO bioavailability, and early lesion development in ApoE$^{-/-}$ mice .

2.8.4.3. Oxidized lipids – central players of Atherogenesis

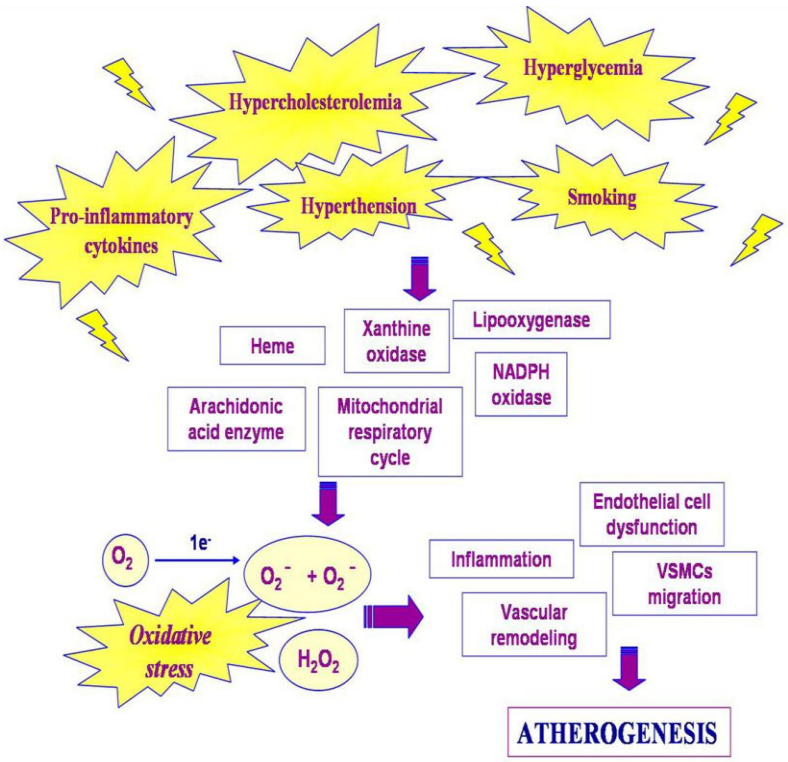
The etiology of atherosclerosis is complex, but it is well known that an increase in uptake, entrapment, and deposition of lipids, especially oxidized low-density lipoprotein (LDL), plays a pivotal role in the development of atherosclerosis. Since the first proposal of the LDL oxidation hypothesis for atherosclerosis by Steinberg and his colleagues in 1989, ample evidence have been presented supporting the hypothesis that oxidative modification of LDL is the key initial event for the progression of atherosclerosis (Tabas et al. 2007; Steinberg, 2009; Hansson & Hermansson, 2011).

Oxidized LDL is capable of a wide range of toxic effects and vessel wall dysfunctions that are characteristically and consistently associated with the development of atherosclerosis. Oxidized LDL particles exhibit multiple atherogenic properties, which include uptake and accumulation of lipids, as well as pro-inflammatory, immunogenic, apoptotic and cytotoxic activities, induction of the expression of adhesion molecules on endothelial cells, promotion of monocyte differentiation into macrophages, production and release of pro-inflammatory cytokines and chemokines from macrophages (Rodella & Favero, 2013).

Several different types of fatty acid oxidation products have been reported to be present in human atherosclerotic lesions. Like fatty acids, cholesterol can undergo enzymatic and non-enzymatic oxidation to a range of oxysterols, and a number of studies reported the presence of oxysterols in organic extracts of human aortas (Brown & Jessup, 1999; Stocker & Keaney, 2004). In addition to lipid oxidation, there is also good evidence for protein oxidation in human atherosclerotic lesions. Oxidative damage to proteins may result from electrophilic (2*e*-) and radical (1*e*-) reactions, e.g., initiated by electron leakage, metal-ion-dependent reactions, auto-oxidation of lipids and sugars, and breakdown products of lipid oxidation. Compared with normal arteries, human carotid endarterectomy samples contain higher concentrations of several amino acid oxidation products (i.e., dopa, *o*-tyrosine,*m*-tyrosine, hydroxyleucine, and hydroxyvaline) indicative of $OH^{\cdot}$ mediated protein oxidation. In addition, such lesions contain elevated levels of *o,o*-dityrosine suggestive of the involvement of HOCl-mediated reactions (Fu, Davies et al. 1998; Stocker & Keaney, 2004).

The presence of oxidatively modified lipids and proteins in lesion site strongly predicting a major role of oxidative stress in development and progression of atherosclerosis stress (Heinecke, 1998). The analysis of plaque composition has revealed products of protein and lipid oxidation, such as chlorinated, nitrated amino acids, lipid hydroperoxides, short-chain aldehydes, oxidized phospholipids, F2α-isoprostanes and oxysterols (Brooks et al, 1970; Stocker & Keaney, 2004). The antibodies generated against LDL modified by malondialdehyde, 4-hydroxynoneal, copper, gave immmuno staining of lipid rich area of atherosclerotic lesion (Madamanchi et al. 2005; Mueller et al. 2005).

In the presence of oxidative stress, the ability of the endothelial cells to maintain the endothelium can become infiltrated by lipids and leucocytes, which initiates an inflammatory response and produces the initial atherosclerotic lesion of a fatty streak. This process is considered an integral part of the pathophysiology of atherosclerosis.



Role of ROS, oxidative stress and inflammation in atherosclerosis. [O2: oxygen; O2 -: superoxide; H_2O_2: hydrogen peroxide; VSMC: vascular smooth muscle cell]. (Rodella & Favero, 2013).

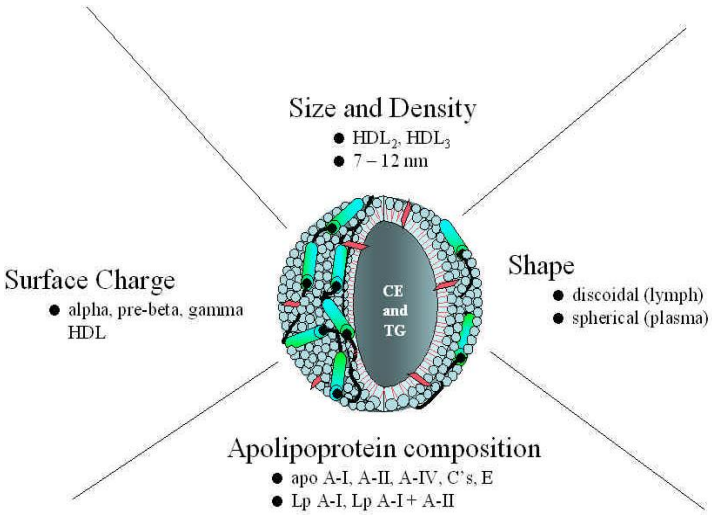
The pathophysiological mechanisms of atherosclerosis are complicated and the integrated picture of the disease process is not yet complete. It is widely recognized that oxidative stress, lipid deposition, inflammation, vascular smooth muscle cells differentiation and endothelial dysfunction play a critical role in the formation, progression and eventually rupture of the atherosclerotic plaque. Indepth knowledge of the various pathogenic mechanisms involved in atherosclerosis can help in formulating preventive and therapeutic strategies and devising pharmaceutical and lifestyle modifications for reducing mortality.

2.9. High Density Lipoprotein and Atherogenesis

Many prospective epidemiologic studies have indicated that a decreased HDL-C level is a significant independent risk factor for heart disease. Moreover, epidemiologic studies also showed that each 1 mg/dl decrease in LDL cholesterol level results in about a 1-2% reduction in CHD risk, and each 1 mg/dl increase in HDL cholesterol level results in about a 3- 4% reduction in CHD risk (Asztalos ,Schaefer, 2003). HDL has antioxidant, anti-inflammatory and anti-thrombotic properties, which contribute to its atheroprotective effect.

2.9.1. HDL Structure, composition and Heterogeneity

HDL is a class of heterogeneous lipoprotein containing approximately equal amounts of lipid and protein. HDL particles are characterized by high density (>1.063 g/mL) and small size (Stoke's diameter =5 to 17 nm). In human plasma, HDL is a heterogeneous collection of particles ranging in diameter from 7-12 nm and density 1.063-1.21 g/ml (Lund et al. 2003; Phillips, 2013). The predominant species of HDL are spherical microemulsion particles in which a core of neutral cholesteryl ester (CE) and triacylglycerol (TG) is encapsulated by a monolayer of phospholipid (PL), unesterified (free) cholesterol (FC) and protein. The protein and PL constituents comprise approximately 50 and 25%, respectively, of the mass of such particles with the CE, FC and TG components making up the remainder. Larger less dense HDL particles have a higher lipid to protein mass ratio. The various HDL subclasses vary in quantitative and qualitative content of lipids, apolipoproteins, enzymes, and lipid transfer proteins, resulting in differences in shape, density, size, charge, and antigenicity (Phillips, 2013).



Factors contributing to the structural heterogeneity of HDL particles. Variations in the surface charge lead to subclasses that can be separated due to differences in electrophoretic mobility. Differences in apolipoprotein composition permit isolation of HDL particles containing either apo A-I alone (Lp A-I) or apo A-I plus apo A-II by immunoaffinity methods. HDL particles can also be distinguished by their shape: spherical plasma HDL particles contain a neutral lipid core whereas the discoidal HDL that occur in lymph do not (Lund et al, 2003).

Plasma high-density lipoproteins (HDLs) are small, dense, protein-rich particles as compared to other lipoprotein classes; roughly half of total HDL mass is accounted for by lipid components. Phospholipids predominate in the HDL lipidome, accounting for 40 to 60% of total lipid, with lesser proportions of cholesteryl esters (30 to 40%), triglycerides (5 to 12%) and free cholesterol (5 to 10%). Lipidomic approaches have provided initial insights into the HDL lipidome with identification of >200 individual molecular lipids species in normolipidemic HDL (Kontush et al. 2013). Plasma HDL particles however reveal high levels of structural, compositional and functional heterogeneity. Cholesterol is the most characteristic component of the

HDL lipidome as, in the form of HDL-C, it represents a major independent negative risk factor for cardiovascular disease. The major lipid classes present in HDL are phospholipids sphingolipids, steroids, cholesteryl esters, triglycerides, and minor lipids.

Apo A-I is the major protein component of HDL. Approximately 70% of total plasma HDL protein is apoA-I (which is present in normolipidemic human plasma at ~130 mg/dL) and it is located in essentially every HDL particle. The second most abundant protein is apoA-II, which comprises 15-20% of total plasma HDL protein, but this component is not present in all HDL particles. ApoA-I and apoA-II are the "scaffold" proteins that primarily determine HDL particle structure. Other members of the exchangeable apolipoprotein gene family that are associated with HDL include apoA-IV, apo-C's and apoE; these proteins comprise ≤ 10% of HDL protein and do not significantly affect overall particle structure. Small populations of HDL particles containing mainly either apoA-IV or apoE exist in normal human plasma and also in plasma from apoA-I-deficient subjects (Phillips, 2013). HDL also contains minor apoproteins such as Apo D, Apo M, and enzymes such as paraoxonase (PON) 1, platelet-activating factor acetylhydrolase (PAF-AH), and glutathione peroxidase 1; lipid transfer proteins such as lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) (Bindu et al. 2011). Proteomic analysis reveals that HDL particles actually contain over 50 different apoproteins (Razaea et al. 2006). Unlike all the non-HDL lipoproteins [VLDL, IDL, lipoprotein (a) [Lp (a)], LDL], HDL particles do not contain apolipoprotein B (apoB). ApoA-I, the primary structural HDL-associated protein, is produced in the liver and intestine (jejunum) (Eggerman et al. 1991).

Apo A1 accounts for almost all of the cholesterol quantified in the clinical laboratory as HDL-C (Assmann & Gotto Jr, 2004). Besides being present in spherical HDL particles, apoA-I is also a critical component of nascent HDL formed by the action of ATP binding cassette transporter A1 (ABCA1). The structures of these discoidal HDL particles, which contain a segment of PL bilayer, are largely determined by the properties of the apoA-I molecule. It is well established that the C-terminal domain of the human apoA-I molecule plays a critical role in lipid binding because deletion of this domain drastically reduces the level of binding. In addition to the above lipid-bound forms, some 5-10% of the apoA-I in human plasma is present in a lipid-free/poor state, designated preβ1-HDL (Phillips, 2013).

Knowledge of HDL structure at the molecular level is critical for understanding how this lipoprotein achieves the multiple functions. The amphipathic α-helix is the structural motif that enables apoA-I to achieve this functionality. The repeating amphipathic α-helical segments are critical for the ability of exchangeable apolipoproteins to interact with PL and stabilize HDL particles. The amphipathic α-helices in apoA-I are well adapted to bind to a polar/nonpolar interface, such as the PL-water interface. Such particles contain a segment of phospholipid bilayer and are stabilized by two apoA-I molecules that are arranged in an anti-parallel, double-belt, and conformation around the edge of the disc shielding the hydrophobic phospholipid acyl chains from exposure to water. The apoA-I molecules are in a highly dynamic state and they stabilize discoidal particles of different sizes by certain segments forming loops that detach reversibly from the particle surface. The flexible apoA-I molecule adapts to the surface of spherical HDL particles by bending and forming a stabilizing trefoil scaffold structure. The above

characteristics of apoA-I enable it to partner with ABCA1 in mediating efflux of cellular phospholipid and cholesterol, and the formation of a heterogeneous population of nascent HDL particles (Philip, 2013).

2.9.2.Atheroprotective functions of HDL

There are several well-documented functions of HDL that may explain the ability of these lipoproteins to protect against atherosclerosis.

2.9.2.1. Reverse cholesterol transport and HDL Biogenesis

Reverse cholesterol transport [RCT], originally proposed by Glomset (1968) is currently understood as the physiologic process by which cholesterol in peripheral tissues is transported by HDL to the liver for excretion in the bile and feces. It is believed to be a critical mechanism by which HDL exert a protective effect on the development of atherosclerosis. Cholesterol efflux from intimal macrophage foam cells may protect against the progression and complications of atherosclerotic vascular disease. Most peripheral cells and tissues (except those in steroidogenic organs) cannot catabolize cholesterol and can only dispose of it by effluxing it to extracellular acceptors such as HDL. Cholesterol is effluxed from arterial macrophages to extracellular HDL-based acceptors through the action of transporters such as ABCA1 and ABCG1 (Rader et al. 2009).

Apo A1 plays a major role in RCT. Lipid-free apo A-I or lipid-poor pre–β-HDL particles produced in the intestine or liver or shed during lipolysis of triglyceride-rich lipoproteins initiate efflux of phospholipids and cholesterol from cell membranes in a process facilitated by phospholipid transfer protein. The nascent

form of circulating HDL rich in Apo AI, termed discoidal pre-β HDL, removes FC and PL from peripheral cells throughout the body by interacting with a membrane associated protein ubiquitously expressed in peripheral tissues, known as ATP-binding cassette transporter 1 (ABCA1). Cholesterol in these nascent discoidal HDL particles is then esterified by lecithin-cholesterol acyltransferase (LCAT). Cholesteryl esters readily move to the core of HDL particles, producing a steady gradient of free cholesterol and enabling HDL to accept cholesterol from various donors. The reciprocal exchange of cholesteryl ester for triglycerides mediated by CETP moves the bulk of the cholesteryl esters to lipoprotein remnant particles, which are subsequently cleared by the liver. At the same time, HDL becomes enriched with triglycerides, which are substrates for Hepatic lipase (HL). The concerted action of CETP-mediated cholesteryl ester transfer and HL-mediated hydrolysis of triglycerides and phospholipids helps to form the smaller HDL particles that are the preferred binding partners for scavenger receptor type B1 (SR-B1), the major HDL receptor on hepatocytes. The binding of HDL with SR-B1 mediates the selective uptake of cholesteryl esters that have not undergone CETP-mediated transfer to apo B–containing particles. Lipid-free apolipoproteins or lipid-poor pre–β-HDL are formed in reactions catalyzed by PLTP, CETP, and HL. Thus, RCT can be envisioned as a cycle in which acceptors of cellular cholesterol (ie, apo A-I, pre–β-HDL) are perpetually regenerated to undertake their function of inducing cholesterol efflux (Assmann and Gotto, 2004).

Experiments suggest that disruption of one or more steps in reverse cholesterol transport results in accelerated atherosclerosis, whereas over expression of

pivotal proteins in reverse cholesterol transport, such as apo A-I, PLTP, LCAT, and SR-B1, exerts atheroprotective effects (Eckardstein et al. 2000; Furbee et al. 2002).

ATP-binding cassette protein (ABC) transporters: As a member of a large family of membrane transporters, ABCA1 may move cellular lipids across the bilayer in a process requiring hydrolysis of adenosine triphosphate (ATP). ABCA1 is instrumental for the de novo synthesis of HDL in the liver and small intestine by mediating the efflux of phospholipids and cholesterol to apoA-I that is produced in these organs. Patients with Tangier Disease, an autosomal recessive disorder characterized by two non-functional ABCA1 alleles and extremely low levels of HDL-C, exemplify the significance of ABCA1 in HDL-C metabolism (Bodzioch et al. 1999). The major cholesterol contribution to HDL generation presumably comes from ABCA1 in hepatocytes. As macrophages comprise a very small portion of peripheral cells in the body, the cholesterol efflux contributed by these cells may be too low to affect the efficiency of the overall reverse cholesterol transport. However, ABCA1 in macrophages may have an antiatherogenic effect that is independent of plasma HDL.

Along with ABCA1, ABCG1 and ABCG4 drive further cholesterol removal from peripheral cells. As opposed to ABCA1, both ABCG1 and ABCG4 will promote efflux of cholesterol to lipid-rich HDL particles, but not to lipid-poor apoA-I. ABCG1 promotes efflux of PL and FC from macrophages to mature HDL-C rather than pre-β HDL (Kennedy et al. 2005). Macrophages deficient in ABCG1 also have impaired FC efflux and accumulate excess cholesterol (Out, 2006). Taken together,

these data suggest that both ABCA1 and ABCG1 are potential therapeutic targets to raise HDL-C and promote RCT.

Transcription of both ABCA1 and ABCG1 is regulated by members of a steroid superfamily of nuclear receptors known as the Liver X receptor/Retinoid X receptor (LXR/RXR) heterodimer. When activated by oxysterols from FC this heterodimer stimulates ABCA1 and ABCG1 gene expression, thereby enhancing cholesterol efflux (Vaughan & Oram. 2005; Venkateswaran et al. 2000). The heterodimer is also regulated by the activity of peroxisome proliferator-activated receptors (PPAR) α and γ, which are closely linked to insulin resistance and the metabolic syndrome (Anderson et al. 2004).

HDL-C catabolism: As CE accumulate in its central core, pre-β HDL-C matures into larger HDL-C particles known as HDL-3 and HDL-2. These larger molecules undergo hepatic catabolism and excretion in bile. HDL-C catabolism is mediated by 4 mechanisms: 1) hepatic uptake of larger HDL-C particles via hepatic scavenger receptor B1 (SR-B1) receptors for excretion as bile, 2) metabolism of mature HDL-C by hepatic lipase (HL) to smaller particles devoid of lipid and rich in Apo AI, 3) renal uptake of smaller HDL-C particles mediated by apo-E receptors such as cubulin, or 4) LDLr-mediated hepatic uptake of LDL-C and VLDL-C after acquiring CE via CETP activity (Moestrup & Koz. 2000; Lewis, 2006). HDL metabolism induces multiple beneficial, often overlapping, atheroprotective activities .

2.9.2.2. HDL and Antioxidative Mechanisms

The two main hypotheses of atherogenesis that have survived decades of research are the reverse cholesterol transport hypothesis and the oxidation hypothesis. Both hypotheses assign a central role to LDL in initiating atherogenesis and to HDL in mitigating the process. A growing body of evidence suggests that HDL exerts part of its antiatherogenic effect by counteracting LDL oxidation (Navab et al. 2004, 2004a). Inhibition of LDL oxidation by HDL is usually attributed to the high content of antioxidants in this lipoprotein; to antioxidative properties of apo A-I; and to the presence of several enzymes, such as paraoxonase (PON), platelet activating factor acetylhydrolase (PAF-AH), and glutathione peroxidase (GPX), which prevent LDL oxidation or degrade its bioactive products. Apo A-I was shown to reduce peroxides of both phospholipids and cholesteryl esters and to remove hydroperoxyeicosatetraenoic acid (HPETE) and hydroperoxyoctadecadienoic acid (HPODE), which are products of 12-lipoxygenase, from native LDL.

One of the main antioxidant/antiinflammatory functions of HDL is mediated by a transport mechanism that binds and carries away oxidant molecules. HDLs are major carriers of plasma lipid hydroperoxides in animal models of atherosclerosis and in humans. HDLs associated enzymes are able to remove EO6-positive oxidized phospholipids. As a consequence, HDL has the capacity to inhibit the oxidative modification of low-density lipoprotein (LDL) in a process that reduces the atherogenicity of these lipoproteins (Navab et al. 2005).

2.9.2.3. Anti-inflammatory properties of HDL

The role of inflammation in atherogenesis has been well established by a number of studies demonstrating accumulation of macrophages derived from circulating monocytes in atheromatous plaques. Anti-inflammatory effects of HDL-C include: 1) neutralization of lipopolysaccharide-induced tumor necrosis factor alpha (TNF-α) release 2) inhibition of complement activation 3) inhibition of vascular cell adhesion molecules (VCAM) and monocyte chemotactic protein (MCP-1), which are known to mediate monocyte-endothelial cell interaction and 4) induced expression of the anti-inflammatory cytokine transforming growth factor-β2 by HDL3 (Dimayuga et al. 1999 ; Barter et al. 2004). In vitro studies have shown that HDL inhibit monocyte transmigration in response to oxidized LDL.This property appears to be related to paraoxonase and platelet-activating factor acetyl hydrolase on HDL and is reduced in acute inflammatory states as a consequence of the HDL accumulating serum amyloid A

There are several reports of the effects of infusing rHDL into humans (Barter et al. 2004). These studies highlight the ability of a single infusion of rHDL to raise plasma HDL and improve vascular reactivity. Studies using recombinant apolipoprotein AI liposomes have shown that direct infusion can effectively reduce established atheromatous plaques in animals and in coronary patients (Chiesma et al. 2002; Chiesa et al & Sirtori 2003; Nissen et al. 2003].

These studies, raise the possibility that some of the noncholesterol transport functions of HDL may be of pathophysiological importance. It was concluded that the beneficial properties of apoA-I and the apoA-I mimetic peptide are linked both by their ability to reduce lipoprotein lipid oxidation and to enhance reverse cholesterol

transport. The antioxidant and antiinflammatory properties of HDL may be as important as its cholesterol efflux function in terms of protecting against the development of atherosclerosis.

2.9.2.4. Antithrombotic effects of HDL

HDL is also associated with anti-thrombotic and profibrinolytic effects. HDL inhibits platelet aggregation by blocking thromboxane-A2 (TXA2) and PAF activity, while stimulating nitric oxide (NO) and PGI2 synthesis (Saku et al. 1985; Naqvi et al. 1999). In the Atherosclerosis Risk in Communities (ARIC) study, HDL-C levels inversely correlated with circulating von Willebrand factor (vWF) levels, suggesting that HDL may prevent synthesis of this pro-thrombotic protein. HDL also enhances the anti-thrombotic activity of protein C and protein S (Griffin et al. 1999). HDL may also promote fibrinolysis by downregulating plasminogen activator inhibitor-I (PAI-I) and by upregulating tissue plasminogen activator (t-PA). HDL transports various sphingolipids may directly or indirectly contribute antithrombotic activity. Thus, in addition to its cholesterol-transporting properties, HDL favorably regulates endothelial cell phenotype and reduces the risk of thrombosis.

HDL Therapeutics: Approaches to raise HDL-C levels and subsequently promote RCT include lifestyle modifications [Exercise and weight loss, dietary modification, smoking cessation, moderate alcohol consumption] , standard pharmacologic therapy [niacin, fibrates and statins] and several emerging therapeutics [HDL-C delipidation therapy exogenous Apo AI mimetics enhances RCT via the ABCA1 pathway (Navab et al. 2004), CETP inhibition, LXR/RXR agonists, selective and non-selective PPAR agonists and drugs targeting HDL-C catabolism are among some of the novel

emerging therapies harnessing the anti-atherogenic, anti-oxidant, anti-inflammatory and pro-endothelial functions of HDL-C based on metabolic targets involved in RCT. However, trials evaluating HDL-C targeted therapies are limited, in part due to a lack of pharmacologic agents specifically designed to raise HDL-C and our limited ability to measure HDL-C effectiveness. Given the strong body of evidence that demonstrates the important role of HDL in preventing lipid oxidation and the downstream monocyte-mediated inflammatory response, HDL molecules or peptides containing domains of HDL that are beneficial may serve as therapeutic agents that can slow atherosclerotic events and augment or even replace our current therapeutic modalities (Kapur et al. 2008)

2.9.3.Modified HDL and atherogenesis

HDL is a plasma lipoprotein heterogeneous in origin, size, composition and function. In contrast with other lipoproteins, many physiological functions of HDL influence the cardiovascular system in favorable ways unless HDL is modified pathologically. The strong inverse relationship between HDL-C level and risk for coronary artery disease has been attributed to different mechanisms. HDL is best known as a key player in reverse cholesterol transport. The other atheroprotective functions of HDL that have more recently attracted attention include its anti-inflammatory, anti-oxidant and vasodilatory properties. In addition, HDL has been shown to possess anti-apoptotic, antithrombotic and anti-infectious functions among other actions. However, these activities of HDL are compromised in many pathological states associated with inflammation.

It is apparent that many patients with ‘normal’ or even ‘elevated’ plasma HDL experience clinical events (Pearson, 2006). Furthermore, the recent clinical trial ILLUMINATE, which targets to increase plasma HDL levels with a new selective cholesteryl ester transfer protein (CETP) inhibitor, Torcetrapib, was prematurely terminated because of an increase in all-cause mortality despite an increase in HDL-C levels (Nissen et al. 2007). These disappointing results suggest that HDL may not always be atheroprotective and in some conditions, it paradoxically enhances the process of atherosclerosis.

2.9.3.1. Dysfunctional HDL as an atherogenic particle

Although epidemiological studies have consistently demonstrated an inverse association between plasma HDL, as well as apoA-I concentration, and the risk of myocardial infarction, a subset of patients with high plasma HDL concentrations have enhanced rather than reduced atherosclerosis**.** van Lenten et al (1995) reported that during an acute-phase response in animals or humans following surgery, HDL properties changed to become proinflammatory. These observations have formed the basis for subsequent studies evaluating the proinflammatory properties of HDL. These acute phase responses are temporary. As they subside, HDL reverses its function and becomes protective. How an acute phase response becomes chronic is not fully understood. It has been proposed that modification of HDL may lead to changes in its antiatherogenic properties or even result in an actual promotion of atherogenic events. HDL is known to undergo dramatic modification in structure and composition as a result of the concerted actions of the acute-phase response and inflammation (Khovidhunkit et al. 2004). As a result,

HDL particles progressively lose normal biological activities and acquire altered properties. Such altered HDL particles have been termed “dysfunctional HDL” (Navab et al. 2001) and HDL has been proposed to possess “chameleon-like properties” (Van Lenten et al. 2001). HDL can be dysfunctional with total loss of function or functionally altered with a deficiency in normal HDL function.

At basal state, functional HDL shows high levels of antioxidants, active antioxidant proteins, and antioxidant enzymes with anti-inflammatory activity. However, when antioxidant and anti-inflammatory functions of HDL are overwhelmed by pathological processes such as inflammation, HDL is converted into a dysfunctional, proinflammatory particle that cannot promote cholesterol efflux or prevent LDL oxidation. Evidence shows that many pathological processes associated with systemic inflammation including chronic heart disease, metabolic syndrome, chronic kidney disease, infections, and rheumatic diseases are characterized by the presence of dysfunctional or proinflammatory HDL (Ansell, et al. 2007).

It is of interest that HDL recovered from different participants often exhibits marked heterogeneity in its in-vitro functional properties (Ansell et al. 2003) examined the characteristics of HDL sampled from patients who developed CHD despite very high HDL-C levels (i.e., HDL ≥84 mg/dl). The difference in the anti-inflammatory potential of HDL was marked: those individuals who had CHD despite supernormal levels of HDL-C had uniformly proinflammatory HDL. They proposed that in setting of both vascular and nonvascular systemic inflammation, including

CHD, diabetes, surgery and influenza infection, the atheroprotective effects of HDL can markedly diminish, even to the point where it becomes proinflammatory. Several similar observations have been reported. Gowri et al. (1999) reported the decreased protection against LDL oxidation by HDL from poorly controlled diabetic patients, due to the abnormal composition of HDL and White et al. (2008) demonstrated that, in contrast to promoting eNOS activity, HDL from diabetic patients actually inhibits eNOS activity because of the abnormally high level of myristic acid in HDL. A number of studies (Ashby et al. 2001; Van Lenten et al. 1995; Van Lenten et al. 2001) showed the functional changes of HDL during acute phase responses in humans, rabbits, and mice. All these studies indicate that the dysfunctional form of HDL is associated with alterations in lipid or protein content of circulating HDL.

In patients with stable CHD or acute coronary syndrome, studies indicate that the endothelial repair capacity of HDL is attenuated (Besler et al. 2011). Evidence suggests that endothelial protein kinase C (PKC) βII activation is increased in HDL in patients with CHD and contributes to reduced NO production, endothelial dysfunction, inhibition of Akt-dependent endothelial NO synthase (NOS)-activating phosphorylation, and promotion of endothelial inflammatory activation. There was, however, no difference in the macrophage cholesterol efflux capacity of HDL between patients with CAD and healthy subjects, thereby supporting the involvement of endothelial LOX-1–dependent PKCβII activation in preventing endothelial NO production (Besler et al. 2011). Thus, these altered effects of HDL on endothelial NO production in individuals with CHD may predispose to the development and progression of cardiovascular disease.

Several animal models associated with dysfunctional HDL were established. Berard et al. (1997) developed a transgenic mouse model overexpressing human LCAT, the major enzyme promoting the esterification of free cholesterol to cholesteryl ester and packaging cholesterol into the core of HDL. The transgenic mice have elevated HDL and increased diet-induced atherosclerosis, and the observation shows the abnormal HDLs in both composition and function, and corresponding ineffective transport of HDL-C to the liver and impaired reverse cholesterol transport. Using SR-BI-null mice, numerous studies (Van Eck et al. 2007; Gong et al. 2003; Li et al. 2002) demonstrate that despite a marked increase in HDL concentration, the mice developed enhanced atherosclerosis. These genetically manipulated mouse models provide in-vivo evidence for dysfunctional HDL as a potential mechanism leading to increased atherosclerosis in the presence of high plasma HDL levels.

Evidence for the presence of modified HDL [cross-linked apoproteins] in atherosclerotic tissues has been reported (Artola et al. 1997). Furthermore, by using histochemical and immunoblot analyses (Nakano et al. 2003; Matsunaga et al. 2002), these modified lipoproteins were detected in atheromatous plaques of the abdominal aorta (Nakajima et al. 2000) and in the sera from patients with chronic renal failure (Taumura, 2001). Two independent groups have shown that chlorotyrosine (a specific product of HOCl) modified HDL (ClTyr-HDL) is present in human atherosclerotic tissue and human plasma (Bergt et al. 2004; Zheng et al. 2004). The same groups have also shown that plasma ClTyr-HDL levels are increased in patients with CVD, suggesting that circulating levels of modified HDL represent a unique marker for clinically significant atherosclerotic disease. Also, nitrotyrosine-modified HDL

(NO_2Tyr-HDL) levels are increased in patients with CVD. These observations suggest that dysfunctional HDL can be generated *in vivo* and promote atherosclerosis.

HDL acts as an anti-inflammatory molecule in healthy individuals. However, in those with chronic illnesses such as diabetes that are characterized by systemic oxidative stress and inflammation, HDL may actually promote the inflammatory response. HDL particles can vary in size, density, composition, and functional properties influencing their association with atherosclerosis (Lewis & Rader, 2005). Lenten and colleagues (Van Lenten et al. 1995), reported that during an acute-phase response in animals or humans following surgery, HDL properties changed to become proinflammatory. These observations have formed the basis for subsequent studies evaluating the proinflammatory properties of HDL. These acute phase responses are temporary. As they subside, HDL reverses its function and becomes protective. How an acute phase response becomes chronic is not fully understood. When inflammation occurs, the acute phase response leads to the conversion of HDL into a proinflammatory form. If inflammation persists, the acute phase response becomes chronic and leads to the persistence of proinflammatory HDL. This change is central to the process of atherogenesis.

HDL is known to undergo dramatic modification in structure and composition as a result of the concerted actions of the acute-phase response and inflammation (Khovidhunkit et al. 2004b). The close association between inflammation, oxidative stress, dyslipidemia, obesity, HT and atherosclerosis suggests that such HDL alterations play a significant role in disease progression. As a result, HDL particles progressively lose normal biological activities and acquire altered

properties. Such altered HDL particles have been termed “dysfunctional HDL” (Navab et al. 2001) and HDL has been proposed to possess “chameleon-like properties” (Van Lenten et al. 2001). HDL can be dysfunctional (with total loss of function) in cell-based or cell-free assays aimed at measuring anti-inflammatory activity (Navab et al. 2001; Ansell et al. 2003), whereas measurements of antioxidative activity (Hansel et al. 2004; Nobecourt et al. 2005) or cholesterol efflux capacity (Banka et al. 1995; Cavallero et al. 1995) reveal a deficiency in normal HDL function rather than a complete dysfunction.

At basal state, functional HDL shows high levels of antioxidants, active antioxidant proteins, and antioxidant enzymes with anti-inflammatory activity. However, when antioxidant and anti-inflammatory functions of HDL are overwhelmed by pathological processes such as inflammation, HDL is converted into a dysfunctional, proinflammatory particle that cannot promote cholesterol efflux or prevent LDL oxidation (Navab et al. 2009). This dysfunctional HDL shows decreased levels and activities of anti-inflammatory and antioxidant factors, such as Apo A-I and PON1. Dysfunctional HDL contains oxidized phospholipids and proinflammatory proteins, such as serum amyloid A (SAA) and ceruloplasmin. Evidence shows that many pathological processes associated with systemic inflammation including chronic heart disease, metabolic syndrome, chronic kidney disease, infections, and rheumatic diseases are characterized by the presence of dysfunctional or proinflammatory HDL (Ansell et al. 2007, Kontusch & Chapman, 2006). An oxidative environment is produced when an acute-phase response occurs as a result of nonspecific immunity. Thus, HDL appears to be part of the innate

immune system and can be either proinflammatory or anti-inflammatory depending on the presence or absence of an acute-phase response and systemic inflammation.

2.9.3.2.HDL modification: Accumulating data suggest that HDL can easily be modified and lose its antiatherogenic activities through multiple mechanisms. Based on the nature of modification, it can be classified into three types (a) spontaneous oxidative modification, due to the presence of free metal ions and free radicals in the atherosclerotic plaques, similar to the oxidation of LDL (b) enzyme-induced modification, including myeloperoxidase (MPO), chymase-tryptase, matrix metalloproteinases (MMPs), PMN-associated enzyme, endothelial lipase, and so on; these enzymes can degrade or oxidize apolipoproteins without significant changes in lipid moiety, or alternatively induce apolipoprotein cross-linking and lipid oxidation and (c) metabolic modification, such as glycation that occurs under hyperglycaemic conditions, and acute phase reactants-induced modification during inflammation and so on (Feng & Li, 2009). These studies suggest that under some conditions, HDL can readily be modified to lose its atheroprotective properties and become dysfunctional or even atherogenic. Therefore, we should not only evaluate HDL-C level but also measure HDL function while we predict the risk of CVD.

HDL are susceptible to structural modifications mediated by various mechanisms including oxidation, glycation, homocysteinylation or enzymatic degradation (Feretti et al. 2006). Structural alterations of HDL may affect their functional and atheroprotective properties. Oxidants, such as hypochlorous acid, peroxyl radicals, metal ions, peroxynitrite, lipoxygenases and smoke extracts, can alter both surface and core components of HDL. The formation of lipid peroxidation derivatives, such

as thiobarbituric acid reactive substances, conjugated dienes, lipid hydroperoxides and aldehydes, is associated with changes of physical properties (fluidity, molecular order) and of apoprotein conformation. Non-enzymatic glycation, generally associated with lipoxidation, leads to form irreversible complexes called advanced glycation end products. These HDL modifications are accompanied with altered biological activities of HDL and associated enzymes, including paraoxonase, CETP and LCAT (Nobecourt et al. 2007). Homocysteine-induced modification of HDL is mediated by homocysteine-thiolactone, and can be prevented by a calcium-dependent thiolactonase/paraoxonase. Tyrosylation of HDL induces the formation of dimers and trimers of apo AI, and alters cholesterol efflux. Phospholipases and proteolytic enzymes can also modify HDL lipid and apoprotein structure. HDL modification induces generally the loss of their anti-inflammatory and cytoprotective properties.

The underlying mechanisms responsible for generating the dysfunctional HDL and the chemical and structural changes of HDL remain largely unknown. One important pathway may be oxidative damage to HDL. Compositional changes in apoA-I have been identified in serum from atherosclerosis patients, and in specimens taken from atherosclerotic lesions (Zheng et al. 2004). These changes appear to be produced by the effect of myeloperoxidase (MPO) in the artery wall, one of the enzymes released by macrophages in the atherosclerotic process. Since MPO is found associated with HDL in atherosclerotic lesions, it appears that MPO and HDL interact directly within the inflammatory, cholesterol-laden, atherosclerotic lesions. MPO modifies HDL in humans by oxidation of specific amino acid residues in apolipoprotein A-I, which impairs cholesterol efflux through ATP-binding cassette transporter A1 and contributes to atherogenesis. Oxidation per se does not always

generate dysfunctional HDL populations. While tyrosyl radical oxidation of HDL can augment cholesterol efflux, oxidation and subsequent nitration of HDL can have the opposite effect. In addition to an adverse effect on HDL function, myeloperoxidase also inhibits endothelial cell function considering the strong negative effect it has on flow-mediated dilation of the brachial artery. Undurti et al.(2009) suggest that Apo A-I oxidation by MPO results in the loss of HDL-mediated, antiapoptotic, and anti-inflammatory activities .

Inflammation induces major changes in HDL levels and composition. Inflammatory cytokines such as TNF-α and interleukin-6 (IL-6) enhance expression levels of SAA and group IIA secretory phospholipase A2 (sPLA2-IIA), altering apolipoprotein content and levels (Bindhu et al. 2011). Myeloperoxidase is a key inflammatory mediator of macrophages and other leukocytes, and systemic inflammation is thought to convert HDL to a dysfunctional form that loses its antiatherogenic effects. The pro-oxidant acute-phase reactants namely serum amyloid A [SAA] and ceruloplasmin are associated with the formation of proinflammatory HDL along with Apo-j, also called clusterin (Van Lenten et al. 2001).

During acute and chronic inflammation, the content and functions of HDL can change drastically converting atheroprotective HDL to proatherogenic HDL. The acute-phase HDLs are depleted in cholesterol esters but enriched in free cholesterol, triglycerides, and free fatty acids, but none of them can participate in reverse cholesterol transport or antioxidative property. The major protein in HDL, Apo A-I, might be reduced because of decreased Apo A-I synthesis, accelerated HDL catabolism, and Apo A-I replacement by SAA. The acute phase proteins are mainly

the C-reactive protein (CRP), SAA and haptoglobin (Hp) which are released by the hepatocytes after cytokine stimulation.SAA is a pro-oxidant acute-phase reactant associated not only with disabling the anti-inflammatory role of HDL but also with creation of proinflammatory HDL (Khovidhunkt et al. 2004). CRP mainly of hepatic origin, and circulating levels can be induced to increase up to 1000-fold in the presence of inflammation. Like CRP, elevated plasma levels of SAA represent an important, although weaker, cardiovascular risk factor (Kontush & Chapman, 2006) .

Watanabe et al. (2007) demonstrate that the association of haemoglobin [Hb] with HDL also plays an important role in the modulation of HDL function (Watanabe et al. 2007). Hb was found differentially associated with HDL from coronary heart disease patients compared with healthy controls. The data suggest that Hb contributes to the proinflammatory nature of HDL in mouse and human models of atherosclerosis and may serve as a novel biomarker for atherosclerosis. The loss of function of HDL may be the direct result of its oxidative modification. Hb can oxidize ApoA1 (Salvatore, Cigliano et al. 2007). HDL is dysfunctional in haptoglobulin [hp] type 2-2 gentotype in type 2 diabetic patients. More over the replacement of LCAT binding site in ApoA1 with hb-hp complex and subsequent oxidative modification of LCAT site in ApoA1 also make HDL dysfunctional. HDL in Hp 2-2 diabetes may actually be proatherogenic and prothrombotic by limiting NO bioavailability.

HDL lipid composition might equally be altered during inflammation. Enrichment in TG with depletion of CE in the HDL core is the most frequent abnormality of HDL lipid composition. In addition, HDL triglyceride content can also be increased in hypertriglyceridemia as a consequence of elevated CETP activity.

CETP-mediated replacement of cholesteryl esters by triglycerides in the HDL core results in decreased plasma HDL-C, which is another feature of the acute-phase response. Similar elevation in HDL-TG, decrease in HDL-C, and increase in inflammatory markers are observed in the postprandial phase. Acute-phase HDL also contains elevated levels of nonesterified fatty acids, lysophosphatidylcholines, and isoprostanes compared with normal HDL; in addition, CE levels are decreased. As part of the acute-phase response, activities of HDL-associated enzymes including PON1, PAF-AH, LCAT, CETP, and phospholipid transfer protein (PLTP) can be compromised, made dysfunctional or both (Navab et al. 2004).

Alterations occurring in HDL composition and metabolism due to inflammation are intimately associated with impaired biological activities. Enrichment of HDL with SAA results in increased HDL binding to macrophages, decreased cholesterol efflux from macrophages, and increased selective uptake of CE by macrophages. Recently, McGillicuddy et al. (2009) provided evidence in humans and mice indicating that acute-phase HDL enriched in Serum amyloid A induced by acute endotoxaemia have an impaired capacity to remove cholesterol from lipid loaded macrophages (foam cells). The antioxidative activities of HDL might equally become impaired in the presence of inflammation due to the replacement of Apo A-I by SAA and altered enzymatic activities. Indeed, antioxidative deficiency of HDL relative to LDL oxidation by artery wall cells is observed in the acute phase, concomitant with decreases in the activity of PON1 and PAF-AH. All these mechanisms might limit the capacity of HDL to inactivate oxidized phospholipids, resulting in their elevated accumulation in LDL. These altered HDLs are

proinflammatory enhancing LDL oxidation and attracting monocytes to engulf the oxidized LDLs.

According to studies in patients with CHD, HDL is not only ineffective as an anti-inflammatory and antioxidant but is actually a proinflammatory and pro-oxidant promoting LDL oxidation. A study by Corsetti et al. (2011). suggested that raised HDL cholesterol levels and raised CRP levels may result in increased risk of cardiovascular disease. The Thrombogenic Factors and Recurrent Coronary Events (THROMBO) postinfarction study by Corsetti et al. (2008) showed the same results in a subgroup of nondiabetic patients with high CRP levels who showed recurrent risk with increasing HDL cholesterol levels. Extending these studies to a healthy population (Prevention of Renal and Vascular End-Stage Disease study) to determine whether primary coronary risk acted similarly identified a high-risk subgroup at high HDL cholesterol and CRP levels with presumptive evidence for large HDL particles. It also identified a second high-risk group with high CRP levels and low HDL levels as expected from many previous studies (Corsetti et al. 2010). Subgroup patients had low levels of lipoprotein-associated phospholipase A2 (Lp-PLA2) and large HDL particles.

The molecular changes and mechanisms that promote anti-inflammatory HDL conversion to proinflammatory HDL are currently unknown. HDL might play major roles in the transport and metabolism of lipid hydroperoxides in vivo and that these processes contribute to its cardioprotective effects. The transfer of lipid hydroperoxides between HDL and LDL appears to be too slow to substantially influence the distribution of these compounds in plasma (Bowry et al. 1992).

Moreover, plasma is rich in antioxidant defense mechanisms, and LDL and HDL turn over rapidly in that compartment (half-lives of 3–4 days). These observations make it unlikely that either lipoprotein is oxidized in plasma or that LDL directly transfers lipid hydroperoxides to HDL in plasma. So, how might HDL's lipid oxidation products originate? One intriguing possibility is that HDL acquires them at sites of inflammation and then transports them back into plasma (Bergt et al 2004). Studies by Terasaka et al.(2007/ 2008) demonstrate that both macrophages and endothelial cells export 7-ketocholesterol (a cytotoxic cholesterol oxidation product) to HDL by a pathway involving the cholesterol transporter ABCG1.

Model system studies indicate that hepatocytes can efficiently extract lipid hydroperoxides from HDL. Moreover, macrophages are the cellular hallmark of the atherosclerotic lesion, indicating that atherosclerosis is a chronic inflammatory disease. These observations suggest that HDL protects endothelial cells and macrophages from lipid-oxidation products by transporting those toxic substances to the liver, as originally proposed for cholesterol.

It is noteworthy that HDL isolated from humans with established CHD contains much higher levels of chlorotyrosine than does HDL of apparently healthy control subjects (Bergt et al. 2004; Zheng et al. 2004). Chlorotyrosine is a specific oxidation product of the heme protein myeloperoxidase, and macrophages in human atherosclerotic lesions express high levels of MPO (Shao et al. 2010). Myeloperoxidase can trigger lipid peroxidation by a variety of pathways, by generating tyrosyl radical, for example. When apolipoprotein A-I (apoA-I) is chlorinated by myeloperoxidase, it loses its ability to remove cholesterol from cells

by the ABCA1 pathway (Shao et al. 2010; Bergt et al. 2004), which normally is an important conduit for cholesterol efflux from macrophages. These observations suggest that macrophages could generate a dysfunctional form of HDL that contains oxidized lipids and proteins. Thus, the inflamed atherosclerotic lesion is one potential location where oxidation could be biologically and clinically important.

The attenuated atheroprotective properties of HDL raise the possibility of an indirect putative proatherogenic effect of these particles. The role of HDL in inflammation is more complex. In its basal state, HDL is anti-inflammatory, but during acute inflammation HDL become pro-oxidant. HDL can also undergo oxidative modification in vivo and HDL lipids are equally or even more susceptible to oxidation than those of LDL. Compared to LDL, relatively little is known about the role of HDL oxidation in atherogenesis. There is also little information on relative susceptibility to oxidation of different HDL subclassess. Oxidative modification of HDL might have important consequences concerning the efficiency of HDL in promoting cholesterol efflux from the peripheral cells (RTC) and in inhibiting LDL oxidation. Therefore, studying the mechanisms of the HDL oxidation might have potential pathophysiological significance.

It has now been repeatedly shown that HDL can develop frankly proinflammatory characteristics in association with multiple diseases and, in particular, with coronary heart disease. In fact, most patients with CHD and other associated inflammatory diseases routinely express a proinflammatory HDL phenotype. Independent of measured HDL-C levels, even a 'normal' individual can express a dysfunctional HDL phenotype and may become transiently, or

progressively, pro-oxidant in the presence of systemic stress or infection or localized vascular inflammation. As such, recognizing and determining the anti-inflammatory function or the proinflammatory dysfunction of a patient's HDL will likely become more important clinically. Better understanding of the dysfunctional aspects of HDL metabolism will lead to improved predictive accuracy for CVD risk and disease expression and may also provide new strategies for the prevention and treatment of at-risk CHD patients. Understanding the relationship between inflammation and HDL function will be an area of active research in the field of atherosclerosis within the coming years.

MATERIALS AND METHODS

3.1. Materials

Sodium Chloride, Sodium hydroxide, Potassium bromide, Potassium dihydrogen orthophosphate, Disodium hydrogen phosphate, Hydrochloric acid, Ethylene diamine tetra acetic acid (EDTA), Isopropanol, Methanol, Pyridine, n-butanol, Chloroform, Ethanol, Copper sulphate, Isoamyl alchohol, Dinitrophenyl hydrazine (DNPH), Trichloroacetic acid, acetic acid, Butylated hydroxy toluene, Glycine, Bromophenol Blue, Sodium azide, Hematoxylin, Coomassie blue, Phenol etc. were purchased from Merck Pharmaceuticals. Dichlorofluoresceine diacetate, Diphenyl iodonium, PD98059, SB208530, SP600125, N-acetyl cysteine, RPMI 1640, Histopaque, Dulbaccos-phosphate buffered saline [D-PBSA], Bovine serum albumin, Propidium iodide, Hoechst 33342, triton-X-100, Triz Base, Sodium dodesyl sulphate, Beta- mercaptoethanol, Tetramethylethylenediamine [TEMED], Ammonium persulphate, Acrylamide, Bis-acrylamide, Oil Red O, Ethidium bromide, Phenyl acetate, Trypan blue, Gelatin, Nitroblue tetrazolium, Dimethyl sulphoxide, Gentamycine, Streptomycine, Sodium citrate, Sucrose, Penicillin, Calcium chloride, Sodium bicarbonate, Paraformaldyhyde, Riboflavin, Folin's reagent, Glycerol, PEG_{8000}, DNAse, Thiobarbituric acid, Guanidine Hydrochloride, Agarose, Filter membranes etc. were purchased from Sigma St. Louise, New Jersey. Syringe filter was purchased from Millipore. Antibodies were purchased from Santacruze. Tissue culture plates were purchased from Nunc, Denamrk. PCR primers were synthesized and purchased from CalBiochem. MMLV –reverse transcriptase, RNasin, dNTPs, Taq

DNA polymerase, Trizol reagent were purchased from Promega Corporation, USA. TNF-alpha and IL-10 kit were purchased from Thermo scientific, Rockford, USA. Polypropylene tubes for culture purpose were purchased from BD Bioscience. Reagent kits for Cholesterol and Triglycerides were purchased from ASPEN lab, Delhi and Phospholipid kit from FAR srl, Verona, Italy.hsCRP reagent kit was obtained from hyphen Biomed, France.

3.2.Methods

3.2.1.Blood sample collection

Fasting blood samples (~10 ml) were collected from apparently healthy volunteers of the hospital staff (total sample size~72) with informed consent and with Institutional review board approval. Samples were subjected to low speed centrifugation at 1500-x g for 15 min at room temperature for separating serum for different analyses.

3.2.2. Analytical methods

3.2.2.1. Estimation of total cholesterol in serum: Serum total cholesterol was quantitated by the recommended enzymatic method (CHOD-POD) using reagent kits. [ASPEN lab, Delhi].

Principle- This method follows determination of cholesterol after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneamine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinders reaction)

Cholesterol ester + H_2O $\xrightarrow{\text{Cholesterol esterase}}$ Cholesterol + fatty acid

Cholesterol +O_2 Cholesterol oxidase $\rightarrow$ Cholesterol-3-one + H_2O_2

2 H_2O_2 + 4- aminoantipyrine + Phenol Peroxidase $\rightarrow$ quinoneamine+ H_2O

Procedure: 1 ml reagent was mixed with 10 μl of serum /standard and incubated for 10 minutes at 37^0C. The absorbance was read within 60 min against reagent blank at 505nm using UV-VIS spectrophotometer [Shimadzu] and calculated the values against standard values.

3.2.2.2. Estimation of triglycerides in serum: Serum triglycerides were quantitated by the recommended enzymatic method (GPO-POD) using reagent kits [ASPEN lab, Delhi].

Principle: Determination of triglycerides after enzymatic splitting with lipoprotein lipase. The colour indicator is quinonamine which is generated from 4-aminoantipyrine and 4- chlorophenol by H_2O_2 under the catalytic action of peroxidase.

Triglycerides + LPL $\longrightarrow$ glycerol + fatty acids

Glycerol + ATP + Glycerol 3 Kinase $\longrightarrow$ glycerol- 3-phosphate + ADP

Glycerol- 3 –phosphate + O_2 + Glycerol 3 phosphate oxidase $\longrightarrow$ Dihydroxy acetone phosphate + H_2O_2

$2H_2O_2$ + amino antipyrine + 4-chlorophenol + Peroxidase $\longrightarrow$ Quinonamine + HCl + 4 H_2O

Procedure : 1 ml reagent was mixed with 10 μl of serum or standard incubated for 10 minutes at 37^0C. The absorbance was read within 60 min against reagent blank at 505 nm using UV-VIS spectrophotometer and calculated the value against standard values.

3.2.2.3. Estimation of HDL-C in serum: HDL-C was assayed by the precipitation method (modified method of Izzo et al. 1981).

Reagents required:

1. Polyethylene glycol 8000 (200 mM/l,pH10); (2) 1N NaOH (3)Glycine Buffer 0.2M,pH 10.

Procedure: HDL-C was estimated by measuring the cholesterol content in the serum-supernatant obtained after selective precipitation of all the apo-B containing lipoproteins, including VLDL, LDL, Lp(a) using PEG_{8000}. The assay was performed as follows. About 200 μl of serum was added to equal amount of PEG reagent. The sample was mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. The clear supernatant was collected and its cholesterol content was quantitated by CHOD-POD method as described above.

3.2.2.4. Quantitation of LDL-C in serum

Serum LDL-C was calculated from triglycerides, total cholesterol and HDL-C using Friedewald formula as

LDL-C= total cholesterol- (TG/5+HDL-C) mg%

3.2.3.1. Isolation of HDL (d= 1.06- 1.21) by ultracentrifugation

1. Solution 1- 23 % NaCl, density 1.14 gm/ml

2. Solution 2- NaCl/KBr , density 1.36 g/ml

Procedure: Fresh serum was collected from healthy subjects and subjected to density gradient ultra-centrifugation using Beckman Optima TLX 120 ultracentrifuge with a fixed angle rotor type 120.2. Briefly, the density of 0.5 ml serum was adjusted to 1.087 by adding solution 1. The sample was centrifuged at 4,36000 x g for ~ 2.5 hr at 15 ^{0}C. The top ~0.5 ml fraction was discarded and the bottom fraction of the sample was collected. Density of the bottom fraction was again adjusted to 1.21g/ml by adding solution 2 and made the final density to d-1.21 g/ml and centrifuged at 4,36,000 xg for ~ 2.5 hr. The upper fraction containing HDL was collected. To facilitate the visualization of lipoprotein bands after centrifugation, the density-adjusted plasma in one of the tube was stained with Coomassie blue (5% w/w) before centrifugation. The isolated HDL was dialyzed against PBS (pH 7.4) at 4 ^{o}C extensively and the purity was checked by polyacrylamide disc gel electrophoresis (3.75%)

3.2.3.2. Isolation of LDL

1. Solution 1- 0.9% NaCl and EDTA, density- 1.003 g/ml

2. Solution 2 - 16.7% NaCl , density-1.10 g/ml

Procedure: LDL fraction was isolated from serum by the standard sequential ultra centrifugation method by using salt solutions of various densities using Beckman

Optima TLX 120 ultracentrifuge with a fixed angle rotor type of 120.2. Briefly, density of 0.5 ml serum was adjusted to d=1.006 g/ml with 0.9% NaCl and loaded into rotor and centrifuged for 2.5 hr at 4,36000 x g at 15 ^{0}C and the top layer (~0.5 ml, VLDL, IDL) was removed. Serial preparation of individual lipoprotein fractions was accomplished by readjusting the bottom fractions after each run with the appropriate density salt solutions. The density of the bottom fraction was adjusted to 1.06 g/ml with solution 2 and centrifuged for 2.5 hr at 4,36000 x g .Top fraction containing LDL was separated at a density of 1.063 g/ml with a syringe from top of the tube and dialyzed extensively against PBS, pH (7.4) at 4 ^{o}C. The purity of the isolated fractions was checked by 3.75% polyacrylamide gel electrophoresis.

3.2.3.3. Assay of total proteins: Total protein was measured by Lowry's method (Lowry et al. 1951). Proteins react with copper in alkaline solution and reduce phosphomolybdic- phosphtotungstic acid in the Folins reagent with the formation of a blue colour that can be measured at 750 nm.

Reagents

(1) 0.1 N NaOH; (2) 1% Sodium citrate; (3) Solution A : 2 gm Na_2CO_3 in 100 ml of 0.1 N NaOH; (4) Solution B: 0.5 gm CuSO4 in 100 ml of 1% sodium citrate; (5) Solution C: (prepare freshly) : mix solution B:A in the ratio 1:50 (6) Solution D Folin's reagent [1N] (7) Protein standard: stock- 2 mg/ml BSA.

Procedure: 1 ml reagent C was mixed with 20 μl sample or standard. After incubating for 10 minutes at room temperature, 0.1 ml Folin's reagent was added.

Absorbance was measured after 30 minutes at 750 nm in a UV-VIS spectrophotometer and the values calculated against standard.

Concentration of protein = Test Absorbance/Standard absorbance x100 mg%

3.2.3.4. Polyacrylamide disc gel electrophoresis [PAGE] of isolated lipoprotein

Solution A: 9.15 gm Trisma base was dissolved in 12 ml of 1N HCl. After adding 0.11 ml TEMED the solution was diluted to 25 ml with distilled water (pH to 8.9)

Solution B: 1.495 gm Trisma base was dissolved in ~ 9 ml 1 N HCl. After adding 0.575 ml TEMED the solution was diluted to 25 ml with distilled water (pH 6.6).

Solution C: 2.4 gm acrylamide and 0.063 gm bis acrylamide (63 mg) were dissolved in 15 ml DW and diluted to to 25ml. The solution was filtered and stored at 4^0C in dark bottles.

Solution D: 2.4 gm acrylamide and 0.063 gm bis acrylamide (63 mg) were dissolved in 15 ml DW and diluted to 25ml. The solution was filtered and stored at 4^0C in dark bottle

Solution E : 2 mg Riboflavin was mixed in 25 ml water, filtered and stored at 4^0C in dark bottle

Solution F: 8 gm Sucrose was dissolved in 20 ml water (40%) and stored at 4^0C

solution G: 0.14 gm ammonium persulphate was dissolved in 10 ml water (14%).Working solution - 0.14%

Solution H : Stock Sudan black 100 mg/ml

Separating Gel: The solutions A: C: G were mixed in a ratio of 1: 3: 4 .

Concentrating Gel: The solutions B: D: E: F were mixed in a ratio of 1:2:1:1

Sample Gel: Mix the solutions B: D: E: F: H_2O in a ratio of 1:2:1:1:3. Eight part of this sample gel was mixed with one part of sudan black.

3.75% polyacrylamide disc gels were cast in glass tubes (5mm ID, 9cm). Briefly 1 ml of the separating gel solution was loaded in each tube and layered with water. The gels were kept for ~ 30 minutes under visible light for polymerization. After removing the water layer, 0.1 ml separating gel solution was layered and kept for ~30 minutes for polymerization. Then 200 μl of sample gel with dye was layered. To this, dialysed HDL fraction [~300 μg protein/tube] was added, mixed well by inversion and allowed to prestain for lipids with sudan black for ~ 30 minutes . The gel tubes were then inserted into electrophoretic apparatus and subjected to electrophoresis for ~35 minutes using Tris- glycine buffer pH (7.2) at constant current of 5 mA/tube. The gels were visualized for resolution of lipoproteins and scanned with an Image scanner (Amersham Biosciences, USA).

3.2.3.5. Kinetics of LDL oxidation

Procedure: The isolated LDL fraction (100μg/ml) was subjected to oxidation in the presence of different concentration of copper from a low concentration of 0.5 μM/L to a higher concentration of 5μM/L in PBS pH 7.4 [20mM] at 37^0C for different time intervals up to 3 hours. The progress of oxidation was monitored as formation of

conjugated dienes at every 30 minutes by measuring the increase in absorbance at 234 nm using an UV- VIS spectrophotometer.

3.2.3.6. Assay of antioxidant capacity of HDL

Procedure: LDL oxidation [100 μg/ml] was initiated by adding $Cu2^{+}$ at 5 μM/ml in PBS pH 7.4 as described above. LDL oxidation was also carried out in the presence of HDL at equal protein concentration [1:1] to determine the ability of HDL to inhibit LDL oxidation. The progress of LDL oxidation [with the presence or absence of HDL] was measured as an increase in conjugate diene formation at 234nm in a Shimadzu UV-VIS Spectrophotometer at different time intervals and the percentage inhibition of LDL oxidation was calculated.

3.2.3.7. Assay of serum lipidperoxides (TBARS assay) (Ohkawa et al. 1979)

Reagents: (1) 8.1 % SDS (2) 20 % acetic acid (3) 0.8 % Thiobarbituric acid (4) n-butanol-pyridine reagent (15:1 ratio)

Procedure : Briefly 0.2 ml of sample was mixed with 0.2 ml 8.1 % SDS ,1.5 ml of acetic acid and 1.5 ml of 0.8% TBA and diluted to 4 ml with water. The test tubes with sample were kept at 95^{0}C for one hour and cooled under tap water. 1 ml of water and 5 ml of n-butanol-pyridine were added to each tube, shaken vigourosly and then centrifuged at 4000 rpm for 10 minutes. The organic layer was collected and the absorbance was recorded at 532 nm in a UV-VIS spectrophotometer against n-butanol blank. The MDA content was calculated using the extinction coefficient for MDA, 1.56×10^{5} m^{-1},$cm^{-1.}$

3.2.3.8. Assay of serum protein carbonyls

Reagents

(1) 20% TCA(2) 2M HCl (3) 0.2 % DNPH in 2M HCl (4) 6 M guanidine HCl (5) Ethyl acetate: ethanol (1:1)

Procedure: Protein carbonyles were quantitated by the method described by Levine et al.(1990) using dinitrophenyl hydrazine [DNPH]. After precipitation of serum proteins with 20% TCA, the protein pellets were suspended in DNPH solution and incubated with shaking at 5 min interval at room temperature for one hour. Proteins were then reprecipitated with 20% TCA and washed once with 10% TCA and thrice with ethanol/ethyl acetate mixtur (1:1) to remove lipids and excess DNPH.The precipitate was dissolved in 6 M guanine hydrochloride and the absorbance was recorded at 370 nm in UV-VIS spectrophotometer. The data were expressed as μm of carbonyl groups/liter serum by using a molor absorption coefficient of 21,000 mol^{-1} liter cm^{-1} for the DNPH derivatives.

3.2.3.9. Assay of serum paraoxonase activity PON-1 (Lorenz et al. 1979)

Reagents (1) Tris acetate buffer (100 mM/L,pH 7.8); (2) Phenyl acetate (10 mM/L); (3) $CaCl_2$ (200mM/L)

Procedure: Paraoxonase activity was measured using phenyl acetate as substrate. Briefly, 10 μl of serum was added to 2.5 ml of a reaction mixture consisting of 4 mM/L phenyl acetate in Tris acetate buffer, pH 7.8, containing 20mM/L calcium chloride. The rate of hydrolysis of phenyl acetate was monitered at 270 nm using a

UV-VIS spectrophotometer and PON-1 activity [kU/L] was calculated using a molar absorption coefficient at 270nm of 1310 liter/Mol^{-1} cm^{-1}.

3.2.3.10. Assay of Serum hs-CRP

CRP level was measured using the Zymutest CRP kit,a highly sensitive 'one step' sandwich ELISA technique, as described by manufacturer's instruction (Hyphen Biomed , France).

First, the immunoconjugate, a goat polyclonal antibody [specific for human CRP],coupled to horse-radish peroxidase (HRP) was introduced into the microwell, coated with a polyclonal antibody [F (ab') 2fragments] specific for CRP. Then, the diluted sample [1:100] and standards were added, and incubated for one hour at room temperature. When present, CRP reacted with the immunoconjugate. Following a washing step,the peroxidase substrate tetramethyl benzidine [TMB] in the presence of hydrogen peroxide was added. After incubation for 5 minutes, the reaction was stopped with 0.45M sulfuric acid. The absorbance was recorded after 10 minutes at 450nm in a spectrophotometer.The amount of colour developed is directly propotional to the concentration of CRP.

3.2.4.1. Oxidative modification of HDL

Reagents (1) 20mM/L PBS, pH.7.4 (2) Copper sulphate stock - 2mM/L $CuSO_4.5H_2O$ in PBS (3) Working solution - 200 μM $CuSO_4$ solution

Procedure: HDL (1 mg protein/ml in PBS) was oxidized with $CuSO_4$ at two different concentrations (a) 5μM /L for 12 hour at 37^0C (mild oxidative condition–m.oxHDL)

(Ren et al .2010) and (b) 20 μM /L for 24–36 h at 37^0C (a strong oxidative condition–oxHDL) (Callegari et al. 2006) 1 mg LDL was also oxidized with 5μM copper for 24 hours. The oxidized lipoproteins were extensively dialyzed in PBS containing 0.03 mM EDTA for 24 hours with three buffer changes. The extent of oxidative modification was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Ohkawa (1979) and TBARS were expressed as malondialdehydes nM/mg protein. The samples were sterilized by filtering through 0.22 micron size syringe filter and kept at -80^{o}C.

3.2.4.2. Monocyte cell isolation and culture

Procedure: Human peripheral blood mononuclear cells were isolated from blood of healthy volunteers using Histopaque 1077 based density gradient centrifugation. The buffy coat formed at the interface was collected, washed twice with PBS pH 7.4 and finally with RPMI 1640 medium. The pellet was resuspended in RPMI medium. The cells ($1x10^6$/ml) were then seeded on to culture dishes and incubated for 2 hours for adherence in an atmosphere of 5% CO_2 at 37^{o}C. Non-adherent cells were removed by washing and the monocytes adhered to dishes were maintained in serum-free RPMI 1640 medium supplemented with penicillin (100 U/I), streptomycin (100 mg/l) and gentamycin (100 mg/l) for 24 hours. The cell were characterised by an immunocytochemical method for CD14 surface marker. Above 85% of cells were found to be CD14 positive. Cell viability, assessed by Trypan blue exclusion test, was found to be greater than 95%. These cells represent monocytes in an early stage of macrophage differentiation and are thus referred to as monocytes-macrophages in the text.

3.2.4.3. Estimation of cell viability by trypan blue exclusion test

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension [1 x 10^6 cells/ml] was mixed with dye [0.1 mL of trypan blue, 0.4 % in PBS. pH 7.2] , loaded to the counting chamber [hemocytometer] and then examined under a microscope at low magnification. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. Cell viability should be at least 95%.

3.2.4.4. Cell treatment: Cells were maintained in culture as described above and then treated with a medium containing PBS alone or with HDL (both native and oxidized forms-m.oxHDL and oxHDL) at varying concentrations 10 to 200 μg protein/ml and cultured for 24 to 48 hours**.** Cell culture supernatant was collected after the treatment and cells were dislodged by 3 mm EDTA treatment and total cell protein was determined by Lowry's method.

3.2.4.5. Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species were measured by DCFH method (Zhang et al. 2010). Measurement of ROS was based on ROS-mediated conversion of non-fluorescent 2', 7'- Dichlorofluoresceine diacetate (DCFH-DA) into fluorescent DCFH.

Reagents: (1) DCFH stock(1 mM /L) - 2.5 mg DCFH in 10 ml DMSO and kept in dark tubes. (2) Working DCFH – 10 μM/ml

Procedure: Monocytes–macrophages after treatment with HDL were incubated with DCFH-DA (10μM/ml) in medium at 37^{o}C for 45 min. After washing with PBS, DCFH- fluorescence of the cells from each well was measured in a fluorescence microplate reader (Biotek FLX 800) at an excitation wavelength of 485 nm and emission at 528 nm. The intensity of fluorescence reflects the extent of oxidative stress

3.2.4.6. Assay of TNF-α

Concentrations of TNF-α in cell culture supernatants were determined using enzyme linked immunosorbent assay (ELISA) kits from Thermo scientific, Rockford, USA, according to the manufacturer's protocol.

Briefly, 50 μl standard diluent and 50 μl of standard or sample was dispensed into each well coated with corresponding antibody for TNF-α and incubated for half an hour at room temperature. After washing the wells,100 μl of biotinylated antibody reagent was added to each well and incubated for one hour at room temperature. After washing the wells, 100 μl of streptavidin-HRP reagent was added to each well and again incubated for 30 min at room temperature. Then TNB substrate solution [100 μl] was added and allowed for enzymatic reaction to develop in the dark for 30 minutes. The reaction was stopped by adding 100 μl of stop solution and the absorbance was measured at 450 nm using an ELISA microtitre plate reader (Biotek instruments).

3.2.4.7. Activity of matrix metalloproteinases (MMP9 & -2)

Activity of MMPs secreted by the cells to the culture medium was determined by gelatin zymography (Galis et al. 1994). For this, 7.5 % SDS-PAGE polymerized together with gelatin (1 mg/ml) was used. Based on protein concentration, cell culture supernatants, [20~ µl] after mixing with 10 µl of sample buffer [0.4M/L tris HCl, pH 6.8,containing 5% SDS, 20% glycerol. 0.03% bromophenol blue] were loaded on gel and subjected to electrophoresis at 120 V for 1.5 h. After electrophoresis, the gels were treated with 2.5 % Triton-X 100 for 30 min and subsequently incubated with substrate buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, and 0.02 % NaN_3, pH 7.5) at 37^0C for 72 hrs. The gels were then stained with Coomassie blue R-250. The image was recorded using an image scanner (Amersham Biosciences). Gelatinolytic activity was quantified using quantity one program (BioRad).

3.2.4.8. Oil Red O staining of cells for neutral lipid accumulation:

After treatment with oxHDL, monocytes-macrophages were washed with PBS and fixed by 4% PBS-buffered formaldehyde for 10 minutes. Cells were then stained with Oil Red O (0.06%) for 30 minutes. A quick wash was first given with 60 % isopropanol and subsequently cells were washed with PBS for three times. Counter stain the cells with haematoxylin [100mg/L] for 5 minutes.Stained cells were examined under a microscope [Olympus IX70 inverted- microscope, Barcelona, Spain] at 40 X magnifications. Cells with more than 6 or large Oil Red O-positive droplets were counted. Percentage of lipid-accumulated cells was then calculated.

3.2.4.9. Propidium iodide [PI] staining for measuring cell death

Monocytes-macrophages after treatment with oxHDL were first stained with propidium iodide (PI stains dead cells only) and subsequently stained with cell-permeable Hoechst 33342 (stains all cells) (Serra-Perez, 2008). PI and Hoechst were added at a final concentration of 1 μg /ml and 0.5 μg/ml respectively. Micrographs of three to six random fields per dish were obtained in an Olympus IX70 inverted-microscope (Barcelona, Spain) with the appropriate filter sets for PI (green) and Hoechst 33342(UV). PI- and Hoechst 33342-stained nuclei were counted on micrographs manually and the percentage of dead cells was calculated by dividing the number of PI-positive nuclei by the total number of nuclei (Hoechst-positive) for each micrograph

3.2.4.10. Immunocytochemistry for ABCG1 and CD36 expression

Freshly isolated monocytes-macrophages ($1x10^6$ cells/ml) were incubated with oxHDL (100μg/ml) for 24 hours. After that cells were fixed with 4% formaldehyde for 15 minutes at 4^0 C. The fixed cells were blocked with 0.5% BSA for 30 minutes. After washing, cells were incubated with rabbit primary antibody against ABCG1 or CD36 (1:100 dilution) for 16 hours and washed. The cells were then incubated with anti rabbit secondary antibody conjugated with FITC (1:100 dilution) for 1.5 hours and cells were counter stained with propidium iodide (0.5μg/ml) and examined under a fluorescence microscope [Olympus, Barcelona] with appropriate filter. Photographs were taken with 20x magnification.

3.2.4.11. RNA isolation from monocytes- macrophages

Monocytes – macrophages ($1x10^7$ cells/ml) were incubated with oxHDL or native HDL(100μg/ml) for 24 hours. Untreated cells were kept as control. After treatment with HDL, RNA was isolated by Trizol (Invitrogen) reagent Total RNA was extracted in chloroform, precipitated with isopropanol, and washed in 70% ethanol. Genomic DNA contamination of RNA sample was removed by DNase treatment (RNAase free, amplification grade; Sigma) and further purified by phenol chloroform extraction and quantified in spectrophotometer at 260 and 240 nm.

3.2.4.12. Agarose gel electrophoresis for RNA

RNA isolated was subjected to agarose gel [1.5 % gel] electrophoresis using TBE buffer[Tris Borate EDTA buffer [0.5 M pH 8.0] containing ethidium bromide. RNA sample in 4 μl loading buffer was applied to the gel and electrophoresis was carried out at 80 V for 1hour. The gel was viewed under a UV illuminator and imaged the gel under a Gel Doc system (BioRad).

3.2.4.13. cDNA preparation for RT-PCR analysis

Conditions used for cDNA preparation:

cDNA Synthesis - Each RNA sample was reverse transcribed to cDNA with MMLV reverse transcriptase using Oligo dT. Briefly, 3 μl oligo dT was added to 2 μg RNA and incubated for 50 min at 70^0C. The sample was then transferred to a reaction mixture containing dNTP (200mM), RNasin (0.7 units), MMLV-RT, and RT buffer and incubated at 37^0C for an hour and subsequently at 90^0C for 5 min. The prepared

cDNA was stored at -20^{0}C. Human-specific primers for the genes were designed by NCBI prime blast. The sequences of oligonucleotide primers used were:

1. LXR alpha	Forward primer-	5' TGCGTCGCAAGTGCCAGGAG 3'
	Reverse primer	5' TGTTGCTGGGCAGCGACGAG 3'
2. ABCG1	Forward primer	5'ACCAGCCCAGCGCCAAACTC3'
	Reverse primer	5' GCGAGGTGATGCGCAGGTG3'
3. CD36	Forward primer'	5'CTGGCAACAAACCACACACTGGA3'
	Reverse primer	5'TGACAGCCCCAGCGATGAGC 3'
4. GAPDH	Forward primer	5' GTCGCCAGCCGAGCCACATC3'
	Reverse primer	5' CGCCACAGTTTCCCGGAGGG 3'

Reverse trascriptase PCR was performed using standard conditions. PCR amplification was performed in a total volume of 20 µl containing of the cDNA as template. The mixture was incubated for 2 min at 50^{0}C and 10 min at 95^{0}C and then subjected to thermal cycles, each involving denaturation at 95^{0}C for 15 sec and annealing/extension at 60^{0}C for 1 min.

3.2.4.14. NADPH oxidase activity measurement in monocytes-macrophages

The superoxide generating activity of oxHDL was determined by nitro blue tetrazolium (NBT) assay, which detects reduction of NBT to formazan by superoxide. The assay was performed based on the method of (Hua et al. 2000) with a slight

modification. The cells were treated with oxHDL at 100μg/ml for 24 hours. One set of cells were pre-treated with DPI (10μM) for 1 hour before oxHDL treatment. After treatment cells were incubated with 0.04% NBT at 37°C for 30 min and then formazan-containing cells were monitored. The percentage of NBT positive cells was calculated. Photographs were taken using microscope [Olympus IX70 inverted-microscope, Barcelona, Spain] at 40 X magnification.

3.3. Statistics

Statistical analysis was carried out by GraphPad prism statistical software. One-way / two way ANOVA was employed to assess the variation among groups in the form of means. Difference between the means was assessed with the students t test. The correlation coefficient was also used to strength of any association between different variables. Value of 'p' less than 0.05 was considered statistically significant.

RESULTS

4.1. Identification of the prevalence of functionally altered HDL in subjects

Fasting blood samples were collected from apparently healthy volunteers (total sample size ~72) from the hospital staff for isolation of HDL fractions for studying its functional properties. The basic characteristics of the subjects studied were provided in Table I.

Table I. Basic characteristics of study subjects

Sample size	72
Sex (Males/Females)	23/49
Age	40 ± 16
BMI (Wt in Kg/ Ht in m^2)	24 ± 4
Total cholesterol (mg/dl)	198 ± 35
Triglycerides (mg/dl)	124 ± 61
HDL-C (mg/dl)	43 ± 8
LDL-C	130 ± 34
Glucose (mg/dl)	93 ± 20

Among these subjects, seven were found to have metabolic syndrome and they have more than three of the following risk factors for heart disease, such as dyslipidemia [high cholesterol and/or triglycerides and low HDL-cholesterol]; high blood pressure, and obesity. The remaining 65 volunteers have normal lipid profile and no risk factors such as obesity, hypertension and diabetes mellitus, for CHD.

4.1.1. Isolation of HDL and LDL

In order to study the functionality, HDL and LDL fractions were isolated from blood samples collected from the volunteers by density gradient ultracentrifugation. The isolated lipoproteins were dialyzed against PBS at 4 ^{o}C and the purity was checked by polyacrylamide disc gel electrophoresis (3.75%)

4.1.2. Purity of isolated lipoprotein

The purity of isolated lipoproteins was checked with PAGE and dot blot analysis and the results were given in Fig. 1. HDL fractions isolated by ultracentrifugation resolved as a single band in PAGE and confirmed the absence of other lipoproteins, i.e. LDL and VLDL. In dot blot analysis, antibodies against apoA1,and apoB were used to detect the presence of HDL and or for the presence of LDL [as contaminant]. HDL fraction showed the presence of apoA1only. Both the dot blot assay and PAGE of isolated HDL confirmed its purity.

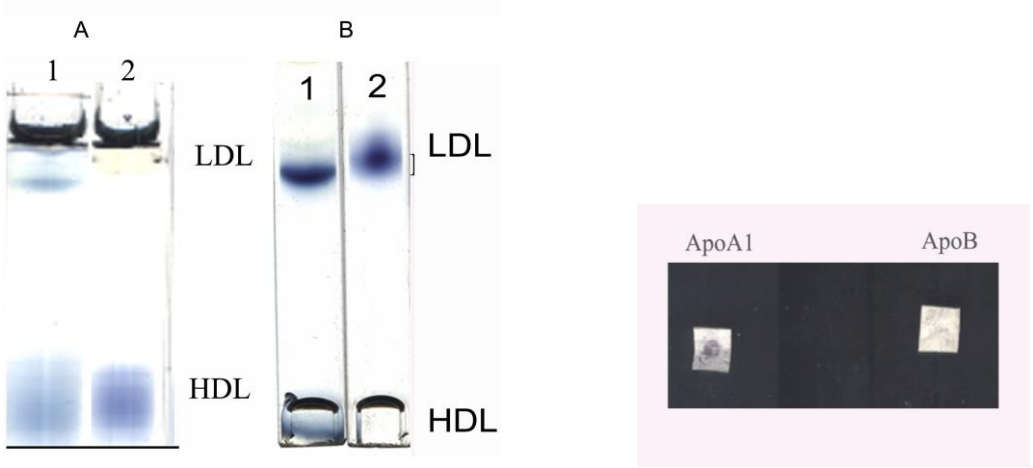


Figure 1. *Disc gel electrophoretic pattern of serum lipoproteins on 3.75% polyacrylamide gels. Panel A) lane 1- serum lane 2) ultracentrifugally isolated HDL fraction. panel B) lane 1 –Serum lane 2- ultracentrifugally isolated LDL fraction. Panel C) Dot blot immuno assay of isolated HDL. Dot blot assay was performed using antibodies against ApoA1 and ApoB.*

4.1.3. Functional property of HDL

HDL is a potential antiatherogenic agent as it can inhibit LDL oxidation. Hence the functionality of isolated HDL particle was assessed by its antioxidant ability to inhibit LDL oxidation. To achieve this the oxidation kinetics of LDL was first carried out.

4.1.3.1. Oxidation kinetics of Low Density Lipoprotein

The oxidation kinetics of LDL was first carried out using different copper concentration and the results were given in **Fig 2A & 2.B.** Time course generation of conjugated dienes (CD) from LDL with different concentrations (0.5 to 5 μm) of copper sulphate was assayed. At a copper concentration of 5μM , LDL oxidation initiated after a lag period of ~ one hour showed a propagation phase and produced maximum content of CD at 3.0 hr compared to other lower copper concentrations. Whereas at lower copper concentrations the corresponding lag periods before the initiation of LDL oxidation were found to be longer, more than one hour, and CD productions at 3.0 hr were comparatively lower[as lower absorbance] than that of higher copper concentrations. So for further LDL oxidation studies copper at a concentration of 5μM was used.

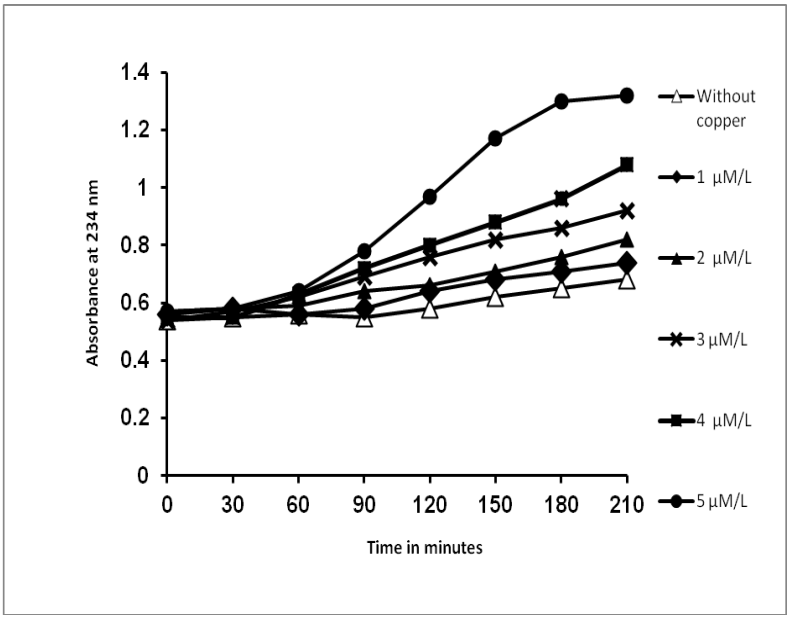


Figure 2 A. *Time course generation of conjugated dienes (CD) from LDL with different concentration of copper. LDL [0.1mg protein/ml] was incubated with copper sulphate [0.5µM/L to 5µM/L] at 37ºC for the indicated time period. CD was measured by an increase in absorbance at 234nm for every 30 min. Data are the mean ± SD of 6 independent experiments.*

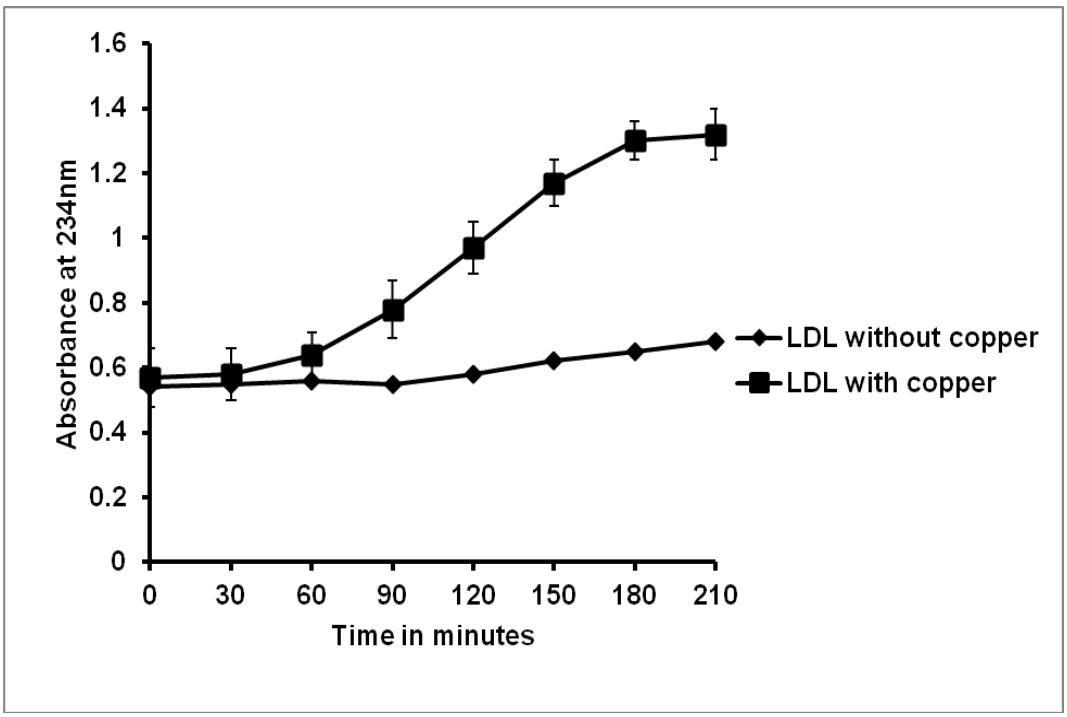


Figure 2 B. *LDL oxidation kinetics with* 5μM/L *copper sulphate. LDL [0.1mg protein/ml] was incubated with* 5μM *copper sulphate at 37ºC for the indicated time period. CD was measured by an increase in absorbance at 234nm for every 30 min. Data are the mean ± SD of 6 independent experiments.*

Fig.2. denotes a typical LDL oxidation curve with 5μM copper sulphate. Copper mediated LDL oxidation begins first with a lag phase, which indicates the resistance capacity of LDL to initiate oxidation due to the presence of antioxidants in LDL. The second phase of oxidation is the propagation phase that results in maximum CD formation. Third phase of oxidation represents a saturation point in LDL oxidation after that no further increase in CD production was observed. The increase in conjugated diene during the propagation phase was reported to be mainly due to the formation of cholesteryl linoleate hydroperoxides (Lass et al. 1996).

4.1.3.2.*The antioxidant potential of HDL*

4.1.3.2.1. Effect of Copper on HDL oxidation

HDL is known for its antioxidant property. But like LDL, it can undergo oxidative modification. The susceptibility of HDL particle to in- vitro oxidation with copper sulphate was assessed using copper at varying concentrations from 0.5 micro molar to 10 micro molar copper concentration. Fig.3 indicated the oxidation potential of HDL. It was observed that unlike LDL, HDL was able to resist oxidation induced by copper sulphate even at a higher concentration of 10μM/L for more than 3 hrs.

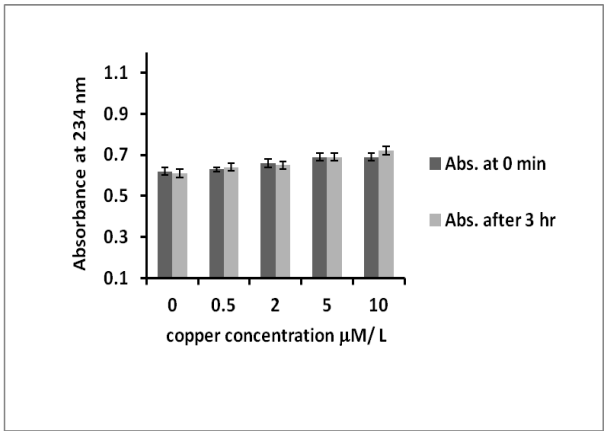


Figure 3. *Effect of different concentration of copper on HDL oxidation. HDL at a protein concentration of 0.1mg/ml was incubated with copper sulphate at different concentrations [0.5μM/L to 10μM/L] at 37ºC for 3hr. CD formation was measured as increase in absorbance at 234nm. Data are the mean ± SD of 6 independent experiments.*

4.1.3.2.2. Effect of HDL concentration on LDL oxidation

Varying concentrations of HDL isolated from healthy subjects [having normal lipid profile] were co-incubated with LDL [0.1mg/ml] and copper sulphate [5μM/L] to monitor their antioxidant potential. HDL exhibited significant antioxidant

property and inhibited LDL oxidation in a dose-dependent manner. As evident from Fig.4, HDL at equal protein concentration with LDL [1:1] or at higher HDL concentration showed maximum inhibitory capacity against LDL oxidation. For the rest of studies, HDL and LDL at equal protein concentrations were used to assess the antioxidative capacity of HDL .

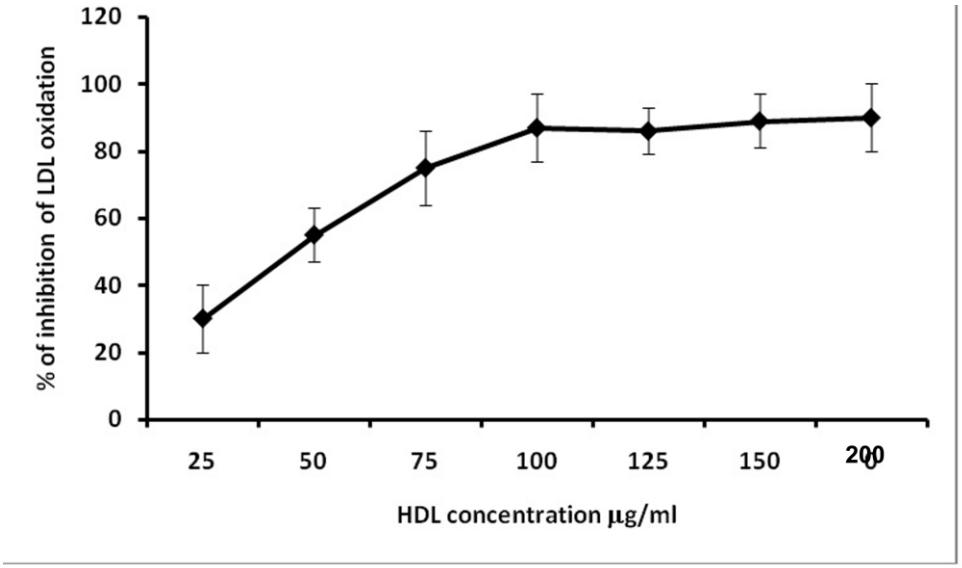


Figure 4. *Effect of different concentration of HDL on LDL oxidation. LDL at a protein concentration of 0.1mg/ml was incubated with HDL at different concentrations from 25 µg/ml to 200 µg/ml with 5µ M/L copper sulphate at 37ºC for 3 hr. CD formation was measured as an increase in absorbance at 234nm and expressed as % inhibition of LDL oxidation by HDL. Data are the mean ± SD of 6 independent experiments.*

The protective antioxidant effect of HDL against LDL oxidation induced by copper was shown in Fig.5. Oxidation of LDL gave rise to typical conjugated diene vs. time –curve. However, co-incubation of LDL with HDL at equal protein concentration effectively resisted LDL oxidation up to 3 hrs. This result clearly demonstrated the antioxidant potential of human HDL.

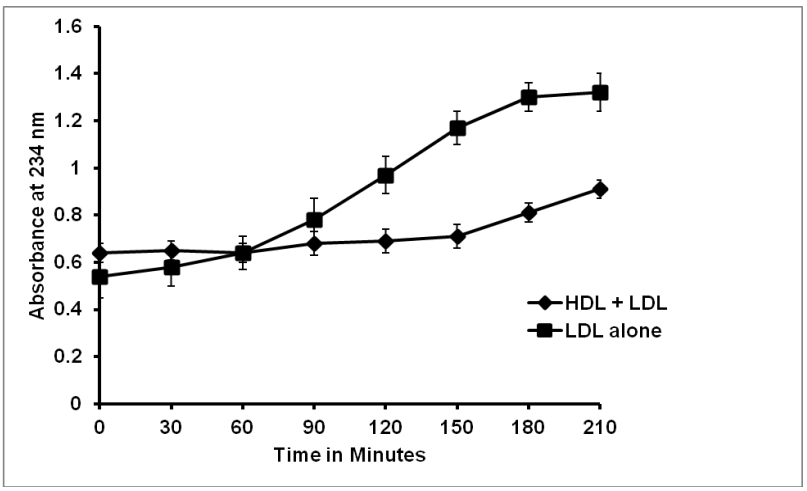


Figure 5. *The antioxidant effect of HDL on LDL oxidation. LDL [0.1mg protein/ml] alone or with HDL [0.1mg protein/ml] was incubated with 5μM copper sulphate and the kinetics of oxidation was measured as increase in absorbance due to CD formation at 234nm for 3 hrs. Data are the mean ± SD of 6 independent experiments.*

4.1.4. Antioxidative capacity of HDL in healthy subjects and in subjects with metabolic syndrome

The functional property of HDL particles, isolated from 72 subjects, were studied using the in vitro assay as mentioned above, where the antioxidant capacity of HDL to inhibit LDL oxidation was measured. The antioxidant capacity [as % inhibition of LDL oxidation] of HDL was found to be varied from person to person. HDL from majority of healthy volunteers exhibited remarkable antioxidant property [mean% inhibition of LDL oxidation: 77±15 (group1). However, HDL showed decreased antioxidant property (less than 50% inhibition of LDL oxidation) in few healthy subjects [n=8, mean% inhibition of LDL oxidation : 38± 7 (group II); group I vs II : $p < 0.01$] as well as in those subjects with metabolic syndrome (MetS) [n=7, mean % inhibition of LDL oxidation : 33 ± 10 (group III); group I vs III, $p < 0.01$]. These subjects were grouped into different groups

according to their HDL's antioxidant ability, as group I with HDL having more than 50% antioxidant capacity to inhibit LDL oxidation and group II & III [subjects with MetS] with HDL showing less than 50% inhibition of LDL oxidation; and different parameters were analyzed in blood samples for comparison.

4.1.5. Lipid profile, oxidative stress and inflammation status

Lipid profile, activity of paraoxonase-1 [an antioxidant enzyme associated with HDL], oxidative stress markers [protein carbonyls and lipid peroxides], inflammation marker [hsCRP] were analyzed in these three groups of subjects - group I with HDL having more than 50% antioxidant capacity; group II & III with HDL having less than 50% antioxidant capacity; and presented in Table II. Lipid profile was found to be normal in Group I subjects with HDL showing marked antioxidant property. When compared to group I, HDL in group II & III [MetS] subjects exhibited inadequate antioxidant capacity to inhibit LDL oxidation. Subjects in group II and III showed lower mean levels of HDL-C compared to group I. Group III subjects with MetS were found to have dyslipidemia as well as higher BMI and lower activity of paraoxonase-1 compared to group I & II. In addition both group II & III subjects with less antioxidant property of HDL showed higher oxidative stress and inflammation as evidenced by higher concentration of serum lipid peroxides, protein carbonyls and hsCRP. The lacks of adequate antioxidant function of HDL in these subjects were found associated with excess systemic oxidative stress and inflammation. However, HDL's antioxidant property did not show any correlation to the level of HDL-C [r.

0.486759]. Neither LDL-C nor TG was correlated with HDL dysfunction in this study.

Table II, Lipid profile and markers of oxidative stress and inflammation in subjects having different antioxidant property of HDL

	HDL's antioxidant capacity		
	>50%	**<50%**	**<50%**
	Group1	**Group II**	**Group III [MetS]**
Sex (Males/Females)	16/41	3/5	4/3
Age	37± 13	38±11	43±8
BMI (Wt in Kg/ Ht in m^2)	23 ± 4	23± 2	27± 3
Total cholesterol (mg/dl)	181± 29	193± 21	220± 26
TG(mg/dl)	91 ± 50	128± 65*	153± 40 *
LDL-cholesterol (mg/dl)	115± 22	127± 21	149± 28
HDL-cholesterol (mg/dl)	48 ± 10	40± 9*	40± 6*
Glucose (mg/dl)	85 ± 12	92± 8	106± 36
Paraoxonase –1(kU/L)	119 ± 26	131 ± 37	91±18
hsCRP (mg/L)	15 ± 3	45 ± 9 *	40±10*
Protein carbonyls (nM/ml serum)	11 ± 1.8	16 ±2.9 *	15 ±1.5 *
Lipid peroxides (μM/L)	1.8 ± 0.6	6 ± 1.6*	6±2.2*

Values are the mean ± SD; * p <0.05 group 1 vs. group II or group III

4.2. Invitro induction of dysfunctionality in HDL using an oxidative system and its functional characterization

HDL particle can undergo structural alterations during conditions like acute phase response and oxidative stress and transform into functionally altered form. Although dysfunctional HDL has been implicated in the pathogenesis of atherosclerosis, the underlying pathways remain poorly understood. It is proposed that ROS, myeloperoxidases, and metal ions can induce dysfunctionality in HDL by oxidative modifications. Being a heterogeneous particle, oxidative modification of its components may affect its function. However, the pro-atherogenic pathways undergone by oxidized HDL remain poorly understood.

Local monocyte activation inside the atherosclerotic plaque acts as a focus for foam cell formation and inflammation as it involves increased secretion of inflammatory cytokines and metalloproteinases together with the production of ROS. To stimulate the efflux of cholesterol from macrophage- foam cells as well as to initiate other antiatherogenic functions in endothelium, HDL should make contact with these cells. Since monocyte plays a crucial role in atherogenesis the current study was aimed to investigate the influence of both native and oxHDL on monocytes-macrophages functions relevant to atherogenesis. Most of the previous studies were centered on the defective role of HDL in reverse cholesterol transport. However HDL has several other important cardioprotective functions including antiinflammatory/antioxidant response that need to be explored in great detail. The present study was therefore to investigate the influence of in vitro oxidized HDL on

pro-inflammatory response and ROS generation in monocytes-macrophages, the key cells involved in atherogenesis.

4.2.1. Oxidative modification of HDL

Oxidative modification in HDL was induced by incubating HDL with $CuSO_4$ at two different concentrations (a) 5μM for 12 hours at 37^0C (mild oxidative condition -m.oxHDL) and (b) 20 μM for 24 to 36 hours at 37^0C (a strong oxidative condition-oxHDL. The extent of HDL oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehydes. The MDA concentrations were <1.5 nM/mg protein for native HDL (nHDL), 3-5 nM/mg protein for HDL oxidized under mild oxidation condition and 60 -80 nM/mg protein for HDL oxidized with 20μM $CuSO_4$.

4.2.2 Investigation of the effect of oxHDL on monocytes-macrophages function relevant to atherosclerosis

4.2.2.1. Human PBMC culture

Human blood monocytes were isolated and cultured under standard conditions. Cells were maintained in serum-free RPMI medium for 24 hrs. The morphology of the cells was monitored under microscope.

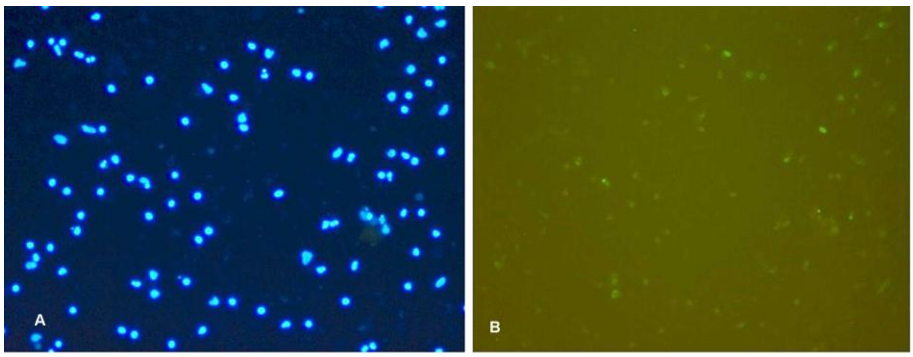


Figure 6*. The CD14+ cells of isolated PBMC. PBMC cells (1x10 ^{6}) were stained with FITC labeled CD14 marker and fluorescent image was taken with Hoechst as conter stain (blue and green filter). A- Hoechst staining of cells. B- corresponding FITC image showing CD14+ cells.*

Cell viability, assessed by Trypan blue exclusion test, was found to be greater than 95%.

4.2.2.2. OxHDL induces ROS production in monocytes-macrophages

To study whether oxHDL induced any oxidative stress, monocytes–macrophages were treated with native (nHDL), m.oxHDL and oxHDL at different concentrations (10–200 μg protein/ml) and the ROS generated were quantitated with DCFH fluorescence. The results (Fig.7) showed that oxHDL treatment induced more ROS production in monocytes–macrophages than that of m.oxHDL and native HDL in a concentration-dependent manner and was found maximum at 200 μg/ml ($p<0.01$]. Significant increase in ROS production was observed with oxHDL at concentration of 50 μg/ml, whereas a similar increase in ROS formation was observed with m.oxHDL only at a concentration of 200 μg/ml. Native HDL did not cause any rise in the ROS.

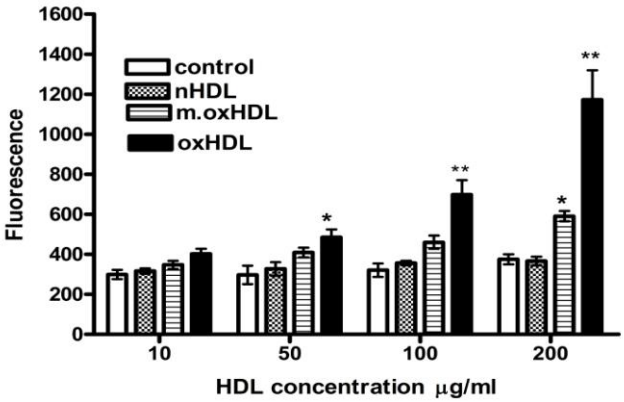


Figure 7. *Effects of oxHDL on ROS in mo-Mø. Cells (2x10^4/ml) were cultured under standard condition and treated with native HDL, mildly oxHDL, or oxHDL at varying concentrations 10, 50, 100, and 200 μg/ml medium for 24 hrs. Untreated cells were kept as control and the levels of reactive oxygen species in cells were measured as ROS-mediated DCFH fluorescence. Data are the mean ± SD of three independent experiments, each well represent triplicate **p <0.01, *p< 0.05 native HDL versus mildly oxHDL or native HDL versus oxHDL.*

Additional experiments were carried out to find out the time-dependent changes in ROS formation induced by oxHDL in monocytes-macrophages. A significant increase in ROS production was observed from 4 hrs and showed a steady increase as time increases (Fig.8).

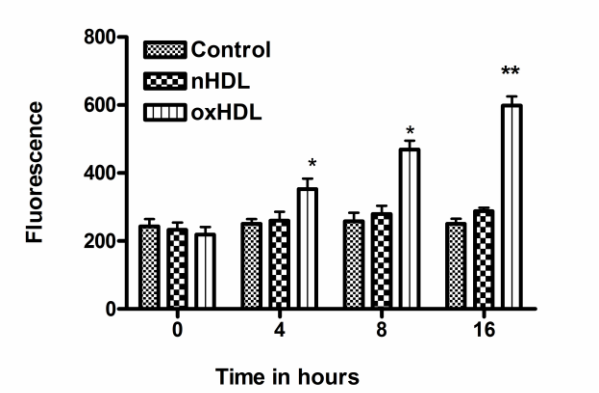


Figure 8. *Time- dependent formation of ROS by oxHDL. Cells (2x10^4 cells/well) were incubated with oxHDL, or native HDL at 50 μg/ml protein concentration for 4, 8 and 16 hrs. Uutreated cells were kept as control. Intracellular ROS formation was measured as DCFH fluorescence. Data are mean ± SD of three independent experiment each well represent triplicate. p value *<0.05,**<0.01 oxHDL vs native HDL*

Next experiments were carried out to compare the effect of oxHDL with oxLDL. LDL (1 mg/ml) was oxidized with 5 µM copper sulphate for 24 hrs and the extent of LDL oxidation was measured as TBARS (MDA: 60-80 nm/mg protein). It was evident that oxLDL induced more ROS than oxHDL at equal protein concentrations [Fig.9], indicating that oxLDL is more powerful in inducing ROS than oxHDL.

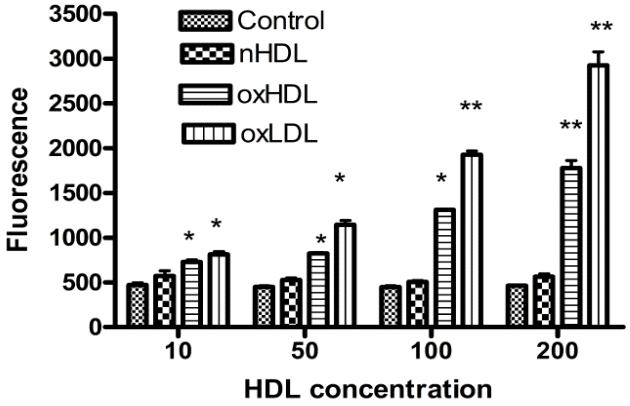


***Figure 9**. Comparative effect of oxHDL and oxLDL in ROS production in mo-Mø. Cells (2x10^4/ml) were cultured under standard condition and treated with native HDL, oxHDL or oxLDL at varying concentrations 10, 50, 100, and 200 µg/ml medium for 24 hrs. Untreated cells were kept as control and the levels of reactive oxygen species in cells were measured as ROS- mediated DCFH fluorescence. Data are the mean ± SD of three independent experiments each well represent triplicate ** p<0.01, *p<0.05 natHDL vs oxHDL or natHDL vs. oxLDL*

4.2. 2.3. oxHDL induces release of MMP-9 and MMP-2 in monocytes–macrophages

Under oxidative stress or pro-inflammatory stimuli, monocytes–macrophages get activated. The production of metalloproteinases is a marker of monocytes–macrophages inflammatory response and activation or differentiation. Monocytes were kept under standard culture medium for up to 4 days. Gelatinolytic activity was

measured in the culture medium every 24 hr [Fig.10]. MMP-9 activity was found to be increased as the days of culture progressed indicating their basic characteristics.

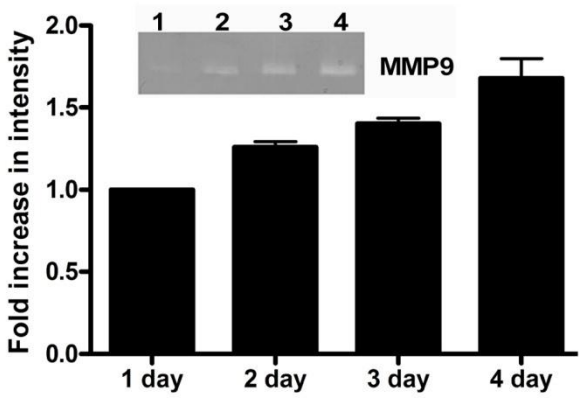


Figure 10. *MMP-9 activity in cultured mo-Mø. 1 x 10^6 /ml of cells were cultured under standard conditions and MMP-9 activity was assayed in the medium up to 4 days by gelatine zymography on 7.5 % gel. Gelatinolytic activity of MMP- 9 appeared as transparent bands on a blue background. The image was digitally captured and the bands were quantified by densitometry using Adobe Photoshop and a histogram analysis program. Results are expressed as means $\pm$ SD[relative intensity to day 1] three independent experiments. Inset shows zymogram of a representative experiment where lane 1,2,3,4 indicates MMP-9 released during the days 1,2,3,4 of monocyte culture.*

To study whether oxHDL has any influence on MMP production in monocytes–macrophages, the cells were treated with native HDL, m.oxHDL, and oxHDL at different concentrations (50, 100, and 200 μg/ml) for 24 hrs. The MMPs released in cell culture supernatant were assessed by gelatin zymography. Gelatinolytic activity was observed as clear zones corresponding to 92 kDa for MMP9 (Fig.11 A) and 72 kDa for MMP2 (Fig.11 B) in cells treated with oxHDL compared to that of native HDL. Gelatinolytic activity of MMP2 was observed after 24 hrs of culture in oxHDL treated cells (Fig.11 B). Cells exposed with native HDL or

m.oxHDL showed only basal level expression of MMP9 at 24 hr treatment (Fig.11C).

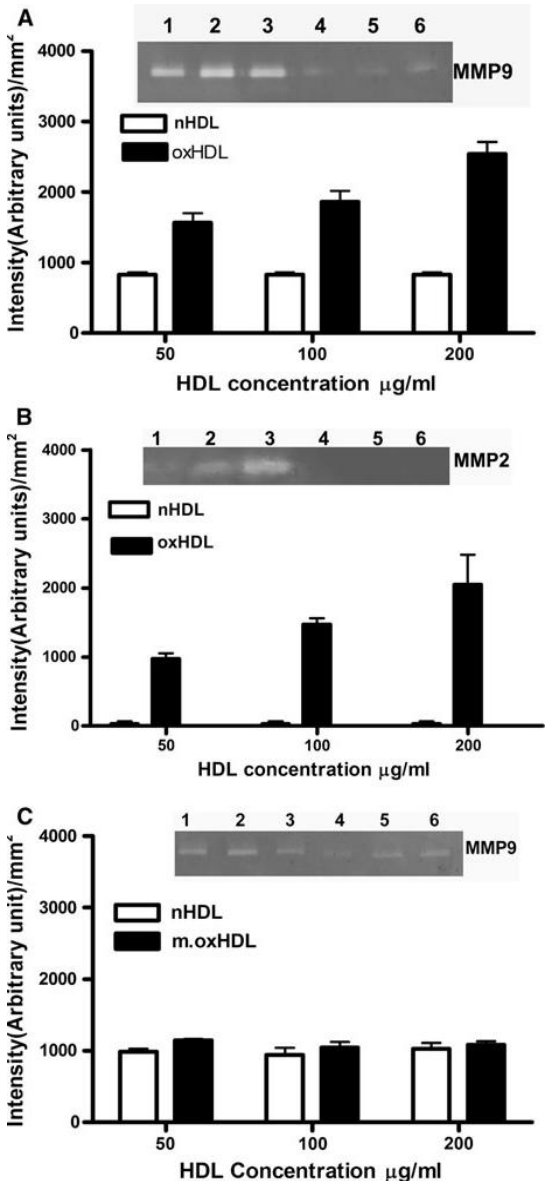


Figure 11. *Effect of oxHDL on MMP production in mo-Mø. 1 x 10^6/ml of cells were cultured under standard condition and treated with different concentrations of oxHDL, mildly oxHDL and native HDL for 24 hrs. MMPs secreted into the medium were assayed by gelatine zymography in 7.5 % gel. Gelatinolytic activity of 92 kDa MMP-9 and (Fig.11.A) and 72 kDa MMP-2 (Fig.11.B) bands were quantified by Quantity one programme (BioRad) and expressed as arbitrary units of intensity/mm^2 in corresponding graphs. Results are expressed as mean ± SD of three independent experiments. Inset in A and B shows zymogram of a representative experiment where lanes 1–3 in each figure indicates gelatinolytic activity of cells treated with oxHDL at 50, 100, 200 μg/ ml concentration and lane 4–6 represent gelatinolytic activity of*

cells treated with native HDL at 50, 100, 200 μg/ml. Fig.11C- inset shows zymogram of cells treated with mildly oxidized HDL, where lane 1–3 represent cells treated with m.oxHDL and lane 4–6 represent cells treated with native HDL and corresponding intensity in graph.

Additional experiment showed that oxHDL induced MMP-9 activity in a time-dependent manner [Fig.12.] in monocytes-macrophages.

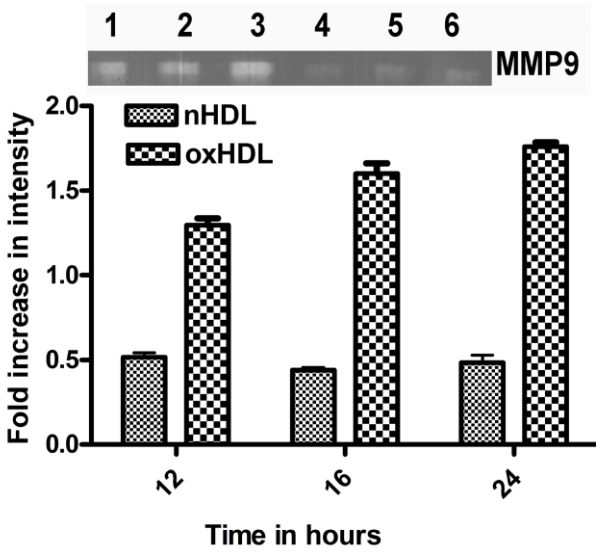


Figure 12. *oxHDL induced MMP9 in a time dependent manner in mo-Mø. 1 x 10^6 /ml of cells were cultured under standard condition and treated with oxHDL or native HDL (100μg/ml protein) for different time period as indicated. MMP-9 secreted into the medium was assayed by gelatin zymography on 7.5 % gel. Gelatinolytic activity of MMP- 9 was quantified by densitometry. Inset shows zymogram of a representative experiment where the zymogram shows the gelatinolytic activity of cells treated with oxHDL(lane 1-3) or native HDL (lane 4-6) for 12 hrs, 16 hrs, 24 hrs*. Correspondong intensity *are expressed as means ± SD in graph [relative intensity to 12 hrs] of three independent experiments.*

Further experiments were carried out to compare the effect of oxHDL on MMP 9 activity with that of oxLDL (Fig.13). The result showed that both oxHDL and LDL induced almost similar MMP activity.

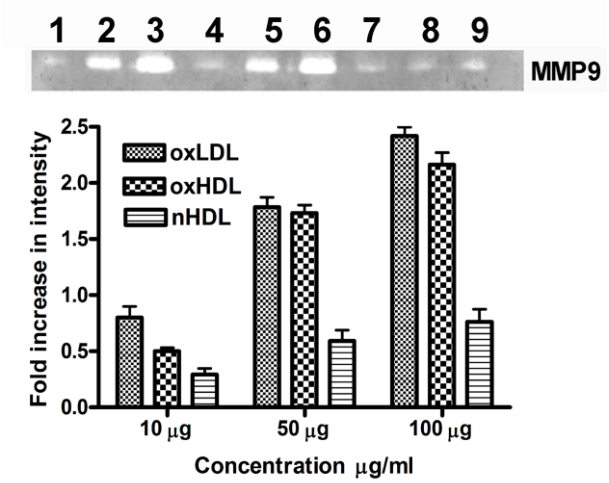


Figure 13. *Comparative effect of oxHDL and oxLDL in inducing MMP-9 activity in mo-Mø. Cells (1x10^6 cells/ml) were incubated with oxLDL (Lane 1,2,3) oxHDL (Lane 4,5,6) and natHDL (lane7,8,9) at 10,50,100μg/ml concentration for 24 hrs and the activity of MMP-9 was assessed by gelatin zymography. Gelatinolytic activity of MMP- 9 was quantified by densitometry as described above and expressed as intensity in graph. Inset shows zymogram of a representative experiment. Data are the mean± SD of three independent experiments .*

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4.2.2.4. OxHDL induces TNF-alpha production in monocytes–macrophages

Monocytes–macrophages were treated with oxHDL to see its influence on TNF-alpha production - a marker of monocyte- macrophage inflammatory response. Cells were cultured under standard conditions and treated with native HDL, m.oxHDL, and oxHDL for 24 hrs at a minimum concentration of 50 μg/ml that produces significant release of ROS in monocytes– macrophages. The release of TNF-alpha was quantitated in culture supernatant by ELISA method. The result showed that oxHDL treatment induced significant production of TNF-alpha ($p<0.01$) in monocytes–macrophages, whereas treatment of cells with m.oxHDL and native HDL showed no such effect on TNF-alpha production (Fig.14).

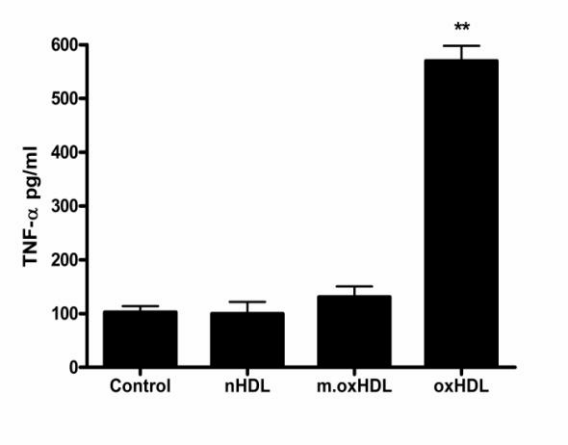


Figure 14. *Effect of oxHDL on TNF-alpha production in mo-Mø. Cells (1 x 10^6 cells/ml) were cultured under standard condition and treated with native HDL, mildly oxHDL, or oxHDL at a concentration of 50 μg/ml medium for 24 hrs. Cells treated with PBS alone were kept as control. The release of TNF-alpha in culture supernatant was measured by ELISA method. Data are the mean ± SD of three independent experiments, each well represents duplicate. **p<0.01native HDL versus oxHDL.*

4.2.2.5. Oxidized HDL induces cell death in monocytes-macrophages

To examine whether oxHDL induces cell death, monocytes-macrophages after treatment with oxHDL (100 μg/ml) for 24 and 48 hrs were subjected to propidium iodide staining. oxHDL did not induce cell death at a significant level compared to native HDL at 24 hrs of treatment. However, treatment of cells with oxHDL for 48 hours induced significant cell death [p<0.05[about 28% cell death] indicating the cytotoxic effect of oxHDL(Fig.15).

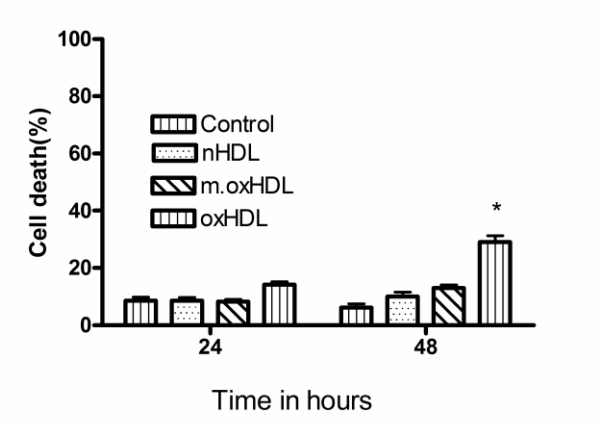


Figure 15. *Cytotoxic effect of oxHDL at 24 and 48 hrs in mo-Mø. Cells (1x10^6 Cells/ml) were incubated with 100 μ/ml oxHDL, m.oxHDL or native HDL for 24 hrs and 48 hrs and stained with propidium iodide (stains dead cells) and cell-permeable Hoechst 33342(stains all cells) at a final concentration of 1μg /ml and 2.5 μg/ml respectively.Untreated cells were kept as control. Micrographs of three to six random fields per dish were obtained in an Olympus IX70 inverted-microscope (Barcelona, Spain) with the appropriate filter sets for PI (green) and Hoechst 33342(UV). PI- and Hoechst 33342-stained nuclei were counted on micrographs manually and the percentage of dead cells was calculated by dividing the number of PI positive nuclei by the total number of nuclei (Hoechst positive) for each micrograph. The values are the representative results of three independent experiments performed. 'P' *<0.05 native HDL vs. oxHDL, native HDL vs. m.oxHDL.*

In addition, experiments showed that oxHDL induced cell death was found to be concentration-dependent and the cytotoxic effects of HDL was significantly less than that of oxLDL (Fig .16]

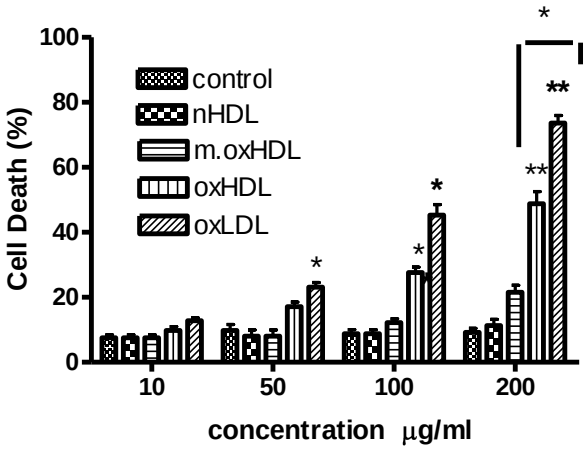
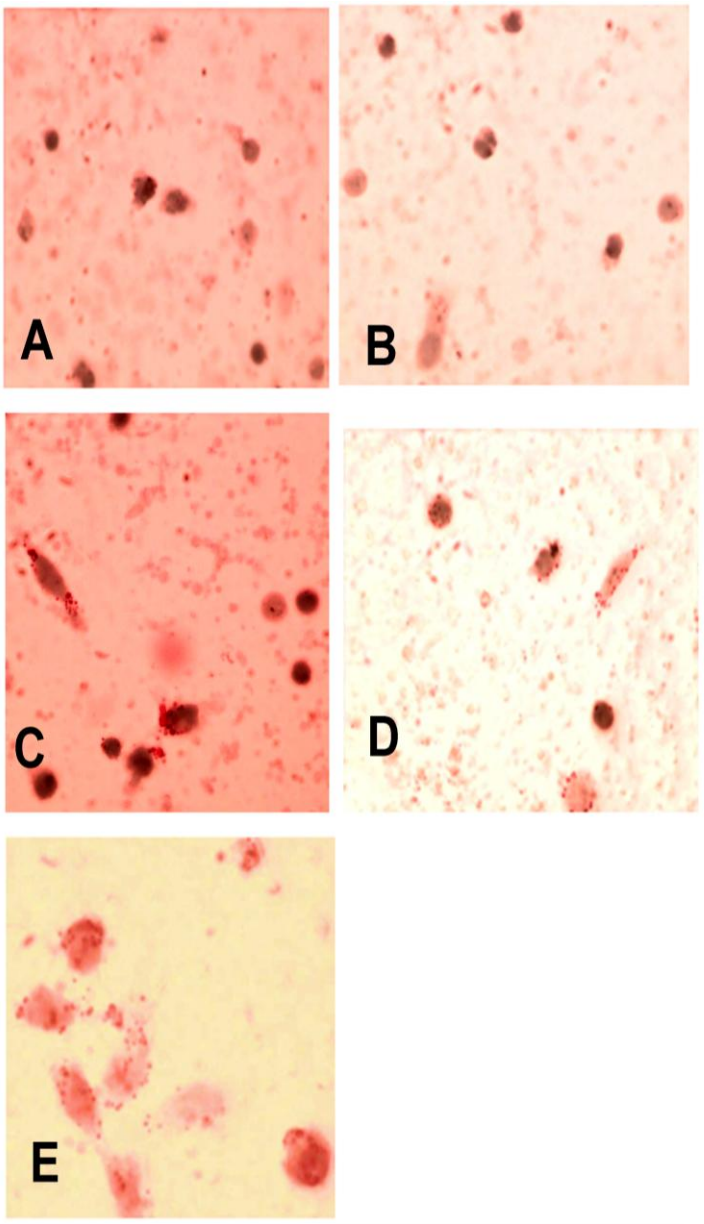


Figure 16. *Cytotoxic effect of oxHDL in a concentration dependent manner. Cells 1x10^6 Cells/ml) were incubated with 10,50,100,200 μg/ml native HDL, oxHDL, m.oxHDL, oxLDL for 48 hrs and stained with propidium iodide and cell-permeable*

*Hoechst 33342 as described above. Untreated cells were kept as control. Micrographs were obtained with appropriate filters. Percentage of cell death was calculated manually as number of PI positive cells and negative cells. The values are the representative results of three independent experiments performed. P **<0.01, * P<0,05 native HDL vs. oxHDL. oxHDL vs.oxLDL.*

4.2.2.6. Oxidized HDL promotes neutral lipid accumulation in monocytes-macrophages

Formation of foam cells is a crucial event in the atherogenic mechanisms. In the activated state monocytes-macrophages engulf modified lipids through scavenger receptors and lead to accumulation of lipids in cells, foam cells. In this study we examined the role of oxHDL on lipid accumulation in monocytes-macrophages. Cells were cultured for two days and were exposed to native HDL, m.oxHDL or oxHDL [100 μg/ml] for 24 hours. The cells were stained with Oil Red O. It was observed that oxHDL treatment induced more accumulation of lipids in the cells ($p < 0.05$) compared to that of native HDL or m.oxHDL (Fig.17) suggesting its positive influence on cell activation and lipid uptake. Experiments were also carried out with oxidized LDL [TBARS content 60-80nm/mg protein] under same condition. When compared to oxHDL, lipid accumulation was found to be remarkably higher with oxLDL.


A
B
C
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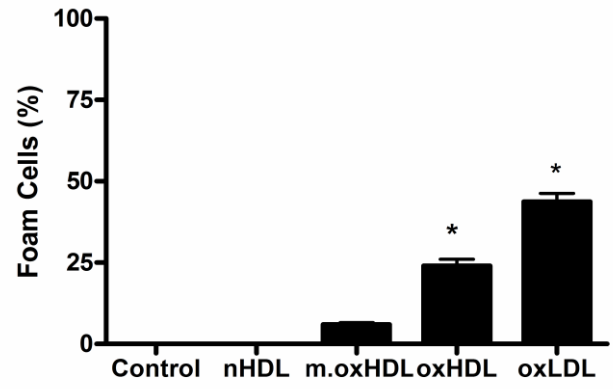
Foam Cells (%)
100
75
50
25
0
*
*
Control
nHDL
m.oxHDL
oxHDL
oxLDL

Figure 17. *Effect of oxHDL on intracellular lipid accumulation in mo-Mø. 1x10^6 cells/ml were cultured for two days and incubated with 100 μg/ml each of native HDL, mildly oxHDL, oxHDL and oxLDL for 24 hrs. Cells treated with PBS alone were kept as control. Inset: Oil Red O staining for lipid accumulation. Cells with more than 6 Oil Red droplets were counted and presented as percentage of lipid-accumulated cells. The values are mean ± SD of three independent experiments. *p<0.05 oxHDL vs native HDL or oxHDL vs. oxLDL.*

4.2.2.7. Influence of oxHDL on genes responsible for lipid homeostasis

Macrophages have important roles in lipid homeostasis and are central to the pathogenesis of atherosclerosis. Liver X receptors (LXRs) are key transcriptional regulators of genes involved in lipid homeostasis and inflammation and are determinants of atherosclerosis susceptibility. ATP-binding cassette transporters (ABC) – ABCA1 and ABCG1 are membrane cholesterol transporters and have been implicated to mediate cholesterol efflux from cells to apolipoprotein A-I, the major protein constituent of HDL and mature HDL respectively. LXR is the major regulator of ABCA1, ABCG1, and apoE. Scavenger receptor CD36 also play an important role in lipid accumulation. We have demonstrated that oxHDL induced intracellular lipid accumulation, oxidative stress and inflammatory response in monocytes-macrophages. Since LXR signaling is critical for lipid homeostasis, we next examined the expression of LXR, ABCG1 and CD36 in monocytes-macrophages in response to oxHDL treatment.

4.2.2.7.1. oxHDL induces expression of LXR, ABCG1 and suppresses CD36 gene in monocytes-macrophages

Cells were treated with oxHDL at 100μg/ml for 24 hrs. Total RNA was isolated from cells using Trizol reagent and the isolated RNA was quantitated

spectrophotometrically at 260 nm. The quality of RNA preparation was assessed by electrophoresis on 1.5% agarose gel [Fig.18].

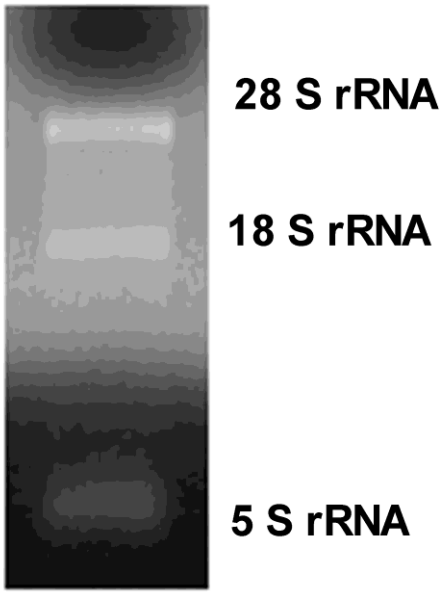

Figure 18. *Agarose gel electrophoresis of RNA isolated from mo-Mø. Total RNA was isolated from 1x10^7 cells/ml using Trizol reagent and the isolated RNA was subjected to agarose gel[1.5 %] electrophoresis for 1 hrs at 80 volts. The gels were viewed in a Gel-Doc system and documented. The figure illustrates a representative agarose gel of isolated RNA.*

About 2 μg RNA was used to prepare cDNA using oligo DT primer. The gene expression level of LXR,ABCG1,CD36 were checked by reverse transcriptase- PCR. PCR amplification was carried out using appropriate primers. GAPDH was used as an internal control. The PCR products were visualized by electrophoresis on 1.5% agarose gels. Gels were documented in a gel documentation system. The results [Fig.19] showed that oxHDL treatment enhanced the expression level of both LXR, and ABCG1, while the expression of CD36 was down regulated. In cells exposed to native HDL, the expression level of CD 36 remains unchanged.

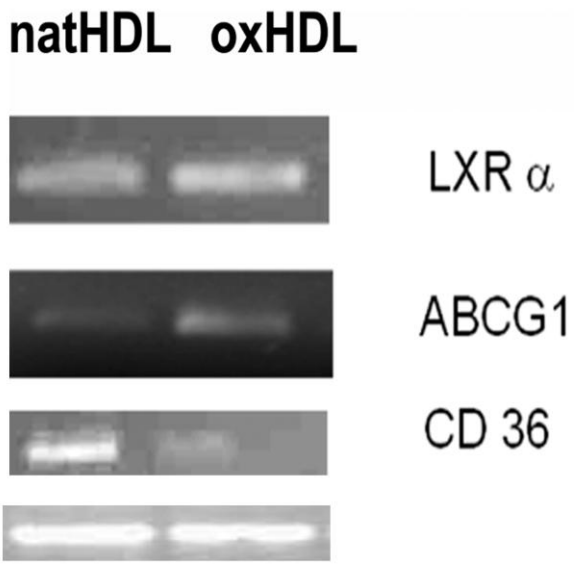

Figure 19. *Induction of LXR,ABCG1, and CD36 expression in mo-Mø by oxHDL. Cells (1x 10^7 cells/ml) were treated with oxHDL or native HDL for 24 hrs. Total RNA was isolated from cells using Trizol reagent and cDNA prepared. The gene expression were analyzed by RT-PCR. GAPDH was used as internal control. The data are representative of three independent experiments.*

4.2.2.7. 2. oxHDL increases ABCG1 expression in monocytes-macrophages

Macrophage ATP-binding cassette transporter -ABCG1 has been shown to promote cholesterol and phosphoipid efflux to mature HDL in vitro. The deficiency of reverse cholesterol transport mechanism can influence atherogenesis. ABCG1 expression is reported to be upregulated during cholesterol uptake in macrophage by LXR agonists. But the role of oxHDL in ABCG1 expression in macrophage is unknown. Immunocytochemical analysis were carried out to assess the effect of oxHDL on the expression of ABCG1 on monocytes-macrophages [Fig.20] ABCG1 protein expression was found to be enhanced in cells treated with oxHDL, whereas native HDL or medium alone did not influence ABCG1 expression.

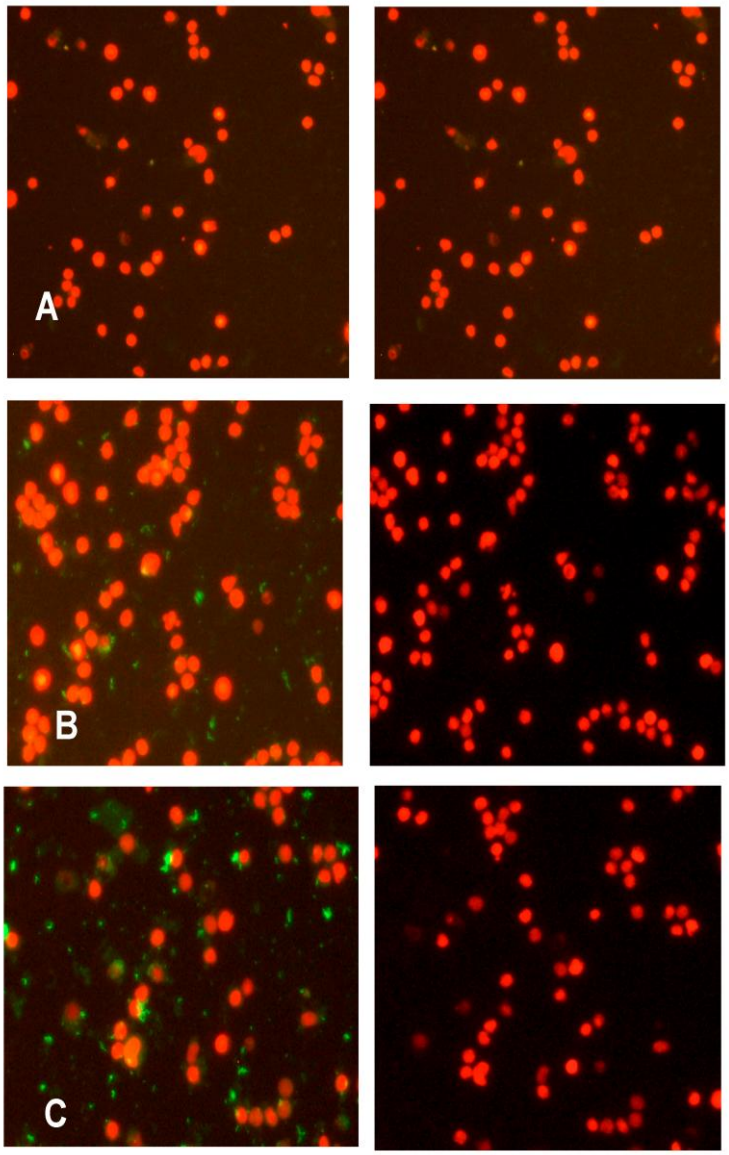

Figure 20. *Effect of oxHDL on ABCG1 surface expression. mo-Mø (1x10^6 cells/ml) were incubated with oxHDL or native HDL (100μg/ml) for 24 hrs. Untreated cells were kept as control. After fixing and washing, cells were incubated with rabbit primary antibody against ABCG1 (1:100 dilution) for 16 hours and subsequently with FITC conjugated anti rabbit secondary antibody (1:100 dilution) for 1.30 hrs. Cells were examined under fluorescence microscope with appropriate filter. The figure represents- -left panel FITC staining of ABCG1 and' right panel corresponding PI staining. Photographs were taken with 20 x magnification. -A represent cells treated with PBS alone, B represents cells treated with natHDL and- C cells treated with oxHDL. Data are representative of three independent experiments.*

4.2.2.7. 3.oxHDL decreases CD 36 expression in monocytes-macrophages

CD36, is a macrophage receptor for oxLDL and has been proven to play a critical role in atherosclerotic- foam cell formation. Its expression is regulated by many factors including oxidized LDL and HDL. Here, we performed immunocytochemical analysis to investigate the role of oxHDL on the expression of CD36 on monocytes-macrophages [Fig.21]. CD36 protein expression was found to be decreased in cells treated with oxHDL.

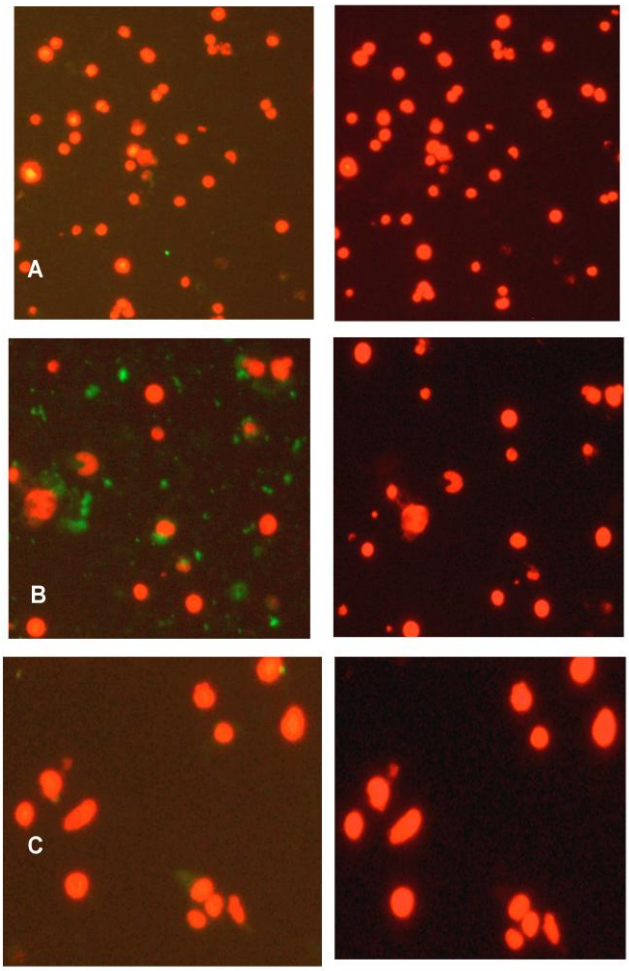

Figure 21. *Effect of oxHDL on CD36 surface expression. mo-Mø (1x10^6 cells/ml) were incubated with oxHDL or native HDL(100μg/ml) for 24 hrs.Untreated cells were kept as control. After fixing and washing, cells were incubated with rabbit primary antibody against CD36 (1:100 dilution) for 16 hrs and then with FITC conjugated anti-rabbit secondary antibody (1:100 dilution) for 1.30 hrs. The cells*

were examined under a fluorescence microscope with appropriate filter. The figures represents- -left panel FITC stained CD 36 and right panel corresponding PI staining. In figure A represent cells treated with PBS alone, B represents cells treated with natHDL and C represents cells treated with oxHDL. Data are representative of three independent experiments.

Treatment of cells with oxHDL showed an enhanced gene expression of LXR -α and ABCG1 and decreased expression of CD36 as evidenced by RT-PCR analyses while treatment with native HDL showed only basal level expression of the above genes. This suggests that oxHDL-induced oxidative stress and lipid accumulation in monocytes-macrophages might act as an adaptive stimuli for lipid homeostasis in cells. Similar response was observed in the surface expression levels of ABCG1 and CD36. LXR activation represents a mechanism to prevent macrophage foam cell formation. However, adequate cholesterol efflux through ABCG1 to the acceptor ,i.e oxHDL, might not be taking place as HDL is modified and thus may result in lipid accumulation in these cells.

4.3. Role of NADPH oxidase in oxHDL-mediated generation of ROS and gelatinase B in monocytes-macophages

Current study proves that oxHDL can induce ROS formation in monocytes-macrophages that mediate various signaling pathways leading to macrophage inflammatory response, as evidenced by the increased production of TNF-α and MMP-9. This finding demonstrates for the first time that oxHDL can induce MMP-9 secretion in monocytes-macrophages. This suggests that unlike native HDL, oxHDL may promote matrix degradation by enhancing the release of MMP9 from arterial monocytes-macrophages, favoring atherosclerotic plaque destabilization and rupture.

ROS have been implicated in the pathogenesis of virtually every stage of vascular lesion formation in atherosclerosis. There are several potential sources of ROS in most cells, including the mitochondria, NADPH oxidases (NOX), cytochrome P450-based enzymes, xanthine oxidase and uncoupled nitric oxide synthases. Although multiple enzymes and processes can contribute to oxidative stress, recent studies indicate that a multi component NADPH oxidase is a major source of ROS production in vascular cells. NADPH oxidase is an inducible electron transport system found in cells that transfers reducing equivalents from NADPH to oxygen resulting in O_2^- generation. Once generated, superoxide rapidly dismutates to hydrogen peroxide, either spontaneously, particularly at low pH, or catalyzed by superoxide dismutase(SOD). It has emerged as a major source of oxidative stress in the artery wall, particularly in artery disease including atherosclerosis. Since NADPH oxidases appear to be especially important for redox signaling inn atherogensis, the next objective of the study was to determine whether NADPH oxidase is involved in oxHD-induced ROS formation in monocytes-macrophages.

4.3.1. OxHDL induces NADPH oxidase activation and ROS production in monocytes-macrophages.

Here, we investigated whether oxHDL-induced ROS was mediated through NADPH oxidase. For this an inhibitor for this oxidative enzyme [diphenyleneiodonium chloride (DPI)] and free radical scavengers were used to assess their role in oxHDL- mediated ROS formation. As shown in Figure 22, pre-treatment with inhibitor of NADPH oxidase [DPI] or ROS scavenger NAC or BHT markedly inhibited oxHDL-induced ROS [DCFH fluorescence intensity]. DPI inhibited ROS

formation by ~60%, relative to oxHDL, suggesting that NADPH oxidase plays a significant role in oxHDL induced ROS formation.

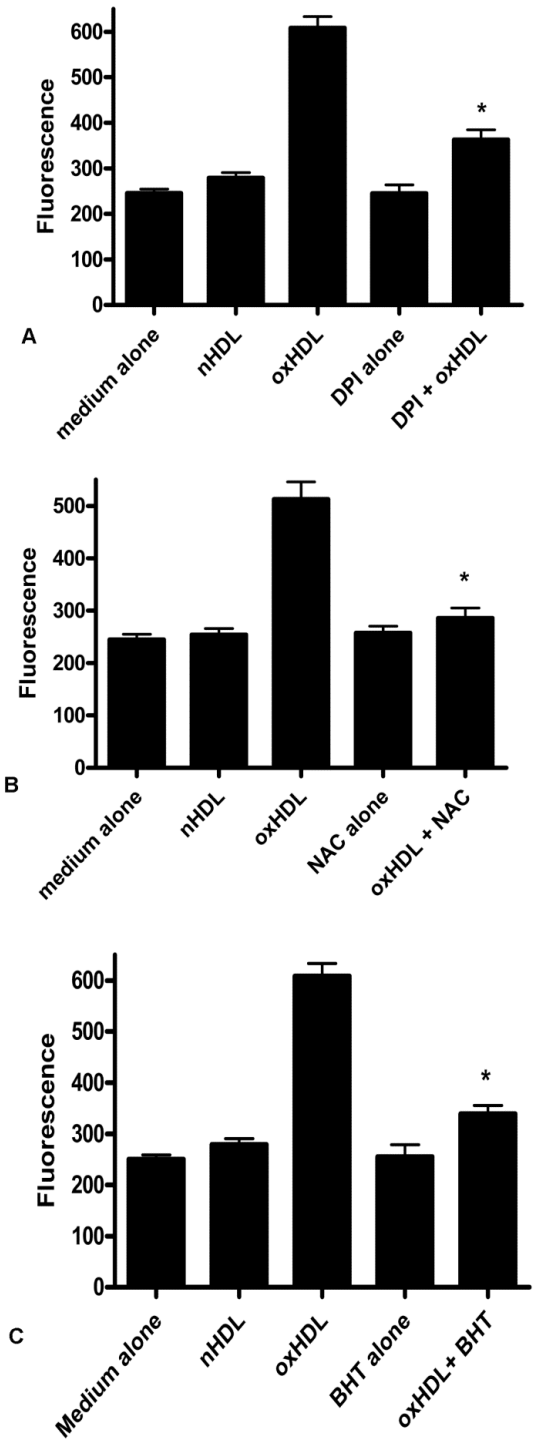

Figure 22. *Effects of DPI & antioxidants on oxHDL- induced generation of ROS in mo-Mø. Cells (2x10^4/ml) were cultured under standard condition and pre-treated with*

DPI (10 μM), NAC (10 mM), BHT (80 μM) *or medium alone for 1 hrs. Cells were then treated with native HDL or oxHDL at 100 μg/ml medium for 24 hours. Intracellular ROS generation was measured as ROS- mediated DCFH fluorescence intensity. Data are the mean ± SD of three independent experiments each well represent triplicate. *p<0.05 oxHDL vs. oxHDL+DPI ,oxHDL+NAC ,or oxHDL+BHT.*

Additional experiments were carried out to assess NADPH oxidase activity [superoxide generating activity] based on nitro blue tetrazolium (NBT) reduction assay, which detects reduction of NBT to formazan by superoxide. The number of NBT positive cells was calculated and expressed as percentage [Fig. 23]. Cells treated with oxHDL showed deeply stained granules [~55%], indicating enhanced NADPH oxidase activity, while pre-treatment with DPI, restored oxHDL-mediated NBT reduction to control levels. This results confirmed that NADPH oxidase activation is involved during oxHDL-induced ROS generation.

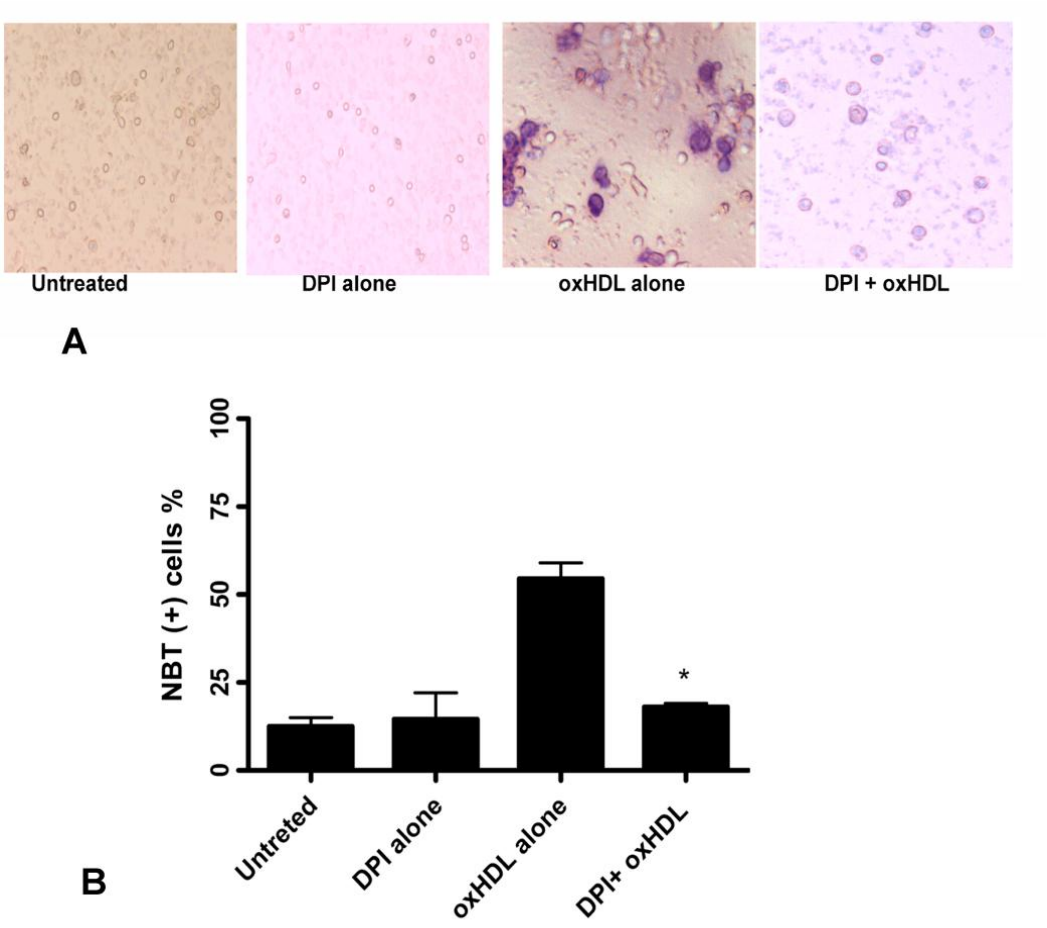

Figure 23. *NADPH oxidase activity in oxHDL-induced ROS formation in* ***mo-Mø.*** *Cells after treatment as described above were subjected to NBT reduction assay. NBT (1.6 mg/ml) was added and incubated at 37°C for 45 min. (A). Micrographs show NBT tests from cells treated with DPI alone, oxHDL and cells pre-treated with DPI before oxHDL treatment. (B) More than 200 cells were counted under microscope from the above assay, and the percentage of formazan-containing cells was calculated and represented.* Data are the Mean ±SD *p <0.05

4.3.2. Role of NADPH oxidase/ROS in mediating oxHDL-induced gelatinase formation in monocytes-macrophages

The production of gelatinase is a marker of monocytes- macrophages inflammatory response and activation. We next investigated the role of NADPH oxidase in oxHDL-induced gelatinase B formation in monocytes-macrophages.

Cells were treated with DPI- [NADPH oxidase inhibitor] for 1 hr and then exposed to oxHDL for 24 hrs. Pre-treatment of cells with DPI significantly prevented oxHDL induced gelatinise B induction [$p < 0.05$], indicating the involvement of NADPH oxidase/ ROS in oxHDL- induced gelatinase B production (Fig.24).

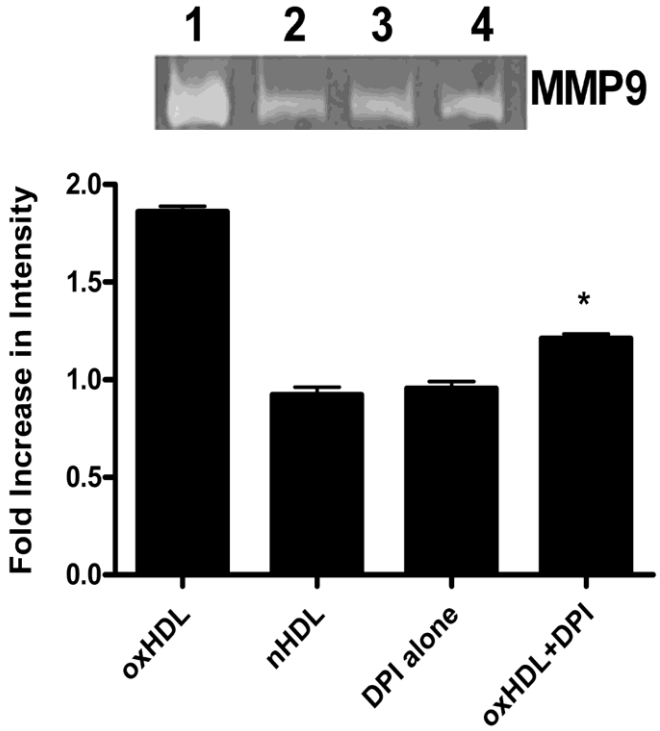

Figure 24. *NADPH oxidase-derived ROS mediate MMP-9 formation in mo-Mø. 1 x 10^6 /ml of cells were cultured under standard condition. Cells were pre-treated with DPI (10 μM) for 1 hour and followed by native HDL or oxHDL(100 μg/ml) treatment for 24 hours. Gelatinases secreted into the medium were assayed by gelatine zymography in 7.5 % gel. The image was digitally captured and the bands were quantified by densitometry using Adobe Photoshop and a histogram analysis program. Results are expressed as means $\pm$ SD [relative intensity to native HDL] of three independent experiments *$p<0.05$. Inset shows zymogram of a representative experiment where lane 1&2 indicates gelatinolytic activity of cells treated with oxHDL and native HDL , 3&4 represent gelatinolytic activity of cells pre-treated with DPI alone, and DPI+ oxHDL respectively .*

ROS may act as a potent source for the induction of gelatinase B in monocyte-macrophages. Next experiments were undertaken to assess the effect of free radical

scavengers such as N-acetyl cysteine (NAC) and butylated hydroxy toluene (BHT), on the release of gelatinase B from monocytes-macrophages. The cells were pre-treated with NAC (10 mM) and BHT (80 μM) for 1 hr and then subjected to treatment with oxHDL (100 μg/ml) for 24 hours. As shown in Fig.25 oxHDL induced gelatinase B release in monocytes-macrophages. Whereas pre-treatment with NAC and BHT effectively inhibited the formation MMP-9 activity. These findings indicated the involvement of NADPH oxidase/ ROS in oxHDL-induced formation of gelatinase B in monocyte-macrophages.

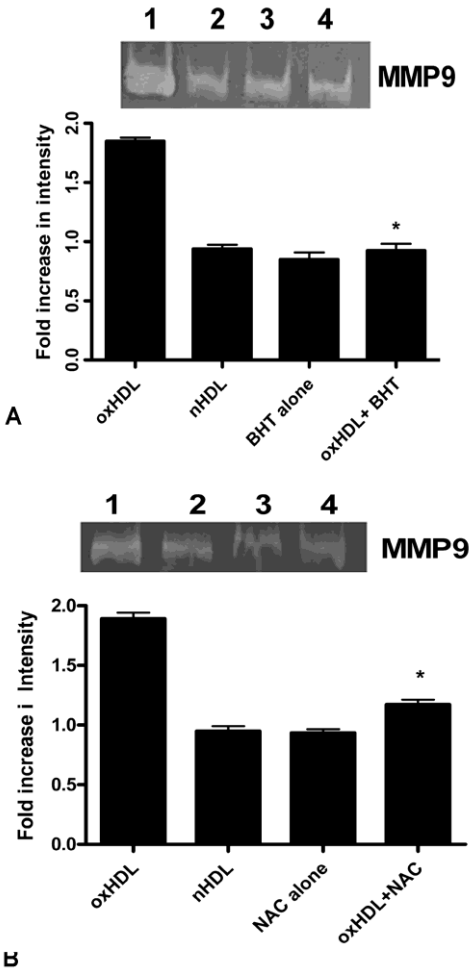

Figure 25. *Antioxidants-NAC and BHT inhibit oxHDL-induced MMP-9 in mo-Mø. 1 x 10^6 /ml of cells were cultured under standard condition and pre-treated with NAC*

(10 mM) and BHT (80 μM) for 1 hr , followed by treatment with oxHDL or native HDL [100μg/ml] for 24 hrs. Gelatinases secreted in the medium were assayed by gelatin zymography in 7.5 % gel. The image was digitally captured and the bands were quantified by densitometry using Adobe Photoshop and a histogram analysis program. Results are expressed as means ± SD[relative intensity to native HDL] three independent experiments. Inset shows zymogram of a representative experiment where lanes 1 &2 in figure A & B indicate gelatinolytic activity of cells treated with oxHDL and native HDL. Lane 3 & 4 in fig A represent gelatinolytic activity of cells treated with BHT , and BHT+ oxHDL respectively. In figure B lane 3 & 4 represent cells treated with NAC and , NAC+oxHDL.

4.3.3. MAPK Signaling Pathways in regulation of MMP9 Expression

ROS up-regulates MMP9 expression. However, the regulatory mechanisms of MMP9 expression and activity are not well established. The low amount of ROS generated has been shown to be important in redox signaling in numerous cellular processes such as cell growth, apoptosis, migration, and extracellular matrix remodeling. The magnitude and location of the rise in intracellular ROS may serve as important determinants of signal transduction pathway activation. Several members of the mitogen-activated protein kinase (MAPK) family are redox sensitive. The goal of this study is to determine what signaling pathways are responsible for oxHDL-induced expression of MMP9 in monocytes- macrophages.

4.3.4.OxHDL-induced ROS, stimulate gelatinase formation via ERK1/2 and JNK-MAPK signaling pathways

To determine if MAPK signaling pathways were playing a role in oxHDL/ROS-induced MMP-9 expression in monocytes-macrophages, experiments were carried out in the presence of chemical inhibitors specific to JNK [SP600125], p38 [SB203580] and ERK1/2-[PD98059] MAPK pathways. After pre-treatment with these inhibitors cells were exposed to oxHDL and MMP9 enzymatic activity was

determined by gelatin zymography. Analysis of the effects of these inhibitors on MMP9 formation showed that both ERK1/2 and JNK inhibitors significantly suppressed oxHDL-induced formation of MMP-9 from monocytes-macrophages [Fig.26.A & B]. However, blocking p38 MAPK activation with SB 203580, did not elicit any effect on oxHDL- induced MMP9 release (Fig 26.C). This preliminary finding indicates the significant contribution of ERK1/2 and JNK- MAPK pathways in oxHDL-mediated gelatinase B formation in monocyte-macrophages.

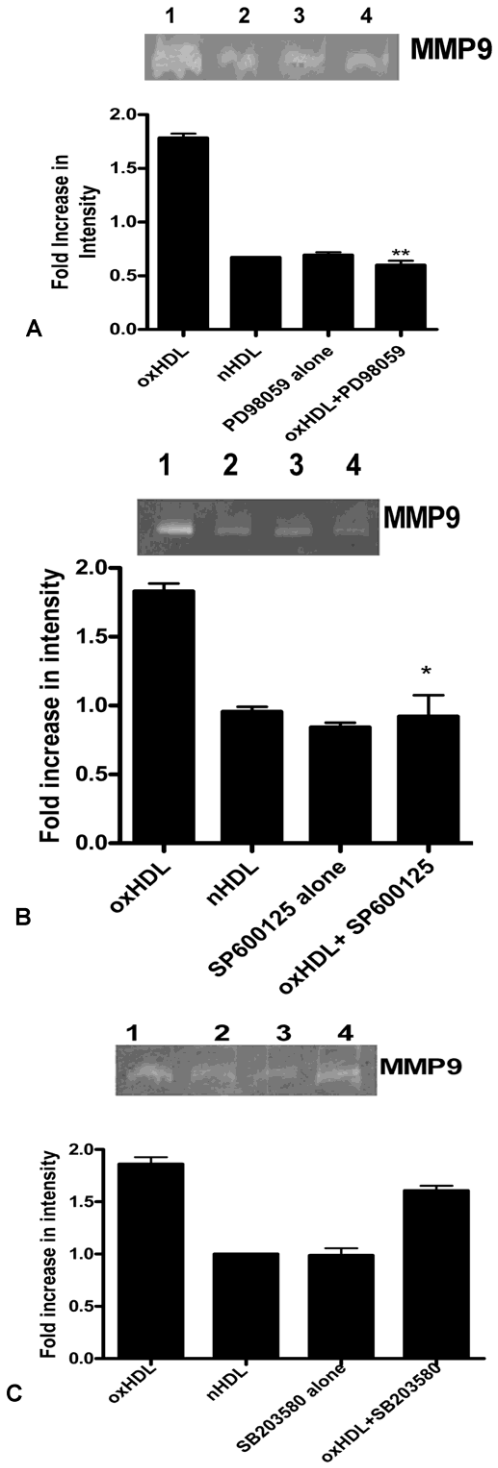

Figure 26. *Effect of MAP Kinases inhibitors on oxHD-induced MMP production in mo-Mø. 1 x 10^6 /ml of cells under culture were pre-treated one hr with inhibitors specific to ERK1/2- [PD98059 ,JNK [SP600125], p38 [SB203580] and] and then incubated with oxHDL or native HDL (100μg/ml) for 24 hrs. MMP-9 activity in the medium was assayed by gelatin zymography in 7.5 % gel. The image was digitally captured and the bands were quantified by densitometry using Adobe*

Photoshop and a histogram analysis program. Results are expressed as means ± SD [relative intensity to native HDL] three independent experiments done in duplicate. Insets shows zymogram of a representative experiment. Lanes 1-2 in each figure indicates gelatinolytic activity of cells treated with oxHDL and native HDL alone (100 μg/ml), respectively. Lane 3 &4 in each figure represents cells treated with inhibitor alone and inhibitor + oxHDL. Results are expressed as means ± SD three independent experiments done in duplicate.

We have demonstrated that oxidized HDL- induced inflammatory response in monocytes-macrophages by enhanced production of ROS and MMP-9. The current results demonstrate that NADPH oxidase activity is required for ROS production and subsequent expression of gelatinase B in monocytes-macrophages,which could be effectively inhibited by pre-treatment of cells with DPI and antioxidants-NAC and BHT. Further, the study provides evidence for the involvement of MAP Kinase family- ERK1/2 and JNK , in oxHDL-induced expression of gelatinase expression.

DISCUSSION

5.1. Identification of the prevalence of functionally altered HDL in subjects

Although high HDL-C levels are considered to be cardioprotective in nature, it is known to undergo dramatic modification in structure, composition and biological functions. Several clinical studies have identified individuals with a significant atherosclerotic burden despite normal or elevated levels of HDL-C(Baron, 2007). Conversely some populations with very low levels of HDL-C have paradoxically lower rates of heart disease **(Calabresi & Franceschini ,** 1997). Further, elevations in HDL-C due to mutations or polymorphisms in genes that regulate HDL remodeling [cholesteryl ester transfer protein (CETP)] or clearance [scavenger receptor type BI (SR-BI)] have not been clearly linked to vascular protection (**Zhong et al.** 1996). Hence recent attention has turned to the functionality, rather than the quantity of HDL-C as a factor in determining the overall cardio-protective properties. In the present study, the antioxidant effect of HDL against LDL oxidation was investigated. The results showed that the antioxidant property varied widely with different HDL particles irrespective of the level of HDL-C, suggesting that the occurrence of functionally deficient HDL may come in conditions of elevated HDL-C. HDL from majority of healthy volunteers showed remarkable antioxidant property. However, in few cases, HDL exhibited inadequate antioxidant ability to inhibit LDL oxidation. These subjects were also found to have enhanced systemic oxidative stress and inflammation compared to healthy subjects. Although the exact reason for this

functional deficiency in HDL is not known, these findings indicate that systemic inflammation, oxidative stress, and/or other unknown factors might impair HDL function resulting in it being a less efficient anti-atherogenic agent. This preliminary finding observed in healthy volunteers is in agreement with previous reports, which showed decreased protection by HDL from poorly controlled type 2 diabetic subjects against LDL oxidation (Gowry et al. 1999) and a reduction of HDL antioxidant property in haemodialysis patients against oxidative stress (Morena et al. 2011). It has also been reported that in human subjects with obstructive sleep apnea, HDL was significantly less able to retard LDL oxidation than HDL from control patients with similar HDL cholesterol levels (Hansson, 2005). Nobecourt et al. 2005) have also reported that the defective antioxidative activity observed in small dense HDL3 particles in type 2 diabetes was intimately linked to oxidative stress, glycemia and hypertriglyceridaemia.

Atherosclerosis can be considered as a chronic inflammatory disease driven by the progressive incorporation of the cytotoxic byproducts of lipid and phospholipid oxidation into a changing monocyte population at the cellular level of the arterial endothelium (Ross, 1999). HDL is associated with a rich core of proteomes [apolipoproteins, such as apoA-1, apoA-II, apoA-IV, apo C, apoE and apoJ], and accompanied by powerful antioxidative enzymes such as paraoxonase, PAF-AH, glutathione selenoperoxidase. Normal functional HDL has high levels of active anti-oxidant proteins and enzymes with high antioxidant potential and has antiinflammatory activity. HDL due to its intrinsic physicochemical properties, exhibits potent antioxidant property thereby it protects and reverts oxidation of LDL. LDL oxidation is considered to be an early event in the formation of atherosclerotic

lesions that promotes inflammation in the artery wall. HDL protects LDL particles against oxidative stress in tissues and can function as a trap for oxidized lipids, removing them from circulation by transfer to the liver for excretion, which would be protective. By limiting LDL oxidation, HDL plays a key antiinflammatory role in slowing atherogenesis. High antioxidant and antiinflammatory activities of HDL are associated with protection from CVD. However, not all HDL is functionally similar. HDL from those subjects with systemic inflammation did not effectively prevent LDL oxidation, suggesting that functionally deficient HDL may play a role in promoting arterial inflammation. The current study provides evidence for the differential antioxidant properties of HDL in subjects and this may have important consequences for the understanding of the protective antiatherogenic action of HDL in vivo. Determining HDL function may also identify subjects with normal or low HDL-C levels that are at particularly high risk for cardiovascular events. Further detailed studies involving more healthy subjects are essential to explore the factors causing dysfunctionality in HDL in healthy volunteers.

Recent reports regarding functional changes in HDL mainly pointed out on the structural modification of HDL components (Kontush & Chapaman, 2006). Also the replacement of HDL component is another factor that generates functionally altered HDL. It appears that there is no definitive common structural feature that determines HDL functionality. Even though there are several evidences showing the prevalence of dysfunctional HDL in patients with acute coronary syndromes (Paneni et al. 2012; Dodani, 2008; McMahon et al. 2009), the underlying mechanism, which converts the normal HDL to a dysfunctional stage and its involvement in atherosclerosis development, are still unclear. HDL has been shown to have a variety of functions

that may contribute to its cardiovascular protective effects, including promotion of macrophage cholesterol efflux [RCT], antiinflammatory and antioxidative effects. The close association between inflammation, oxidative stress, dyslipidemia, and atherosclerosis suggests that such HDL functional alterations might play a significant role in disease progression. Few studies are available in human populations investigating involvement of vascular inflammation and oxidative stress-related dysfunctional transformation of HDL in establishing CVD. High level of HDL-C usually has lower risk for heart disease. Whereas some people who have high HDL-C levels still get heart attacks and suffer from other CVD (Sharma et al. 2009). In acute and chronic inflammation (e.g .influenza A), the content and functions of HDL can change drastically converting atheroprotective HDL to proatherogenic HDL (Paoletti, 2004). HDL from many CVD patients was found to be pro-inflammatory, thus increasing monocyte chemotaxis in response to LDL, unlike the HDL from healthy controls that reduced monocyte chemotaxis (Smith, 2010). The complexity of HDL structure and function demands more investigation to better understand HDL metabolism, function and its regulation in humans.

HDL is today regarded as one of the most important protective factors against atherosclerosis. The present study demonstrates that all HDL isolated from healthy volunteers are not same in quality i.e.antiatherogenic property and provides evidence for the presence of functionally altered HDL,even in conditions of normal HDL-C, which have significantly impaired ability to prevent LDL oxidation, a key component of the atheroprotective function of HDL. The close association between oxidative stress and inflammation suggests that such functionally altered HDL might play a role in atherogenesis. The serum HDL-C level does not predict the *functionality* of HDL

and thus point out the need for assessment of HDL functionality to provide additional information for better predicting the cardiovascular risk associated with HDL.

5.2. Invitro induction of dysfunctionality in HDL using an oxidative system and its functional characterization

Atherosclerosis is considered an inflammatory disease that induces a prooxidant environment and oxidized lipids play a crucial role in its progression. Though plasma HDL concentration is inversely related to cardiovascular diseases, HDL oxidation, and other modifications can occur in the vascular wall (Shao et al. 2010) and contribute to atherogenesis. It is not yet clearly understood whether oxidative modification of HDL has a role in the pathogenesis of atherosclerosis. One potential mechanism generating dysfunctional HDL involves oxidative modification of apoA1, the major protein of HDL. HDL can be modified into a dysfunctional and proinflammatory form by different ways and the impact of this modified HDL induced changes remain unknown. This study analyzed the effect of copper induced-oxidized HDL on monocytes–macrophages functions, particularly on monocyte inflammatory response.

The present in vitro study demonstrates that treatment of monocytes–macrophages with oxHDL induces proinflammatory response as evidenced by increase in ROS production, the release of cytokines like TNF-α, and matrix degrading enzymes such as MMP9 and MMP2. Copper-induced oxidative modification of HDL (with TBARS as MDA concentration of 60–80 nM/mg protein) resulted in a significant increase in ROS production in monocytes–macrophages in a

concentration dependent manner. However, treatment with mildly oxHDL (except at very high concentration of 200 μg/ml) and native HDL produced no such response, thereby establishing oxidative modification of HDL as a primary cause for oxidative stress, which largely depends on the level of oxidative modification. This study also shows that oxHDL is able to induce MMP2 (Gelatinase- A) and MMP9 (Gelatinase-B) in monocytes–macrophages. The role of oxHDL on MMP secretion from monocytes–macrophages has not been previously reported. These findings are consistent with the reports that showed inflammatory cytokines and oxidized lipids can induce MMP activity in cells (Xu et al. 1999; Rajavashisth et al. 1999, Huang et al.2001). MMPs are capable of degrading virtually all the components of extracellular matrix. Several lines of evidence support the potential role of MMPs in human atherosclerosis and plaque disruption (Agewall .2006). This study suggests that unlike native HDL and mildly oxHDL, oxHDL may promote matrix degradation by enhancing the release of MMP-9 and MMP-2 from arterial monocytes–macrophages, favoring atherosclerotic plaque destabilization and rupture.

TNF-α is a pleiotropic cytokine produced primarily by activated monocytes–macrophages. TNF-α is emerging as one of the key cytokines that exerts adverse effects during atherosclerosis. It can augment the local inflammatory response, alter lipid homeostasis and increase the uptake of cholesterol ester (Napolitano & Bravo, 2005;Persson .2008). Atherogenic cytokines can down regulate ABCA1 in cultured foam cells via the activation of NF-kB, a key nuclear transcription factor involved in atherogenesis that regulate a variety of genes involved in inflammatory response (Mei et al. 2007). Here, oxHDL was found to be an inducer of TNF-α in monocytes–

macrophages. The exact cellular mechanisms associated with these effects of oxHDL remains to be elucidated. It is possible that the oxidative stress induced by oxHDL in monocytes–macrophages led to proinflammatory response. This response in the artery wall can recruit other cell types also and can contribute to the development of complex lesions.

The oxidative stress exerted by oxHDL could also be cytotoxic and promote cell death as evidenced by the increase in the percentage of dead cells observed in PI staining of cells after exposure to oxHDL for 48 hrs. This is in agreement with the findings of Keller et al (Keller et al. 2000) that oxHDL exacerbates oxidative stress and neuronal cell death. ROS play important roles in regulation of cell survival. In general, balanced level of ROS have a wide variety of cellular regulatory functions such as acting as intracellular signaling molecule, cell survival and growth. But abnormal level of ROS can induce cell death. ROS can trigger apoptosis either indirectly, through damage to DNA, lipids, and proteins or more directly by ROS-mediated activation of signaling molecules. Such proapoptotic signaling of ROS may occur through activation of MAP kinases, such SAPK/JNK, ERK1/2, and p38.

Even though oxHDL is proven to be cytotoxic and proinflammatory, controversy exists on the role of oxHDL in cellular functions mostly because of the heterogeneity in structure of HDL. HDL modifications can be achieved by different means such as non-enzymatic modifications due to the presence of free metal ions in atherosclerotic plaque, cell-associated inflammatory enzymes, association with acute phase protein, and metabolic modifications that can lose its antiatherogenic activities (Kontush & Chapman, 2006; Shao et al. 2010; Stadler et al. 2004). Copper and iron

may be important modulators of lipid peroxidation. In this study, a mild oxidative condition in HDL does not elicit significant inflammatory response suggesting that low degree of oxidation in HDL may not be deleterious, while extreme oxidation induces deleterious effects leading to intracellular lipid deposition. Pirillo et al. (Pirillo et al. 2007) reported that mild oxidized HDL increases the cholesterol efflux property, whereas oxidation proceeds; HDL loses its cholesterol efflux properties and promotes lipid accumulation in cells. Macrophages in the intima-media express scavenger receptors that bind oxidized lipids and form foam cells. Thorne et al. (Thorne et al. 2007) have reported that the uptake of oxHDL by CD36 on macrophages accelerates foam cell formation. In consistence with these findings, our result also showed that treatment of monocytes–macrophages with oxHDL increased the lipid accumulation suggesting its proatherogenic role.

Macrophages have important roles in both lipid metabolism and inflammation and are central to the pathogenesis of atherosclerosis. LXRs are key transcriptional regulators of genes involved in lipid homeostasis and inflammation and are determinants of atherosclerosis susceptibility. Native HDL and its apolipoproteins can promote cholesterol efflux from macrophage- foam cells via the ATP-binding cassette transporters- ABCA1 and ABCG1. The ability of HDL to promote efflux of cholesterol from peripheral cells and transport to the liver was well recognized as an antiatherogenic mechanism. ABCA1 promotes cholesterol and phospholipid efflux from cells to lipid-poor apoA-1 but not to mature HDL particles, while ABCG1 promotes cholesterol efflux to HDL and other lipoprotein particles but not to lipid-poor apoA-1(Oram et al. 2000; Wang et al. 2004). CD36, belongs to class B scavenger receptor family, is a macrophage receptor for oxLDL and has

been proven to play a critical role in atherosclerotic foam cell formation. Experiments addressing the role of LXR, ABCG1, and CD36 have provided following results. Treatment of cells with oxHDL showed an enhanced gene expression of LXR -α and ABCG1 and suppressed expression of CD36 as evidenced by RT-PCR analyses while treatment with native HDL showed only basal level expression of the above genes. This suggests that oxHDL-induced oxidative stress and lipid accumulation in monocytes-macrophages might act as an adaptive stimuli for lipid homeostasis in cells as evidenced by enhanced expression of LXR-α and ABCG1 for cholesterol efflux and supressed expression of CD36 [a strong receptor of modified lipids] to inhibit further lipid uptake. Similar response was observed in the protein expression levels of ABCG1 and CD36. LXR activation represents a mechanism to prevent macrophage foam cell formation. However, adequate cholesterol efflux through ABCG1 to the acceptor ,i.e oxHDL, might not be taking place and thus resulting in observed lipid accumulation in these cells. Calvo et al (1998) have reported that human CD36 is a high affinity receptor for native lipoproteins HDL,LDL and VLDL and for OxLDL and AcLDL, suggesting that binding of lipoproteins to CD36 might contribute to the regulation of lipid metabolism, and to the pathogenesis of atherosclerosis. A variety of cell surface glycoproteins (SR-A, MARCO, CD68, CD36, and SR-BI), collectively designated as scavenger receptors, contribute to the uptake of modified lipoproteins.

Thorne et al. 2007 have shown that CD36 is a receptor for oxHDL. OxHDL can down-regulate both the mRNA and surface protein expression of CD36 on

human peripheral macrophages (Ren et al.2010). In consistence with these reports current findings also demonstrated that treatment of monocytes-macrophages with oxHDL increased the neutral lipid accumulation suggesting its proatherogenic role.

Oxidation, particularly oxidative modification of LDL within the artery wall and its subsequent unregulated uptake by macrophages, has been postulated to be an important event in disease development (Heinecke, 1998). A range of reactive species can oxidize lipoproteins, including HDL in vitro, but the nature of oxidants under in vivo condition is controversial. Fu et al(1998) have demonstrated the presence of elevated levels of specific protein oxidation products in advanced human lesions and identified metal ions (high iron and copper) as possible catalysts for these species. These findings are consistent with the hypothesis that high iron and copper levels may contribute as an independent factor for atherosclerosis, a multifactorial disease, and its sequelae. In the current study the severity of oxidative stress and inflammation induced by oxHDL compared with that of oxLDL was studied. Even though the MDA content in oxHDL was comparable to that of oxLDL, the proinflammatory response induced by oxHDL was less compared to that of oxLDL. Several studies cited the proatherogenic activities of oxLDL and the current findings demonstrate that oxHDL is also act as proatherogenic during oxidative modifications. Although dysfunctional HDL is implicated in the pathogenesis of cardiovascular disease, the underlying pathways of its formation and its effect on cellular environment remains poorly understood. Observations of Shao et al. 2010) show that levels of MDA-protein adducts were elevated in HDL isolated from human atherosclerotic- lesions and apoA-I co-localized with acrolein adducts in such lesions. This peroxidative modification of HDL might specifically modify HDL function in

vivo. This study shows that, copper-mediated oxidation caused accumulation of MDA in HDL, thereby converting it into a pro-inflammatory particle that stimulates the production of ROS, TNF-alpha, MMP-9, and MMP-2 as well as formation of foam cells in cultured monocytes–macrophages—a possible way of modified HDL-mediated proatherogenic actions.

In conclusion, this study demonstrates that following in vitro oxidative modification, HDL loses its atheroprotective functions and exerts proinflammatory response by releasing TNF-alpha, MMP-9, and MMP-2 as well as promotes oxidative stress in human monocytes–macrophages. The generation of oxHDL in vivo might therefore be regarded as atherogenic.

5.3. Role of NADPH oxidase in oxHDL-mediated generation of ROS and gelatinaseB in monocytes-macophages

The antiatherogenic effects of HDL has been attributed to its role in reverse cholesterol transport, its effects on endothelial cells and its antioxidant and antiinflammatory properties. However, HDL can undergo oxidative modifications under certain conditions like acute phase response and inflammatory state, mainly by ROS, inflammatory enzymes and/or metal ions (Kontush & Chapman, 2006). There are evidences for the presence of oxidized HDL in vivo (Pennathur, Bergt et al. 2004; Shao, Oda et al. 2006) and for the proatherogenic actions of modified HDL (Van Lentan et al. 2007; Undurti, Huang et al. 2009). Since oxidative modification can occur in HDL, we have investigated the effect of copper-oxidized HDL on monocytes-macrophages function and reported its pro-inflammatory response as

evidenced by enhanced production of ROS, TNF-α and gelatinase (Soumyarani & Jayakumari, 2012). Here we attempted to delineate the molecular pathways associated with oxidized HDL induced formation of ROS and gelatinase in mo.mac. The results indicated that NADPH oxidase-derived ROS and subsequent activation of MAP Kinases-ERK1/2 and JNK, are involved in oxHDL-induced gelatinase expression in monocytes-macrophages.

ROS mediate various signaling pathways that underlie vascular inflammation in atherogenesis, endothelial dysfunction, lesion progression and ultimate plaque rupture. In the vasculature, NADPH oxidase-derived ROS are thought to be involved in nitric oxide inactivation, growth and cell division, kinase activation, activation of matrix metalloproteinases, activation of transcription factors and gene expression, extracellular matrix regulation, endothelial cell proliferation and migration, and neointimal formation (Bedard & Krause, 2007). NADPH oxidase is the major source of ROS in macrophages, which contribute to the pathogenesis of atherosclerosis. This study examined the role of NADPH oxidase-activation in response to oxHDL and the results provided new insights into the mechanism of oxHDL action to regulate the production of ROS and gelatinase and thus exaggerated inflammatory response. Using inhibitor studies with DPI [NADPH oxidase inhibitor] and free radical scavengers [NAC & BHT] as well as NBT reduction assay for NADPH oxidase activity, this study showed the involvement of NADPH oxidase/ ROS generation and subsequent release of gelatinase B in monocytes-macrophages. Thus we suggested that oxHDL-induced formation of ROS and gelatinase were , at least in part, mediated by NADPH oxidase activation in monocytes- macrophages.

NADPH oxidase is a multi-subunit family of enzymes. The enzyme catalyzes the one-electron reduction of molecular oxygen using NADPH as an electron donor, generating superoxide radicals $O2^{-}$. Once generated, superoxide dismutates into hydrogen peroxide, either spontaneously, particularly at low pH, or facilitated by superoxide dismutase and H_2O_2 may subsequently be converted into a variety of active oxygen species, such as singlet oxygen and hydroxyl radicals (Van Heerebeek et al. 2002). Several studies have shown a key role for vascular NADPH oxidase isoforms in the development of human atherosclerosis (Guzik et al. 2000; Azumi et al. 1999). Interestingly, phagocytic NADPH oxidase seems to play also a key role in the development and progression of atherosclerotic lesion (Sorescu et al. 2002; Azumi et al. 2002). Zalba et al found that enhanced NADPH oxidase dependent superoxide production stimulates MMP9 in monocytes and that this relationship may be relevant in the atherosclerotic process (Zalba et al. 2007). Inflammatory stimuli such as TNF alpha , IL-1beta and oxidized lipids can induce MMP production in cells through ROS pathway. NADPH oxidase mediated ROS found as a potential stimulator of gelatinase expression in monocytes-macrophages in response to oxHDL in human monocyte-macrophages. We cannot discarded other sources ROS in oxHDL treated cells.

To establish the contribution of NADPH oxidase to ROS production, NADPH activation was inhibited by means of DPI, the most commonly used NADPH oxidase inhibitor. Although DPI abolished NADPH oxidase-mediated ROS formation, it can also inhibit mitochondrial respiratory complex 1, and other flavo-enzymes such as nitric oxide synthase and xanthine oxidase (O'Donnell et al. 1993). To some extent, the lack of specificity of DPI can be addressed by using specific inhibitors of other sources of ROS. Hence to examine the direct involvement of NADPH oxidase in

oxHDL –induced ROS production further experiments have to be carried out either using more specific NADPH oxidase inhibitor [likes gp91-dstat] or using mitochondrial stain [mitotrackers] or specific inhibitors for mitochondria and other ROS sources to rule out their involvement.

Oxidative damage in various tissues may be controlled or prevented by enzymic and nonenzymic antioxidant defense systems. In the present study, we investigated the effect of NAC, and BHT- powerful free radical scavengers, on oxHDL-induced formation of ROS and gelatinase in monocytes-macrophages. Both NAC and BHT blocked, the increased generation of ROS and gelatinase induced by oxHDL. This may account for their potency in scavenging ROS and reducing oxidative stress. NAC directly inactivates ROS and hypochlorite by conjugation or reduction to form NAC radicals. A mechanism of suppression of NADPH oxidase subunits by NAC has also been reported. NAC can exert benefits as a precursor to the antioxidant, glutathione and modulating inflammatory pathways (Dean et al. 2011). This suggests that use of effective antioxidant/antiinflammatory therapies may provide a promising approach in attenuating oxHDL induced oxidative stress and inflammatory response.

Having established the existence of a relationship between oxHDL/ROS and MMP9 up-regulation, the next objective was to determine which pathways could be signaling MMP-9 expression. There is abundant evidence for activation of elements of the MAP kinases system by NADPH oxidases-derived ROS (Bedard & Heinz Krause, 2007). However, the precise redox-sensitive steps involved in kinase activation in response to oxHDL-induced NADPH oxidase/ ROS production is

presently unknown. We tested whether oxHDL-induced ROS can trigger MAP Kinases in monocytes-macrophages for the release of gelatinase. Using specific inhibitors for MAP Kinase pathways, this study demonstrated that oxHDL-stimulated ERK1/2 and JNK, but not p38 MAP Kinase activation and enhanced gelatinase activity in monocytes-macrophage. ERK MAPK is strongly activated by growth factors, as well as many other stimuli that mediate cell proliferation, differentiation, and survival (*Dent et al.* 2003). In contrast, JNK and p38 MAPK cascades are strongly activated by cellular stresses, as well as by proinflammatory agents such as endotoxin, IL-1, and TNF-α (*Roberts & Cowsert* , 1998; *Finch et al.* 2001).

This study suggests that for induction of gelatinase activity by oxHDL in monocytes-macrophage both ERK1/2 and JNK –MAPK pathways seems to be essential. This reflects the effects of the different components in oxHDL such as proteins or lipids, on monocytes-macrophage and subsequent modulation of observed cellular functions. However, the precise pathways leading to ERK1/2 and JNK activation by oxHDL and/or subsequent gelatinase expression are still unclear. Further elucidation of how ERK1/2 and JNK execute its function in generation of gelatinase will help us understand completely the role of oxHDL in monocytes-macrophage inflammatory response. This finding is in consistent with the report of Cohen et al. that indicates that in trophoblastic cells, TNF-α activates two different pathways leading to MMP9 expression: (a) Erk1/2 pathway which in turn initiates NF-κB activation and (b) SAPK/JNK pathway that activates AP-1 (Cohen et al. 2006). MAPK intracellular signaling pathways have been demonstrated to play a central role in regulating a wide range of inflammatory responses in many different cell types. Activation of JNK/c-Jun and ERK1/2 MAPK signal transduction pathways

leads to activation of murine peritoneal macrophages (Biswas,& Sodhi, 2002). With this view, our findings suggest that these activated MAPK- ERK1/2 and JNK pathways could mediate mo.mac activation and the production of MMP9 in response to oxHDL.

It is thought that the balance between distinct MAPK pathways regulates cell growth, differentiation, survival, and death. Taken together, these results indicate cooperation of multiple MAPK pathways in the regulation of MMP transcription in response to different cell specific signals. Further detailed studies are needed to define the relationship between MAPK activation and oxHDL-stimulated production of gelatinase in these cells.

The upregulation of gelatinase expression can occur through redox-sensitive MAP kinase activation, or through transcription factors, including NFκB, AP-1, which contain redox-sensitive, low-p$K_{\mathbf{a}}$ cysteine residues in their DNA binding domain. NF-κB has long been recognized as a redox-sensitive transcription factor. The preliminary data from our study showed that oxHDL-induced gelatinase expression was not regulated by NF-kB, as inhibition of NF-kB by specific inhibitor was not able to significantly reduce oxHDL-mediated gelatinase expression and an immunocytochemical assay for p65 nuclear translocation also confirmed that NF-kB is not activated during oxHDL treatment results not shown]. Research in recent years has suggested that MAPK signaling pathways and AP-1 could be involved in MMP9 up-regulation (Wang et al. 2009). Other research findings have also shown the link between MMP9 and AP-1. Based on the current view on the importance of stress-activated MAPKs in the regulation of MMP9 expression, it is assumed that activation of AP-1 transcription factor by these MAPKs might be responsible for oxHDL-

stimulated enhanced MMP9 activity in monocytes-macrophages. Since the AP-1 and NF-κB are known regulators of MMP-9 promoter, detailed research are needed to explore the precise contribution of signaling pathways in monocytes-macrophages. Although MMP9 plays a significant role in the pathology of atherosclerosis, the signaling required for MMP9 up-regulation has yet to be fully elucidated.

Inflammation and oxidative stress have been recognized as major contributors to atherogenesis through their effects on lipoprotein metabolism and arterial wall biology. Matrix metalloproteinses play an important role in the homeostasis of extra cellular matrix and inflammation. They contribute both in the formation as well as in the destabilization of atherosclerotic plaque. Inflammatory stimuli such as TNF-α, IL-1β, and oxidized lipids can induce MMP production in cells through a reactive oxygen species pathway (Wang, et al. 2009). Inoue, et al. 2001 have found enhanced MMP activity in aortic endothelial cells through NADPH oxidase system mediated by lysophosphatidyl choline. In agreement to this, the present study identified oxHDL-induced ROS as a potential stimulator of gelatinase expression in monocytes-macrophage. In addition, oxHDL trigger ERK1/2 and JNK -MAP Kinase signaling pathway as one mechanism for enhanced gelatinase formation. Pre-treatment of cells with DPI [NADPH oxidase inhibitor] and antioxidants –NAC & BHT could significantly reduce the production of NADPHoxidase/ROS and MMP9 induced by oxHDL. This finding showing that DPI attenuated both ROS and MMP-9 secretion induced by oxHDL in human blood monocytes-macrophages, demonstrates for the first time to our knowledge that oxHD-mediated NADPH oxidase/ROS production stimulates MMP9 activity in these cells. MMPs are key enzymes that regulate tissue

remodelling through turnover of the extracellular matrix in both normal and pathological conditions. Strong evidence indicates that various MMP/TIMP imbalances are crucial elements in the disease processes. While inhibition of MMPs has been suggested as a therapeutic approach in several inflammatory conditions, a complete understanding of the biology of these complex enzymes is essntial before considering them as therapeutic targets.

Plasma MMP9 levels correlate with the presence of atherosclerosis and represent an independent risk factor for atherothrombotic events (ie, coronary heart disease events and cerebrovascular disease. Functional alteration in HDL could be one of the contributing factors for the excessive release of MMP9. Recent findings from this laboratory have shown enhanced gelatinase activity - both MMP9 & MMP2, in plasma of subjects having dysfunctional HDL compared to those having functional HDL, indicating the functional importance of HDL in assessing the risk for CVD. The research findings on oxHDL-induced ROS and MMP9 activity in monocytes-macrophages have implications for potential therapies that aim to reduce the amount of MMP9 or inflammatory response in patients. In fact, MMP9 emerges as a potential mediator of the pro-atherosclerotic actions of phagocytic NADPH oxidase in both symptomatic and asymptomatic subjects. In this regard the NADPH inhibitor may be a better target for vascular diseases. At the same time NAC is considered as a powerful anti-oxidant and it exhibit different mechanisms of antioxidant action.

HDLs are highly heterogeneous in size, physicochemical properties, metabolism, and in their anti-atherogenic functions .HDL has an array of anti-

atherogenic mechanisms, including antioxidative and anti-inflammatory properties. HDL can decrease superoxide production and inactivate neutrophil NADPH oxidase, a respiratory burst enzyme, which is an important source of ROS in the vessel wall. However, under inflammatory conditions HDL undergoes functional alteration and loses its atheroprotective properties. In the microenvironment of atheroma, HDL can be converted to a pro-atherogenic particle and could elicit pro-inflammatory response in vascular cells. Unlike native functional HDL ,the presence of functionally altered HDL in vivo might propagate the atherogenic mechanism as enhanced production of ROS and gelatinase B. Further research is vital to better understand the complexity of cellular redox reactions mediated by functionally altered HDL[oxHDL],as such pro-inflammatory responses are likely to play a central role in the development of atherosclerotsis. In principle, protection against such deleterious effects can be by prevention, interception and repair. A better understanding of these processes may lead to development of a new class of antioxidants targeted to specific subcellular sites, for treatment and prevention of CVD. Synthetic peptide analogs of the amphipathic helixes of apoA-I, [ApoA-I mimetic peptides] offer an attractive approach for HDL therapy to improve atheroprotective properties.

In summary, this results demonstrated that oxHDL-induced ROS production in human peripheral blood monocytes-.macrophages through NADPH oxidase, in turn initiated the activation of ERK1/2 and JNK MAPK pathways and enhanced gelatinase expression. These findings suggest that oxHDL enhanced oxidative stress and inflammatory responses in monocytes-macrophages via activation of, at least in part, NADPH oxidae-induced ROS and subsequent stimulation of MAPK-kinase signaling

pathways. These results provide new insights into the mechanisms of oxHDL action on monocytes-marcophages to regulate the expression of gelatinase B and thus exaggerate the inflammation responses.

SUMMARY AND CONCLUSION

Coronary artery disease is the leading cause of death worldwide. Although a large proportion of CHD is preventable, they continue to increase mainly because preventive measures are inadequate. A vast body of experimental and clinical evidence has identified arterial inflammation as the basic mechanism of atherogensis, finally leading to the development of atherosclerotic- plaque vulnerability and rupture. Although high HDL-C levels are considered to be cardioprotective in nature, recent pharmacological findings have raised doubts about the beneficial effects of raising HDL-C level. HDL is known to undergo dramatic modification in structure, composition and biological functions. Functionally altered HDL is generally thought of as not cardioprotective even if HDL-C is present in high levels. Clearly we need to gain a better understanding of HDL heterogeneity and function as determinants of cardiovascular disease risk. It is hypothesized that high prevalence of dysfunctional HDL could be a contributing factor to the excessive risk of CHD. However, the pro-atherogenic pathways exerted by functionally altered HDL remain poorly understood.

The major focus of the current study is identification of the prevalence of functionally altered HDL in healthy subjects. This study also investigated how the intrinsic function of monocyte, the key cell type involved in the development of atherosclerotic lesion, might be influenced by its interaction with functionally altered HDL particles to gain insights into the cellular mechanism of action. To stimulate the efflux of cholesterol from macrophage-foam cells as well as to initiate other antiatherogenic functions in endothelium, HDL should make contact with these cells.

Therefore it is important to determine the effect of functionally altered HDL on monocytes- macrophage functions relevant to atherogenesis.

In order to study the functionality, HDL fractions were isolated from blood samples collected from healthy volunteers by ultracentrifugation. The antioxidant functionality of HDL was assessed in terms of its ability to inhibit LDL oxidation in vitro. In majority of healthy volunteers having normal lipid profile, HDL showed remarkable antioxidative property to resist the oxidation of LDL. However, this antioxidant property varied widely with different HDL particles irrespective of the level of HDL-C. HDL isolated from few volunteers and also from those with metabolic syndrome exhibited inadequate antioxidant ability to inhibit LDL oxidation. These subjects were found to have enhanced systemic oxidative stress and inflammation as evidenced by higher concentration of serum lipid peroxides, protein carbonyls and hsCRP. Although the exact reason for this functional deficiency in HDL is not known, these findings indicate that systemic inflammation, oxidative stress , and/or other unknown factors might impair HDL function leading to a less efficient antiatherogenic agent. These finding point to the need for assessment of HDL function rather than measuring HDL-C for better predicting the cardiovascular risk associated with HDL.

The attenuated atheroprotective-antioxidant property of HDL observed in subjects raise the possibility of an indirect putative proatherogenic effect of these particles. To investigate this possibility, in vitro oxidative modification was induced in HDL particle using copper sulphate [mild and severe oxidation conditions]. The extent of oxidation was quantitated by measurement of lipid peroxides [MDA]. The

next objective was to investigate the influence of both native and oxidatively modified HDL (oxHDL) on monocytes-macrophages functions relevant to atherogenesis.

Human peripheral blood mononuclear cells were isolated, cultured under standard conditions, and incubated with native or oxidized HDL at varying concentrations for different time intervals. Treatment of cells with oxHDL for 24 hrs enhanced the production of ROS in a concentration- dependent way, while native HDL had no such effect. Further, the release of TNF-α, MMP-9 and MMP-2 was found to be remarkably higher in cells incubated with oxHDL at 24 hrs than that of mildly oxHDL and native HDL. These findings indicate that oxidatively modified HDL induces proinflammatory response and oxidative stress in human monocytes-macrophages. The current study demonstrated for the first time that unlike native HDL and mildly oxHDL, oxHDL could induce MMP9 activity in human monocytes-macrophages. oxHDL may promote matrix degradation by enhancing the release of gelatinase from arterial monocytes-macrophages, thereby favoring atherosclerotic plaque destabilization and rupture. The extent of oxidative modification occurred in HDL-lipids and -proteins must be a determining factor for the overall pro-inflammatory and proatherogenic activities observed for oxHDL related to native HDL.

Since NADPH oxidase appear to be especially important for redox signalling, the next objective of this study was to determine whether NADPH oxidase is involved in oxHDL-induced ROS formation in monocytes-macrophages. Using inhibition studies with DPI [NADPH oxidase inhibitor] and free radical scavengers [NAC &

BHT] as well as NBT reduction assay for NADPH oxidase activity, this study demonstrated the involvement of NADPH oxidase-mediated ROS generation and subsequent release of gelatinase B in monocytes-macrophages.

Having established the existence of a relationship between oxHDL/ROS and MMP9 up-regulation, the next objective was to determine which ROS-mediated pathways could be signaling MMP9 expression. The precise redox-sensitive steps involved in kinase activation in response to oxHDL-induced NADPHoxidase/ ROS production is presently unknown. The possible involvement of stress kinases on oxHDL-induced gelatinase formation in monocytes-macrophages, was next examined using chemical inhibitors specific to JNK, p38 and ERK1/2- MAPK pathways. Examination of the effects of these inhibitors on MMP9 formation showed that both ERK1/2 and JNK inhibitors, but not p38 inhibitor, significantly suppressed oxHDL-induced formation of MMP9 from monocytes-macrophages. Taken together, these results showed that oxHDL-induced ROS formation and gelatinase expression in mo.mac via NADPH oxidase/ROS-ERK1/2 & JNK signalling-MMP9. This reflects the effects of the different components in oxHDL such as proteins or lipids, on monocytes-macrophages and subsequent modulation of observed cellular functions. It is thought that the balance between distinct MAPK pathways regulates cell growth, differentiation, survival, and death. Further elucidation of how ERK1/2 and JNK execute its function in generation of gelatinase will help us understand completely the role of oxHDL in monocytes-macrophages inflammatory response. In addition, the exact cell sensors that recognize oxHDL to mediate the observed inflammatory response remains to be elucidated. Although MMP9 plays a significant role in the pathology of atherosclerosis, the precise signaling pathways required for MMP9 up-

regulation has yet to be fully elucidated. The present study identified oxHDL-induced ROS as a potential stimulator of gelatinase expression in monocytes-macrophages , thus highlighting the altered character of HDL.

This is the first report to our knowledge that oxidative modification in HDL can induce MMP-9 activity through, at least in part, NADPH oxidase/ROS in monocytes-macrophages and promote inflammatory response. We cannot discard other sources of ROS and/or oxHDL/ ROS effects on other MMPs in human monocytes. Available evidence substantiates that plasma MMP9 levels correlate with the presence of atherosclerosis and represent an independent risk factor for atherothrombotic events. Functional alteration in HDL could be one of the contributing factors for the excessive release of MMP9. Further research is vital to ***better understand*** the complexity of cellular redox reactions mediated by functionally altered HDL, as such proinflammatory responses are likely to play a central role in the development of atherosclerosis.

In conclusion, the functional assay of HDL demonstrates that all HDL are not same in quality i.e.antioxidant-atheroprotective property. Blood levels of HDL-C do not predict the functional heterogeneity of HDL and point out the need for functional assay of HDL for better predicting the cardiovascular risk. Further investigations, demonstrate that following in vitro oxidative modification, HDL loses its atheroprotective functions and exerts proinflammatory response by releasing TNF-α, MMP9, and MMP2 as well as promotes oxidative stress [ROS] in human peripheral blood monocytes-macrophages. This study also reveals that oxHDL mediated the production of ROS and MMP9 in mo.mac through NADPH oxidase-induced ROS,

which in turn stimulate MAPK-ERK1/2 and JNK activation and gelatinase expression. These results provide new insights into the mechanisms of action of oxidatively modified HDL on monocytes-macrophages- the key cell type involved in atherogenesis, to stimulate the generation of gelatinase B and thus exaggerated the inflammatory response. However, monocytes-macrophages exhibited no such responses with mildly oxHDL and native HDL. The generation of oxHDL in vivo might therefore be regarded as possibly atherogenic.

FUTURE DIRECTIONS

- The major challenge associated with primary prevention of coronary artery diseases involves early and accurate detection of CAD in asymptomatic individuals at high cardiovascular risk. Present study demonstrated the presence of functionally deficient HDL in subjects having systemic inflammation.It is now known that not all HDL populations maintain a consistent anti-atherogenic profile *in vivo*. This heterogeneity may result from differences in quantitative and qualitative content of lipids, apolipoproteins and enzymes. Additional experiments will be needed to charecterize the HDL particle in detail, to distinguish functional from dysfunctional HDL, their role in cellular oxidative stress, inflammation, foam cell formation, and to delineate the molecular mechanism of action in order to provide new insights into the potential anti atherogenic or even pro-atherogenic character of HDL .
- Cellular recognition of functionally altered HDL is an unknown phenomenon. The role of specific receptors that recognize oxHDL may also be addressed in future studies.

- Future research has to be focused on whether the functional assay of HDL may help identify subjects at high risk for future CV events. There is need to identify sub-clinical CAD using atherosclerosis surrogate markers like common carotid artery intima-media thickness in subjects that will help identify high-risk subjects for early preventive strategies to reduce future risk of CAD. It is also of interest whether any of the HDL raising agents such as mimetic peptides, can influence HDL quality.

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List of publication from the Thesis

1) **V. S. Soumyarani & N. Jayakumari.** Oxidatively modified high density lipoprotein promotes inflammatory response in human monocytes–macrophages by enhanced production of ROS, TNF-α, MMP-9, and MMP-2. Mol Cell Biochem. 2012 ;366(1-2):277-85.doi: 10.1007/s11010-012-1306-y.

2) **V.S. Soumyarani and N. Jayakumari**. Oxidized HDL Induces Cytotoxic Effects: Implications for Atherogenic Mechanism.
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